Potent synergy of DHODH and SREBP inhibition in acute myeloid leukemia via disruption of cholesterol and lipid metabolism

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Abstract

Acute myeloid leukemia (AML) remains difficult to cure, in part related to strong genetic and functional heterogeneity between and within individual patients. Metabolic reprogramming is emerging as an important feature of AML cells, allowing exploration of alternative treatment strategies. Here, we describe a novel DHODH inhibitor, JNJ-74856665, that showed strong efficacy in a subset of AML samples. In a multi-omics approach, by combining label-free quantitative proteome data with drug sensitivity data in bone marrow stromal co-cultures in a large cohort of primary AML patient samples we identified that sensitivity to DHODH inhibition (DHODHi) is linked to cholesterol and lipid metabolism. DHODHi resulted in an accumulation of cholesterol, mitochondrial reactive oxygen species (ROS) and lipid peroxidation. LC-MS/MS-based lipidomics studies revealed that DHODHi resulted in a strong increase in polyunsaturated fatty acids and triglycerides, which are the primary lipid species stored in lipid droplets (LD). We hypothesized that this might be the consequence of increased ROS and lipid peroxidation levels, prompting the cell to detoxify such toxic lipid species by storing them in LD. Indeed, we could observe a marked increase in LD formation upon DHODHi. The transcriptional regulator SREBF2, known to control cholesterol and lipid metabolism, was up-regulated in DHODHi sensitive AML, and a strong synergy was observed between the combination of both DHODHi and the SREBP inhibitor dipyridamole. Our data indicate that it would be interesting to further explore combined DHODH and SREBP inhibition as a therapeutic target option in AML.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease, in which a block in differentiation leads to the accumulation of immature myeloid blasts. Despite ongoing improvements in diagnosis and therapies, the treatment of AML remains challenging with the '3+7' chemotherapy backbone serving as the basis for current treatment.

A promising addition to cancer therapy is the recent emergence of dihydroorotate dehydrogenase (DHODH) inhibition, which is the rate limiting step in the *de novo* pyrimidine synthesis pathway.

As an important step in the pathway, it converts dihydroorotate (DHO) to orotate, a substrate for uridine monophosphate (UMP) that can be further converted to other pyrimidine nucleotides needed in proliferating cells.[1] In AML, DHODH was identified as one of the main targets to overcome the block in differentiation.[2-4] Here, by integrating proteogenomic data with our *ex vivo* drug screening platform in primary AML patient samples, we studied the effect of a novel DHODH inhibitor, JNJ-74856665.[5] By integration of this dataset, we aimed to gain insight into which AML subtypes benefit the most from DHODH inhibition (DHODHi). We also wanted to explore synergistic drug combinations. We identified that sensitivity to DHODHi is linked to cholesterol and lipid metabolism, which could be targeted with dipyridamole, and combination treatment resulted in strong synergistic cell death as a consequence of reactive oxygen species (ROS) and lipid ROS accumulation, combined with impaired cholesterol metabolism and lipid detoxifi cation.

Methods

Primary samples

Acute myeloid leukemia blasts from peripheral blood or bone marrow from untreated patients and normal bone marrow (NBM) samples were obtained from healthy individuals at the University Medical Center Groningen after informed consent. The protocol was approved by the Medical Ethical Committee, in accordance with the Declaration of Helsinki. Mononuclear cells (MNC) were isolated via Lymphoprep™ separation and cryopreserved. Next-generation sequencing was performed to obtain mutation status of primary AML cells using the TruSight Myeloid Sequencing Panel (Illumina) or exome sequencing. Neonatal cord blood (CB) samples were obtained from healthy full-term pregnancies at the obstetrics departments at the Martini Hospital and University Medical Center Groningen.

Cell culture

The AML cell lines THP1 (DSMZ: ACC-16) and HL60 (DSMZ: ACC-3) cells were cultured in RPMI 1640 with 10% FCS and 1% P/S. MS5 murine stromal cells (DSMZ: ACC-441) were cultured in alpha-MEM (Lonza) with 10% FCS and 1% P/S. All cell cultures were kept at 37°C and 5% CO_2. Inhibitor JNJ-74856665 was provided by Janssen Biologics BV for all DHODHi experiments. Diyridamole, Dilazep and Betulin were obtained from MedChemExpress (Monmouth Junction, NJ, USA). Atorvastatin and Rosuvastatin were obtained from Axon Medchem (Groningen, NL).

DHODH inhibitor screen in primary acute myeloid leukemia samples

Cryopreserved MNC of AML patients were co-cultured in Gartner's medium supplemented with G-CSF (Amgen), N-Plate (TPO) (Amgen) and IL-3 (Sandoz) (all 20 ng/mL) on MS5, which were confluent plated on 0.1% gelatin-coated 96-well plates and pre-treated with Mitomycin C. 100,000 MNC were plated per well, and after two days recovery cells were treated with DMSO or 0.3, 3.00, 30.0 and 300 nM JNJ-74856665 inhibitor for seven days. On day 7, cells were stained with CD45-PECy7 (BioLegend; 304016), CD14-PE (BioLegend; 325606), CD11b-FITC (ImmunoTools; 21279113X2), and DAPI (ThermoScientific) in a 96-well plate, and were incubated for 30 minutes (min) at 4°C. Fluorescence measurements were taken using a MACSQuant® X Flow Cytometer (Miltenyi Biotec).

DHODH inhibitor screen in healthy samples

Healthy MNC were isolated from NBM by a density gradient using Lymphoprep™ (STEMCELL™ Technologies). Stem cells were isolated using the CD34 Microbead Kit (Miltenyi). After 24 hour (hr) recovery in Stemline® II hematopoietic medium (Merck; #S0192) supplemented with 1% P/S, SCF, FLT3-L and N-plate (TPO) (Amgen) (all 100 ng/mL), $CD34^+$ NBM cells were treated with DMSO or 0.3, 3.00, 30.0, and 300 nM JNJ-74856665 inhibitor for seven days and co-cultured on MS5 in Gartner's medium supplemented with cytokines.

Flow cytometry data analysis inhibitor screen

All flow data were analyzed using Flow Jo™ (BD BioSciences). Counts, percentages and median fluorescence intensities were exported for further analysis. To calculate area under the curve (AUC) values, $DAPI^-/CD45^{dim}$ (primary samples) or $DAPI^-$ (cell lines) counts were normalized to the DMSO control. Next, AUC was calculated using trapezoid rule integration computed by the trapz(z) function in the R package caTools. CD11b median fluorescence intensity (MFI) values were normalized to DMSO control.

Inhibitor combination treatments

Acute myeloid leukemia cell lines, primary AML patient cells, and $CD34^+$ CB cells were treated with DMSO or 0.3, 3.00, and 30.0 nM JNJ-74856665 inhibitor for three days in combination with Dipyridamole (DMSO or 1, 5, and 10 µM), Betulin (DMSO or 5, 10, and 20 µM), Dilazep (DMSO or 0.1, 1, and 10 µM), Atorvastatin (DMSO or 2, 10, and 20 µM) or Rosuvastatin (DMSO or 0.3, 3, and 30 µM). After three days of incubation, cells were stained with Annexin-V APC, CD11b-FITC, CD14-PE and 7AAD for DHODH and Dipyridamole treated cells. The other combinations were stained with Annexin-V APC and DAPI. All staining was performed in a 96-well plate and incubated for 30 min at 4°C. Fluorescence measurements were taken using a MACSQuant® X Flow Cytometer (Miltenyi Biotec). Flow data were analyzed using Flow Jo™ (BD BioSciences) and ZIP scores were calculated using SynergyFinder 3.0.[6]

Reactive oxygen species, BODIPY cholesterol, neutral lipid and mitochondrial measurements

After treatment, 100,000 cells were taken and washed twice with PBS. Next, cells were resuspended in medium containing ROS–DCF-DA (10 µM) (Merck; #35845), the mitochondrial superoxide probe MitoSOXTM (5 µM) (ThermoScientific; #M36008) or C11-BODIPY (lipid ROS) (Thermofisher; #D3861) or cells were resuspended in PBS containing BODIPY-cholesterol (MedChemExpress; #HY-125746), BODIPY™ 493/503 (Invitrogen™; #D3922), Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) (Thermofisher; #T669) or MitoTracker GreenTM (Thermofisher; #M7514). Additionally, verapamil hydrochloride (12.5 µM) was added to all staining procedures to prevent efflux of the mitochondria-related probes. Fluorescence measurements were taken using a MACSQuant® X Flow Cytometer (Miltenyi Biotec) or a NovoCyte Quanteon (Agilent). All flow data were analyzed using Flow Jo™ (BD BioSciences). For each sample, a minimum of 10,000 events were acquired inside the viable gate.

Lipidomics

To prepare samples for LC-MS/MS, to each 75 µL cell suspension, 200 µL methanol containing 1000x diluted EquiS-

PLASH internal standard (Avanti) was added and vortexed well. Then, 625 µL MTBE (methyl tert-butyl ether) was added and incubated for 1 hr on a shaker at 900 rpm. 100 µL water was added and incubated on a shaker at 900 rpm for 10 min. Extracts were centrifuged at 1,000 x g for 10 min and the upper organic phase was collected in a separate tube. The lower phase was re-extracted with 250 µL solvent mixture (MTBE/methanol/water 10:3:2.5 v/v/v) and incubated on a shaker for 30 min at 900 rpm. Extracts were centrifuged at 1,000 x g for 3 min and the upper organic phase was added to the organic phase of the first extraction. The combined organic phases were concentrated under a heated nitrogen stream (50°C) and subsequently dried in a vacuum centrifuge at 30ºC. Dried lipid pellets were dissolved in 25 µL CH3Cl/methanol/water 60:30:4.5 v/v/v and with 75 µL isopropanol/acetonitrile/water 2:1:1 v/v/v.

Results

Primary AML patient samples were analyzed for mutation status (Illumina Trusight sequencing; N=26) (*Online Supplementary Table S1*), full label-free quantitative proteome (LC-MS/MS; N=17[7]), and for DHODH inhibitor (DHODHi) sensitivity on MS5 bone marrow stromal cells (N=26). The inhibitor was tested at a range of 0.3-300 nM, and after seven days the effects on proliferation, viability, and differentiation were determined by flow cytometry (Figure 1A, *Online Supplementary Figure S1*). *Ex vivo* culturing of primary AML samples can be challenging and, therefore, stromal co-cultures were used because of their superior long-term support of primary AML patient samples compared to liquid culture conditions,[8-11] and only samples were included where the viability could be maintained throughout the experiments (*Online Supplementary Figure S1B, C*). AUC values were determined based on viable DAPI-/CD45^{dim} blast counts, which revealed a strong but heterogeneous response to the inhibitor (Figure 1B, C). An induction of differentiation based on increased CD11b expression was also noted in some (N=8; AML28, 32, 31, 30, 8, 29, 14, and 6) but not all AML patient samples. Not all samples in which proliferation and viability were most strongly inhibited responded by inducing differentiation, suggesting that these processes are not directly linked (Figure 1B). One of the functions of DHODH entails the rate limiting step in *de novo* pyrimidine synthesis, which is up-regulated in proliferating cells in response to the increased demand of nucleotides needed for DNA synthesis. Indeed, while we observed a trend towards inverse correlations between the proliferation rate of unperturbed cells and AUC values, this did not reach significance (*Online Supplementary Figure S1D*), suggesting that other functions downstream of DHODH might also play an important role. No significant correlations were observed between mutation status and response to DHODHi, but larger cohort sizes may be needed to identify such associations (*Online Supplementary Figure S1E*). At lower concentrations, healthy CD34^{+} cells derived from normal bone marrow were less sensitive compared to AML samples (Figure 1D). To gain a better understanding of the protein expression programs that underlie DHODH inhibitor sensitivity, we analyzed the full proteome of primary AML patients included in the drug screen (N=17).[12] We calculated Pearson correlation coefficients between the quantitative proteome dataset and AUC values of the DHODHi (Figure 2A). Subsequently, the ranked Pearson correlation coefficient list was used for gene set enrichment analysis (GSEA), which showed that DHODHi sensitive AML samples were enriched for signatures related to 'cholesterol metabolism', 'biosynthesis of unsaturated fatty acids', 'glutathione conjugation', 'membrane lipid biosynthetic process', 'PPAR signalling pathway', and 'cholesterol biosynthesis'. Indeed, sterol regulatory binding transcription factor 2 (SREBF2), which controls expression of genes related to cholesterol synthesis and genes associated with lipid synthesis and detoxification, such as SCD1[13-16] and DGAT1/2,[17,18] was up-regulated in AML samples with high sensitivity to DHODHi (Figure 2A). These data suggest that AML samples with an increased cholesterol metabolism and/or lipid metabolism would be most dependent on DHODH activity. Reversely, insensitive AML samples were enriched for signatures like 'MYC targets', 'KEGG spliceosome', and 'KEGG Ribosome' (Figure 2B, C).

To further explore potential downstream mechanisms that mediate DHODHi sensitivity, we treated AML cell lines HL60 and THP1 with the inhibitor. Proliferation was impaired in both cell lines, albeit HL60 cells were slightly more sensitive compared to THP1 (*Online Supplementary Figure S1F, G*). This was accompanied by an increase in apoptosis as determined by Annexin V staining, as well as an induction of differentiation (*Online Supplementary Figure S1F, G*). We also observed a significant increase in the amount of total cholesterol accumulation in the cells upon DHODHi, possibly as a consequence of reduced proliferation (Figure 3A). In addition, DHODH is suggested to regulate reactive oxygen species (ROS) production and ferroptosis.[19] Thus, we wondered if DHODHi would change ROS levels in HL60 and THP1 cells. Mitochondrial superoxide levels (MitoSox) and lipid ROS (BODIPY-C11) were significantly increased in both cell lines after treatment with DHODHi (Figure 3B). Cytoplasmic ROS levels were only significantly increased in HL60 cells upon DHODHi. As expected, mitochondrial activity as determined by TMRE was decreased upon DHODHi as a consequence of impairing the electron transport chain (ETC), while the total number of mitochondria remained unchanged (Figure 3B).

We wondered whether similar or different mechanisms would underly sensitivity in these two AML models, as we previously showed that the metabolism of these cell lines is quite different, with HL60 being more glycolytic and THP1 were more oxidative phosphorylation (OXPHOS)-driv-

en.[20,21] Indeed, THP1 cells displayed higher basal levels of mitochondrial and cytoplasmic ROS, while total lipid ROS levels were comparable (Figure 3C). From these data, we hypothesized that the effects of DHODHi in THP1 cells might derive primarily from inhibition of the ETC as compared to HL60 cells. We then performed single sample GSEA analyses on the top 8 DHODHi-sensitive AML samples focusing on gene sets associated with metabolism. Although all sensitive samples had cholesterol and lipid metabolism as commonly enriched terms (Figure 2A-C), differences were also seen (*Online Supplementary Figure S2*). Specifically, some of the DHODHi sensitive cells were more enriched

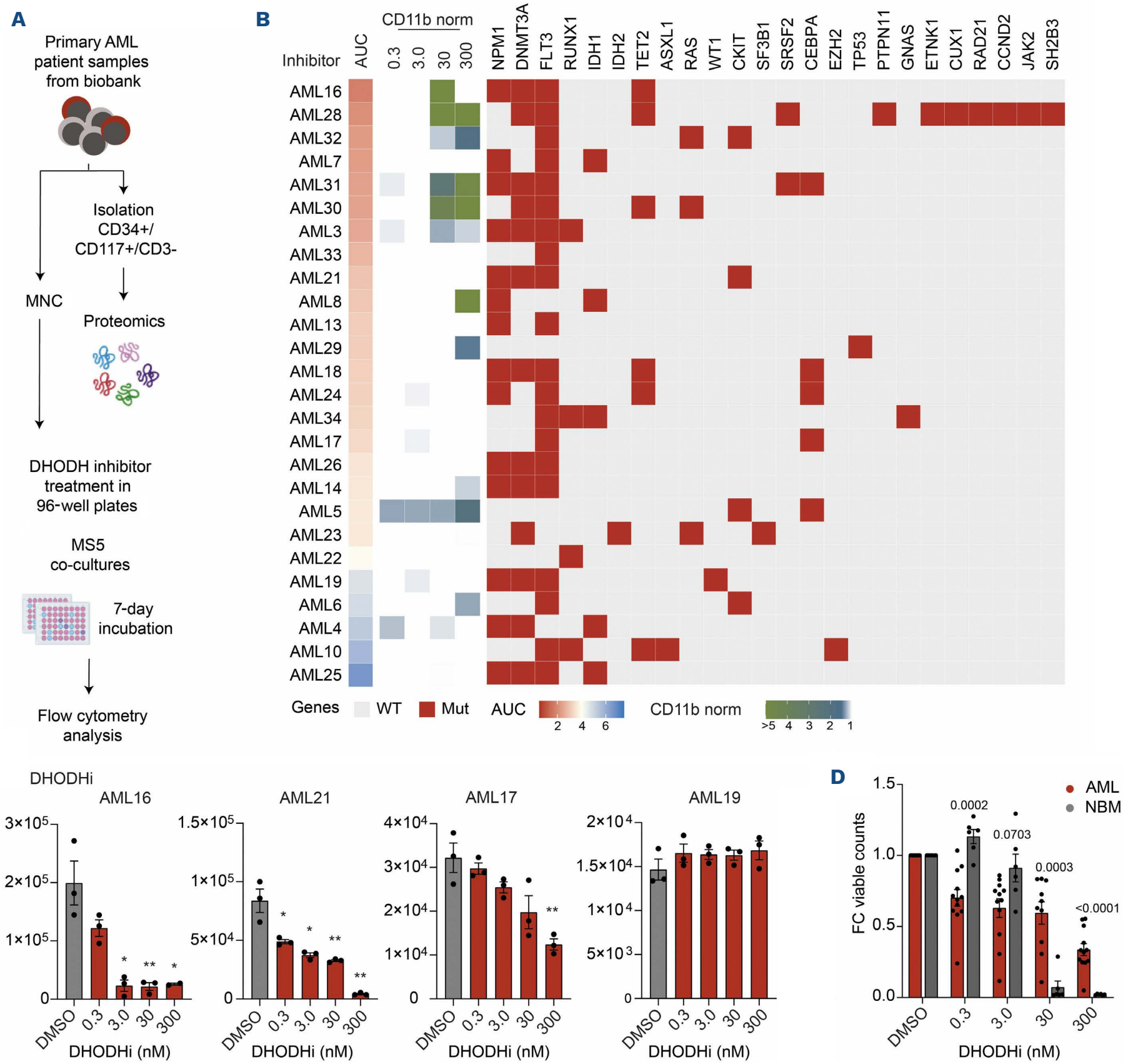

Figure 1. Sensitivity to DHODH inhibition of primary acute myeloid leukemia samples co-cultured on MS5. (A) Schematic visualization of the DHODH inhibitor drug screen in primary acute myeloid leukemia (AML) patient samples co-cultured on MS5 cells for seven days. (B) Area under the curve (AUC) values, which were calculated using the trapezoid method, and normalized median fluorescence intensity (MFI) values of CD11b from DHODH treated primary AML cells (N=26) with various mutations co-cultured on MS5 cells. (C) Dose-dependent effects on cell viability upon DHODHi treatment of AML16, AML21, AML17 and AML19 under MS5 culture conditions. (D) Fold change of viable cell counts of primary AML samples and healthy CD34⁺ cells of normal bone marrow (NBM) treated with DHODHi both co-cultured on MS5 cells. Statistical analysis by unpaired Student *t* test. **P*>0.05, ***P*>0.01. MNC: mononuclear cells; mut: mutated; WT: wild-type.

for processes related to mitochondrial respiration and ETC, while others were more enriched for glycolysis. Additionally, a subset of samples showed specific enrichment for lipid droplet formation. Together, these data indicate that distinct processes downstream of DHODH might underly sensitivity in individual AML.

To explore this further, we performed LC-MS/MS-based lipidomics studies in both AML models in the absence or presence of DHODHi. These data revealed a profound impact of DHODHi on the lipidome (Figure 4, *Online Supplementary Figure S3*), with most notably a strong increase in triglycerides (TG). In THP1 cells, but not HL60 cells, a strong decrease in acylcarnitines (CAR) was observed upon DHODHi (*Online Supplementary Figure S3A, B*), whereby baseline CAR levels were much higher in THP1 (*Online Supplementary Figure S3C, D*). Once again, this is in line with the notion that THP1 cells are more OXPHOS-driven,[21] in part via fatty acid oxidation (FAO), which was impaired by inhibiting the ETC upon treatment with DHODH inhibitors. A significant increase in cholesterol esters (CE) was also observed in HL60 (Figure 4A, B), but levels went down in THP1 cells, while THP1 did display higher basal levels compared to HL60 (*Online Supplementary Figure S3B, D*). A remarkable shift in the degree of lipid chain unsaturation

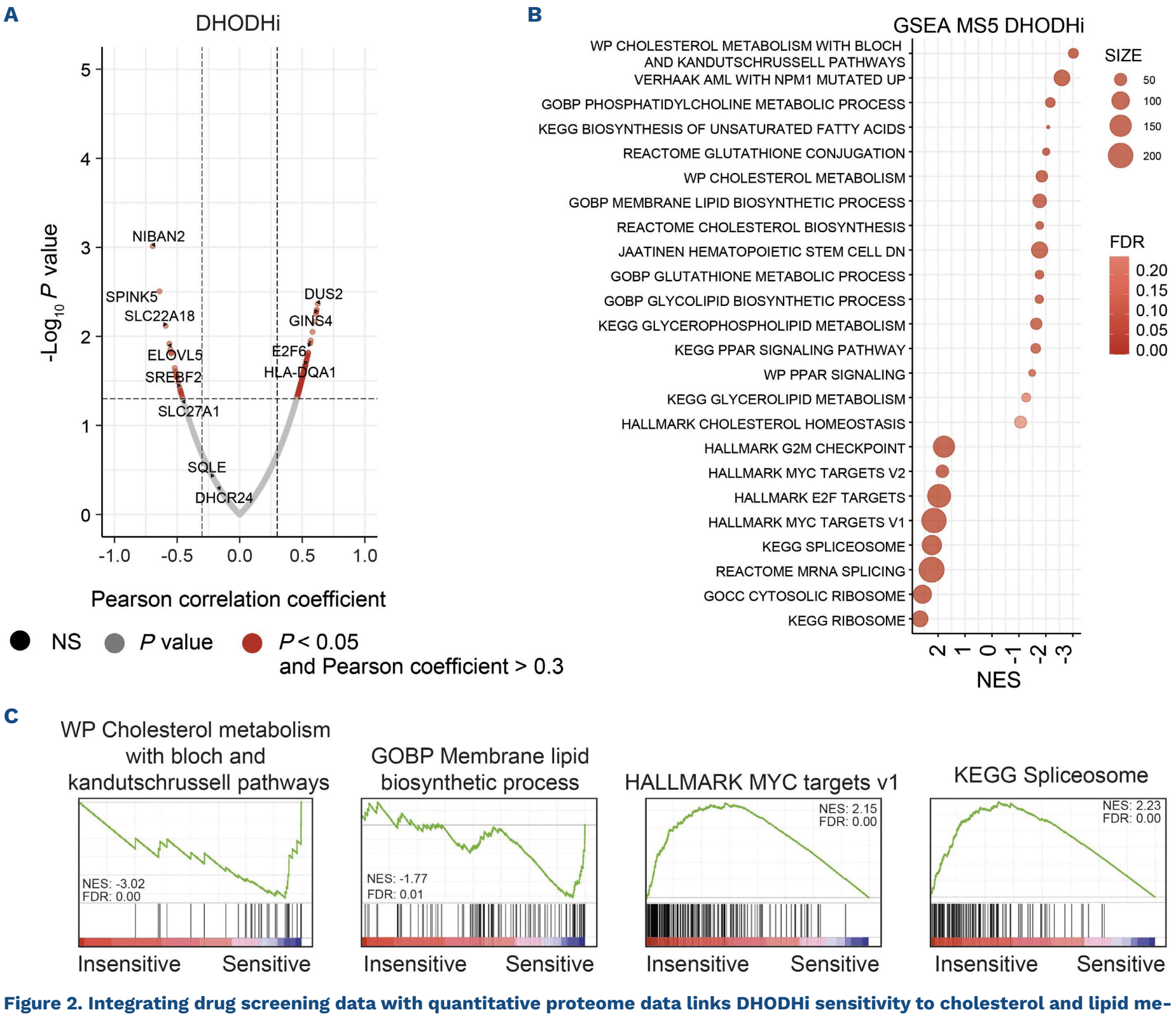

Figure 2. Integrating drug screening data with quantitative proteome data links DHODHi sensitivity to cholesterol and lipid metabolism. (A) Volcano plot of the Pearson correlation coefficient and -log10 *P* values of the proteome dataset *versus* area under the curve (AUC) values of DHODHi-treated primary acute myeloid leukemia samples. (B) Dotplot of gene set enrichment analysis (GSEA) signatures enriched in sensitive and less sensitive primary acute myeloid leukemia (AML) patient samples. (C) Highlighted gene set enrichment signatures from panel (B). FDR: false discovery rate; GSEA: gene set enrichment analysis; NS: not significant.

was observed, with an increase in polyunsaturated fatty acids (PUFA) upon DHODHi (Figure 4C, *Online Supplementary Figure S3E*). When ROS levels increase, PUFA run the risk of becoming peroxidated, resulting in ferroptosis.[22] TG are the primary lipid species stored in lipid droplets (LD), [23,24] and we, therefore, hypothesized that the increase in TG upon DHODHi could be a consequence of increased ROS and lipid peroxidation levels, promoting detoxification by storing toxic lipid species in LD. Indeed, we observed a significant increase in TG PUFA (Figure 4D, *Online Supplementary Figure S3F*) as well as the formation of LD upon DHODHi (Figure 4E, *Online Supplementary Figure S3G*).

One of the key regulatory transcription factor families involved in cholesterol and lipid metabolism is the family of sterol regulatory element-binding proteins (SREBP), and, as outlined above, we noted a significant upregulation of SREBF2 in cells that are most sensitive to DHODHi (Figure 2A). It has been shown that the phosphodiesterase inhibitor dipyridamole, an antiplatelet agent approved by the US Food and Drug Administration,[25] can inhibit the activation of SREBP and their target genes.[26] Therefore, we wanted to explore whether treatment with dipyridamole would enhance the inhibitory effect of DHODHi. Single treatment experiments indicated that HL60 cells are slightly sensitive, but THP1 cells are not sensitive to dipyridamole alone at low concentrations (Figure 5A, B). When treating THP1 cells with higher concentrations of dipyridamole (up to 40 μM), we did see a significant effect on proliferation (*Online Supplementary Figure S4A*). Baseline cholesterol levels were also higher in THP1 compared to HL60 (Figure 5C, *Online Supplementary Figure S3C, D*), and a reduction in cholesterol levels could only be achieved in HL60 upon

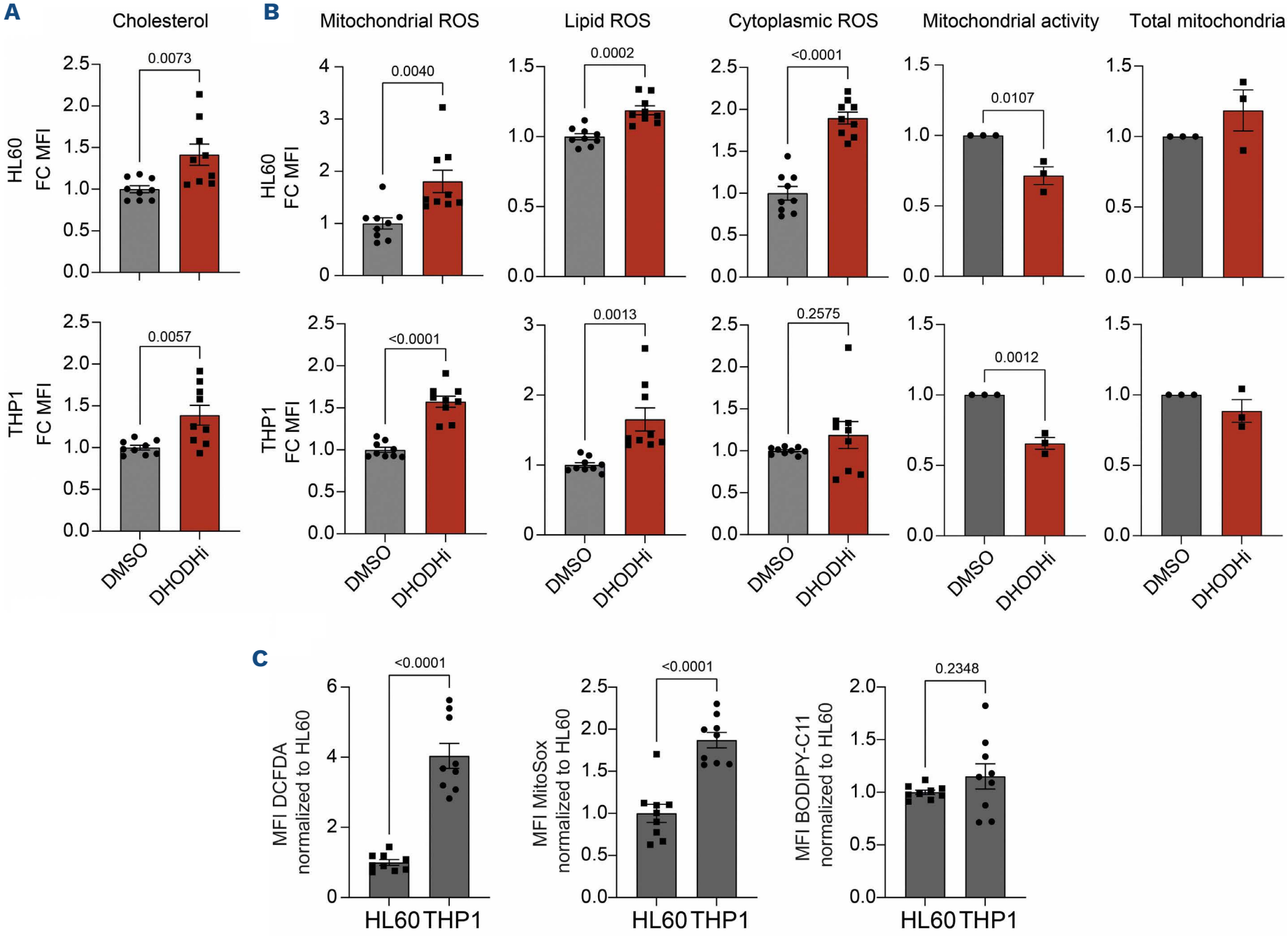

Figure 3. DHODHi increases intracellular cholesterol, reactive oxygen species and lipid reactive oxygen species levels while mitochondrial activity is reduced. (A) Mean fluorescence intensity (MFI) fold change of cholesterol (BODIPY-cholesterol) in 4-day DMSO or DHODHi-treated (3 nM) HL60 and THP1 cells (N=4 independent experiments). (B) MFI fold change of cytoplasmic reactive oxygen species (ROS) (DCFDA), mitochondrial superoxide levels (MitoSox), lipid ROS (BODIPY-C11), mitochondrial activity and total mitochondria (Mitotracker green) in 4-day DMSO or DHODHi-treated (3 nM) HL60 and THP1 cells. (C) Baseline cytoplasmatic ROS, mitochondrial ROS and lipid ROS in HL60 and THP1 cells. Data are normalized to HL60 values. Bar graphs represent the mean ± standard error of mean of at least three independent experiments. Statistical analysis by unpaired Student *t* test.

dipyridamole treatment alone at low concentrations (Figure 5C). In addition, the amount of cholesterol in THP1 cells was decreased upon treatment with higher concentrations of dipyridamole (*Online Supplementary Figure S4B*). In line with this, we also noticed higher mRNA expression levels of genes involved in the cholesterol metabolism pathway in THP1 cells when compared to HL60 (*Online Supplementary Figure S4C*), which was confirmed by quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) for *DHCR7* and *HMGCS1* (Figure 5D). Treatment with dipyridamole resulted in reduced expression of *DHCR7* and *HMGCS1*, even though this occurred within 6 hr in HL60 and only after 96 hr in THP1 (Figure 5E).

Combination treatment with both DHODHi and dipyridamole showed strong synergy in both cell lines with ZIP scores of 27.6 and 32.5 for the HL60- and THP1-treated cells, respectively, as determined by SynergyFinder[27] where a ZIP score >10 was considered synergistic (Figure 5A, B). Combination treatment resulted in high levels of apoptosis, and induction of differentiation was also observed (Figure 5F). Similar results were obtained in primary AML patient samples. AML3, AML6, AML14 and AML27 were co-cultured on MS5 and were treated with single agents or combinations. There was no effect on proliferation with dipyridamole as single treatment in 2 of the 4 primary AML samples, AML3 and AML6, while slight reductions in proliferation were seen in AML14 and AML27 (*Online Supplementary Figure S4D*). But, similar to our observations in cell lines, the combination treatment of DHODHi and dipyridamole was strongly synergistic in all cases with ZIP scores of 35.5, 29.1, 27.0, and 11.1, respectively (*Online Supplementary Figure S4D*). After these four initial samples an additional 10 patient samples were analyzed in combination treatment studies. Again, combination treatment resulted in strongly reduced cell counts and an induction of differentiation (*Online Supplementary Figure S4E*). Normal $CD34^+$ cells were less sensitive to combination treatment of DHODH and SREBP inhibition, particularly at lower DHODH inhib-

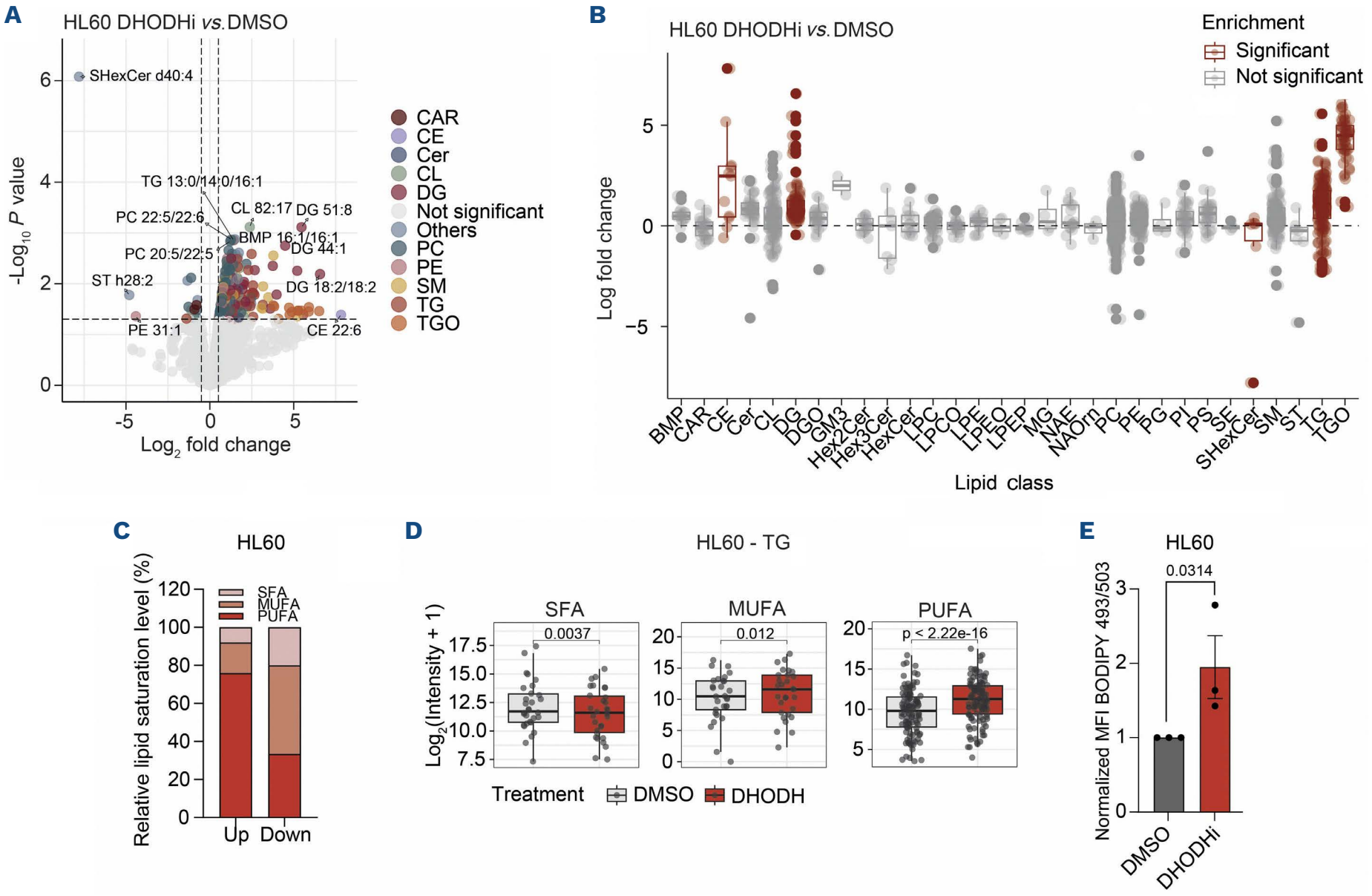

Figure 4. LC-MS/MS-based lipidomics on DHODHi-treated HL60 cells reveals a profound impact on lipid metabolism. (A) Volcano plot showing the differentially expressed lipids of HL60 cells treated with 3 nM of DHODHi *versus* DMSO for 24 hours. Classes are depicted by colors in the legend. (B) Enrichment analysis for lipid classes comparing DHODHi *versus* DMSO treated cells. (C) Total number and relative saturation levels of up- and down-regulated differentially expressed lipids in DHODHi-treated cells. (D) Triglycerides (TG) abundances in DMSO and DHODHi-treated HL60 cells divided by saturation class. (E) Median fluorescence intensity (MFI) fold change of neutral lipids (BODIPY 493/503) in DMSO and DHODHi-treated HL60 cells. Statistical analysis by paired Student *t* test (D) and unpaired Student *t* test. (E). *P>0.05.

itor concentrations (*Online Supplementary Figure S5A*), without consistent induction of differentiation (*Online Supplementary Figure S5B, C*). Previous studies have also discussed the combination treatment of DHODH and dipyridamole, which was positioned as a known inhibitor of the nucleoside/nucleotide transport channels hENT1/2 (SLC29A1-A2).[28] To address this further, we evaluated the efficacy of DHODHi in combination with dilazep, an ENT1 inhibitor. While combination treatment was additive in HL60 cells, it was synergistic in THP1 and 2 primary AML

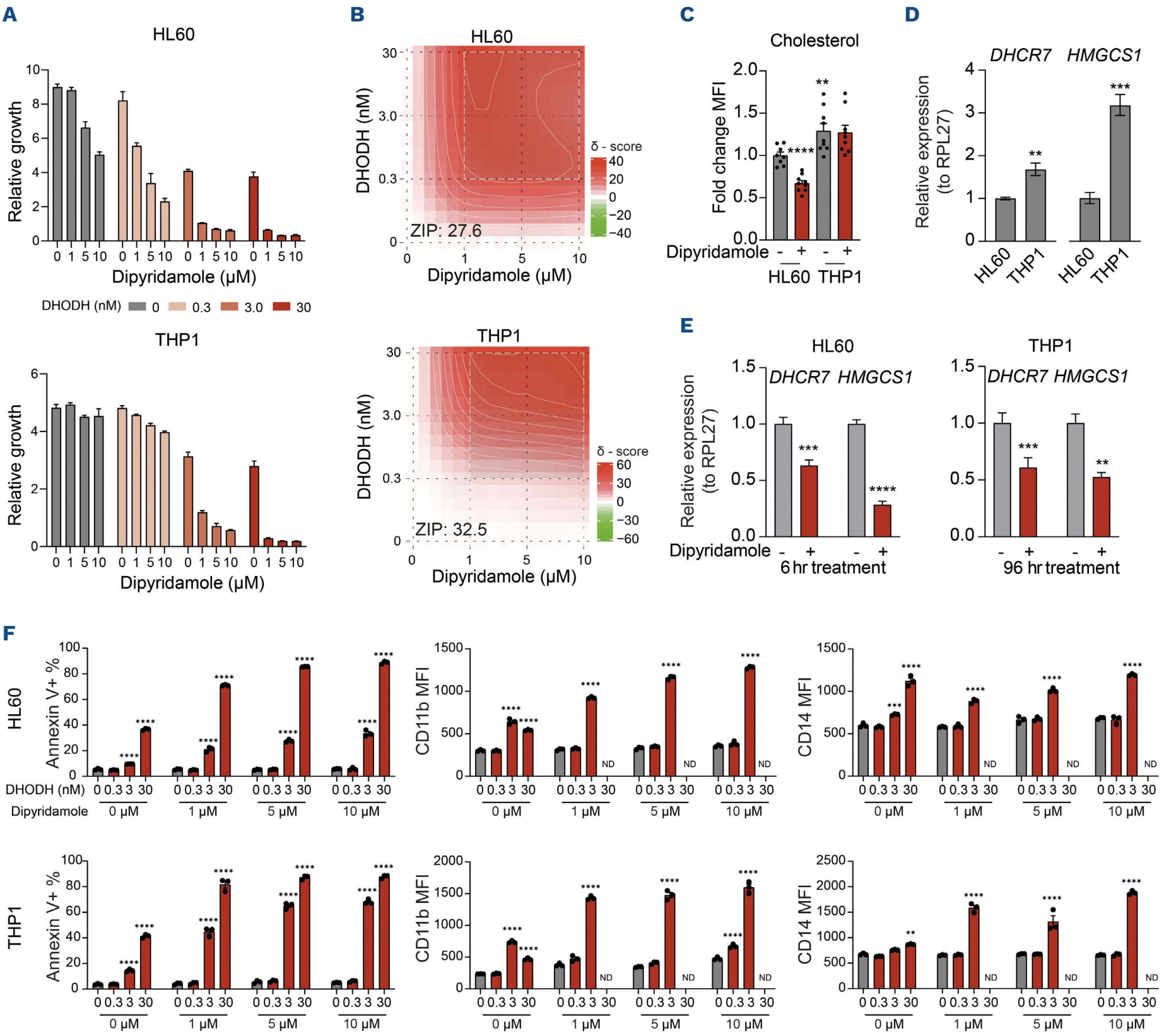

Figure 5. Combination treatment with DHODHi and dipyridamole results in strong synergism in acute myeloid leukemia cell lines and primary acute myeloid leukemia samples. (A) Impact on relative growth of HL60 and THP1 cells treated for three days with DMSO, DHODHi (0.3, 3.0, and 30 nM) and/or dipyridamole (1, 5, and 10 μM). Representative of three independent experiments. (B) Synergy distribution plots of data shown in panel (A). (C) Median fluorescence intensity (MFI) fold change in cholesterol (BODIPY-cholesterol) in 4-day DMSO or 5 μM dipyridamole-treated HL60 and THP1 cells (N=4 independent experiments). Data are normalized to HL60 DMSO values. (D) mRNA expression of DHCR7 and HMGCS1 in HL60 and THP1 cells. Data are normalized to HL60 values. Representative of three independent experiments. (E) mRNA expression of DHCR7 and HMGCS1 in HL60 and THP1 cells treated with DMSO or 5 μM dipyridamole for 6 and 96 hours (hr), respectively. Data are normalized to DMSO. Representative of three independent experiments. (F) Annexin V percentage, CD11b expression, and CD14 expression of HL60 and THP1 cells treated with DMSO, DHODHi, and dipyridamole for three days. Representatives of three independent experiments. Synergy scores were calculated using the Zero interaction potency model. Bar graphs represent the mean ± standard error of mean of at least three independent experiments. Statistical analysis by unpaired Student *t* test. ***P*>0.01, ****P*>0.001, *****P*>0.0001.

samples, albeit that ZIP scores were not as high as with the DHODH/dipyridamole combination (*Online Supplementary Figure S6A, B*). Clearly, the salvage pathway might also play a role downstream of SREBP, although to a lesser extent in HL60 cells, possibly due to their glycolytic-driven metabolism. To further address the potential link with cholesterol metabolism, we used the specific inhibitors rosuvastatin and atorvastatin, which target HMG-CoA reductase, crucial for cholesterol synthesis. Combination treatment of these inhibitors together with DHODHi had an additive effect on the synergistic action (*Online Supplementary Figure S6C-F*), but, again, with lower ZIP scores compared to the DHODH/dipyridamole combination. Interestingly, inhibition of cholesterol metabolism in combination with DHODHi displayed stronger effects in HL60 compared to THP1, again highlighting the heterogeneity in metabolic vulnerabilities across different AML subtypes.

Discussion

By combining label-free quantitative proteome data with drug sensitivity screens in primary AML patient samples co-cultured on bone marrow stroma, we show here that sensitivity to the DHODH inhibitor JNJ-74856665 is linked to cholesterol and lipid metabolism. The transcriptional regulator SREBF2 was up-regulated in DHODHi sensitive AML, and a strong synergy was observed between DHODH inhibition and the SREBP inhibitor dipyridamole. Our data indicate that several pathways downstream of dipyridamole might account for the synergistic actions together with DHODHi, including its control over cholesterol metabolism, over nucleoside/nucleotide import, and over lipid detoxification via LD formation.

The DHODH enzyme exerts several functions in the cell. It controls pyrimidine nucleotide synthesis by catalyzing the oxidation of dihydroorotate to orotate, the base precursor of pyrimidines.[29] During this reaction, the electron acceptor ubiquinone is reduced to ubiquinol, thereby providing electrons from the ETC complex I/II to complex III and thereby maintaining mitochondrial OXPHOS and ATP production. Furthermore, it has been shown that DHODH controls protein O-linked N-acetylglycosylation via the generation of UDP-GlcNAc.[3] Restored differentiation in AML upon DHODHi was attributed, at least in part, to depletion of UDP-GlcNAc leading to decreased O-linked N-acetylglycosylation.[3] Indeed, we observed strong heterogeneity in responses toward inhibition of DHODH across a panel of primary AML patient samples. While some samples responded strongly in terms of loss of proliferation and viability, others were much less sensitive. Within our cohort of 26 samples, sensitivity was not associated with a specific genetic subtype of AML. We initially hypothesized that sensitivity might be linked to proliferation, and while we, indeed, observed a trend between sensitivity and relative growth levels during our culture periods, this did not reach significance, leaving open the possibility that other characteristics would underly DHODHi sensitivity.

We and others have shown that metabolic programs can differ considerably between AML subtypes.[11,20,21,30-34] By linking DHODHi sensitivity to protein expression programs, we identified that the most sensitive AML were characterized by high cholesterol and lipid metabolism. The transcription factor family of sterol regulatory element-binding proteins (SREBP) controls expression of enzymes involved in cholesterol and lipid metabolism,[17] including HMGCS1, HMGCR1, SQLE, DHCR7/24, as well as SCD1, which converts saturated fatty acids into mono-unsaturated acids (MUFA), and DGAT1/2, which is essential for LD formation, thereby playing an important role in the detoxification of lipids. Based on these initial findings, and since SREBF2 was among the up-regulated proteins in DHODHi-sensitive AML, we tested efficacy of dipyridamole in combination with inhibition of DHODH and uncovered potent synergistic effects. We wondered whether the underlying mechanisms explaining these synergistic effects would be similar across different AML subtypes, and, therefore, performed extensive lipidomics studies and utilized more specific inhibitors to inhibit cholesterol metabolism or the nucleotide salvage pathway by inhibiting ENT importers. For this, we used two AML model systems: the glycolytic cell line HL60 and the OXHPOS-driven cell line THP1. In both cell lines, inhibition of DHODH resulted in increased levels of PUFA, as well as increased levels of TG. The latter is indicative for LD formation, as we have also observed that inhibition of DGAT1 results in strong reductions of TG.[35] These data would suggest that the increase in ROS and lipid ROS levels as a consequence of DHODHi would drive a cellular response by detoxifying these toxic lipid species in LD, which would be counteracted by SREBP inhibition. These data are in line with a recent publication in which DHODHi in $CD8^+$ T cells also resulted in increased ferroptosis and PUFA accumulation.[36] While mitochondrial ROS was increased upon DHODHi in both HL60 and THP1 cells, cytoplasmic ROS levels were only increased in HL60 cells, even though lipid ROS, LD, and TG levels were increased in both models. The reason behind this remains unclear, but our observations suggest that the timing of measuring ROS and lipid ROS can be critically important, whereby at later timepoints all ROS species may have already been incorporated into lipids. We speculate that this might have been the case in our THP1 measurements, but further studies are clearly required.

While part of the phenotypes of the DHODHi/dipyridamole combination could be phenocopied by combining DHODHi with inhibitors against cholesterol metabolism or the pyrimidine salvage pathway, ZIP scores were never as high as those seen with the DHODHi/dipyridamole combination. Furthermore, slight differences between AML models were observed, whereby THP1 was more sensitive to the combination with ENT inhibitors while HL60 cells were more

sensitive to combined inhibition of cholesterol metabolism. These observations highlight the heterogeneity across the AML landscape, but also further underline the potential of SREBP inhibition together with DHODHi, as several important pathways can be affected.

The impact of DHODHi and its combination with dipyridamole on myeloid differentiation was also noted in a subset of AML cases, in line with data by Branstrom *et al.*[37] where 2 out of 5 tested primary AML patient samples with DHODH inhibitor Emvodostat showed differentiation. There was no clear correlation between the impact of DHODHi on proliferation and differentiation. While differentiation was affected in some cases, this was not consistently observed among all strong responders in terms of proliferation and apoptosis. The number of samples in which differentiation was induced was relatively small in our cohort, which did not allow further functional studies of the mechanisms that might underly these observations.

A limitation of the current study is the lack of *in vivo* models and future studies will be required to evaluate efficacy of the DHODHi/dipyridamole combination *in vivo* in patient-derived xenograft models. Clinical trials with DHODH inhibitors (e.g., JBZ-001 [*NCT06801002*]; BAY2402234 [*NCT03404726*]) showed acceptable tolerability, although some of these trials were stopped prematurely due to insufficient efficacy, pinpointing the need for combination treatments. Additionally, dipyridamole is widely used in the clinic as a well-tolerated antiplatelet drug. It is typically used at 75-100 mg four times daily, reaching plasma concentrations of 0.5-1.9 ug/mL, which is in the low nM range, as also used in our experiments. Whether there is also a therapeutic window in primary patients remains to be determined in follow-up studies, but our data indicate that it would be interesting to further explore combined DHODH and SREBP as a therapeutic target option in AML.

Disclosures

AK, CP and UP are current employees of Janssen Research & Development and may own stock/stock options in Johnson & Johnson. All of the other authors have no conflicts of interest to disclose.

Contributions

SMH and JJS conceived and designed the study; SMH, DS, NLR, MG, DPM, FAJvdH and JJS performed experiments, analyzed and interpreted data, performed statistical analysis, and drafted the article; AK, CP and UP provided compounds; AK, CP, UP and GH interpreted data; GH provided patient samples and clinical data; SMH and JJS wrote the paper which was reviewed by all authors. All authors gave final approval of the submitted manuscript.

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Data-sharing statement

All data are available in the manuscript or upon request.

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