Title
Co-targeting of convergent nucleotide biosynthetic pathways for leukemia eradication

Permalink
https://escholarship.org/uc/item/4ds709f7

Journal
Journal of Experimental Medicine, 211(3)

ISSN
0022-1007

Authors
Nathanson, DA
Armijo, AL
Tom, M
et al.

Publication Date
2014-03-01

DOI
10.1084/jem.20131738

Peer reviewed
The ability to reprogram cellular metabolism, a hallmark of cancer first noted long ago (Warburg et al., 1927) and recently reappreciated, is essential for tumor progression (Hanahan and Weinberg, 2011). Although cancer-initiated metabolic reprogramming processes are promising therapeutic targets (Vander Heiden, 2011), the existence of alternative, compensatory biosynthetic pathways presents a significant challenge for developing such therapies. For example, in lipid metabolism, cancer cells scavenge extracellular lipids as an alternative to energy-requiring pathways (Reichard, 1988). The DNP uses glucose and amino acids to generate ribonucleotide di- and triphosphates (NDPs), which are converted to deoxyribonucleotide triphosphates (dNTPs) required for DNA replication and repair can be produced by the de novo pathway (DNP) or by the nucleoside salvage pathway (NSP). However, the role of the NSP in dCTP production and DNA synthesis in cancer cells is currently not well understood. We show that acute lymphoblastic leukemia (ALL) cells avoid lethal replication stress after thymidine (dT)-induced inhibition of DNP dCTP synthesis by switching to NSP-mediated dCTP production. The metabolic switch in dCTP production triggered by DNP inhibition is accompanied by NSP up-regulation and can be prevented using DI-39, a new high-affinity small-molecule inhibitor of the NSP rate-limiting enzyme dC kinase (dCK). Positron emission tomography (PET) imaging was useful for following both the duration and degree of dCK inhibition by DI-39 treatment in vivo, thus providing a companion pharmacodynamic biomarker. Pharmacological co-targeting of the DNP with dT and the NSP with DI-39 was efficacious against ALL models in mice, without detectable host toxicity. These findings advance our understanding of nucleotide metabolism in leukemic cells, and identify dCTP biosynthesis as a potential new therapeutic target for metabolic interventions in ALL and possibly other hematological malignancies.

Pharmacological targeting of metabolic processes in cancer must overcome redundancy in biosynthetic pathways. Deoxycytidine (dC) triphosphate (dCTP) can be produced both by the de novo pathway (DNP) and by the nucleoside salvage pathway (NSP). However, the role of the NSP in dCTP production and DNA synthesis in cancer cells is currently not well understood. We show that acute lymphoblastic leukemia (ALL) cells avoid lethal replication stress after thymidine (dT)-induced inhibition of DNP dCTP synthesis by switching to NSP-mediated dCTP production. The metabolic switch in dCTP production triggered by DNP inhibition is accompanied by NSP up-regulation and can be prevented using DI-39, a new high-affinity small-molecule inhibitor of the NSP rate-limiting enzyme dC kinase (dCK). Positron emission tomography (PET) imaging was useful for following both the duration and degree of dCK inhibition by DI-39 treatment in vivo, thus providing a companion pharmacodynamic biomarker. Pharmacological co-targeting of the DNP with dT and the NSP with DI-39 was efficacious against ALL models in mice, without detectable host toxicity. These findings advance our understanding of nucleotide metabolism in leukemic cells, and identify dCTP biosynthesis as a potential new therapeutic target for metabolic interventions in ALL and possibly other hematological malignancies.

The ability to reprogram cellular metabolism, a hallmark of cancer first noted long ago (Warburg et al., 1927) and recently reappreciated, is essential for tumor progression (Hanahan and Weinberg, 2011). Although cancer-initiated metabolic reprogramming processes are promising therapeutic targets (Vander Heiden, 2011), the existence of alternative, compensatory biosynthetic pathways presents a significant challenge for developing such therapies. For example, in lipid metabolism, cancer cells scavenge extracellular lipids as an alternative to energy-requiring de novo fatty acid biosynthesis (Kauffman et al., 2011). In amino acid metabolism, glycine and serine required for tumor growth can be produced de novo and can also be scavenged from the extracellular environment (Jain et al., 2012; Maddocks et al., 2013).

Nucleotide metabolism also involves redundant and convergent biosynthetic pathways. Deoxyribonucleotide triphosphate (dNTP) pools required for DNA replication and repair can be produced by the de novo pathway (DNP) or by the nucleoside salvage pathway (NSP; Fig. 1 A; Reichard, 1988). The DNP uses glucose and amino acids to generate ribonucleotide di- and triphosphates (NDPs), which are converted to...
Targeting switchable nucleotide synthesis in leukemia | Nathanson et al.

deoxyribonucleotide diphosphates (dNTPs) by ribonucleotide reductase (RNR). The same dNTPs can also be produced via the NSP (Reichard, 1988), starting with extracellular deoxyribonucleosides (dNs) which are imported in the cell via specialized transporters. The first enzymatic steps in the cytosolic NSP are catalyzed by two kinases: thymidine kinase 1 (TK1) phosphorylates thymidine (dT), while deoxycytidine (dC) kinase (dCK) phosphorylates dC, deoxyadenosine (dA), and deoxyguanosine (dG; Reichard, 1988). The relevance of these two NSP kinases for dNTP production in normal and malignant cells is yet to be defined. Because dN substrates for the NSP kinases are absent from most cell culture media, it has been assumed that the NSP is dispensable for DNA replication (Xu et al., 1995). However, recent in vivo findings have challenged this assumption. For example, we reported impaired hematopoiesis in dCK−/− mice due to dCTP pool deficiency, resulting in replication stress (RS), S-phase arrest, and DNA damage in hematopoietic progenitors (Toy et al., 2010; Austin et al., 2012). Analyses of dCK−/TK1− double-knockout mice showed that NSP-derived dCTP synthesis is required to compensate for the inhibition of de novo dCTP production (Austin et al., 2012; Fig. 1 A). The mechanism of DNP inhibition involves allosteric regulation of RNR-mediated reduction of cytidine diphosphate (CDP) to dCTP by dTTP produced via TK1 from endogenous dT (Austin et al., 2012; Fig. 1 A). Production of dNTPs by the NSP may be therapeutically relevant in cancer. For example, the ability of cancer cells to switch their dCTP synthesis from the DNP to the NSP may explain why dT given as a single dCTP-depleting agent showed limited efficacy in clinical trials (Chiuten et al., 1980; Kufe et al., 1980, 1981). If correct, this hypothesis suggests
that a combination of dT (to inhibit DNP-mediated dCTP production) and a dCK inhibitor (to co-target dCTP production by the NSP) would be more efficacious in killing tumor cells than either treatment alone. Here, we investigate this possibility in the context of acute lymphoblastic leukemia (ALL). We demonstrate that co-targeting both de novo and salvage pathways for dCTP biosynthesis is well tolerated in mice and is efficacious in T-ALL and B-ALL models. We also describe a positron emission tomography (PET)–based assay to noninvasively monitor in vivo pharmacological targeting of dCTP biosynthesis in cancer cells.

RESULTS
dC salvage via dCK prevents dT-induced lethal RS in T-ALL cells

Treatment with dT increases cytosolic dTTP concentration, resulting in allosteric inhibition of dCTP production via the DNP (Fig. 1 A; Reichard, 1988). Accordingly, in CCRF-CEM (CEM) human T-ALL cells, dT increased dTTP and decreased dCTP in a dose-dependent manner (Fig. 1 B). Early S-arrest (Fig. 1 C) was induced by concentrations of dT as low as 50 µM, which increased dTTP ~20-fold and reduced dCTP ~5-fold (Fig. 1 B). Supplementation of CEM cultures with 2.5 µM dC completely prevented dT-induced S-phase arrest (Fig. 1 C). Addition of dC did not prevent S-phase arrest in CEM cells treated with the RNR inhibitor hydroxyurea, 5-FU (5-fluorouracil), or cisplatin (Fig. 1 D), indicating that dC salvage via dCK prevents dT-induced lethal RS in T-ALL cells.

To study the role of dCK in the prevention of dT-induced S-phase arrest by dC addition, we generated CEM dCKlow cells (Fig. 1 E) using a dCK-targeted shRNA vector. Knocking down dCK reduced [3H]-dC uptake by ~95% (Fig. 1 F) and decreased cytosolic dCTP levels by ~30% (Fig. 1 G) but did not perturb normal cell cycle progression (Fig. 1 H). Supplementation of cell culture media with 2.5 µM dC restored the dCTP pool in dT-treated dCKlow cells to ~55% of its baseline value but had no effect on dT-induced dCTP pool depletion in dCKwt cells (Fig. 1 G). Consequently, dC addition prevented dT-induced S-phase arrest only in CEM dCKwt cells (Fig. 1 C) and not in CEM dCKlow cells (Fig. 1 H). Accordingly, in the presence of both dT and dC, only dCKlow and not dCKwt CEM cells displayed the following: activation of the RS response marker Chk1 phosphorylated on Ser345 (pChk1; Fig. 1 I); induction of DNA damage, as determined by activation of Chk2 phosphorylated on Thr68 (pChk2; Fig. 1 J); pH2.A.X staining by flow cytometry (Fig. 1 J), as well as by comet assay (Fig. 1 K); and apoptosis (Fig. 1 L). Thus, down-regulation of dCK expression in CEM cells abolished their ability to compensate for dT-mediated inhibition of dCTP production via the DNP, resulting in dCTP depletion, stalled DNA replication, RS, DNA damage, and apoptosis.

In T-ALL cells, dT triggers a metabolic switch to NSP-dCTP production and up-regulates dC salvage

To investigate the biochemical mechanism by which the NSP compensates for dT-mediated DNP inhibition, we quantified the contributions of each dCTP biosynthetic pathway to both the free cytosolic dCTP and the dCTP incorporated into the DNA. CEM cells were incubated for 12 h with [U-13C]-glucose, the substrate for the DNP, and with [U-13C/15N]-dC, the substrate for the NSP (Fig. 2 A). Heavy isotope–labeled dCTP species were detected by combined liquid chromatography–tandem mass spectrometry in the multiple reaction-monitoring mode (LC/MS/MS-MRM). Mass additions between 3 and 8 identified dCTP produced from [U-13C]-glucose via the DNP to NSP-mediated dCTP biosynthesis in T-ALL cells and up-regulates the NSP. (A) Schematic of the [U-13C]-glucose and [U-13C/15N]-dC stable isotope labeling approach used to determine the source (DNP or NSP) of the free dCTP pool and of the dCTP incorporated into the DNA of CEM cells treated with various dT concentrations. (B) dCTP derived from [U-13C]-glucose (DNP) and [U-13C/15N]-dC (NSP) in the free dCTP pool and incorporated into the DNA of CEM cells after 12 h of incubation with stable isotope–labeled DNP and NSP precursors, in the presence or absence of dT. Values are the mean of absolute peak area/106 cells ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with 0 µM dT control. Data are representative of n = 2 independent experiments. (C) Quantification of dCK kinase activity in CEM cells at baseline and after 8 h of treatment with 50 µM dT. Data are representative of n = 2 independent experiments. Values are mean ± SEM. ***, P < 0.001. (D) Quantification of the uptake of [3H]-labeled dC by CEM cells at baseline and after 4 h of treatment with 50 µM dT. Data are representative of n = 2 independent experiments. Values represent mean ± SEM. ***, P < 0.001.
into the DNA. During the 0–4-h timeframe, dCTP decreased several-fold in both CEM dCKwt and dCKlow xenografts and then started to recover as plasma dT dropped to baseline values.

In vivo, salvage of endogenous dC rescues T-ALL cells from RS induced by dT treatment

To examine whether findings from cell culture studies (Figs. 1 and 2) can be recapitulated in vivo, s.c. CEM dCKwt and dCKlow xenografts were established in NOD SCID gamma (NSG) mice. Plasma dT peaked at ~1.5 mM, 2 h after treatment with a single dT injection (2 g/kg, intraperitoneally) and then rapidly declined to baseline values (~10 µM) at 8 h (Fig. 3 A). Intratumoral dTTP increased in both dCKwt and dCKlow tumors for at least 4 h after dT administration (Fig. 3 A). In dCKwt tumors, dT induced a slight and transient up-regulation of pChk1 at the 2- and 4-h time points (Fig. 3 B). In marked contrast, a more pronounced and sustained pChk1 up-regulation was induced by dT treatment in dCKlow tumors (Fig. 3 B). These findings suggest that dCK is required to enable CEM cells to resist RS induced by dT treatment in vivo.

To understand the role of dCK in dCTP production and utilization in tumors from dT-treated mice, we measured the free dCTP pool and incorporation of NSP-produced dCTP into the DNA. During the 0–4-h timeframe, dCTP decreased several-fold in both CEM dCKwt and dCKlow xenografts and then started to recover as plasma dT dropped to baseline values.
preventing RS induction. Moreover, similar to in vitro findings (Fig. 2 B), dT treatment in vivo increases the incorporation of NSP-produced dCTP into tumor DNA. To determine if the increase in the utilization of the NSP-produced dCTP for DNA synthesis in tumors from dT-treated mice is also associated with an up-regulation of the NSP as shown in vitro (Fig. 2, C and D), we took advantage of 18F-L-FAC (1-[(2-deoxy-2-18F-fluoroarabinofuranosyl)cytosine], a fluorinated dC analogue (Radu et al., 2008; Shu et al., 2010). 18F-L-FAC crosses the cell membrane via nucleoside transporters and accumulates specifically in dCK-expressing cells by a phosphorylation-dependent mechanism (Fig. 3 F); dCK-dependent phosphorylated 18F-L-FAC retention in living animals can be imaged and quantified noninvasively by PET. As anticipated, dCKlow tumors accumulated ~40% less 18F-L-FAC than dCKwt tumors (Fig. 3 F). 4 h after dT treatment, 18F-L-FAC accumulation increased by ~20% in dCKwt tumors (Fig. 3 G). 18F-L-FAC accumulation also increased in...
The synergy between dT treatment and pharmacological dCK inhibition, we screened to examine whether the NSP can be exploited therapeutically. Development of DI-39, a small molecule, high affinity dCK inhibitor that occupies the substrate binding site of the kinase, may provide a new therapeutic strategy in ALL. Because the NSP is required to prevent dT-induced RS in the dCKlow tumor and dCKnull leukemia cell line L1210-10K (Jordheim et al., 2004) was treated with increasing concentrations of DI-39 far above those required to inhibit dCK activity or to kill CEM cells, the synergistic effect of DI-39/dT combination was synergistic, reducing dCTP in CEM cells by 70% (Fig. 5 F). Although in the presence of dC, neither dT nor DI-39 alone induced RS or apoptosis in CEM cells, the DI-39/dT combination triggered both RS and apoptosis, as measured by Annexin V staining (Fig. 5 H). Notably, when the dCK-null leukemia cell line L1210-10K (Jordheim et al., 2004) was treated with increasing concentrations of DI-39 far above those required to inhibit dCK activity or to kill CEM cells when combined with dT, it did not induce apoptosis, further supporting the selectivity of DI-39 for dCK (Fig. 5 I). The DI-39/dT combination also induced RS (Fig. 5 J) and apoptosis (Fig. 5 K) in four other ALL cell lines (Jurkat, MOLT-4, K562, K562D).
To further investigate the effects of DI-39 on tumor dCTP metabolism, 5.5 h after treatment with dT and/or DI-39, CEM tumor-bearing mice were pulsed for 30 min with [U-\(^{13}\)C/\(^{15}\)N]-dC. LC/MS/MS-MRM was used to quantify label incorporation into DNA. Analogous to our dCK knock-down results (Fig. 3 F), DI-39 significantly reduced [U-\(^{13}\)C/\(^{15}\)N]-dC incorporation into the DNA of CEM cells (Fig. 6 E). Moreover, the DI-39/dT combination promoted RS in CEM tumors, as indicated by pChk1 up-regulation (Fig. 6 F). Together, these findings indicate that DI-39 efficiently inhibits tumor dCK activity in vivo for up to 12 h, the DI-39/dT combination induces RS in CEM cells in vivo, and PET imaging provides a useful PD companion biomarker for DI-39.

To further investigate the effects of DI-39 on tumor dCTP metabolism, 5.5 h after treatment with dT and/or DI-39, CEM tumor-bearing mice were pulsed for 30 min with [U-\(^{13}\)C/\(^{15}\)N]-dC. LC/MS/MS-MRM was used to quantify label incorporation into DNA. Analogous to our dCK knock-down results (Fig. 3 F), DI-39 significantly reduced [U-\(^{13}\)C/\(^{15}\)N]-dC incorporation into the DNA of CEM cells (Fig. 6 E). Moreover, the DI-39/dT combination promoted RS in CEM tumors, as indicated by pChk1 up-regulation (Fig. 6 F). Together, these findings indicate that DI-39 efficiently inhibits tumor dCK activity in vivo for up to 12 h, the DI-39/dT combination induces RS in CEM cells in vivo, and PET imaging provides a useful PD companion biomarker for DI-39.

**DI-39 inhibits tumor dCK activity in vivo and promotes RS when combined with dT**

To evaluate DI-39 in vivo, we determined its pharmacokinetics (PK) in plasma and in tumor tissues. The plasma half-life of DI-39 was ~50 min (Fig. 6 A), and detectable amounts of drug (~15 nM) were present in tumor tissues 8 h after single dose administration (Fig. 6 B). To correlate the amount of DI-39 in plasma and tumor at 2, 4, 8, and 12 h after administration of the drug with the pharmacodynamic (PD) effect of DI-39 (i.e., inhibition of tumor dCK activity), we performed \(^{18}\)F-FAC PET/CT scans of CEM tumor-bearing mice at these time points (Fig. 6 C). DI-39 (50 mg/kg, administered intraperitoneally) reduced \(^{18}\)F-FAC accumulation in tumors by ~30% for up to 8 h (Fig. 6 D). This level of reduction was comparable with that obtained in the dCK knockdown model (Fig. 3 E). The timing of recovery of tumor dCK activity, determined with PET, after DI-39 administration, indicates that sustained target inhibition could be obtained by administering DI-39 every 12 h. Notably, this information could not be obtained from conventional plasma PK measurements (Fig. 6 A).

To further investigate the effects of DI-39 on tumor dCTP metabolism, 5.5 h after treatment with dT and/or DI-39, CEM tumor-bearing mice were pulsed for 30 min with [U-\(^{13}\)C/\(^{15}\)N]-dC. LC/MS/MS-MRM was used to quantify label incorporation into DNA. Analogous to our dCK knock-down results (Fig. 3 F), DI-39 significantly reduced [U-\(^{13}\)C/\(^{15}\)N]-dC incorporation into the DNA of CEM cells (Fig. 6 E). Moreover, the DI-39/dT combination promoted RS in CEM tumors, as indicated by pChk1 up-regulation (Fig. 6 F). Together, these findings indicate that DI-39 efficiently inhibits tumor dCK activity in vivo for up to 12 h, the DI-39/dT combination induces RS in CEM cells in vivo, and PET imaging provides a useful PD companion biomarker for DI-39.

**Pharmacological co-targeting of DNP and NSP dCTP biosynthesis with DI-39 and dT blocks the growth of T-ALL xenografts in mice**

The therapeutic efficacy of the DI-39/dT combination was first tested in mice bearing established s.c. CEM xenografts. Only the combination therapy dramatically reduced tumor burden in these mice, as indicated by end point tumor sizes (Fig. 7 A) and weights (Fig. 7 B). In addition, TUNEL staining from harvested tumors indicated significant induction of DNA breaks only with the DI-39/dT combination (Fig. 7 C). In contrast to findings shown in Fig. 4, dT treatment alone...
The combination therapy is effective against a primary B-ALL systemic model and has minimal effects on the normal hematopoietic progenitor pool.

We next assessed the efficacy of the DI-39/dT combination therapy against short-term cultures of murine BCR-ABL (p185), Arf−/− pre–B ALL cells (p185BCR-ABL/Arf−/−; Williams et al., 2006; Boulos et al., 2011). Although primary B-ALL cells were sensitive in culture to the DI-39/dT combination, they required fourfold more dT than the CEM T-ALL cell line for optimal induction of apoptosis (Fig. 8 A). This finding is consistent with previous clinical observations that B-ALL cells are less sensitive to dT treatment than T-ALL cells (Kufe et al., 1980). To evaluate the efficacy of dT and/or DI-39 in an in vivo B-ALL model, firefly luciferase–marked p185BCR-ABL/Arf−/− cells were inoculated intravenously in NSG mice. 11 d after inoculation, bioluminescence imaging (BLI) of firefly luciferase–marked p185BCR-ABL/Arf−/− ALL-bearing NSG mice treated with vehicle or 50 mg/kg DI-39 revealed substantial
systemic disease with focal BM and spleen localization (Fig. 8 B). Although dT (2 g/kg) treatment significantly reduced BLI signals in BM and spleen, the addition of DI-39 had a more pronounced effect than dT alone (Fig. 8, B and C). To confirm the BLI findings, we also analyzed the leukemia burden in BM by flow cytometry using CD19 (a B cell marker which, in NSG mice, is present only on the leukemia cells; Fig. 8 D). Treatment with dT induced a significant decrease in the percentage of p185BCR-ABL+/−/− ALL cells relative to vehicle-treated mice (Fig. 8 D). The addition of DI-39 resulted in an additional ~2-fold reduction in the percentage of leukemic cells compared with dT alone (Fig. 8 D). These findings, using primary p185BCR-ABL+/−/− cells, indicate that the DI-39/dT combination is effective against an aggressive in vivo B-ALL model.

In parallel with analyses of BM-resident leukemic cells, we also assessed the effects of the combination therapy on the hematopoietic progenitor pool. We analyzed the Lineage− Sca−1+ c-Kit+ (LSK) HSC population, as well as short-term (ST), long-term (LT), and multipotent progenitor (MPP) hematopoietic progenitor cells. With the exception of a minor decrease in the percentage of LSK upon dT treatment (Fig. 8 E), there were no significant changes between control and treated groups (Fig. 8, E and F; and Fig. S1, A and B). Therefore, the combination therapy preferentially targets BM-resident leukemia cells while sparing normal hematopoietic progenitors. In addition, DI-39 alone or in combination with dT, when administered twice a day for 7 d in NSG mice, did not affect body weight (Fig. 8 G) and had no detectable effects on RBCs, hemoglobin, platelets, or neutrophils (Fig. 8 H).

Partial inhibition of dCK in hematopoietic tissues prevents hematological toxicity from dT and DI-39
To further investigate the potential hematological toxicity of the combination therapy, we took advantage of our dCK−/− mice (Austin et al., 2012). This approach allowed us to directly compare the effects on the hematopoietic system induced by complete loss of dCK function in the dCK−/− mice with the effects induced pharmacologically in dCK wild-type mice (dCK+/−) by DI-39 and dT. In the erythroid lineage, the DI-39/dT combination induced markedly less DNA damage and genotoxicity in the dCK−/− mice, as measured by pH2A.X staining (Fig. 9 A) and the micronucleus assay, respectively (Fig. 9 B), than did dCK gene elimination alone in dCK−/− mice. These findings indicate that pharmacological inhibition of dCK activity by DI-39, alone or in combination with dT treatment, is better tolerated than complete elimination of dCK enzymatic activity by genetic dCK gene inactivation.

DISCUSSION
We demonstrate here a requirement for a functional NSP in T-ALL and B-ALL cells to prevent dCTP pool insufficiency, RS, and apoptosis after pharmacological inhibition of de novo dCTP synthesis. We introduce DI-39, a new small molecule inhibitor of dCK; dCK is the kinase required for the compensatory metabolic switch, triggered by dT-mediated DNP inhibition, to NSP-dependent dCTP biosynthesis. We elucidate how DI-39 inhibits dCK by obtaining a high-resolution crystal structure of the inhibitor–dCK complex. We demonstrate the therapeutic efficacy of co-targeting both the DNP and NSP dCTP biosynthetic pathways, using in vivo models of T-ALL and B-ALL, without detectable toxicity against normal hematopoietic progenitors. We also describe a companion pharmacodynamic PET assay of dCK enzyme activity, which allows noninvasive in vivo imaging of pharmacological interventions targeting dCTP biosynthesis.

Selectivity of the DI-39/dT combination therapy for leukemic cells relative to normal hematopoietic progenitors
Our current working model to explain the mechanism and observed selectivity of the combination therapy for leukemia
cells relative to normal hematopoietic progenitors is depicted schematically in Fig. 9 (C and D). According to this model, pharmacological co-targeting of the DNP (by dT) and of the NSP by DI-39 is highly effective at inducing lethal RS against T- and B-ALL cells and has minimal effects on normal hematopoietic cells. As indicated by 18F-FAC PET imaging of dCK activity (Fig. 6 D and Fig. 9 D), DI-39 induced partial inhibition of dCK in normal BM cells compared with the complete loss of dCK activity in dCK−/− mice (Toy et al., 2010; Austin et al., 2012). The residual dCK activity in BM cells after DI-39 treatment may be sufficient to prevent the more substantial reductions observed for the dCTP pools of hematopoietic progenitors in the dCK−/− mice. This model of low or absent toxicity due to partial inhibition of the therapeutic target is reminiscent of recent work in which hypomorphic ATR suppression was lethal to tumor tissues exposed to oncogenic stress yet had only minimal toxicity to normal tissues (Bartek et al., 2012; Schoppy et al., 2012). Furthermore, the enhanced susceptibility of ALL cells to a reduced supply of dCTP could reflect the inherent inability of these leukemic cells to mount an efficient RS response. Although additional studies are required to precisely identify the defects in cell cycle checkpoints that increase the susceptibility of ALL cells to RS induced by dNTP insufficiency, when compared with normal hematopoietic progenitor cells, we note the presence of inactivating TP53 mutations in several tested ALL cell lines. In this context, it has been suggested that, in normal cells with wild-type p53, the skewing in dNTP pools induced by inhibition of de novo pyrimidine synthesis by PALA (N-(phosphonacetyl)-L-aspartate) creates reversible DNA damage that is sufficient to activate p53 and induce the expression of proteins that provide protective arrest at multiple cell cycle checkpoints (Hastak et al., 2008). In cancer cells with defects in p53 or in its downstream effectors, failure to arrest DNA synthesis when pyrimidine dNTP pools are depleted leads to irreversible DNA damage that eventually causes apoptosis (Hastak et al., 2008).

**Potential clinical implications**

High avidity for dT has been previously identified as a potential metabolic liability of certain cancers, leading to clinical studies using high dT doses as a potential therapeutic (O’Dwyer et al., 1987). Prolonged (over 5 d) dT infusions have shown responses in heavily pretreated T-ALL and cutaneous T cell lymphoma patients, with the side effects encountered being tolerable, manageable, and reversible (Chu et al., 1980; Kufe et al., 1980, 1981). However, therapeutic responses to dT in these patients were, in general, limited and transient, potentially reflecting the ability of the NSP, via dCK, to compensate for the dCTP-depleting effect of dT. Because potent small molecule inhibitors of dCK have recently been described (Yu et al., 2010; Murphy et al., 2013), future clinical studies can determine if the anti-leukemic activity of dT reported in T-ALL and cutaneous T cell-lymphoma patients can be significantly improved by pharmacological blockade of the dC salvage pathway.

**Companion diagnostics for therapies targeting dCTP biosynthetic pathways in cancer**

The data presented here provide examples of both in vivo and in vitro companion diagnostics (or biomarkers) that could assist the clinical translation of the DI-39/dT combination therapy. For example, direct assessments of temporal changes in tumor dCK activity in vivo with PET were more useful than conventional plasma pharmacokinetic measurements for identifying the optimal schedule for the DI-39/dT combination therapy (Fig. 6). Because our PET assays for monitoring dCK activity have already been translated to humans (Schwarzenberg et al., 2011), approaches similar to those described in our preclinical experiments could be used in future clinical trials to noninvasively monitor dCK inhibition in target tissues in vivo. Up-regulation of pChkl and pH2A.X levels by leukemia cells upon DI-39/dT treatment (Fig. 6 F) could provide additional pharmacodynamic biomarkers of DNA damage, as shown previously for PARP inhibitors (Fong et al., 2009). Furthermore, because the efficacy of the DI-39/dT therapy depends on the capacity of tumors to cells to take up large amounts of dT and convert it to dTTP, PET imaging using 18F-FLT (3’-deoxy-3’-fluorothymidine), a probe for dT metabolism (Shields et al., 1998), may enable the identification of tumors with unusually high avidity for dT. Thus, 18F-FLT PET may match the proposed definition of a predictive or enrichment biomarker (de Bono and Ashworth, 2010) for dT-based therapies.

**Regulation of the NSP by the DNA damage response (DDR) pathway**

Our in vitro (Fig. 2 C) and in vivo (Fig. 3, E and F) data indicate that, in CEM T-ALL cells, dT treatment up-regulated the activity of the NSP. Although NSP up-regulation by dT treatment may result from a decrease in the negative feedback by dCTP on dCK activity (Datta et al., 1989), additional mechanisms could also be involved. For example, dCK activity is increased by treatment with DNA damaging agents that do not affect dCTP production via the DNP (Ooi et al., 1996; Casper et al., 2003). Moreover, dCK activation after DNA damage involves phosphorylation of the kinase on serine 74 (Yang et al., 2012). This serine is part of an SQ/TQ motif, which is a typical phosphorylation site for ATM and ATR kinases in the DDR pathway. Indeed, dCK has been identified as a direct target of these kinases (Matsuoka et al., 2007). Therefore, after DNA damage induced by high dose dT and, potentially, by other genotoxic therapies, dATR pathway may promote NSP up-regulation via posttranslational regulation of dCK to expand dNTP pools and facilitate DNA repair. If correct, this model provides a rationale for testing dCK inhibitors in combination with radiation therapy and other genotoxic therapies.

In summary, our results provide new insight into the nucleotide metabolism of leukemic cells and also demonstrate a new therapeutic strategy to overcome the redundancy and adaptability of nucleotide metabolism in ALL and, possibly, in other hematological malignancies in which uncontrolled expansion of the dTTP pool by dT treatment results in a potential metabolic liability. Similar approaches that fit within the
conceptual framework of targeting non-oncogene addiction (Luo et al., 2009) may be applicable to other redundant biosynthetic pathways that provide survival advantages to tumor cells.

**MATERIALS AND METHODS**

**Cell lines and culture conditions.** Human cell lines CCRF-CEM, Jurkat, MOLT-4, RSR-4;11, and TF-1 were obtained from American Type Culture Collection. NALM-6 and L1210-10K cells were a gift from M. Teitell (UCLA) and C. Dumontet (Université ´Clermont Bernard Lyon I, Lyon, France), respectively. All cell lines were maintained in 5% FBS in RPMI-1640 and were grown at 37°C, 20% O2, and 5% CO2.

**Animals.** Mice were bred and housed under specific pathogen-free conditions and were treated in accordance with the UCLA Animal Research Committee protocol guidelines. The dCK−/− were generated and bred as previously described and backcrossed to C57BL/6 mice for n = 7 generations (Toy et al., 2010; Austin et al., 2012). Age-matched (5–12 wk old) WT and dCK−/− littermates were used to assess KS induction by dT in BM myeloid cells.

**Reagents, antibodies, immunoblotting, and flow cytometry.** dT, 2′-dC, hydroxyurea, 5-FU, and cisplatin (all Sigma-Aldrich) were prepared in DMSO or water. Lentiviral shRNA constructs against dCK and nontargeting control were from Sigma-Aldrich. For cell culture assays, dCK inhibitors were reconstituted in DMSO. Immunoblotting was performed as previously described (Austin et al., 2012). The following antibodies and reagents for immunoblotting were purchased: phospho-Chk1 Ser-345, phospho-Chk2 Thr-68, Chk1, Chk2, anti–mouse HRP-conjugated IgG, and anti–rabbit HRP-conjugated IgG (Cell Signaling Technology); dCK and β-actin (Sigma-Aldrich); and TK1 (Abcam). Bound antibody was detected using chemiluminescence immunoblotting detection reagents (Thermo Fisher Scientific). Isolation and FACs phenotyping of hematopoietic stem cells, EryA, and myeloid was performed as previously described (Austin et al., 2012). The p185−/−;Bcr-Mis/Arf−/− cells were identified using an anti–CD19 (APC) antibody. For cell cycle analyses, total DNA content was determined using 1 µg/ml DAPI or 20 µg/ml propidium iodide containing 5 µg/ml RNase A. Annexin V staining was performed according to the manufacturer’s protocol (BD). For the micronucleus assay, isolated BM cells were stained with the following antibodies (eBiosciences): Ter119 PerCP-Cy5.5 (TER-119), CD71 APC (R17217), CD45 PE-Cy7 (30-F11), CD61 PE (2C9.G3), and CD11b APC-eFluor780 (M1/70). Bound antibodies were detected using chemiluminescence immunoblotting detection reagents (Thermo Fisher Scientific). Isolation and FACs phenotyping of hematopoietic stem cells, EryA, and myeloid was performed as previously described (Austin et al., 2012). The p185−/−;Bcr-Mis/Arf−/− cells were identified using an anti–CD19 (APC) antibody. For cell cycle analyses, total DNA content was determined using 1 µg/ml DAPI or 20 µg/ml propidium iodide containing 5 µg/ml RNase A. Annexin V staining was performed according to the manufacturer’s protocol (BD). For the micronucleus assay, isolated BM cells were stained with the following antibodies (eBiosciences): Ter119 PerCP-Cy5.5 (TER-119), CD71 APC (R17217), CD45 PE-Cy7 (30-F11), CD61 PE (2C9.G3), and CD11b APC-eFluor780 (M1/70). Cells were stained, washed, and fixed with Cytofix/Cytoperm solution (BD). Cells were then washed and stained with 1 µg/ml DAPI in PBS/2% FBS. All flow cytometry data were acquired on a four-laser LSRII cytometer (BD) and analyzed using FlowJo (Tree Star).

**Measurements of dT and DI-39 pharmacokinetics in mice.** NOD SCID gamma (NSG) mice were injected with 2 mg/kg dT intraperitoneally. 75 µl of whole blood was obtained at 0, 2, 4, and 8 h through retro-orbital sinus bleeds using heparinized capillary tubes. Whole blood was immediately centrifuged at 3,000 × g for 5 min to isolate serum. 30 µl of serum was mixed with 1 ml methanol/acetonitrile (1:9), vortexed for 2 min, and centrifuged at 14,000 × g for 4 min at 4°C. Extraction was repeated and the pooled supernatant was dried under vacuum centrifugation. The residue was dissolved in 100 µl water, filtered, and eluted through a microBondapak C18 column (Waters) under a gradient mobile phase from 2 to 50% methanol over 10 min at a flow rate of 1.5 ml/min. dT was detected by absorbance intensity (254 nm), and peak areas were normalized to the internal standard and tumor weight.

The experiment using CCRF-CEM cells to measure the uptake of DI-39 in cell culture followed a similar protocol as the one described above. CCRF-CEM cells were cultured in 5% FBS in RPMI-1640 media supplemented with 1 µM DI-39 for 10, 30, 40, and 60 min before cell extraction. For some samples, the media with 1 µM DI-39 was removed and the cells were washed three times in PBS before adding fresh media without DI-39 for 60 min. The cells were extracted and homogenized in 1 ml ice-cold acetonitrile/water (50/50, vol/vol) containing the internal standard as mentioned before. The cell extract was left overnight at 4°C on a shaker and the next day centrifuged at 20,000 × g for 10 min. 700 µl of the supernatant was transferred to a clean tube and was evaporated to dryness in a vacuum centrifuge. The residue was reconstituted in 100 µl acetonitrile/water (50/50, vol/vol). For plasma measurements, ~100 µl of blood was collected through a retro-orbital sinus bleeds using capillary blood collection tubes. Samples were centrifuged at 20,000 × g for 5 min, and 30 µl of the supernatant was transferred into a clean tube. The sample was mixed with 500 µl ice-cold acetonitrile/water (50/50, vol/vol) containing the internal standard and processed in the same way as the tumor homogenates. Calibration standards were prepared by spiking working stock solution of DI-39 in tumor homogenates and plasma from untreated mice to give the following range: 0.02–20 pmol/µl. 5-µl samples were injected onto a reverse phase column (ZORBAX Rapid Resolution High Definition [RRHD] Eclipse Plus C18 [Agilent Technologies], 2.1 × 50 mm, 1.8 µm) equilibrated in water/acetonitrile/formic acid, 95/5/0.1, and eluted (200 µl/min) with an increasing concentration of solvent B (acetonitrile/formic acid, 100/0.1, vol/vol: min%/acetonitrile; 0/5, 0/5, 2/9, 8/80, 9/80, 10/5, and 12/5). The effluent from the column was directed to an electro spray ion source (Jet Stream; Agilent Technologies) connected to a triple quadrupole mass spectrometer (6460 QQQ, Agilent Technologies) operating in the positive ion MRM mode. The ion transitions for DI-39 and DI-70 (m/z 483.3 and 550.2) were monitored. The MRM analysis was performed as previously optimized conditions. The DI-39 peak areas were normalized to the internal standard and tumor weight.

**Incorporation of stable isotope–labeled glucose and dC into the free dCTP pool and into DNA.** CEM cells were transferred into RPMI supplemented with 5% dialyzed FCS containing 10 µM uniformly labeled [U-13C6/15N4]-dC (Cambridge Isotopes), 2 g/liter of uniformly labeled [U-13C6]-glucose (Cambridge Isotopes), and 0, 50, or 250 µM dT. For the dNTP analysis, the cells were extracted overnight at ~20°C with 75% methanol. The extracts were then heated in boiling water for 3 min, pelleted, and the supernatants were transferred and dried under vacuum centrifugation. For DNA analysis, cells were collected and genomic DNA was extracted using the Quick-gDNA MiniPrep kit (Zymo Research). Genomic DNA was then digested to nucleosides using the DNA Degradase Plus kit (Zymo Research).

For the in vitro studies, tumors-bearing mice were injected with 200 µl of 2.5 mM [U-13C6/15N4]-dC 30 min before sacrifice. Tumors were harvested, mechanically digested into single cells, and cell counts were obtained. DNA extraction was performed as described above.
DNA hydrolysis samples were diluted 1/1 with solvent A (water/formic acid, 100/0.2, vol/vol) and analyzed using a modified version of a previously reported method (Cohen et al., 2009) in which 10-µl aliquots of the solution were injected onto a porous graphite carbon column (Thermo Hypersil, 100 × 2.1 mm, 3-µm particle size) equilibrated in solvent A and eluted (300 µl/min) with an increasing concentration of solvent B (acetonitrile/min%/vol B; 0/0, 6/60, 6/100, 9/100, 9/1.0, and 10/0). The effluent from the column was directed to Jet Stream-connected 6460 QQQ (Agilent Technologies) operating in the positive ion MRM mode. After verification of retention times using authentic standards, the peak areas of the M→H−→fragment ion transitions for the dC isotopomers (M1, 228.1→112.1; M2, 229.1→112.1; M3, 230.1→112.1; M4, 231.1→112.1; M5, 233.1→112.1; M6, 234.1→113.1; M7, 235.1→114.1; M8, 236.1→115.1; M9, 237.1→116.1; M10, 238.1→117.1; M11, 239.1→118.1; M12, 240.1→119.1) were recorded with instrument manufacturer-supplied software (MassHunter; Agilent Technologies) and normalized to cell number. The dC isotopomers of M1 through M4 for the DNP and M1 through M2 for the NSP were detected and used for data analysis.

For free dNTP analysis, a modified version of the same previously reported method (Cohen et al., 2009) was used in which dried samples were redissolved in 100 µl solvent C (5 mM hexylamine and 0.5% mM diethylamine, pH 10.0) and 10-µl aliquots were injected onto porous graphite carbon column (Hypercarb, 150 × 2.1 mm, 3-µm particle size; Thermo Fisher Scientific) equilibrated in solvent C and eluted (150 µl/min) with an increasing concentration of solvent D (acetonitrile/min%/vol B; 0/0, 5/0, 5/60, 25/100, 30/100, 30/1.0, and 40/0). The effluent from the column was directed to the same instrument described above, operating in the negative ion mode. After verification of retention times using authentic standards, the intensities of preselected (M→H+)→fragment ion transitions for various dCTP isotopomers (M1, 466.0→159.0; M2, 467.0→159.0; M3, 468.0→159.0; M4, 469.0→159.0; M5, 470.0→159.0; M6, 471.0→159.0; M7, 472.0→159.0; M8, 473.0→159.0; M9, 474.0→159.0; M10, 475.0→159.0; M11, 476.0→159.0; M12, 477.0→159.0; M13, 478.0→159.0) were recorded, again with instrument manufacturer-supplied software (MassHunter), and normalized to cell number. The dCTP isotopomers of M1 through M4 for the DNP and M1 through M2 for the NSP were detected and used for data analysis. The M1 and M2 isotopomers were not detected.

dNTP pool measurements. Intracellular dNTP pool measurements were conducted as previously described (Austin et al., 2012).

Comet assay. The comet assay was performed according to the CometAssay reagent kit protocol (Trevigen) under alkaline conditions. For quantification, four random sections of each slide containing >100 cells were imaged and Olive Tail Moment obtained using Cometscore (TriTek) software.

Generation of luciferase viral construct, and retroviral gene transduction. The gene encoding humanized secreted Gaussia luciferase (GLuc), pCMV-GLuc-1 (Nanolight Technology), was subcloned into the MSCV-IRES-GFP retroviral vector. Phoenix-Ampho cells were transfected with the generated vector using Lipofectamine transfection reagent (Invitrogen). 48 h after transfection, virus was harvested and used to transduce CEM dCK+ and CEM dCK− cells. GFP-positive cells were sorted with a FACSAria II cell sorter (BD).

Crystallization of DI-39 in complex with dCK and UDP. The 4C S74E dCK variant used for crystallographic studies was expressed and purified as described in Nomme et al. (2014). Crystallization, x-ray data collection, and refinement were also performed as described in Nomme et al. (2014). In brief, crystals of dCK in complex with UDP, MgCl2, and a 2.5-fold excess of the DI-39 inhibitor were grown using the hanging drop vapor diffusion method at 12°C. The reservoir solution contained 0.9–1.5 M trisodium citrate dehydrate and 25 mM Heps, pH 7.5. Diffraction data were collected at the Advanced Photon Source, Argonne National Laboratory on Life Sciences-Collaborative Access Team (LS-CAT) beamlines 21 ID-C.

Mouse xenograft tumor models and treatments. CEM xenograft tumors were developed in 8–12-wk-old female NSG mice by implanting 2 × 106 CEM dCK−-sGluc-GFP and/or dCK−-sGluc-GFP cells in 100 µl of equal volume Matrigel (BD) and RPMI i.v. in the flanks. Tumor growth was monitored daily by caliper measurements [(length × width)²/2] and blood Gaussia luciferase (GLuc) assay (Tannous, 2009). 10 µl of blood was collected via tail vein nick and mixed with 2 µl of 50 mM EDTA. 1 µl of blood was mixed with 99 µl PBS and transferred to a 96-well OptiPlate (Perkin Elmer). 100 µl of 20 µM coelenterazine substrate was mixed and luciferase activity was measured using a plate luminescence microplate reader SpectraMax L (Molecular Devices). Systemic tumor models were established by intravenous injection of 106 CEM dCK−-sGluc-GFP or dCK−-sGluc-GFP in 100 µl RPMI. 2 g/kg dT was administered in saline and DL-39 in a mixture of 1.4% DMSO and 40% Captisol (Ligand Pharmaceuticals) mixture.

TUNEL assay. Tumors from CEM xenografts were harvested and fixed overnight in 10% buffered formalin solution. Samples were then paraffin-embedded and 5-µm sections were mounted on glass slides. TUNEL staining was performed according to the manufacturer’s protocol (Roche). Stained slides were subsequently scanned on a ScanScope AT (Aperio) and analysis was conducted using Tissue Studio 64 (Dual) 3.5 (Definiens AG).

dCK kinase and uptake assays. These assays were performed as previously described (Shu et al., 2010).

Peripheral blood counts. All mice were anesthetized and whole blood was obtained through cardiac puncture. For peripheral blood counts, samples were collected in tubes containing EDTA and submitted to UCLA Division of Lab Animal Medicine for analysis.

PET imaging. PET/CT studies were performed as previously described (Radu et al., 2008; Shu et al., 2010).

Pharmacokinetic studies of DL-39 in mice. This assay was performed as previously described (Murphy et al., 2013).

Statistical analyses. All statistics are presented as averages of biological replicates ±SEM, unless indicated. P-value significances were calculated from multiple replicates within a dataset representative of multiple independent experiments, as indicated, using one sample Student’s t test function in Prism 5 (GraphPad Software).

Online supplemental material. Fig. S1 shows the FACS gating strategy to identify hematopoietic progenitor populations quantified in Fig. 8 (E and F). Table S1 shows data collection and refinement statistics for the DL-39-dCK crystal structure. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131738/DC1.

We thank Larry Pang for his assistance with PET/CT imaging studies, the UCLA Biomedical Cyclotron for producing the PET probes used in this study, and Christopher Ryan with his help with the UCNS/MS-MRM assays. We also thank Dr. Nagichettiar Satyamurthy for his advice and expertise on the synthesis of dCK inhibitors, and Drs. Norman Hardman, Ken Herrmann, and Ting-Ting Wu for critically reading the manuscript. We acknowledge the UCLA Jonsson Comprehensive Cancer Center and the UCLA Institute for Molecular Medicine for their use of their equipment and Dr. Jonathan Said for help with immunohistochemistry. D.A. Nathanson, J.M. Murphy, and T.M. Le are supported by the UCLA Scholars Program. C.G. Radu, M.E. Phelps, and J. Czernin are co-founders of Sofie Biosciences, a molecular diagnostic company. They hold equity in Sofie Biosciences. The University of California also holds equity in Sofie Biosciences. C.G. Radu and J. Czernin are among the authors declare the following competing financial interests: C.G. Radu, M.E. Phelps, and J. Czernin are co-founders of Sofie Biosciences, a molecular diagnostic company. They hold equity in Sofie Biosciences. The University of California also holds equity in Sofie Biosciences. C.G. Radu and J. Czernin are among...