CLINICAL INVESTIGATION

Challenges of combining cytotoxic chemotherapy and tyrosine kinase inhibitors

Single-agent tyrosine kinase inhibitors (TKIs) have significantly improved patient outcomes across multiple tumor subtypes. However, TKI therapy is rarely curative. Early optimism of combining TKIs with cytotoxic chemotherapy failed to produce substantial results in numerous randomized studies. This article highlights potential missteps and improvements in combination therapy thus far, while shedding light on potential improvements for future combinations.

Keywords: gemcitabine • nucleoside transporters • tyrosine kinase inhibitors

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Lymphomas were not only early discoveries of cancer, but were also some of the first malignancies treated with cytotoxic chemotherapy [1]. Although single agents such as nitrogen mustard showed early improvements, this strategy was not curative until chemotherapeutics were combined. By targeting multiple cellular functions, combination therapy was able to irreversibly hinder further disease proliferation. This same approach of combining conventional cytotoxics with differing targets has substantially improved both relapse-free survival in many solid tumor malignancies treated adjuvantly, as well as overall survival in metastatic disease [1]. However, as more chemotherapeutics were combined, there became a trend of increasing toxicity compounded by diminishing improvements in outcomes. In the emerging era of targeted therapies, the vast majority of combinations of conventional cytotoxic and targeted therapies have failed, as have almost all combinations of targeted therapies.

Increasing knowledge of critical cell signals in many malignancies led to development of tyrosine kinase inhibitors (TKIs). By often targeting more specific, upregulated and mutated signaling pathways, TKIs are a large step towards improving outcomes in malignancies such as chronic myelogenous leukemia (CML), non-small-cell lung cancer (NSCLC) and hepatocellular carcinoma [2]. However, similarly to early results with single-agent cytotoxic chemotherapy, single-agent TKI improvements are often short lived. This limitation led to the question of combination therapy with cytotoxic chemotherapy and TKIs.

Diseases that assimilated TKIs early on often did not have a backbone of cytotoxic chemotherapy, such as CML and hepatocellular carcinoma. Alternatively, although cytotoxic chemotherapy is the standard treatment of advanced NSCLC, erlotinib and gefitinib, two different TKIs targeting EGFRs, were each found to be superior to cytotoxic chemotherapy in patients harboring an *EGFR* mutation [3,4]. This improvement spawned some of the earliest combinations considered for TKIs and chemotherapeutics. In a preclinical xenograft study, Higgins *et al.* showed that erlotinib titrated to the maximum tolerated dose in combination with either cisplatin or gemcitabine alone had improved antitumor activity as compared with any of the three drugs alone [5]. A similar study with gefitinib in NSCLC xenografts also showed improved activity compared with individual drugs [6]. Both of these combinations proceeded to large randomized controlled trials, but neither was able to substantially improve clinical

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outcomes compared with standard platinum-doublet chemotherapy [7,8]. Subsequent NSCLC studies also missed their mark [9,10].

Clinical trials of TKI–chemotherapy combinations

Outside of NSCLC, other examples of failed TKI–chemotherapy combinations demonstrate the difficulty of translating bench results to clinical outcomes. Although a recent Phase II trial found improved antitumor activity of erlotinib with chemoradiotherapy in advanced cervical cancer, a large majority of other combination studies, particularly with EGFR TKIs, have not fared so well [11]. Aside from NSCLC studies, a meta-analysis of several advanced pancreatic cancer trials showed mixed results for erlotinib in combination with gemcitabine [12]. In advanced biliary tract cancers, erlotinib combined with gemcitabine and oxaliplatin failed to extend survival despite improved objective response rates [13].

Multitargeted antiangiogenic TKIs have had substantial success as single agents, which also prompted enthusiasm regarding combination therapy. Axitinib was combined with gemcitabine in pancreatic cancer. Despite impressive Phase II data, a large Phase III trial compared with placebo failed to improve outcomes and led to significantly increased toxicity [14,15]. Sunitinib was combined with docetaxel and subsequently capecitabine in metastatic breast cancer patients, with neither combination showing any improvements in survival [16,17]. The majority of these clinical trials were buoyed by strong preclinical work that showed significant improvements in antitumor activity, leading to significant curiosity as to what went wrong.

Nucleosides & nucleoside transporters

Nucleosides, divided into pyrimidines and purines, are building blocks for nucleic acids such as DNA and RNA. Others such as adenosine, a purine, are also vital in energy metabolism, thereby affecting multiple downstream physiological processes. Despite being required in regular cellular functioning, certain cells, such as bone marrow stem cells, leukocytes and platelets, are incapable of synthesizing nucleosides [18]. Instead, these cells have developed nucleoside salvage pathways wherein extracellular nucleosides are transported into the cell.

Human nucleoside transporters (hNTs) are predominantly organized into three concentrative and four equilibrative nucleoside transporters (hCNT1–3 and hENT1–4, respectively) [19]. Multiple hNTs are present in nearly all tissue types, with hENT1 being the most predominant [20,21]. Together, hNTs are critical to cell survival. In fact, mutations that inactivate hENT3 lead to H-syndrome, characterized by heart anomalies, hearing loss, hypogonadism and pigmented hypertrichotic dermatosis with insulin-dependent diabetes [22]. While hCNTs typically allow for nucleosides to enter cells through the utilization of sodium gradients, hENTs are membrane transport proteins that allow for bidirectional flow. hENTs are typically divided based on their sensitivity or insensitivity to the potent vasodilator nitrobenzylmercaptopurine ribonucleoside, such as with hENT1 and hENT2, respectively.

Regulation of hNTs is poorly understood. TNF-α appears to be able to increase hCNT2 and hCNT3 mRNA levels. Hypoxia also appears to increase various forms of both hENTs and hCNTs. However, constitutive activity is present in areas that are most critical, such as in the bowels for absorption of ingested nucleosides, in kidneys for reabsorption and in the liver for nucleoside metabolism.

Nucleoside analogs

Nucleoside analogs were created in order to closely mimic physiological nucleosides and disrupt cellular processes. However, these chemotherapeutics have a low molecular weight and are very hydrophilic and therefore rely on hNTs for entry into cells. Once they enter the cell, they become phosphorylated and either block further enzymatic cascades or inhibit DNA/RNA replication. Examples of these drugs include fludarabine, capecitabine and gemcitabine.

Interestingly, gemcitabine appears to be a common chemotherapeutic used in many of studies analyzing the utility of combined TKI–chemotherapy regimens in solid organ tumors. As a cytidine nucleoside analog, gemcitabine is transported into cells via hENT1/2 and hCNT1/3 [23]. Once there, it undergoes multiple phosphorylation steps (outlined with TKI effects in Figure 1). As gemcitabine diphosphate, it is a potent inhibitor of ribonucleotide reductase, leading to its increased DNA incorporation [24]. It will then lead to DNA strand breaks through disruption of topoisomerase-1 function. Gemcitabine triphosphate can also be incorporated into RNA, leading to the cytotoxicity of cells not actively undergoing proliferation [25].

Demonstrations of nucleoside analog reliance on hNTs can be found in preclinical studies. Inhibitors of hENTs such as dipyridamole (DP) significantly increased gemcitabine resistance in multiple tumor cell cultures [26]. Alternatively, transfection of cDNA encoding hCNT1 has been shown to increase gemcitabine toxicity in pancreatic cancer cells [27]. As such, hNT expression often predicts for tumor sensitivity to nucleoside analogs. Other examples include acute myeloid leukemia patients with increased hCNT3 expression and pancreatic cancer patients with a higher

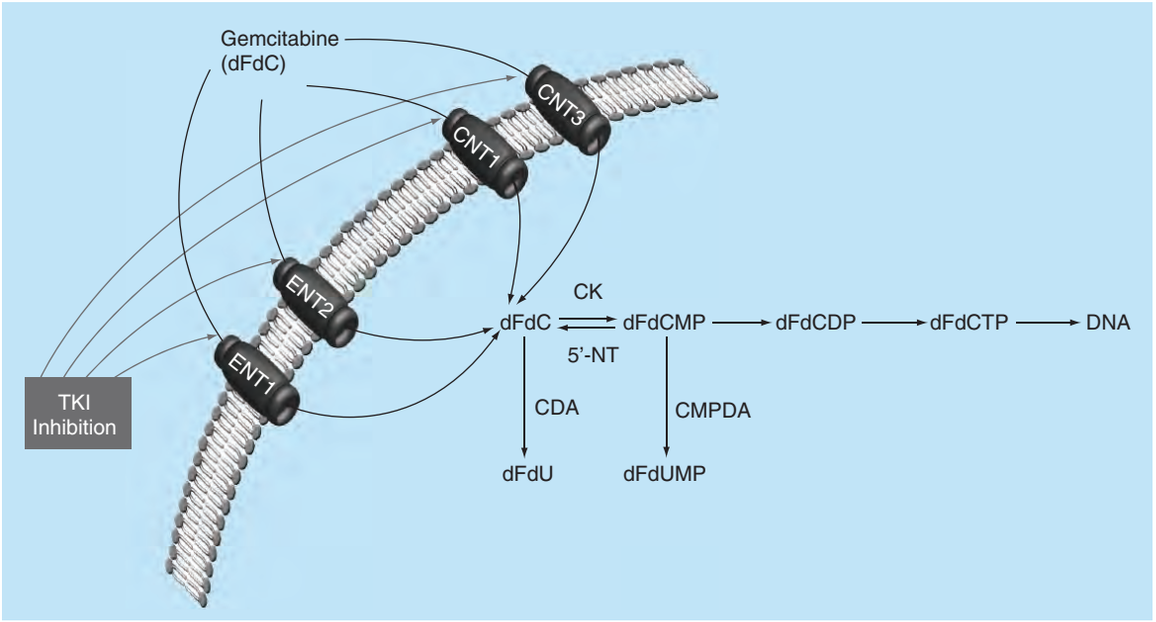

Figure 1. Gemcitabine, a nucleoside analog, and the steps required for chemotherapeutic uptake into a target cell, conversion to an active form and the effect of tyrosine kinase inhibitors on human nucleoside transport channels.

5′-NT: 5′-nucleotidase; CDA: Cytidine deaminase; CK: Cytidine kinase; CNT: concentrative nucleoside transporter 1 and 3; CMPDA; Cytidine monophosphate deaminase; dFdC: difluorodeoxycytidine; dFdCDP: difluorodeoxycytidine monophosphate; dFdCMP: difluorodeoxycytidine monophosphate; dFdCTP: difluorodeoxycytidine triphosphate; dFdU: difluorodeoxyuridine; dFdUMP: difluorodeoxyuridine monophosphate; ENT: Equilibrative nucleoside transporter 1 and 2; TKI: Tyrosine kinase inhibitor.

prevalence of hENT1. In both situations, hNT expression predicted for better survival with nucleoside analogs [28,29]. However, hCNT3 expression in chronic lymphocytic leukemia appeared to predict for fludarabine resistance [30]. Consequently, exceptions to the rule exist.

TKIs & hNTs

By design, TKIs compete with ATP at growth factor receptor regulatory pockets. As a consequence, a number of TKIs were found to interact with ATP-binding cassette transporter-mediated multidrug resistance proteins and inhibit P-glycoprotein, thereby enhancing the cytotoxic effects of certain chemotherapeutic agents [31–33]. These findings drove significant enthusiasm for combination therapy.

However, given that TKIs target the nucleoside moiety in ATP, TKIs may also bind to hNTs that recognize and transport nucleosides into cells. In fact, this observation was coincidentally first discovered by Huang *et al.* while investigating erythroid cell differentiation with p38 MAPK inhibitors [34]. An unexpected lack of cell proliferation made the authors question whether MAPK inhibitors blocked nucleoside transport. Using radiolabeled cytarabine and uridine, leukemic cell cultures were grown in nucleoside-rich media with increasing concentrations of MAPK inhibitors. Intracellular uptake of cytarabine and uridine was poor across all types of MAPK inhibitors at nearly all concentrations and across hENT subtypes.

This same group hypothesized that this effect may occur with multiple protein kinase inhibitors, given similar mechanisms of action at targeting nucleoside moiety receptors [35]. Applying a similar set of cell culture studies analyzing the cellular uptake of radiolabeled uridine and cytarabine, various TKIs were tested. Although the degree of inhibition was highly variable, all classes of TKIs were associated with reduced intracellular nucleoside levels, including inhibitors of MAPK, EGFR, BCR-ABL kinase, PKC and cyclin-dependent protein kinases. By this demonstration, the authors were able to conclude that protein kinase inhibitor dysregulation of hNTs was not related to function, but was representative of a structural component.

Interest in these findings waned until recently, when interest in combinations of TKIs and chemotherapy developed. CML is characterized by translocations of chromosomes 9 and 22, leading to the BCR-ABL fusion protein [36]. Imatinib, a TKI inhibiting BCR-ABL protein function, became standard when it was shown to improve survival compared with cytarabine and IFN-α. Other TKIs, such as nilotinib, have since been developed and have been

shown to have greater potency and specificity compared with imatinib [36]. Because a subset of CML patients still have poor responses and short durations of remission despite TKI therapy, interest in combining TKIs and cytarabine has resurfaced. Multiple cell culture studies in fact demonstrated synergism between imatinib and cytarabine [37,38]. However, these preclinical models suffered from a design flaw: use of imatinib levels below therapeutic plasma concentrations. Naud *et al.* have since published similar findings to Huang *et al.* [39]. As suggested by therapeutic improvements of nilotinib compared with imatinib, nilotinib also demonstrated stronger inhibition of thymidine and cytarabine uptake into CML cells in culture.

However, these studies appeared to be contradicted by two different xenograft studies of erlotinib and gefitinib in combination with chemotherapy in which synergy was found [5,6]. Both of these studies led to multiple large Phase III randomized studies, of which benefits were either small or insignificant [7–9]. Similarly, vandetanib (another EGFR TKI) combined with cisplatin and gemcitabine in pancreatic cancer was found to be intolerable [40]. Damaraju *et al.* sought to explain these findings by tracking both uridine and gemcitabine intracellular concentrations as well as cytotoxicity in NSCLC cells grown with erlotinib or gefitinib and in a pancreatic cell line grown with vandetanib [41]. Multiple interesting findings were borne out of these studies. First, reduced uridine and gemcitabine intracellular concentrations in each of the cell lines were reversed when TKIs were removed from culture. Lower hENT1 plasma membrane expression was also observed when cells were grown in the presence of TKIs, but was subsequently restored once TKIs were removed. The authors were also able to characterize the effects of each TKI on specific hNTs. Finally, through cytotoxicity assessments, while confirming antagonism in the concurrent use of TKIs and gemcitabine, synergism was noted when they were given sequentially. Specifically, gemcitabine followed by TKI therapy increased cell death in each of the NSCLC cell lines. Perhaps this synergism is what is demonstrated by a recent NSCLC trial, FASTACT2, in which gemcitabine-based chemotherapy is interspersed sequentially by erlotinib [42]. Particularly in those harboring an *EGFR*-activating mutation, intercalated treatment improved both progression-free and overall survival.

Damaraju *et al.* have since continued examining this relationship between hNTs and TKIs with three other oral multitargeted TKIs: pazopanib, axitinib and sunitinib [43]. In this instance, using pancreatic cacner, NSCLC and renal cell cancer cultures, each of these TKIs inhibited hNTs, especially hENT1, leading to poor uptake of uridine or gemcitabine. Similarly to the three other EGFR TKIs, inhibition occurred at levels seen within tumors clinically and therefore likely speaks to a relevant interaction. Other studies are underway examining other TKIs. A current list of TKIs and which hNTs they impact can be found in Table 1.

Inflammation, cellular stress & hNTs

Kinases in the MAPK family participate in inflammation and stress response. The JNK and p38 pathways are particularly activated by cellular stresses [44]. Using murine models, Leisewitz *et al.* found that cells significantly activate JNK as a response to gemcitabine or cytarabine exposure [45]. This led to downstream reduction of murine ENT1 mRNA and promoter activity. These results imply that cancer cells can utilize JNK stress pathways to downregulate hNTs in humans and thereby lead to nucleoside analog drug resistance. Furthermore, JNK activation can occur in response to inflammation, particularly via nitric oxide [46,47]. Consequently, inflammation may also play a role in both hNT function and nucleoside analog chemotherapy response.

Table 1. Various tyrosine kinase inhibitors and reported human nucleoside transporter inhibition.

Tyrosine kinase inhibitor	Area of activity	Target	hNT inhibited	Ref.
Erlotinib	Lung, cervix	EGFR	hENT1, hCNT3	[41]
Gefitinib	Lung	EGFR	hENT1, hCNT1	[41]
Vandetanib	Lung, mesothelioma	EGFR	hENT1, hENT2, hCNT1–3	[40]
Sunitinib	RCC, GIST	Multiple; VEGF	hENT1, hENT2, hCNT1–3	[43]
Pazopanib	RCC, sarcoma	Multiple; VEGF	hENT1, hENT2, hCNT1–3	[43]
Axitinib	RCC, lung	Multiple; VEGF	hENT1, hENT2, hCNT1–3	[43]
Imatinib	CML, GIST	BCR-ABL	hENT1	[39]
Nilotinib	CML	BCR-ABL	hENT1	[39]

CML: Chronic myelogenous leukemia; GIST: Gastrointestinal stromal tumor; hNT: Human nucleoside transporter; RCC: Renal cell cancer.

hNT inhibition & other chemotherapeutics

Although many negative interactions exist, knowing that TKIs can inhibit hNTs can also be taken advantage of. Folic acid is necessary for DNA synthesis, particularly for thymine synthesis [48]. Examples of antifolate chemotherapeutics include ralitrexed and pemetrexed [49,50]. Cell death is induced by the inhibition of nucleoside production. However, a mechanism of resistance is to salvage preformed extracellular nucleosides [51]. Moreover, this salvage pathway can actually lead to higher disease activity when compared with cells that are required to undergo rate-limiting enzymatic reactions in order to produce their own nucleosides.

As mentioned previously, DP can inhibit the activity of hENT1 and hENT2. In doing so, DP potentiates the antitumor activity of pemetrexed and methotrexate *in vitro* [52,53]. However, vasodilatory effects and generally poor results *in vivo* failed to find a therapeutic case for wider DP usage [54]. Various DP analogs had been present before in order to avoid vasodilatory side effects. Smith *et al.* were able to demonstrate synergism between DP analogs and pemetrexed *in vitro* and *in vivo* with murine models [55].

Li *et al.* demonstrated in cell line studies that similar synergism can be found when combining pemetrexed with erlotinib both concurrently and sequentially, regardless of *EGFR* mutation status [56]. Notably, the order of therapeutics was important, as antagonism was found when pemetrexed followed erlotinib. Erlotinib can lead to G1-phase cell cycle arrest, which would prevent cells proceeding to the S-phase, in which they would be most sensitive to pemetrexed. A similar finding was noted in mesothelioma cell lines when vandetanib was combined with pemetrexed [57]. Following confirmation in a Phase I setting, multiple Phase II studies have also demonstrated the efficacy of this combination as a second-line therapy in advanced NSCLC [58,59]. Quite encouragingly, Dittrich *et al.* were able to demonstrate an improvement in overall survival with this Phase II study [58]. Together, these studies suggest that EGFR TKIs likely inhibit the same pemetrexed-resistance mechanism as DP analogs by blocking nucleoside salvage. As such, a better understanding of hNTs could not only identify negative interactions, but also areas for therapeutic improvement.

Conclusion & future perspective

As with combination cytotoxic chemotherapy regimens, trial and error will occur. Although many of the TKI–chemotherapy combination studies have failed to achieve their goals, it should not deter us from continuing to pursue such investigations. However, these trials likely suffered from insufficient preclinical modeling prior to proceeding to the clinical trial. The effects of TKIs on hNTs is a prime example of an underappreciated off-target effect that has led to poor outcomes when combined with nucleoside analogs such as gemcitabine and capecitabine. Alternatively, this interaction can be utilized well, such as in the case of pemetrexed with vandetanib or erlotinib, for which early Phase II data may even suggest improvements in overall survival. This difference of interactions of pemetrexed versus nucleoside analogs with TKIs highlights the need to understand the molecular mechanisms underlying negative clinical trials. A full appreciation of existing and upcoming TKIs with respect to pharmacological structures and off-target effects may in fact avoid such negative outcomes, perhaps suggesting a broader involvement by clinical pharmacologists. Consequently, similarly to early combination cytotoxic chemotherapeutic regimens that were able to find cures for leukemias and lymphomas, combination therapy with next-generation therapeutics such as TKIs is the future.

Executive summary

- Multiple clinical trials have attempted to improve advanced malignancy outcomes by combining tyrosine kinase inhibitors (TKIs) and cytotoxic chemotherapy.
- However, improvements on outcomes have either been small or insignificant.
- A common chemotherapeutic used across these studies is gemcitabine, a nucleoside analog.
- TKIs by design competitively bind to the nucleoside binding site on many of their target receptors/kinases, thereby inhibiting them. The structural similarity of TKIs to ATP has led to cross-reactivity with human nucleoside transporters (hNTs).
- In doing so, TKIs in fact block the mechanism by which gemcitabine and other nucleoside analogs enter tumor cells.
- Robust *in vitro* models have demonstrated this detrimental effect and should serve as a cautionary tale for future TKI–chemotherapy combinations.
- Blocking hNTs can also have synergistic effects in cases such as pemetrexed – a folate analog antimetabolite – in which hNTs allow tumor resistance through nucleoside salvage pathways.
- Careful consideration and strong preclinical investigation should guide future clinical trials combining TKIs with cytotoxic chemotherapy, especially nucleosides.

Financial & competing interests disclosure

MB Sawyer and VL Damaraju are named on a patent regarding the combination of chemotherapy and targeted therapy that is owned by Alberta Health Services. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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