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SHORT COMMUNICATION

Imatinib inhibition of fludarabine uptake in T-lymphocytes

Erica L. Woodahl · Joanne Wang · Shelly Heimfeld · Aaron G. Ren · Jeannine S. McCune

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Abstract
Purpose We investigated the potential drug–drug interaction between imatinib and fludarabine, which may be concomitantly administered in chronic myeloid leukemia (CML) patients receiving fludarabine-based conditioning for allogeneic hematopoietic cell transplantation (HCT). Imatinib is an inhibitor of human equilibrative transporters (hENTs), which are responsible for the intracellular uptake of fludarabine.
Methods Intracellular accumulation of fludarabine triphosphate (F-ara-ATP), the active metabolite of fludarabine, was measured in $CD4^+$ and $CD8^+$ T-lymphocytes isolated from healthy volunteers, which were treated in vitro with fludarabine alone, and in the presence of either imatinib or NBMPR, a known hENT inhibitor.
Results Imatinib significantly inhibited F-ara-ATP accumulation in $CD4^+$ and $CD8^+$ T-lymphocytes in a concentration-dependent manner. The observed imatinib inhibition was comparable to inhibition observed with NBMPR. The inhibition of F-ara-ATP by imatinib is likely due to inhibition of nucleoside transporters hENT1 and hENT2.
Conclusions There is significant in vitro drug interaction between imatinib and fludarabine. This effect may be of important consideration in patients receiving fludarabine-based conditioning prior to HCT.

E. L. Woodahl · J. S. McCune (✉)
Department of Pharmacy, University of Washington,
Box 357630, Seattle, WA 98195, USA
e-mail: jmccune@u.washington.edu

J. Wang · A. G. Ren
Department of Pharmaceutics,
University of Washington, Seattle, WA, USA

E. L. Woodahl · J. Wang · S. Heimfeld · J. S. McCune
Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Keywords Imatinib · Fludarabine · Chronic myeloid leukemia · Hematopoietic cell transplantation · Nucleoside transporters

Abbreviations

CML	Chronic myeloid leukemia
HCT	Hematopoietic cell transplantation
F-ara-ATP	Fludarabine triphosphate
NBMPR	Nitrobenzylthioinosine
CD73	Ecto-5′-nucleotidase
hENT	Human equilibrative nucleoside transporter
hCNT	Human concentrative nucleoside transporter
dCK	Deoxycytidine kinase
dNT-1	Deoxynucleotidase-1
AK	Adenylate kinase
NDK	Nucleoside diphosphate kinase
CN-II	5′-Nucleotidase

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder affecting 1–2 out of every 100,000 people in the United States annually, and accounts for 15% of all leukemias found in adults [7, 21]. The tyrosine kinase inhibitor imatinib (Gleevec®) has rapidly become the standard frontline treatment of CML [1]. However, some CML patients have incomplete responses or relapse while on imatinib and are subsequently treated with allogeneic hematopoietic cell transplantation (HCT) [1, 4, 17].

The use of the purine nucleoside analog fludarabine in HCT conditioning has dramatically increased over the past 10 years and has become a key component of both myeloablative and nonmyeloablative conditioning regimens [6]. Fludarabine must undergo intracellular uptake

and metabolism to form its active metabolite, fludarabine triphosphate (F-ara-ATP), which inhibits ribonucleotide reductase and DNA polymerase and ultimately leads to cellular apoptosis in both actively dividing and resting cells [8]. As represented in Fig. 1, fludarabine is administered as the monophosphate prodrug and is rapidly dephosphorylated in plasma to fludarabine by serum phosphatase and ecto-5′-nucleotidase (CD73) [8]. Intracellular uptake of fludarabine is mediated by several nucleoside transporters, including the nitrobenzylthioinosine (NBMPR)-sensitive human equilibrative nucleoside transporter 1 (hENT1), the NBMPR-insensitive human equilibrative nucleoside transporter 2 (hENT2), and the human concentrative nucleoside transporter 3 (hCNT3) [8, 16]. Once inside the cell, fludarabine is sequentially phosphorylated to the monophosphate (F-ara-AMP), diphosphate (F-ara-ADP), and triphosphate (F-ara-ATP) forms by deoxycytidine kinase (dCK), adenylate kinase (AK), and nucleoside diphosphate kinase (NDK), respectively [20]. The F-ara-AMP can also be dephosphorylated to fludarabine by 5′-nucleotidase (CN-II) and deoxynucleotidase-1 (dNT-1). Several lines of evidence suggest that variable levels of cellular uptake and activation of fludarabine in lymphocyte subsets ($CD4^+$ and $CD8^+$ cells) may significantly influence outcomes in patients receiving fludarabine-based conditioning for allogeneic HCT [3, 15].

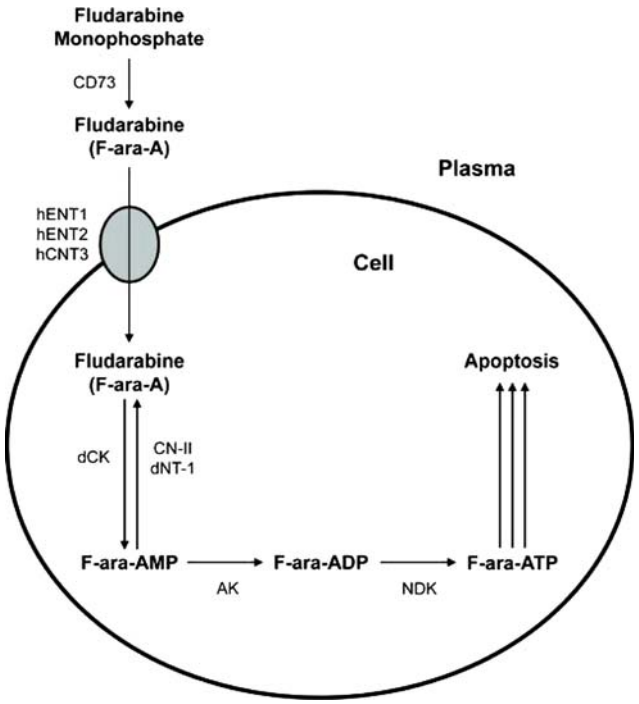

Fig. 1 Intracellular disposition of fludarabine. The schematic displays the intracellular uptake and metabolism of fludarabine to the active metabolite fludarabine triphosphate (F-ara-ATP). Abbreviations: *CD73* ecto-5′-nucleotidase, *hENT1* human equilibrative nucleoside transporter 1, *hENT2* human equilibrative nucleoside transporter 2, *hCNT3* human concentrative nucleoside transporter 3, *dCK* deoxycytidine kinase, *CN-II* 5′-nucleotidase, *dNT-1* deoxynucleotidase-1, *AK* adenylate kinase, and *NDK* nucleoside diphosphate kinase

Imatinib and several other protein kinase inhibitors have been reported to inhibit hENT1 in a cell culture model [10]. Inhibition of human equilibrative transporters (hENTs) is expected to lead to decreased intracellular F-ara-ATP formation and accumulation, ultimately resulting in decreased cytotoxicity in T-lymphocytes. Thus, inadequate F-ara-ATP accumulation in the recipient's $CD4^+$ and $CD8^+$ T-lymphocytes could lead to increased risk of graft rejection. Concomitant use of imatinib and fludarabine is likely in CML patients who are receiving fludarabine-based conditioning for HCT. Thus, it was our goal to investigate potential drug–drug interactions associated with simultaneous exposure of imatinib and fludarabine in $CD4^+$ and $CD8^+$ T-lymphocytes.

Materials and methods

Isolation of T-lymphocytes from healthy volunteers

A mononuclear-enriched apheresis product was obtained from six healthy volunteers and $CD4^+$ and $CD8^+$ T-lymphocytes were enriched from this product using anti-$CD4^+$ or anti-$CD8^+$ magnetic microbeads (Miltenyi Biotec Inc., Auburn, CA) and an autoMACS™ automatic magnetic cell sorter (Miltenyi Biotec Inc.) following the manufacturer's instructions. Final purities were >90% in all cases (data not shown).

Fludarabine triphosphate accumulation in T-lymphocytes

F-ara-ATP accumulation was measured independently in 1×10^6 purified $CD4^+$ or $CD8^+$ cells within 2 h of cell isolation. Cells were incubated in fludarabine (5 μM) in RPMI 1640 media (not containing phenol red) for 4 h at 37°C in the presence or absence of inhibitors. This fludarabine concentration approximates the maximum concentration achieved after administration of fludarabine 30 mg/m^2, a dose which is frequently used in hematopoietic cell transplant conditioning regimens [15, 19]. Inhibitor concentrations of imatinib were used at 5 and 10 μM, which are the expected peak plasma concentrations achieved after 400 mg (i.e., the usual starting dose) and 800 mg (i.e., the dose used in patients with nonresponsive or resistant disease) doses of imatinib [1, 18]. Imatinib inhibition of hENTs was compared with a known and potent inhibitor of hENTs, NBMPR (150 nM and 50 μM). Controls were incubated with fludarabine alone and incubated with fludarabine in the presence of the vehicle used for imatinib and NBMPR (0.5% DMSO). All incubations were carried out

in triplicate. After fludarabine incubation, cells were washed twice in ice-cold phosphate buffered saline, centrifuged, and solubilized in 1 M perchloric acid. Samples were frozen at −70°C prior to F-ara-ATP quantitation.

LC–MS quantitation of fludarabine triphosphate in T-lymphocytes

After thawing, the sample was centrifuged and the supernatant was neutralized to pH ~7 with 1 M potassium bicarbonate.The F-ara-ATP was quantitated using the LC–MS method described previously [11], with the following modifications. YMC-Pack Hydrosphere C18 column (3 μm; 2.0 mm × 150 mm) was used with a flow rate of 0.225 ml/min and column temperature at 30°C (Waters, Milford, MA). The mobile phase consisted of 20 mM ammonium formate pH 8 (A) and methanol (B) with the following gradient: initially 96.5% A and 3.5% B, to 12% B at 5 min, and then 3.5% B at 8 min. Chloroadenosine triphosphate was used as an internal standard. This F-ara-ATP quantitation assay is highly sensitive and provides a limit of detection equivalent to 50 fmol F-ara-ATP.

Statistical analysis

Student's two-sided *t* test was used to evaluate differences between two sets of data. The *P* values <0.05 were considered statistically significant.

Results

The average F-ara-ATP accumulation in $CD4^+$ or $CD8^+$ T-lymphocytes in the presence of imatinib and NBMPR, normalized to percent of the fludarabine-only control, are shown in Fig. 2. The concentrations of fludarabine and imatinib used in these experiments were consistent with plasma concentrations after therapeutic dosing [18]. For all concentrations of both of the inhibitors, F-ara-ATP accumulation was significantly lower compared to control in both $CD4^+$ and $CD8^+$ T-lymphocytes ($P < 0.0001$). Exposure to the vehicle control of 0.5% DMSO had no effect on F-ara-ATP accumulation.

Imatinib significantly inhibited F-ara-ATP accumulation in both $CD4^+$ and $CD8^+$ T-lymphocytes, and there appeared to be significant concentration-dependent inhibition of F-ara-ATP accumulation with increasing concentrations of imatinib in T-lymphocytes. In $CD4^+$ cells, the F-ara-ATP accumulation, as a percent of control accumulation, was 48.3 ± 8.9 and 31.4 ± 8.6% in the presence of 5 and 10 μM imatinib, respectively ($P < 0.05$); and in $CD8^+$ cells, F-ara-ATP accumulation was 45.4 ± 9.3 and 30.2 ± 9.1% in the presence of 5 and 10 μM imatinib, respectively ($P < 0.05$).

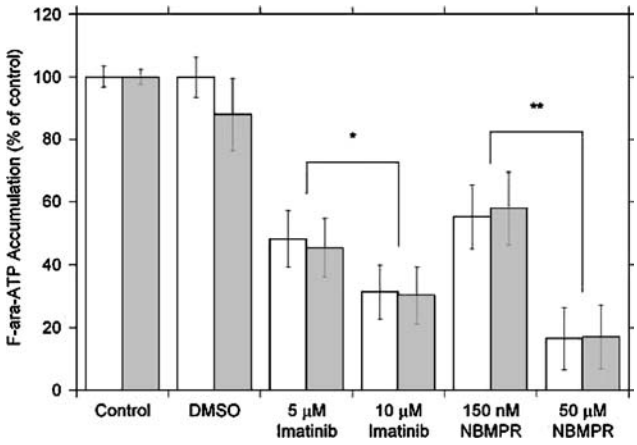

Fig. 2 Inhibition of intracellular accumulation of fludarabine triphosphate by imatinib in $CD4^+$ and $CD8^+$ T-lymphocytes. $CD4^+$ cells are represented by *open bars* and $CD8^+$ cells represented by *shaded bars* (error bars represent SD for $n = 6$ healthy volunteers). The control bars refer to incubation with fludarabine alone and the DMSO bars refer to the incubation of fludarabine with the vehicle control used for imatinib and NBMPR (0.5% DMSO). Inhibition of F-ara-ATP accumulation was performed at two concentrations for each inhibitor: 5 and 10 μM imatinib, and 150 nM and 50 μM NBMPR. F-ara-A accumulation was significantly inhibited in all inhibitor incubations, compared to control ($P < 0.0001$). *Significant concentration-dependent inhibition by imatinib in both $CD4^+$ and $CD8^+$ cells ($P < 0.05$). **Significant concentration-dependent inhibition by NBMPR in both $CD4^+$ and $CD8^+$ cells ($P < 0.0001$)

We compared the inhibition of F-ara-ATP accumulation by imatinib to inhibition by NBMPR, a potent and specific inhibitor of hENT1 (K_i = 1–10 nM), which at higher concentrations (>10 μM) also inhibits hENT2 [13]. A concentration-dependent inhibition of F-ara-ATP accumulation was also observed with NBMPR, with 50 μM NBMPR significantly inhibiting F-ara-ATP accumulation in both $CD4^+$ and $CD8^+$ T-lymphocytes compared to 150 nM NBMPR ($P < 0.0001$). Imatinib inhibited F-ara-ATP accumulation to a similar degree as the inhibition observed by NBMPR.

Discussion

Our data suggest that concomitant exposure to imatinib and fludarabine will inhibit F-ara-ATP accumulation in $CD4^+$ and $CD8^+$ T-lymphocytes. Lower accumulation would be expected to result in decreased apoptosis in these T-cells, which could increase a HCT recipient's likelihood of experiencing graft rejection. Future studies should evaluate the apoptotic effect of fludarabine alone compared to fludarabine and imatinib in $CD4^+$ and $CD8^+$ T-lymphocytes to strengthen the significance of our findings. In fact, CML patients do exhibit a higher graft rejection rate compared to other leukemia patients receiving fludarabine/total body irradiation as conditioning for HCT from a matched unrelated donor graft [2, 3]. However, the concomitant adminis-

tration of imatinib was not described in these reports. Chronic phase CML patients are often treated with imatinib immediately prior to or during fludarabine-based conditioning regimens to lower the tumor burden at the time of non-myeloablative HCT [12]. Insufficient immune suppression in the patients is considered the major factor causing the higher rejection rate, and based upon these data, consideration should be given for discontinuing imatinib prior to fludarabine administration.

NBMPR is a tight-binding, selective inhibitor of nucleoside transport. Human ENTs vary in their susceptibility to NBMPR inhibition. The NBMPR-sensitive hENT1 is inhibited in cells at the nanomolar range, while inhibition of NBMPR-insensitive hENT2 requires much higher concentrations (micromolar) [5, 9, 22]. Therefore differential sensitivity to NBMPR inhibition can elucidate the hENT isoform contribution to substrate uptake. At the lower (nanomolar) concentration of NBMPR, we saw significant reduction in F-ara-ATP accumulation in both $CD4^+$ and $CD8^+$, due to inhibition of hENT1. At the higher (micromolar) concentration of NBMPR, we observed an even greater magnitude of inhibition of F-ara-ATP accumulation, suggesting that hENT2 also has a role in fludarabine uptake in $CD4^+$ and $CD8^+$ lymphocytes.

Imatinib, used at clinically relevant concentrations, inhibited F-ara-ATP accumulation in $CD4^+$ and $CD8^+$ lymphocytes in a concentration-dependent manner similar to what was observed with the concentration-dependent inhibition by NBMPR. The lower concentration (5 μM) of imatinib decreased intracellular accumulation of F-ara-ATP to the same degree as nanomolar concentrations of NBMPR, indicating that imatinib is a potent inhibitor of hENT1. Increasing the imatinib concentration (10 μM) further potentiated its inhibitory effect to a degree that can not be solely explained by hENT1 inhibition, suggesting that imatinib may also affect hENT2-mediated fludarabine uptake. However, we cannot exclude the possibility that some of the inhibitory effect of imatinib may be due to inhibition of some of the kinases involved in the sequential phosphorylation of fludarabine. Since the mechanism of hENTs inhibition by imatinib is not known, the time for return to normal function of hENTs after imatinib exposure is unclear. However, given the half-life of imatinib (19.3 ± 4.4 h), imatinib should be stopped at least 5 days before beginning fludarabine administration to allow for adequate elimination of imatinib. The recovery of F-ara-ATP accumulation in T-lymphocytes after imatinib exposure will be the basis of future studies.

Our data clearly show that imatinib inhibits F-ara-ATP accumulation in $CD4^+$ and $CD8^+$ T-lymphocytes obtained from healthy volunteers, which is suggestive of an adverse drug–drug interaction between imatinib and fludarabine. The inhibition of hENT1 by imatinib, and potentially hENT2, may be of clinical significance during concomitant administration of imatinib and fludarabine in CML patients. However, the effect of imatinib on F-ara-ATP accumulation in T-lymphocytes from CML patients remains to be evaluated. Decreased accumulation of F-ara-ATP in T-lymphocytes in the presence of imatinib may affect cellular apoptosis in patients prior to HCT, which may lead to decreased engraftment. Another study has shown, using lower concentrations of fludarabine and imatinib, that the combination of fludarabine and imatinib intensifies the antiproliferative effect on granulocyte–macrophage progenitor cells (CFU-GM) isolated from patients with CML [14]. However, in this study, the CFU-GM cells isolated from bone marrow were cultured for 14 days, and it is not clear how this would impact expression of hENTs. In addition, the relative expression of hENTs in $CD4^+$ and $CD8^+$ T-lymphocytes versus CFU-GM progenitor cells has not been demonstrated.

In conclusion, we observed a significant in vitro drug–drug interaction between imatinib and fludarabine. Imatinib inhibits fludarabine uptake into $CD4^+$ and $CD8^+$ T-lymphocytes and that the inhibition is likely due to inhibition of nucleoside transporters hENT1 and hENT2. This effect may be of particular importance in patients receiving fludarabine-based conditioning regimens prior to HCT.

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