STIMULUS REPORT

blood advances

Efficacy of combined CDK9/BET inhibition in preclinical models of *MLL*-rearranged acute leukemia

Hannah McCalmont,[1,*] Ka Leung Li,[2,*] Luke Jones,[1] John Toubia,[2] Sarah C. Bray,[3] Debora A. Casolari,[2] Chelsea Mayoh,[1] Saumya E. Samaraweera,[2] Ian D. Lewis,[4] Rab K. Prinjha,[5] Nicholas Smithers,[5] Shudong Wang,[6] Richard B. Lock,[1,†] and Richard J. D'Andrea[2,†]

[1]Children's Cancer Institute, School of Women's and Children's Health, University of New South Wales Sydney, Sydney, NSW, Australia; [2]Centre for Cancer Biology, SA Pathology and University of South Australia, Adelaide, SA, Australia; [3]Registry of Older South Australians, South Australian Health and Medical Research Institute, Adelaide, SA, Australia; [4]Adelaide Oncology and Haematology, Adelaide, SA, Australia; [5]Medicines Research Centre, GlaxoSmithKline, Stevenage, United Kingdom; and [6]Drug Discovery and Development, Cancer Research Institute, University of South Australia, Adelaide, SA, Australia

Key Points

- Cyclin-dependent kinase 9 and bromodomain and extraterminal inhibitors are synergistic in MLL-rearranged leukemia.
- Multiple AML driver genes are downregulated by the combined therapy suggesting broad applicability for this subtype.

Introduction

Chromosomal rearrangements of the lysine methyltransferase 2A (*KMT2A* or *MLL*) gene are observed in ~10% of all acute leukemias, with particularly high frequency (~80%) in infant acute lymphoblastic leukemia (ALL),[1] where, despite aggressive chemotherapy, patients still experience poor outcome and long-term side effects.[2] Mixed lineage leukemia (MLL) rearrangements (MLL-r) also indicate particularly poor outcomes for patients with acute myeloid leukemia (AML).[3] Mechanistically, MLL-r frequently generates fusion proteins involving partners that function in the super elongation complex,[4] the result of which is aberrant recruitment to MLL target genes of the positive transcription elongation factor b (PTEFb), composed of cyclin-dependent kinase 9 (CDK9) as the catalytic subunit.[5] CDK9 positively regulates transcription elongation through phosphorylation of serine 2 of RNA polymerase II (RNAPII).[6] Given the central role of CDK9 in the leukemic MLL-r gene-expression program,[7] and the well-described ability of CDK9 inhibitors to reduce levels of the short-lived prosurvival protein MCL1,[8] a number of CDK9 inhibitors have been selected for clinical trials focusing on acute leukemias, including those with MLL-r.[8,9] In MLL-r leukemia, the bromodomain and extraterminal (BET) family member bromodomain-containing 4 (BRD4)[10] acts to recruit PTEFb to superenhancers and together with CDK9 drives increased expression of many oncogenes including *MYC*.[11,12] The roles of CDK9 and BRD4 in MLL-r leukemias present a strong case for testing inhibitors of these proteins in combination as a potential treatment of MLL-r acute leukemias.

Methods

The patient-derived xenograft (PDX) model was established by engrafting infant MLL-r ALL or adult MLL-r AML cells into female NSG mice. Recipient mice were treated with either vehicle, iBET-151 (15 mg/kg; intraperitoneally; 5 days for 2 weeks), CDKI-73 (25 mg/kg; orally; 14 days), or the combination. These doses do not show hematological toxicity (supplemental Table 1). Detailed methods including methods for in vitro assays are provided in the supplemental Methods.

Results and discussion

We first investigated the combination of a CDK9 and BET inhibitor in vitro using cells derived from a panel of established infant MLL-r ALL PDX,[13] and AML PDX cells carrying the *MLL-MLLT3* rearrangement (AML-18) (supplemental Table 2).[14] Combination treatment using CDKI-73[15] and JQ1[16] resulted in further reduction of viable cells compared with either single drug treatment of all samples (Figure 1), with the Bliss-additive curve indicating synergy. We treated 3 MLL-r AML cell lines (MV4;11,

Submitted 14 June 2019; accepted 27 December 2019; published online 23 January 2020. DOI 10.1182/bloodadvances.2019000586.

*H.M. and K.L.L. are joint first authors.

†R.B.L. and R.J.D. are joint senior authors.

All raw data and count files reported in this article have been deposited in the Gene Expression Omnibus database (accession number GSE129449).

The full-text version of this article contains a data supplement.

© 2020 by The American Society of Hematology

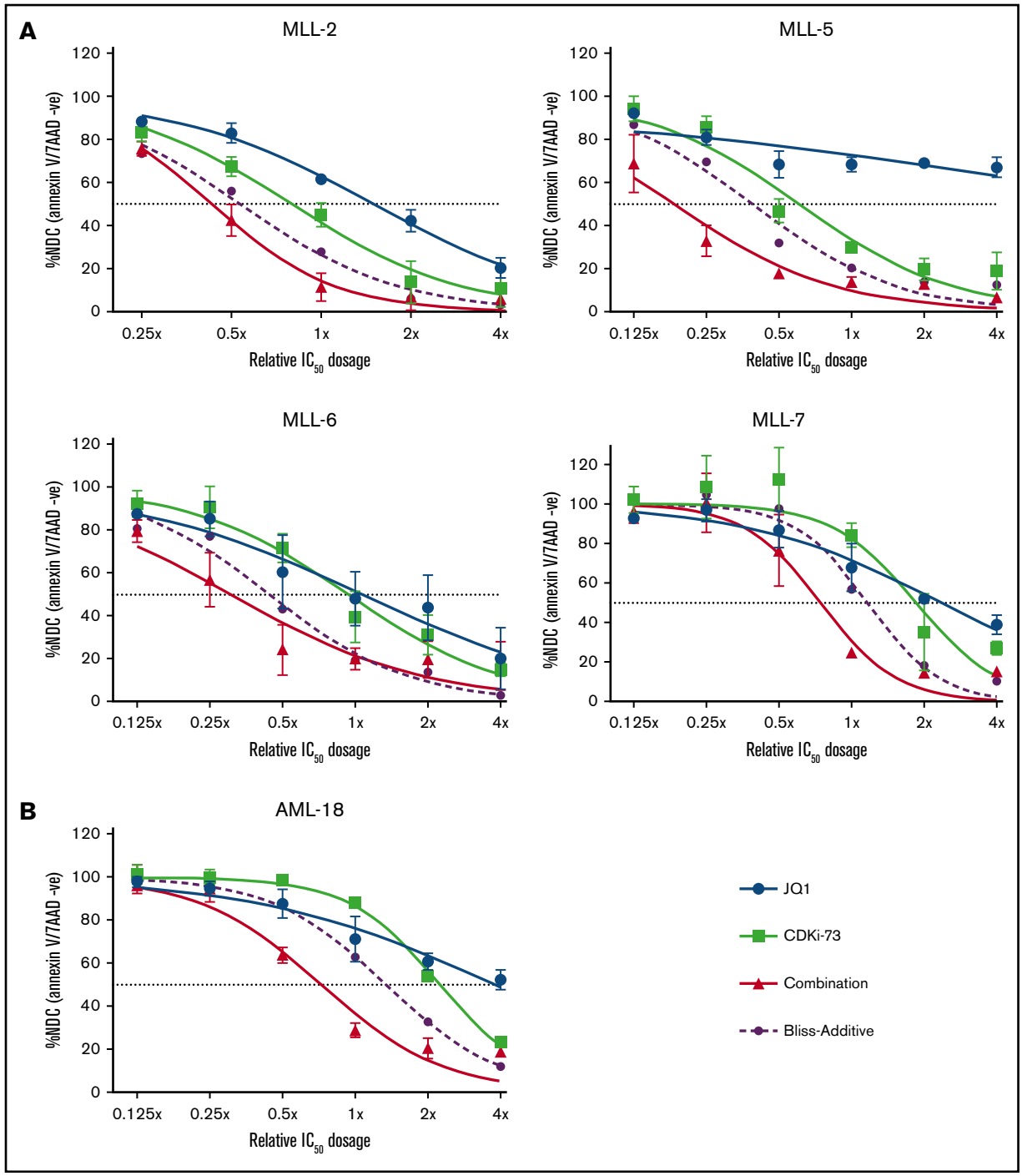

Figure 1. Synergistic activity of combined CDK9/BET inhibition in MLL-r PDXs in vitro. PDX cells from 4 MLL-ALL (n = 2) (A) and 1 MLL-AML (n = 3) (B) were treated for 48 hours, with single treatment of JQ1 (blue), CDKI-73 (light green), or the combination (red). Viability was measured by annexin V and 7-aminoactinomycin D (7AAD) staining and flow cytometry analysis. The double-negative (annexin V and 7-AAD negative) population (viable cells) of treated samples is plotted as a percentage of the no drug control (NDC). Bliss-additive effect is indicated by the purple dashed lines. Viability below this curve indicates synergy. IC_{50}, 50% inhibitory concentration.

MOLM-13, THP1) and a non–MLL-r ALL cell line (697; supplemental Table 2) with a combination of CDKI-73 and the BET inhibitor iBET-151, which has improved pharmacokinetic properties over JQ1. For all cell lines, we observed synergistic cell killing (supplemental Figure 1), consistent with the synergy not being limited to MLL-r.[17]

We next investigated the efficacy of this CDKI-73/iBET-151 combination treatment in vivo following engraftment of infant ALL and adult AML MLL-r PDXs (MLL-2 and AML-18, respectively) in NSG mice. For MLL-2, single treatments delayed disease progression with respect to event-free survival (EFS),

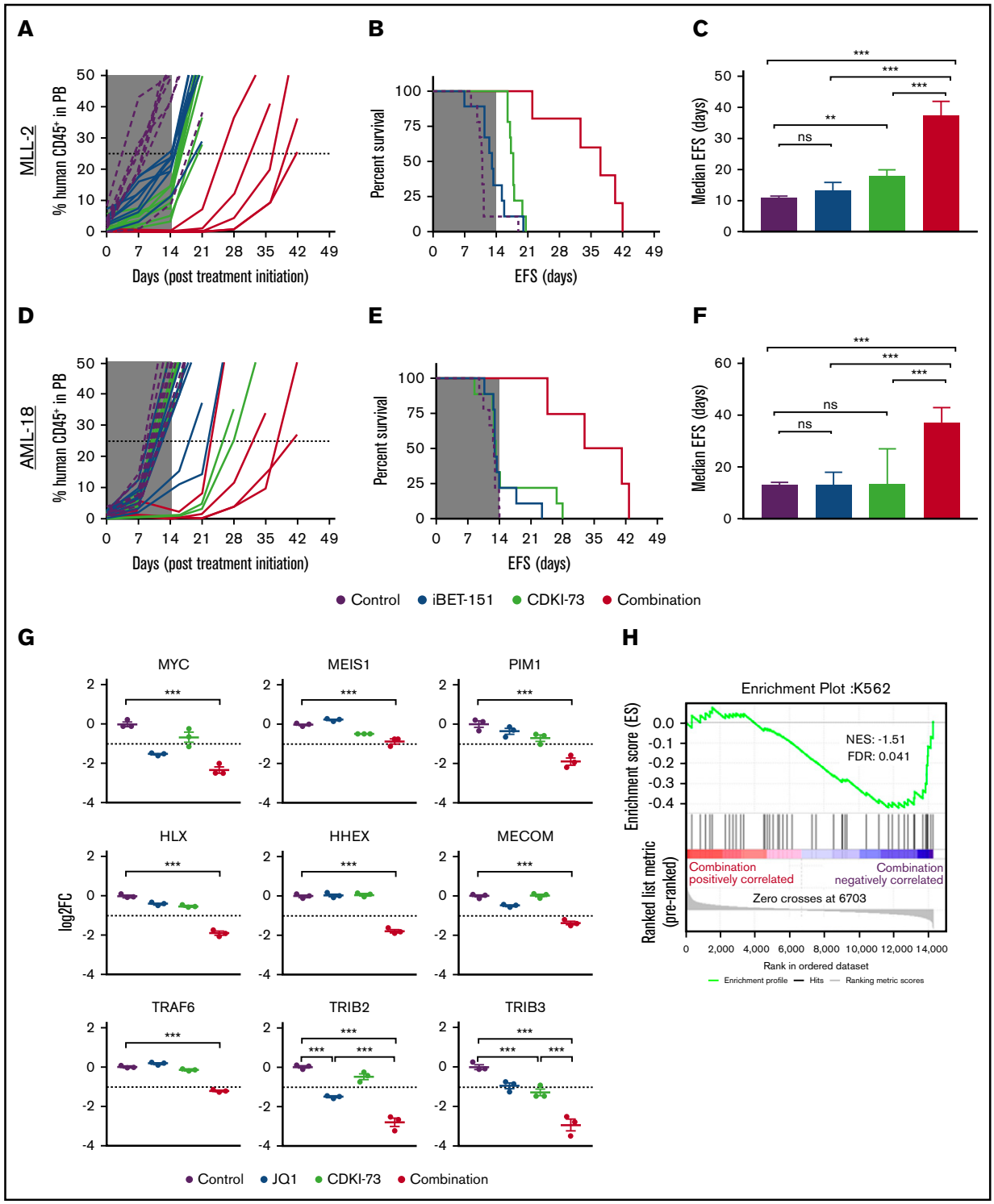

Figure 2. In vivo efficacy of combined CDK9/BET inhibition against MLL-r ALL and AML PDXs. Human CD45$^+$ cells (percentage of huCD45$^+$) in the peripheral blood (PB) of engrafted mice (left panels; A,D), EFS curves (middle panels; B,E), and median EFS of engrafted recipients (right panels; C,F, error bars depict 95% confidence interval) for MLL-ALL (A-C, n = 9 per treatment group) and MLL-AML (D-F, n = 9 per treatment group). Mice were treated with vehicle (purple), iBET-151 (blue), CDKI-73 (light green), or the combination (red) for 14 days as indicated (gray shading indicates treatment duration). (G) Messenger RNA expression of selected genes measured by RNA-seq in AML-18 treated with CDKI-73, JQ1, or in combination for 4 hours (error bars depict standard error, Tukey multiple comparison test; $^{**}P < .01$, $^{***}P < .001$; dashed line indicates log fold change (FC) less than or equal to −1 cutoff for negatively differentially expressed genes). (H) Gene-set enrichment analysis using the ranked gene-expression list as determined by RNA-seq for the comparison of combination treatment vs vehicle in AML-18 cells. Plot shows significant negative enrichment for transcription factor genes associated with superenhancers in K562 cells.[22] No significant enrichment was observed for this gene set with ranked gene expression from single treatments (supplemental Table 4). FDR, false discovery rate; NES, normalized enrichment score; ns, not significant.

however, improvement in median EFS was ≤7 days (Figure 2A-C). Neither single agent significantly delayed the progression of AML-18 (Figure 2D-F). For both PDX models, the combination treatment resulted in a significant increase in EFS compared with vehicle and single treatments (Figure 2B,E) and led to progression delays of 26.2 and 24.3 days for MLL-2 and AML-18, respectively (Figure 2C,F). At the end of the treatment period (day 14), a profound reduction of leukemic burden in the bone marrow, spleen, and peripheral blood compartments was observed in mice engrafted with AML-18 and treated with the combination, compared with mice treated with vehicle control or each single agent (supplemental Figure 2).

Immunoblots of lysates from the spleens of MLL-2–engrafted mice showed that all treatments resulted in depletion of MYC and BCL2 (supplemental Figure 3) as in previous reports.[18] Consistent with on-target activity of CDKI-73, we observed reduced RNAPII-Ser2 phosphorylation with the single treatment (29%), which was further reduced (49%) in the combination. Most strikingly, combination treatment resulted in further reduction of the antiapoptotic protein MCL1, and increased levels of cleaved caspase 3, compared with either single agent alone, consistent with increased cell killing. The decrease of MCL1 is a potential mechanism for the enhanced MLL-2 in vivo response with the combination treatment, and is consistent with targeting of the MCL1 superenhancer.[11]

Downregulation of BCL2 family prosurvival proteins was not observed for the AML-18 PDX (data not shown). To investigate the mechanism for synergy in AML-18, gene-expression changes induced by treatment of the PDX cells in vitro for 4 hours with CDKI-73, JQ1, or the combination were determined by RNA sequencing (RNA-seq). Combination treatment resulted in significant downregulation of the hallmark MLL target genes *MYC* and *MEIS1* (Figure 2G). The reduction in *MYC* expression is likely to be a significant contributor to the synergy observed in the MLL-r AML given that a BRD4- and CDK9-dependent MYC superenhancer is essential for maintenance of MLL-MLLT3–driven AML in mouse models.[11,19,20] Another report also shows that combining a BET inhibitor with alternative CDK9 inhibitors synergistically repressed MYC in an MLL-AML cell line; however, this was not investigated in primary AML.[21] To define other key myeloid oncogenic drivers, downregulation of which may contribute to the synergistic response, we determined genes that were uniquely downregulated in the combination treatment relative to control, or were downregulated by either single agent and in the combination (supplemental Figure 4A; supplemental Table 3). Through comparison with the DisGeNet AML database (supplemental Figure 4B-C; supplemental Table 3), multiple genes known to induce or promote AML were identified displaying enhanced downregulation in the combination treatment (Figure 2G). Of these, *PIM1*, *HLX*, *TRAF6*, and *TRIB3* show similar responses in MV4;11 and MOLM-13 cell lines to that observed in AML-18 (supplemental Figure 5). Gene-set enrichment analysis showed, in combination treatment only, significant negative enrichment of transcription factor genes associated with superenhancers in the K562 myeloid leukemia cell line, but not in $CD34^+$ cells, $CD14^+$ cells, or nonhematopoietic tissue (Figure 2H; supplemental Table 4).[22] Although it has been suggested that the CDK9/BET inhibitor combination may act via a global effect on transcriptional elongation,[21] our results are most consistent with reduced expression by the combination treatment of multiple AML driver genes through targeting of myeloid leukemia superenhancers. Indeed, *PIM1*, *HLX*, and *TRIB3* are linked to myeloid superenhancers and *TRIB3* in particular has a very high superenhancer ranking in K562 cells.[11,22]

Our results highlight the potential of CDK9 inhibitors to act synergistically with transcriptional targeted therapies applicable to MLL-r acute leukemia, for example, BET, DOT1L, and Menin inhibitors.[23] The synergy observed here for 2 PDX models of acute leukemia supports testing of the CDK9/BET inhibitor combination in MLL-r leukemia in the relapsed refractory setting or as an alternative to chemotherapy in high-risk cases. Such a tailored strategy has been successful in acute promyelocytic AML, where combination treatment with all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) has dramatically improved outcome and replaced chemotherapy.[24] A key question will be whether this therapeutic approach reduces disease relapse, which is the major cause of poor survival outcomes in aggressive AML subtypes. However, it is very difficult to model clinical relapse in PDX models, as clonal evolution can differ compared with that in the patient.[25] Thus, clinical testing will be required to establish whether this combination therapy is effective in improving survival for MLL-r acute leukemia patients.

Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia (NHMRC; fellowships APP1059804 and APP1157871 [R.B.L.], and NHMRC program grant APP1091261 [R.B.L.]), a Tour de Cure research grant (17-UNSW-RS-01) (R.B.L. and R.J.D.), grants from the Ray and Shirl Norman Foundation, the Royal Adelaide Hospital Research Foundation, the Contributing Hematologist Committee (R.J.D., I.D.L., and S.W.), and an Industry Development grant from Bioinnovation SA (R.J.D. and S.W.).

Authorship

Contribution: H.M., K.L.L, L.J., S.C.B., D.A.C., S.E.S., I.D.L., R.B.L., and R.J.D. designed experiments and analyzed data; H.M., K.L.L., and L.J. performed the experiments; C.M., J.T., and K.L.L. performed bioinformatics analysis; N.S. and R.K.P. reviewed data and edited the manuscript; S.W. provided CDKI-73 and reviewed the manuscript; and R.J.D. and R.B.L. supervised the research and prepared the manuscript.

Conflict-of-interest disclosure: S.W. is shareholder of, holds patents with, and receives royalties from Le Sun Pharm Ltd. R.K.P. and N.S. are employees and shareholders of GlaxoSmithKline, which is carrying out clinical development of epigenetic inhibitors. The remaining authors declare no competing financial interests.

ORCID profile: K.L.L., 0000-0001-5674-4621.

Correspondence: Richard J. D'Andrea, Acute Leukaemia Laboratory, Centre for Cancer Biology, University of South Australia, GPO Box 2471, Adelaide, SA 5001, Australia; e-mail: richard.dandrea@unisa.edu.au; and Richard B. Lock, Children's Cancer Institute, PO Box 81, Randwick, NSW 2031, Australia; e-mail: rlock@ccia.org.au.

References

1. Mann G, Attarbaschi A, Schrappe M, et al; Interfant-99 Study Group. Improved outcome with hematopoietic stem cell transplantation in a poor prognostic subgroup of infants with mixed-lineage-leukemia (MLL)-rearranged acute lymphoblastic leukemia: results from the Interfant-99 Study. *Blood*. 2010;116(15):2644-2650.
2. Pieters R, Schrappe M, De Lorenzo P, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. *Lancet*. 2007;370(9583):240-250.
3. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424-447.
4. Meyer C, Burmeister T, Gröger D, et al. The MLL recombinome of acute leukemias in 2017. *Leukemia*. 2018;32(2):273-284.
5. Mohan M, Lin C, Guest E, Shilatifard A. Licensed to elongate: a molecular mechanism for MLL-based leukaemogenesis. *Nat Rev Cancer*. 2010;10(10):721-728.
6. Bacon CW, D'Orso I. CDK9: a signaling hub for transcriptional control. *Transcription*. 2019;10(2):57-75.
7. He N, Chan CK, Sobhian B, et al. Human polymerase-associated factor complex (PAFc) connects the super elongation complex (SEC) to RNA polymerase II on chromatin. *Proc Natl Acad Sci USA*. 2011;108(36):E636-E645.
8. Gojo I, Sadowska M, Walker A, et al. Clinical and laboratory studies of the novel cyclin-dependent kinase inhibitor dinaciclib (SCH 727965) in acute leukemias. *Cancer Chemother Pharmacol*. 2013;72(4):897-908.
9. Zeidner JF, Karp JE. Clinical activity of alvocidib (flavopiridol) in acute myeloid leukemia. *Leuk Res*. 2015;39(12):1312-1318.
10. Yang Z, Yik JH, Chen R, et al. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol Cell*. 2005;19(4):535-545.
11. Lovén J, Hoke HA, Lin CY, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell*. 2013;153(2):320-334.
12. Itzen F, Greifenberg AK, Bösken CA, Geyer M. Brd4 activates P-TEFb for RNA polymerase II CTD phosphorylation. *Nucleic Acids Res*. 2014;42(12):7577-7590.
13. Richmond J, Carol H, Evans K, et al. Effective targeting of the P53-MDM2 axis in preclinical models of infant MLL-rearranged acute lymphoblastic leukemia. *Clin Cancer Res*. 2015;21(6):1395-1405.
14. Lee EM, Yee D, Busfield SJ, et al. Efficacy of an Fc-modified anti-CD123 antibody (CSL362) combined with chemotherapy in xenograft models of acute myelogenous leukemia in immunodeficient mice. *Haematologica*. 2015;100(7):914-926.
15. Rahaman MH, Yu Y, Zhong L, et al. CDKI-73: an orally bioavailable and highly efficacious CDK9 inhibitor against acute myeloid leukemia. *Invest New Drugs*. 2019;37(4):625-635.
16. Filippakopoulos P, Qi J, Picaud S, et al. Selective inhibition of BET bromodomains. *Nature*. 2010;468(7327):1067-1073.
17. Baker EK, Taylor S, Gupte A, et al. BET inhibitors induce apoptosis through a MYC independent mechanism and synergise with CDK inhibitors to kill osteosarcoma cells. *Sci Rep*. 2015;5(1):10120.
18. Moreno N, Holsten T, Mertins J, et al. Combined BRD4 and CDK9 inhibition as a new therapeutic approach in malignant rhabdoid tumors. *Oncotarget*. 2017;8(49):84986-84995.
19. Lancho O, Herranz D. The myc enhancer-ome: long-range transcriptional regulation of MYC in cancer. *Trends Cancer*. 2018;4(12):810-822.
20. Bahr C, von Paleske L, Uslu VV, et al. A Myc enhancer cluster regulates normal and leukaemic haematopoietic stem cell hierarchies [published correction appears in *Nature*. 2018;558:E4]. *Nature*. 2018;553(7689):515-520.
21. Gerlach D, Tontsch-Grunt U, Baum A, et al. The novel BET bromodomain inhibitor BI 894999 represses super-enhancer-associated transcription and synergizes with CDK9 inhibition in AML. *Oncogene*. 2018;37(20):2687-2701.
22. Hnisz D, Abraham BJ, Lee TI, et al. Super-enhancers in the control of cell identity and disease. *Cell*. 2013;155(4):934-947.
23. Chan AKN, Chen CW. Rewiring the epigenetic networks in *MLL*-rearranged leukemias: epigenetic dysregulation and pharmacological interventions. *Front Cell Dev Biol*. 2019;7:81.
24. Sanz MA, Fenaux P, Tallman MS, et al. Management of acute promyelocytic leukemia: updated recommendations from an expert panel of the European LeukemiaNet. *Blood*. 2019;133(15):1630-1643.
25. Klco JM, Spencer DH, Miller CA, et al. Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. *Cancer Cell*. 2014;25(3):379-392.