Inhibition of nucleoside transporters by tyrosine kinase inhibitors and its effects on chemotherapy efficacy

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Nucleoside transporters (NTs) are essential for transport of physiologic nucleosides and anticancer nucleoside analogues. There are 7 NTs 5 of which play roles in cellular membrane transport namely human equilibrative nucleoside transporter 1 (hENT1), hENT2, human concentrative nucleoside transporter 1 (hCNT1), hCNT2, and hCNT3. Several studies have demonstrated roles for hENT1 in gemcitabine activity in pancreatic cancer where tumors that have low hENT1 levels have a poor response to gemcitabine compared to tumors with high hENT1 levels. Many clinical trial studies with tyrosine kinase inhibitors (TKIs) and a nucleoside analogue backbone have failed or been disappointing. Our group has discovered a possible explanation for the disappointing results of combination regimens consisting of a TKI and a nucleoside analogue. We have found that TKIs have an unappreciated pharmacologic effect in that TKIs are potent NT inhibitors. NT inhibitory properties may mean that it will be difficult to combine some TKIs with anticancer nucleoside drugs. Careful attention to scheduling TKIs and nucleosides may allow successful combinations of some TKIs and nucleoside anticancer drugs but for some other TKIs due to their long half-lives it may be impossible to successfully schedule them with nucleosides.

Keywords: nucleoside transporters; gefitinib; erlotinib; vandetanib; gemcitabine; pancreatic cancer; non-small cell lung cancer

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Introduction

Nucleoside transporters (NTs) are essential membrane proteins responsible for mediating transport of naturally occurring nucleosides across cellular membranes [1, 2]. They also play important roles in uptake of nucleoside analog chemotherapeutic agents such as gemcitabine, cytarabine, and fludarabine [2-4]. This review will cover relationships between NTs and chemotherapy potency, mechanisms by which tyrosine kinase inhibitors (TKIs) inhibit NTs, and the clinical relevance of TKI co-administration with chemotherapeutic regimens that include nucleoside drugs whose cellular uptake is dependent on functional NTs.

Nucleoside transporters

There are two families of NTs in humans, equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) [1]. ENTs transport nucleosides independent of sodium gradients whereas CNTs require a sodium gradient for passage of nucleosides [5, 6].

There are four human ENTs (hENT1-4) that have been identified by molecular cloning and functional expression of genes [4,5,7-9]. hENT1-4 are classified based on their functional characteristics. hENT1/2 exhibit markedly different sensitivities to nitrobenzylmercapturine ribonucleoside (NBMPR), a highly potent and specific nucleoside transport...
inhibitor [9, 10]. hENT1 is sensitive to NBMPR at nanomolar concentrations and is widely expressed by mammalian cells whereas hENT2 is insensitive to NBMPR at nanomolar concentrations [8-10]. Dilazep and dipyridamole, both vasodilator drugs are potent inhibitors of hENT1 and hENT2 [11, 12]. These drugs exert more effects on hENT1 than hENT2 [11, 12]. Both hENT1 and hENT2 are selective for purine and pyrimidine nucleosides and are responsible for transport of nucleoside analogs [13, 14]. hENT2 also transports nucleobases [13].

Both hENT3 and hENT4 are insensitive to NBMPR and are weakly inhibited by dilazep and dipyridamole [5, 7, 15]. hENT3 is found primarily in intracellular membranes and transports both purine and pyrimidine nucleosides, whereas hENT4, which also transports serotonin, transports adenosine in brain and cardiac tissues [7, 15].

There are three human CNTs (hCNT1-3) all of which are sodium dependent and transport nucleosides against their concentration gradients [1]. hCNT1 is selective for pyrimidine nucleosides and also transports adenosine [16, 17]. hCNT2 is selective for purine nucleosides and also transports uridine, and hCNT3 transports both pyrimidine and purine nucleosides [18-20]. Thiopyrimidine 2'-deoxynucleoside and its ribonucleoside are two high affinity inhibitors of hCNT1 that have been identified [21].

**Nucleoside analog chemotherapies and hENT1**

Gemcitabine, cytarabine, and fludarabine are examples of pyrimidine and purine nucleoside analogs used in cancer chemotherapy [2, 4, 22]. Gemcitabine is active against many different types of cancers such as non-small cell lung cancer (NSCLC), head and neck, ovarian, breast, bladder and pancreatic cancers [2-4, 23, 24]. Cytarabine and fludarabine have activity against many types of leukemias and lymphomas [25-27]. For nucleoside chemotherapeutic agents to exert their effects intracellularly they must first be taken up by cells [4, 28, 29] and must require the presence of NTs, most importantly hENT1. In fact, hENT1 protein and mRNA expression, and presumably also activity, have been shown to correlate with gemcitabine effectiveness in patients with pancreatic cancer [24].

A retrospective study investigating hENT1 levels in patients who received palliative gemcitabine chemotherapy for advanced pancreatic cancer showed that there was a significant median overall survival advantage (of nine months) in patients who had high hENT1 expression compared to those who did not [30]. In addition, a study of adjuvant gemcitabine therapy in resected pancreatic cancer patients showed that high levels of hENT1 or hCNT3 were associated with longer overall survival [31]. This has been supported by in vitro data, which illustrated that hENT1 mRNA levels correlated with the extent of gemcitabine inhibition of gastrointestinal cell line growth [24, 32]. Higher hENT1 levels were associated with increased gemcitabine activity [24, 32]. Damaraju et al. have summarized evidence that many nucleoside analog drugs require NTs in plasma membranes to be active [4]. Cell lines that do not have NTs exhibit resistance to gemcitabine [28]. In the clinical setting, this would mean that a lack of available nucleoside transporters or dysfunctional NTs could result in nucleoside analog drug failure. This has been seen in cell lines when a NT inhibitor is present. NBMPR prevents intracellular uptake of nucleosides by hENT1 [5]. Protein kinase inhibitors have also been shown to inhibit nucleoside uptake. Huang et al. showed that co-incubation of human leukemia K562 cells with CPEC (cyclopentenyl cytosine), a nucleoside analog, and SB203580, a p38 MAP kinase inhibitor, resulted in decreased cellular uptake of CPEC [13]. Other protein kinase inhibitors such as epidermal growth factor receptor (EGFR) TKIs and BCR-ABL TKIs have also been tested and shown to behave similarly to SB203580 [33].

**Tyrosine kinase inhibitors (TKIs)**

Gefitinib is an EGFR TKI that blocks cancer cell growth and mitosis [34, 35]. Laboratory data suggested that EGFR may be a target for treating NSCLC [36]. This was validated in the clinical setting when gefitinib was shown to be highly effective in treating advanced NSCLC. IDEAL-1 and IDEAL-2 were two landmark trials that investigated gefitinib’s efficacy and safety in patients with previously treated advanced and metastatic NSCLC [34, 37]. Results from both IDEAL-1 and IDEAL-2 showed that gefitinib had significant antitumor activity, improved response rates and reduction in patient symptoms [34, 37]. IDEAL-1 showed median overall survival times of 7.6 and 8.0 months for gefitinib at 250 mg/day and 500 mg/day respectively [37]. IDEAL-2 showed similar results confirming gefitinib as an effective treatment option for NSCLC [34].

**TKI inhibition of nucleoside analogs**

Results from IDEAL-1 and IDEAL-2 lead to the hypothesis that at least an additive or possibly synergistic effect should be seen with the combination of gefitinib and standard chemotherapy protocols (platinum-based doublets) for treating NSCLC. This hypothesis was tested in the INTACT 1 and 2 trials. INTACT 1 was a clinical trial of gefitinib in combination with gemcitabine and cisplatin for treatment of advanced NSCLC [38]. However, results of the trial were negative and did not show a survival difference between treatment groups. For the gefitinib dose of 500
mg/day, 250 mg/day, and placebo groups, median survival was 9.9, 9.9, and 10.9 months respectively, median time to progression were 5.5, 5.8, and 6.0 months respectively, and response rates were 49.7, 50.3, and 44.8% respectively. The INTACT 2 trial reported similar findings with gefitinib in combination with paclitaxel/carboplatin [39].

Lack of synergistic or additive effects between gefitinib and standard chemotherapy is not limited to NSCLC. A study of urothelial cancer showed similar response rates between standard chemotherapy with gemcitabine and cisplatin and gefitinib versus standard chemotherapy plus placebo [40]. The lack of synergistic or additive activity is not isolated to gefitinib alone. Erlotinib, another TKI, has also been studied in combination with gemcitabine for treatment of advanced pancreatic cancer [41]. Overall response rates were not significantly different and median survival benefit was only 0.33 months in favor of erlotinib plus gemcitabine compared to gemcitabine alone [41]. Other TKIs have been studied and have also been shown to be incompatible with anticancer nucleoside analogs. Imatinib and nilotinib, both inhibitors of the Bcr-Abl fusion protein, have been implicated in blocking cytarabine uptake in chronic myeloid leukemia (CML) [42].

A hypothesis for lack of synergistic or additive effects between TKIs and nucleoside analog based chemotherapeutic agents is inhibition by TKIs of nucleoside uptake into cells. Naud et al. showed that imatinib inhibited [3H]thymidine uptake in K562 cells by 76% at a 10 μM [42]. Similar responses to imatinib were also observed in MEG-01 (megakaryoblastic leukemia) cell lines. Cytarabine uptake was inhibited by 50% and 78% when K562 cells and MEG-01 cells respectively were treated with imatinib [42]. Furthermore, a study by Woodahl et al. showed that imatinib reduced fludarabine uptake as well [43].

Nilotinib is a more potent drug than imatinib for CML treatment [44]. Therefore it is not surprising that nilotinib was shown to be a stronger inhibitor of cytarabine uptake in vitro. It prevented uptake of [3H]thymidine in K562 cells by 97% at 10 μM, similar to what was achieved with dipyridamole [42]. With MEG-01 cells, nilotinib at 10 μM blocked entry of [3H]thymidine by 96% [42]. At the same concentration, nilotinib inhibited entry of cytarabine by 94% and 96% in K562 and MEG-01 cells respectively [42].

A study by Damaraju et al. showed that three TKIs (erlotinib, gefitinib, and vandetanib) inhibited uptake of [3H]uridine in yeast cells as well as in human lung and pancreatic cancer cell lines [45]. Similar results were seen when uridine was replaced with gemcitabine [45]. In addition, exposing cell lines to erlotinib, gefitinib or vandetanib resulted in a decrease in hENT1 activity [45]. This suggests that TKIs cause a direct inhibition of NTs [45]. When cells were allowed to recover in drug free media, there was a reversal in the uptake pattern and increased hENT1 activity [45].

Damaraju et al. also showed that TKIs decreased expression of cell surface hENT1 [45]. A fluorescent hENT1 probe was used to measure the amount of hENT1 sites on cell surfaces. When cells were treated with TKIs, there was decreased staining of hENT1 sites with 5′-S-[2-(6-aminohexanamido)]ethyl-6-N-(4-nitrobenzyl)-5′-thioadenosine-fluorescein-5-yl isothiocyanate (SAHENTA-FITC) [45]. The staining pattern was reversed once cells were allowed to recover in drug free media.

Multi-targeted TKIs were also studied in an investigation of interactions of TKI inhibitors axitinib, pazopanib, and sunitinib with gemcitabine [46]. [3H]Uridine uptake in yeast cells producing recombinant human nucleoside transporters was inhibited by TKIs [46]. Pazopanib, sunitinib, and axitinib inhibited hENT1 by 50% compared to untreated cells [46]. Besides hENT1, inhibition of hENT2, hCNT1, hCNT2, and hCNT3 by all three TKIs was also observed [46].

The above studies suggest that concurrent administration of TKIs with nucleoside analogs will decrease treatment efficacy in patients. We hypothesize that in designing TKIs to mimic the chemical structure of ATP and therefore bind to ATP regulatory pockets of target enzymes and receptors that TKIs resemble the nucleoside adenosine. TKIs structural resemblance to adenosine leads to TKI binding to various nucleoside transporters competing with natural nucleosides and nucleoside analogs thus preventing their transport into cells. It is interesting to note the broad nature of TKI inhibition in that prior to these studies no single compound had been shown to inhibit both ENTs and CNTs.

Clinical Relevance

Based on observations described above, it was hypothesized that the sequence of chemotherapy administration has a profound effect on drug efficacy. This was investigated in vitro with cells that were pretreated with TKIs before gemcitabine, pretreated with gemcitabine before TKIs, or treated with both agents concurrently [45]. Cytotoxicity was greatest when gemcitabine was administered first followed by TKIs [45]. However, results from TKIs administered prior to or concurrent with gemcitabine varied in efficacy, ranging from additive to antagonistic [45].

In renal cell carcinoma cell lines treated with both TKI inhibitors and gemcitabine, there was decreased
accumulation of gemcitabine [46]. However, adding gemcitabine prior to TKI inhibitors resulted in increased cytotoxicity [46]. This study showed that the sequence of TKI inhibitors with nucleoside analogs was important.

A sequential administration of chemotherapy and TKI was studied clinically in locally advanced and metastatic NSCLC patients with known and unknown EGFR status. Patients were treated with gemcitabine/platinum doublet and then given either intercalated erlotinib or placebo on a regular interval [47]. Results from the study showed improved progression free survival of chemotherapy and intercalated erlotinib compared to platinum doublet alone [47]. Thus, TKIs can increase cytotoxic potency if administered after standard chemotherapy regimens.

Conclusions

TKIs given in combination with nucleoside chemotherapy have failed in multiple studies. The reason for the failure to improve treatment outcomes we believe is likely due to effects of TKIs inhibiting NTs. Nucleosides and most nucleoside analogs need specific transporters for uptake into cells. As far as we know, TKIs are the only drugs to inhibit both hENTs and hCNTs. In summary, TKIs given prior to or concurrently with nucleoside analog chemotherapy would attenuate the cytotoxic response. However, if TKIs are administered following nucleoside analog chemotherapy, there would be a synergistic effect. The practical implication would be that the sequence of administration of these drugs is important to the overall efficacy of the cancer treatment.

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Conflict of interest

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

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