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Antileukemic Efficacy of BET Inhibitor in a Preclinical Mouse Model of MLL-AF4$^+$ Infant ALL

Michela Bardini[1,2], Luca Trentin[3], Francesca Rizzo[1], Margherita Vieri[1], Angela M. Savino[1], Patricia Garrido Castro[4,5], Grazia Fazio[1,2], Eddy H.J. Van Roon[4], Mark Kerstjens[4], Nicholas Smithers[6], Rab K. Prinjha[6], Geertruy Te Kronnie[3], Giuseppe Basso[3], Ronald W. Stam[4,5], Rob Pieters[5], Andrea Biondi[1,2], and Gianni Cazzaniga[1]

Abstract

MLL-rearranged acute lymphoblastic leukemia (ALL) occurring in infants is a rare but very aggressive leukemia, typically associated with a dismal prognosis. Despite the development of specific therapeutic protocols, infant patients with *MLL*-rearranged ALL still suffer from a low cure rate. At present, novel therapeutic approaches are urgently needed. Recently, the use of small molecule inhibitors targeting the epigenetic regulators of the MLL complex emerged as a promising strategy for the development of a targeted therapy. Herein, we have investigated the effects of bromodomain and extra-terminal (BET) function abrogation in a preclinical mouse model of MLL-AF4$^+$ infant ALL using the BET inhibitor I-BET151. We reported that I-BET151 is able to arrest the growth of MLL-AF4$^+$ leukemic cells *in vitro*, by blocking cell division and rapidly inducing apoptosis. Treatment with I-BET151 *in vivo* impairs the leukemic engraftment of patient-derived primary samples and lower the disease burden in mice. I-BET151 affects the transcriptional profile of *MLL*-rearranged ALL through the deregulation of *BRD4*, *HOXA7*/*HOXA9*, and *RUNX1* gene networks. Moreover, I-BET151 treatment sensitizes glucocorticoid-resistant *MLL*-rearranged cells to prednisolone *in vitro* and is more efficient when used in combination with HDAC inhibitors, both *in vitro* and *in vivo*. Given the aggressiveness of the disease, the failure of the current therapies and the lack of an ultimate cure, this study paves the way for the use of BET inhibitors to treat *MLL*-rearranged infant ALL for future clinical applications. *Mol Cancer Ther; 17(8); 1705–16.* ©*2018 AACR.*

Introduction

Acute lymphoblastic leukemia (ALL) carrying the *MLL* rearrangement is a rare but very aggressive disease most frequently occurring in infant patients under the age of 1 year at diagnosis. It is characterized by a dismal prognosis and typically associated to therapy resistance and high incidence of relapse (1). The t(4;11)/MLL-AF4$^+$ is the most recurrent translocation and almost exclusively associated to an early pro-B phenotype. Despite the development and implementation of specific therapeutic protocols (2–4), infant patients with *MLL*-rearranged leukemia still suffer from a low cure rate. Therefore, at present, new therapeutic approaches are urgently needed to improve the overall outcome of these patients.

Alteration of the epigenome has a crucial role in human cancer. Many studies have extensively demonstrated that the mechanism driving to MLL leukemia is mainly ascribable to the alteration of the chromatin structure induced by the MLL fusion, leading to the broad deregulation of a variety of target genes (5–7). Therefore, *MLL*-rearranged infant leukemia may be considered indeed the paradigmatic example of an epigenetic disease and an optimal candidate for novel therapeutic interventions using epigenetic agents.

Several studies reported the use of specific small molecule inhibitors targeting different epigenetic regulators taking part to the MLL complex and functionally involved in the recruitment of the transcriptional machinery, such as, the histone methyltransferase DOT1L, the bromodomain and extra-terminal (BET) proteins or the cofactor Menin (7–11). The BET family of epigenetic adaptors (including BRD2, BRD3, and BRD4) bind to specific acetylated residues of the histone core through their bromodomains and mediate the assembly and the recruitment of the super-elongation complex to promote transcription (7). By blocking the bromodomains, the BET inhibitors are able to abrogate the function of BET proteins and in particular BRD4. The abrogation of BET functions using small molecule inhibitors was proven effective in hematological malignancies (9, 10, 12–14), and clinical trials with chemically different BET inhibitors are currently ongoing (15). More recently, the superior efficacy of BET inhibitors when used in combination with HDAC inhibitors (HDACi) has been reported (16, 17). The first two studies showing the antileukemic efficacy of BET inhibition

[1]Centro Ricerca Tettamanti, Pediatric Clinic, University of Milano-Bicocca, Fondazione MBBM, Monza, Italy. [2]Department of Medicine, University of Milano-Bicocca, Milano, Italy. [3]Department of Woman and Child Health, University of Padua, Padua, Italy. [4]Department of Pediatric Oncology/Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. [5]Princess Maxima Center for Pediatric Oncology, Utrecht, the Netherlands. [6]Epinova DPU, Immuno-Inflammation Therapy Area, GlaxoSmithKline, Medicines Research Centre, Stevenage, Hertfordshire, England, United Kingdom.

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A. Biondi and G. Cazzaniga contributed equally to this article.

Corresponding Author: Michela Bardini, Università di Milano-Bicocca, Monza 20900, Italy. Phone: 39-2333661; Fax: 39-039-2332267; E-mail: michela.bardini@unimib.it

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were published back-to-back in Nature 2011 in two different mouse models of MLL-AF9$^+$ AML, either by using an RNA interference strategy or small molecule inhibitors (9, 10). However, at present, most of the studies were focused on *MLL*-rearranged AML and mainly used retroviral transduction models, human cell lines or patient-derived primary samples exposed to BET inhibitors *in vitro* (9, 10, 14). Less is known about *MLL*-rearranged ALL, the most frequent type of leukemia occurring below the age of 1 year, and in particular, studies using xenotransplantations of patient-derived *MLL*-rearranged ALL primary samples are missing. Additionally, with regard to the survival benefit given by BET inhibitors administration to mice, different studies reported discordant results (9, 18).

Herein, we evaluate the efficacy of BET inhibition in MLL-AF4$^+$ infant ALL, in a preclinical mouse model of xenotransplantation, as a possible alternative approach for the treatment of these young patients with high-risk leukemia.

Materials and Methods

Study approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee and the scientific board of the Interfant Protocol (www.oncauvergne.fr/index.php?option=com_docman&task=doc_download&gid=944&Itemid). The t(4;11)/MLL-AF4$^+$ infant ALL patients used in this study were enrolled in the Interfant-06 Treatment Protocol (EuraCT Number: 2005-004599-19; ref. 4). Samples were collected after having obtained the signed informed consent from the patients' parents. The mononuclear cells were obtained from the Clinic of Pediatric Oncology BioBank in Padova in accordance with a material transfer agreement. The clinical characteristics of patients are reported in Supplementary Table S1. The animal experiments were performed in the Animal Facility at the University of Milano-Bicocca under the approval of the National Ministry of Public Health protocol nr. 64/2014-PR and in compliance with the National law Dlgs n.26/2014 and the European Directive 2010/63/UE. Xenotransplantation assays and *in vivo* bioluminescence imaging for the combination study were performed at the Erasmus MC Animal Facility, Rotterdam, under the approval no. EMC 3388 (103-14-03).

Cell lines

The SEM, RS4;11, MV4;11, KOPN8, and K562 human cell lines were purchased from DSMZ (www.dsmz.de/de) and maintained in complete RPMI medium. The ALL-PO cell line was established in our laboratory from a 3-month-old female infant patient with t(4;11)/MLL-AF4$^+$ ALL through serial transplantations into SCID mice (19) and maintained in complete RPMI advanced medium. The luciferase-transduced SEM cell lines (SEM-SLIEW) were kindly provided by Dr. Stam, Dep. of Pediatric Oncology/Hematology, Erasmus Medical Center, Rotterdam. Cells were maintained in liquid culture for a maximum of 30 passages. Cells were periodically tested and authenticated by phenotype analysis and RT-PCR detection of the fusion gene; mycoplasma was tested by PCR-based analysis.

Compounds

I-BET151 (GSK1210151; ref. 9) was provided by GlaxoSmithKline under a material transfer agreement. EC_{50} values are reported in Supplementary Table S2. Prednisolone was purchased from Bufa BV. The HDACis ITF2357 (Givinostat, PubChem CID: 52914048, patent US 6034096 Example 12) and LBH589 (Panobinostat) were purchased from Selleckchem.

Cell cycle, proliferation, and apoptosis analysis

For cell-cycle analysis, cells were resuspended in 350 μL of ice-cold saline solution containing glucose 1.1 g/L, NaCl 8 g/L, KCl 0.4 g/L, $Na_2HPO_4.2H_20$ 0.2 g/L, KH2PO4 0.15 g/L, and EDTA 0.2 g/L, passed through a 21G needle several times, fixed and permeabilized with 1 mL of EtOH (to a final concentration of 70%), stained with 500 μL of propidium Iodide 10 μg/mL (Sigma-Aldrich) + 25 μL of RNAse 1 mg/mL (Calbiochem) for 1 hour at 4°C in the dark and run by FACS. Analysis was performed using the FlowJo software.

For analysis of cell proliferation, cells were prelabeled with 0.2 μL of carboxyfluorescein succinimidyl ester (CFSE) probe at the final concentration of 1 μmol/L using the CellTrace CFSE Cell Proliferation Kit (Life Technologies) for 15 minutes at room temperature in the dark, incubated for 5 additional minutes with 1 mL of fresh culture medium, washed, then plated in the presence of I-BET151 or DMSO and analyzed by FACS.

For analysis of apoptosis, cells were stained with annexin V/7-AAD (Apoptosis/Necrosis Detection Kit, Enzo Life Sciences) and anti-human CD45 and CD19 antibodies (Becton Dickinson). The percentage of annexin V/7-AAD$^+$ apoptotic cells was evaluated by FACS analysis gating on the CD45$^+$CD19$^+$ human population.

In vivo experiments

The freshly cultured SEM cell line or the thawed cells from primary patients (diagnosis or relapse) were transplanted intravenously into 6- to 12-week-old NOD.CB17-Prkdc^{scid}/NcrCrl (NOD/SCID) mice. Mice were checked daily and weighted twice a week to score weight loss as an early sign of illness or toxicity. Mice were culled at the same time point for analysis of engraftment, or sacrificed when becoming moribund. Engraftment was analyzed by FACS using anti-human CD45-APC (Becton Dickinson), anti-human CD19-PECy5 (Becton Dickinson), anti-mouse CD45-PE (eBioscience), and DAPI (Calbiochem). The cutoff level for positivity was set to 0.1% human CD45$^+$ cells (>0.1% hCD45$^+$) of the live total mononuclear cells in the bone marrow (BM). For *in vivo* imaging, SEM-SLIEW cells were transplanted into NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) recipients. *In vivo* bioluminescence imaging was performed at the Applied Molecular Imaging Facility of Erasmus Medical Center in Rotterdam using an IVIS Spectrum Imaging System (Perkin Elmer) after intraperitoneal injection with RediJect D-Luciferin Bioluminescent Substrate (Perkin Elmer). An injectable solution containing 3 mg/mL of I-BET151 in 5:95 v/v DMSO: (10%) w/v, Kleptose HPB in (0.9%/g) saline, pH5.0 was freshly prepared every week for intraperitoneal (i.p.) administration to mice, according to the manufacturer's instructions. Mice transplanted with SEM cell line were injected daily with I-BET151 30 mg/kg for 2 weeks, according to a previous study (9). In mice transplanted with patient-derived samples, as primary cells have a slower kinetics of engraftment compared with SEM and the treatment needed to be protracted for a longer period, the dose of I-BET151 was reduced to 15 mg/kg and the treatment schedule adjusted to 5-day-on/2-day-off up to 7 weeks, which was much better tolerated and showed no toxicity or long-term side effects, also in

agreement with later studies (18, 20). For *in vivo* combination studies, an injectable solution containing 1 mg/mL of LBH589 was dissolved in 0.9% NaCl saline solution. Mice were injected i.p. with I-BET151 15 mg/kg and LBH589 5 mg/kg on the opposite flanks, according to a 5-day-on/2-day-off schedule, until they showed signs of overt disease or become moribund. The *in vivo* data were analyzed by Mann–Whitney nonparametric statistical test. Survival analysis was performed by Kaplan–Meier survival curve (log-rank Mantel–Cox test).

Gene expression profiling

The diagnostic or relapse sample from 3 MLL-AF4$^+$ infant ALL patients (Pat.1, Pat.3 and Pat.4) were expanded *in vivo* through serial transplantations into NOD/SCID mice. The BM cells were collected from leukemic mice (primary, secondary, or tertiary recipients) 12 to 26 weeks after transplantation. Only those samples showing a robust engraftment (>70% hCD45$^+$ cells in the BM) were selected for GEP analysis. A total of 17 patient-derived xenograft samples were available: $n = 8$ from Pat.1, $n = 4$ from Pat.3, and $n = 5$ for Pat.4. Each sample was exposed to I-BET151 10 μmol/L or DMSO *ex vivo* for 6 hours. RNA was extracted with TRIzol (Invitrogen Life Technologies) from paired I-BET151- or DMSO-treated samples, quantified using Qubit HS-RNA Assay kit (Invitrogen Life Technologies) and checked for quality using the RNA 6000 NANO Assay kit on Agilent Bionalyzer (Agilent). A total of 28 samples ($n = 16$ I-BET151 and $n = 12$ DMSO) were of enough quantity/quality to be processed for GEP. Gene expression analysis was performed using the Affymetrix GeneChip Human Genome U133 Plus 2.0 array and the Affymetrix GeneChip 3′ IVT PLUS reagent kit according to the manufacturer's instructions.

Data analysis was performed using Bioconductor and R packages. Probe level signals were converted to expression values using the robust multiarray averaging (RMA) algorithm. Gene array data were deposited in NCBI's Gene Expression Omnibus (GEO) accession number GSE79057. Raw data were analyzed using the significance analysis of microarray (SAM) algorithm and/or the Shrinkage *t* statistic by setting a false discovery rate (FDR) <0.01. The genes commonly identified with both SAM and Shrinkage *t* test analyses were intersected with those found to be differentially expressed in all the three patients, and the resulting probe set was defined as the "I-BET151 signature" (Supplementary Table S3).

The gene ontology (GO) analysis of the I-BET151 signature was performed using DAVID. The analysis of Upstream Transcriptional Regulator was performed using the Ingenuity Pathway Analysis (IPA, Qiagen). The Gene Set Enrichment Analysis (GSEA) was performed using the "Cancer Hallmark" or "C2cgp" molecular signatures databases with FDR *q* value <0.05 (Supplementary Table S4).

MTS assay

MLL-rearranged cell lines were seeded on a 96-well plate (5×10^5 cells/mL) and treated with different concentrations of prednisolone and/or I-BET151. MTS assay was performed at day 4 after treatment, using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) on a VersaMax Microplate Reader (Molecular Devices).

Additional information available in Supplementary Methods.

Results

I-BET151 inhibits the growth of MLL-AF4$^+$ ALL cells, blocks cell division, and induces apoptosis

The effect of BET function abrogation was first assessed in three different human ALL cell lines carrying the MLL-AF4 fusion (namely, SEM, RS4;11, ALL-PO), using the BET inhibitor I-BET151. Treatment with I-BET151 inhibits the growth of MLL-AF4$^+$ cells *in vitro*, while sparing K562 cells (Fig. 1A). I-BET151 affects the cell cycle, as shown by a decrease of cells in S-phase and an increase in G_0–G_1 phase (Fig. 1B) and blocks the mitotic cell division of MLL-AF4$^+$ cells (Fig. 1C). Finally, I-BET151 rapidly induces apoptosis in *MLL*-rearranged cell lines treated *in vitro* (Fig. 1D), as well as in patient-derived xenograft samples (both at diagnosis and relapse) exposed to the compound *ex vivo* (Supplementary Fig. S1).

Treatment with I-BET151 impairs the engraftment and the disease burden of MLL-AF4$^+$ infant ALL *in vivo*

The efficacy of I-BET151 treatment *in vivo* was evaluated in a xenotransplantation mouse model of MLL-AF4$^+$ ALL. At first, we used the human MLL-AF4$^+$ ALL cell line SEM. One week after transplantation, mice were treated with I-BET151 or the vehicle (DMSO) every day for 2 weeks and then culled for the engraftment analysis. Strikingly, we observed that all the mice in the control group were positively engrafted, while in the BM of treated mice, the leukemic engraftment was not detectable, or below the cutoff level of positivity (≤0.1% hCD45$^+$; Fig. 2A).

Next, we sought to investigate the antileukemic effect of I-BET151 in primary samples. We transplanted the BM cells (diagnostic samples) from two infant patients with MLL-AF4$^+$ ALL (Pat.1 and Pat.2) into primary NOD/SCID mice, waited for the engraftment to occur, then treated the animals with either I-BET151 or DMSO for 5 to 7 weeks according to a 5-day-on/2-day-off schedule. All the mice were culled at the same time for the engraftment analysis in the BM. We observed a reduced leukemic engraftment and a lower disease burden in the group of mice treated with I-BET151 compared with controls (Fig. 2B and C). The status of the disease was checked in transplanted mice before the treatment was started, through BM aspiration (Supplementary Fig. S2). In mice transplanted with Pat.1, a small but clear population of leukemic cells was detectable in the BM aspirates, although at very low level (approximately 0.1% hCD45$^+$; Supplementary Fig. S2A). Instead, mice transplanted with Pat.2 showed a much more consolidated leukemia engraftment (>1% hCD45$^+$, Supplementary Fig. S2B). Nevertheless, even if the treatment was commenced in bulk disease, I-BET151 was proven effective in lowering the disease burden of MLL leukemia (Fig. 2C). Serial transplantation experiments were performed using a population of BM cells derived from Pat.1 after two rounds of expansion *in vivo*, therefore highly enriched for leukemia-initiating cells (L-ICs). Again, we observed that treatment with I-BET151 significantly impaired the leukemic potential of MLL-AF4$^+$ L-ICs in serially transplanted mice (Fig. 2D).

A low-level residual leukemia was still detectable in some mice despite the treatment. To address whether it might have arisen from I-BET151–resistant L-ICs, the BM cells from 4 primary mice showing residual leukemia after one round of treatment *in vivo* were retransplanted in limiting dilution conditions (approximately 12×10^3 hCD45$^+$ cells/mouse) into secondary recipients (Supplementary Fig. S3A). I-BET151 was administrated to serially

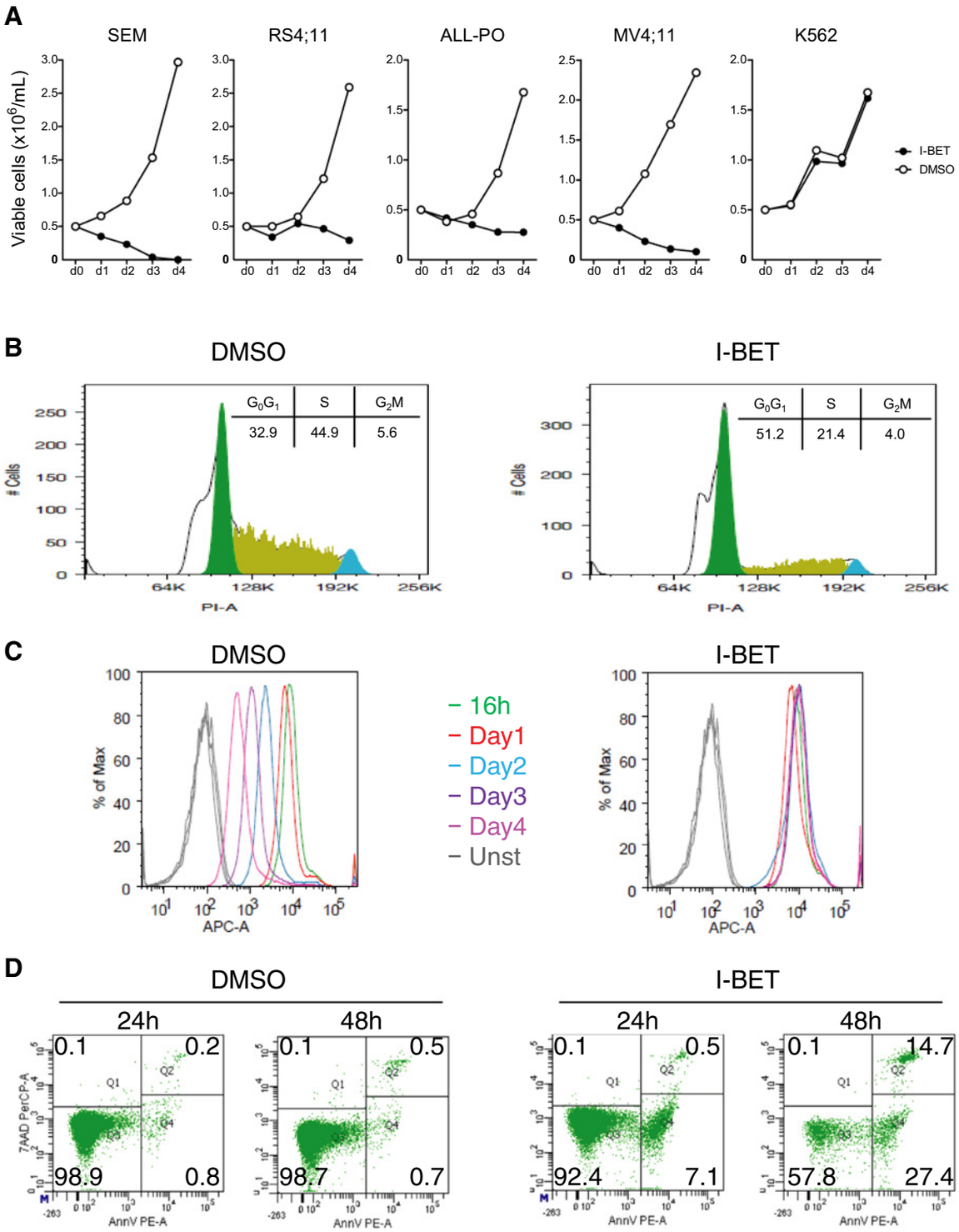

Figure 1.

I-BET151 treatment inhibits the growth of MLL-AF4$^+$ cells *in vitro*. **A,** Growth inhibition assay. Different MLL-AF4$^+$ cell lines are shown (SEM, RS4;11, ALL-PO, MV4;11); K562 was used as control. The number of viable cells was evaluated through viability dye staining and light microscopy cell counting. **B,** Cell-cycle analysis. The percentage of cells after 24 hours of treatment in each phase is reported, showing an increase of G_0–G_1 phase (+55.6%), a decrease of S-phase (−52.3%) and G_2–M phase (−28.6%). Data refer to one cell line are shown, representative of different MLL-AF4$^+$ cell lines. **C,** Proliferation assay using CSFE cell trace staining. Data from one representative cell line are reported. **D,** Analysis of apoptosis. Numbers indicate to the percentage of cells in each quadrant, gated on live cells. I-BET, I-BET151 10 μmol/L; DMSO, vehicle. Data from one representative cell line are reported.

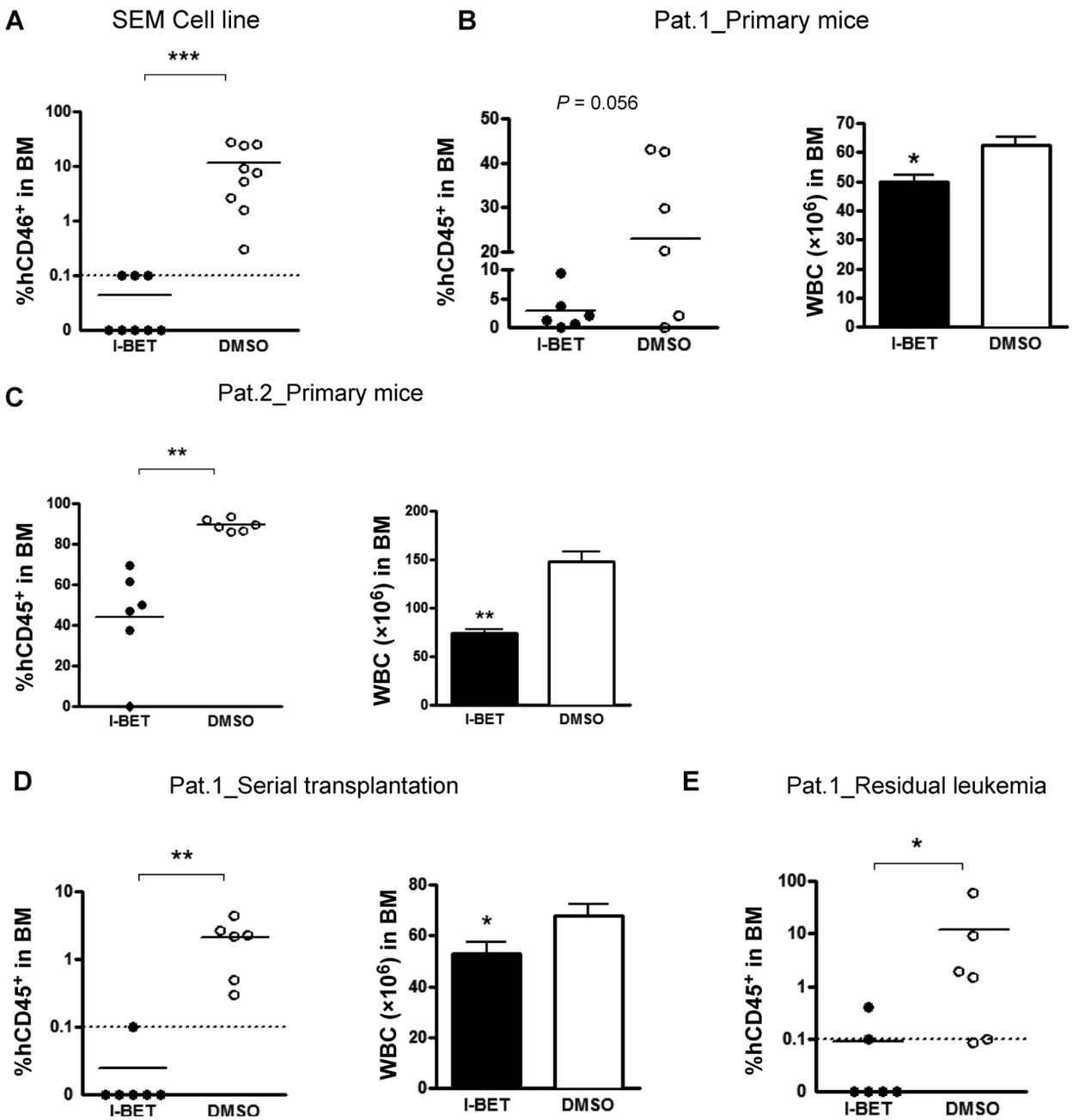

Figure 2.

I-BET151 treatment impairs the engraftment of MLL-AF4$^+$ ALL cells *in vivo*. **A,** Analysis of engraftment in primary NOD/SCID mice transplanted with SEM cell line (9×10^6 cells/mouse) treated with I-BET151 30 mg/kg. Treatment started 1 week after transplantation. The leukemic engraftment (% hCD45$^+$) was measured by FASC analysis in the bone marrow (BM) of transplanted mice, 2 weeks after treatment. **B,** Analysis of engraftment and cellularity in primary mice transplanted with the diagnostic sample from patient 1 (Pat.1, see also Supplementary Fig. S2A) treated with I-BET151 15 mg/kg. **C,** Analysis of engraftment and cellularity in primary mice transplanted with the diagnostic sample from patient 2 (Pat.2, see also Supplementary Fig. S2B) treated with I-BET151 15 mg/kg. The only mouse which was negative at the endpoint analysis (diamond-shaped dot) was indeed found to be positively engrafted (1% hCD45$^+$ cells) in the BM aspirate after 3 weeks of treatment; however, the leukemic engraftment was completely cleared by the end of the treatment. **D,** Analysis of engraftment and cellularity in tertiary mice serially transplanted with Pat.1 treated with I-BET151 15 mg/kg. The cells were expanded twice *in vivo*, then serially transplanted into tertiary recipient mice ($n = 12$, 2×10^6 cells/mouse). Treatment started 1 week after transplantation. The endpoint analysis of engraftment was performed after 5 weeks of treatment. **E,** Analysis of engraftment in mice transplanted with low-level residual leukemic cells in limiting dilution conditions (see also Supplementary Fig. S3) treated with I-BET151 15 mg/kg. The dashed line indicates the cutoff level for positivity in BM (>0.1 %hCD45$^+$). WBC: white blood cells count. I-BET: I-BET151; DMSO: vehicle.

transplanted mice for 5 weeks, then the treatment was stopped for another 5 weeks follow-up period, and the mice were culled for analysis of engraftment at late time point (Supplementary Fig. S3B). Importantly, the engraftment of MLL-AF4$^+$ residual leukemic cells was highly impaired in I-BET151–treated mice compared with controls (Fig. 2D), suggesting that L-ICs giving rise to residual leukemia were still responsive to treatment upon serial transplantation and reexposure to the compound *in vivo*.

I-BET151 treatment changes the MLL-AF4 transcriptional profile through the deregulation of *BRD4*, *HOXA7/9*, and *RUNX1* gene networks

The transcriptional profile of MLL-AF4$^+$ ALL cells treated with I-BET151 was investigated by gene expression analysis. Through a comparative statistical approach (Fig. 3A), we identified 601 probe sets consistently modulated upon I-BET151 treatment, consisting of 189 upregulated and 216 downregulated genes (Fig. 3B; Supplementary Table S3). This was referred hereafter as the "I-BET151 signature."

Gene ontology analysis revealed that the genes identified in the I-BET151 signature were mainly involved in the nucleosome core assembly and organization, post-transcriptional regulation, and chromatin remodeling (Fig. 3C), thus confirming the expected activity of I-BET151 as an epigenetic agent.

Further analyses were performed to identify the putative upstream transcriptional regulators of the genes included in the I-BET151 signature. As expected, *BRD4* was found as an upstream transcriptional regulator (Fig. 3D), being BRD4 the

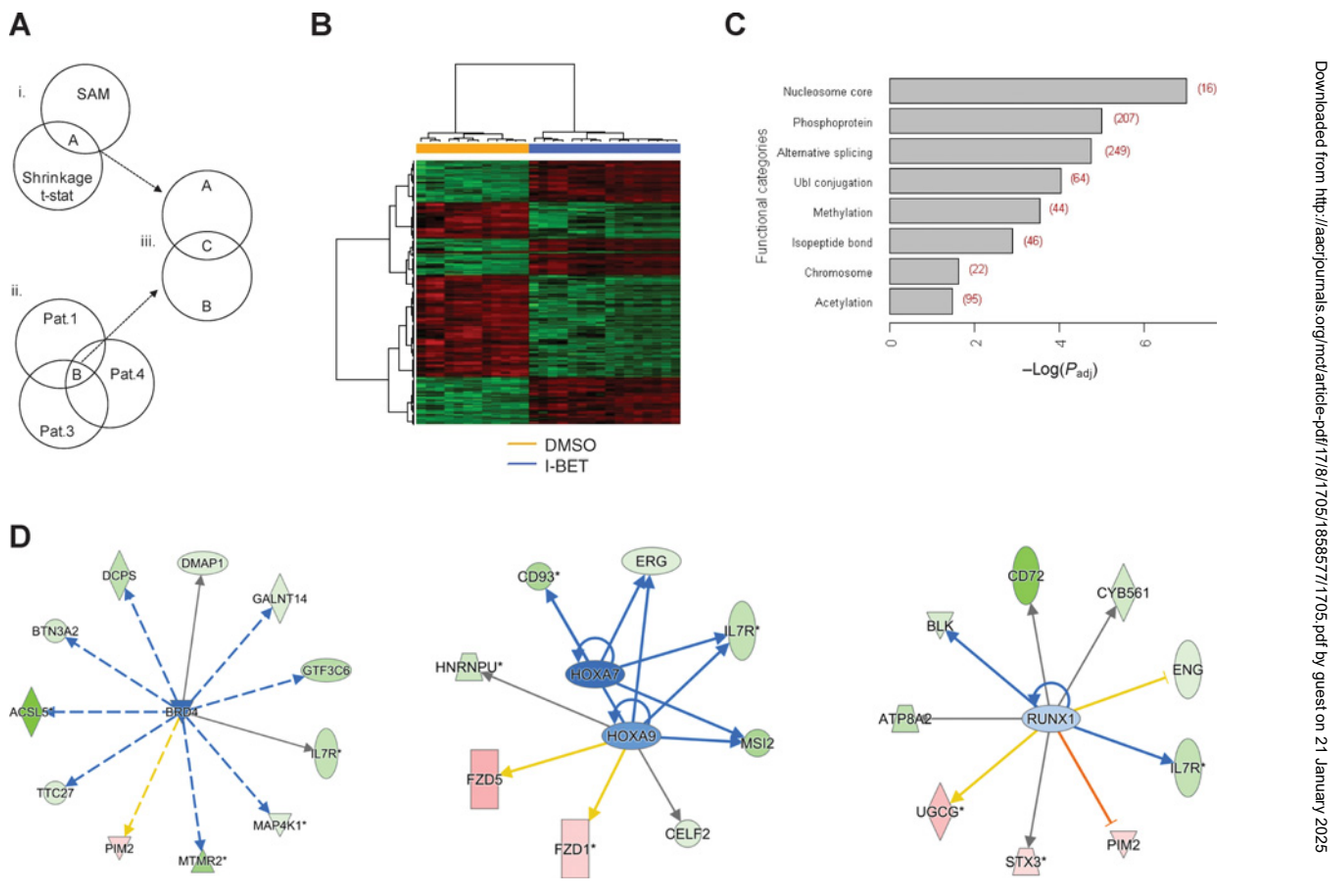

Figure 3.

I-BET151 changes the transcriptional profile of patient-derived xenograft samples though the downregulation of BRD4, HOXA7/9, and RUNX1 gene networks. **A,** Overview of the comparative statistical tests used for GEP analysis. (i) Raw data were analyzed by SAM and Shrinkage *t* test analysis (FDR *q* <0.01) by comparing I-BET151 treated versus the controls (DMSO). Common deregulated probe sets resulting from both the methods were identified (**A**). (ii) Each patient (Pat. 1, 3, and4) was analyzed individually by Shrinkage *t* test analysis (FDR *q* <0.05) and commonly deregulated probe sets were identified (**B**). (iii) Probe sets A were intersected with probe sets B to identify the common deregulated probes (C), here referred as the "I-BET151 signature." **B,** Unsupervised clustering analysis of the 601 probe sets within the I-BET151 signature (red, upregulated; green, downregulated). I-BET: I-BET151; DMSO: vehicle. **C,** Gene ontology analysis (DAVID) of the I-BET151 signature. Gene categories with FDR *P* value <0.05 are reported. The number of probe sets listed in each category is indicated in brackets. **D,** Analysis of upstream transcriptional regulators of the I-BET151 signature. The predicted upstream regulators and their downstream targets are depicted in the inner and outer circles, respectively. The different colors indicate the level of expression of upstream regulators (blue, downregulated) or downstream targets (green, downregulated; red, upregulated), and the color intensity reflects the degree of regulation (from mild to strong). The line shapes and colors indicate the predicted relationship between the downstream targets and their upstream regulators (dashed, indirect interaction; solid, direct interaction; blue, leads to inhibition; yellow, inconsistency between the predicted relationship and the state of the downstream target; grey, unpredicted effect; orange, leads to activation; arrow line, stands for "acts on"; plain line, indicates an inhibitory effect). Asterisks (*) indicate that multiple identifiers in the dataset file map to a single gene.

main target of I-BET151. More intriguingly, two members of the HOXA family (namely, *HOXA9* and *HOXA7)* and *RUNX1* also emerged as predicted upstream transcriptional regulators of the I-BET151 signature (Fig. 3D). Similarly to *BRD4*, also *HOXA7/9* and *RUNX1* gene networks resulted to be downmodulated after I-BET151 treatment, as revealed by the analysis of upstream regulators.

The GSEA showed that two signatures upregulated in *MLL*-rearranged leukemia ("Mullighan signature 1 and 2") were enriched in DMSO controls, while the gene targets downregulated by MLL-AF4 fusion ("Gaussmann") were enriched in I-BET151 treated samples (Supplementary Table S4), consistent with the MLL-AF4 transcriptional program being reversed upon I-BET151 treatment. These data support the efficacy of I-BET151 for the treatment of MLL-AF4$^+$ infant ALL and further strengthen the robustness of our signature.

cMyc, *Bcl2*, and *CDK6* were previously identified as direct targets of BRD4 in *MLL*-rearranged AML (9). In particular, *CDK6* is also a direct target of MLL fusion and crucially required for the maintenance of *MLL*-rearranged leukemia (21, 22). *CDK6* was found in our I-BET151 signature as significantly downregulated upon I-BET151 treatment (Supplementary Table S3) and validated in independent experiments both *in vitro* and *ex vivo* (Supplementary Fig. S4). Although *c-Myc* and *Bcl2* were not included in our I-BET151 signature when specifically investigating their expression by qPCR, we could observe that, alike CDK6, c-Myc and Bcl2 transcripts were downregulated in diagnostic or relapse patient-derived xenograft samples exposed to the compound *ex vivo* (Supplementary Fig. S4A). Additionally, the binding of BRD4 to the transcriptional starting site of *CDK6*, *c-Myc*, and *Bcl2* was decreased in MLL-AF4$^+$ human ALL cell lines upon I-BET151 treatment *in vitro* as shown by ChIP analysis (Supplementary Fig. S4B), consistent with those genes being direct targets of BRD4. Finally, the expression of the proteins was not detectable by Western blot in the BM of mice treated with I-BET151 *in vivo* compared with controls (Supplementary Fig. S4C). Moreover, the GSEA of the I-BET151 signature revealed that *c-Myc* targets were enriched in DMSO control samples, whereas proapoptotic genes were enriched in I-BET151 treated samples (Supplementary Table S4), thus supporting our functional data *in vitro* and further corroborating the hypothesis that those pathways are crucially targeted by BET inhibitors.

Few genes, newly identified in our I-BET151 signature, were selected based on the current literature as potentially involved in MLL leukemia and validated in independent experiments, such as *IL7R*, *CD93*, *MSI2*, and *CD72*. *IL7R* emerged from the upstream regulator analysis of our I-BET151 signature as a common downstream target of BRD4, HOXA7/9, and RUNX1 (Fig. 3D). Treatment with I-BET151 downregulates IL7R expression (both mRNA and protein) in MLL-AF4$^+$ ALL cell lines *in vitro* as well as in diagnostic or relapse PDX samples *ex vivo*, and the activation of IL7R downstream effector phospho-STAT-5 was also impaired (Supplementary Fig. S5A), in agreement with a previous study (13). Similarly, the downregulation of *CD93*, *MSI2*, and *CD72* upon I-BET151 exposure was validated in independent experiments, both *in vitro* and *ex vivo* (Supplementary Fig. S5B–S5D).

I-BET151 sensitizes *MLL*-rearranged ALL cell to prednisolone *in vitro*

Typically, *MLL*-rearranged infant ALL cells are more resistant to glucocorticoids compared with other childhood B-ALL (23). However, the GSEA of our I-BET151 signature revealed that genes involved in the xenobiotic metabolism and estrogen response (early and late) were enriched in I-BET151-treated samples, whereas genes involved in the inflammatory response were enriched in DMSO controls (Supplementary Table S4). These findings suggest that treatment with BET inhibitors might potentiate the effect of other compounds used in standard chemotherapy, for example the glucocorticoids with anti-inflammatory activity (i.e., prednisone). Additionally, the activation of the estrogen/glucocorticoids-related transcriptional signatures, such as the progesterone receptor (PGR) signaling, was also predicted by the upstream regulator analysis in I-BET151-treated samples (Fig. 4A).

According to these data, we sought to investigate whether I-BET151 was able to potentiate the effect of prednisone, a glucocorticoid agent with anti-inflammatory activity currently used in the standard therapy. Two *MLL*-rearranged ALL cell lines highly resistant to glucocorticoids (according to their EC_{50} values), namely, SEM and KOPN8, carrying the t(4;11)/MLL-AF4 or the t(11;19)/MLL-ENL translocation respectively, were used for combination studies *in vitro*. Cells were treated with I-BET151 alone or in combination with prednisolone (the active form of prednisone), and MTS assay was performed 4 days after treatment. In both cell lines, we observed that the combinatorial effect of I-BET151 and prednisolone treatment was higher than the predicted sum of the effects given by the single compounds (Fig. 4B), consistent with the hypothesis that I-BET151 sensitizes glucocorticoids-resistant *MLL*-rearranged cells to prednisolone.

The antileukemic activity of I-BET151 is enhanced when combined to HDACis

The GSEA of the I-BET151 signature showed that the gene targets upregulated by HDAC ("Heller" probe set) and genes involved in the response to HDACi such as Azacytidin, Trichostatin A, and Romidepsin ("Zhong," "Chiba," "Kim," "Della," "Kabayashi" probe sets) were found enriched in I-BET151-treated samples, while the genes downregulated by HDAC ("Heller" probe sets and "Peart" proliferation cluster) were found enriched in DMSO controls (Supplementary Table S4), thus providing a rational for the use of BET and HDACis in combination. Two different pan- HDACi compounds were used for combination studies *in vitro* and *in vivo*, namely, ITF2357 (Givinostat) and LBH589 (Panobinostat). The administration of I-BET151 and ITF2357 leads to an increased apoptosis *in vitro* and the combinatorial effect resulted to be either synergic or additive in different *MLL*-rearranged ALL cell lines (Fig. 5A). The SEM cell line, stably transduced with the luciferase reporter gene (SEM-SLIEW), was transplanted *in vivo* into immunodeficient NSG mice. Three days after transplantation, the engraftment was confirmed by *in vivo* imaging (Supplementary Fig. S6A), and mice were randomized in three groups receiving DMSO, I-BET151 alone, or I-BET151 and LBH589 until they develop the clinical signs of overt leukemia, become moribund and were euthanized. Besides a significant improvement of survival in the group of mice treated with I-BET151 alone (28 days) compared with DMSO controls (21 days), a further prolonged survival was observed in mice receiving the combination therapy (34 days) (Fig. 5B; Supplementary Fig. S5B), and the cellularity was significantly lower in the BM of mice treated with both I-BET151 and LBH589 compared with I-BET151 alone (Fig. 5B).

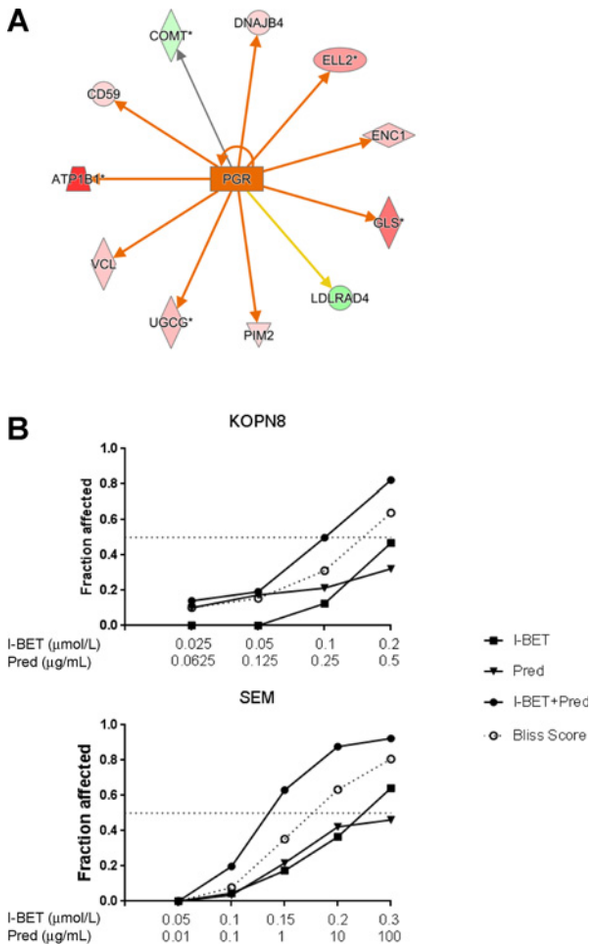

Figure 4.

I-BET151 sensitizes *MLL*-rearranged ALL cell lines to prednisolone. **A,** Analysis of upstream transcriptional regulators showing the predicted activation of the progesterone receptor (PGR) in samples treated with I-BET151. The predicted upstream regulator PGR and its downstream targets are depicted in the inner and outer circles, respectively. The different colors indicate the expression level of upstream transcriptional regulators and downstream targets (green, downregulated; red, upregulated), and the color intensity reflects the degree of regulation (from mild to strong). The line shapes and colors indicate the predicted relationship between the downstream targets and the upstream regulators (solid, direct interaction; orange, leads to activation; yellow, inconsistency between the predicted relationship and the state of the downstream molecule; grey, unpredicted effect). Asterisks (*) indicate that multiple identifiers in the dataset file map to a single gene. **B,** MTS assay showing the combinatorial effect of I-BET151 and prednisolone in SEM and KOPN8 cell lines *in vitro*. The different concentrations of I-BET151 and prednisolone are reported below. The fraction affected refers to the percentage of dead cells at 4 days after treatment and was calculated as the mean value of 2 replicates normalized on the untreated controls. Dashed lines indicate the EC-50, corresponding to fraction affected 0.5. The Bliss score (open dot line) reports the predicted additive effects of the two compounds used in combination, calculated accordingly to the Bliss formula: $FA_1 + FA_2 - (FA_1 \times FA_2)$, where FA_1 and FA_2 are the fraction affected (FA) by the single treatment with compound 1 and 2. I-BET, I-BET151; Pred, prednisolone.

Discussion

In the present study, we reported that BET inhibition: (i) inhibits the growth of *MLL*-rearranged ALL cells by blocking cell division and rapidly inducing apoptosis; (ii) impairs leukemia engraftment and prolongs mice survival; (iii) changes the MLL-AF4 transcriptional profile through the deregulation of MLL-AF4 fusion, *BRD4*, *HOXA7/9*, and *RUNX1* gene networks; (iv) sensitizes glucocorticoids-resistant *MLL*-rearranged cell lines to prednisolone; and (v) has a superior effect when combined to HDACis.

In a xenotransplantation mouse model, using both a human cell line and patient-derived primary diagnostic samples transplanted *in vivo*, we demonstrated that treatment with I-BET151, as a single agent, exerts an antileukemic effect on MLL-AF4$^+$ ALL cells, being able to lower the disease burden, impair the leukemia engraftment and prologue mice survival. Notably, despite the different kinetics of engraftment of the two patients used for xenotransplantations, and regardless of whether the treatment is started in a minimal disease setting (Pat.1) or in bulk leukemia (Pat.2), I-BET151 has proven effective against MLL-AF4$^+$ infant ALL *in vivo*. The latter scenario better reflects the clinical setting in patients and points out to a possible application to the clinical practice. Our results clearly show that I-BET151 impairs the *in vivo* repopulating capacity of a MLL-AF4$^+$ cell population enriched for L-ICs in serially transplanted mice. Furthermore, we observed that MLL-AF4$^+$ L-ICs which escaped the treatment and gave rise to residual leukemia were still responsive to I-BET151 upon reexposure to the compound *in vivo*. Differently from the previous experiments, where the mice were culled for analysis of engraftment right after one cycle of treatment, in the latter experiment, the engraftment was analyzed at late time point, after one round of treatment a long period of stop therapy. Importantly, we also demonstrated that relapse samples are sensitive to BET inhibition, as I-BET151 exposure *ex vivo* was able to induce apoptosis in xenograft samples collected from mice transplanted with the relapse of MLL-AF4$^+$ infant ALL patients. Taken together, these data provide the rationale for a possible use of I-BET151 as a first line compound during the induction phase for the treatment of *MLL*-rearranged infant ALL patients, or for the treatment of refractory cases.

Treatment with I-BET151 induces a broad alteration of the MLL-AF4$^+$ transcriptional profile. The analysis of the gene expression profile allowed us to identify a specific signature of genes deregulated upon I-BET151 treatment and provided further insights into the molecular mechanism of I-BET inhibition. The genes identified in the I-BET151 signature were mainly involved in the nucleosome and chromatin modification, thus confirming the expected epigenetic activity of I-BET151, and *BRD4* (but not other members of BET family) was identified as a predicted upstream transcriptional regulator of the I-BET151 gene signature, pointing to BRD4 as the main target of BET inhibitor in agreement with the current literature. Interestingly, *HOXA9*, *HOXA7*, and *RUNX1* were also identified as upstream transcriptional regulators of the I-BET151 signature. *HOXA* genes are well known direct targets of MLL fusions and directly involved in the maintenance of *MLL*-rearranged leukemia (24). The *RUNX1* proto-oncogene was also found to be a putative target of MLL fusion, highly expressed in MLL-AF4$^+$ ALL patients compared with normal BM cells (25, 26), with a role in leukemic transformation and survival (27–29). We can speculate that I-BET151 indirectly deregulates the *BRD4*, *HOXA7/9*, and *RUNX1* gene networks by acting on their downstream targets, without affecting the expression of these master transcriptional regulators, which indeed remained unchanged in MLL-AF4$^+$ ALL cells upon I-BET151 treatment.

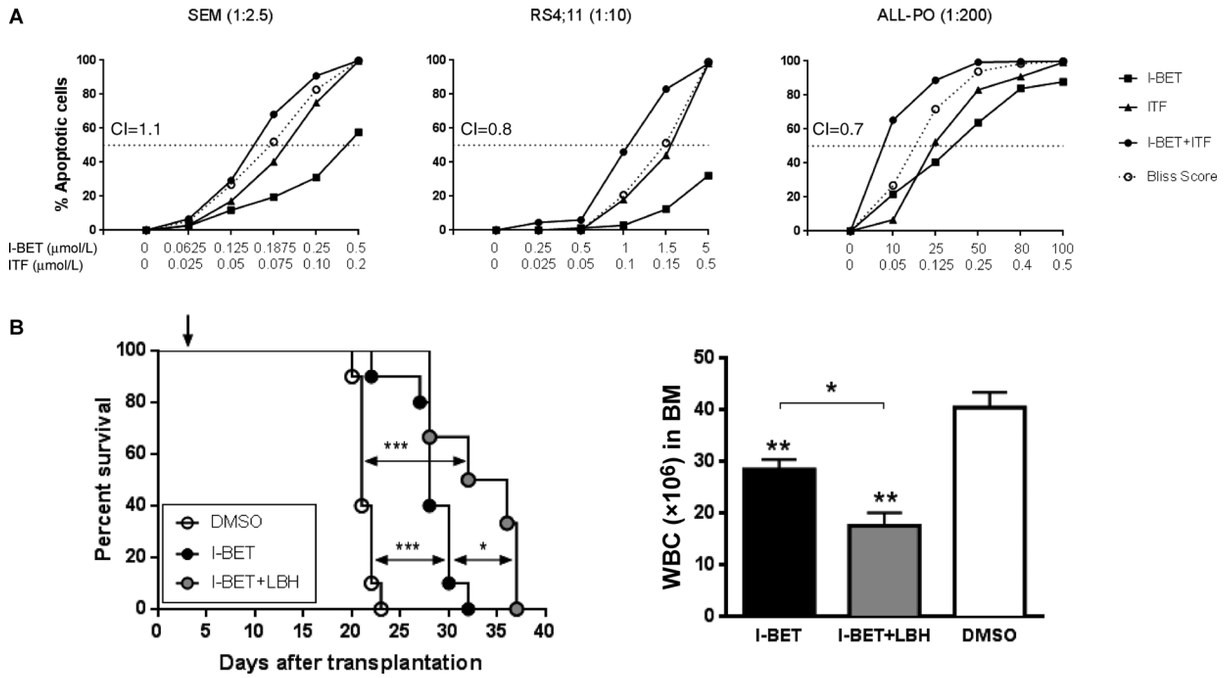

Figure 5.

I-BET151 treatment is more effective when used in combination with HDACi. **A,** Analysis of apoptosis in different *MLL*-rearranged cell lines treated with I-BET151 and/or ITF2357 *in vitro*. Graphs show the percentage of annexin V/7-AAD$^+$ apoptotic cells detected by FACS analysis 72 hours after treatment and refer to the mean values calculated on 3 replicates. The different concentrations of I-BET151 and ITF2357 are reported below, and the ITF:I-BET constant ratio is indicated in brackets. Dashed lines indicate the EC_{50}, corresponding to the 50% of apoptotic cells. The Bliss score (open dot lines) is also reported. The combination index (CI) was calculated using the Compusyn software (the two compounds are synergic if CI < 1, additive if CI ≈ 1). I-BET, I-BET151; ITF, ITF2357. **B,** Survival curve of NSG mice ($n = 30$) transplanted with SEM-SLIEW cells (10^7 cells/mouse) and treated *in vivo* with vehicle (DMSO), I-BET151 alone (15 mg/kg) or in combination with LBH589 (5 mg/kg). The black arrow indicates when the treatment was started (day 3). Four mice of the I-BET + LBH group died prematurely after 3 to 10 days of treatment and were censored. Median survival DMSO, 21 days ($n = 10$); median survival I-BET, 28 days ($n = 10$); median survival I-BET + LBH, 34 days ($n = 6$). The three groups' survival curves were compared with the log-rank Mantel–Cox test ($P < 0.0001$). The analysis of cellularity (WBC, mean ± SEM) in the BM of mice treated with I-BET ($n = 5$), IBET + LBH ($n = 4$), and DMSO ($n = 10$) at the endpoint is also reported. I-BET, I-BET151; LBH, LBH589; DMSO, vehicle.

Our findings also support the existence of a cross-regulation mechanism between the *BRD4* and *HOXA7/9* gene networks. This is in agreement with recent studies reporting the functional interdependence of the BRD4/P-TEFb and DOT1L axes, which regulate two dichotomous groups of MLL targets with distinct biological functions: the translational regulators (like *c-Myc*) and the transcriptional regulators (as the *HOX* genes), respectively (30, 31).

Novel genes were identified in this study as downregulated upon I-BET151 treatment and predicted targets of *BRD4*, *HOXA7/9*, or *RUNX1*, such as *IL7R*, *CD93*, *MSI2*, and *CD72*. These genes were previously reported in literature as associated to leukemia, albeit not yet demonstrated to have a functional role in leukemogenic transformation driven by MLL fusion or involved in BET inhibitor response. Further studies will be required to elucidate their potential impact in *MLL*-rearranged infant ALL. *CD93* was recently reported as a marker of actively cycling nonquiescent subset of cells enriched for L-ICs activity in *MLL*-rearranged AML (32). The RNA-binding protein Musashi 2 (*MSI2*) regulates the translation of several targets, including *c-Myc* (33) and was found to be essential for *MLL*-rearranged AML (33, 34). Also, *MSI2* was reported as a direct target of *HOXA9* (35) in agreement with our upstream regulator analysis. *CD72* regulates the BRC signaling and is involved in the activation and proliferation of normal and malignant B lymphocytes (36, 37). This antigen was found to be specifically overexpressed in infant ALL patients carrying the *MLL* rearrangements compared with other subgroups of pediatric ALL (38).

The crucial involvement of *c-Myc* in the response to treatment with BET inhibitors has been extensively reported in a variety of tumors. *c-Myc* was not found as differentially expressed by GEP analysis, perhaps due to the stringency of the analysis applied. However, by qPCR and Western blotting analysis of xenograft samples treated *ex vivo* and *in vivo*, we could confirm that *c-Myc* expression was affected by I-BET151 treatment. In our study, we have also demonstrated that the RNA-binding protein *MSI2* is downregulated after treatment with BET151. Recent studies have shown that *MSI2* promotes *c-Myc* translation in *MLL*-rearranged AML (33), and treatment with BET inhibitor efficiently depletes therapy-resistant MSI2$^+$ cells in pancreatic cancer (39). In opposite, the MAX dimerization protein 1 (*MXD1*) was found among the genes upregulated upon I-BET151 treatment, in agreement with another study in human ALL cell lines carrying *CRLF2* mutations treated with the BET inhibitor JQ1 (13). c-Myc forms heterodimers with other transcription factors, for example MAX, to bind the DNA and promote the transcription of distinct programs. MXD1 acts as a transcriptional repressor

and a tumor suppressor by competing with c-Myc for the binding to MAX, thus preventing c-Myc/Max dimer formation. It was previously shown that treatment with BET inhibitors leads to a significant change in the transcription of many (but not all) genes of the *c-Myc*–related transcriptional network (40), and few studies raised the possibility that BET inhibitors can repress the tumor progression by targeting c-Myc cofactors (41, 42). In favor of this hypothesis, *MXD1*, but not *c-Myc*, was found as an upstream transcriptional regulator of our I-BET signature (M. Bardini and L. Trentin; unpublished observations), pointing to *MXD1* as a mediator of I-BET151–induced downregulation of *c-Myc* targets. We can speculate that, beside (or in addition to) the downregulation of c-Myc transcript induced by I-BET151, other (not transcriptional) regulation mechanisms might possibly play a role, for example the post-transcriptional regulation of c-Myc translation through the RNA-binding protein MSI2 and/or the functional inhibition of c-Myc transcriptional activity mediated by MXD1.

It can be argued that treatment with I-BET151 alone was not sufficient to completely eradicate the disease in mice. The combination of different drugs is indeed a pillar for cancer therapy, to increase the suboptimal response to monotherapy and to circumvent the acquisition of resistance to single agents. Infant patients with *MLL*-rearranged ALL are typically refractory to treatment and frequently experience relapses (1, 4). Resistance to BET inhibition was proven to arise in the stem cell compartment (20, 43) and might be attributed, at least in part, to the transcriptional plasticity and the clonal heterogeneity of the *MLL*-rearranged L-ICs (43, 44). To envision a possible combination therapy for MLL-AF4$^+$ infant ALL, we sought to evaluate the effect of I-BET151 together with prednisolone or with another class of epigenetic drugs, the HDACis. *MLL*-rearranged infant ALL samples were proven to be more resistant to glucocorticoids compared with other subgroups of pediatric ALL (23). Prednisone is a glucocorticoid agent administrated to patients in multiagent chemotherapy during the induction phase, and the early response to prednisone *in vivo* was considered a predictive factor for patient stratification (4). We reported here that I-BET151 administration induces the upregulation of genes involved in the glucocorticoids receptor signaling and sensitizes glucocorticoids-resistant *MLL*-rearranged cell lines to the active form of prednisone *in vitro*, thus providing the rationale for the use of I-BET151 as a coadjuvant agent during induction phase. Our GSEA analysis revealed an increase in the drug metabolism, a suppression of inflammation and an activation of glucocorticoids response in samples treated with I-BET151. We can speculate that these mechanisms might possibly explain the observed sensitization to prednisolone induced by I-BET151, although additional studies will be required to better address this hypothesis. HDACis were previously identified as active compounds against *MLL*-rearranged cells (45) and their use in *MLL*-rearranged leukemias has shown antileukemic activity (46, 47). For the first time in the present study, the efficacy of a combination treatment with BET and HDACis was reported for *MLL*-rearranged infant ALL. The HDACi compounds used in this study (ITF2357 and LBH589) are nonselective pan- HDACis with a strong inhibitory activity against all HDAC enzymes (class I, II, and IV) and currently used in clinical trials. I-BET151 and HDACi seem to act synergistically *in vitro* and mice receiving both the compounds *in vivo* display a longer survival compared with I-BET151 alone. In agreement with other studies (48), we also observed that I-BET151 and HDACi induce similar changes in the transcriptional profile, consistent with these two epigenetic compounds affecting common targets, perhaps explaining, at least in part, the enhanced effect of their combination. The use of BET inhibitor in combination with HDACi has been already reported in several tumors including leukemia and the molecular mechanisms of their synergistic effect has been partially elucidated (16, 17, 49). HDACi redistributes the histone acetylation marks, mistargets BET proteins away from target active enhancers (49) and affects transcriptional elongation (46, 49); while BET inhibitors abrogate transcription by selectively displacing BRD4 from super enhancers and interfering with the BRD4-mediated transcriptional pause release (31, 50, 51). Recently, novel BRD4/HDAC dual inhibitors were generated to successfully target a human MLL-AF4$^+$ AML cell line (52).

In conclusion, overall our data demonstrate that I-BET151, alone or in combination with HDACi, exerts an antileukemic effect in a preclinical mouse model of MLL-AF4$^+$ infant ALL and increases the sensitivity to glucocorticoids. Given the aggressiveness of the disease, the relative low improvements achieved by the current therapies and the lack of an ultimate cure, this study is particularly relevant and paves the way for future clinical applications.

Disclosure of Potential Conflicts of Interest

R.K. Prinjha has ownership interest (including patents) in GlaxoSmithKline. N. Smithers is an employee and shareholder of GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M. Bardini, N. Smithers, G.T. Kronnie, R.W. Stam, G. Cazzaniga

Development of methodology: F. Rizzo, G. Fazio

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Bardini, F. Rizzo, A.M. Savino, P.G. Castro, G. Fazio, E.H.J. Van Roon, M. Kerstjens, R.W. Stam, G. Cazzaniga

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Bardini, L. Trentin, F. Rizzo, A.M. Savino, P.G. Castro, G.T. Kronnie

Writing, review, and/or revision of the manuscript: M. Bardini, L. Trentin, P.G. Castro, N. Smithers, R.K. Prinjha, G. Basso, R.W. Stam, R. Pieters, G. Cazzaniga

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.K. Prinjha,

Study supervision: G.T. Kronnie, G. Basso, A. Biondi, G. Cazzaniga

Other (use of the BET inhibitor in terms of route of administration, dose, target engagement, vehicle, and dose level): N. Smithers

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