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Tretinoin Enhances the Effects of Chemotherapy in Juvenile Myelomonocytic Leukemia Using an Ex Vivo Drug Sensitivity Assay

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ABSTRACT

PURPOSE Juvenile myelomonocytic leukemia (JMML) is an aggressive pediatric malignancy with myelodysplastic and myeloproliferative features. Curative treatment is restricted to hematopoietic stem-cell transplantation. Fludarabine combined with cytarabine (FLA) and 5-azacitidine (AZA) monotherapy are commonly used pre-transplant therapies. Here, we present a drug screening strategy using a flow cytometry–based precision medicine platform to identify potential additional therapeutic vulnerabilities.

METHODS We screened 120 dual- and 10 triple-drug combinations (DCs) on peripheral blood (n = 21) or bone marrow (n = 6) samples from 27 children with JMML to identify DCs more effectively reducing leukemic cells than the DCs' components on their own. If fewer leukemic cells survived a DC ex vivo treatment compared with that DC's most effective component alone, the drug effect was referred to as cooperative. The difference between the two resistant fractions is the effect size.

RESULTS We identified 26 dual- and one triple-DC more effective than their components. The differentiation agent tretinoin (TRET; all-trans retinoic acid) reduced the resistant fraction of FLA in 19/21 (90%) samples (decrease from 15% [2%-61%] to 11% [2%-50%] with a mean effect size of 3.8% [0.5%-11%]), and of AZA in 19/25 (76%) samples (decrease from 69% [34%-100+%] to 47% [17%-83%] with a mean effect size of 16% [0.3%-40%]). Among the resistant fractions, the mean proportion of $CD38^+$ cells increased from 7% (0.03%-25%; FLA) to 17% (0.3%-38%; FLA + TRET) or from 10% (0.2%-31%; AZA) to 51% (0.8%-88%; AZA + TRET).

CONCLUSION TRET enhanced the effects of FLA and AZA in ex vivo assays with primary JMML samples.

ACCOMPANYING CONTENT

Data Supplement

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INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare and aggressive pediatric malignancy with features of both myelodysplasia and myeloproliferation. In most patients, one of five canonical RAS pathway genes (*PTPN11*, *NRAS*, *KRAS*, *NF1*, or *CBL*) is mutated. Treatment recommendations for children with newly diagnosed JMML are based upon associated prognostic features of these driver mutations and include allogeneic hematopoietic stem-cell transplantation (HSCT), the only established curative approach.[1-3]

Although HSCT is considered the standard of care yielding long-term survival in approximately 50% of patients, the potential impact of pre-HSCT therapy for cytoreduction has not been prospectively studied. A retrospective, single-institution study demonstrated a trend toward improved outcomes in patients who achieved a molecular response with chemotherapy before HSCT.[4] When it is administered, the choice of pre-HSCT therapy is mainly at the discretion of the treating physician. Currently, the most commonly used therapies before conditioning for HSCT are moderately intensive chemotherapy (particularly fludarabine + cytarabine [FLA]) or AZA monotherapy.[3-5] For such regimens, a significant reduction in mutant allele frequency was positively correlated with improved 5-year progression-free survival after HSCT ($P = .12$).[4] Yet, with only 33% (7/21) of patients experiencing such molecular responses,[4] there remains room for improvement.

CONTEXT

Key Objective

Juvenile myelomonocytic leukemia (JMML) is an aggressive pediatric malignancy for which curative treatment is restricted to hematopoietic stem-cell transplantation. Fludarabine combined with cytarabine (FLA) and 5-azacitidine (AZA) monotherapy are commonly used pre-transplant therapies; however, most patients still proceed to transplant with active disease. Leveraging a high-throughput, flow cytometry–based predictive precision medicine platform (PPMP), we sought to identify novel, more effective pre-transplant regimens using primary JMML specimens.

Knowledge Generated

We identified 26 dual- and one triple-drug combination that were more effective than their components. Particularly intriguing was the finding that tretinoin (TRET; all-trans retinoic acid) reduced the FLA and AZA ex vivo resistant fractions and upregulated CD38 on substantial proportions of surviving FLA + TRET and AZA + TRET treated cells.

Relevance

The PPMP-generated data demonstrate that the addition of TRET to current pre-transplant therapies would potentially enhance treatment efficacy.

As of 2023, only AZA is approved by the US Food and Drug Administration for patients with newly diagnosed JMML.[5] Herein, we demonstrate the use of a predictive precision medicine platform (PPMP) for high-throughput ex vivo drug screening in combination with primary JMML specimens to identify treatment regimens potentially superior to those currently in use. To our knowledge, this is the first large-scale study systematically screening for novel drug vulnerabilities in primary JMML cells.

METHODS

Detailed Methods are provided in the Data Supplement.

Patients

Ex vivo drug profiling was successfully performed on 27 patients diagnosed with JMML. Their clinical characteristics, treatment history, and response to treatment are summarized in Table 1 and detailed in the Data Supplement (Table S1).

Ethics

The study design was approved by the Institutional Review Board of the University of California, San Francisco in accordance with the Declaration of Helsinki. All specimens were obtained with the patient's or guardian's written informed consent during routine clinical assessments.

Ex Vivo Drug Sensitivity Testing

Ex vivo drug sensitivity assays with bone marrow (BM) aspirate or peripheral blood (PB) specimens were performed at Notable Labs (Foster City, CA) using a high-throughput, automated custom flow cytometry–based PPMP.[6,7]

Data Analysis

Ex vivo drug sensitivity was evaluated by normalizing the number of leukemic cells remaining in each treatment condition to the mean number of live leukemic cells in the dimethyl sulfoxide (DMSO) control, yielding ex vivo resistant fractions, that is, fractions of leukemic cells surviving the ex vivo treatment at particular drug concentrations. To enrich for effects occurring in multiple patients, we only selected treatment conditions tested on at least five samples, resulting in 58 unique drugs and 130 drug combinations (DCs), composed of 120 dual- and 10 triple-DCs (Data Supplement [Table S2]).

One-sided Wilcoxon signed-rank tests were carried out to determine if a DC induced a more substantial reduction of the resistant fraction than all of its components on their own. The Bonferroni correction was applied to adjust for multiple comparisons at the DC level, with a significance level set to $\alpha = .05/n$, whereby n represents the number of comparisons conducted per DC (n = 2 for dual-DCs, comparing A *v* A + B and B *v* A + B; n = 3 for triple-DCs, comparing A + B *v* A + B + C, A + C *v* A + B + C, and B + C *v* A + B + C; Figs 1A and 1B). *Effect sizes* were determined for each sample represented by the DC to quantify the degree of DC-mediated change of the resistant fraction by subtracting the resistant fraction of the DC from the lowest resistant fraction of its components. A positive value indicates a cooperative effect, suggesting the combination was most effective in reducing the resistant fraction.[8,9] Conversely, a negative value indicates an antagonistic effect, suggesting the combination was less effective in reducing the resistant fraction compared with the most effective component (Data Supplement [Fig S1]). If the DC and its components had resistant fractions >1, the effect size was defined as 0.

TABLE 1. Clinical Characteristics of the 27 Patients From Whom Samples Were Profiled

Characteristic	Analysis Cohort (N = 27)
Age, months, median (range)	14 (1-114)
Sex, No. (%)	
Female	9 (33)
Male	18 (67)
Sample type, No. (%)	
Bone marrow	6 (22)
Peripheral blood	21 (78)
Disease status, No. (%)	
De novo	22 (81)
Relapsed/refractory	5 (19)
Treatment regimen, No. (%)	
Fludarabine, cytarabine	11 (41)
5-azacitidine, fludarabine, cytarabine	2 (7)
Fludarabine, cytarabine, ruxolitinib	1 (4)
Cytarabine, daunorubicin, etoposide	1 (4)
Busulfan, fludarabine, melphalan	1 (4)
5-azacitidine, venetoclax	1 (4)
5-azacitidine	4 (14)
Trametinib	2 (7)
6-mercaptopurine	2 (7)
Unknown	1 (4)
None	1 (4)
Clinical response, No. (%)	
CR	4 (14)
PR	12 (44)
SD	4 (15)
PD	5 (19)
Unknown	1 (4)
Never treated	1 (4)
No. of patients with >1 mutation (%)	6 (22)
PTPN11	8 (24)
KRAS	7 (21)
NRAS	5 (15)
CBL	4 (12)
NF1	4 (12)
ASXL1	2 (6)
JAK3	1 (3)
SETBP1	1 (3)
SH2B3	1 (3)

NOTE. Age represents months at the time of sample collection.
Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; SD, stable response.

Next-Generation Sequencing

Sequencing results were available as part of standard-of-care testing in the majority of samples. In patients without clinical sequencing results, genomic DNA samples were sequenced targeting more than two dozen genes known to be recurrently mutated in JMML as previously described.[4]

RESULTS

Patient Characteristics

The median age of the patient cohort was 14 months (range, 1-114 months) and consisted of 18 males (67%) and nine (33%) females. Among the 27 patients, 22 (81%) had de novo and five (19%) relapsed/refractory (R/R) disease. The most commonly used therapy regimen after sample collection was FLA with or without AZA, administered to 13 of 27 patients. Other treatments included other multiagent chemotherapy, AZA monotherapy, and no therapy. In addition to JMML-associated RAS pathway mutations (*PTPN11*, *KRAS*, *NRAS*, *CBL*, and *NF1*), *ASXL1*, *JAK3*, *SETBP1*, and *SH2B3* mutations were identified (Table 1 and Data Supplement [Fig S2]). The majority (78%) of specimens were PB and 22% were BM. For one patient (filtered out of main analysis for missing a large portion of the combination conditions), both PB and BM aspirate samples were available and tested, demonstrating a strong agreement in ex vivo drug sensitivity across 70 treatment conditions (Pearson's $r = 0.99$; Data Supplement [Fig S3]).

Identification of DCs More Effective Ex Vivo Than Their Components

We sought to identify DCs superior to their components in inducing cytotoxic or cytostatic effects in leukemic JMML cells in ex vivo culture. Of the 130 DCs, 27 (26 dual and one triple), representing 15 unique drugs, demonstrated such an effect (Figs 1C and 1D, Data Supplement [Tables S3 and S4]).

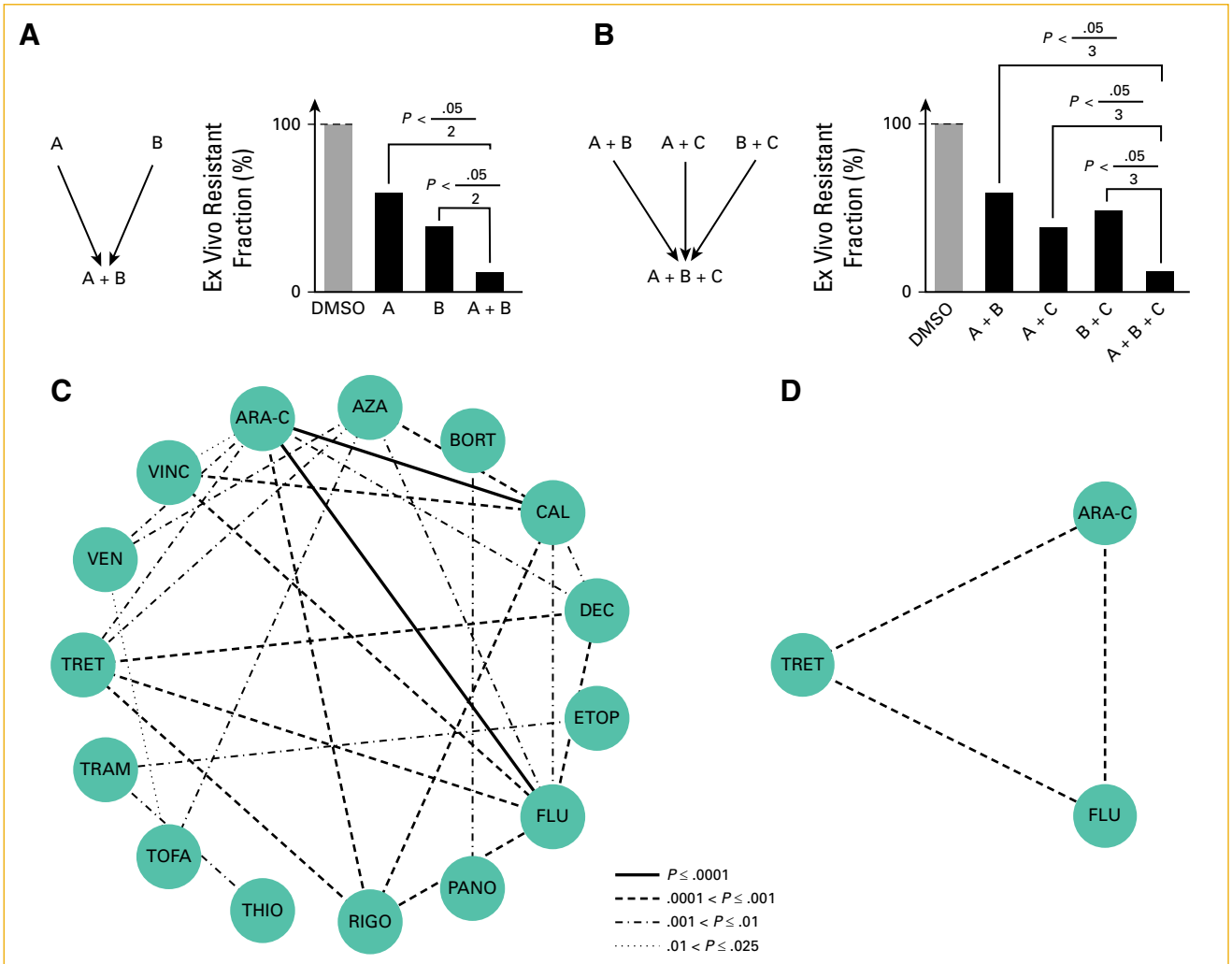
Drug Classes

We ranked the 130 DCs and their corresponding, mechanism of action-based, drug classes by the extent they reduced the leukemic cell fraction. Effective combinations include cytotoxic chemotherapeutic agents such as fludarabine, cytarabine, gemcitabine, doxorubicin, rigosertib, or vincristine (Fig 2). Similarly, drug classes representing cytotoxic therapies (such as antibiotics/anthracyclines, vinca alkaloids/vinca alkaloid-like agents, or antimetabolites) reduced the leukemic cell fraction more extensively as single agents in ex vivo culture than compounds such as differentiating agents or steroids (Fig 3).

Drug classes (over)represented in the 26 dual-DCs include combinations between antimetabolites (cytarabine, fludarabine, and thioguanine), differentiating agents (calcitriol and tretinoin [TRET]), vinca alkaloid/vinca alkaloid–like agents (rigosertib[10,11] and vincristine), and hypomethylating agents (5-azacitidine, decitabine; Data Supplement [Fig S4]).

Effect Types and Sizes

Effect types for the 130 DCs are provided in the Data Supplement (Fig S5). Given the clinical relevance of FLA and AZA, we performed additional analyses on these



Drug	Abbreviation	Drug Class
5-Azacitidine	AZA	Hypomethylating agent
Bortezomib	BORT	Proteasome inhibitor
Calcitriol	CAL	Differentiating agent
Cytarabine	ARA-C	Antimetabolite
Decitabine	DEC	Hypomethylating agent
Etoposide	ETOP	Topoisomerase inhibitor
Fludarabine	FLU	Antimetabolite
Panobinostat	PANO	HDAC inhibitor

Drug	Abbreviation	Drug Class
Rigosertib	RIGO	Vinca alkaloid/vinca alkaloid–like
Thioguanine	THIO	Antimetabolite
Tofacitinib	TOFA	Protein kinase inhibitor
Trametinib	TRAM	Protein kinase inhibitor
Tretinoin	TRET	Differentiating agent
Venetoclax	VEN	BCL-2 inhibitor
Vincristine	VINC	Vinca alkaloid/vinca alkaloid–like

FIG 1. Strategy to identify DCs significantly reducing the ex vivo resistant fractions of their components. (A) Principle to identify dual DCs with hypothetical example. A + B, consisting of the components A and B, is significantly more effective in reducing the ex vivo leukemic fraction than A or B on their own, that is, A + B yields a lower ex vivo resistant (resistant) fraction than A or B. (B) Principle to identify triple-DCs with hypothetical example. A + B + C, consisting of the components A + B, A + C, and B + C, is more effective in reducing the resistant fractions than A + B, A + C, or B + C. (C) Network graph specifying the 26 dual combinations (representing 15 unique drugs and nine drug classes), connecting drugs (nodes) with lines. (D) Network graph specifying the triple combination, connecting drugs with lines. For both (C) and (D), line thickness corresponds to the mean significance (*P*) value of the A + B versus A and A + B versus B tests, with thicker lines indicating higher significance (lower *P* values). BCL-2, B-cell lymphoma 2; DCs, drug combinations; DMSO, dimethyl sulfoxide; HDAC, histone deacetylase.

combinations to understand the ex vivo effect of adding TRET. For FLA + TRET (Fig 4) and AZA + TRET (Fig 5), most of the effects were cooperative, observed in 90% and 76% of samples, respectively, as detailed below.

Inclusion of TRET lowered the ex vivo resistant (resistant) fraction of FLA for 19 of 21 patients (90%) with a mean effect size of 3.8% (range, 0.5%-11%; Fig 4A). For samples demonstrating this cooperative effect, the inclusion of

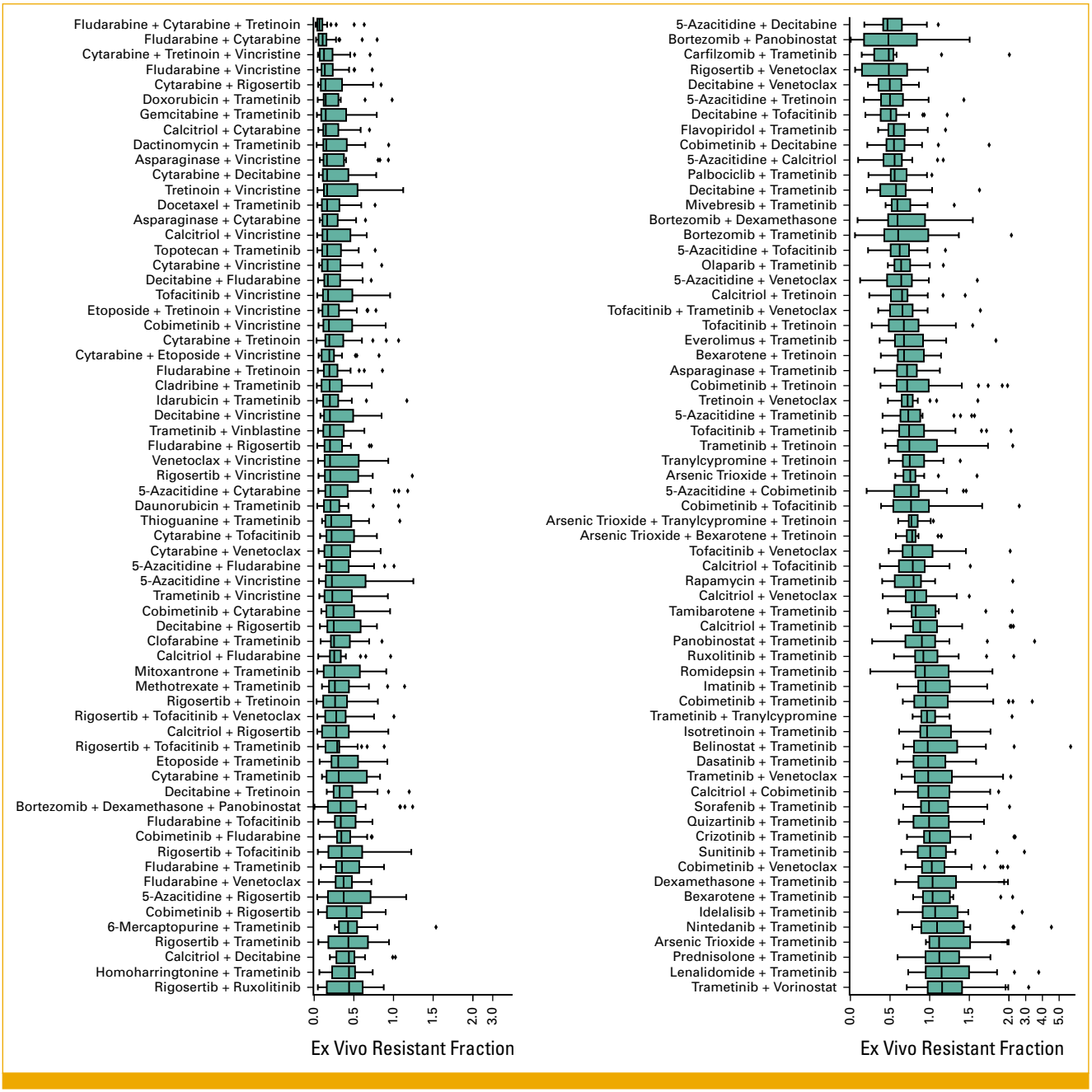


FIG 2. Ranking of the 130 DCs on the basis of their ex vivo effectiveness. Shown are box plots of the ex vivo resistant fractions of the 130 DCs (120 dual and 10 triple) included in the analysis, ordered top to bottom on the basis of increasing median resistant fraction values and thereby reduced ex vivo effectiveness. Each drug is represented by at least five samples (patients). Left and right sides of the boxes represent the first (Q1) and third (Q3) quartiles, respectively. Left and right whiskers indicate the outermost data points no further away from the boxes than 1.5 times the IQR (Q3-Q1) subtracted from Q1, or added to Q3, respectively. Note that the two most effective combinations are FLA (fludarabine + cytarabine) + tretinoin and FLA. Ex vivo resistant fraction values ≥1 indicate lack of an ex vivo antileukemic effect. DCs, drug combinations.

TRET reduced the FLA-resistant fraction by 4%, from 15% to 11%. When only including samples with cooperative effects *and* FLA-resistant fractions >5% (n = 14), the mean effect size was 5% (range, 2%-11%). Relative to the FLA treatment condition, that is, with the FLA resistant fraction set to 100%, TRET reduced the mean resistant fraction in these samples by 30% (30% for de novo [n = 9] and 31% for R/R [n = 5]).

Inclusion of TRET lowered the AZA-resistant fraction for 19 of 25 patients (76%) with a mean effect size of 16% (range, 0.3%-40%; Fig 5A). For samples demonstrating

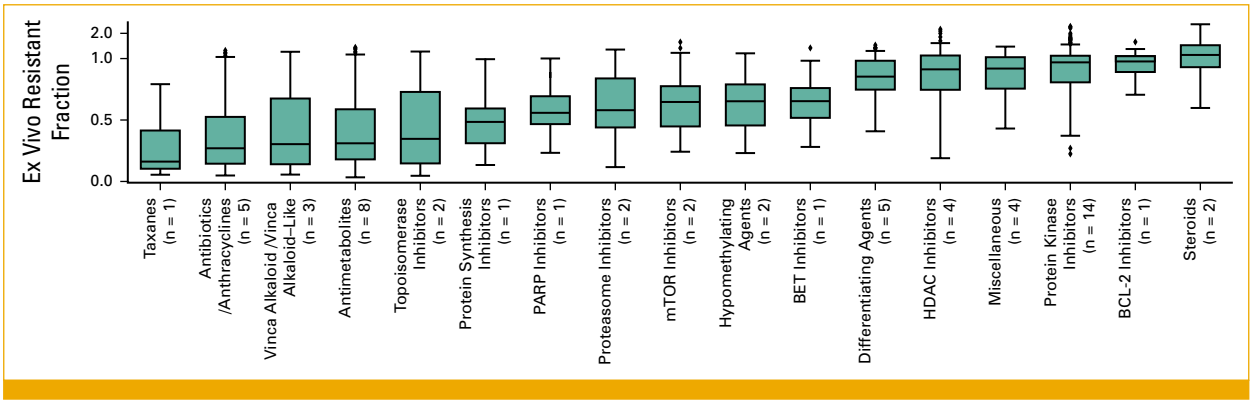


FIG 3. Drug classes representing the 58 drugs included in the analysis. Drugs were classified based on their mechanisms of action. Shown are box plots of the ex vivo resistant fractions for the 17 drug classes representing the 58 drugs included in the analysis, ordered left to right on the basis of increasing median resistant fraction values. This order reflects the reduced ex vivo effectiveness of the drugs when used as single agents. The assignment of the drugs to their classes is found in the Data Supplement (Table S6). Top and bottom of the boxes represent the third (Q3) and first (Q1) quartiles, respectively. Top and bottom whiskers indicate the outermost data points no further away from the boxes than 1.5 times the IQR (Q3-Q1) added to Q3, or subtracted from Q1, respectively. Ex vivo resistant fraction values ≥1 indicate lack of an ex vivo antileukemic effect. BCL-2, B-cell lymphoma 2; BET, bromodomain and extra-terminal domain; HDAC, histone deacetylase; mTOR, mammalian target of rapamycin; PARP, poly (ADP-ribose) polymerase.

this cooperative effect, the inclusion of TRET reduced the AZA-resistant fraction by 22%, from 69% to 47%. All samples with cooperative effects had AZA-resistant fractions >5%. Relative to the AZA treatment condition (100%), TRET reduced the mean resistant fraction in these samples by 31% (33% for de novo [n = 17] and 13% for R/R [n = 2]).

Bliss Independence Testing

To validate the effect size calculations, we compared our results with Bliss independence scores, thereby using a classic approach used to identify synergistic drug relationships. The Bliss model predicts DC effects by assuming drugs act independently of one another, yielding differences between expected and observed effects defined as synergy (positive Bliss score) or antagonism (negative Bliss score).[12,13] However, our approach determines the difference in the resistant fractions between a DC and its most effective component, thereby identifying cooperative or antagonistic effects on the basis of the DC's relative performance. As shown in Figure 5B for AZA + TRET, effect size is positively correlated with Bliss score (Pearson's $r = 0.67$), with 80% (20/25) of samples assigned to the same effect type as by our method (ie, cooperative for positive or antagonistic for negative Bliss scores). For FLA + TRET, the correlation is weaker (Pearson's $r = 0.23$; Fig 4B). However, all samples with a cooperative effect by our method had a positive Bliss score and thereby would have been assigned the same effect type (ie, cooperative) had we only used the Bliss approach. Furthermore, although one sample was classified as antagonistic by our method yet demonstrated a positive Bliss score, the sample had the second-lowest Bliss score among all samples (Fig 4B). Taken together, these data demonstrate concordance between our method and the Bliss approach.

TRET Induced Differentiation in AZA-Treated Samples

We sought to understand whether TRET could provide anticancer activity beyond the reduction of leukemic cell numbers shown in Figures 2, 4A, and 5A, similarly to its in vivo role in cell differentiation in acute promyelocytic leukemia (APL) or its ex vivo roles in differentiation and reduction of the clonogenic potential of myeloid blasts reported for EVI-1+ acute myeloid leukemia (AML).[14,15] Although there was no difference in the relative proportions of (more differentiated) CD11b+ cells in the FLA + TRET compared with the FLA-resistant populations (Fig 4C), TRET in combination with AZA induced differentiation as seen by increased proportions of CD11b+ cells (Fig 5C).

TRET Upregulated CD38 on Ex Vivo Treatment Refractory Cells

CD38 is a therapeutic target in several hematologic malignancies.[16] Interestingly, its gene contains a retinoic acid response element in the first intron conferring responsiveness to ATRA.[17,18] Similarly to ex vivo cultures with primary AML cells,[17] TRET induced the upregulation of CD38 in primary JMML samples relative to the DMSO control (Figs 4D and 5D). The mean proportion of $CD38^+$ cells among the surviving cells in samples with a cooperative effect increased from 7% (0.03%-25%; FLA) to 17% (0.3%-38%; FLA + TRET; Fig 4E) and from 10% (0.2%-31%; AZA) to 51% (0.8%-88%; AZA + TRET; Fig 5E), resulting in mean ex vivo resistant $CD38^-$ fractions of 9% (1%-49%) for FLA + TRET (Fig 4F) and 23% (4%-55%) for AZA + TRET (Fig 5F).

Mutation Status and Effect Size

To investigate the association between gene mutation status and effect size for FLA + TRET and AZA + TRET, we

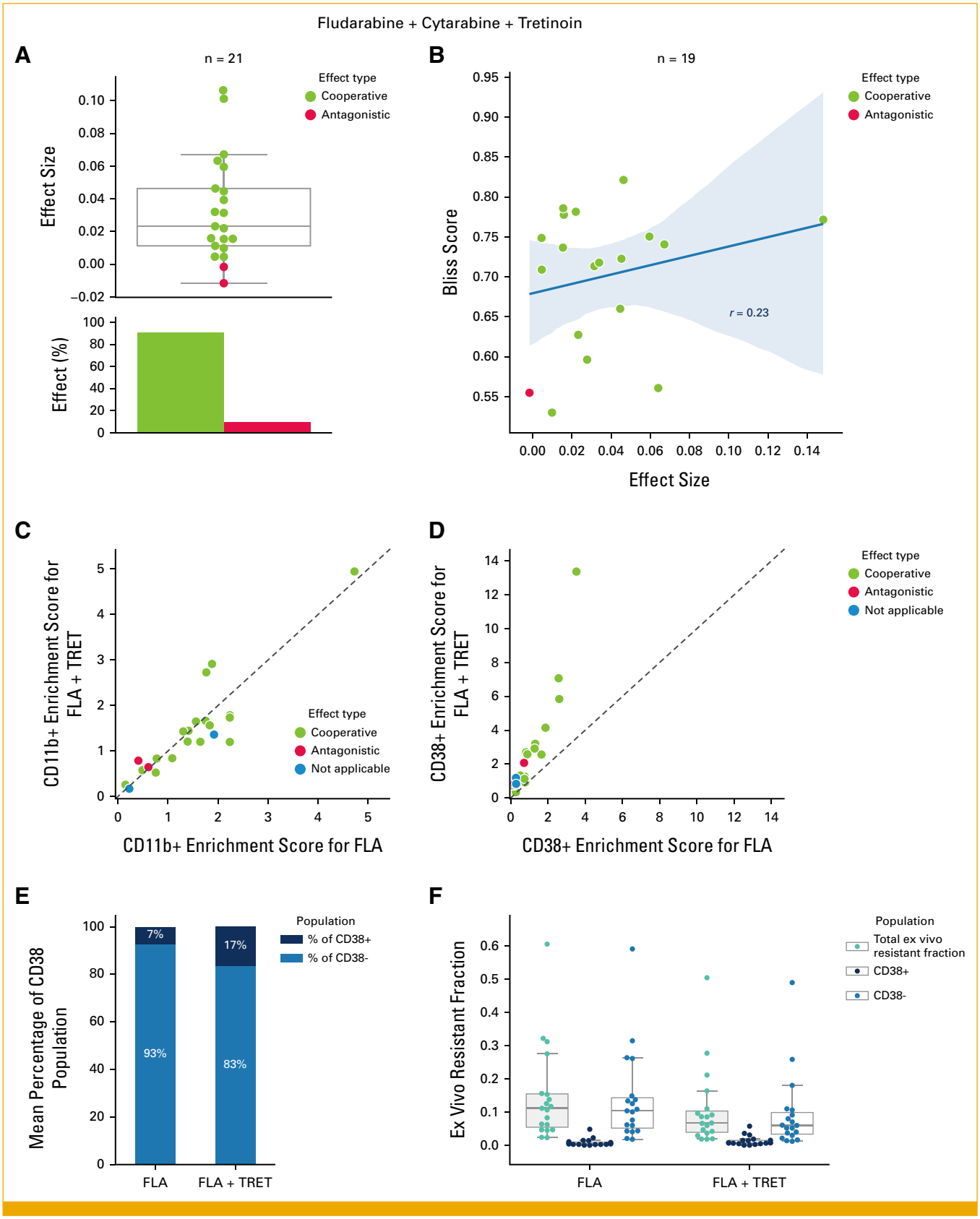


FIG 4. Quantification of the FLA + TRET effect. (A) Box plot with an overlapping swarm plot for FLA + TRET (top) displaying the distribution of individual effect sizes for both effect types (cooperative in green and antagonistic in red), with each dot representing a patient. Top and bottom of the boxes represent the third (Q3) and first (Q1) quartiles, respectively. Top and bottom whiskers indicate the outermost data points no further away from the boxes than 1.5 times the IQR (Q3-Q1) added to Q3, or subtracted from Q1, respectively. (continued on following page)

FIG 4. (Continued). The bar plot (bottom) shows the relative percentage of patients per effect type. Cooperative effects were observed for 19 of 21 (90%) patients for FLA + TRET (mean effect size 3.8%, range 0.5%-11%). (B) Regression plot demonstrating a positive relationship between effect size and Bliss score with Pearson's $r = 0.23$. Each point on the plot, colored by effect type, represents an evaluable patient; shaded region indicates 95% CI. Scatterplot illustrating enrichment scores reflecting the fraction of (C) CD11b+ and (D) $CD38^+$ cells under drug treatment relative to the fraction of CD11b+ or $CD38^+$ cells under vehicle (DMSO) treatment. Therefore, an enrichment score of >1 indicates a higher ratio of CD11b+ or $CD38^+$ cells to total leukemic cells after ex vivo drug treatment than in the DMSO control. The line of identity reflects no change between the two dose conditions. Points above or below the line indicate an increase or reduction, respectively, of the enrichment score for FLA + TRET compared with FLA alone. (E) Stacked bar plot illustrating the mean relative proportions of $CD38^+$ and $CD38^-$ within the FLA versus FLA + TRET resistant fractions ($P = .0000029$) in samples demonstrating a cooperative effect. (F) Box plot with overlapping swarm plot displaying the distribution of individual ex vivo resistant fractions of FLA and FLA + TRET in samples demonstrating a cooperative effect. Each point represents a sample, color indicates the population. FLA, fludarabine + cytarabine; TRET, tretinoin.

categorized genes into RAS pathway, epigenetic modulators (EPI), and JAK-STAT pathway (Data Supplement [Table S5]). We focused on EPI and JAK-STAT pathway mutations since all patients had at least one RAS pathway mutation. Using ordinary least squares regression analysis, we found that gene mutation status explained 34.4% of the variation in effect size for FLA + TRET ($R^2 = 0.344$) and 32.5% for AZA + TRET ($R^2 = 0.325$). EPI pathway mutations were associated with higher effect sizes by AZA + TRET ($P = .007$). The Data Supplement (Fig S6A and S6B) illustrates the distribution of effect sizes for both DCs, grouped by pathway.

Relationship Between Clinical Responses and Ex Vivo Resistant Fractions

To identify whether the PPMP correctly predicted treatment outcomes, we compared the ex vivo resistant fractions after drug treatment with the clinical outcomes of those patients (Table 1) that were treated with the same regimens as their leukemic cells ex vivo (n = 13). As demonstrated in the Data Supplement (Fig S7A), noncytotoxic (AZA, venetoclax + AZA) regimens resulted in less pronounced reduction of the ex vivo leukemic fractions (ie, higher resistant fractions) than cytotoxic (FLA, FLA + AZA) treatments. When focusing only on patients treated with FLA or AZA, we observed that the resistant fractions of responders (partial response or complete response [CR]) were substantially lower than those of the nonresponders (progressive disease or stable disease; $P = .04$). All (3/3) patients treated with AZA were nonresponders. By contrast, 82% (9/11) of patients treated with FLA were responders. Applying a resistant fraction cutoff of 0.3, all (3/3) AZA nonresponders and 89% (8/9) of the FLA responders are identified as nonresponders and responders, respectively (Data Supplement [Fig S7B]).

DISCUSSION

We have previously demonstrated that the screening platform used can predict treatment responses to various regimens used in hematologic malignancies, particularly in adult and pediatric AML.[6,7,19] For this study in JMML, however, the main emphasis was on identifying JMML cell vulnerabilities beyond those currently being used clinically rather than predicting treatment outcome. The PPMP used for this study assesses drug responses on a per-cell basis using flow cytometry and thus provides additional value compared with approaches based on total cells such as CellTiter-Glo or MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assays, which cannot distinguish between malignant and nonmalignant cell populations.

Our data suggest that the addition of TRET to FLA—a regimen frequently used as pre-HSCT therapy—reduces ex vivo leukemic cell counts. Interestingly, the Children's Oncology Group studied another retinoid (13-cis retinoic acid [CRA]) in combination with FLA as pre-HSCT therapy (ClinicalTrials.gov identifier: NCT00025038).[1] CRA is a stereoisomer of all-trans retinoic acid (ATRA, TRET) with a 100-fold lower affinity for binding to retinoid acid receptors compared with TRET.[20-22] However, although their mechanisms of action differ, some of the effects attributed to CRA possibly also occur with TRET since CRA can be converted to TRET, and vice versa, TRET to CRA.[21,23,24] In a trial (ClinicalTrials.gov identifier: NCT00025038), CR rates of 79% (27/34; based on white blood cells [WBCs] only) and 35% (12/34; based on WBCs and organomegaly combined) were reported for patients who received FLA + CRA as first-line pre-HSCT therapy.[1] In the study presented here, where patients were not treated with a retinoid in combination with FLA, we observed a CR rate after FLA of only 27% (3/11; Data Supplement [Table S1]). This raises the question of whether CRA in the trial (ClinicalTrials.gov identifier: NCT00025038) had contributed to the higher CR rate and whether TRET would have exerted a similar effect, although factors such as low numbers of patients treated with FLA and potentially different definitions of CR may confound this assumption.

Although TRET added to FLA only reduced the mean resistant fraction of FLA by 4%, the effect was substantially larger in combination with AZA, where in addition to a 22% reduction of the mean resistant fraction, a 41% increase in the proportion of CD11b+ (more mature) cells occurred. This observation is consistent with those by others, demonstrating in AML/APL an upregulation of CD11b after in vivo or ex vivo treatment with ATRA (TRET).[14,25,26]

We also assessed CD38 in the FLA + TRET and AZA + TRET ex vivo resistant fractions and observed a substantial increase in the relative proportions of $CD38^+$ cells compared with

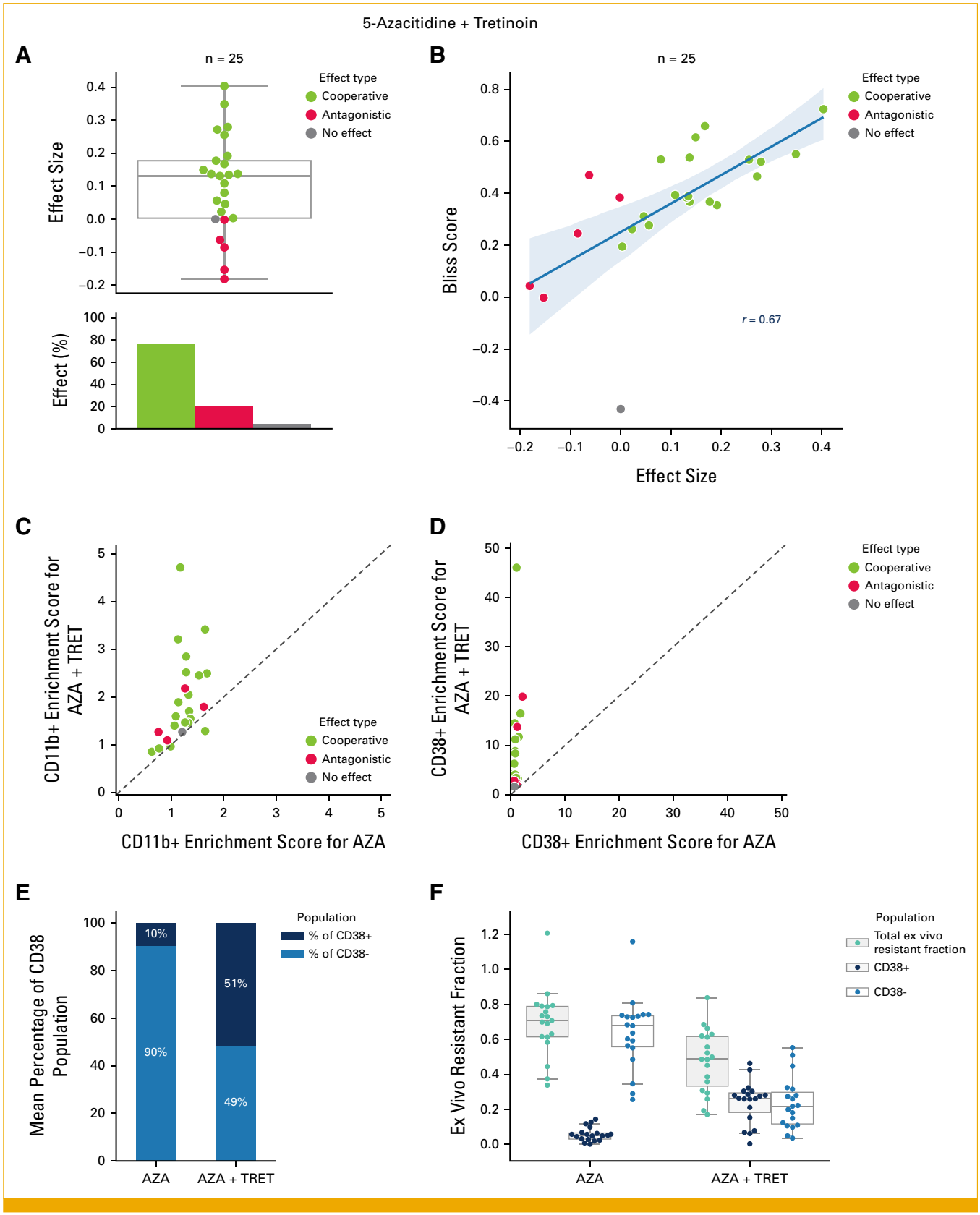


FIG 5. Quantification of the AZA + TRET effect. (A) Box plot with an overlapping swarm plot for AZA + TRET (top) displaying the distribution of individual effect sizes for both effect types (cooperative in green, antagonistic in red, and no effect in gray), with each dot representing a patient. Top and bottom of the boxes represent the third (Q3) and first (Q1) quartiles, respectively. Top and bottom whiskers indicate the outermost data points no further away from the boxes than 1.5 times the IQR (Q3-Q1) added to Q3, or subtracted from Q1, (continued on following page)

FIG 5. (Continued). respectively. The bar plot (bottom) shows the relative percentage of patients per effect type. Cooperative effects were observed for 19 of 25 (76%) patients for AZA + TRET (mean effect size 16%, range 0.3%-40%). (B) Regression plot demonstrating a positive relationship between effect size and Bliss score with Pearson's $r = 0.67$. Each point on the plot, colored by effect type, represents an evaluable patient; shaded region indicates 95% CI. Scatterplot of (C) CD11b+ and (D) $CD38^+$ enrichment scores comparing AZA with AZA + TRET. See legend of Figure 4 for details. (E) Stacked bar plot illustrating the mean relative proportions of $CD38^+$ and $CD38^-$ within the AZA versus AZA + TRET resistant fractions ($P = .0000000037$) in samples demonstrating a cooperative effect. (F) Box plot with overlapping swarm plot displaying the distribution of individual ex vivo resistant fractions of AZA and AZA + TRET in samples demonstrating a cooperative effect. Each point represents a sample, color indicates the population. AZA, 5-azacitidine; TRET, tretinoin.

treatments with only FLA or AZA, respectively. This finding suggests that—theoretically—adding a CD38-targeted agent might further reduce the leukemic cell numbers.

Whether or not to add TRET to current chemotherapy regimens would, among others, depend on the expected toxicity it would add. In pediatric APL, TRET has been associated with differentiation syndrome (retinoic acid syndrome), pseudotumor cerebri, and hyperleucocytosis.[27] However, a combination of ATRA and arsenic trioxide was shown to be noninferior to standard chemotherapy with less toxicity in pediatric patients with APL treated on the Children's Oncology Group phase III clinical trial AAML1331.[28]

In contrast to conventional combination analyses, we chose to screen drugs using single-dose points on the basis of clinically achievable concentrations, rather than using dose ranges. This strategy allowed us to screen a larger number of drugs and DCs, although it carries the risk that the concentrations may be suboptimal and/or that not all combinations with superior effectiveness compared with their components may have been identified. Furthermore, we identified 130 DCs that have the potential to exert more substantial cancer cell–directed effects than their components, although it remains unknown if the ex vivo effects are also biologically meaningful in the absence of clinical correlation. Additional study limitations include small sample sizes, as we could not pragmatically screen all 130 DCs on samples from all 27 patients. However, each combination needed to be tested on at least five samples from five unique patients to be included in the analysis presented here, thereby reducing the chance of incorrectly classifying combinations as more effective than their components. Finally, this study evolved over time, affecting factors including the flow cytometry panels (marker changes) and the flow cytometry staining process. Therefore, some degree of data drift may have occurred.

In summary, we have demonstrated the potential utility of a high-throughput drug screening platform in identifying therapeutic combinations for patients with JMML. Our correlative data suggest that addition of TRET to FLA or to AZA might enhance the desired cytoreduction in children with JMML that has correlated with superior post-HSCT response rates.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Stieglitz E, Ward AF, Gerbing RB, et al: Phase II/III trial of a pre-transplant farnesyl transferase inhibitor in juvenile myelomonocytic leukemia: A report from the Children's Oncology Group. Pediatr Blood Cancer 62:629-636, 2015
2. Mayerhofer C, Niemeyer CM, Flotho C: Current treatment of juvenile myelomonocytic leukemia. J Clin Med 10:3084, 2021
3. Gupta AK, Meena JP, Chopra A, et al: Juvenile myelomonocytic leukemia-A comprehensive review and recent advances in management. Am J Blood Res 11:1-21, 2021
4. Hecht A, Meyer J, Chehab FF, et al: Molecular assessment of pre-transplant chemotherapy in the treatment of juvenile myelomonocytic leukemia. Pediatr Blood Cancer 66:e27948, 2019
5. FDA approves azacitidine for newly diagnosed juvenile myelomonocytic leukemia. US Food and Drug Administration. 2022. https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-azacitidine-newly-diagnosed-juvenile-myelomonocytic-leukemia
6. Spinner MA, Aleshin A, Santaguida MT, et al: Ex vivo drug screening defines novel drug sensitivity patterns for informing personalized therapy in myeloid neoplasms. Blood Adv 4:2768-2778, 2020
7. Strachan DC, Gu CJ, Kita R, et al: Ex vivo drug sensitivity correlates with clinical response and supports personalized therapy in pediatric AML. Cancers 14:6240, 2022
8. Geary N: Understanding synergy. Am J Physiol Endocrinol Metab 304:E237-E253, 2013
9. Duarte D, Vale N: Evaluation of synergism in drug combinations and reference models for future orientations in oncology. Curr Res Pharmacol Drug Discov 3:100110, 2022
10. Kowalczyk JT, Wan X, Hernandez ER, et al: Rigosertib induces mitotic arrest and apoptosis in RAS-mutated rhabdomyosarcoma and neuroblastoma. Mol Cancer Ther 20:307-319, 2021
11. Jost M, Chen Y, Gilbert LA, et al: Pharmaceutical-grade rigosertib is a microtubule-destabilizing agent. Mol Cell 79:191-198.e3, 2020
12. Demidenko E, Miller TW: Statistical determination of synergy based on Bliss definition of drugs independence. PLoS One 14:e0224137, 2019
13. Tang J, Wennerberg K, Aittokallio T: What is synergy? The Saariselkä agreement revisited. Front Pharmacol 6:181, 2015
14. Horna P, Zhang L, Sotomayor EM, et al: Diagnostic immunophenotype of acute promyelocytic leukemia before and early during therapy with all-trans retinoic acid. Am J Clin Pathol 142:546-552, 2014
15. Verhagen HJMP, Smit MA, Rutten A, et al: Primary acute myeloid leukemia cells with overexpression of EVI-1 are sensitive to all-trans retinoic acid. Blood 127:458-463, 2016
16. Naik J, Themeli M, de Jong-Korlaar R, et al: CD38 as a therapeutic target for adult acute myeloid leukemia and T-cell acute lymphoblastic leukemia. Haematologica 104:e100-e103, 2019
17. Farber M, Chen Y, Arnold L, et al: Targeting CD38 in acute myeloid leukemia interferes with leukemia trafficking and induces phagocytosis. Sci Rep 11:22062, 2021
18. Kishimoto H, Hoshino S, Ohori M, et al: Molecular mechanism of human CD38 gene expression by retinoic acid: Identification of retinoic acid response element in the first intron. J Biol Chem 273:15429-15434, 1998
19. Jacoby MA, Welch JS, Westervelt P, et al: Predictive precision medicine platform accurately predicts individual patient response to AML treatments to maximize outcomes. Cancer Res 83, 2023 (suppl 7; abstr 4342)
20. Takahashi Y, Moiseyev G, Chen Y, et al: An enzymatic mechanism for generating the precursor of endogenous 13-cis retinoic acid in the brain. FEBS J 278:973-987, 2011
21. Hunsu VO, Facey COB, Fields JZ, et al: Retinoids as chemo-preventive and molecular-targeted anti-cancer therapies. Int J Mol Sci 22:7731, 2021
22. Özpolat B: Acute promyelocytic leukemia and differentiation therapy: Molecular mechanisms of differentiation, retinoic acid resistance and novel treatments. Turk J Haematol 26:47-61, 2009
23. Breitman TR, Selonick SE, Collins SJ: Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci U S A 77:2936-2940, 1980
24. Hofmann V, Berens ME, Moreillon MC, et al: Effects of 13-cis retinoic acid and Ara-C on differentiation and proliferation of non-promyelocytic acute myelogenous leukemia. Leuk Res 9:1367-1371, 1985
25. Fazi F, Zardo G, Gelmetti V, et al: Heterochromatic gene repression of the retinoic acid pathway in acute myeloid leukemia. Blood 109:4432-4440, 2007
26. Klobuch S, Steinberg T, Bruni E, et al: Biomodulatory treatment with azacitidine, all-trans retinoic acid and pioglitazone induces differentiation of primary AML blasts into neutrophil like cells capable of ROS production and phagocytosis. Front Pharmacol 9:1380, 2018
27. Mahmoud HH, Hurwitz CA, Roberts WM, et al: Tretinoin toxicity in children with acute promyelocytic leukaemia. The Lancet 342:1394-1395, 1993
28. Kutny MA, Alonzo TA, Abla O, et al: Assessment of arsenic trioxide and all-trans retinoic acid for the treatment of pediatric acute promyelocytic leukemia: A report from the Children's Oncology Group AAML1331 trial. JAMA Oncol 8:79-87, 2022