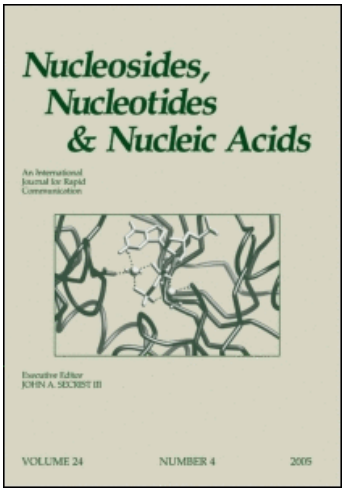

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IMATINIB-RESISTANT CML CELLS HAVE LOW ENT ACTIVITY BUT MAINTAIN SENSITIVITY TO GEMCITABINE

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□ *Philadelphia chromosome-positive chronic myelogenus leukemia (CML) is widely treated with imatinib mesylate (imatinib), a potent inhibitor of the Bcr-Abl tyrosine kinase. However, resistance to this compound remains a concern. Current treatment approaches include combinations of imatinib with nucleoside analogs such as gemcitabine, which requires equilibrative nucleoside transporters (ENTs) for uptake, to overcome this resistance. Here we report that imatinib treatment decreased ENT1-dependent activity and mRNA expression. Although, imatinib-resistant cells showed decreased levels of both ENT1 and ENT2 activity and expression, these cells remained sensitive to gemcitabine, suggesting that nucleoside analogs can be used as adjunctive therapy.*

Keywords Imatinib; equilibrative nucleoside transporter; drug resistance; gemcitabine

INTRODUCTION

The underlying cause of chronic myeloid leukemia (CML) is the product of Philadelphia chromosome (Ph), the Bcr-Abl fusion protein, which presents a constitutively activated Abl tyrosine kinase activity. Treatment of Ph positive (Ph^+) CML has centered on strategies that suppress the Ph^+ cells or the Ph-associated Bcr-Abl molecular abnormalities.[1,2] CML is widely treated with the Bcr-Abl selective tyrosine kinase inhibitor, imatinib mesylate (imatinib, Gleevec, STI571).[3,4] However, resistance to imatinib, particularly in the advanced phases of CML and Ph^+ acute lymphoblastic leukemia (ALL) is very common. Thus, it is crucial to develop new strategies of treatment to overcome or avoid the development of resistance. Current treatment approaches include the combination of treatments using imatinib and compounds such as interferon-α (IFN-α), hemoharringtonine (HHT) and nucleoside analogs such as cytarabine and gemcitabine.[5–8]

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Mammalian equilibrative nucleoside transporters (ENTs) are facilitative transporters broadly selective for pyrimidine and purine nucleosides and are essential for the modulation of nucleoside concentration and nucleoside analog availability. Uptake of clinically important nucleoside analogs (e.g., gemcitabine, capecitabine, cytarabine (ara-C)) is mediated primarily by Equilibrative Nucleoside Transporter 1 (hENT1) in human cells.[9] The development of resistance to both gemcitabine and capecitabine correlates strongly with a deficiency of ENT1 expression in human breast cancer cells[10] and resistance to ara-C has been attributed to reduced expression of ENT1 in acute myeloid leukemia cells.[11]

We report here that Bcr-Abl mouse cells, 32Dp185 cells, treated with imatinib showed decreased mENT1 transport activity and mRNA expression in a time-dependent manner. Interestingly, although the imatinib-resistant cells showed low ENT activity and expression, these cells remained sensitive to the nucleoside analog gemcitabine. Thus, our results show that resistance to imatinib may not alter the sensitivity to nucleoside analogs, suggests that cotreatment with nucleoside analogs and imatinib in CML may prove effective in reducing the development and consequences of resistance to the latter agent.

MATERIALS AND METHODS

Cell Culture and Reagents

Mouse 32Dp185 cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were maintained in the presence 5% CO_2 at 37^{o}C. NBMPR (nitrobenzymercaptopurine, 6-[(40 nitrobenzyl)thio]-9-(b-D-ribofuranosyl)purine), was from Sigma (Sigma-Chemical Co., St. Louis, MO, USA). Uridine ([5,6-^{3}H], 35-60 Ci/mmol) and[^{3}H]-NBMPR 40 Ci/mmol were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA., USA). STI-571 (Imatinib mesylate, Figure 1a) was from Toronto Research Chemicals (North York, ON, Canada). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was obtained from Promega (Madison, WI, USA) and the assay was performed according to the manufacturer's instructions.

Generation of the Imatinib-Resistant 32Dp185 Cell Line (32Dp185 STI-R)

Mouse 32Dp185 cells were exposed to increasing concentrations of imanitib for several weeks (0.1–1 μM). The cells were maintained at a constant imanitib final concentration of 1 μM in RPMI 1640 supplemented

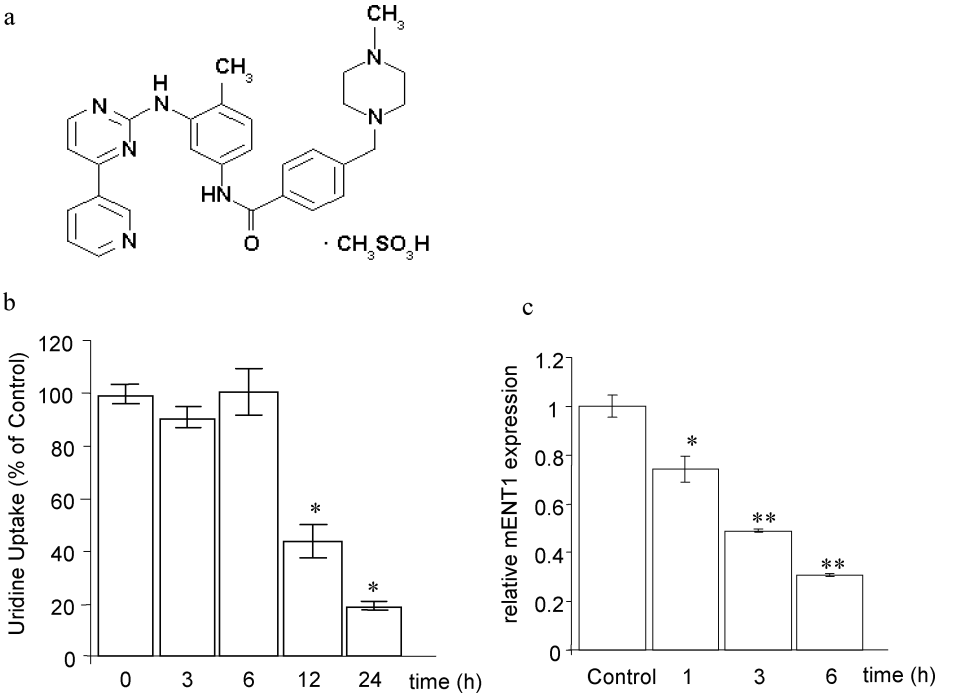

FIGURE 1 Imantinib decreased ENT1-dependent uridine uptake and mRNA expression. (a) Structure of imatinib mesylate. 32Dp185 cells were incubated with 1 μM imatinib for the times indicated. The values shown in the graphs represent means ± S.D. of data from a representative experiment (n = 3). (b) ^{3}[H]-Uridine uptake was measured as described in the Materials and Methods section. (c) Real Time PCR was performed to quantify the relative change in mENT1 transcript after treatments for the times indicated. Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$ Student's t test).

with 10% heat inactivated FBS and 100 units/ml penicillin and 100 mg/ml streptomycin.

ENT1- and ENT2-Dependent[^{3}H]-Uridine Uptake Assay

Equal amounts of cells were used for the uptake of uridine. The assay was performed as described previously.[12]After the uptake assay the cells were lysed in 800 μl lysis buffer containing 20 mM Tris-HCl pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol and 2 mM EDTA. After centrifugation at 10,000g for 10 minutes at 4^{o}C, 600 μl of supernatant were use to measure for radioactivity and 200 μl were used to measure protein concentration.

Real Time PCR

Total RNA was extracted using Trizol (Invitrogen, USA) according manufacturer's instructions. Two μg RNA were used for cDNA synthesis using SuperScriptII reverse transcriptase (Invitrogen) according to manufacturer's

protocol. Expression level for mENT1 was quantified by real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The PCR reaction mixtures contained 900 nM each of the sense primer (5'ATGGCAAGGGCTCAATGG-3') and antisense primer (5′-TGGAGTAAGCGGGCATCAGT-3′) for mENT1, sense primer (5′-GCTGTGCCTTGTGTTGGTCTT-3′) and antisense primer (5′-ATGGCTGTGATGGCAGGAA-3′) for mENT2 or 900 nM each of the

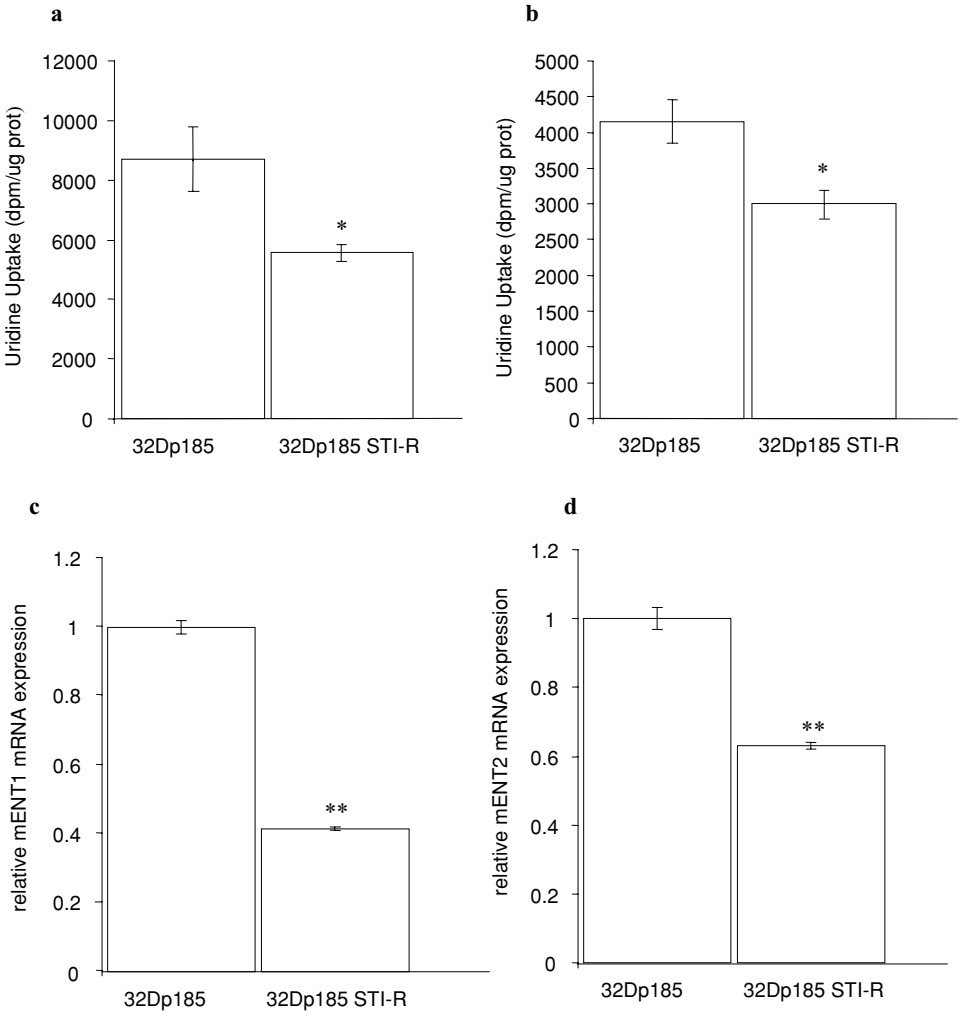

FIGURE 2 Imatinib-resistant cells exhibit decrease ENT1- and ENT2-dependent uridine uptake and mRNA expression. The values shown in the graphs represent means ± S.D. of data from a representative experiment (n = 3). (a) ENT1-dependent ^{3}[H]-uridine uptake and (b) ENT2-dependent ^{3}[H]-uridine uptake was measured in 32Dp185 and 32Dp185 STI-R cells as described in Materials and Methods. Real Time PCR was performed to quantify the relative amounts of (c) mENT1 and (d) mENT2 transcripts. Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$ Student's *t* test).

sense primer (5′-TGAAGCAGGCATCTGAGGG-3′) and antisense primer (5′-CGAAGGTGGAAGAGTGGGAG-3′) for the mouse housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, mGAPDH. One hundred ng cDNA and SYBR Green PCR master mix (Applied Biosystems) was also added to reaction mixtures. After initial 10 minutes incubation at 95°C, cDNA was amplified using increasing cycles of 95°C for 15 seconds and 60°C for 1 minute. Transcript levels and fold change in mRNA between treatments were determined as previously described.[13]

RESULTS

Imatinib Decreased ENT1 Transport Activity and Expression

In order to investigate the effects of imatinib on ENT1-dependent uridine uptake, we treated 32Dp185 cells with 1 μM imatinib. Our results showed a time-dependent decrease in ENT1-dependent transport activity (Figure 1b). Quantitative RT-PCR (Q RT-PCR) analysis of ENT1 mRNA expression showed a similar time-dependent down-regulation, suggesting that the diminished transport activity was due to a repression in ENT1 expression (Figure 1c).

Imatinib-Resistant Cells Show Reduced ENT1 Transport Activity and Expression

To test if ENT1 activity and expression were affected in imatinib-resistant cells, we developed an imatinib-resistant cell line derived from 32Dp185 cells (32Dp185 STI-R), as described in Materials and Methods. ENT1- and ENT2-dependent uridine uptake was diminished in the 32Dp185 STI-R cells (60 and 40%, respectively) compared to the parental cells (Figures 2a and 2b).Moreover, Q RT-PCR data indicated that this decrease in uridine uptake was most likely due to a diminished mRNA expression of both ENT isoforms (Figures 2c and 2d).

Imatinib-Resistant Cells Are Less Sensitive to Nucleoside Analogs Treatments

Nucleoside analogs as gemcitabine and araC are potential drugs to be used alone or in combination with imatinib for the treatment of CML.[6,7] These nucleoside analogs are transported into cells mainly through ENTs. To investigate whether imatinib-resistant cells showed reduced sensitivity to gemcitabine (structure shown in Figure 3a), we incubated 32Dp185 and 32Dp185 STI-R cells with 1 μM gemcitabine for 12 hours and performed MTT assays. The results in Figure 3b show that both cell lines were equally sensitive to gemcitabine treatment.

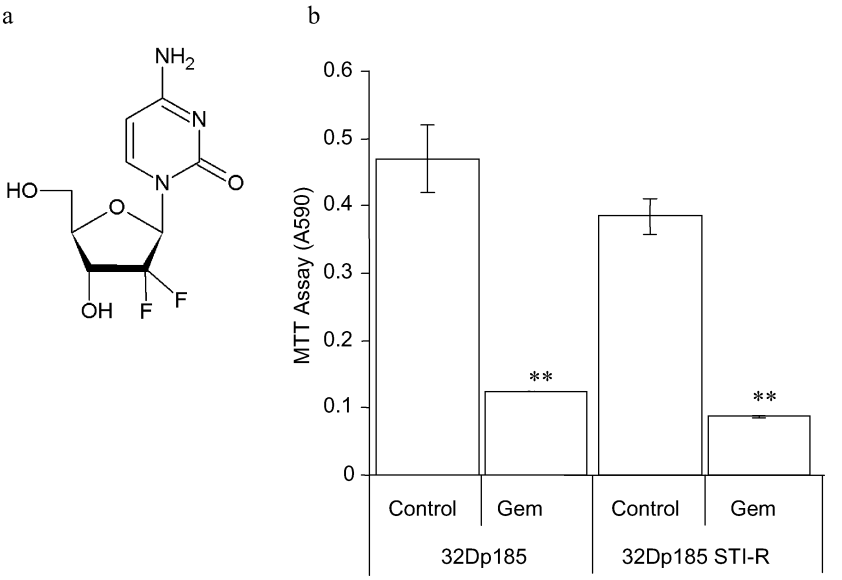

FIGURE 3 Imatinib-resistant cells exhibit the same sensitivity to nucleoside analogs. (a) Structure of gemcitabine. (b) MTT assay was performed in 32Dp185 and 32Dp185 STI-R cells treated for 12 hours with 1 μM gemcitabine (Gem). The values shown are means ± S.D. of data from a representative experiment (n = 3). Asterisks indicate statistical significance (**p < 0.01 Student's *t* test).

DISCUSSION

The results presented here define an important role of Bcr-Abl kinase in ENT1 expression and ENT1-dependent uridine transport. We showed that imatinib treatment down regulated ENT1 expression as well as the ENT1-dependent uridine uptake. Moreover, the imatinib-resistant cell line, 32Dp185 STI-R, exhibited decreased ENT1 and ENT2 activities and expression, suggesting that Bcr-Abl leukemic cells that are resistant to imatinib will also be resistant to nucleoside analogs. However, contrary to our prediction, we observed a similar rate of cell death in imatinib-resistant cells when exposed to the nucleoside analog gemcitabine. These results suggest that in imatinib-resistant cells a compensatory mechanism may exist for gemcitabine uptake when ENT1 and ENT2 are downregulated. Alternatively the amount of ENT-dependent activity remaining in these cells is sufficient to accommodate gemcitabine uptake.

The concentrative nucleoside transporters (CNTs) transport nucleosides against their concentration gradients by coupling the inward transport of nucleosides to the electrochemical Na+ gradients.[4] While CNT1 and CNT2 expression is limited to certain tissues including kidney, intestine and liver, CNT3 is found in a wider variety of tissues, suggesting that it plays multiple roles in nucleoside homeostasis.[15] Both CNT1 and CNT3 can transport gemcitabine with high affinity.[16,17] Moreover, it was previ-

ously reported that expression of hCNT1 in pancreatic cancer cells with a stable expression of hENT1 were more sensitive to gemcitabine treatments than cells with lower expression of CNT1 and the same levels of hENT1.[18] Thus, it is possible that CNTs may contribute to the uptake of gemcitabine under our experimental conditions.

We report here that resistance to imatinib induced an ENT1 and ENT2 expression down-regulation; however, the sensitivity to the nucleoside analog gemcitabine was maintained. These results suggest that nucleoside analogs may be used as a combination therapy in imatinib-resistant CML.

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