ATR inhibition facilitates targeting of leukemia dependence on convergent nucleotide biosynthetic pathways

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Leukemia cells rely on two nucleotide biosynthetic pathways, de novo and salvage, to produce dNTPs for DNA replication. Here, using metabolomic, proteomic, and phosphoproteomic approaches, we show that inhibition of the replication stress sensing kinase ataxia telangiectasia and Rad3-related protein (ATR) reduces the output of both de novo and salvage pathways by regulating the activity of their respective rate-limiting enzymes, ribonucleotide reductase (RNR) and deoxycytidine kinase (dCK), via distinct molecular mechanisms. Quantification of nucleotide biosynthesis in ATR-inhibited acute lymphoblastic leukemia (ALL) cells reveals substantial remaining de novo and salvage activities, and could not eliminate the disease in vivo. However, targeting these remaining activities with RNR and dCK inhibitors triggers lethal replication stress in vitro and long-term disease-free survival in mice with B-ALL, without detectable toxicity. Thus the functional interplay between alternative nucleotide biosynthetic routes and ATR provides therapeutic opportunities in leukemia and potentially other cancers.
Unabated proliferation is a hallmark of cancer which requires new DNA synthesis from deoxyribonucleotide triphosphates (dNTPs). However, cellular dNTP levels only suffice to sustain a few minutes of DNA replication indicating that dNTP pools are produced ‘on demand’ via tightly regulated biosynthetic pathways. These deoxynucleotide biosynthetic pathways, termed de novo and salvage, rely on distinct carbon and nitrogen sources. De novo pathways use glucose and amino acids to produce ribonucleotide diphosphates (rNDPs) which are converted into deoxyribonucleotide diphosphates (dNDPs) by ribonucleotide reductase (RNR), a two-subunit enzyme complex upregulated in most cancers. Salvage pathways convert preformed ribonucleosides, deoxyribonucleosides and nucleobases into nucleotides through the actions of metabolic kinases and phosphoribosyltransferases. Amongst nucleoside salvage kinases, deoxycytidine kinase (dCK) has the broadest substrate specificity, encompassing both purine and pyrimidine nucleosides. While tumors are thought to predominantly rely on de novo pathways to produce nucleotides, scavenging of preformed nucleosides via dCK and other salvage kinases may also play important roles in the economy of nucleotide metabolism in cancer cells. Many of the cell lines included in the Cancer Cell Line Encyclopedia express dCK at higher levels than the corresponding normal tissues. Increased tumor dCK expression relative to matched normal tissues also occurs in patient samples, as evidenced by RNASeq data from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) and/or DI-82, a high-affinity dCK inhibitor (dCKi) developed by our group. At various time points following G1 release, cells were pulsed for 1 h with U-13C6-glucose and U-13C9,15N3-deoxyuridine (EdU) to analyze cell cycle kinetics by flow cytometry. Six hours after release from G1, ~25% of cells in the untreated and single drug treated groups advanced into early S-phase (designated as S1, in blue, Fig. 1a). In contrast, only 16% of cells treated with both VE-822 and dCKi entered S1. Twelve hours after release from G1 arrest, 16% fewer VE-822-treated cells entered the later part of S-phase (designated as S2, in red, Fig. 1b) compared to untreated cells. While at this time point dCK inhibition alone did not affect the number of cells that progressed beyond early S phase, progression to late S-phase was significantly impeded (Supplementary Fig. 1b). Co-inhibition of ATR and dCK decreased the percentage of cells that reached S2 by fivefold relative to untreated cells (Fig. 1b). The effects of ATR inhibition on cell cycle kinetics were partially rescued by nucleotide supplementation, in a dCK-dependent manner (Supplementary Fig. 1c, d).

To further investigate the functions of dCK and ATR at the G1/S transition in CEM cells, a non-targeted liquid chromatography mass spectrometry (LC-MS) assay was used to determine the utilization of labeled [U-13C6]glucose and [U-13C9,15N3]deoxyxycytidine, the main substrates for de novo and salvage nucleotide metabolism in cancer cells (Fig. 1b). The effects of ATR inhibition on cell cycle kinetics were partially rescued by nucleotide supplementation, in a dCK-dependent manner (Supplementary Fig. 1c, d).

**Results**

**ATR and dCK Co-inhibition impairs G1/S transition.** Human T-ALL cells CCRF-CEM (CEM) express dCK and exhibit constitutive phosphorylation of the ATR effector kinase CHEK1 on Serine 345 (pS345, Supplementary Fig. 1a), a marker of replication stress. CHEK1 pS345 levels are reduced following exposure to VE-822, a specific ATR inhibitor (Supplementary Fig. 1a). To investigate whether ATR inhibition increases the dependence of T-ALL cells on dCK activity at the G1/S transition, CEM cells were synchronized in G1 using Palbociclib, a CDK4/6 inhibitor, and then released into media containing VE-822 and/or DI-82, a high-affinity dCK inhibitor (dCKi) developed by our group. At various time points following G1 release, cells were pulsed for 1 h with U-13C6-glucose and U-13C9,15N3-deoxyuridine (EdU) to analyze cell cycle kinetics by flow cytometry. Six hours after release from G1, ~25% of cells in the untreated and single drug treated groups advanced into early S-phase (designated as S1, in blue, Fig. 1a). In contrast, only 16% of cells treated with both VE-822 and dCKi entered S1. Twelve hours after release from G1 arrest, 16% fewer VE-822-treated cells entered the later part of S-phase (designated as S2, in red, Fig. 1b) compared to untreated cells. While at this time point dCK inhibition alone did not affect the number of cells that progressed beyond early S phase, progression to late S-phase was significantly impeded (Supplementary Fig. 1b). Co-inhibition of ATR and dCK decreased the percentage of cells that reached S2 by fivefold relative to untreated cells (Fig. 1b). The effects of ATR inhibition on cell cycle kinetics were partially rescued by nucleotide supplementation, in a dCK-dependent manner (Supplementary Fig. 1c, d).

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Effects of ATR and dCK inhibition on G1-S transition and substrate utilization for dCTP biosynthesis. a, b Flow cytometry analysis of EdU incorporation in CEM T-ALL cells treated with VE-822 (1 μM) and/or dCKi (DI-B1, 1 μM) for 6 a and 12 h b following release from G1 arrest, respectively. Bar graphs summarize the percentage of cell populations in S1 (early S-phase) and S2 (mid to late S-phase) at 6 and 12 h (mean ± s.d., n = 2, one-way ANOVA, Bonferroni corrected). Plots are representative of two independent experiments. c Comparison of metabolite labeling by [13C6]glucose in CEM T-ALL cells treated with VE-822 and/or dCKi for 12 h following release from G1 arrest. Number of metabolites exhibiting alterations in [13C6]glucose labeling greater than 15% with significance at a false discovery rate ≤ 20% are indicated. d Percent glucose labeling of ribonucleotides intermediates in the de novo dCTP biosynthesis (mean ± s.d., n = 6, one-way ANOVA, Bonferroni corrected). e Workflow for targeted LC-MS/MS-MRM analysis of dCTP incorporated into newly replicated DNA using a triple quadrupole mass spectrometer. See text for details and Supplementary Fig. 4 for the LC-MS/MS-MRM analysis of dCTP pools. f, g Contributions of the de novo and salvage pathways to dCTP pools f and dCTP incorporated into newly synthesized DNA g in CEM cells treated with VE-822 and/or dCKi after release from G1 arrest (mean ± s.d., n = 3). Results are representative of two independent experiments. NT = Not treated, V = VE-822, D = dCKi, V + D = VE-822 + dCKi. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ATR ataxia telangiectasia and Rad3-related protein, EDU S'-ethynyl-2'-deoxyuridine, FDR false discovery rate, RNR ribonucleotide reductase, CTPS1/2 CTP synthase 1/2, RSP ribose 5-phosphate, OMP orotidine monophosphate, rCDP cytidine diphosphate, CTP cytidine triphosphate, UTP uridine triphosphate, dCTP deoxycytidine triphosphate, LC-MS/MS-MRM liquid chromatography tandem mass spectrometry operating in multiple reaction monitoring.

biosynthesis, respectively. Of the 166 metabolites identified in CEM cells treated with VE-822 and/or dCKi, 105 metabolites found in all four treatment groups contained glucose-derived 13C atoms. While ATR inhibition did not decrease glucose uptake and labeling of glycolytic intermediates (Supplementary Fig. 2), it significantly decreased glucose utilization for 29 other metabolites (Fig. 1c and Supplementary Data 1). These metabolites included intermediates such as rUTP, rCTP and rCDP in the de novo dCTP biosynthesis (Fig. 1d). These data indicate that ATR inhibition impacts glucose utilization for de novo nucleotide biosynthesis. However, several deoxyribonucleotides, including dCTP, were below the limit of detection of the non-targeted LC-MS approach, raising the concern that the sensitivity of this assay is not sufficient to measure the contribution of the salvage
Table 1 Rates of de novo and salvage produced dCTP incorporation into newly replicated DNA

| Treatment | De novo | Salvage | Ratio (salvage/de novo) |
|-----------|---------|---------|------------------------|
| Not treated | 0.20 ± 0.01 | 0.17 ± 0.01 | 0.83 |
| VE-822 | 0.17 ± 0.01 | 0.14 ± 0.01 | 0.85 |
| dCKi | 0.23 ± 0.01 | 0.019 ± 0.004 | 0.082 |
| VE-822 + dCKi | 0.19 ± 0.01 | 0.022 ± 0.002 | 0.12 |

*Slope ± standard error of regression line in arbitrary unit.

Effects of ATR inhibition on RRM2 and dCK. To investigate the molecular mechanisms underlying the metabolic consequences of ATR and dCK inhibition, global changes in protein expression were assessed in CEM cells using quantitative nano-LC tandem MS (nLC-MS/MS) (Fig. 2a). Chemical isotope coding following reductive dimethylation of peptide N-termini and lysine primary amines with differential stable isotopes (light:medium:heavy) was used to compare expression of experimental (VE-822, dCKi and VE-822 + dCKi) samples to controls (untreated, NT) by mixing equal proportions for triplex nLC-MS/MS quantitative analyses. The data set was filtered for proteins identified in all treatment groups in three independent experiments with coefficients of variation <20%. This yielded 1757 proteins with relative fold changes in treated vs. untreated cells ranging from 0.45 to 1.83. Of these, ~3% (46 proteins) displayed statistically significant (as determined by one-way ANOVA and false discovery rate cutoffs) fold changes in expression (>20%) in at least one treatment group (Fig. 2b and Supplementary Data 2). Protein levels of both RNR subunits, RRM1 and RRM2, as well as thymidylate synthase decreased by more than 20% following ATR inhibition (Fig. 2c). Changes in the expression of de novo enzymes observed in synchronous cells also occurred with ATR inhibition in asynchronous CEM cells (Fig. 2d and Supplementary Data 3), thereby arguing against the possibility of experimental artifacts introduced by Palbociclib-mediated cell cycle synchronization. The reduction in RRM2 levels induced by ATR inhibition was accompanied by an ~50% decrease in the phosphorylation of RRM2 on threonine 33 (pT33) (Fig. 2e), a phosphosite previously linked to the RRM2 stability.

ATR inhibition decreased dNTP levels in several solid tumor-derived cell lines and has been linked to reduced RRM2 levels. However, it remains unclear to what degree RRM2 protein levels are rate-limiting for de novo dCTP biosynthesis. To further investigate the relationship between RRM2 protein levels and dCTP biosynthesis, we knocked down RRM2 in CEM cells using shRNA (Supplementary Fig. 6a). RRM2 levels in the CEM shRNA-RRM2 cells were reduced by 35–50%, as determined by quantitative nLC-MS/MS and intracellular flow cytometry analyses (Supplementary Fig. 6a, b). CEM shRNA-RRM2 cells exhibited ~30% lower incorporation of de novo synthesized dCTP into newly replicated DNA compared to control cells (Supplementary Fig. 6c); a response comparable with the effects of pharmacological ATR inhibition (Fig. 1g). These findings suggest that the RRM2 regulation by ATR is an important determinant of de novo dCTP biosynthesis in T-ALL cells. Since ATR inhibition reduced RRM2 levels by only 20%, it is likely that there are
other mechanisms by which ATR regulates de novo dCTP biosynthesis. These additional mechanisms could include reduced levels of the large RNR subunit, RRM1 (Figs. 2c, d), and/or changes in yet to be identified regulatory PTMs in RRM1 and RRM2 that are modulated directly or indirectly by ATR signaling.

In contrast to the reduced RRM1 and RRM2 levels in asynchronous CEM cells treated with VE-822 (1 µM) for 12 h (mean ± s.d., n = 3, one-way ANOVA, Bonferroni corrected). (f, left panel) Salvage produced \([^{13}C_9,^{15}N_3]dCMP\) in asynchronous CEM cells treated with VE-822 or dCKi for 12 h (mean ± s.d., n = 3, one-way ANOVA, Bonferroni corrected). (f, right panel) Relative levels of dCK pS74, after normalized to dCK protein level from NT, in asynchronous CEM cells treated with VE-822 (1 µM) for 12 h (mean ± s.d., n = 3, one-way Student’s t-test). (g) Summary of the observed effects of ATR and dCK inhibition in CEM cells. | Partial decrease/inhibition | Nearly complete inhibition | Increase | No change | ND not determined. NT = Not treated, V = VE-822, D = dCKi, V + D = VE-822 + dCKi. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. nLC-MS/MS nano liquid chromatography tandem mass spectrometry, RRM1 ribonucleotide reductase subunit 1, RRM2 ribonucleotide reductase subunit 2, TYMS thymidylate synthase, dCK deoxycytidine kinase, dCMP deoxycytidine monophosphate.

**Increased salvage dCTP biosynthesis by RNR inhibition.** To identify the most potent clinically relevant RNR inhibitors that could be used to target the remaining de novo nucleotide biosynthetic activity in ATR inhibited CEM cells we evaluated four compounds, each with a distinct mechanism of action: 3-AP, hydroxyurea (HU), gallium maltolate (GaM), and thymidine (dT) (Fig. 3a). Amongst these, 3-AP was the most...
**Fig. 3** 3-AP potently inhibits RNR and enhances salvage nucleotide biosynthesis. **a** Mechanisms of action of four RNR inhibitors. The two RNR subunits, RRM1 (α) and RRM2 (β) form a catalytically active αβ2 complex. Each RRM1 subunit contains two allosteric regulatory sites (the specificity and activity sites), as well as the active site, where nucleotide reduction occurs. The active form of the RRM2 dimer (holo-β2) houses the di-iron cofactor and the tyrosyl radical (Y-O•). 3-AP forms a complex with Fe2+ which interferes with the regeneration of the tyrosyl radical in RRM2 therefore promoting the formation of an inactive met-β small subunit which retains its di-iron center7. Hydroxyurea (HU) scavenges the RRM2 tyrosyl radical and depletes the di-iron center to form an inactive apo-β form. Gallium maltolate (GaM) releases Ga3+ which mimics Fe3+ and disrupts the RRM2 di-iron center. Thymidine (dT) is converted via the salvage pathway to thymidine triphosphate (dTTP) which binds to the allosteric specificity site on RRM1 to favor GDP reduction over pyrimidine (CDP and UDP) reduction, thereby resulting in dCTP insufficiency.

**b** Effects of RNR inhibitors on cell cycle progression. CEM cells were incubated for 24 h with indicated concentrations of RNR inhibitors followed by cell cycle analyses using flow cytometry. Shown in bold red are the concentrations of each RNR inhibitor required to induce a greater than 45% increase in the S-phase population, indicative of S-phase arrest due to nucleotide insufficiency. Cell cycle plots are representative of two independent experiments. See Supplementary Fig. 8 for quantification.

**c** LC-MS/MS-MRM analysis of dCTP biosynthesis in CEM cells treated with 500 nM 3-AP for 12 h (mean ± s.d., n = 3). NT not treated. CMPK1 uridine-cytidine monophosphate kinase 1, NME1/2 nucleoside diphosphate kinase 1/2, dC 2'-deoxycytidine, dCMP deoxycytidine monophosphate, dCDP deoxycytidine diphosphate.
potent, as indicated by induction of S-phase arrest at concentrations as low as 0.5 µM (Fig. 3b and Supplementary Fig. 7). In contrast, 60–100-fold higher concentrations of HU, GaM and dT were required to induce S-phase arrest. The effects of 3-AP on the utilization of \([^{13}C_6]\)glucose and \([^{13}C_5, ^{15}N_3]\)dC for nucleotide biosynthesis in CEM cells were investigated (Fig. 3c), using the targeted LC-MS/MS-MRM assay. 3-AP doubled the rCTP and rCDP pools, likely reflecting an inefficient conversion of these pools to dCDP via RNR. However, the most significant change in 3-AP treated cells was a ~19-fold reduction in the incorporation of de novo produced dCTP into DNA. Along with its effects on de novo biosynthesis, 3-AP triggered a compensatory upregulation of the salvage pathway. Salvage dC nucleotide pools doubled in size following 3-AP treatment, and the incorporation of salvage-produced dCTP into DNA increased by >1.5-fold, thereby providing a potential mechanism of resistance to RNR inhibition by 3-AP (Fig. 3c).

**Triple combination induces lethal replication stress in vitro.** We next quantified the impact of combined ATR, dCK and RNR inhibition on the de novo and salvage dCTP biosynthesis in asynchronous CEM cells (Fig. 4a and Supplementary Data 4). ATR inhibition decreased the \([^{13}C_6]\)glucose labeling of the rCDP pool by 40% (Fig. 4a, rCDP panel), an effect similar to that observed in the synchronous model (Fig. 1d). In contrast, RNR inhibition increased the size of the rCDP pool (Fig. 4a, rCDP panel, 3-AP). While neither ATR nor RNR inhibition alone had a
The statistically significant impact on the de novo contributions to the dCDP, dCMP and dCTP pools, these pools were nearly abolished when both ATR and RNR were inhibited simultaneously. However, the salvage biosynthetic contributions to the dCMP, dCDP and dCTP pools remained substantial in the absence of the dCKi. In fact, RNR inhibition, alone or combined with ATR inhibition, increased the salvage contributions to the dCMP, dCDP and dCTP pools by approximately twofold, indicative of a compensatory mechanism. dCK inhibition abolished this adaptive mechanism to augment these deoxyribonucleotide pools. Consequently, the rate of dCTP incorporation into DNA was lowest when all three enzymes were inhibited (Fig. 4a, DNA panel).

Persistent nucleotide insufficiency triggers replication stress characterized by the accumulation of single-stranded DNA (ssDNA) at stalled replication forks, followed by DNA double-stranded breaks. To investigate these events we used flow cytometry and antibodies against ssDNA as an indicator of...
Fig. 6 The triple combination therapy is effective and well-tolerated in a systemic primary B-ALL model. a Plasma pharmacokinetic parameters for 3-AP (15 mg kg$^{-1}$), VE-822 (40 mg kg$^{-1}$) and dCKi (50 mg kg$^{-1}$) in C57BL/6 mice (n $\geq$ 3) after single dose oral co-administration (mean $\pm$ s.d., n $\geq$ 3). b Doses and schedules for the triple combination therapy of leukemia bearing mice. c, d Bioluminescence images c and quantification of whole body radiance d of leukemia bearing mice treated with the combination therapy (treated, n = 5) or vehicle (control, n = 5) at indicated days after tumor inoculation. See also Supplementary Figs 11-13. e, f Kaplan-Meier survival analysis e and body weight measurements f of leukemia bearing mice treated with the combination therapy (treated, n = 5) or vehicle (control, n = 5). Median survival for the control group was 14 days after treatment initiation, whereas median survival for the treated group remains undefined (Mantel–Cox test). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. q.d. once/day; b.i.d. twice/day

ATR inhibition alone is marginally effective in vivo. To investigate the in vivo efficacy and tolerability of co-targeting alternative nucleotide biosynthetic pathways and ATR, we used a previously described primary BCR-ABL-expressing Arf-null pre-B (p185BCR-ABL$^{-/-}$) model which is difficult-to-treat and thought to be representative of the human disease$^{31,33}$. When compared with 31 cancer cell lines of different origins, p185BCR-ABL$^{-/-}$ cells were amongst the most sensitive to ATR inhibition by VE-822, with an IC$_{50}$ value of $\sim$300 nM (Fig. 5a). However, despite its high potency in culture against pre-B-ALL cells, VE-822 alone was only marginally efficacious in vivo. C57BL/6 mice inoculated with luciferase expressing p185BCR-ABL$^{-/-}$ cells succumbed to disease within 17 days; all VE-822 treated...
mice died of leukemia within 38 days after inoculation (Fig. 5b–d). We then investigated whether targeting the activities of de novo and salvage pathways can improve the efficacy of ATR inhibition in p185BCR-ABLArf−/− cells. Similar to the findings in the human T-ALL cells, targeting these biosynthetic pathways along with ATR was necessary to achieve maximal induction of cell death (Fig. 5e) and complete inhibition of cell growth (Supplementary Fig. 9a, b).

**Triple combination eradicates B-ALL in vivo.** To translate the above cell culture findings into an in vivo setting, we developed a new drug formulation consisting of PEG-200, Transcutol, Labrasol and Tween-80 blended in a ratio of 5:3:1:1 to solubilize three different drugs (3-AP, VE-822 and dCKi) and achieve therapeutically relevant plasma concentrations via oral delivery (Fig. 6a). Based on plasma pharmacokinetic parameters, 3-AP and dCKi were administered twice/day while VE-822 was administered once/day (Fig. 6b). Treatment was initiated on day 7 post inoculation of pre-B-ALL when mice showed evidence of systemic disease, as indicated by whole body bioluminescence imaging (BLI, Fig. 6c, top row, right panel). While mice in the control group succumbed to disease within 17 days, mice in the combination treatment group had significantly lower disease burden on day 17 (Fig. 6c, d). All treated mice remained disease-free for 442 days after treatment withdrawal on day 42 (Fig. 6e). The combination therapy was well-tolerated, as indicated by maintenance of body weight during treatment (Fig. 6f) and long term survival (over 1 year and currently ongoing) without any detectable pathology. We also assessed the efficacy of the combination therapy when all three components were administered once daily. Although this therapeutic scheme appeared to be slightly less efficacious than the twice/day schedule for 3-AP and dCKi, it was well tolerated and four out of five mice had no detectable disease 313 days after treatment withdrawal (Supplementary Fig. 10). Importantly, removing the dCKi from the combination therapy significantly reduced the therapeutic efficacy in vivo (Supplementary Fig. 11), a result consistent with cell culture findings (Figs 4 and 5e, Supplementary Figs 8 and 9).

While BCR-ABL tyrosine kinase inhibitors are becoming standard care for patients with Philadelphia chromosome positive ALL, therapeutic resistance in pre-B-ALL is common and is caused by the rapid emergence of the T315I BCR-ABL kinase domain (gatekeeper) mutation32, 56. To test the combination of VE-822, dCKi and 3-AP against kinase inhibitor resistant ALL we generated p185BCR-ABLArf−/− T315I mutant cells by exposing leukemia bearing mice to dasatinib and harvesting drug-resistant cells from bone marrow (Supplementary Fig. 12a–c). Mice were inoculated with the T315I-positive cells and treated with the combination therapy (Supplementary Fig. 12d–g). The combination therapy was effective against the highly aggressive dasatinib resistant in vivo pre-B-ALL model with 13 out of 20 mice being disease-free over 365 days post inoculation of leukemia cells (Supplementary Fig. 12). To determine whether mice that did not achieve complete remissions harbor ALL cells that have acquired resistance to the triple combination, we harvested leukemia cells from the bone marrow of the moribund mice. These cells were then used to test the efficacy of the combination treatment in cell culture, and compare it with the original dasatinib-resistant p185BCR-ABLArf−/− pre-B-ALL cells. The harvested leukemia cells responded to the combination treatment as well as did the parental cells (Supplementary Fig. 13). One potential reason for the incomplete response in some of the treated mice is the rapid engraftment of p185BCR-ABLArf−/− T315I + pre-B-ALL cells in the brain coupled with the suboptimal penetrability of 3-AP and potentially, dCKi across the blood–brain-barrier.

**Discussion**

Here we show that ATR inhibition in leukemia cells reduces the output of both de novo and salvage pathways. However, significant remaining activities of both pathways were sufficient to prevent ATR inhibition-induced DNA replication shutdown in cell culture, and permit disease-induced lethality in a systemic mouse model of pre-B-ALL. Combining ATR inhibition with inhibitors of de novo (RNR) and salvage (dCK) rate-limiting enzymes led to rapid accumulation of ssDNA, a hallmark of replication stress22, followed by extensive DNA damage, caspase-8 and PARP cleavage, and apoptosis. This synthetically lethal combination therapy was well-tolerated in vivo and promoted long-term disease-free survival in mice with systemic p185BCR-ABLArf−/− pre-B-ALL, as well as in a mouse model of targeted-therapy (dasatinib) pre-B-ALL resistance. Collectively our results quantify the control exerted by ATR on convergent nucleotide biosynthetic routes, and provide the rationale to co-target both signaling (ATR) and metabolic (RNR and dCK) mechanisms in acute leukemia for optimal therapeutic efficacy.

In mammalian cells dCTP and other dNTPs are present in low concentrations (1–50 pmol of dCTP/10⁶ cells), amounts far below those required to complete one round of genome duplication (~1089 pmol of dCTP/10⁶ cells)14, 57. This apparent discrepancy between dNTP supply and demand can be explained by a model in which dNTP production is tightly coupled with utilization for DNA synthesis—an ‘on demand’ model. According to this model, even small disruptions of dNTP production could significantly impact DNA integrity, unless the demand for dNTPs is reduced by preventing new origin firing, a process regulated by ATR and its effector kinases43. This prediction is supported by the synthetic lethality observed with ATR inhibition and pharmacological targeting of the de novo and salvage pathways observed in cell culture (Fig. 5e and Supplementary Figs 8, 9 and 13) and in vivo (Fig. 6c–f, Supplementary Figs 10–12).

To what extent are our findings of differential utilization of de novo and salvage pathways in leukemia applicable to solid tumors? An examination of three patient-derived primary cells point to significant nucleotide biosynthetic diversity in solid tumors (Supplementary Fig. 14a). HK-374 glioblastoma multiforme cells58 preferentially use the de novo pathways to generate most of their dATP, dGTP and dCTP pools, with only the dTTP pool displaying a significant salvage component. A distinct nucleotide biosynthetic profile is present in two melanoma patient-derived primary cells, M299 and M417 (Supplementary Fig. 14a). In M299 cells, the salvage contribution to all four dNTPs exceeds 50%. Except for dATP, a similarly increased reliance on salvage biosynthesis was evident in M417 cells (Supplementary Fig. 14a). In contrast, de novo pyrimidine biosynthesis is almost completely absent in M417 cells, with greater than 98% of the dTTP and dCTP pools originating from salvage mechanisms. However, the defect in dNTP production could significantly impact DNA integrity, unless the demand for dNTPs is reduced by preventing new origin firing, a process regulated by ATR and its effector kinases43. For example, in a model of ATR inhibition in solid tumors, a synthetic lethality observed with ATR inhibition and pharmacological targeting of the de novo and salvage pathways observed in cell culture (Fig. 5e and Supplementary Figs 8, 9 and 13) and in vivo (Fig. 6c–f, Supplementary Figs 10–12).
(e.g. predominant de novo, predominant salvage, and both de novo and salvage) in solid tumors. Such biomarkers could be provided by positron emission tomography imaging using new probes for nucleotide metabolism developed by us59, 60 as well as by others61.

PTMs can have profound and dynamic effects on the activity of metabolic networks62. Both dCK63 and RRMD64, 65 contain multiple PTMs. Among the four phosphorylation sites reported for dCK66, phosphorylation on serine 74 (pS74) is an important determinant of dCK substrate specificity and catalytic activity67. Both ATR and ATM have been reported to phosphorylate dCK on S74 in response to replication stress and DNA damage respectively28, 64. Our data show that the ATR inhibitor VE-822 reduces dCK activity by ~33%, an effect likely attributed to a two-fold decrease in dCK pS74 (Fig. 2f). However, it remains unknown what kinases are responsible for the remaining dCK pS74 present in VE-822-treated leukemia cells. ATM is a potential candidate, given that phosphorylation of CHEK2 on T68 is detected in ATR inhibited cells (Fig. 4c). Further studies are needed to confirm this hypothesis. In this context, it may also be informative to analyze the activity of dCK following ATR inhibition in tumors harboring inactivating mutations in ATM65–67. Concerning RNR regulation, the observed consequences of ATR inhibition in T-ALL cells include the following: a decrease in RRMD protein levels (Fig. 2c, d), reduced RRMD protein levels (Fig. 2c, d), and a significant reduction in RRMD PT33 (Fig. 2e). A CDK-mediated phosphorylation event which promotes RRMD protein degradation occurred through interactions with the SCF-Cycdin F ubiquitin ligase complex68. It is currently unknown whether the reduction in RRMD levels by ATR inhibition in leukemia cells occurs via a post-translational mechanism concerning protein stability and/or by a transcriptional mechanism downstream of E2F family member69, 70. Further studies are needed to determine the significance of the observed reductions in RRMD protein levels following ATR inhibition (Fig. 2c, d) and to identify the mechanism(s) responsible for this effect.

Our results provide quantitative insights into alterations of nucleotide biosynthetic pathways induced in leukemia cells by inhibiting ATR and rate-limiting de novo (RRMD) and salvage (dCK) enzymes. These findings support a new therapeutic strategy that uses existing inhibitors to exploit the dependency of leukemia cells on de novo and salvage biosynthetic pathways and replication stress response mechanisms. Further refinements in this strategy and expanding its applicability beyond leukemia may come from follow-up studies to define clinically applicable companion biomarkers capable of delineating nucleotide biosynthetic and replication stress subtypes that are predictive of responses in human tumors.

**Methods**

**Cell culture and culture conditions.** Leukemia cell lines: CCRF-CEM, EL4, Jurkat, Molt-4, CEM-R, THP-1, HL-60, TF-1, MV-4-11, HH, HuT 78, K-562; ovarian cancer cell lines: Hep-T30, PA-1, Caov-3, OCAR-5, IGROV-1, A2780; and pancreatic adenocarcinoma cell lines: BxPC-3, Mia PaCa-2, Hs 766 T, AsPC-1 and Panc-1 were obtained from American Type Culture Collection (ATCC). Various cell lines were kindly gifted: Nalm-6 (Michael Tetteli, UCLA), p185RhoA-Arf5cre; pre-B cells (N. Boulus, CERN Foundation), L3.6pl (David Dawson, UCLA), KPC (Guido Eibl, UCLA), and patient-derived primary cells of leukemia CCG332 (Yong-Mi Kim, USC). All hepatocellular carcinoma cell lines were gifts from Ali Zarrinpars (Univ. of Florida): SNU-475, PRC/PRF/5, SNU-449, Hep 3B, Hep G2, and SK-HEL-1. Patient-derived primary cells of glioblastoma (HK-374) and melanoma (M299 and M417) were derived in the labs of Drs. Kornblum (UCLA) and Ribas, respectively. With a few exceptions, cell lines were cultured in RPMI-1640 (Corning) containing 10% fetal bovine serum (FBS, Omega Scientific) and were grown at 37 °C, 5% CO2 and 5% O2. p185RhoA-Arf5cre; pre-B cells were cultured in RPMI-1640 containing 10% FBS and 0.1% β-mercaptoethanol. HK-374 was cultured in DMEM-F12 (Invitrogen) containing B27 supplement (Life Technologies), 20 ng ml−1 basic fibroblast growth factor (bFGF; Peprotech), 50 ng ml−1 epidermal growth factor (EGF; Life Technologies), penicillin/streptomycin (Invitrogen), Glutamax (Invitrogen) and 5 μg ml−1 heparin (Sigma-Aldrich). All cultured cells, except HK-747, were incubated in antibiotic free media and were regularly tested for mycoplasma contamination using MycoAlert kit (Lonza) following the manufacturer’s instructions, except that the reagents were diluted 1:4 from their recommended amount.

**Proliferation assays.** Cells were plated in 384-well plates (1000 cells/well for suspension cell lines and 500 cells/well for adherent cell lines in 30 μl volume). Drugs were serially diluted to the desired concentrations and an equivalent volume of DMSO was added to vehicle control. Ten microliter of the 4x diluted drugs were added to each well. Following 72 h incubation, ATR content was measured using CellTiter-Glo reagent according to manufacturer’s instructions (Promega, CellTiter-Glo Luminescent Cell Viability Assay), and analyzed by SpectraMax luminescence (Molecular Devices). IC50, concentrations required to inhibit proliferation by 50% compared to DMSO treated cells, were calculated using Prism 6.0 h (Graphpad Software).

**Cell cycle kinetics.** CEM T-ALL cells were synchronized in G1 phase, and then treated as indicated. Cells were pulsed with Edu 1 h before collection at different time points. Cells were fixed 4% paraformaldehyde, permeabilized with perm/wash reagent (Invitrogen), stained with Azide-AF647 (using click-chemistry, Invitrogen; Click-it Edu Flow cytometry kit, #C16634) and FcCycle-Violet (Invitrogen), and then analyzed flow cytometry (a detailed description is available in the Supplementary Information).

**Metabolite profiling.** See Supplementary Information for details on sample preparation, data collection and analysis.

**Global proteome and phosphoproteomic analyses.** See Supplementary Information for details on sample preparation, data collection and analysis.

**ssDNA and pH2A.X Measurements.** Treated CEM T-ALL cells were fixed with ice-cold methanol/PBS (6:1 v/v). Staining with F7-26 ssDNA monoclonal antibody (mouse) was performed according to manufacturer’s instructions (EMD Millipore, #MAB3299) followed by secondary antibody IgM-P. Subsequently, cells were stained with the phospho-Histone H2AX (pS139 H2AX) specific antibody conjugated to FITC (EMD Millipore, #05-636) and DAPI, and then analyzed by flow cytometry (a detailed description is available in the Supplementary Information).
Western blots. See Supplementary Information for details on sample preparation and antibody information. Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 15.

Apoptosis assay. Apoptosis and cell death were assayed using Annexin V-FITC and PI according to manufacturer’s instructions (FTTC Annexin V Apoptosis Detection Kit, BD Sciences, #556570).

In vivo leukemia models and treatment regimes. All animal studies were approved by the UCLA Animal Research Committee (ARC). For development of systemic leukemia, 5$	imes$10^5 C57BL/6 female mice were injected intravenously with 50,000 luciferase expressing pIRES-PLC-β1Δ69 +/− pre-B-ALL cells.12, 31. The leukemic burden was monitored using bioluminescence imaging. All drug treatments were performed in an oral formulation consisting of PEG-200, Transcutol, Labrasol and Tween-80 blended in a ratio of 5:3:1:1. The oral gavage volume was 50 μl per dose (a detailed description is available in the Supplementary Information).

Pharmacokinetic measurements. 3-AP, dCKi, VE-822 plasma concentrations were assessed at 0.5, 1, 2, 4, 6, 12 and 24 h following oral administration. Blood samples were collected in heparin-EDTA tubes by the retro-orbital technique and spun down to collect the supernatants. Four parts of methanol were added to plasma samples to precipitate proteins, and the supernatant was collected. Twenty microliter samples were injected onto a reverse phase column equilibrated in water 0.1% formic acid for LC-MS/MS-MRM analysis in positive ion mode. Plasma concentrations were determined by comparison to previously generated standard curves. A population pharmacokinetic modeling (NONMEM v. 7.2) with first-order conditional estimation (FOCE) was used to characterize the G0(t) (half-life), and area under the curve (AUC) for 3-AP, dCKi, and VE-822. 3-AP and VE-822 were best described with a one-compartment model while dCKi was best described by a two-compartment model. All three drugs were well described with first order absorption and a proportional error model. Clearance and volume were scaled by weight.

FACS analyses. All flow cytometry data were acquired on a five-laser LSRII cytometer (BD), and analyzed using the FlowJo software (Tree Star). See Supplementary Information for details on propidium iodide, cell cycle by EdU, pH2A.X, ssDNA, phospho-histone 3 and Annexin V staining.

Animal studies. Mice were housed under specific pathogen-free conditions and were treated in accordance with UCLA Animal Research Committee protocol guidelines. All C57BL/6 female mice were purchased from the UCLA Radiation Oncology breeding colony. VE-822 (ApexBio, 40 mg kg$^{-1}$) 3-AP (ApexBio, dosage as indicated) and DI-82 (dCKi, Sundia Pharmaceuticals, 50 mg kg$^{-1}$) were administered by intraperitoneal (i.p.) injections or oral gavage to recipient animals. For oral administration, single agent or combination of drugs were solubilized in a formulation consisting of the following: PEG-200: Transcutol: Labrasol: Tween-80 mixed in 5:3:1:1 ratio. For i.p. administration, the drugs were solubilized in PEG-400 and 1 mM Tris-HCl in a 1:1 (v:v) ratio. Dasatinib (LC Laboratories) was solubilized in 80 mM citric acid (pH 3.1) and was administered at a dose of 10 mg kg$^{-1}$ by oral gavage. 2 x 10^3 luciferase expressing pIRES-PLC-β1Δ69 +/− pre-B-ALL cells were injected intravenously into C57BL/6 female mice for leukemia induction. The treatment was started 6 or 7 days after the intravenous inoculation of leukemia initiating cells, when animals had developed a significant leukemic burden as monitored by bioluminescence imaging (IVIS Bioluminescence Imaging scanner). The dosing schedules are indicated in the text and figure legends. The mice were observed daily and those that became moribund were killed immediately. Kaplan–Meier curves and bioluminescence quantifications were generated using Prism 6.0 h (Graphpad Software).

Bioluminescence imaging (BLI). Mice were anesthetized with 2% isoflurane followed by intraperitoneal injection of 50 μl (50 mg ml$^{-1}$) substrate D-luciferin (Sigma, #D9994). The mice were imaged with the IVIS 100 Bioluminescence Imaging scanner 10 min after luciferin administration. All mice were imaged in groups of five with 1-minute exposure time, and the images were acquired at low binning.

Statistical analyses. Data are presented as means ± s.d. with indicated biological replicates. Comparisons of two groups were calculated using indicated unpaired or paired conditional test (FOCE) to calculate the G0(t) and area under the curve (AUC) for 3-AP, dCKi, and VE-822. 3-AP and VE-822 were best described with a one-compartment model while dCKi was best described by a two-compartment model. All three drugs were well described with first order absorption and a proportional error model. Clearance and volume were scaled by weight.

All statistical analysis and generated graphs were performed either in R or Graphpad Prism 6.0 h. The rates of dCTP incorporation into the DNA were determined using linear regression with procedure GLM in SAS (v. 9.4). DNA % label was transformed using a shifted log addition of 1 due to the presence of zeros in the data set. Rates were calculated using the slope of the regression line for the log transformed data and are presented in the table with the associated standard errors, along with the ratio between the rate of incorporation into DNA by the de novo and salvage pathways (Table 1).

Data availability. The proteomic and phosphoproteomic data have been deposited in the Proteome Xchange database under accession code PXD006702. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author upon request.

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Additional information
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Competing interests: The authors declare the following competing financial interest(s): C.G.R. and J.C. are co-founders of Trethera Corporation. They and the University of California hold equity in Trethera Corporation. The University of California has patented additional intellectual property for small molecule dCK inhibitors invented by C.G.R., J.C., S.P. and T.M.L. This intellectual property has been licensed by Trethera Corporation.

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