Erlotinib, gefitinib and vandetanib inhibit human nucleoside transporters and protect cancer cells from gemcitabine cytotoxicity

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Potential Conflict of Interest:
Drs. Damaraju and Sawyer are listed on a patent “Combination treatments for cancer” held by Alberta Health Services

List of abbreviations:
NTs, nucleoside transporters; h, human; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; NBMPR, nitrobenzylmercaptopurine ribonucleoside (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine); EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor; SAHENTA-FITC , 5'-S-[2-(6-aminohexanamido)]ethyl-6-N-(4-nitrobenzyl)-5'-thioadenosine-fluorescein-5-yl isothiocyanate; IC50, concentration of test compound that inhibited growth of treated cells by 50% relative to untreated cells; TKIs, tyrosine kinase inhibitors.

Contributions
T.S., D.M. and M.K. conducted transport assays, cytotoxicity studies, yeast inhibition experiments and confocal microscopy studies. V.L.D designed and analyzed experiments and wrote the manuscript. M.B.S. led the research team and M.B.S. and C.E.C. critically reviewed the manuscript.

Translational relevance:
Tyrosine kinase inhibitors (TKIs) 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 transporters.
transporter 1 (hENT1) nitrobenzylmercapturine ribonucleoside (NBMPR), and speculated that failures of combination of TKIs with nucleoside chemotherapy may be due to TKIs inhibiting human nucleoside transporters (hNTs) 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 GI cancer combination chemotherapy for which the backbone is either capecitabine or gemcitabine, known nucleoside permeants of hNTs.
Abstract:

Purpose: Combinations of tyrosine kinase inhibitors (TKIs) 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 (hENTs, hCNTs) 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 h reduced hENT1 activity which was reversed by subsequent incubation in drug free media for 24 h. Greater cytotoxicity was observed when gemcitabine was administered prior to 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 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 NSCLC patients with epidermal growth factor receptor (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) (2). In a phase II trial in advanced urothelial carcinoma patients treated with gemcitabine/cisplatin and concurrent gefitinib, response rates were not significantly different from gemcitabine/cisplatin alone (3). Gemcitabine and gefitinib were evaluated in advanced pancreatic cancer patients (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 urothelial carcinoma patients did not result in increased response rates compared to the control arm of gemcitabine/cisplatin (5). Vandetanib was initially evaluated in advanced NSCLC patients and the combination of vandetanib with gemcitabine/cisplatin was not tolerated in these patients (6).
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 (hNTs) 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 epidermal growth factor 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).
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Materials and Methods

Materials:
Nitrobenzylmercaptopurine ribonucleoside (NBMPR), dilazep, unlabeled nucleosides and other chemicals were obtained from Sigma Chemical Company (Mississauga, ON). Tritiated nucleosides were purchased from Moravek Biochemicals (Brea, CA). Tissue culture (96- and 12-well) plates and flasks were from VWR International (Mississauga, ON). Cell culture media and fetal bovine serum (FBS) were from Gibco BRL (Burlington, ON). Ecolite was from ICN Pharmaceuticals (Montreal, PQ). Dojindo Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies, Inc (Rockville, MD). Erlotinib, gefitinib and vandetanib were from LC Laboratories (Woburn, MA).

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 (Manassas, VA). Cell lines were sent to DDC Medical (Fairfield, OH) 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 Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 10% glucose. All cultures were kept at 37°C in 5% CO2/95% air and sub-cultured at two to three 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 μM respectively (13-15). Earlier studies have shown that gefitinib concentrations in tumor tissues
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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 μM in cell lines and 300 μM in yeast model systems.

**Uridine transport in Saccharomyces cerevisiae:**

*Saccharomyces cerevisiae* yeast were separately transformed with plasmids (pYPbENT1, pYPbENT2, pYPbCNT1, pYPbCNT2, or pYPbCNT3) encoding hNTs (hENT1, hENT2, hCNT1, hCNT2, or hCNT3, respectively) as described elsewhere (17, 18). Uptake of 1 µM [3H]uridine (Moravek Biochemicals, Brea, CA) into yeast was measured as previously described (18, 19) using the semi-automated cell harvester (Micro96 HARVESTER; Skatron Intruments, Lier, Norway). Yeast were incubated at room temperature (RT) with graded concentrations (0-0.3 mM) of test compounds in the presence of 1 µM [3H]uridine in transport buffer (pH 7.4) containing 20 mM Tris, 3 mM K2HPO4, 1 mM MgCl2, 1.4mM CaCl2, and 5 mM glucose with 144 mM NaCl hereafter termed transport buffer. Yeast were incubated with 1 µM [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., San Diego, CA) to obtain the concentration of test compound that inhibited uridine uptake by 50% relative to that of untreated cells (IC50).
values). Each IC<sub>50</sub> 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 (10<sup>5</sup>/well) were seeded in 12-well plates and on the third day, uptake of [³H]nucleosides was measured at RT in 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 time points 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 (0–100 µM) at 0, 25, 50 or 100 µM of either erlotinib, gefitinib or vandetanib using 30-s 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/10<sup>6</sup> 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 µM [³H]uridine for one min or 1 µM [³H]gemcitabine in sodium containing transport buffer for 60 min in absence or presence of 25 µM TKI (erlotinib, gefitinib or vandetanib) or 100 µM dilazep (an inhibitor of hENT1 and hENT2) and processed as described above for uptake assays.

To determine a suitable time point 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
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treated with vandetanib for 0, 2, 4, 6, 12 and 24 h exposures followed by 24 h recovery in drug-
free medium. Decreased uridine transport activities were observed as early as 2 h and the effect
was maximal at 24 h (data not shown). We used 24 h exposures for subsequent experiments with
all four cell lines. Cells were treated for 0 or 24 h with or without 5 µM erlotinib, gefitinib or
vandetanib (A549, AsPC-1 cells) or 2.5 µM erlotinib, gefitinib or vandetanib (H292, H1975
cells) and allowed to recover for 24 h 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'-S-[2-(6-aminohexanamido)]ethyl-6-N-(4-nitrobenzyl)-5'-thioadenosine-fluorescein-
5-y1 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 h 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 phosphate
buffered saline (PBS), (ii) stained with 100 nM SAHENTA-FITC in sodium buffer for 30 min at
RT, (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 X/1.3 oil DIC M27 lens with an argon laser 488 and a photomultiplier tube
detector. Images were collected as 12 bit with 1024 x 1024 dimensions with pixel dwell time of
1.27 µ sec 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 h. Cells were then exposed to graded
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concentrations of gemcitabine, vandetanib or gefitinib in the absence or presence of 1 µM NBMPR (inhibits hENT1) or 25 µM dilazep (inhibits hENT1/2) for 72 h 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 x IC50 values as described earlier (21). For sequential treatments, cells were treated with either drug for 24 h, followed by drug free media for 24 h and subsequent 72 h treatment with the other drug; simultaneous treatments were performed for 72 h after 48 h 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, Ferguson, MO). 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 $[^3]$H|uridine uptake by erlotinib, gefitinib and vandetanib in *Saccharomyces cerevisiae* and in cell lines

Erlotinib, gefitinib and vandetanib (chemical structures are shown in Figure 1 A-C) were assessed for their relative abilities to inhibit transport of $[^3]$H|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 Figure 1 D-F and for each, inhibition of $[^3]$H|uridine transport was observed at µM 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 $[^3]$H|uridine uptake experiments in sodium containing or sodium free media with or without 100 nM NBMPR (inhibits hENT1), or 100 µM dilazep (inhibits hENT1/2) or 1 mM 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 Figure 2 A-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
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yeast radiotracer experiments, they were tested in the four cell lines to evaluate their effects on hENT1-mediated uptake of $[^3\text{H}]$uridine and results from only A549 cells are shown here in detail (Figure 2 D-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 $[^3\text{H}]$uridine uptake. Analysis of results using Line-Weaver Burk plot (Figure 2 G-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 µM $[^3\text{H}]$uridine (1 min) and long-term uptake of 10 µM $[^3\text{H}]$gemcitabine (1 h), which assessed intracellular accumulation of gemcitabine and its metabolites, were measured after incubation in the absence or presence of 25 µM erlotinib, gefitinib, or vandetanib or 100 µM dilazep, a potent inhibitor of both hENT1 and hENT2-mediated activities (23). In $[^3\text{H}]$uridine uptake inhibition studies, the antibodies cetuximab and bevacizumab were tested at three different amounts (10, 50 and 100 µg/ml). The antibodies did not inhibit $[^3\text{H}]$uridine uptake (data not shown). In contrast the three TKIs inhibited uridine uptake (Figure 3 A) and gemcitabine accumulation (Figure 3 B) 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 µM erlotinib, gefitinib or vandetanib for
0 or 24 h with or without recovery in drug free media for 24 h and changes in uptake of 
[^3H]uridine were measured to monitor changes in hENT-mediated activity (Figure 3 C-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 h. To determine if reduced NT activity was due to altered protein 
levels, we used SAHENTA-FITC (a non-permeable 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 h and, after recovery in drug free media for another 24 h, 
were stained with 100 nM SAHENTA-FITC and processed as described in the Materials and 
Methods section. Figure 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 if 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 h 
to graded gemcitabine concentrations (0-100 µM) in the absence or presence of 25 µM dilazep. 
Protection against gemcitabine toxicity was observed (Figure 4 A) in the presence of dilazep 
confirming previous observations in other human cancer cell lines (24) that NT mediated
EGFR and VEGFR TKIs inhibit human nucleoside transporters permeation is important for gemcitabine toxicity. Similar experiments conducted with gefitinib and vandetanib (Figure 4B) in A549 cells showed no effects of dilazep on gefitinib or vandetanib cytotoxicity. Erlotinib, gefitinib and vandetanib toxicities (0-100 µM) were assessed over 72 h in all four cell lines and IC\textsubscript{50} values (mean ± S.E) are summarized in Table 2.

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

Results described in Figure 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 prior to 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 prior to gemcitabine, (ii) pretreated with gemcitabine prior to TKIs, or (iii) treated with both agents together as described in Methods. Isobologram and combination-index 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 Figure 4 C-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
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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 enhance cytotoxic effects of multiple anti-cancer 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) (9) by imatinib were shown earlier. A more recent study showed inhibition of cytarabine 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 based on 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
EGFR and VEGFR TKIs inhibit human nucleoside transporters 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 h.

Pharmacological 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 1b dose escalation study of erlotinib and capecitabine in colorectal patients. Van Custem et al. (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-fluorouridine when erlotinib and capecitabine were administered together. In a study of erlotinib with capecitabine in breast cancer patients (33), the $C_{\text{max}}$ (ng/ml) of capecitabine when administered with docetaxel was 6274 ng/ml but when combined with erlotinib was only 3934 ng/ml, thus suggesting an effect of erlotinib on pharmacokinetics of capecitabine in plasma. Such interaction may also result in lower clearance of nucleoside drugs – e.g., Goss et al. showed that gemcitabine clearance was significantly reduced in the presence of cediranib ($p > 0.02$) (34). The above studies clearly show adverse pharmacokinetic interactions.
EGFR and VEGFR TKIs inhibit human nucleoside transporters 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) (35), wherein untreated stage IIIB/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 chemotherapy improved PFS vs. 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 µM) and mean tumor levels were 7.5 µg/g (17 µM), approximately 42 fold higher (36). In two related studies median plasma gefitinib levels were 1064 ng/ml on day 8 (37) and tumor levels were shown to be approximately 40 fold higher than plasma levels (22.7 versus 0.52 µM) (38). In a phase I study at the 300 mg/day dose of vandetanib, steady state mean vandetanib concentrations were approximately 1000 ng/ml (2.1 µM) (13). Vandetanib administered at doses
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up to 300 mg/day resulted in mean steady-state plasma levels of 2.2 µM (range of 1.6-6.3 µM) (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.
Acknowledgements:

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References:


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Table 1: Summary of IC$_{50}$ values for inhibition of uridine transport in yeast and cell lines.

Inhibition of [³H]uridine uptake by TKIs was assessed in yeast producing each of the five recombinant hNTs in concentration-effect experiments as described in 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$_{50}$ values (mean ± S.E) are listed below.

<table>
<thead>
<tr>
<th>Transporter (Yeast)</th>
<th>Erlotinib</th>
<th>Gefitinib</th>
<th>Vandetanib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (µM ± S.E)</td>
<td></td>
<td></td>
</tr>
<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>

Cell lines (hENT1)

<table>
<thead>
<tr>
<th></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>
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Table 2: Cytotoxicity of gemcitabine and TKIs to pancreatic and lung cancer cell lines.

All four cell lines were treated with gemcitabine alone or in combination with TKIs as described in the Methods section. IC\textsubscript{50} values (mean ± S.E) for gemcitabine and TKIs were derived from 72-h exposure experiments and are listed below. 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.

<table>
<thead>
<tr>
<th>Drug</th>
<th>A549</th>
<th>H292 (IC\textsubscript{50} values ± S.E)</th>
<th>H1975</th>
<th>AsPC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine (nM)</td>
<td>2.2 ± 0.2</td>
<td>3.0 ± 0.5</td>
<td>1.0 ± 0.3</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>Erlotinib (µM)</td>
<td>&gt;100</td>
<td>60.0 ± 8.0</td>
<td>7.0 ± 0.8</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>Gefitinib (µM)</td>
<td>3.5 ± 0.9</td>
<td>3.4 ± 1.4</td>
<td>6.0 ± 0.5</td>
<td>11.0 ± 3.0</td>
</tr>
<tr>
<td>Vandetanib (µM)</td>
<td>3.0 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>4.0 ± 0.1</td>
<td>4.0 ± 0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>A549</th>
<th>H292 CI values at Fa\textsubscript{50}</th>
<th>H1975</th>
<th>AsPC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine first</td>
<td>0.9</td>
<td>0.6</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Vandetanib first</td>
<td>3</td>
<td>1.0</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Simultaneously</td>
<td>0.3</td>
<td>1.1</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Gemcitabine first</td>
<td></td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gefitinib first</td>
<td></td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simultaneously</td>
<td></td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gemcitabine first</td>
<td></td>
<td>0.4</td>
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<td></td>
</tr>
<tr>
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<td></td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simultaneously</td>
<td></td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shown are CI values at Fa\textsubscript{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.
Figure legends:

**Figure 1:** Structures of erlotinib, gefitinib and vandetanib and their effects on $[^3]$H]uridine uptake by recombinant hENT1 in yeast cells. Structures of erlotinib, gefitinib and vandetanib are shown in panels A-C. Yeast cells were incubated with 1 µM $[^3]$H]uridine for 10 min in the absence or presence of increasing concentrations (0-300 µM) of erlotinib, gefitinib or vandetanib (panels D-F). Shown are representative experiments performed with six replicates per concentration and data are expressed as mean ± S.E. values. Uptake values represent % uridine uptake in the presence of TKIs relative to that in its absence (control). Error bars are not shown where S.E values are smaller than the size of the symbol. Each experiment was repeated three times.

**Figure 2:** Effects of erlotinib, gefitinib and vandetanib on $[^3]$H]uridine uptake in cultured cells. Panels A to C show uptake of 10 µM $[^3]$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 µM $[^3]$H]uridine in A549 cells are shown in panels D-F. Competition effects of 0 (■), 5 (▲), 25 (▼) or 100 (♦) µM of erlotinib, gefitinib or vandetanib on uridine uptake rates are shown in panels G-I. Values with mean ± S.E. are shown in each panel. Each experiment was repeated three times.

**Figure 3:** TKI effects on $[^3]$H]uridine uptake and $[^3]$H]gemcitabine accumulation in cultured cells. Incubations with 10 µM $[^3]$H]uridine for 1 min (panel A) or 1 µM $[^3]$H]gemcitabine for 60 min (panel B) were conducted in the absence or presence of 25 µM of erlotinib, gefitinib, or vandetanib or 100 µM dilazep and cell-associated radio activity 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. Panels C and D show effects of 24 h treatment with TKIs on hENT1 activity in A549, AsPC-1 (panel C) and in H292, H1975 (panel D) before and after 24
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h recovery in TKI-free media; cells were pretreated with 5 µM erlotinib, gefitinib or vandetanib for 24 h and uptake of 10 µM [³H]uridine (2 min) was measured in untreated (control) and treated cells immediately or 24 h after incubation in drug-free media (24 h recovery). % Control values with mean ± S.E. are shown. Each experiment was repeated three times. Panel E shows 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 h with erlotinib, gefitinib, or vandetanib (# 3, 5, 7) followed by 24 h recovery after TKI treatment (# 4, 6, 8) as described in Methods. Bright staining on membranes indicates binding of SAHENTA-FITC to hENT1 sites. Scale bar shown is 20 µm.

**Figure 4**: Effects of dilazep on gemcitabine and TKI cytotoxicity. In panel A, A549 cells were exposed to graded concentrations (0-100 µM) of gemcitabine in the absence (○) or presence of 25 µM dilazep (●) for 72 h. Cell viability was measured by CCK-8 assay kit as described in Methods. In panel B, A549 cells were treated with gefitinib or vandetanib in the absence (○) or presence of 25 µM dilazep (●) for 72 h. Plotted are % controls values relative to untreated cells and representative experiments with mean ± S.D. are shown in each panel. Each experiment was repeated three times.

Panels 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. Combination index (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.
Figure 3
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Erlotinib, gefitinib and vandetanib inhibit human nucleoside transporters and protect cancer cells from gemcitabine cytotoxicity

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