A Novel LSD1 Inhibitor T-3775440 Disrupts GFI1B-Containing Complex Leading to Transdifferentiation and Impaired Growth of AML Cells


Abstract

Dysregulation of lysine (K)-specific demethylase 1A (LSD1), also known as KDM1A, has been implicated in the development of various cancers, including leukemia. Here, we describe the antileukemic activity and mechanism of action of T-3775440, a novel irreversible LSD1 inhibitor. Cell growth analysis of leukemia cell lines revealed that acute erythroid leukemia (AEL) and acute megakaryoblastic leukemia cells (AMKL) were highly sensitive to this compound. T-3775440 treatment enforced transdifferentiation of erythroid/megakaryocytic lineages into granulomonocytic-like lineage cells. Mechanistically, T-3775440 disrupted the interaction between LSD1 and growth factor–independent 1B (GFI1B), a transcription factor critical for the differentiation processes of erythroid and megakaryocytic lineage cells. Knockdown of LSD1 and GFI1B recapitulated T-3775440–induced transdifferentiation and cell growth suppression, highlighting the significance of LSD1–GFI1B axis inhibition with regard to the anti-AML effects of T-3775440. Moreover, T-3775440 exhibited significant antitumor efficacy in AEL and AMKL xenograft models. Our findings provide a rationale for evaluating LSD1 inhibitors as potential treatments and indicate a novel mechanism of action against AML, particularly AEL and AMKL.

Introduction

The hematopoietic system is maintained by stem cell self-renewal and a continuous hierarchical differentiation process as well as lineage commitment (1). Many types of transcription machinery function as key regulators of terminal differentiation. Dysregulated hematopoietic cell differentiation and improper stem cell maintenance have frequently been associated with acute myelogenous leukemia (AML; refs. 2, 3). Although developments in chemotherapeutics and bone marrow transplantation have led to significant progress in the treatment of leukemia, the prognosis of patients with relapsed AML remains a significant problem. Hence, research and development aimed at the identification of novel targeted anti-AML agents are needed.

Lysine (K)-specific demethylase 1A (LSD1) is a flavin adenine dinucleotide (FAD)-dependent histone demethylase that demethylates di- and monomethylated K4 on histone H3 (4). Histone modification is often associated with the activation or repression of adjacent gene transcription. LSD1 is known to form a complex with corepressor proteins, namely CoREST and HDAC1, to augment its gene repressor activity (5, 6). In hematopoietic cells, LSD1 also physically interacts with growth factor–independent 1 (GFI1) or growth factor–independent 1B (GFI1B), which are transcriptional repressors and critical regulators of hematopoietic cell lineage development and differentiation (7, 8). Hematopoietic lineage-specific conditional LSD1 knockdown and knockout models have shown that the loss of LSD1 results in hematopoietic stem cell expansion and inhibits terminal granulomonocytic, erythroid, and megakaryocytic lineage differentiation, thus highlighting the importance of LSD1 in normal hematopoiesis (9, 10).

LSD1 is significantly overexpressed in a number of hematologic malignancies, including AML, and has gained attention as a potential therapeutic target in AML (11). Small molecule- or siRNA-mediated inhibition of LSD1 activity has been shown to induce differentiation in AML cells (11, 12). Synergistic growth
suppression effects were observed in non–acute promyelocytic leukemia AML treated with a combination of all-trans retinoic acid and LSD1 inhibition (13). A reversible LSD1 small-molecule inhibitor SP2509 sensitized AML cells to the pan-HDAC inhibitor panobinostat (14). However, the molecular mechanism underlying the mode of action of LSD1 inhibitors in AML remains to be fully elucidated.

In this study, we describe the antileukemic activities and mechanism of action of our novel, selective, and potent LSD1 inhibitor in AML cell subsets. Our LSD1 inhibitor disrupted the LSD1–GFI1B interaction, which in turn induced the transcriptional derepression of GFI1B target genes and consequent transdifferentiation and thereby exhibited antileukemic efficacy in AML-expressing AEL and acute megakaryoblastic leukemia (AMKL) cell lines. Our results suggest the potential of LSD1 inhibition via small-molecule inhibitors as a novel strategy for the treatment of certain types of AML with a poor prognosis.

Materials and Methods

Cells and reagents

The human leukemia cell lines TF-1a and HEL92.1.7 were obtained from ATCC in 2008 and 2011, respectively. CMK11–5 and M07e were obtained from JCRB Cell Bank and DSMZ, respectively in 2014. All cells were grown in RPMI1640 plus 5% CO2. Information about other cell lines and media is available in Supplementary Table S1.

Bioinformatics

To determine differentially expressed genes (DEG) between T-3775440–treated and control-treated cells, microarray data were subjected to a 2-sample comparison. DEGs with a t test P < 0.01 and fold change at least of 2 were extracted.

Immunoprecipitation

The chromatin fraction was used for immunoprecipitation. Cells were harvested and lysed in lysis buffer [20 mmol/L HEPES (pH 7.5), 25% glycerol, 0.2 mmol/L MgCl2, 0.5% NP-40, and 1 mmol/L PMSE] containing a protease inhibitor. After centrifugation, the insoluble fraction was resuspended in micrococcal nuclease (MNase) buffer [20 mmol/L HEPES (pH 7.5), 0.3 mol/L sucrose, 100 mmol/L KCl, 2 mmol/L MgCl2, 1 mmol/L CaCl2, 0.1% Triton X-100, and 1× protease inhibitor cocktail] containing Mnase and incubated for 10 minutes. After centrifugation, the supernatant was harvested as the chromatin fraction. For immunoprecipitation, lysates were incubated with protein G (GE Healthcare) to exclude nonspecific protein binding. Subsequently, antibodies against LSD1 or GFI1B were added to the lysates for a 4-hour incubation, followed by an additional 2-hour incubation with protein G. Finally, protein G was removed using wash buffer 1 [20 mmol/L HEPES (pH 7.5), 100 mmol/L KCl, 5 mmol/L MgCl2, 0.2 mmol/L EDTA, 10% glycerol, 0.1% Tween-20] and wash buffer 2 [20 mmol/L HEPES (pH 7.5), 300 mmol/L KCl, 5 mmol/L MgCl2, 0.2 mmol/L EDTA, 10% glycerol, 0.1% Tween-20] twice each, followed by heating for 10 minutes at 70°C in 4× SDS loading buffer (Wako Pure Chemical Co.).

Western blotting

Whole-cell extracts were prepared in 1× Laemmli sample buffer (Tris-HCl 125 mmol/L, pH 7.5, 1% SDS, 20% glycerol). Whole-cell extracts or immunoprecipitates were fractionated by SDS-PAGE, and separated proteins were transferred to membranes using an iBlot Transfer Stack (nitrocellulose) and iBlot Gel Transfer Device (Invitrogen/Thermo Fisher Scientific). After incubation with StartingBlock T20 (PBS) Blocking Buffer (Pierce Biotechnology), the membranes were labeled overnight for quantitative RT-PCR analysis: CD86 (Hs01567026_m1), FCRLA (Hs00893173_m1), GAPDH (Hs02758991_g1), GATA1 (Hs01085823_m1), GFI1B (Hs01062469_m1), GYPA (Hs00266777_m1), JTCAM (Hs00355885_m1), KDM1A(LSD1) (Hs01002741_m1), and PI6 (Hs00542137_m1).

For the microarray analysis, total RNA was purified as described, and quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was labeled and hybridized to Agilent SurePrint G3 Human Gene Expression 8 × 60K arrays at the Macrogen Company. Microarray data have been deposited in NCBI GEO (accession number: GSE87580).

Cell proliferation assay

To assess cell proliferation and viability in human leukemia cell lines, compounds were added at 24 hours after cell seeding. After the incubation period, cells were lysed with CellFitter-Glo (Promega), and luminescent signals were detected using an ARVO Cell proliferation assay in Supplementary Table S1. No authentication was done by the references used in the analysis were generated from data published by the Immunological Genome Project and are available in the NCBI GEO database (GSE15907). The methods used to extract cell type–specific signatures were described elsewhere (17). Signature data were originally generated for mouse genes and mapped onto human gene symbols for this analysis.
with primary antibodies, followed by incubation with horse-radish peroxidase-conjugated secondary antibodies (Cell Sig-naling Technology). Subsequently, membranes were incubated with ImmunoStar Zeta (Wako) and scanned using an Image-Quant LAS-3000 (Fujifilm).

The following antibodies were used for immunoprecipitation or Western blot analyses: LSD1 (pAb-067-050; Diagenode s.a.), GFI1B (immunoprecipitation: 5849, Cell Signaling Technology; Western blotting: sc28356, Santa Cruz Biotechnology), CoREST (immunoprecipitation: 616242; BD Biosciences), and β-actin (A3854; Sigma-Aldrich).

**Tumor xenograft models**

Female C.B17/1cr-scid/scid Jel mice (CLEA Japan, Inc.) were maintained under specific pathogen-free conditions and used in compliance with the guidelines of the Takeda Institutional Ani-mal Care and Use Committee, in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. AML cells (2 × 10⁶ cells) in Matrigel were inoculated subcutaneously into the left flanks of 6- to 7-week-old mice (day 0). Mice were randomized when the mean tumor volume reached approximately 150 to 350 mm³. Subsequently, mice were treated with vehicle or T-3775440 once daily on a 5 days on/2 days off schedule for 2 weeks. Twice weekly, tumors were measured with vernier calipers, and tumor volumes were calculated as follows: (length × width²)/2. The percentage treated/control ratio (T/C%) was calculated by dividing the change in tumor volumes in vehicle-treated mice by the change in volumes in vehicle-treated mice.

**Statistical analysis**

Where appropriate, the Dunnett-type test, one-tailed Williams t test, and Aspin–Welch t test were used to calculate P values with EXSUS version 8.0.0 software (CAC Croit). P values of <0.05 were considered statistically significant.

**Results**

**Erythroleukemia and megakaryoblastic leukemia cells are highly sensitive to T-3775440**

Previously, the monoamine oxidase (MAO) inhibitor tryan-cypromine (2-PCPA) was found to inhibit LSD1 demethylase activity. Therefore, we selected 2-PCPA as the starting entity for our medicinal chemistry effort and developed T-3775440 (Fig. 1A), which contains a cycloprenylamine moiety to bind to FAD within the catalytic core of the enzyme (18). T-3775440 demonstrated irreversible inhibition of recombinant human LSD1, with a kₐₑₑₑₑ/Kᵦ value of 1.7 × 10⁵ (sec⁻¹ M⁻¹⁻¹). T-3775440 was highly selective for LSD1 relative to other monoamine oxidases (e.g., MAO-A and MAO-B), with an IC₅₀ value of 2.1 nmol/L (Fig. 1B).

To take advantage of this selective inhibitor, we first determined its anticancer activity in vitro. Previously, LSD1 inhibi-tors were reportedly effective against AML cell lines and primary AML cells (12–14). We subjected a panel of 27 leukemia cell lines, including 22 AML cell lines, a chronic myeloid leukemia cell line, and four T-cell acute lymphoblastic leukemia (T-ALL) cell lines, to proliferation screening (Supplementary Table S1). Although small-molecule inhibitors of epigenetic enzymes tend to require sustained incubation to induce phenotypic changes, T-3775440 blocked the proliferation of several cell lines as quickly as day 3 of treatment (Fig. 1C). Of interest, four of five AEL cell lines and three of four AMKL cell lines exhibited clear responses to T-3775440 following a 3-day treatment. In con-trast, T-ALL cell lines were insensitive to T-3775440 (Fig. 1C, Supplementary Fig. S1A). A Western blot analysis of p27 and PARP cleavage revealed that 48-hour T-3775440 treatment induced cell-cycle arrest and apoptosis in AEL (TF-1a, HEL92.1.7) and AMKL cell lines (CMK11-5, M07e; Fig. 1D; Supplementary Fig. S1B). Consistent with changes in protein levels, a flow cytometric cell-cycle analysis revealed that T-3775440 treatment increased the G₁ and sub-G₁ phase populations in TF-1a and CMK11-5 cells (Fig. 1E and F). These results indicate that T-3775440 exhibits rapid antiproliferative and proapoptotic activities against AEL and AMKL cells.

**Treatment with T-3775440 induces transdifferentiation in AML cells**

In previous reports, pharmacologic inhibition or knockdown of LSD1 induced myeloid differentiation in association with altered marker expression and morphologic changes in AML cells (12-14). Consistent with this observation, T-3775440 treatment induced morphologic changes in TF-1a and HEL92.1.7 cells, as exemplified in Fig. 2A. To investigate the gene expression program regulated by LSD1 in AML cells, we compared the transcriptomes of control and T-3775440-treated HEL92.1.7 AEL cells and CMK11-5 AMKL cells in a microarray analysis. T-3775440 treatment increased the signal intensities of 1277 probes (≥2-fold) in HEL92.1.7 and 793 probes in CMK11-5 (Supplementary Fig. S2A). In contrast, fewer downregulated (≤2-fold) probe sets were detected: 282 probes in HEL92.1.7 and 289 probes in CMK11-5 (Supplementary Fig. S2B). To interpret the biological significance of these posttreatment gene expression changes, we employed a NextBio data mining framework (www.nextbio.com). Interestingly, commonly upregulated genes in both HEL92.1.7 and CMK11-5 cells were significantly enriched within SPI1-binding site gene sets (Supplementary Table S2). SPI1, also known as PU.1, is a DNA-binding transcription factor that plays a critical role in myeloid development and activates lineage-specific gene expression (19), our finding suggests that T-3775440 induces the ectopic upregulation of myeloid lineage genes in erythroid and meagakaryocytic lineage cells. We also conducted a gene set enrichment analysis using the same microarray data. In HEL92.1.7 cells, T-3775440 treatment significantly upregulated the monocytic lineage gene signature but downregulated the erythroid gene signature (Fig. 2B and C). Similarly, upon T-3775440 treatment, CMK11-5 tended to lose its original features, which were indicative of a megakaryocytic gene signature, and acquired both natural killer cell and monocytic lineage gene signatures (Supplementary Fig. S2C–S2E). To further address whether these morphologic changes were associated with immunophenotypic changes, we evaluated the expression levels of 242 surface molecules on AML cell lines. Notably, the gran-ulocyte/macrophage markers CD86 and CD11b were commonly upregulated on both TF-1a and HEL92.1.7 cells in response to T-3775440 treatment (Fig. 2D; Supplementary Fig. S3A; Supplementary Table S3), whereas the erythroid markers CD235a and CD71 were downregulated by this treatment (Fig. 2E; Supplementary Fig. S3B; Supplementary Table S4). In CMK11-5 cells, CD86 mRNA expression was also clearly upregulated by T-3775440 in a concentration-dependent manner, although only
a modest increase in cell surface CD86 expression was observed (Supplementary Fig. S3C–S3F). Taken together, these results suggest that T-3775440 converts AML cell lineages from the original erythroid or megakaryocytic lineage to myeloid-like lineages.

Figure 1. T-3775440 leads to cell growth inhibition in AML cell lines. A, Chemical structure of T-3775440. B, In vitro enzymatic activity and selectivity of T-3775440. C, In vitro proliferation assay of 27 leukemia cell lines. Cells were treated with T-3775440 for 3 days. Red bars, AEL cell lines; green bars, AMKL cell lines; and blue bars, T-ALL cell lines. D, TF-1a and CMK11-5 cells were treated with the indicated concentration of T-3775440 for 24 or 48 hours. Treated cells were subsequently harvested and lysed to prepare total lysates. Immunoblotting analyses were performed to determine the expression levels of p27, cleaved PARP, and β-actin in the cell lysates. E and F, TF-1a (E) and CMK11-5 cells (F) were treated with T-3775440 at concentrations of 50 and 100 nmol/L, respectively, or DMSO (control). Cells were harvested 48 hours later, and cell-cycle profiles were analyzed using flow cytometry.

Growth inhibition induced by T-3775440 is attributed to enforced transdifferentiation
The results of morphologic, transcriptomics, and immunophenotyping analyses implicated that T-3775440 induces the transdifferentiation of AEL and AMKL cells from their respective
erythroid and megakaryocytic lineages. To test whether lineage conversion was involved in T-3775440–induced growth inhibition in AML cell lines, we subjected TF-1a cells to flow cytometric sorting and washout analysis. After a 3-day T-3775440 treatment, cells were sorted according to CD86 expression levels and cultured without T-3775440 (Fig. 3A and B). Cell populations expressing moderate or high levels of CD86 grew more slowly than did cells expressing low levels

Figure 2.
Treatment with T-3775440 induces features of morphologic differentiation in cultured AML cells. A, TF-1a and HEL92.1.7 cells were respectively treated with 100 nmol/L and 1 μmol/L of T-3775440 for 72 hours. Representative images of cytospin preparations are shown. B and C, Gene set enrichment analysis plots demonstrate the upregulation of monocytic signature genes (B) and downregulation of erythroid signature genes (C) in HEL92.1.7 cells treated with or without 100 nmol/L of T-3775440 for 24 hours. D and E, TF-1a cells were treated with 50 nmol/L of T-3775440 for 72 hours, after which the expression of 242 surface molecules was analyzed via flow cytometry. Representative surface marker expression levels are described for CD86 (D) and CD235a (E). ITC, isotype control.
of CD86 (Fig. 3B). Growth suppression was sustained for up to 7 days after compound removal, suggesting profound effects of transdifferentiation on TF-1a cell growth. Because T-3775440 upregulated a substantial number of genes controlled by the myeloid master regulator transcription factor SPI1 (Supplementary Table S2), we knocked down SPI1 to further address whether T-3775440–dependent transdifferentiation was associated with growth inhibition (Supplementary Fig. S4A). In Figure 3.

Myeloid gene expression correlates with and is required for the growth-inhibitory activity of T-3775440. A and B, TF-1a cells were treated with 1 μmol/L of T-3775440 for 72 hours. Cells were sorted on the basis of CD86 expression intensity (A) and further cultivated without T-3775440. Proliferation rates were measured at the indicated time points (B). DMSO indicates cells treated with DMSO only and subsequently cultivated further. C–F, TF-1a cells were treated with SPI1-specific or control siRNA and replated with or without 100 nmol/L of T-3775440. Twenty-four hours after compound or vehicle control treatment, cells were harvested for RNA purification. Changes in CD86 (C) and GYP4 (D) expression were measured by qRT-PCR. Values, means of triplicate samples ± SDs. Asterisks denote *P < 0.01 (**) and **P < 0.001 (***) as determined by a Dunnett-type test. E, Apoptosis induction in a dose–response manner was measured in TF-1a cells treated with T-3775440 for 24 hours using Caspase-Glo 3/7. RLU, relative luciferase unit. F, Cell proliferation was measured by CellTiter-Glo following siRNA-mediated SPI1 knockdown and T-3775440–induced growth inhibition in TF-1a cells.
control cells, T-3775440 treatment increased CD86, ITGAM, FCRLA, and SPI1 expression and decreased GYPB (encodes CD235a) and GATA1 expression; in contrast, these T-3775440–dependent increases and decreases in gene expression were attenuated in SPI1 knockdown cells (Fig. 3C and D; Supplementary Fig. S4A–S4D). Furthermore, SPI1 depletion partially rescued cells from T-3775440–mediated apoptosis (Fig. 3E) and growth suppression (Fig. 3F). These data suggest that T-3775440 suppresses AEL cell growth through a transdifferentiation-dependent mechanism in which SPI1-regulated genes play key roles.

T-3775440 treatment inhibits the association of LSD1 with GFI1B, and knockdown of GFI1B and LSD1 phenocopies the antileukemic activity of T-3775440

To gain further insight into the molecular mechanism underlying T-3775440–mediated growth suppression, we tested the effects of this inhibitor on LSD1-containing protein complex. As mentioned previously, LSD1 forms complex with the corepressor proteins CoREST and GFI1B in GFI1B-expressing cells (7). As shown in Fig. 4A, T-3775440 treatment disrupted the LSD1–GFI1B association in a concentration-dependent manner, although the LSD1–CoREST association was retained. At its N-terminus, GFI1B contains a conserved snail-Gfi-1 (SNAG) domain that is critical for recruiting LSD1 (7). To test whether T-3775440 directly disrupts the LSD1–GFI1B interaction, we used recombinant LSD1 and a GFI1B SNAG domain peptide to develop a surface plasmon resonance (SPR) biosensing assay. GFI1B peptide binding to LSD1 significantly elevated the SPR signal, which was diminished by T-3775440 pretreatment (Fig. 4B). The publicly available LSD1 inhibitor GSK-LSD1 yielded similar effects (Supplementary Fig. S5). These results indicate that these irreversible cyclopropylamine derivative LSD1 inhibitors directly disrupt the LSD1–GFI1B interaction. We further assessed the effect of T-3775440 on the LSD1–GFI1B complex at the chromatin level using a chromatin immunoprecipitation assay. We focused on the promoter region of PI16, which was reported to be directly regulated by LSD1 in AML cells (20). T-3775440 decreased LSD1 binding but not GFI1B binding and increased the level of dimethylated H3K4 at the PI16 locus (Fig. 4C; Supplementary Fig. S6A–S6D). In contrast, LSD1 binding to the RPL30 gene locus remained unaffected by treatment with T-3775440 (Supplementary Fig. S6E). These results suggest that T-3775440–mediated dissociation of the LSD1–GFI1B complex occurs on the chromatin of selected target genes.

To evaluate whether the effect of T-3775440 on AML cells was mediated by disruption of this complex, we examined the effects of an siRNA-mediated knockdown of either LSD1 or GFI1B in TF-1a cells (Supplementary Fig. S7A and S7B). Single knockdown of either LSD1 or GFI1B led to increased CD86, ITGAM, FCRLA, and PI16 expression and reduced GYPB and GATA1 expression; however, the effects on cell morphology or cell proliferation were modest (Fig. 5A, B, and D; Supplementary Fig. S7C–S7F). Because the LSD1–GFI1B complex represses the GFI1B promoter (20), LSD1 knockdown led to an increase in GFI1B expression (Supplementary Fig. S7B). Therefore, we speculated that dual gene knockdown would more closely recapitulate the effect of T-3775440 and induce

Figure 4.

T-3775440 disrupts the LSD1–GFI1B–CoREST complex. A, TF-1a cells were incubated with DMSO or 10 or 100 nmol/L of T-3775440 for 24 hours. Chromatin fractions were prepared and subjected to immunoprecipitation (IP) of GFI1B-containing complexes. An immunoblotting analysis was conducted to detect the indicated proteins. B, SPR sensorgrams of the interaction between a GFI1B peptide and LSD1 (gray) and between the GFI1B peptide and LSD1 after treatment with 10 µmol/L T-3775440 (red). RU, resonance units. C, TF-1a cells were treated with 100 nmol/L T-3775440 or DMSO control for 24 hours. After treatment, chromatin was crosslinked and sonicated, and chromatin immunoprecipitation was performed using the indicated antibodies. Chromatin immunoprecipitated DNA was used as a quantitative PCR template to determine relative enrichment on the PI16 promoter. Values, means of triplicate samples ± SDs.

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more robust effects on cell differentiation and proliferation. Indeed, depletion of both LSD1 and GFI1B additively augmented CD86 upregulation (Fig. 5A), induced nuclear segmentation (Fig. 5C; Supplementary Fig. S8), and significantly reduced proliferative capacity relative to either single-siRNA treatment (Fig. 5D), thus confirming the phenotypic interaction between differentiation and growth suppression in TF-1a cells. These results suggest that the effects of T-3775440 are mediated mainly by inhibiting the formation of LSD1–GFI1B-containing complex.

T-3775440 affects later stages of hematopoiesis

The effects of T-3775440 on human erythroid and myeloid progenitor proliferation were evaluated in MethoCult GF H84434, using bone marrow from 3 healthy volunteers. T-3775440 had no significant observable effect on myeloid progenitor numbers (Supplementary Fig. S9A–S9F). In contrast, significant inhibition of erythroid progenitor proliferation was observed at IC_{50} values ranging between 0.1 and 0.14 μmol/L (Supplementary Fig. S9D–S9F). These results suggest that T-3775440 exerts more specific effects at later stages of hematopoietic stem cell proliferation and differentiation, including the BFU-E stage.

T-3775440 exerts tumor growth suppression in in vivo mouse xenograft models of AML

We next conducted an in vivo efficacy study of T-3775440, using mouse subcutaneous xenograft models of AML cell lines. Corroborating our in vitro observation, T-3775440 upregulated CD86 mRNA expression in tumor xenografts of HEL92.1.7 cells in a dose-dependent manner following the oral administration of single doses ranging from 3 to 30 mg/kg (Fig. 6A). To investigate target engagement of this compound in tumors, we tested PI16 expression levels as a direct biomarker
PI16 suppression was dramatically reversed by T-3775440 treatment both in vivo and in vitro (Fig. 6B).

In a TF-1a (AEL) tumor xenograft model, T-3775440 exhibited significant antitumor effects, with 15-day T/C values of 15.6% and <0% at doses of 20 and 40 mg/kg, respectively (Fig. 6C). T-3775440 also exhibited potent antitumor effects in an additional AEL model of HEL 92.1.7 (D), and an AMKL model of CMK11-5 (E) xenografts. Values, mean tumor volumes ± SDs (n = 5). Asterisks denote P < 0.05 (**), and P < 0.025 (***) as determined by a one-tailed Williams test (C and D) or Aspin-Welch t test (E).

In accordance with a previous report in which conditional LSD1 knockdown was tested in mice (9), we found that in mice, T-3775440 treatment resulted in a transient reduction in platelets, followed by a significant rebound (Supplementary Fig. S10A); this was considered a mechanism-based adverse effect of LSD1 inhibition. In contrast, there was no obvious effect on red blood cell counts following a single administration (Supplementary Fig. S10B). On a dosing schedule comprising 5 days on/2 days off, a statistically significant difference in body weight was observed between vehicle- and T-3775440–treated tumor xenograft model mice at higher doses (data not shown). However, efficacious T-3775440 doses were tolerated in all subcutaneous tumor xenograft models. These results demonstrate that T-3775440 possesses profound anti–AEL and anti–AMKL activity in both in vivo xenograft and in vitro models.

**Discussion**

AEL and AMKL are rare subtypes of AML that account for approximately 3% and 1% of adult AML cases, respectively (21, 22). However, both subtypes are aggressive and refractory to conventional therapies and have very poor prognoses, with...
and AMKL patients, respectively (23, 24). In addition, no recurrent cytogenetic abnormalities are specific to these diseases, and therefore, it is challenging to develop molecular targeting therapeutics, such as all-trans retinoic acid, which was discovered for acute promyelocytic leukemia harboring the PML-RARA fusion gene (25, 26). Hence, these leukemia subtypes require the development of novel therapeutics that differ from conventional therapeutics with respect to the mode of action.

Here, we found that T-3775440, a potent and selective inhibitor of LSD1, suppressed growth and/or induced death in a subset of human AML cell lines that were mainly categorized as AEL or AMKL (Fig. 1C). This lineage selectivity clearly distinguishes T-3775440 from conventional chemotherapeutics, such as cytara- bine or anthracyclines. On the basis of analyses of morphologic, gene expression, and immunophenotyping changes, we demonstrated that T-3775440 treatment directed erythroid and megakaryoblastic leukemia cells toward transdifferentiation into granulomonocytic-like lineage cells, as evidenced by nuclear segmentation, microarray analyses, and CD86 expression in TF-1a cells (Fig. 2). Of note, after compound treatment, cells that expressed higher levels of CD86 exhibited more severely impaired proliferation than did cells with lower levels of CD86, suggesting a close linkage between transdifferentiation and T-3775440-mediated growth inhibition (Fig. 3A and B). Furthermore, knockdown of SPI1, which encodes the critical hematopoietic transcription factor for myeloid differentiation PU.1, reversed not only T-3775440-dependent transdifferentiation but also cell growth suppression and apoptosis (Fig. 3C–F). These results further indicate that the transdifferentiation plays a critical role in T-3775440-mediated cell growth inhibition/apoptosis.

In addition, we found that T-3775440 disrupted the LSD1–GFI1B interaction in AEL cells (Fig. 4A). GFI1B, a zinc finger protein that functions as a transcriptional repressor, is essential for the generation of definitive erythroid and megakaryocytic lineages (27). According to Saleque and colleagues, the epige- netic regulation of hematopoietic differentiation by GFI1B depends on the cofactors CoREST and LSD1 (7). These cofactors are recruited to the promoter regions of GFI1B target genes in a process mediated by the interaction between LSD1 and the SNAG domain of GFI1B, and inhibition of this process perturbs erythroid and megakaryocytic differentiation (8). Regarding AML, GFI1B overexpression has been observed in erythroid and megakaryocytic leukemia (28). These lines of evidence strongly suggest that GFI1B and its interaction with LSD1 participate in the pharmacologic effects of T-3775440. Indeed, analogous to observations in T-3775440-treated cells, GFI1B knockdown derepressed SPI1 target genes, including CD86, leading to inhibited cell growth (Fig. 5A and D). In addition, double knockdown of GFI1B and LSD1 had stronger effects on CD86 induction and cell growth, compared with each single knockdown. These results support the hypothesis that the effects of T-3775440 are medi- ated through disruption of the LSD1–GFI1B interaction. Recently, GFI1B was reported to directly regulate SPI1 gene expression (29). Indeed, T-3775440 induced the upregulation of SPI1 in AEL cells (Supplementary Fig. S4A). Thus, the LSD1–GFI1B–SPI1 axis is thought to play an important role in T-3775440-mediated transdifferentiation and growth suppression. Although the molecular mechanism by which T-3775440 affects the LSD1–GFI1B interaction remains to be elucidated, it is assumed that T-3775440, a derivative of translypolypromine (2-PCPA), covalent- ly binds to FAD in a manner similar to other 2-PCPA ana- logous inhibitors (30) and that the resultant FAD adduct might hinder the interaction between LSD1 and the SNAG domain of GFI1B.

In recent years, several LSD1 inhibitors containing various scaffolds have been generated and subjected to efficacy evaluation (11, 31, 32). Among them, ORY-1001 and GSK2879552 have entered clinical trials for the treatment of patients with AML (EudraCT number: 2013-002447-29, ClinicalTrials.gov identifier: NCT02177812). Similar to T-3775440, both inhibitors harbor a cyclopropylamine moiety and irreversibly inhibit the catalytic activity of LSD1 (31, 33). During the preparation of this article, 6- or 12-day treatment courses with GSK2879552 and other cyclopropylamine derivatives were reported to suppress the growth of a diverse range of AML cell lines, irrespective of subtype (33, 34). Similarly, T-3775440 was more profoundly efficacious and exhibited a broader anti-AML spectrum after prolonged treatment (data not shown). We observed relatively rapid (3 days of treatment) responses to T-3775440 in GFI1B-expressing AEL and AMKL cell lines. The rapid effects of our inhibitor may also underscore the significant impact of LSD1–GFI1B complex disruption and subsequent transdifferentiation in GFI1B-dependent leukemia cells. McGrath and colleagues recently reported that the irreversible LSD1 inhibitor RN-1, another cyclopropylamine derivative, exerted relatively strong effects on AEL and AMKL cells (20). Although these authors did not discuss these effects in great detail, both our and their findings suggest that AEL and AMKL cells are generally suscepti- ble to cyclopropylamine-derived LSD1 inhibitors. Therefore, it would be very interesting to investigate the clinical effi- cacies of cyclopropylamine derivatives, such as ORY-1001 and GSK2879552, in patients with AEL and AMKL.

During the course of our in vivo evaluation, we observed a considerable reduction in platelet counts in T-3775440-treated mice. This observation is consistent with a previous report in which conditional LSD1 knockdown in adult mice led to severe thrombocytopenia (9, 10). A colony-forming cell assay of normal human bone marrow cells revealed the selective activity of T-3775440 at a later stage, during the transition from GFI1B–GEF2/MYB toward BFU-E (Supplementary Fig. S9). These results suggest that during hematopoesis, T-3775440 targets the common erythro- megakaryocytic progenitor population, which is known to express high levels of GFI1B (35). Prominent thrombocytopenia is likely due to the relatively short half-lives of platelets, compared with red blood cells. Importantly, the effect of T-3775440 on platelets was reversible and is expected to be manageable by platelet transfusion in clinical settings.

Although numerous attempts have been made to identify novel oncogenic driver mutations and possible mutation-relat- ed cancer vulnerabilities in AMLs, the available therapeutic options and the number of actionable oncogenic drivers have remained limited. AEL and AMKL have not been reported to possess such driver mutations and therefore, diseases remain with a major unmet medical need. In this study, we demon- strated that T-3775440, a novel inhibitor of LSD1, disrupted the LSD1–GFI1B interaction, leading to cell transdifferentiation and consequent cell growth inhibition and/or apoptosis induc- tion. Moreover, T-3775440 exhibited significant antitumor efficacy in in vivo models of acute erythroleukemia and AMKL. Our findings provide a rationale for the testing of T-3775440 as therapeutics.
a potential treatment with a novel mechanism of action for AML, particularly acute erythroleukemia and AMKL, and suggest the possibility of enforced cell transdifferentiation as a novel therapeutic strategy to override the differentiation block and self-renewal capability of AML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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