LSD1 and GCN5 inhibition facilitates Am80 (tamibarotene)-mediated differentiation therapy in RARA-high non-APL AML

Materials and Methods

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Materials and Methods:

Cell lines, primary cells, and cell culture

Six AML cell lines (Kasumi-1, HL-60, OCI-AML2, THP-1, MV4-11, and OCI-AML3) and 69 primary AML (n=67), and high risk MDS (n=2) samples were analyzed (Suppl. Table 1). AML cell lines were purchased from DMSZ, maintained under standard conditions, and subjected to specified treatments with 0.1 µM ATRA, 0.1 µM Am80, 0.1 µM of the LSD1 inhibitor GSK-LSD1, and 100 µM of the GCN5 inhibitor MB3. Primary human AML samples were isolated from peripheral blood mononuclear cells using StemSep columns (STEMCELL Technologies, Canada), cultured in Stemline II Medium (Sigma, Hamburg, Germany), and treated with 1 µM ATRA, 1µM Am80, 0.1 µM of the LSD1 inhibitor GSK-LSD1, and 100 µM of the GCN5 inhibitor MB3. Their use was approved by the respective institutional ethics committees (approval numbers: 4753-04/16 and BB 199/20), obtained with the donor's informed consent, and conducted in accordance with the Declaration of Helsinki.

Cell analyses

Surface marker expression was assessed by flow cytometry using a BD LSRFortessa™ after 3 days of treatment. To prevent non-specific Ig Fc-receptor binding, cells were pre-blocked with Human TruStain FcX before staining with anti-CD11b-FITC (BioLegend, #301330) and anti-CD117-BV711 (BioLegend, #313230). Final analysis was performed using FLOWJO software. Cell viability was assessed using the CellTiter-Glo® luminescent cell viability assay (Promega), according to the manufacturer's protocol. Cells were initially seeded at a density of 10,000 cells/ml and subsequently treated with the indicated drug concentrations.

RNA-seq

Cells were harvested and total RNA was isolated using the ZR RNA MiniPrep Kit (Zymo Research). Isolated RNA was processed using the Illumina TruSeq Stranded mRNA sample preparation kit according to the

manufacturer's instructions. The quality of the libraries was assessed using an Agilent 2100 BioAnalyzer, and global gene expression profiling was carried out using a HiSeq2500 sequencer in a 51-cycle/single-end/high-output mode.

Analysis of gene array data was performed using Galaxy, and raw counts were used as input to the DESeq2 package using default settings to quantify candidate genes.

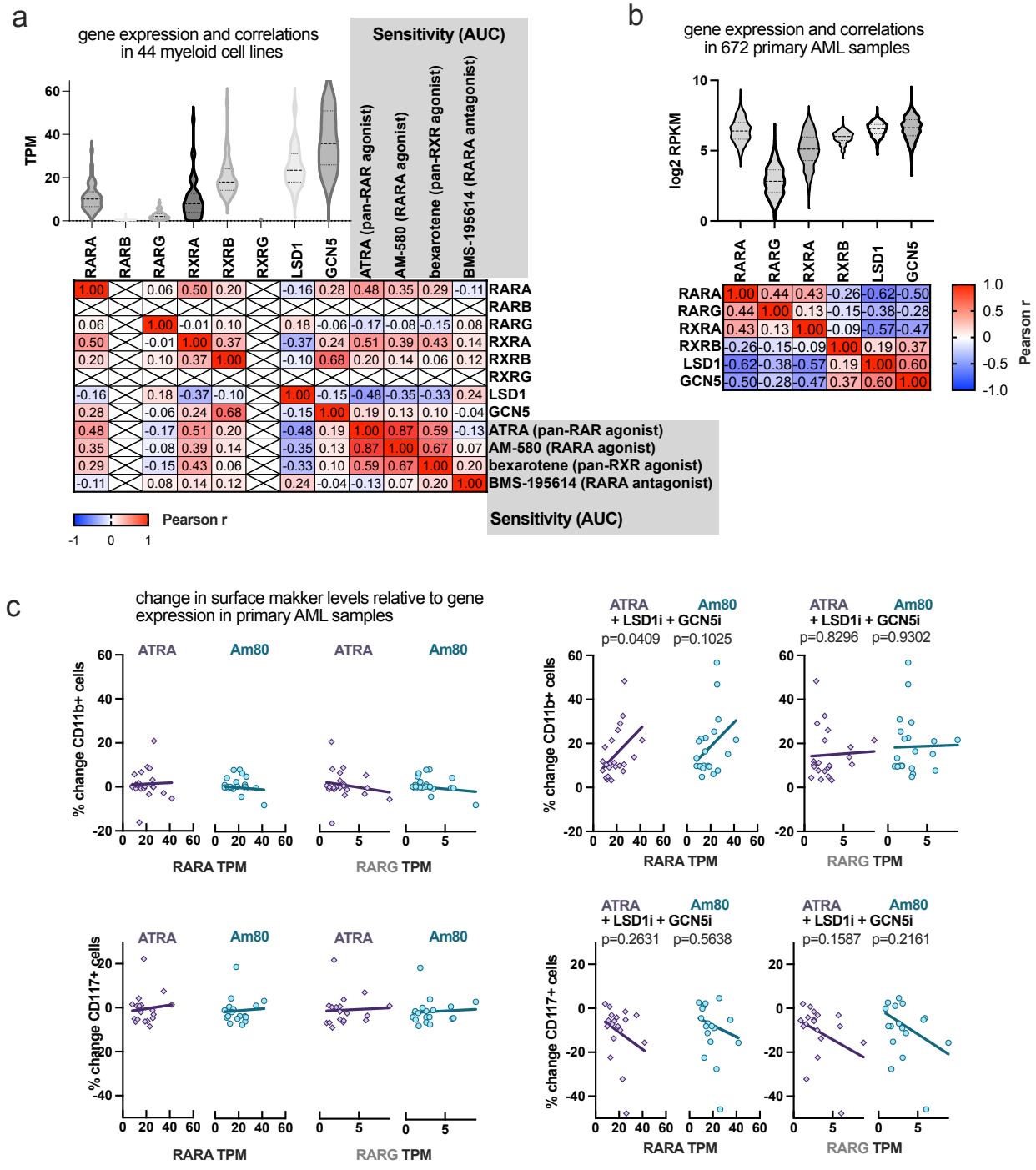
The gene expression TPM values of protein-coding genes for the 44 cell lines were obtained from the DepMap database (depmap.org). Values are inferred from RNA-seq data using the RSEM tool and are reported after log2 transformation, using a pseudo-count of 1; log2(TPM+1).

Statistics

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, unless otherwise noted. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using the GraphPad Prism software (version 9; GraphPad Software Inc.).

Supplementary Figures

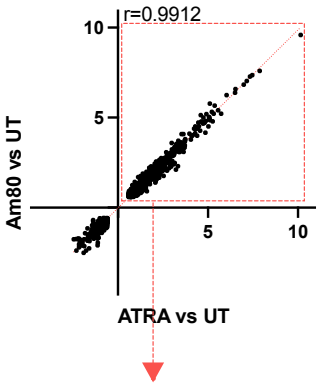
Supplementary Figure 1



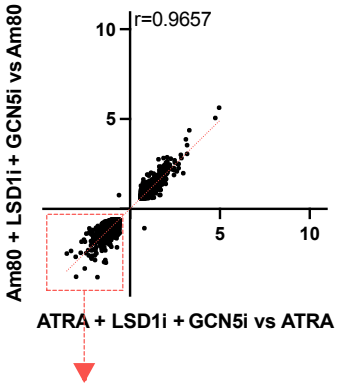
a Upper panel: Gene expression (TPM) of RARs, RXRs, LSD1 and GCN5 in 44 myeloid cell lines. Lower panel: Pearson correlation matrix. **b** Upper panel: Gene expression (TPM) of selected, expressed RARs, RXRs, LSD1 and GCN5 in 672 primary AML. Lower panel: Pearson correlation matrix. **c** Pearson correlation of RARA and RARG gene expression with the change in CD11b and CD117 surface marker levels following 3-day treatment of primary AML samples with ATRA (1µM) or Am80 (1µM) alone (left panel) or in combination with the LSD1 inhibitor GSK-LSD1 (0.1µM) and the GCN5 inhibitor MB3 (100µM) (right panel).

Supplementary Figure 2

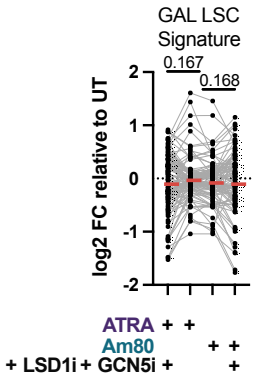
a Gene expression in OCI-AML3



b Gene expression in OCI-AML3



d



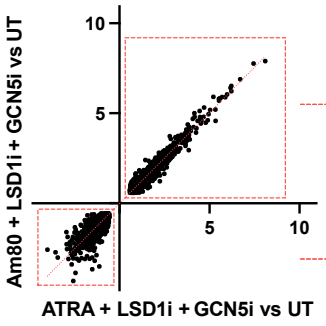
	ATRA	Am80
CYP26B1	10.15	9.59
CYP26A1	7.87	7.60
ENSG00000288927	7.46	7.36
PTGES	7.33	7.28
HIC1	7.15	7.04
TGM2	6.99	6.83
DHRS3	6.53	6.58
ENSG00000272482	6.51	6.38
MPPED1	6.03	6.25
LINC02766	5.73	5.22
ENSG00000262810	5.57	5.40
TRIM31	5.37	5.66
SPIB	5.31	5.21
ENSG00000263674	5.27	4.79
RHBDL3	5.22	5.32
CLEC17A	5.11	4.85
NTN4	5.10	5.78
SLC6A12	5.09	4.91
CYP4F22	5.08	4.98
FGR	5.06	5.03
CRYGN	5.03	4.50
CXCR5	4.91	5.19
SHE	4.87	4.82
SLC2A14	4.85	4.16
WDR86	4.82	4.71
RARB	4.75	5.00
TNFSF15	4.73	4.66
PGLYRP2	4.68	4.77
TRABD2A	4.62	4.77
LBP	4.60	5.13

	ATRA	Am80
MESP1	-3.53	-2.50
RIMBP3	-3.29	-2.34
ENSG00000274104	-3.04	-2.67
LINC02723	-3.01	-3.79
RCOR2	-2.75	-2.45
TSTD1	-2.71	-1.83
SPHK1	-2.57	-1.72
POU2F2	-2.53	-3.32
LINC01686	-2.52	-1.84
PAQR7	-2.44	-2.47
CD300E	-2.44	-1.38
ENSG00000280161	-2.34	-2.31
EXTL1	-2.30	-2.60
SPATC1	-2.29	-1.34
SNHG25	-2.27	-1.09
HSPA12B	-2.24	-1.23
EMILIN1	-2.18	-2.04
OXCT2P1	-2.18	-2.69
MT-TS2	-2.18	-1.79
TMEM160	-2.16	-1.42
ENSG00000288856	-2.15	-2.77
TBX4	-2.15	-2.32
KLHL30	-2.14	-1.96
PRDM11	-2.13	-1.29
HLA-G	-2.13	-1.35
ADAM19	-2.11	-1.92
PLEKHG5	-2.11	-2.62
NGFR	-2.08	-1.90
TCAF1P1	-2.08	-1.18
STRA6	-2.08	-1.42

e

Epigenetic Factors	RARA Spearman's r
KDM1A	-0.7021
EHMT2	-0.6991
TRIM28	-0.6951
SMARCC1	-0.6864
PRMT7	-0.6697
LAS1L	-0.6547
PRMT5	-0.6456
PRMT1	-0.6352
SET	-0.6339
ZRANB3	-0.6276
APEX1	-0.6223
CHD1L	-0.6131
TRUB2	-0.6090
KAT2A (GCN5)	-0.6089
SMARCA4	-0.6068
RUVBL2	-0.6066
EHMT1	-0.6021
U2AF2	-0.6005
MTA1	-0.5983
HDAC2	-0.5957
WDR5	-0.5951
ARID1B	-0.5925
EXOSC2	-0.5895
NPM1	-0.5878
ZMYM3	-0.5846

c



GO:BP terms ATRA and Am80 + LSD1i + GCN5i vs Untreated

TOP 200 uprgeulated genes

term_name	term_id	adjusted p
negative regulation of retinoic acid receptor signaling pathway	GO:0048387	3.27E-06
response to external stimulus	GO:0009605	8.48E-06
regulation of retinoic acid receptor signaling pathway	GO:0048385	2.18E-05
regulation of multicellular organismal process	GO:0051239	9.13E-05
inflammatory response	GO:0006954	1.07E-04

TOP 200 downregulated genes

term_name	term_id	adjusted p
negative regulation of multicellular organismal process	GO:0051241	7.82E-03
negative regulation of developmental process	GO:0051093	1.40E-02
embryonic lung development	GO:1990401	4.42E-02
negative regulation of cell differentiation	GO:0045596	8.34E-02
tube development	GO:0035295	9.79E-02

a Genes upregulated by ATRA and Am80. **b** Genes downregulated following the addition of the LSD1 and GCN5 inhibitors to ATRA and Am80. **c** Biological Processes (GO terms) enriched in the TOP 200 up or downregulated genes following ATRA or Am80 in combination with LSD1 and GCN5 inhibitors. The analysis was performed using the g:Profiler tool. **d** Differential expression of leukemic stem cell gene sets following treatments. Log2 FC relative to untreated cells of genes in the marker-defined gene set GAL_LEUKEMIC_STEM_CELL_UP (gsea-msigdb.org). **e** Table of epigenetic modifiers whose expression is significantly negatively correlated with RARA in 672 primary AML.

Supplementary Tables

Supplementary Table 1: Patient Characteristics

Characteristics	Patients (N=69)
Median age (range)	60 years (25 to 91 years)
Sex (m/f)	27/42
FAB (%)	
M0	2 (2,9)
M1	9 (13)
M2	8 (11,6)
M4	14 (20,3)
M5	5 (7,2)
M6	1 (1,4)
Not classified	30 (43,5)
Type (%)	
Primary	41 (59,4)
Secondary	14 (20,3)
Relapsed	11 (15,9)
Therapy-related	1 (1,4)
MDS	2 (2,9)
Not classified	0 (0)
Blasts in peripheral blood	
>50%	34 (49,3)
<50%	32 (46,4)
Not determined	3 (4,3)
Cytogenetics (%)	
normal	31 (44,9)
complex	9 (13)
t8;21	3 (4,3)
inv16	2 (2,9)
Other	24 (34,8)
Mutations (% tested positive)	
FLT3-ITD	26 (37,7)
NPM1	19 (27,5)
IDH1	2 (2,9)
IDH2	9 (13)
RUNX1-RUNX1T1	3 (4,3)
CEBPA	7 (10,1)
KMT2A-PTD	7 (10,1)
PML-RARA	0 (0)
CBFb-MYH11	4 (5,8)