Cancer Therapy: Preclinical

Erlotinib, Gefitinib, and Vandetanib Inhibit Human Nucleoside Transporters and Protect Cancer Cells from Gemcitabine Cytotoxicity

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Abstract

Purpose: Combinations of tyrosine kinase inhibitors (TKI) with gemcitabine have been attempted with little added benefit to patients. We hypothesized that TKIs designed to bind to ATP-binding pockets of growth factor receptors also bind to transporter proteins that recognize nucleosides.

Experimental Design: TKI inhibition of uridine transport was studied with recombinant human (h) equilibrative (E) and concentrative (C) nucleoside transporters (hENT, hCNT) produced individually in yeast. TKIs effects on uridine transport, gemcitabine accumulation, regulation of hENT1 activity, and cell viability in the presence or absence of gemcitabine were evaluated in human pancreatic and lung cancer cell lines.

Results: Erlotinib, gefitinib and vandetanib inhibited [3H]uridine transport in yeast and [3H]uridine and [3H]gemcitabine uptake in the four cell lines. Treatment of cell lines with erlotinib, gefitinib, or vandetanib for 24 hours reduced hENT1 activity which was reversed by subsequent incubation in drug-free media for 24 hours. Greater cytotoxicity was observed when gemcitabine was administered before erlotinib, gefitinib, or vandetanib than when administered together and synergy, evaluated using the CalcuSyn Software, was observed in three cell lines resulting in combination indices under 0.6 at 50% reduction of cell growth.

Conclusions: Vandetanib inhibited hENT1, hENT2, hCNT1, hCNT2, and hCNT3, whereas erlotinib inhibited hENT1 and hCNT3 and gefitinib inhibited hENT1 and hCNT1. The potential for reduced accumulation of nucleoside chemotherapy drugs in tumor tissues due to inhibition of hENTs and/or hCNTs by TKIs indicates that pharmacokinetic properties of these agents must be considered when scheduling TKIs and nucleoside chemotherapy in combination.

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Introduction

The U.S. Food and Drug Administration (FDA) approved the tyrosine kinase inhibitor (TKI) erlotinib for maintenance of patients with locally advanced or metastatic non–small cell lung cancer (NSCLC) and more recently as a first-line treatment in patients with NSCLC with EGFR (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations. Similarly, gefitinib has been approved for EGFR mutation–positive NSCLC in more than 80 countries worldwide. It showed impressive, single-agent activity and received accelerated FDA approval based on early phase II studies pending definitive randomized studies (1). However, in one of the pivotal randomized studies, gefitinib was combined at doses of 250 or 500 mg/day with gemcitabine/cisplatin with expectations that the two gefitinib combinations would be superior to gemcitabine/cisplatin alone in NSCLC but there were no statistical differences between placebo and treatment arms in terms of progression-free survival (PFS; ref. 2). In a phase II trial in patients with advanced urothelial carcinoma treated with gemcitabine/cisplatin and concurrent gefitinib, response rates were not significantly different from gemcitabine/cisplatin alone (3). Gemcitabine and gefitinib were evaluated in patients with advanced pancreatic cancer (4) and response rates were similar to those observed in a pivotal phase III study in patients with locally advanced or metastatic pancreatic cancer with gemcitabine or gemcitabine and erlotinib with median survival of 5.9 to 6.2 months. Addition of gefitinib to gemcitabine in a different phase II trial in patients with urothelial carcinoma did not result in increased response rates compared with the control arm of gemcitabine/cisplatin (5). Vandetanib was initially evaluated in patients with advanced NSCLC and the combination of vandetanib with gemcitabine/cisplatin was not tolerated in these patients (6).
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Translational Relevance
Tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib have been combined with nucleoside chemotherapy with negative or disappointing results. We noted that vandetanib and other TKIs share structural features with a potent inhibitor of human equilibrative nucleoside transporter 1 (hENT1) nitrobenzylmercaptopurine ribonucleoside (NBMPR), and speculated that failures of combination of TKIs with nucleoside chemotherapy may be due to TKIs inhibiting human nucleoside transporters (hNT), blocking entry of nucleoside drugs. We showed that gefitinib, erlotinib, and vandetanib inhibited hENT1, the hNT necessary for activity of many nucleoside drugs. Not only did TKIs inhibit hNTs directly, they also inhibited hENT1 activity indirectly by reducing hENT1 protein in plasma membranes. hNT inhibition may be responsible for failure to successfully combine nucleosides and TKIs. This previously unnoticed feature of EGFR TKIs likely explains why they have failed in gastrointestinal cancer combination chemotherapy for which the backbone is either capcitabine or gemcitabine, known nucleoside permeants of hNTs.

Combining conventional cytotoxic drugs with novel agents that target key signaling pathways that control cancer cell survival, proliferation, and/or invasion should be a promising approach, and several clinical trials of TKIs (2–6) suggest that there may be unfavorable interactions between TKIs and nucleoside chemotherapy drugs. Although literature reports suggest that TKIs interfere with uptake of nucleoside chemotherapy drugs (7–10), detailed studies of TKI effects on individual human nucleoside transporters (hNT) or a theory to explain why TKIs inhibit hNTs are lacking. Transporters for physiologic nucleosides and nucleoside analogs include human equilibrative nucleoside transporters 1–4 (hENT1–4) and human concentrative nucleoside transporters (hCNT1–3). Detailed summaries of hNTs’ roles in transport of nucleoside drugs can be found in recent reviews (11, 12).

We hypothesized that poor clinical outcomes of combining erlotinib, gefitinib, or vandetanib with nucleoside chemotherapy drugs (e.g., gemcitabine) were due to TKI inhibition of hNTs, resulting in lowered intracellular accumulation of nucleoside drug metabolites and reduced treatment efficacy. To study potential interactions between TKIs and hNTs, we investigated TKI inhibition of uridine transport in yeast cells producing each of recombinant hNTs individually and TKI effects on uridine uptake, gemcitabine accumulation, and cytotoxicity in one pancreatic adenocarcinoma cell line AsPC-1 and three human NSCLC cells that differ in their EGF receptor (EGFR) and KRAS mutation status—i.e., A549 (EGFR wild-type and KRAS mutant), H292 (EGFR wild-type and KRAS wild-type), and H1975 (EGFR L858R, T790M mutation and wild-type KRAS).

Materials and Methods

Materials
Nitrobenzylmercaptopurine ribonucleoside (NBMPR), dilazep, 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. Erlotinib, gefitinib, and vandetanib were from LC Laboratories.

Cell culture
Human lung cancer cell lines A549, H292, and H1975 and the human pancreatic cancer cell line AsPC-1 were obtained from American Type Culture Collection (ATCC). Cell lines were sent to DDC Medical to verify their authenticity and mycoplasma status. Results showed that cell lines were 100% matched to the ATCC panel of markers and were free of mycoplasma. Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, and 10% glucose. All cultures were kept at 37°C in 5% CO2/95% air and subcultured at 2 to 3-day intervals to maintain exponential growth. Transport and cytotoxicity experiments were conducted with cells in exponential growth phase.

Choice of TKI concentrations to be studied
Plasma concentrations of gefitinib, erlotinib, and vandetanib are 0.4, 2.5 and 2.0 μmol/L, respectively (13–15). Earlier studies have shown that gefitinib concentrations in tumor tissues were 42-fold higher than plasma concentrations (15). Similarly, vandetanib tumor concentrations were 30-fold higher in tumor tissues than in plasma (16). Given structural similarities among the three TKIs, we expected similar concentrating effects to be seen with erlotinib and assumed that gefitinib, erlotinib, and vandetanib concentrations were likely to be considerably higher in tumor tissues than in plasma (15, 16). We therefore studied TKI concentrations up to 100 μmol/L in cell lines and 500 μmol/L in yeast model systems.

Uridine transport in Saccharomyces cerevisiae
Saccharomyces cerevisiae yeast were separately transformed with plasmids (pYPHENT1, pYPHENT2, pYPHCNT1, pYPHCNT2, or pYPHCNT3) encoding hNTs (hENT1, hENT2, hCNT1, hCNT2, or hCNT3, respectively) as described elsewhere (17, 18). Uptake of 1 μmol/L [3H]uridine (Moravek Biochemicals) into yeast was measured as previously described (18, 19) using the semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments). Yeast were incubated at room temperature with graded concentrations (0–0.5 mmol/L) of test compounds in the presence of 1 μmol/L [3H]uridine in yeast growth media at pH 7.4. Yeast were incubated with 1 μmol/L [3H]uridine in the absence of...
test compound and the resulting uninhibited (i.e., control) values were used to determine % Control values. 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 (IC50 values). Each IC50 value determination was conducted with nine concentrations and six replicates per concentration and experiments were repeated three times.

Nucleoside transport inhibition in A549, H292, H1975, and AsPC-1 cells

Cells (104/well) were seeded in 12-well plates and on the third day, uptake of [3H]uridine was measured at room temperature in transport buffer (pH 7.4) containing 20 mmol/L Tris, 3 mmol/L K2HPO4, 1 mmol/L MgCl2, 1.4 mmol/L CaCl2, 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, [3H]uridine was added, and uptake was measured over fixed timepoints in the presence or absence of known NT inhibitors or graded concentrations of TKIs. Effects of TKIs on kinetics of uridine uptake were determined in A549 cells at graded concentrations of [3H]uridine (0–1000 μmol/L) at 0, 25, 50, or 100 μmol/L of either erlotinib, gefitinib, or vandetanib using 30-second incubations (established in preliminary experiments to be from linear portions of time courses of [3H]uridine uptake in A549 cells, data not shown). 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/106 cells and graphs generated using the Prism software. Each experiment was conducted two or three times with triplicate measurements.

For [3H]uridine uptake and [3H]gemcitabine accumulation experiments, cells were exposed to either 10 μmol/L [3H]uridine for one minute or 1 μmol/L [3H]gemcitabine in sodium-containing transport buffer for 60 minutes in absence or presence of 25 μmol/L TKI (erlotinib, gefitinib, or vandetanib) or 100 μmol/L dilazep (an inhibitor of hENT1 and hENT2) and processed as described above for uptake assays.

To determine a suitable timepoint for study of recovery of transport activity in cell lines treated with TKIs, a preliminary experiment with A549 cells was conducted in which cells were treated with vandetanib for 0, 2, 4, 6, 12, and 24-hour exposures followed by 24-hour recovery in drug-free medium. Decreased uridine transport activities were observed as early as 2 hours and the effect was maximal at 24 hours (data not shown). We used 24-hour exposures for subsequent experiments with all four cell lines. Cells were treated for 0 or 24 hours with or without 5 μmol/L erlotinib, gefitinib, or vandetanib (A549, AsPC-1 cells) or 2.5 μmol/L erlotinib, gefitinib, or vandetanib (H292, H1975 cells) and allowed to recover for 24 hours in drug-free media after which uridine uptake in untreated and treated cells was measured in drug-free media.

Cell-surface staining and confocal microscopic visualization of hENT1 sites on A549 cells with 5′-[2-(6-aminohexanamido)]ethyl-6-N-(4-nitrobenzyl)-5′-thioadenosine-fluorescein-5-yl isothiocyanate SAHENTA-FITC

Synthesis and use of a fluorescent probe for evaluation of cell surface hENT1 sites was described earlier (20). For evaluation of cell-surface abundance of hENT1, A549 cells grown on cover slip dishes were treated for 0 or 24 hour with or without 5 μM erlotinib, gefitinib or vandetanib and allowed to recover for 0 or 24 h in drug-free media. Cells were (i) washed twice with PBS, (ii) stained with 100 nmol/L 5′-[2-(6-aminohexanamido)]ethyl-6-N-(4-nitrobenzyl)-5′-thioadenosine-fluorescein-3-yl isothiocyanate (SAHENTA-FITC) in sodium buffer for 30 minutes at room temperature, (iii) washed with PBS, and (iv) resuspended in a small volume of PBS for fluorescence visualization. Confocal microscopic analysis was done using a Zeiss 710 LSM with Zeiss Plan-Apochromat 40 ×/1.3 oil DIC M27 lens with an argon laser 488 and a photomultiplier tube detector. Images were collected as 12 bit with 1024 × 1024 dimensions with pixel dwell time of 1.27 μsec and Zen 2011 software was used for analysis of the images.

Cytotoxicity assays

Dojindo Cell Counting Kit-8 (CCK-8) was used to quantify drug induced cytotoxicity. Cells were seeded in 96-well plates and allowed to attach for 24 hours. Cells were then exposed to graded concentrations of gemcitabine, vandetanib, or gefitinib in the absence or presence of 1 μmol/L NBMPR (inhibits hENT1) or 25 μmol/L dilazep (inhibits hENT1/2) for 72 hours after which they were treated with CCK-8 reagent for assessment of cytotoxicity. For evaluation of in vitro synergy of combinations of gemcitabine with any of the TKIs, experiments were based on the individual drug’s IC50 value, with the highest concentration being 8 × IC50 values as described earlier (21). For sequential treatments, cells were treated with either drug for 24 hours, followed by drug-free media for 24 hours and subsequent 72-hour treatment with the other drug; simultaneous treatments were performed for 72 hours after 48 hours in drug-free media. Drug synergy was determined by the isobologram and combination index methods (CI), derived from the median effect principle of Chou and Talalay (22) using the CalcuSyn software (Biosoft). Using data from the growth inhibitory experiments and computerized software, CI values were generated over a range of fraction affected (Fa) levels from 0.05–0.90 (5%–90% growth inhibition). A CI of 1 indicates an additive effect between two agents, whereas a CI < 1 or >1 indicates synergism or antagonism, respectively.
Results

Inhibition of $[3H]$uridine uptake by erlotinib, gefitinib, and vandetanib in Saccharomyces cerevisiae and in cell lines

Erlotinib, gefitinib, and vandetanib (chemical structures are shown in Fig. 1A–C) were assessed for their relative abilities to inhibit transport of $[3H]$uridine by each of the five recombinant hNTs produced in yeast in inhibition experiments to determine $IC_{50}$ values (inhibitor concentrations that produced 50% inhibition of transport). Representative concentration–effect curves for inhibition of hENT1-mediated uridine transport by erlotinib, gefitinib, or vandetanib are shown in Fig. 1D–F and for each, inhibition of $[3H]$uridine transport was observed at micromolar concentrations. $IC_{50}$ values obtained from such experiments with erlotinib, gefitinib, and vandetanib for each of the five recombinant NTs are shown in Table 1, where it is evident that hENT1 was inhibited by all three TKIs, whereas hENT2 and hCNT1/2/3 were inhibited to different extents by the three TKIs. Vandetanib inhibited hENT1, hENT2, hCNT1, hCNT2, and hCNT3, whereas erlotinib inhibited hENT1 and hCNT3 and gefitinib inhibited hENT1 and hCNT1.

To assess TKI inhibition of hNT activity in A549, H292, H1975, and AsPC-1 cells, we first established the type of hNT activities present by conducting $[3H]$uridine uptake experiments in sodium-containing or sodium-free media with or without 100 nmol/L NBMPR (inhibits hENT1), or 100 μmol/L dilazep (inhibits hENT1/2) or 1 mmol/L excess uridine (inhibits hENT1/2, hCNT1/2/3) in each of the cell lines. Uridine uptake activities in A549, H292, and H1975 cells are shown in Fig. 2A–C and the % inhibition was similar with the various NT inhibitors, indicating that uridine uptake was mediated primarily by hENT1. Similar results were seen with AsPC-1 cells (data not shown). Since erlotinib, gefitinib, and vandetanib were identified as hENT1 inhibitors in yeast radiotracer experiments, they were tested in the four cell lines to...
Table 1. Summary of IC\textsubscript{50} values for inhibition of uridine transport in yeast and cell lines

<table>
<thead>
<tr>
<th>Transporter (yeast)</th>
<th>Erlotinib</th>
<th>Gefitinib</th>
<th>Vandetanib</th>
</tr>
</thead>
<tbody>
<tr>
<td>hENT1</td>
<td>34 ± 6</td>
<td>14 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>hENT2</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>hCNT1</td>
<td>160 ± 20</td>
<td>37 ± 11</td>
<td>64 ± 17</td>
</tr>
<tr>
<td>hCNT2</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>hCNT3</td>
<td>11 ± 1</td>
<td>&gt;300</td>
<td>28 ± 9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell lines (hENT1)</th>
<th>Erlotinib</th>
<th>Gefitinib</th>
<th>Vandetanib</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>3.0 ± 0.3</td>
<td>5.0 ± 0.4</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>H292</td>
<td>6.0 ± 3.0</td>
<td>6.0 ± 0.4</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>H1975</td>
<td>6.0 ± 0.2</td>
<td>2.0 ± 0.6</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>ASPC-1</td>
<td>1.6 ± 0.4</td>
<td>2.0 ± 0.4</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

NOTE: Inhibition of [\textsuperscript{3}H]uridine uptake by TKIs was assessed in yeast producing each of the five recombinant hNTs in concentration-effect experiments as described in Materials and Methods. Inhibition experiments were also conducted in cell lines all of which have major intrinsic hENT1 activity and negligible or no CNT or hENT2 activities. IC\textsubscript{50} values (mean ± SE) are listed.

evaluate their effects on hENT1-mediated uptake of [\textsuperscript{3}H]uridine and results from only A549 cells are shown here in detail (Fig. 2D–F). results from the four cell lines are summarized in Table 1.

The nature of the interactions of TKIs with hENT1 was examined in A549 cells by studying effects of fixed concentrations of erlotinib, gefitinib, or vandetanib on kinetics of [\textsuperscript{3}H]uridine uptake. Analysis of results using Line-Weaver Burk plot (Fig. 2G–I) showed the competitive nature of uridine uptake inhibition by TKIs, suggesting that TKIs and uridine bind to the same or overlapping sites on hENT1.

**Erlotinib, gefitinib, and vandetanib inhibit uridine uptake and gemcitabine accumulation in A549, H292, H1975, AsPC-1 cells and reduce cell surface hENT1 staining in A549 cells**

Because of the failure of combination therapies with TKIs and nucleoside analog drugs such as gemcitabine, effects of erlotinib, gefitinib, and vandetanib on uridine uptake and gemcitabine accumulation were examined in all cell lines. Short-term uptake of 1 \(\mu\text{mol}/\text{L}\) [\textsuperscript{3}H]uridine (1 minute) and long-term uptake of 10 \(\mu\text{mol}/\text{L}\) [\textsuperscript{3}H]gemcitabine (1 hour), which assessed intracellular accumulation of gemcitabine and its metabolites, were measured after incubation in the absence or presence of 25 \(\mu\text{mol}/\text{L}\) erlotinib, gefitinib, or vandetanib or 100 \(\mu\text{mol}/\text{L}\) dilazep, a potent inhibitor of both hENT1 and hENT2-mediated activities (23). In [\textsuperscript{3}H]uridine uptake inhibition studies, the antibodies cetuximab and bevacizumab were tested at three different amounts (10, 50, and 100 \(\mu\text{g}/\text{mL}\)). The antibodies did not inhibit [\textsuperscript{3}H]uridine uptake (data not shown). In contrast, the three TKIs inhibited uridine uptake [Fig. 3A] and gemcitabine accumulation [Fig. 3B] in all cells tested.

To further study effects of erlotinib, gefitinib, or vandetanib on hENT1 activity, A549, AsPC-1, H292, and H1975 cells were exposed to 0 or 5 \(\mu\text{mol}/\text{L}\) erlotinib, gefitinib, or vandetanib for 0 or 24 hours with or without recovery in drug-free media for 24 hours and changes in uptake of [\textsuperscript{3}H]uridine were measured to monitor changes in hENT1-mediated activity (Fig. 3C and D). Results indicated a decrease in hENT1-mediated activity after exposure to erlotinib, gefitinib, or vandetanib in all cell lines tested. This decrease in NT activity was reversed after culturing cells in drug-free media for 24 hours. To determine whether reduced NT activity was due to altered protein levels, we used SAHENTA-FITC (a nonpermeable fluorescent hENT1 probe) to measure the abundance of hENT1 sites on cell surfaces as described earlier (20). A549 cells were treated with erlotinib, gefitinib, or vandetanib for 24 hours and, after recovery in drug-free media for another 24 hours, were stained with 100 \(\text{nmol}/\text{mL}\) SAHENTA-FITC and processed as described in the Materials and Methods section. Fig. 3E (panels 1, 2) shows unstained and cell-surface stained hENT1 in untreated cells and decreased staining after treatment with TKIs (panels 3, 5, 7) that recovered after culturing cells in drug-free media (panels 4, 6, 8).

**Cytotoxicity of gemcitabine, erlotinib, gefitinib, or vandetanib to A549, H292, H1975, and AsPC-1 cells**

Cytotoxicity of gemcitabine, erlotinib, gefitinib, or vandetanib was assessed in all four cell lines. Erlotinib was not toxic to A549 cells and therefore was not used in subsequent experiments. Since gemcitabine, gefitinib, and vandetanib were toxic to A549 cells, we assessed whether mediated transport by hENTs as observed previously with gemcitabine (24) contributed to toxicity with TKIs. Cytotoxicity studies were conducted with gemcitabine, gefitinib, or vandetanib in absence and presence of dilazep, an established inhibitor of hENT1/2. A549 cells were exposed for 72 hours to graded gemcitabine concentrations (0–100 \(\mu\text{mol}/\text{L}\)) in the absence or presence of 25 \(\mu\text{mol}/\text{L}\) dilazep. Protection against gemcitabine toxicity was observed (Fig. 4A) in the presence of dilazep confirming previous
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Figure 2. Effects of erlotinib, gefitinib, and vandetanib on \[^{3}H\]uridine uptake in cultured cells. A–C, uptake of 10 \(\mu\)mol/L \[^{3}H\]uridine in A549, H292, and H1975 cells in sodium-containing or sodium-free media without or with inhibitors of hENT activity. Effects of increasing concentrations of erlotinib, gefitinib, or vandetanib on uptake of 10 \(\mu\)mol/L \[^{3}H\]uridine in A549 cells are shown in (D–F). Competition effects of 0 (■), 5 (▲), 25 (▼), or 100 (○) \(\mu\)mol/L of erlotinib, gefitinib, or vandetanib on uridine uptake rates are shown in (G–I). Values with mean ± SE are shown in each panel. Each experiment was repeated three times.

In vitro combination studies with gemcitabine and erlotinib, gefitinib, or vandetanib

Results described in Fig. 3B showed reduced accumulation of gemcitabine in presence of erlotinib, gefitinib, or vandetanib in all cell lines tested. This situation, when translated clinically, would mean reduced effectiveness of gemcitabine of simultaneous administration regimens or when TKIs are administered before gemcitabine. In contrast, we should see equivalent or higher effectiveness when gemcitabine is administered before TKIs. We explored cytotoxicity of simultaneous and sequential administration of gemcitabine with various TKIs in the four cell lines as follows. Individual toxicities of gemcitabine, erlotinib, gefitinib, or vandetanib to A549, H292, H1975, and AsPC-1 cells are summarized in Table 2. For in vitro combination studies cells were either (i) pretreated with TKIs before gemcitabine, (ii) pretreated with gemcitabine before TKIs,
Figure 3. TKI effects on \(^{3}\)H|uridine uptake and \(^{3}\)H|gemcitabine accumulation in cultured cells. Incubations with 10 \(\mu\)mol/L \(^{3}\)H|uridine for 1 minute (A) or 1 \(\mu\)mol/L \(^{3}\)H|gemcitabine for 60 minutes (B) were conducted in the absence or presence of 25 \(\mu\)mol/L of erlotinib, gefitinib, or vandetanib or 100 \(\mu\)mol/L dilazep and cell-associated radioactivity was measured. Values plotted are % control values obtained in the absence of additives and average values from two or more experiments are shown in each panel. (Legend continued on following page.)
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Figure 4. Effects of dilazep on gemcitabine and TKI cytotoxicity. A, A549 cells were exposed to graded concentrations (0–100 μmol/L) of gemcitabine in the absence (○) or presence of 25 μmol/L dilazep (●) for 72 hours. Cell viability was measured by CCK-8 assay kit as described in Materials and Methods. B, A549 cells were treated with gefitinib or vandetanib in the absence (○) or presence of 25 μmol/L dilazep (●, △) for 72 hours. Plotted are % controls values relative to untreated cells and representative experiments with mean ± SD are shown in each panel. Each experiment was repeated three times. C–F, show isobologram analysis of the sequential combination of gemcitabine followed by TKIs in A549, H292, H1975, and AsPC-1 cells. Individual doses of gemcitabine and TKIs to achieve 90% (straight line) growth inhibition (Fa = 0.90), 75% (hyphenated line) growth inhibition (Fa = 0.75), and 50% (on the axes, open circle) growth inhibition (Fa = 0.50) were plotted on x- and y-axes. CI values calculated using CalcuSyn software are represented by points above (indicate antagonism between drugs) or below the lines (indicate synergy). Symbols indicate: (○) for ED50, (●) for ED75, and (△) for ED90, respectively.

or (iii) treated with both agents together as described in Materials and Methods. Isobologram and CI methods developed by Chou and Talalay (22) were used to confirm and quantify the synergism observed with the various combinations of gemcitabine and TKIs. 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. 4C–F. CI values at Fa values of 0.5 for all cell lines are summarized in Table 2. These results indicated that the sequence, gemcitabine followed by TKIs, was synergistic, whereas the sequence TKI followed by gemcitabine or simultaneous treatment ranged from additive to antagonistic thus supporting our hypothesis that inhibition of hNTs by TKIs lead to decreased effectiveness of combinations involving TKIs and nucleoside chemotherapy drugs.

Discussion

TKIs were designed to bind to the ATP regulatory pocket of growth factor receptors and compete with ATP at the binding site. Since adenosine is the nucleoside moiety in ATP, we expected that TKIs may also bind to hNTs that recognize and transport nucleosides and therefore interfere with nucleoside chemotherapy. Interaction of TKIs with ATP-binding domains of ATP-binding cassette (ABC) transporter-mediated multidrug resistance (MDR) proteins in cancer cells (25–27) and inhibition of P-gp activity (28) was shown earlier. Many TKIs modulate both P-gp and ABCG2 activities and

(Continued) C and D, effects of 24-hour treatment with TKIs on hENT1 activity in A549, AsPC-1 (C), and in H292, H1975 (D) before and after 24-hour recovery in TKI-free media; cells were pretreated with 5 μmol/L erlotinib, gefitinib, or vandetanib for 24 hours and uptake of 10 μmol/L [3H]uridine (2 minutes) was measured in untreated (control) and treated cells immediately or 24 hours after incubation in drug-free media (24-hour recovery). % Control values with mean ± SE are shown. Each experiment was repeated three times. E, confocal microscopic analysis of staining of hENT1 sites in A549 cells with SAHENTA-FITC in untreated-unstained (#1), untreated-stained with SAHENTA-FITC (#2), or treated-stained with SAHENTA-FITC for 24 hours with erlotinib, gefitinib, or vandetanib (#3, 5, 7) followed by 24-hour recovery after TKI treatment (#4, 6, 8) as described in Materials and Methods. Bright staining on membranes indicates binding of SAHENTA-FITC to hENT1 sites. Scale bar shown is 20 μm.
enhance cytotoxic effects of multiple anticancer drugs by increasing accumulation of P-gp and ABCG2 substrates (29, 30). Another group of potential target proteins are NTs. Inhibition of hENT1-mediated activity in K562 cells by p38 mitogen-activated protein kinase inhibitors (8) and of murine equilibrative NT 1 (mENT1; ref. 9) by imatinib were shown earlier. A more recent study showed inhibition of cytobate uptake by imatinib and nilotinib (31).

In this study, we showed that hENT or hCNT mediated \(^{[3]H}\)uridine uptake was inhibited to varying extents by erlotinib, gefitinib, and vandetanib at concentrations that are readily achievable in patient plasma, tissue, and tumors. Uridine uptake in A549 cells was inhibited in a competitive manner that suggests binding of TKIs at the nucleoside (permeant) binding site of hENT1. Although erlotinib, gefitinib, and vandetanib interacted with the nucleoside-binding site of hENT1, they do not appear to be transported on the basis of the results of indirect cytotoxicity experiments wherein dilazep protected cells against gemcitabine toxicity but not against TKI toxicity. Direct evidence for TKIs lack of transportability by hNTs requires use of \(^{[3]H}\)TKIs. Erlotinib, gefitinib, and vandetanib inhibited \(^{[3]H}\)uridine uptake and accumulation of \(^{[3]H}\)gemcitabine thus suggesting interference with nucleoside analog chemotherapy when combined with these TKIs. In contrast, EGFR and VEGFR antibodies cetuximab and bevacizumab had no effect on \(^{[3]H}\)uridine uptake in A549 cells (data not shown) thus suggesting that antibodies can be combined with nucleoside drugs without any adverse effects on pharmacokinetics of nucleoside drugs.

Results from cell surface staining of hENT1 levels by SAHENTA-FITC showed reduction in cell surface staining intensity thus suggesting that these TKIs also affected cell surface hENT1 expression in addition to direct inhibition of transport activity thereby causing reduction in uptake of nucleosides in the presence of TKIs. This effect was reversed by culturing cells in absence of erlotinib, gefitinib, or vandetanib for 24 hours.

Pharmacologic evidence from several clinical trials with TKIs and nucleoside chemotherapy (2–6) suggests that there may be unfavorable interactions between TKIs and nucleoside chemotherapy drugs. Changes in pharmacokinetics of nucleoside metabolites of capecitabine in presence of erlotinib were shown in a phase Ib dose escalation study of erlotinib and capcitabine in patients with colorectal cancer. Van Custem and colleagues (32) reported that the mean peak concentration (\(C_{\text{max}}\)) for capecitabine was 27% lower and the mean area under the concentration curve (AUC) was 12% lower in the presence than in the absence of erlotinib. They also noted reduced levels of 5'-deoxy-5-fluourouridine when erlotinib and capcitabine were administered together. In a study of erlotinib with capcitabine in patients with breast cancer (33), the \(C_{\text{max}}\) (ng/mL) of capcitabine when administered with docetaxel was 6,274 ng/mL but when combined with erlotinib was only 3,934 ng/mL, thus

| Table 2. Cytotoxicity of gemcitabine and TKIs to pancreatic and lung cancer cell lines |
|-------------------------------|-----|-----|-----|
| **Drug combination**          | A549 | H292 (IC\(_{50}\) values ± SE) | H1975 |
| Gemcitabine \((\text{nmol/L})\) | 2.2 ± 0.2 | 3.0 ± 0.5 | 1.0 ± 0.3 |
| Erlotinib \((\mu\text{g/mL})\)  | >100 | 60.0 ± 8.0 | 7.0 ± 0.8 |
| Gefitinib \((\mu\text{g/mL})\)  | 3.5 ± 0.9 | 3.4 ± 1.4 | 6.0 ± 0.5 |
| Vandetanib \((\mu\text{g/mL})\) | 3.0 ± 0.2 | 1.0 ± 0.4 | 4.0 ± 0.1 |

<table>
<thead>
<tr>
<th><strong>Drug combination</strong></th>
<th>A549</th>
<th>H292</th>
<th>H1975</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine first</td>
<td>0.9</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Vandetanib first</td>
<td>3</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Simultaneously</td>
<td>0.3</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Gemcitabine first</td>
<td>0.4</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Gefitinib first</td>
<td>3.0</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Simultaneously</td>
<td>3.0</td>
<td>1.2</td>
<td>1.4</td>
</tr>
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</table>

**NOTE:** All four cell lines were treated with gemcitabine alone or in combination with TKIs as described in the Materials and Methods section. IC\(_{50}\) values (mean ± SE) for gemcitabine and TKIs were derived from 72-hour exposure experiments and are listed above. Cytotoxicity of combinations of gemcitabine with TKIs either sequentially or simultaneously was determined in each cell line and CI values at Fa values of 50% are listed below. Shown are CI values at FA\(_{50}\) (50% cytotoxic effect) where CI values significantly less than 1 indicate synergy; values close to 1, an additive effect; and values significantly greater than 1, an antagonistic effect of the 2 agents.
suggesting an effect of erlotinib on pharmacokinetics of capcitabine in plasma. Such interaction may also result in lower clearance of nucleoside drugs—for example, Goss and colleagues showed that gemcitabine clearance was significantly reduced in the presence of cediranib (P > 0.02; ref. 34). The above studies clearly show adverse pharmacokinetic interactions between nucleoside drugs and TKIs that may reduce treatment efficacy when these agents are administered together.

In combination toxicity studies, cells showed greater sensitivity to drug combinations when cells were exposed to gemcitabine for 24 hours followed by erlotinib, gefitinib, or vandetanib as predicted from our uptake inhibition studies. There were mostly additive to antagonistic interactions if the TKI was added first followed by gemcitabine or simultaneously with the exception of vandetanib in A549 and AsPC-1 in simultaneous addition. Synergism observed in the sequential schedule of nucleoside drug followed by TKIs is supported by results from a recent phase III trial (FASTACT-2; ref. 35), wherein untreated stage IIIIB/IV NSCLC patients were randomized and treated with gemcitabine plus platinum followed by intercalated erlotinib or placebo every 4 weeks. Treatment with intercalated combination of erlotinib and chemotherapymproved PFS versus chemotherapy alone as first-line treatment in patients with advanced NSCLC with known and unknown EGFR mutation status.

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, concentrations of erlotinib, gefitinib, or vandetanib achievable in plasma are levels that would inhibit hNT activity and tumor levels are at least 40-fold higher than plasma levels. In a recent study of neoadjuvant breast cancer patients, mean gefitinib plasma concentrations at steady state were 0.18 µg/mL (0.403 µmol/L) and mean tumor levels were 7.5 µg/g (17 µmol/L), approximately 42-fold higher (36). In two related studies, median plasma gefitinib levels were 1,064 ng/mL on day 8 (37) and tumor levels were shown to be approximately 40-fold higher than plasma levels (22.7 vs. 0.52 µmol/L; ref. 38). In a phase I study at the 300 mg/day dose of vandetanib, steady-state mean vandetanib concentrations were approximately 1,000 ng/mL (2.1 µmol/L; ref. 13). Vandetanib administered at doses up to 300 mg/day resulted in mean steady-state plasma levels of 2.2 µmol/L (range of 1.6–6.3 µmol/L; ref. 39) and was shown to accumulate in tumor tissues to much higher levels (16). At these concentrations, we can expect adverse drug interactions between TKIs and nucleoside chemotherapy drugs that are consistent with inhibition of NTs by TKIs.

In summary, we have shown that two EGFR TKIs and one VEGFR/EGFR TKI inhibit hENT1. Inhibition of hENT2, hCNT1/2/3 are varied among the three TKIs, but all three TKIs inhibit at least one hCNT. Furthermore, these TKIs caused a decrease in cell surface abundance of hENT1, a ubiquitous hNT necessary for activity of many nucleoside chemotherapy drugs. Clinical implications of this study are that lower concentrations of nucleosides and their metabolites in plasma and in tumor tissues would be expected when TKIs and nucleoside drugs are administered together. This drug interaction between nucleoside drugs and TKIs will lead to reduced treatment efficacy when these agents are administered together. To obtain the best clinical responses, nucleoside drugs should not be administered concurrently with EGFR and VEGFR TKIs but rather in sequence, with the nucleoside drug (e.g., gemcitabine) followed by TKIs. We suspect that many ATP-competitive TKIs have the potential to inhibit hNTs and that different classes of TKIs inhibit different hNTs with different potencies.

Disclosure of Potential Conflicts of Interest
V.L. Damaraju has ownership interest (including patents) in patent. C.E. Cass is employed (other than primary affiliation, e.g., consulting) as a sole proprietor in CE Cass Consulting and has commercial research grant from Clavis Pharma ASA. M.B. Sawyer has ownership interest (including patents) in a patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: V.L. Damaraju, C.E. Cass, M.B. Sawyer Development of methodology: V.L. Damaraju Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Scriver, D. Mowles, M. Kuzma Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.L. Damaraju, T. Scriver, M.B. Sawyer Writing, review, and/or revision of the manuscript: V.L. Damaraju, A. Ryan, C.E. Cass, M.B. Sawyer Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Scriver, D. Mowles, M. Kuzma Study supervision: V.L. Damaraju, M.B. Sawyer

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Erlotinib, Gefitinib, and Vandetanib Inhibit Human Nucleoside Transporters and Protect Cancer Cells from Gemcitabine Cytotoxicity

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