Supplementary Information

SI Materials and Methods

Silencing of deoxycytidine kinase expression. To silence dCK we used the Silencer Select Validated siRNA for dCK, ID s186, and the Silencer Select Negative Control #1 siRNA (Ambion). Cells were transfected with dCK siRNA by using Nucleofector II and Nucleofector Kit T (Amaxa). Cultured cells were treated according to the Amaxa protocol at a density of 3 x 10^6 cells/100 µl reagent (solution T). For optimization, we used 1 µg to 3 µg of siRNA per sample. To evaluate dCK suppression, total RNA was extracted and analyzed by real-time PCR with a cDNA synthesized by using a primer set designed to amplify exons 5-7 (encompassing the s186 cleavage point of the siRNA). In each sample, the copy number of dCK relative to GAPDH expression was calculated as follows:

2^dCt (dCt = Ct[GAPDH] – Ct[target]).

Real-time PCR. The ABI Prism protocol was used with 0.3 µM of the primers and the following PCR cycles: Denaturation for 15 min at 95 °C followed by 40 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 30 sec.

Primers:

Human ABCG2/BCRP:

(Forward) 5'-ACTGGCTTAGACTCAAGCACA-3'

(Reverse) 5'-ATAGGCCTCACAGTGATAACCA-3'

Human dCK:

(Forward) 5'-ACTCAACCTGCAGAAGGAGA-3'

(Reverse) 5'-AACTTGGCTGGGACATCGA-3'

GAPDH:

(Forward) 5'-TGAACGGGAAGCTCACTGG-3'

(Reverse) 5'-TCCACCACCCTGTTGCTGTA-3'

Clofarabine HPLC. To measure intracellular ^{3}H-labeled clofarabine metabolites, we used a procedure based on that described by Parker et al [4]. The method was modified as follows: buffer A concentration was 2.5 mM (vs. 5 mM) $NH_4H_2PO_4$, pH 2.88, and gradient time was 40 min (rather than 50 min). Clofarabine and its metabolites were analyzed on a Shimadzu HPLC system coupled to an LKB fraction collector and used a Whatman Partisil SAX anion exchange column (10 mm particle size, 250mm X 4.6mm I.D.). Clofarabine and its metabolites were resolved by using a binary gradient system with a 2-buffer mobile phase (buffer A, 2.5 mM $NH_2H_2PO_4$, pH 2.88; buffer B, 750 mM $NH_2H_2PO_4$, pH 3.7). The flow rate was 1.5 ml/min at ambient temperature; 75 fractions were collected over 50 min. The unlabeled standards were clofarabine (2.5 min retention time), GMP (8.9 min), GDP (21.15 min), and dGTP (39.1 min).

Vesicle Transport. Inside-out vesicles prepared from Sf9 cells expressing ABCG2 (Genomembrane, Yokohama, Japan) were used to determine the inhibitory potential of clofarabine, according to the manufacturer's protocol. [^{3}H]methotrexate (10 μmol/L) was used as the reference ABCG2 substrate in the presence of various concentrations of clofarabine. Briefly, tubes containing the vesicles and clofarabine and tubes containing [^{3}H]methotrexate

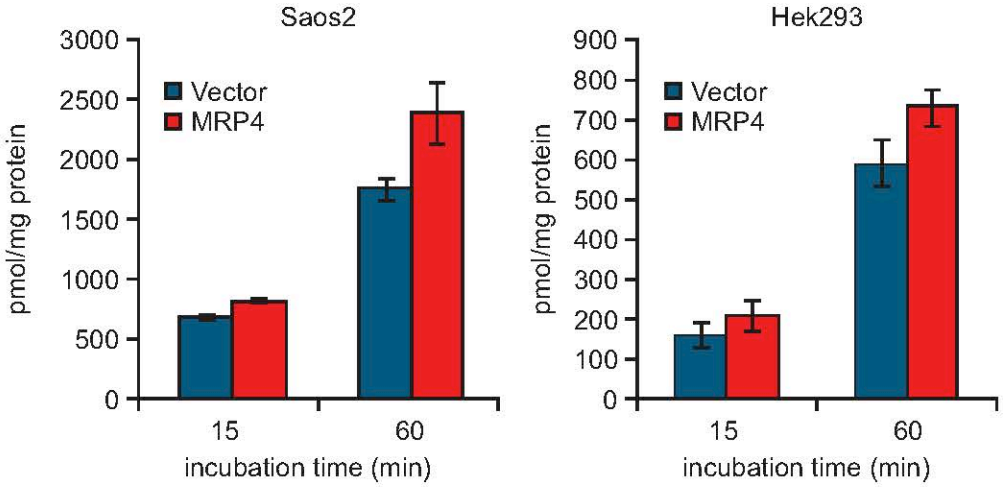
were separately pre-incubated at 37 ºC for 10 min. Tubes were then combined and incubated for 10 min. Cold buffer (200 µL) was added to stop the reaction, and contents were transferred to a 96-well glass-fiber filter plate with a vacuum. Filters were washed 5 times with cold buffer, removed and then combined with scintillation fluid for measurement of total radioactivity.

Supplementary Figure Legends

Fig. S1. MRP4 does not transport clofarabine. Saos2 and HEK293 cells expressing human MRP4 were incubated for the indicated times with radiolabeled [^{3}H]-clofarabine before analysis.

Fig. S2. SiRNA knockdown of dCK mRNA in OCI-AML3 cells. dCK mRNA expression in OCI-AML3 cells transfected with the indicated amounts of dCK siRNA or negative control (NC) siRNA, as a percentage of values in untransfected cells. Cells harvested at the indicated times were analyzed by real-time PCR.

Fig. S3. HPLC analysis of clofarabine effluxed into the media. Saos-2 cells expressing ABCG2 and either dCK or empty vector were incubated with 10µM^{3}H-clofarabine for 60 minutes under ATP-depletion conditions. Subsequently cells were washed with ice-cold PBSX3 followed by addition of warmed (37 ºC) ATP-depletion media. Medium was collected at 60 minutes and subsequently analyzed by HPLC as described in the "Materials and Methods".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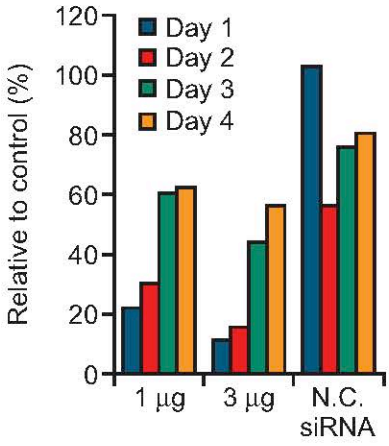
120
100
80
60
40
20
0
Relative to control (%)
Day 1
Day 2
Day 3
Day 4
1 μg
3 μg
N.C.
siRNA

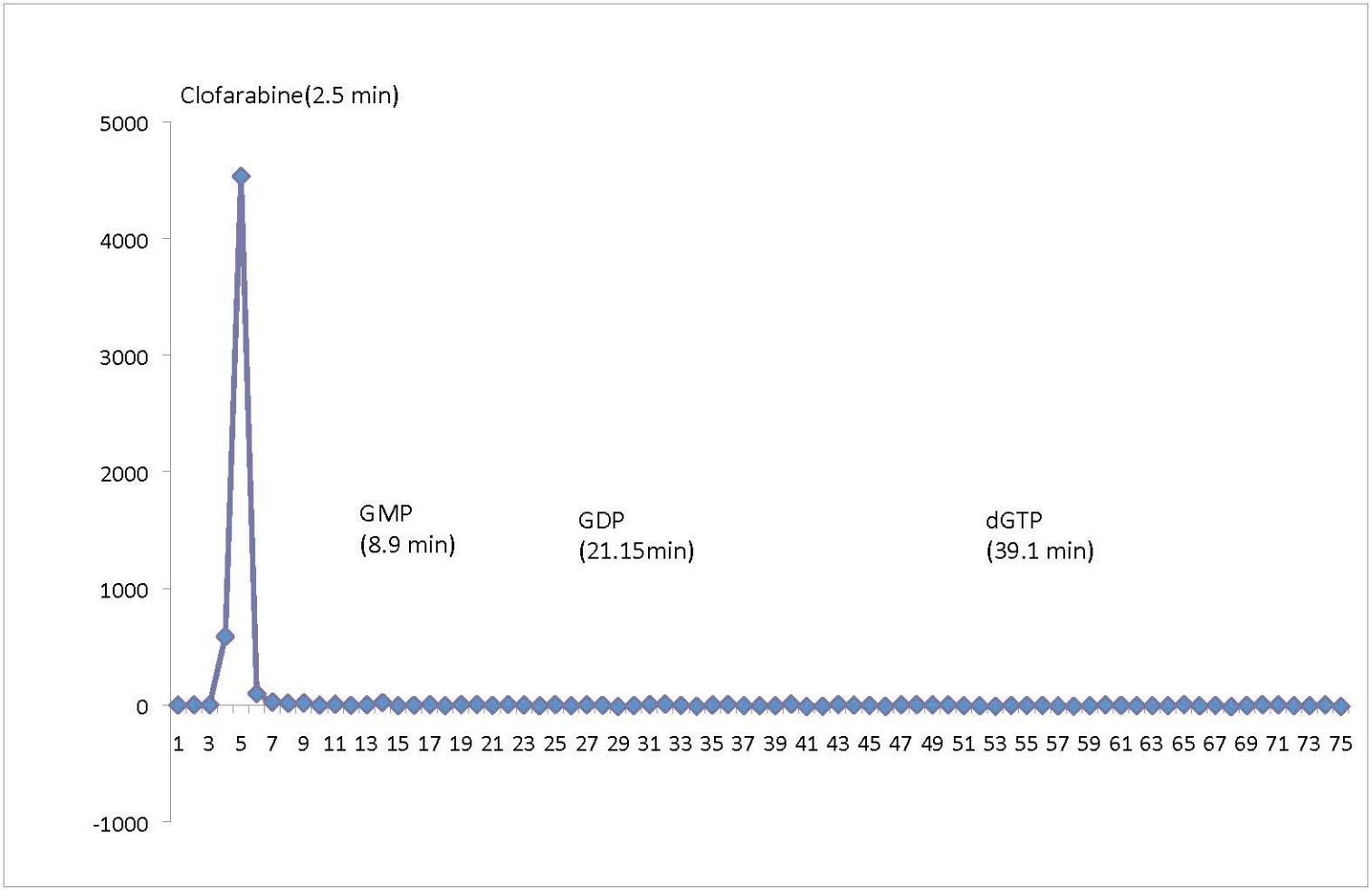

Fig S3. Radioactive profile of Chlorfarabine effluxed from ABCG2 overexpressing Cells. Each three fractions represents 2min of time. The location of the unlabeled standards (GMP, GDP and dGTP) and their average elution time is indicated in parentheses