Interactions of Multitargeted Kinase Inhibitors and Nucleoside Drugs: Achilles Heel of Combination Therapy?

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Abstract

Multitargeted tyrosine kinase inhibitors (TKI) axitinib, pazopanib, and sunitinib are used to treat many solid tumors. Combination trials of TKIs with gemcitabine, a nucleoside anticancer drug, in pancreas, renal, lung, ovarian, and other malignancies resulted in little benefit to patients. TKI interactions with human nucleoside transporters (hNT) were studied by assessing inhibition of [3H]uridine uptake in yeast producing recombinant hNTs individually and in cultured human cancer cell lines. Axitinib, pazopanib, and sunitinib inhibited hENT1 at low micromolar concentrations. In A549, AsPC-1, and Caki-1 cells, [3H]uridine, [3H]thymidine, [3H]gemcitabine, and [3H]fluorothymidine (FLT) accumulation was blocked by all three TKIs. Pazopanib > axitinib ≥ sunitinib inhibited hENT1 with IC₅₀ values of 2, 7, and 29 μmol/L, respectively, leading to reduced intracellular gemcitabine and FLT accumulation. Pretreatment or cotreatment of Caki-1 cells with TKIs reduced cellular accumulation of [3H]nucleosides, suggesting that TKI scheduling with nucleoside drugs would influence cytotoxicity. In combination cytotoxicity experiments that compared sequential versus simultaneous addition of drugs in Caki-1 cells, cytotoxicity was greatest when gemcitabine was added before TKIs. In clinical settings, TKI inhibitor concentrations in tumor tissues are sufficient to inhibit hENT1 activity, thereby reducing nucleoside chemotherapy drug levels in cancer cells and reducing efficacy in combination schedules. An additional unwanted interaction may be reduced FLT uptake in tumor tissues that could lead to aberrant conclusions regarding tumor response.

Introduction

Oral multitargeted tyrosine kinase inhibitors (TKI) have activity towards VEGF receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), stem cell factor receptor (KIT), and other tyrosine kinases. VEGF is a mediator of angiogenesis and contributes to tumor growth and metastasis (1, 2), whereas PDGFR activates growth and survival of vascular smooth muscle cells and recruitment and differentiation of pericytes (3, 4). TKI inhibition of tumor angiogenesis and other signaling pathways associated with tumor development resulted in promising antitumor activity against many solid tumor types including renal cell cancer (RCC), pancreatic cancer, gastrointestinal stromal tumors (GIST), and non–small cell lung cancer (NSCLC; refs. 5–7).

Among oral TKIs approved for clinical use, axitinib (AG-013736), pazopanib (GO78034), and sunitinib (SU11248) are used to treat several solid tumor types. Sunitinib is used to treat advanced RCC, pancreatic neuroendocrine tumors, and GIST (8). Similarly axitinib and pazopanib are used to treat advanced RCC, soft tissue sarcomas, pancreatic, and lung cancers (6, 9–16).

Combinations of conventional cytotoxic drugs such as gemcitabine, a nucleoside analog that targets cells in S-phase of the cell cycle, with novel agents that target key signaling pathways that control cancer cell survival, proliferation, and/or invasion is a promising approach, and has been attempted in several clinical trials (17–21). Clinical trials of combinations of TKIs with gemcitabine have been attempted in pancreatic, bladder, NSCLC, ovarian, and other malignancies with little benefit to patients (9, 22–24). These disappointing results suggest that there may be unfavorable interactions between TKIs and nucleoside chemotherapy drugs.

In humans, nucleoside transport is mediated by two unrelated protein families, the SLC28 family of concentrative nucleoside transporters (CNT) and the SLC29 family of equilibrative nucleoside transporters (ENT; ref. 25). SLC28 and SLC29 families have three human concentrative (hCNT1/2/3) and four human equilibrative members (hENT1/2/3/4), respectively. Nucleosides and nucleoside analog drugs are transported into cells by hENT1 and hENT2 and hCNT1, hCNT2, and hCNT3. Roles of hNTs in transport of nucleoside and nucleoside drugs are summarized in recent reviews (25–27).

Earlier studies indicated that TKIs may interfere with uptake of nucleoside chemotherapy drugs (28–31) and more recently Damaraju and colleagues (32) showed that erlotinib, gefitinib, and vandetanib compete with nucleoside drugs for cellular uptake and hence lead to reduced efficacy of combination treatments in cytotoxicity studies. As TKIs are widely used, in the current study...
we examined interactions of human nucleoside transporters (hNTs) with multitargeted TKIs axitinib, pazopanib, and sunitinib and resulting effects on combination cytotoxicity in cultured human cancer cell lines. To study interactions of TKIs with hNTs, we examined inhibition of \(^{3}H\)uridine transport in yeast cells producing each of five recombinant hNTs individually as well as inhibition of uridine and thymidine uptake and gemcitabine accumulation in three human cancer cell lines, pancreatic adenocarcinoma AsPC-1, NSCLC A549, and RCC Caki-1. We also examined sequential versus simultaneous combination cytotoxicity with gemcitabine and TKIs in Caki-1 cells.

**Materials and Methods**

**Materials**

Nitrobenzylmercaptopurine ribonucleoside (NBMPR), dilazep, dipryidamole, unlabeled nucleosides, and other chemicals were obtained from Sigma Chemical Company. Tritiated nucleosides were purchased from Moravek Biochemicals. Tissue culture (96- and 12-well) plates and flasks were from VWR International. Cell culture media and FBS were from Gibco BRL. Ecolite was from ICN Pharmaceuticals. Dojindo Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies, Inc. Axitinib, pazopanib, and sunitinib were from LC Laboratories.

**Cell culture**

Human cancer cell lines, A549 (NSCLC), AsPC-1 (pancreatic cancer), and Caki-1 (RCC) were obtained from ATCC in 2005, 2001, and 2002, respectively. Cell lines were sent to DDC Medical to verify their authenticity by STR profiling in July 2013 for A549 and AsPC-1 and February 2014 for Caki-1 cells and mycoplasma status. Results showed that A549 and AsPC-1 were 100%, whereas Caki-1 was >80% matched to the ATCC panel of markers and all three were free of mycoplasma. Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L glutamine. All cultures were kept at 37°C in 5% CO\(_2\)/95% air and subcultured at 2- to 3-day intervals to maintain exponential growth. Transport and cytotoxicity experiments were conducted with cells in the exponential growth phase.

**Uridine transport in Saccharomyces cerevisiae**

*Saccharomyces cerevisiae* yeast were transformed with plasmids (pYPHENT1, pYPHENT2, pYPHCNT1, pYPHCNT2, or pYPHCNT3) encoding hNTs (hENT1, hENT2, hCNT1, hCNT2, or hCNT3, respectively) as described elsewhere (33, 34). Uptake of 1 \(\mu\)mol/L \(^{3}H\)uridine (Moravek Biochemicals) into yeast was measured as previously described (34, 35) using the semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments). Yeast were incubated at room temperature with 1 \(\mu\)mol/L \(^{3}H\)uridine in yeast growth media (pH 7.4) in the presence or absence (uninhibited controls) of graded concentrations of test compounds. Uridine self-inhibition was used to determine maximum inhibition of mediated transport.

Concentration–effect curves were subjected to nonlinear regression analysis using Prism software (version 4.03; GraphPad Software Inc.) to obtain the concentration of test compound that inhibited uridine uptake by 50% relative to that of untreated cells (IC\(_{50}\) values). Each IC\(_{50}\) value determination was conducted with nine concentrations and six replicates per concentration and experiments were repeated three times.

**Nucleoside transport inhibition in A549, AsPC-1, and Caki-1 cells**

Cells (100,000/well) were seeded in 12-well plates and on the third day, uptake of \(^{3}H\)nucleosides was measured at room temperature in transport buffer (pH 7.4) containing 20 mmol/L Tris, 3 mmol/L K\(_2\)HPO\(_4\), 1 mmol/L MgCl\(_2\), 1.4 mmol/L CaCl\(_2\), and 5 mmol/L glucose with 144 mmol/L NaCl hereafter termed transport buffer. For uridine uptake assays, cell growth medium was aspirated, cells were washed with sodium or sodium-free buffer, \(^{3}H\)uridine was added and uptake was measured over fixed time points in the presence or absence of established NT inhibitors (NBMPR, dilazep) or TKIs. Sunitinib’s effects on kinetics of uridine uptake were determined in A549 cells at graded concentrations of \(^{3}H\)uridine (0–1,000 \(\mu\)mol/L) at 0, 25, 50, or 100 \(\mu\)mol/L of sunitinib using 30-second incubations from a period during which initial time courses of \(^{3}H\)uridine uptake were shown to be linear. At the end of uptake intervals, permeant-containing solutions were removed by aspiration, and cells were quickly rinsed twice with sodium buffer and solubilized with 5% TritonX-100. Radioactivity in solubilized extracts was measured by liquid scintillation counting. Uptake values were expressed as pmol/10\(^6\) cells and graphs were generated using the Prism software. Each experiment was conducted two or three times with triplicate measurements.

For \(^{3}H\)uridine and \(^{3}H\)thymidine accumulation experiments, A549, AsPC-1, and Caki-1 cells were exposed to either 10 \(\mu\)mol/L \(^{3}H\)uridine or 1 \(\mu\)mol/L \(^{3}H\)thymidine for 60 minutes in the absence or presence of 5 or 25 \(\mu\)mol/L sunitinib or 100 \(\mu\)mol/L dilazep (an inhibitor of hENT1 and hENT2; ref. 36) and processed as described above for uptake assays. Effects of TKIs on 1 \(\mu\)mol/L \(^{3}H\)gemcitabine or 10 \(\mu\)mol/L \(^{3}H\)uridine uptake (15 minutes) into cells were studied in the absence or presence of 10 \(\mu\)mol/L inhibitor.

For sequencing of exposure to nucleosides and TKIs, uptake experiments were conducted as follows. In the first set of experiments, control cells were incubated in buffer without drug for 15 minutes after which 10 \(\mu\)mol/L \(^{3}H\)uridine, 1 \(\mu\)mol/L \(^{3}H\)gemcitabine, or 1 \(\mu\)mol/L \(^{3}H\)fluorothymidine (FLT) was added and uptake was measured for 15 minutes. In the second set of experiments, cells were incubated with 10 \(\mu\)mol/L axitinib, pazopanib, or sunitinib for 15 minutes after which the drug was removed and uptake of 10 \(\mu\)mol/L \(^{3}H\)uridine, 1 \(\mu\)mol/L \(^{3}H\)gemcitabine, or 1 \(\mu\)mol/L \(^{3}H\)FLT was measured for 15 minutes. In the third set of experiments, 10 \(\mu\)mol/L \(^{3}H\)uridine, 1 \(\mu\)mol/L \(^{3}H\)gemcitabine, or 1 \(\mu\)mol/L \(^{3}H\)FLT was added for 15 minutes after which media was removed and 10 \(\mu\)mol/L axitinib, pazopanib, or sunitinib was added. In the last set of experiments, cells were incubated in buffer without any drug for 15 minutes after which the buffer was removed and either 10 \(\mu\)mol/L \(^{3}H\)uridine, 1 \(\mu\)mol/L \(^{3}H\)gemcitabine, or 1 \(\mu\)mol/L \(^{3}H\)FLT was added together with 10 \(\mu\)mol/L axitinib, 10 \(\mu\)mol/L pazopanib, or 10 \(\mu\)mol/L sunitinib and incubated for 15 minutes. At the end of these time points, media was removed and cells were processed for radioactivity as described above.

**Cytotoxicity assays**

Dojindo CCK-8 was used to quantify drug-induced cytotoxicity. A549, AsPC-1, or Caki-1 cells were seeded in
96-well plates and allowed to attach for 24 hours. Cells were then exposed to graded concentrations of sunitinib for 72 hours. Effects of NBMPR on sunitinib toxicity were tested in A549 cells by exposing cells to sunitinib in the absence or presence of 1 μmol/L NBMPR for 72 hours after which they were treated with CCK-8 reagent for cytotoxicity assessment. For evaluation of in vitro synergy of combinations of gemcitabine with the TKIs, experiments were based on the individual drug's IC50 value. For sequential treatments, cells were incubated with either drug for 24 hours, followed by incubation in drug-free media for 24 hours and subsequent incubation for 72 hours with the other drug. Simultaneous or individual treatments were performed for 72 hours. Drug synergy was determined by the isobologram and combination index (CI) methods, derived from the median effect principle of Chou and Talalay (37) using the CalcuSyn software (Biosoft). Using data from growth inhibitory experiments and computerized software, CI values were generated over a range of fraction affected (Fa) levels from 0.05 to 0.90 (5%–90% growth inhibition). A CI value of 1 indicates an additive effect between two agents, whereas a CI value <1 or >1 indicates synergism or antagonism, respectively.

Statistical analysis
One-way ANOVA was used for statistical analysis using GraphPad Prism software.

Results
Inhibition of uridine uptake mediated by recombinant hNTs produced in Saccharomyces cerevisiae
Axitinib, pazopanib, and sunitinib (chemical structures shown in Fig. 1) were assessed for their relative abilities to inhibit [3H]uridine uptake by each of the five hNTs in concentration-dependent inhibition experiments to determine IC50 (inhibitor concentration that produced 50% inhibition of transport) values. A representative concentration–effect curve for sunitinib inhibition of hENT1-mediated uridine transport in yeast is shown in Fig. 2A. IC50 values obtained from such experiments with axitinib, pazopanib, and sunitinib in yeast producing each of the five recombinant NTs are presented in Table 1. For hENT1, pazopanib, sunitinib, and axitinib IC50 values (± S.E.) were 4 ± 0.3, 31 ± 5, and 46 ± 4 μmol/L, respectively. Inhibition of hENT2, hCNT1, hCNT2, and hCNT3 was seen with IC50 values ranging from 80 to 210 μmol/L with the exception of axitinib (hCNT1 and hCNT3) and pazopanib (hENT2) where IC50 values were >300 μmol/L and thus could not be determined.

Inhibition of hENT1 mediated [3H]uridine and [3H]thymidine uptake by sunitinib in human cancer cell lines
A549 cells possess predominantly hENT1 with a minor component of hENT2 activities (32). Sunitinib, which inhibited hENT1 in yeast radiotrace experiments, was tested in A549 cells to evaluate its effects on native hENT1-mediated uptake in human cells. Figure 2B shows inhibition of [3H]uridine uptake in A549 cells by graded concentrations of sunitinib. Similar concentration–effect studies were conducted with axitinib, pazopanib, and sunitinib in A549, AsPC-1, and Caki-1 cells and resulting IC50 values (mean ± SE) are presented in Table 1, and in all situations, significant hENT1 inhibition was observed with IC50 values ranging from 1 to 29 μmol/L.

We examined accumulation of [3H]thymidine in all 3 cell lines by following long-term uptake (1 hour) of 1 μmol/L [3H]thymidine in the absence or presence of 5 or 25 μmol/L of sunitinib or 100 μmol/L dilazep. Both sunitinib concentrations (5 and 25 μmol/L) inhibited thymidine uptake (Fig. 2C) in all cell lines tested, although neither concentration achieved complete inhibition as was seen with 100 μmol/L dilazep.

Effect of TKIs on kinetics of uridine uptake in hENT1-producing yeast
The nature of interaction of TKIs with hENT1 were further examined in hENT1 producing yeast cells by studying effects of fixed axitinib (Fig. 3A), pazopanib (Fig. 3B), or sunitinib (Fig. 3C) concentrations on kinetics of [3H]uridine uptake (Fig. 3A–C). Although analysis of results using a Lineweaver–Burk plot showed
Effects of sequencing of administration of TKIs and nucleosides on nucleoside retention in cells

Our results thus far showed that TKIs interfered with uptake of nucleosides in three model cell lines tested. We examined this further in Caki-1 cells to see whether changes in nucleoside uptake occur when two agents are added separately in sequence or simultaneously together. $[^3]$H]Uridine (10 μmol/L), $[^3]$H]gemcitabine (1 μmol/L), or $[^3]$H]FLT (1 μmol/L) uptake was measured in Caki-1 cells that were either treated without or with 10 μmol/L of each TKI for 15 minutes before or after exposure to radiolabeled nucleoside or during exposure for 15 minutes. Results of sequencing of administration on nucleoside accumulation are shown Fig. 4A–C. Significant inhibition ($P < 0.05$) of nucleoside/drug accumulation occurred when TKIs were combined with uridine or gemcitabine or FLT during simultaneous exposures or were administered before nucleosides, whereas no effects were observed when TKI exposures were after uridine exposure.

Cytotoxicity and synergy studies

Our results presented above suggested that a sequential schedule for combination of nucleoside drugs with TKI inhibitors in which administration of a nucleoside drug followed by a TKI inhibitor would result in better synergy than any other sequence. Sunitinib cytotoxicity was tested in A549 cells in absence or presence of 1 μmol/L NBMPR, a potent and specific inhibitor of hENT1 (Fig. 5A). Results showed that NBMPR had no effect on sunitinib cytotoxicity thus indicating that sunitinib is not a permeant for hENT1. Gemcitabine was equally cytotoxic to A549, AsPC-1, and Caki-1 cells (data not shown) with IC50 values (mean ± SE) of 2.2 ± 0.2, 3.5 ± 0.8, and 4.2 ± 0.1 μmol/L, respectively. In cytotoxicity studies conducted over 72 hours, axitinib, pazopanib, and sunitinib were cytotoxic to A549 cells (Fig. 5B). All three cell lines were sensitive to sunitinib with IC50 values of 5 μmol/L. AsPC-1 was insensitive to axitinib but was sensitive to pazopanib with IC50 value of 35 μmol/L. Caki-1 cells were sensitive to axitinib and pazopanib with IC50 values of 25 and 30 μmol/L.

We explored cytotoxicity of sequential and simultaneous administration of gemcitabine with axitinib or pazopanib in Caki-1 cells. For in vitro combination studies cells were (i) pretreated with axitinib or pazopanib before gemcitabine, (ii) pretreated with gemcitabine before axitinib or pazopanib, or (iii) treated with both agents together or alone as described.
in Materials and Methods. Isobologram and CI methods developed by Chou and Talalay (37) were used to confirm and quantify the synergism observed with various combinations. Isobolograms were constructed for Fa values of 0.50, 0.75, and 0.90, representing 50%, 75%, and 90% growth inhibition, respectively, and are shown in Fig. 5C–F. These results indicated that the sequence, gemcitabine followed by TKIs, was synergistic (CI values at Fa 50% were 0.4 and 0.7 for axitinib and pazopanib; Fig. 5C and D), whereas the sequence TKI followed by gemcitabine (CI values at Fa 50% were 2 and 2.2 for axitinib and pazopanib, Fig. 5E and F) or simultaneous treatment (CI values at Fa 50% were 3 and 2 for axitinib and pazopanib) was antagonistic, suggesting that inhibition of hNTs by TKIs would lead to decreased effectiveness of combinations involving these agents and nucleoside chemotherapy drugs.

### Discussion

Several TKIs (e.g., sunitinib, pazopanib, and axitinib) targeting VEGFR have been approved by regulatory agencies for treatment of RCC and GIST in first-, second- or third-line settings (8, 39–41). In addition, other therapeutic approaches include treatment with sunitinib in advanced pancreatic neuroendocrine tumors (42). In a recent randomized phase II study in 113 patients with advanced pancreatic adenocarcinoma, the combination of gemcitabine with sunitinib did not lead to improved progression-free survival when compared with gemcitabine alone (23). In a phase III study of patients with advanced pancreatic adenocarcinoma, addition of axitinib to gemcitabine did not lead to improved overall survival (9, 12, 24). In an earlier study, we presented results on interactions of erlotinib, gefitinib, and vandetanib with the hNTs (32). This interaction was attributed to similarities of these and other TKIs to the classical hENT1 inhibitor NBMPR with the underlying implication that TKIs, when combined with nucleoside chemotherapy, inhibit hNTs and therefore are likely to interfere with nucleoside chemotherapy cytotoxicity.

In this study, we showed that sunitinib, pazopanib, and axitinib inhibited transport of uridine by recombinant hNTs produced individually in yeast. hENT1 was inhibited at low concentrations by sunitinib, pazopanib, and axitinib, whereas the other hNTs were inhibited at much higher concentrations. Sunitinib inhibited hENT1 and hCNT1; axitinib inhibited hENT1 and hCNT2 and pazopanib inhibited hENT1 and all three hCNTs equally well. In experiments with cultured human cancer cell lines, hENT1-mediated uridine was inhibited by sunitinib, pazopanib, and axitinib with pazopanib inhibiting hENT1 at very low concentrations followed by axitinib and sunitinib. Uridine uptake in hENT1-producing yeast cells was inhibited by axitinib, pazopanib, and sunitinib in a competitive manner that suggests an apparent competitive inhibition of uridine transport inhibition in yeast cell lines all of which have major intrinsic hENT1 activity and negligible or no CNT or hENT2 activities. IC50 values (mean ± SE) are listed above.

![Figure 3. Effects of axitinib or pazopanib on kinetics of [3H]uridine uptake in hENT1-producing yeast. Effects of 0 ( ), 5 ( ), 25 ( ), 50 ( ), or 100 ( ) µmol/L of axitinib (A), pazopanib (B), and sunitinib (C) on uptake of [3H]uridine at various concentrations (0–1,000 µmol/L). Values with mean ± SE are shown in each panel. Each experiment was repeated three times.](Image)

### Table 1.

Summary of IC50 values for uridine transport inhibition in yeast and cell lines

<table>
<thead>
<tr>
<th>Transporter (yeast)</th>
<th>Axitinib</th>
<th>Sunitinib</th>
<th>Pazopanib</th>
</tr>
</thead>
<tbody>
<tr>
<td>hENT1</td>
<td>46 ± 4</td>
<td>31 ± 5</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>hENT2</td>
<td>180 ± 44</td>
<td>215 ± 4</td>
<td>&gt;300</td>
</tr>
<tr>
<td>hCNT1</td>
<td>&gt;300</td>
<td>80 ± 6</td>
<td>170 ± 13</td>
</tr>
<tr>
<td>hCNT2</td>
<td>130 ± 10</td>
<td>200 ± 20</td>
<td>150 ± 12</td>
</tr>
<tr>
<td>hCNT3</td>
<td>&gt;300</td>
<td>130 ± 7</td>
<td>120 ± 3</td>
</tr>
<tr>
<td>Cell lines (hENT1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>7.0 ± 10</td>
<td>26.0 ± 2.0</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>ASPC-1</td>
<td>5.0 ± 0.1</td>
<td>25.0 ± 5.0</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Caki-1</td>
<td>7.0 ± 10</td>
<td>29.0 ± 9.0</td>
<td>2 ± 0.2</td>
</tr>
</tbody>
</table>

NOTE: Inhibition of [3H]uridine uptake by TKIs was assessed in yeast producing Caki-1, ASPC-1, A549, and hENT1-producing yeast. Effects of 0 ( ), 5 ( ), 25 ( ), 50 ( ), or 100 ( ) µmol/L of axitinib (A), pazopanib (B), and sunitinib (C) on uptake of [3H]uridine at various concentrations (0–1,000 µmol/L). Values with mean ± SE are shown in each panel. Each experiment was repeated three times.
uptake by axitinib, pazopanib, and sunitinib although such inhibition could also be achieved by binding to an allosteric site (38). "H"Uridine and "H"Hymidine accumulation in three cell lines was inhibited by 5 and 25 µmol/L sunitinib, respectively. In addition, we tested effects of changing the sequence of administration of nucleosides with TKIs on "H"nucleoside accumulation in Caki-1 cells. Cells were treated with (i) "H"nucleoside alone for 15 minutes, (ii) a combination of "H"nucleoside with individual TKIs for 15 minutes, (iii) individual TKIs for 15 minutes followed by "H"nucleoside, or (iv) combination of TKIs with nucleosides for 15 minutes. Simultaneous as well as sequential (when a TKI was given before a nucleoside) administration of either pazopanib or axitinib with "H"nucleoside resulted in a large decrease in nucleoside accumulation. Caki IC50 values (Table 1) were predictive of results observed wherein pazopanib (IC50 value, 2 µmol/L) had large effects in both dosing schedules followed by axitinib with modest effects, whereas the magnitude of the inhibition by sunitinib (IC50 value, 29 µmol/L) was much less consistent with its IC50 value for inhibition of hENT1 activity.

Although TKIs appeared to be interacting with nucleoside-permeant binding sites of hENT1, they appeared not to be transported by hENT1 based on results of cytotoxicity experiments with sunitinib in the absence or presence of the hENT1 transport inhibitor NBMPR, although direct evidence for their lack of transportability would require measurement of uptake of "H"-labeled TKIs. In combination cytotoxicity studies, Caki-1 cells showed greater sensitivity to drug combinations when they were exposed to gemcitabine for 24 hours followed by either axitinib or pazopanib as predicted from our uptake inhibition studies. Earlier reports indicated interaction of TKIs with ATP-binding domains of ATP-binding cassette (ABC) transporter-mediated multidrug resistance (MDR) proteins in cancer cells (43–45). Inhibition of P-glycoprotein (P-gp) activity (46) resulted in enhanced cytotoxic effects of multiple anticancer drugs by increasing accumulation of P-gp and ATP-binding cassette subfamily G member 2 (ABCG2) substrates (47, 48). Earlier studies showed inhibition of hENT1-mediated activity in K562 cells by p38 MAPK inhibitors (29), and our current and previous results (32) indicate that another group of potential target proteins are hNTs.

Effects of axitinib, pazopanib, and sunitinib inhibition of hNTs on nucleoside chemotherapy efficacy need to be addressed and be made known to medical oncologists. There is pharmacologic evidence that suggests that there have been issues with TKIs and nucleoside chemotherapy (9, 12, 24). Although it is difficult to extrapolate in vitro studies to the clinic, especially with drugs that have such extensive protein binding and accumulation in tumors, TKI inhibitors achieve levels in tissues that could inhibit hENT1. In the phase I study of pazopanib, Hurwitz and colleagues (49) found at a dose of 800 mg daily that pazopanib plasma levels were 103 µmol/L, and we found that pazopanib’s IC50 value for hENT1 inhibition was 2 µmol/L for A549 cells. The FDA-approved product monograph indicates that pazopanib plasma concentrations are 132 µmol/L which would inhibit hENT1 completely thus blocking accumulation of cytotoxic nucleoside drugs. In a study of neoadjuvant breast cancer patients, mean gefitinib plasma levels at steady state were 0.18 µg/mL (0.40 µmol/L) and mean tumor levels were 7.5 µg/g (17 µmol/L), an approximately 42-fold difference.
In patients with lung cancer, gefitinib tumor levels were approximately 40-fold higher than plasma levels (22.7 vs. 0.52 µmol/L; ref. 51). Gotink and colleagues (52) reported tumor sunitinib levels in mice and in patients with RCC treated with sunitinib. Mice were treated with sunitinib for 4 weeks at a dose of 40 mg/kg daily after which sunitinib tumor levels were 9-fold higher than plasma levels (22.7 vs. 0.52 µmol/L; ref. 51). Gotink and colleagues (52) reported tumor sunitinib levels in mice and in patients with RCC treated with sunitinib doses ranging from 37.5 mg/day to 50 mg/day for 4 weeks before tumor biopsy. In patients with RCC, sunitinib tumor levels were 9 µmol/L, 30-fold higher than plasma levels (0.3 µmol/L). We found that sunitinib’s IC50 for hENT1 inhibition in A549 cells was 26 µmol/L with nearly 30% to 40% inhibition of hENT1 at 10 µmol/L sunitinib. Patients with advanced RCC taking axitinib 5 mg twice a day had a maximum observed plasma concentration of 27.8 ng/mL and an area under the plasma concentration time curve at 24 hours of 265 ng/mL (53) giving a concentration average at steady state of 11 ng/mL.

Unlike sunitinib, axitinib levels have not been studied in tumor tissues. Reyner and colleagues (54) studied axitinib levels in normal tissues in mice and found that the highest tissue to plasma partitioning ratio of axitinib was in the liver, around 3.8-fold. Assuming that axitinib would only concentrate 3.8-fold in tumors, the concentration average at steady state in tumors would be 0.1 µmol/L, whereas assuming that it concentrates in tumors like sunitinib, the concentration average at steady state in tumors would be 0.3 µmol/L. It is also important to consider how axitinib and gemcitabine were administered in the clinical trial. Axitinib was administered 0.5 hours before the start of the gemcitabine infusion (14), yielding axitinib levels at the start of the infusion of approximately 10 ng/mL and 2 hours after the end of the gemcitabine (half-life 0.3 hours) infusion of approximately 20 ng/mL including a peak axitinib level of 28.2 ng/mL. It is therefore possible that NTs were exposed to axitinib concentrations sufficient to inhibit hENT1 to some extent.
As our in vitro studies found an IC\textsubscript{50} value for axitinib inhibition of hENT1 of 3 to 7 \( \mu \)mol/L in three cell lines, there could be significant (approximately 20%) hENT1 inhibition in tumors at concentrations less than 2 \( \mu \)mol/L. We cannot rule out effects of TKIs on reduction of cell surface expression of hENT1 as was shown earlier using a fluorescent probe for evaluation of cell surface hENT1 sites (32). Thus, plasma levels from phase I studies of axitinib, pazopanib, and sunitinib taken together with studies of tissue and plasma levels of gefitinib suggest that IC\textsubscript{50} values for hENT1 inhibition of axitinib, pazopanib, and sunitinib are relevant to the clinic.

Preclinical and clinical pharmacologic evidence indicates drug interactions between TKIs and nucleoside chemotherapy drugs that are consistent with inhibition of hENTs by TKIs providing an explanation for the failure of combination therapies with multitargeted TKIs and nucleoside chemotherapy.

Our study raises doubts about \(^{18}\text{F}\)-FLT’s utility as a noninvasive measure of decreased proliferation caused by targeted therapies such as TKIs. Earlier studies by Paproski and colleagues (55) have shown that hENT1 is necessary for uptake and retention of \(^{18}\text{F}\)-FLT into cancer cells. Barthel and colleagues (56) showed in in vivo experiments of 5-fluorouracil in induced fibrosarcoma-1 xenograft mouse that \(^{18}\text{F}\)-FLT was an earlier and more pronounced marker of decreased tumor proliferation than \(^{18}\text{F}\)-fluoro-deoxy-glucose. Liu and colleagues, (57) used \(^{18}\text{F}\)-FLT to study sunitinib effects on solid tumors. Although they noted significant decreases in \(^{18}\text{F}\)-FLT uptake in patients treated with sunitinib, decreases in \(^{18}\text{F}\)-FLT uptake did not correlate with tumor response. We suspect that decreases in \(^{18}\text{F}\)-FLT uptake observed by Liu and colleagues were mostly due to inhibition of \(^{18}\text{F}\)-FLT uptake by sunitinib inhibition of hENT1 rather than to decreased proliferation. In another study by Zhao and colleagues (58), significant changes in FLT uptake were noticed before any change in tumor size. Our studies suggest that \(^{18}\text{F}\)-FLT might not be a reliable marker of tumor proliferation when used with a TKI especially as our earlier study (32) showed that TKIs not only compete with nucleoside binding on hENT1, but also decrease cell surface expression of hENT1.

In summary, we have demonstrated that three multitargeted TKIs, axitinib, pazopanib, and sunitinib, inhibit hENT1, a ubiquitous hNT that is necessary for activity of many nucleoside chemotherapy drugs. We have also shown that these agents decrease accumulation of nucleoside drugs when combined together or when TKIs are given first. Our results suggest that a sequence of gemcitabine followed by TKIs would result in synergistic cytotoxic effects. Implications of this study in the clinic are that when nucleoside chemotherapy drugs are administered concurrently with multitargeted TKIs, attention should be paid to sequence of administration of these agents to achieve better response profiles in patients. In addition, as FLT uptake is also inhibited by these TKIs, early assessment of tumor response by PET imaging should be monitored carefully with concerns about \(^{18}\text{F}\)-FLT’s utility as a noninvasive measure of decreased proliferation of tumors in patients treated with TKI-targeted therapies. We are extending these studies to in vivo tumor-bearing mice with \([^{18}\text{F} ]\)FLT PET imaging studies to validate our in vitro observations.

**Disclosure of Potential Conflicts of Interest**

V.I. Damara\textsuperscript{u} has ownership interest in a patent. M.B. Sawyer received speakers’ bureau honoraria as a travel grant from Pfizer, has ownership interest in a patent regarding methods of combining tyrosine kinase inhibitors and methods for treating their side effects, and is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Kuzma, D. Movles

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.I. Damara\textsuperscript{u}, M.B. Sawyer

Writing, review, and/or revision of the manuscript: V.I. Damara\textsuperscript{u}, C.E. Cass, M.B. Sawyer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Kuzma, D. Movles

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**Grant Support**

This work was supported by the Alberta Cancer Foundation (to M.B. Sawyer, C.E. Cass), the Canadian Cancer Society Research Institute (to C.E. Cass), and AstaRzeneca (M.B. Sawyer).

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Received April 17, 2014; revised October 20, 2014; accepted October 21, 2014; published OnlineFirst December 17, 2014.

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2015;14:236-245. Published OnlineFirst December 17, 2014.

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