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A pilot clinical trial of oral tetrahydrouridine/decitabine for noncytotoxic epigenetic therapy of chemoresistant lymphoid malignancies ☆,☆☆

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A B S T R A C T

One mechanism by which lymphoid malignancies resist standard apoptosis-intending (cytotoxic) treatments is genetic attenuation of the p53/p16-CDKN2A apoptosis axis. Depletion of the epigenetic protein DNA methyltransferase 1 (DNMT1) using the deoxycytidine analog decitabine is a validated approach to cytoreduce malignancy independent of p53/p16. *In vivo* decitabine activity, however, is restricted by rapid catabolism by cytidine deaminase (CDA). We, therefore, combined decitabine with the CDA-inhibitor tetrahydrouridine and conducted a pilot clinical trial in patients with relapsed lymphoid malignancies: the doses of tetrahydrouridine/decitabine used (~10/0.2 mg/kg orally (PO) 2×/week) were selected for the molecular pharmacodynamic objective of non-cytotoxic, S-phase dependent, DNMT1-depletion, guided by previous Phase 1 studies. Patients with relapsed/refractory B- or T-cell malignancies (n = 7) were treated for up to 18 weeks. Neutropenia without concurrent thrombocytopenia is an expected toxicity of DNMT1-depletion and occurred in all patients (Grade 3/4). Subjective and objective clinical improvements occurred in 4 of 7 patients, but these responses were lost upon treatment interruptions and reductions to manage neutropenia. We thus performed parallel experiments in a preclinical *in vivo* model of lymphoma to identify regimen refinements that might sustain DNMT1-targeting in malignant cells but limit neutropenia. We found that timed-alternation of decitabine with the related molecule 5-azacytidine, and combination with inhibitors of CDA and *de novo* pyrimidine synthesis could leverage feedback responses of pyrimidine metabolism to substantially increase lymphoma cytoreduction but with less neutropenia. In sum, regimen innovations beyond incorporation of a CDA-inhibitor are needed to sustain decitabine DNMT1-targeting and efficacy against chemo-resistant lymphoid malignancy. Such potential solutions were explored in preclinical *in vivo* studies.

Introduction

Chemotherapeutic agents that induce apoptosis confer favorable clinical outcomes for many patients with lymphoid malignancies. However, once such patients develop relapsed or refractory disease, outcomes are generally poor and require intensive manuevers, such as stem cell transplantation. Salvage treatments for this patient population should address mechanisms of resistance to initial treatment. One mechanism-of-resistance is inactivation of the master regulators of apoptosis, *p16/CDKN2A* and *TP53* [1-8], by mutation, deletion or epigenetics that confers resistance to multiple lines of therapy that share apoptosis (cytotoxicity) as a common final pathway of action (reviewed in [9-14]). Treatments that do not require the p53/p16-CDKN2A apoptosis-axis are hence needed.

DNA methyltransferase 1 (DNMT1) is an enzyme that transfers methyl groups to specific cytosine nucleotides of genomic DNA, an epigenetic modification linked with gene repression. DNMT1 is recruited to the DNA replication fork during cell S-phase, to recapitulate onto the newly synthesized DNA strand the methylation pattern of the parental strand, maintaining this repression mark through cell division. Additionally, DNMT1 is recruited by sequence-specific DNA-binding proteins (transcription factors) as a coregulator (corepressor), to in this way dynamically mediate gene repression *vs* activation [15-17]. DNMT1 can be depleted from dividing cells using the deoxycytidine analog decitabine or cytidine analog 5-azacytidine. Depletion of DNMT1 has been validated in pre-clinical studies of resistant/relapsed T and B-cell malig-

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nancies [18-28] as being capable of activating maturation-related programs (eg, p27/CDKN1B) [18,29] that terminate malignant cell replication via a p53/p16-independent pathway (reviewed in [30]). Both deoxycytidine and 5-azacytidine are prodrugs that, in order to deplete DNMT1, must be processed through pyrimidine metabolism into a deoxycytidine triphosphate analog which is incorporated into the newly synthesized DNA strand during S-phase. Rate-limiting decitabine and 5-azacytidine processing are deoxycytidine kinase (DCK) and uridine cytidine kinase 2 (UCK2) respectively. Hematopoietic malignancies highly express DCK and UCK2, encouraging several prior applications of these agents to myeloid and lymphoid neoplasms [31,32].

The results of these early phase clinical trials in lymphoid malignancies have been mostly disappointing, with response rates generally <10% [33-39], except in angioimmunoblastic T-cell lymphoma [40], a rare subtype of T-cell malignancy. This disconnect between preclinical and clinical results could reflect another aspect of decitabine and 5-azacytidine metabolism: rapid catabolism *in vivo*, into uridine counterparts that do not deplete DNMT1, by the pyrimidine metabolism enzyme cytidine deaminase (CDA). CDA is highly expressed in liver, gastrointestinal and reticuloendothelial tissue such as the spleen, and shortens the half-life of decitabine and 5-azacytidine *in vivo* to approximately 10 minutes compared to 12 hours *in vitro* [41,42]. Severely abbreviated plasma half-lives are problematic as DNMT1-depletion by decitabine/5-azacytidine is S-phase, and hence exposure-time, dependent. High CDA expression in intestines and liver limit decitabine and 5-azacytidine oral bioavailability [43]. CDA, less expressed in rodents than in primates, furthers the disconnect between pre-clinical and clinical data [44,45].

We therefore conducted a pilot clinical trial of oral decitabine combined with an inhibitor of CDA, tetrahydrouridine (THU), to treat relapsed T- and B-cell malignancies, a first such evaluation. Doses of decitabine ingested orally were ~20% of doses approved for intravenous infusion to treat myeloid malignancies, and chosen to produce a decitabine concentration-time profile when combined with oral THU -low C_{max}, ~2 hour plasma $t_{½}$– ideal for noncytotoxic DNMT1-depletion, as demonstrated in a prior Phase 1 evaluation [43,46,47]. Several patients with relapses after several lines of standard therapy benefitted subjectively and objectively from this treatment, but these benefits were lost upon decitabine dose holidays and reductions to manage treatment-induced neutropenia. We therefore also conducted parallel preclinical studies of alternative decitabine and 5-azacytidine schedules to leverage interactions with pyrimidine metabolism [48], and identified candidate, clinically relevant solutions to increase tumor responses and decrease neutropenia.

Methods

Study design

This was a single-arm, open-label, pilot/proof-of-concept clinical trial of oral decitabine/THU in patients with T- and B-cell malignancies that had progressed on one or more lines of systemic therapy. This overall design was not altered during the course of the study.

Patient population

This clinical trial was conducted under an Investigational New Drug number from the US FDA, reviewed and approved by the Cleveland Clinic Institutional Review Boards, and funded via philanthropy to Cleveland Clinic including from the Leukemia and Lymphoma Society. Written informed consent was obtained prior to treatment in all patients, and all research was conducted within the principles expressed by the Declaration of Helsinki. The study was registered on clinicaltrials.gov: NCT02846935. The treatment population was adult (≥18 years of age) patients with histologically confirmed refractory/relapsed lymphoid malignancies, with progression of disease on one or more prior lines of systemic therapy, measurable disease per response evaluation criteria in solid tumors and ECOG performance status 0-2.

Interventions

Decitabine and THU drug substance were synthesized by Ash Stevens (Detroit, Michigan) and drug product was formulated by KP Pharmaceutical Technologies (Bloomington, IN). Drugs were dispensed in plastic bottles at 4°C. Bottles were opened after equilibration to room temperature. An oral THU dose of ~10 mg/kg was ingested 60 minutes before oral decitabine ~0.2 mg/kg twice a week on consecutive days. THU was supplied as 250 mg/capsules, and decitabine as 5 mg/capsules. Patients weighing 40 to 60 kilograms (kg) were given 2 capsules of each drug, 61 to 80 kg 3 capsules of each drug and 81 kg or higher 4 capsules of each drug. Patients were instructed to take the decitabine capsules ~60 minutes after taking the THU capsules, to generate sufficient time for the intended biological effect of THU of systemic CDA-inhibition. The rationale for the regimen design is shown figuratively (Fig. 1).

For patients with rapidly progressive disease that might benefit from a more intense period of initial therapy, the treatment protocol allowed an induction phase in which drugs were taken for 5 consecutive days (eg, Monday-Friday) in week 1, to be repeated in week 2 if no grade 3 or higher hematologic toxicities occurred. From week 3 onward, drugs were ingested on 2 consecutive days at the same doses, if no grade 3 or higher hematologic toxicities were noted.

Biological mechanisms predict that neutropenia will be problematic, so dose reductions for this were built into the protocol from the beginning: neutrophils $<0.5 \times 10^9$/L would trigger an interruption of study drug until recovery of neutrophil counts to $>1.0 \times 10^9$/L then resumption at a reduced dose (reduction by 1 in the number of capsules of each drug ingested).

Outcomes

The primary end-point was tumor objective response [49]. Secondary end-points included tolerability and safety assessment by toxicity characterization using CTCAE v4.

Sample size

The study planned to enroll patients with 3 separate biologic/histologic subsets of lymphoid malignancy: (1) T-cell lymphoma, (2) Aggressive B-cell lymphoma, (3) Indolent B-cell lymphoma. The study was terminated after enrolling and treating 3 patients with T-cell lymphoma, 4 with aggressive B-cell lymphoma, and 0 patients with indolent B-cell lymphoma ($n = 7$). All 7 treated patients were analyzed with their data reported here.

Clinical pathology tests

Blood counts and blood chemistries were standard clinical pathology tests through the CLIA-certified Clinical Pathology Laboratory at the at the Cleveland Clinic.

Preclinical in vivo *studies of resistant T-cell malignancy*

All experiments were approved by the Cleveland Clinic IACUC and followed approved procedures. A xenotransplant model of peripheral T-cell malignancy was derived from a patient with mycosis

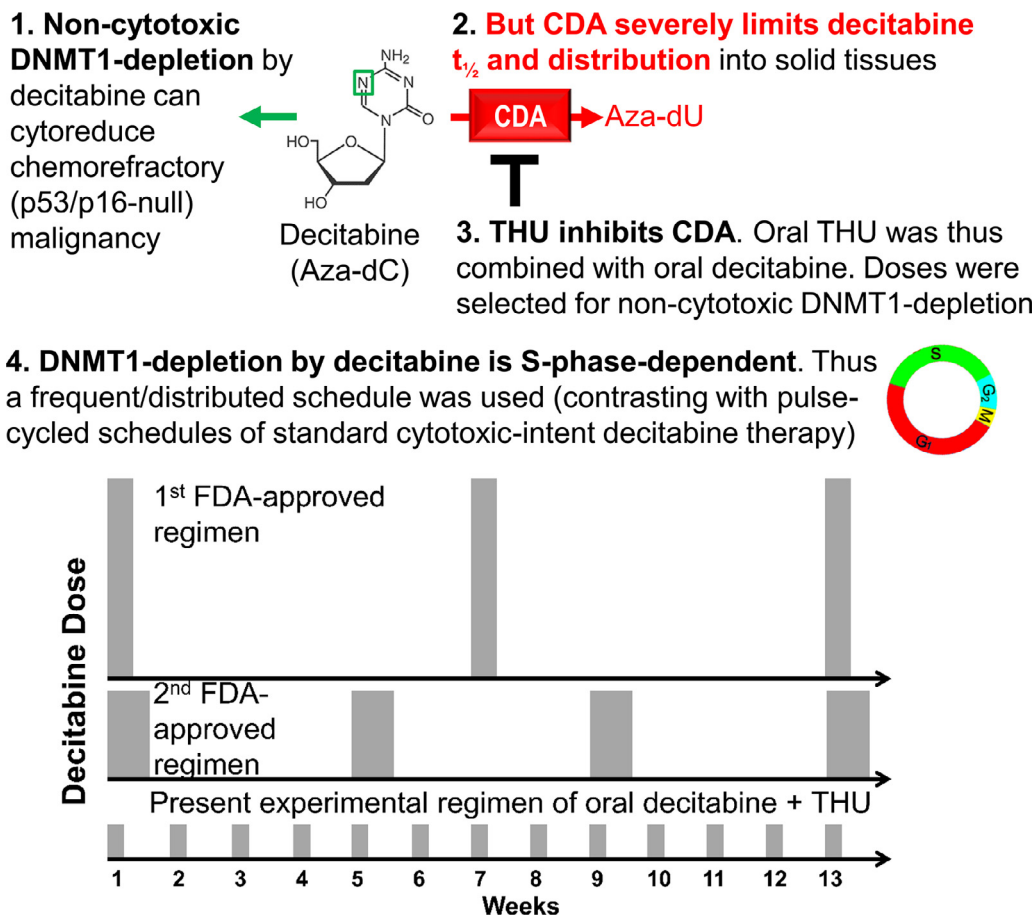

Fig. 1. Rationale for this treatment regimen for relapsed lymphoid malignancy.

fungoides/Sezary's disease relapsed after romidepsin, photopheresis, and mogamulizumab. These cells were subcutaneously injected into the right and left flanks of 6- to 8-week-old immunocompromised NSG mice (0.1×10^6 cells per injection). Mice were randomized to the treatment groups, and tumor volume was assessed by caliper measurement twice weekly throughout the study using the equation: volume (mm^3) = long (mm) × wide [2] (mm)/2. Mice were treated with (1) subcutaneous vehicle 2×/week; (2) intraperitoneal romidepsin 1 mg/kg on day 1, 4, 12, 17; (3) intraperitoneal leflunomide 20 mg/kg on day 1 and day 4 of each week; (4) intraperitoneal THU 10 mg/kg and subcutaneous decitabine 0.1 mg/kg on day 2 of each week together with THU and subcutaneous 5-azacytidine 1 mg/kg on day 5 of each week; (5) a combination of leflunomide and THU/decitabine/5-azacytidine. Mice body weights were recorded weekly and weight percentage during treatment was calculated as 100 × weight/initial weight. Animals were closely monitored and euthanized for signs of toxicity or distress (as defined in the Animal Protocol) or tumor volume ≥1000 mm^3. Euthanasia was by CO_2 inhalation followed by cervical dislocation.

Statistics

Preliminary efficacy signal was to be evaluated in 3 cohorts with differing biology: indolent B-cell lymphoma/CLL; aggressive B-cell lymphoma and T cell lymphoma, with the null hypothesis that the overall response rate is 5% or less versus the alternative that the response rate is 30% or greater. With 12 patients per biologic subset enrolled in this Proof of Concept trial, with alpha = 0.05 and power of 80%, if 3 or fewer individuals of the 12 patients entered achieve an objective response, the treatment would not be considered promising for further study (planned sample size n = 36, actual sample size n = 7 [indolent B-cell lymphoma/CLL n = 0; aggressive B-cell lymphoma n = 4; T cell lymphoma n = 3). Patient and disease characteristics are summarized descriptively and graphical displays show data for individual patients. Distributions of dose and time on treatment are also described. All statistical tests are 2-sided.

All patients enrolled into the study are included in the analyses.

Results

Patient flow and characteristics

Seven patients were screened, eligible, and enrolled at Cleveland Clinic with the first patient starting study drug in May, 2017 and the last patient receiving the last dose of study drug in Jan, 2018. All patients received the intervention (Fig. 1). Results from all patients were analyzed. There were 4 males and 3 females. Their median age was 70 years (range 63-80) (Table). Four had aggressive B-cell malignancies and 3 had peripheral T-cell malignancies (Table). All patients had disease that had relapsed after a median of 4 lines of prior therapy (range 2-9) (Table). All patients had substantial baseline subjective and objective evidence of disease, at nodal and/or extranodal sites, and elevated baseline LDH levels (Table).

Adverse events

The only adverse event definitely attributed to study treatment was neutropenia: grade 3 or 4 neutropenia occurred in all 7 patients (Fig. 3).

Table
Baseline characteristics of study patients.

†	Age, sex	D_X	Disease status	Prior treatments*	Clinical baseline Subjective	Best clinical response Subjective	LDH (baseline/ best)	Oral decitabine dose mpk 2×/wk (+THU)
					Objective	Objective		
1	63M	HL, DLBCL	8th Relapse	ABVD; Brentuximab; R/ICE; ASCT (Bu/Cy/VP); R/Gemcitabine; R/Vinorelbine; Ibrutinib; IMGN529	Fatigue; SOB at rest (needing 3 L/min supplemental O_2); Cough	All symptoms worse (terminal respiratory failure)	267/ Not done	**Wk 1-3:** 0.2
					Lymphadenopathy – Multi-Level; Lung Nodules/Cavitary Mass; Adrenal Mass; Intra-Medullary Disease	Not done		
2	70M	PTCL	3rd Relapse	Brentuximab; ASCT (Bu/Cy/VP); CHOP	Fatigue; Painful axillary lymphadenopathy	**Fatigue decreased; Axillary pain/lymphadenopathy resolved**	237/181	**Wk 1-6:** 0.2 **Wk 7-10:** None **Wk 11-18:** 0.15
					Lymphadenonpathy – Multi-Level/Axillary Necrosis;	**Lymphadenopathy - resolved/decreased/stable**		
3	76FM	DLBCL	4th Relapse	R/CHOP; Ibrutinib; R/Bendamustine; Lenalidomide	Fatigue; Night Sweats; SOBOE; Anorexia; Weight Loss	All symptoms worse	217/172	**Wk 1-4:** 0.2 **Wk 5-6:** None **Wk 7:** 0.1
					Lymphadenopathy – Mediastinal; Lung Nodules/Cavitary Mass;	Lymphadenopathy/Lung Noodules - progressed		
4	69FM	AITL	2nd Relapse	Brentuximab; ASCT (Bu/Cy/VP)	Fatigue; SOB (needing regular thoracocentesis); Night Sweats; Abdominal Distension	**All symptoms resolved**	261/188	**Wk 1-6:** 0.17 **Wk 7-8:** None **Wk 9-12:** 0.09
					Lymphadenopathy – Multi-Level; Lung Nodules; Pleural/Pericardial Effusions; Splenomegaly	**Lymphadenopathy - decreased/stable; Pericardial/L Pleural Effusions - decreased; R Pleural Effusion – progressed; Splenomegaly - decreased**		
5	68M	PTCL	5th Relapse	CHOP/IT MTX; HD MTX; Brentuximab; Pralatrexate; Romidepsin	Fatigue; SOBOE; Fevers; Night Sweats; Early Satiety; CN palsy	Baseline symptoms continued + painful herpes simplex oral lesions	296/156	**Wk 1-2:** 0.21 5X/wk **Wk 3-4:** 0.21
					Lymphadenopathy – Multi-Level; Lung Nodules; Portal Vein Thrombosis/ Ascites/Splenomegaly	**Lymphadenopathy – Resolved retroperitonaeal, Stable/Resolved lung/mediastinal**; Portal vein thrombosis and splenomegaly persistent/worse		
6	80FM	Marginal Zone, DLBCL	9th Relapse	R/ (5 courses); Radiation (3 courses); R/Bendamustine	Mass in R thigh; R lower extremity edema	All symptoms same, new L thigh mass	215/176	**Wk 1-4:** 0.22 **Wk 5-8:** None
					R thigh mass	R thigh mass - stable; New L thigh mass		
7	74M	DLBCL	4th Relapse	Radiation/R/Bendamustine; Radiation; Ibrutinib; R/Lenalidomide	Painful cutaneous nodules lower extremities	**Symptoms resolved**	421/351	**Wk 1-6:** 0.22 **Wk 7-8:** None **Wk 9:** 0.15 **Wk 10**: None
					Cutaneous/fascial masses lower extremities	**Cutaneous/fascial masses resolved**; New L inguinal lymphadenopathy		

ABVD = Doxorubicin, Bleomycin, Vinblastine, Dacarbazine; AITL = Angioimmunoblastic T-cell Lymphoma; ASCT = Autologous Stem Cell Transplant; Bu/Cy/VP = Busulphan, Cyclosphosphamide and Etoposide transplant conditioning; CHOP = Cyclophosphamide, Doxorubicin, Vincristine, Prednisone; DLBCL = Diffuse Large B-cell Lymphoma; ECOG PS = ECOG Performance Score (scale from 0 to 4; 0 = best, with no impairments; 4 = bedridden); HD = High Dose; HL = Hodgkin's Lymphoma; IT = Intra-thecal; MTX = Methotrexate; PTCL = Peripheral T-cell Lymphoma; R/ = Rituximab.

* Prior therapy includes radiation if given separately from chemotherapy; NL = normal; NA = not available); PET and/or CT imaging.

† Prior Rx = number of prior lines of therapy.

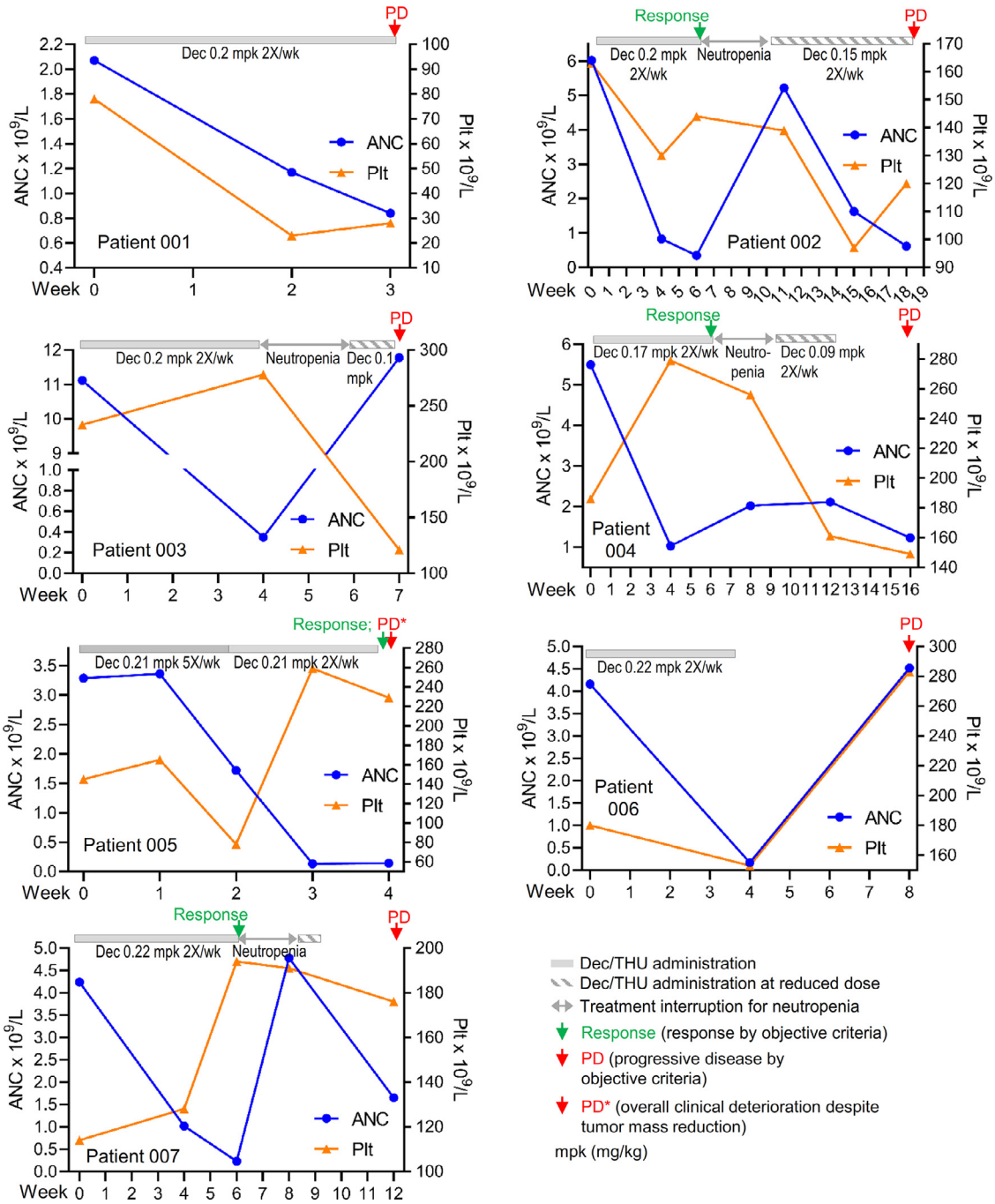

Fig. 2. In most patients, oral THU/decitabine induced neutropenia but not thrombocytopenia, expected with noncytotoxic DNMT1-depletion in bone marrow; initial responses observed in some patients were lost with treatment interruptions/dose-reductions used to manage neutropenia. Mpk = mg/kg of oral decitabine dose (oral decitabine was ingested ~1 hour after ~10 mpk oral THU); PD = progressive disease; *Table summarizes disease status and clinical course.

Tumor burden and other efficacy parameters

Objective decreases in tumor burden (Table), together with subjective clinical improvements (decrease in baseline symptoms), occurred in 4 of 7 patients (Table, Figs. 2, 3). These responses occurred in patients with both relapsed B- and T-cell malignancies (Table, Fig. 2). These objective and subjective improvements were lost with treatment holds and dose-reductions used to manage neutropenia (Table, Fig. 2). An expected indicator of systemic noncytotoxic DNMT1-depletion is a decrease in neutrophil counts with relatively preserved or increased platelet counts and hemoglobin [9,50-52]: this peripheral blood count pattern was observed in 6 of 7 patients (Fig. 2). The exception was patient 1, who had rapidly progressive pancytopenia during the first 3 weeks of therapy likely because of refractory, rapidly progressing diffuse large B-cell lymphoma (Fig. 2). Per protocol, the neutropenia (neutrophils $<0.5 \times 10^9$/L) was managed by treatment holds followed by resumption of therapy at a lower dose, resulting in recovery of neutrophil counts (Fig. 2).

The study was terminated after treatment of 7 patients, when the investigating team judged that the regimen should be redesigned to manage treatment-induced neutropenia without reducing dose below the minimum biologically effective dose.

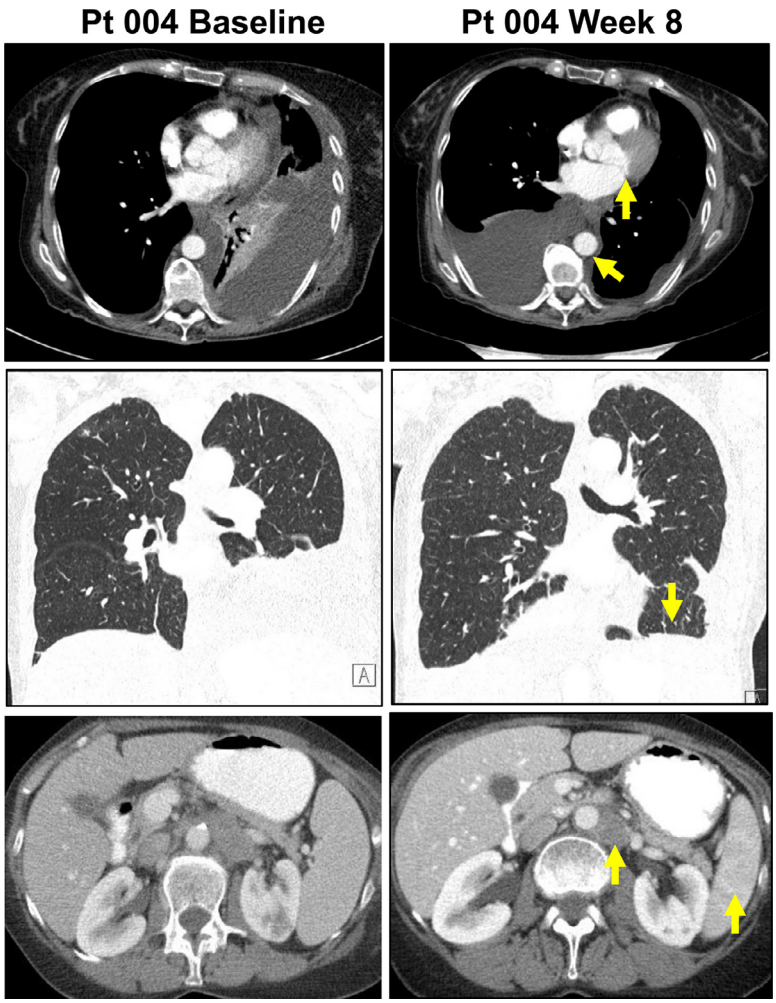

Fig. 3. Example of serial CT-scan results: Patient 004 had disseminated angioimmunoblastic T-cell lymphoma relapsed after first-line Brentuximab vedotin and second-line autologous stem cell transplantation with busulphan/cyclophosphamide/etoposide conditioning. Yellow arrows indicate improvements with THU/decitabine treatment in pericardial/pleural effusions (top, middle panels), periaortic lymphadenopathy (top, bottom panels) and splenomegaly (bottom panel).

Methods of integrating G-CSF supportive care into noncytotoxic DNMT1-depleting therapy

In contrast to chemotherapy, the bone marrow can have preserved or increased cellularity with noncytotoxic decitabine therapy [50,51,53,54]. However, these DNMT1-depleted hematopoietic precursors have decreased ability to switch from default erythroid-megakaryocyte progenitor fate trajectories to granulo-monocytic fates [50,51,53,54] (Fig. 4A). Although G-CSF administration after chemotherapy pulses is standard medical practice, this approach may not be optimal for this different kind of neutropenia [53]. Thus, in mice, we compared administering G-CSF after, or before, THU/decitabine administered for 3 consecutive days per week for 7 weeks (Fig. 4B). G-CSF administration before THU/decitabine was better at preserving peripheral blood neutrophil counts (Fig. 4C). We also examined bone marrow neutrophil content: this was also higher with G-CSF administration before instead of after THU/decitabine (Fig. 4D, E).

Preclinical evaluation of other regimen changes to increase antitumor effects but decrease neutropenia

We previously showed that resistance to decitabine and 5-azacytidine originates from feedback responses of the pyrimidine metabolism network to nucleotide perturbations [48]. Based on these observations, we evaluated in a patient-derived xenograft model of treatment-resistant peripheral T-cell malignancy (Sezary/Mycosis Fungoides) a regimen designed to overcome resistance emerging from metabolism [48] (Fig. 5A): decitabine was alternated with 5-azacytidine ~96 hours apart, and an inhibitor of de novo pyrimidine synthesis 2×/week (leflunomide to inhibit dihydroorotate dehydrogenase), in addition to the CDA-inhibitor, were incorporated into therapy [48] (Fig. 5B). This regimen substantially and significantly decreased tumor locally (subcutaneous tumor mass) and systemically (peripheral blood and bone marrow human CD3+ cells) compared to romidepsin (histone deacetylase inhibitor) as a standard therapy control. The de novo pyrimidine synthesis inhibitor by itself also showed minimal efficacy (time-to-distress ~25 days vs 20 days with vehicle), but clearly synergized with the THU/decitabine/5-azacytidine (almost doubling time-to-distress, to ~75 days vs ~40 days with THU/decitabine/5-azacytidine alone) (Fig. 5C-E). The absence of significant neutropenia, in addition to tumor restriction, enabled long-term administration of the treatment (75 days) in this *in vivo* experiment (Fig. 5C-E).

Discussion

Noncytotoxic DNMT1-depletion by decitabine or 5-azacytidine is scientifically validated to terminate proliferation of p53/p16-

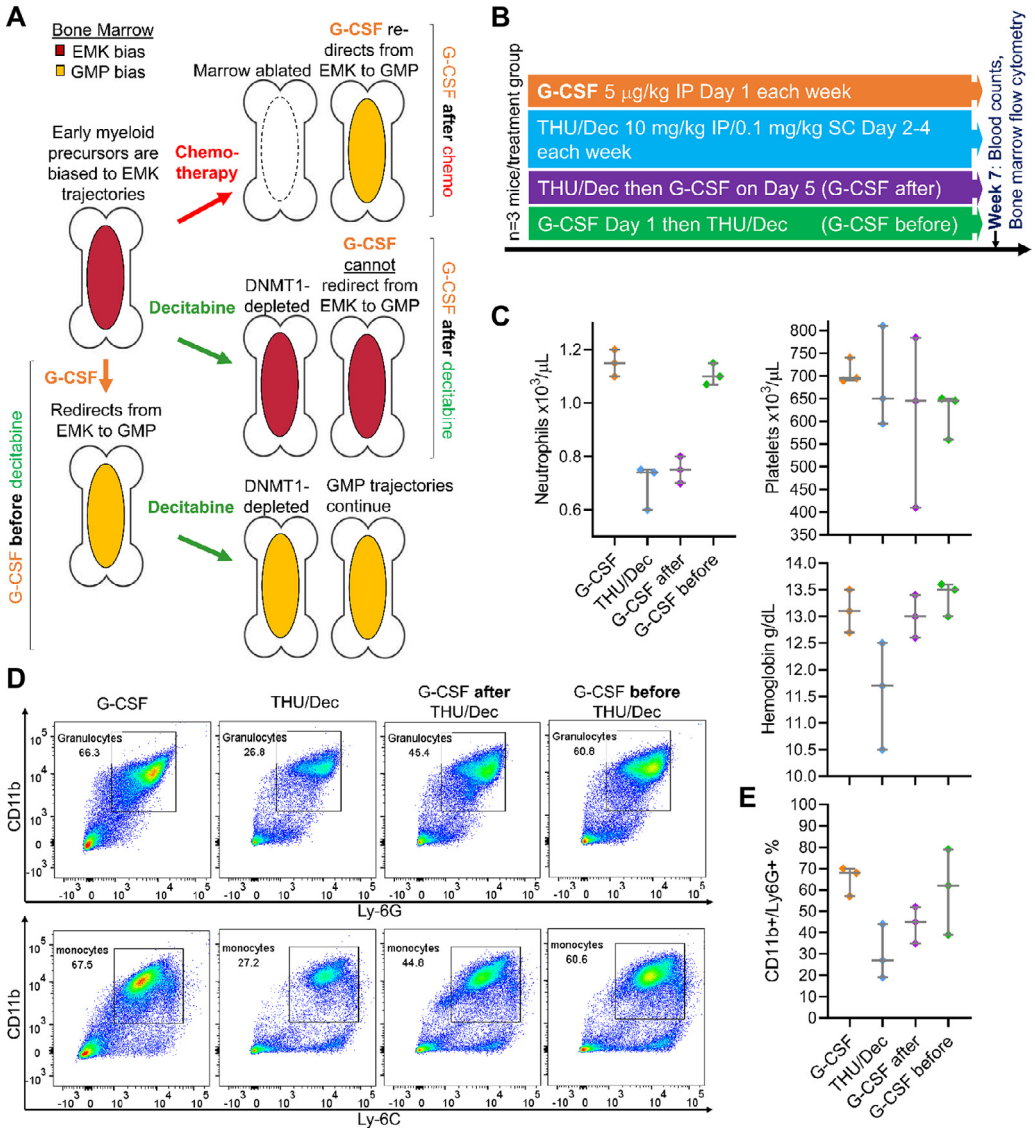

Fig. 4. Noncytotoxic DNMT1-depletion decreases neutrophils but preserves or increases platelets and hemoglobin; this modality of therapy, distinct from chemotherapy, may benefit from granulocyte-colony stimulating factor (G-CSF) administration before, rather than after, therapy. (A) Earliest hematopoietic precursors have master transcription factor expression patterns that favor erythroid-megakaryocyte progenitor (EMK) production. Reprogramming to granulocyte-monocyte progenitors (GMP) requires DNMT1 to turn-off baseline stem cell or EMK master transcription factor circuits. Thus, after decitabine, bone marrow, although cellular, is relatively resistant to G-CSF reprogramming toward GMP. G-CSF before decitabine, however, can more efficiently redirect to GMP. (B) Experiment schema. To evaluate these principles *in vivo*, mice were treated with THU/decitabine (THU/Dec) with G-CSF administered after or before. (C) THU/Dec decreased neutrophils but platelets and hemoglobin were preserved or increased; G-CSF administered before appeared more effective at preserving neutrophil counts. Peripheral blood counts at week 7 by Hemavet. (D) Bone marrow flow cytometry at week 7 to measure proportions of granulocytes and monocytes. (E) Bone marrow flow cytometry data (bone marrow granulocyte percentage) quantified in all the treated mice.

null chemorefractory malignant cells (reviewed in [55]). Rapid catabolism of decitabine and 5-azacytidine by CDA severely limits their plasma half-lives and tissue distributions, and thereby likely their ability to deplete DNMT1 in tumor cells *in vivo* [44,45]. Therefore, in this pilot clinical trial in patients with multiply relapsed B- and T-cell lymphoid malignancies, we combined oral decitabine with the CDA-inhibitor THU. The doses of THU and decitabine were selected for noncytotoxic DNMT1-targeting (low decitabine C_{max}, plasma half-life ~2 hours); this was guided by a previous Phase 1 clinical trial that identified minimal biologically active oral doses for this purpose [56]. Objective reductions in lymphoma tumor burden, together with subjective improvements in symptoms, occurred in 4 of 7 patients, but these responses were lost upon study drug interruptions and dose-reductions used to manage neutropenia, a grade 3/4 side-effect observed in all patients.

Neutropenia is expected with cytotoxic chemotherapy, and high concentrations/doses of decitabine have cytotoxic pancytopenia effects [48,57]. However, low doses/concentrations of decitabine that deplete DNMT1 without cytotoxicity also cause neutropenia, via shunting to other lineages rather than cell killing, redirecting cell production fluxes to platelets, counts of which are thus preserved or increased [50-53,57,58]. What is the mechanism? Previous work provides clues. Hematopoietic lineage-trajectories are governed by master transcription factors. Once DNMT1 is depleted, hematopoietic precursors cannot “switch-off” baseline master transcription factor settings, needed to transition to other lineage-fate trajectories [53]. Thus, noncytotoxic decitabine treatment of hematopoietic stem cells expands hematopoietic stem cells (locks in the stem cell program), even if growth factors for alternative lineage-fates, for example, G-CSF, are subsequently added [51,53,59]. Priming toward megakaryocytic fates is the earliest lineage-fate bias of hematopoi-

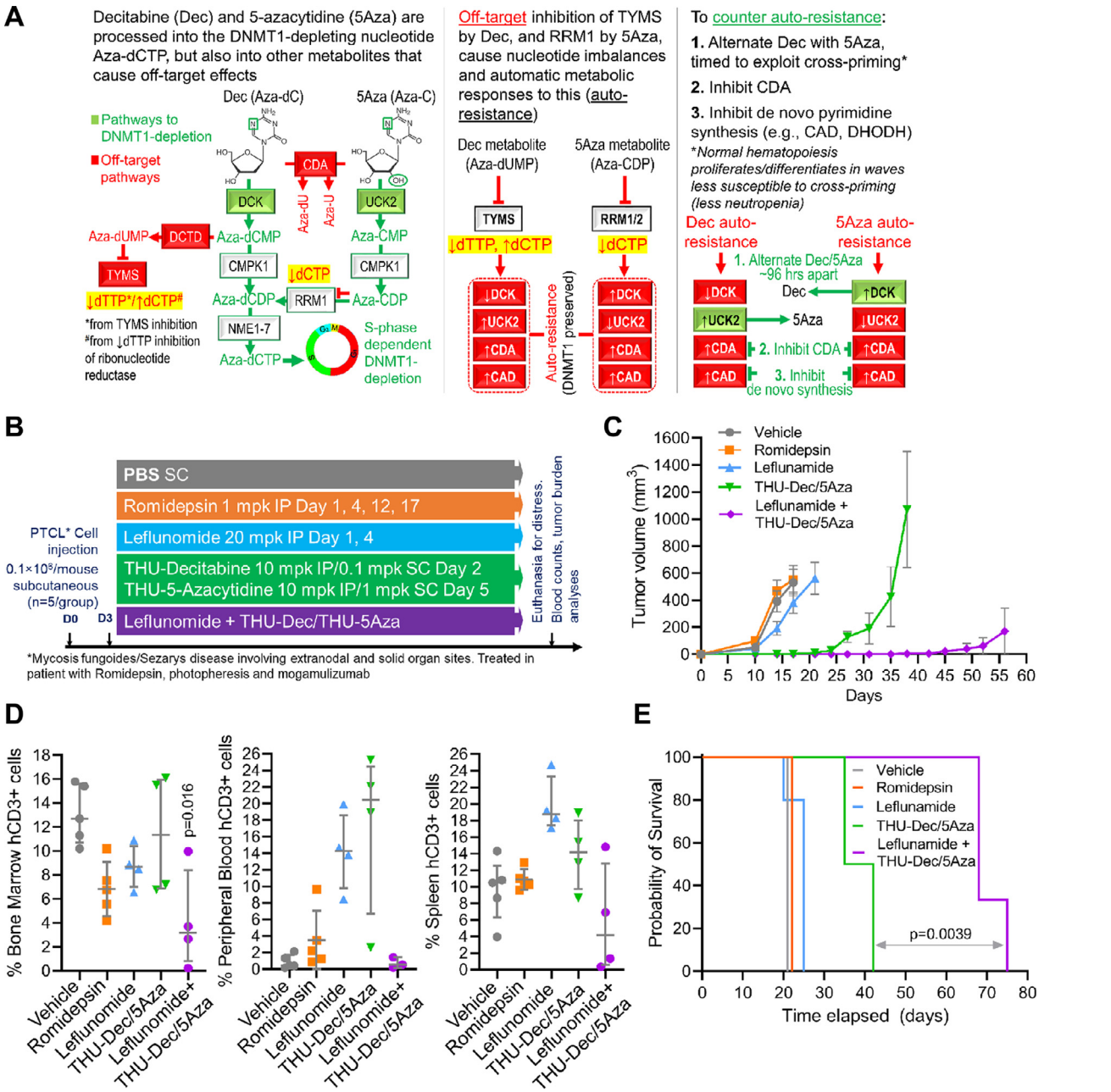

Fig. 5. Local and disseminated CD3+ tumor burden, and time-to-distress, were most substantially improved by alternating decitabine (Dec) with 5-azacytidine (5Aza) and combinations of this with inhibitors of CDA and *de novo* pyrimidine synthesis. (A) Resistance to Dec and 5Aza emerges from feedback responses of pyrimidine metabolism to Dec/5Aza-induced nucleotide imbalances [48]. The evaluated regimen exploits anticipation of these responses to enhance DNMT1-targeting in malignant cells without exacerbating neutropenia. (B) Experimental schema. The xenotransplant model of peripheral T-cell malignancy was derived from a patient with mycosis fungoides/Sezary's disease relapsed after romidepsin, photopheresis, and mogamulizumab. (C) Tumor volume. Mean ± standard error. (D) Disseminated tumor burden measured by flow-cytometry for human CD3+ cells in bone marrow, peripheral blood and spleen after euthanasia. Median ± inter-quartile range. *P*-value 2-sided Mann-Whitney test. (E) Time-to-distress. *P*-values Log-Rank test of THU-Dec/5Aza alone vs THU-Dec/5Aza + Leflunomide.

etic stem cells, documented by master transcription factor expression patterns, DNA methylation and lineage-tracking [54,60-62]. This default setting of normal hematopoiesis, locked-in by non-cytotoxic DNMT1-depletion, may explain preserved or increased platelet counts even as neutrophils/monocytes simultaneously decline [50-53,57,58]. This neutropenia, distinct from that caused by traditional cytotoxic chemotherapy, may require distinct management approaches. *In vivo* in mice, pretreatment with G-CSF before THU/decitabine was better at preserving neutrophil counts then G-CSF administration afterwards. These observations are consistent with prior *in vitro* studies in which G-CSF before, but not after, decitabine was able to promote granulopoiesis [53,59].

The starting doses of THU/decitabine ingested orally (~10/0.2 mg/kg) were close to the minimum biologically effective doses needed to target DNMT1, as shown in a prior Phase 1 clinical trial [56]. This could explain why the dose-reductions used to manage treatment-induced neutropenia correlated with loss of tumor responses. Thus, in future clinical trials, alternative administration schedules, instead of dose-reductions, should be evaluated as an approach to maintain therapeutic DNMT1-targeting but

not exacerbate neutropenia. In designing administration schedules, one key consideration is that DNMT1-depletion by decitabine is S-phase (exposure-time) dependent. Thus, frequent disbursed administration increases the possibilities of overlap between random malignant S-phase entries and drug exposure windows and may be more efficacious than pulse-cycled schedules of administration used for cytotoxic chemotherapy (administration for a few consecutive days followed by extended multiweek intervals needed to recover from toxicity) [48,58,63].

Another consideration is that malignant cells rapidly adapt at a metabolic level to dampen decitabine or 5-azacytidine effect as pyrimidine synthesis compensates to achieve homeostasis [48]. Specifically, decitabine and 5-azacytidine cause nucleotide imbalances [48]. These trigger automatic metabolic compensations that dampen the activity of subsequent doses, and culminate in treatment-resistance [48]. To exploit these consistent and predictable metabolic responses, we alternated the deoxynucleotide analog decitabine with the cytidine analog 5-azacytidine in a preclinical *in vivo* model of mycosis fungoides/Sezary's syndrome, and incorporated into therapy an inhibitor of de novo pyrimidine synthesis as well as THU [48]. These regimen modifications increased tumor cytoreduction without exacerbating neutropenia: malignant cells indefinitely replicate and thus have the opportunity to stabilize metabolic adaptations for resistance, while normal hematopoietic progenitors proliferate/terminally differentiate in successive waves, each treatment naïve. All the agents used in the preclinical experiments are available for clinical evaluation as oral drugs.

Although few patients were treated in this pilot clinical trial, each was a case-study in conventional therapy resistance, having nodal/extranodal disease relapsed after a spectrum of standard treatments, including radiation, high dose chemotherapy/autologous stem cell transplant, and antibody-drug conjugates. It is noteworthy that objective and subjective responses occurred, albeit transiently, to the oral DNMT1-targeting treatment. Responses in this setting, in patients with both B- or T-cell malignancies, are consistent with scientific validation of DNMT1 as a genetics agnostic oncotherapy target. In three patients the lymphoma was primary refractory to THU/decitabine, despite neutropenia indicating DNMT1-targeting in the normal hematopoietic compartment. To understand this primary resistance, the much wider clinical experience with decitabine or 5-azacytidine to treat myeloid malignancies (a standard therapy), can offer useful insights: clinical resistance in the myeloid malignancies was by metabolic configurations in malignant cells that were adverse to decitabine or 5-azacytidine processing into DNMT1-depleting triphosphate nucleotides [48]. Since pyrimidine metabolism is an ancient, fundamental network, we suspect metabolism underlies primary resistance also in lymphoma patients. The regimen modifications evaluated in the pre-clinical model of mycosis fungoides/Sezary's disease are intended to address these mechanisms of resistance, without exacerbating neutropenia.

DNMT1 is one of few targets validated for salvage of p53/p16-null, chemo/radiation-refractory malignancy, warranting clinical evaluation of decitabine to deplete DNMT1, and rational efforts to address its pharmacologic limitations. Here we addressed one such limitation by combining decitabine with THU to inhibit CDA that otherwise severely abbreviates its half-life and solid tissue distribution. The combination of THU with decitabine was pharmacodynamically active: all patients experienced the expected systemic effect of noncytotoxic DNMT1-depletion of neutropenia without thrombocytopenia. The dose reductions used to manage this side-effect, however, also correlated with loss of observed responses. Further regimen refinements, to enable sustainable DNMT1-targeting in malignant cells, but simultaneously limit neutropenia, are needed. Parallel preclinical *in vivo* experiments were thus performed along these lines and suggest directions for further clinical investigation.

References

[1] Pinyol M, Hernandez L, Martinez A, et al. INK4a/ARF locus alterations in human non-Hodgkin's lymphomas mainly occur in tumors with wild-type p53 gene. Am J Pathol 2000;156(6):1987–96.
[2] Vasmatzis G, Johnson SH, Knudson RA, et al. Genome-wide analysis reveals recurrent structural abnormalities of TP63 and other p53-related genes in peripheral T-cell lymphomas. Blood 2012;120(11):2280–9.
[3] Delfau-Larue MH, Klapper W, Berger F, et al. High-dose cytarabine does not overcome the adverse prognostic value of CDKN2A and TP53 deletions in mantle cell lymphoma. Blood 2015;126(5):604–11.
[4] Mak V, Hamm J, Chhanabhai M, et al. Survival of patients with peripheral T–cell lymphoma after first relapse or progression: spectrum of disease and rare long-term survivors. J Clin Oncol 2013;31(16):1970–6.
[5] Coiffier B, Pro B, Prince HM, et al. Romidepsin for the treatment of relapsed/refractory peripheral T-cell lymphoma: pivotal study update demonstrates durable responses. J Hematol Oncol 2014;7:11.
[6] Rozovski U, Benjamini O, Jain P, et al. Outcomes of patients with chronic lymphocytic leukemia and Richter's transformation after transplantation failure. J Clin Oncol 2015.
[7] Moller MB, Gerdes AM, Skjodt K, Mortensen LS, Pedersen NT. Disrupted p53 function as predictor of treatment failure and poor prognosis in B- and T-cell non-Hodgkin's lymphoma. Clin Cancer Res 1999;5(5):1085–91.
[8] Xu P, Liu X, Ouyang J, Chen B. TP53 mutation predicts the poor prognosis of non-Hodgkin lymphomas: evidence from a meta-analysis. PLoS One 2017;12(4):e0174809.
[9] Sauntharajah Y, Sekeres M, Advani A, et al. Evaluation of noncytotoxic DNMT1-depleting therapy in patients with myelodysplastic syndromes. J Clin Invest 2015.
[10] Farooqui MZ, Valdez J, Martyr S, et al. Ibrutinib for previously untreated and relapsed or refractory chronic lymphocytic leukaemia with TP53 aberrations: a phase 2, single-arm trial. Lancet Oncol 2015;16(2):169–76.
[11] Kinzler KW, Vogelstein B. Cancer therapy meets p53. N Engl J Med 1994;331(1):49–50.
[12] Schmitt CA, Lowe SW. Apoptosis is critical for drug response in vivo. Drug Resist Updat 2001;4(2):132–4.
[13] Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74(6):957–67.
[14] Vazquez A, Bond EE, Levine AJ, Bond GL. The genetics of the p53 pathway, apoptosis and cancer therapy. NatRevDrug Discov 2008;7(12):979–87.
[15] Gu X, Hu Z, Ebrahem Q, et al. Runx1 regulation of Pu.1 corepressor/coactivator exchange identifies specific molecular targets for leukemia differentiation therapy. J Biol Chem 2014;289(21):14881–95.
[16] Gu X, Ebrahem Q, Mahfouz RZ, et al. Leukemogenic nucleophosmin mutation disrupts the transcription factor hub that regulates granulomonocytic fates. J Clin Invest 2018;128(10):4260–79.
[17] Di Ruscio A, Ebralidze AK, Benoukraf T, et al. DNMT1-interacting RNAs block gene-specific DNA methylation. Nature 2013;503(7476):371–6.
[18] Peters SL, Hlady RA, Opavska J, et al. Essential role for Dnmt1 in the prevention and maintenance of MYC-induced T-cell lymphomas. Mol Cell Biol 2013;33(21):4321–33.
[19] Hoglund A, Nilsson LM, Forshell LP, Maclean KH, Nilsson JA. Myc sensitizes p53-deficient cancer cells to the DNA-damaging effects of the DNA methyltransferase inhibitor decitabine. Blood 2009;113(18):4281–8.
[20] Guan H, Xie L, Klapproth K, Weitzer CD, Wirth T, Ushmorov A. Decitabine represses translocated MYC oncogene in Burkitt lymphoma. J Pathol 2013;229(5):775–83.
[21] Hassler MR, Klisaroska A, Kollmann K, et al. Antineoplastic activity of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine in anaplastic large cell lymphoma. Biochimie 2012;94(11):2297–307.
[22] Kalac M, Scotto L, Marchi E, et al. HDAC inhibitors and decitabine are highly synergistic and associated with unique gene-expression and epigenetic profiles in models of DLBCL. Blood 2011;118(20):5506–16.
[23] Leshchenko VV, Kuo PY, Jiang Z, Thirukonda VK, Parekh S. Integrative genomic analysis of temozolomide resistance in diffuse large B-cell lymphoma. Clin Cancer Res 2014;20(2):382–92.
[24] Iqbal J, Kucuk C, Deleeuw RJ, et al. Genomic analyses reveal global functional alterations that promote tumor growth and novel tumor suppressor genes in natural killer-cell malignancies. Leukemia 2009;23(6):1139–51.
[25] Kozlowska A, Jagodzinski PP. Inhibition of DNA methyltransferase activity upregulates Fyn tyrosine kinase expression in Hut-78 T-lymphoma cells. Biomed Pharmacother 2008;62(10):672–6.
[26] Ripperger T, von Neuhoff N, Kamphues K, et al. Promoter methylation of PARG1, a novel candidate tumor suppressor gene in mantle-cell lymphomas. Haematologica 2007;92(4):460–8.
[27] Han Y, Amin HM, Frantz C, et al. Restoration of shp1 expression by 5-AZA-2′-deoxycytidine is associated with downregulation of JAK3/STAT3 signaling in ALK-positive anaplastic large cell lymphoma. Leukemia 2006;20(9):1602–9.
[28] Ushmorov A, Leithauser F, Sakk O, et al. Epigenetic processes play a major role in B-cell-specific gene silencing in classical Hodgkin lymphoma. Blood 2006;107(6):2493–500.

[29] Duckworth A, Glenn M, Slupsky JR, Packham G, Kalakonda N. Variable induction of PRDM1 and differentiation in chronic lymphocytic leukemia is associated with anergy. Blood 2014;123(21):3277–85.
[30] Sauntharajah Y, Triozzi P, Rini B, et al. p53-Independent, normal stem cell sparing epigenetic differentiation therapy for myeloid and other malignancies. Semin Oncol 2012;39(1):97–108.
[31] Toy G, Austin WR, Liao HI, et al. Requirement for deoxycytidine kinase in T and B lymphocyte development. Proc Natl Acad Sci U S A 2010;107(12): 5551–5556.
[32] Choi O, Heathcote DA, Ho KK, et al. A deficiency in nucleoside salvage impairs murine lymphocyte development, homeostasis, and survival. J Immunol 2012;188(8):3920–7.
[33] Benton CB, Thomas DA, Yang H, et al. Safety and clinical activity of 5-aza-2′-deoxycytidine (decitabine) with or without Hyper-CVAD in relapsed/refractory acute lymphocytic leukaemia. Br J Haematol 2014;167(3):356–65.
[34] Burke MJ, Lamba JK, Pounds S, et al. A therapeutic trial of decitabine and vorinostat in combination with chemotherapy for relapsed/refractory acute lymphoblastic leukemia. Am J Hematol 2014;89(9):889–95.
[35] Stathis A, Hotte SJ, Chen EX, et al. Phase I study of decitabine in combination with vorinostat in patients with advanced solid tumors and non-Hodgkin's lymphomas. Clin Cancer Res 2011;17(6):1582–90.
[36] Blum KA, Liu Z, Lucas DM, et al. Phase I trial of low dose decitabine targeting DNA hypermethylation in patients with chronic lymphocytic leukaemia and non-Hodgkin lymphoma: dose-limiting myelosuppression without evidence of DNA hypomethylation. Br J Haematol 2010;150(2):189–95.
[37] Stewart DJ, Issa JP, Kurzrock R, et al. Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clin Cancer Res 2009;15(11):3881–8.
[38] Malik A, Shoukier M, Garcia-Manero G, et al. Azacitidine in fludarabine-refractory chronic lymphocytic leukemia: a phase II study. Clin Lymphoma Myeloma Leuk 2013;13(3):292–5.
[39] Clozel T, Yang S, Elstrom RL, et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. Cancer Discov 2013;3(9):1002–19.
[40] Lemonnier F, Dupuis J, Sujobert P, et al. Treatment with 5-azacytidine induces a sustained response in patients with angioimmunoblastic T-cell lymphoma. Blood 2018;132(21):2305–9.
[41] Ebrahem Q, Mahfouz R, Ng KP, Sauntharajah Y. High cytidine deaminase expression in the liver provides sanctuary for cancer cells from decitabine treatment effects. Oncotarget 2012;3(10):1137–45.
[42] Liu Z, Liu S, Xie Z, et al. Characterization of in vitro and in vivo hypomethylating effects of decitabine in acute myeloid leukemia by a rapid, specific and sensitive LC-MS/MS method. Nucleic Acids Res 2007;35(5):e31.
[43] Lavelle D, Vaitkus K, Ling Y, et al. Effects of tetrahydrouridine on pharmacokinetics and pharmacodynamics of oral decitabine. Blood 2012;119(5): 1240–1247.
[44] Shindoh H, Nakano K, Yoshida T, Ishigai M. Comparison of in vitro metabolic conversion of capecitabine to 5-FU in rats, mice, monkeys and humans–toxicological implications. J Toxicol Sci 2011;36(4):411–22.
[45] Dedrick RL, Forrester DD, Cannon JN, el-Dareer SM, Mellett LB. Pharmacokinetics of 1-beta-D-arabinofuranosylcytosine (ARA-C) deamination in several species. Biochem Pharmacol 1973;22(19):2405–17.
[46] Donald Lavelle MG, Pacini Michael, Krauz Lani, Hassan Johara, Ibanez Vinzon, Ruiz Maria A, Pacelli Daisy, Alhandalous Chaher, Phelps Mitch A, Fenner Kathleen, Mahfouz Reda Z, Barnard John, Hsu Lewis L, Gordeuk Victor R, DeSimone Joseph, Sauntharajah Yogen, Molokie Robert E. Combination with Thu to address pharmacologic limitations of decitabine, interim PK/PD from a Phase 1/2 clinical trial of oral Thu-Decitabine in sickle cell disease. Blood 2014;124(21):90.
[47] Terse P, Engelke K, Chan K, et al. Subchronic oral toxicity study of decitabine in combination with tetrahydrouridine in CD-1 mice. Int J Toxicol 2014;33(2):75–85.
[48] Gu X, Tohme R, Tomlinson B, et al. Decitabine- and 5-azacytidine resistance emerges from adaptive responses of the pyrimidine metabolism network. Leukemia 2020.
[49] Cheson BD. Staging and response assessment in lymphomas: the new Lugano classification. Chin Clin Oncol 2015;4(1):5.
[50] Sauntharajah Y, Hillery CA, Lavelle D, et al. Effects of 5-aza-2 '-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood 2003;102(12):3865–70.
[51] Milhem M, Mahmud N, Lavelle D, et al. Modification of hematopoietic stem cell fate by 5aza 2 ' deoxycytidine and trichostatin A. Blood 2004;103(11):4102–10.
[52] Olivieri NF, Sauntharajah Y, Thayalasuthan V, et al. A pilot study of subcutaneous decitabine in beta-thalassemia intermedia. Blood 2011;118(10):2708–11.
[53] Hu Z, Negrotto S, Gu X, et al. Decitabine maintains hematopoietic precursor self-renewal by preventing repression of stem cell genes by a differentiation-inducing stimulus. Mol Cancer Ther 2010;9(6):1536–43.
[54] Izzo F, Lee SC, Poran A, et al. DNA methylation disruption reshapes the hematopoietic differentiation landscape. Nat Genet 2020;52(4):378–87.
[55] Velcheti V, Schrump D, Sauntharajah Y. Ultimate precision: targeting cancer but not normal self-replication. Am Soc Clin Oncol Educ Book 2018(38):950–63.
[56] Molokie R, Lavelle D, Gowhari M, et al. Oral tetrahydrouridine and decitabine for non-cytotoxic epigenetic gene regulation in sickle cell disease: a randomized phase 1 study. PLoS Med 2017;14(9):e1002382.
[57] Negrotto S, Ng KP, Jankowska AM, et al. CpG methylation patterns and decitabine treatment response in acute myeloid leukemia cells and normal hematopoietic precursors. Leukemia 2012;26(2):244–54.
[58] Sauntharajah Y, Sekeres M, Advani A, et al. Evaluation of noncytotoxic DNMT1-depleting therapy in patients with myelodysplastic syndromes. J Clin Invest 2015;125(3):1043–55.
[59] Uchida N, Hsieh MM, Platner C, Sauntharajah Y, Tisdale JF. Decitabine suspends human CD34(+) cell differentiation and proliferation during lentiviral transduction. Plos One 2014;9(8):e104022.
[60] Rodriguez-Fraticelli AE, Wolock SL, Weinreb CS, et al. Clonal analysis of lineage fate in native haematopoiesis. Nature 2018;553(7687):212–16.
[61] Carrelha J, Meng Y, Kettyle LM, et al. Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. Nature 2018;554(7690):106–11.
[62] Psaila B, Mead AJ. Single-cell approaches reveal novel cellular pathways for megakaryocyte and erythroid differentiation. Blood 2019;133(13):1427–35.
[63] Ng KP, Ebrahem Q, Negrotto S, et al. p53 independent epigenetic-differentiation treatment in xenotransplant models of acute myeloid leukemia. Leukemia 2011;25(11):1739–50.