Acid ceramidase promotes drug resistance in acute myeloid leukemia through NF-κB-dependent P-glycoprotein upregulation.

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Abstract

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. More than half of older AML patients fail to respond to cytotoxic chemotherapy, and most responders relapse with drug resistant disease. Failure to achieve complete remission can be partly attributed to the drug resistance advantage of AML blasts that frequently express P-glycoprotein (P-gp), an ATP-binding cassette transporter. Our previous work showed that elevated acid ceramidase (AC) levels in AML contribute to blast survival. Here, we investigated P-gp expression levels in AML relative to AC. Using parental HL-60 cells and drug-resistant derivatives as our model, we found that P-gp expression and efflux activity were highly upregulated in resistant derivatives. AC overexpression in HL-60 conferred resistance to AML chemotherapeutic drugs cytarabine, mitoxantrone and daunorubicin and was linked to P-gp upregulation. Furthermore, targeting AC through pharmacologic or genetic approaches decreased P-gp levels and increased sensitivity to chemotherapeutic drugs. Mechanistically, AC overexpression increased NF-κB activation whereas NF-kB inhibitors reduced P-gp levels, indicating that the NF-κB pathway contributes to AC-mediated modulation of P-gp expression. Hence, our data supports an important role for AC in drug resistance as well as survival and suggests that sphingolipid targeting approaches may also impact drug resistance in AML.

Keywords: MDR1, ABCB1, cancer, sphingolipid, drug therapy, sphingosine-1-phosphate, ceramide

Introduction

Drug resistance is a major barrier to cancer chemotherapeutics (1, 2). Acute myeloid leukemia (AML), the most common acute form of adult leukemia, is no exception (3). AML is a group of disorders characterized by uncontrolled proliferation of immature myeloid cells. Less than 50% of patients will respond to current first-line cytotoxic chemotherapy, and a high percentage of the initial responders will relapse (4). Poor response to chemotherapy and relapse of the disease are associated with multidrug resistance (MDR) in AML patients (3, 5). Hence, a focus on the gene products and mechanisms involved in MDR should be paramount in AML therapeutics.

P-glycoprotein (P-gp), a member of the ATP-binding cassette transporters, is a 170 kDa transmembrane protein that exports a broad range of hydrophobic substrates (6-8). P-gp is also known as multidrug resistance protein 1 (MDR1) and the gene encoding P-gp is *ABCB1*. P-gp mediates efflux of a diverse range of drugs due to the conformational flexibility of its structure (9). P-gp expression strongly correlates with a drug resistant phenotype in several different types of cancer (3, 10). P-gp was reported to be abundantly expressed in AML blasts from patients with poor prognoses (5, 11). Furthermore, high P-gp expression correlated with poor response to chemotherapy (12-14). While several clinical trials for direct P-gp inhibitors were conducted, the outcomes were not promising due to toxicity of the inhibitors or inadequate dosing to effectively combat drug resistance (3, 15). Hence, there is an unmet need to identify novel strategies to combat drug resistance, and sphingolipid-targeting agents that impair AML blast survival as well as drug resistance may represent an alternative and efficacious approach to address this need.

Sphingolipids are a diverse group of lipids that are important for many cellular processes (16). Known functions of sphingolipids include key roles in structural integrity of cells, signaling pathways, cell cycle progression, migration and survival (17-19). Ceramide, a pro-death sphingolipid, is metabolized by ceramidases to sphingosine and free fatty acid. Sphingosine is then phosphorylated by sphingosine kinases to form sphingosine 1-phosphate (S1P), a bioactive molecule involved in survival mechanisms (17). We

and others have shown that the dysregulation of the sphingolipid pathway, including the elevated expression of acid ceramidase (AC) and sphingosine kinase 1 in AML patient samples, promotes AML blast survival (20-23). Targeting AC has been a subject of interest in several different types of cancer, including AML (24-29). Recent studies have also shown that the sphingolipid enzyme glucosylceramide synthase (GCS) may upregulate P-gp transcription and that targeting GCS sensitizes cells to chemotherapeutic drugs (30, 31). Hence, sphingolipid enzymes may influence drug resistance in addition to cell survival.

The present study explored the hypothesis that elevated AC induces P-gp expression to increase drug resistance in AML. We demonstrated that elevated AC is associated with increased P-gp expression and confers drug resistance to AML chemotherapeutic agents. Genetic or pharmacologic manipulation of AC reduced P-gp levels and sensitized cells to chemotherapeutic drugs. We also showed that AC overexpression increased NF-κB activation while exogenous S1P increased MDR1 transcript levels. Furthermore, sphingosine kinase or NF-κB inhibitors suppressed P-gp levels, which suggests the involvement of this pathway in AC-mediated P-gp expression. Taken together, these studies show a novel role of AC in regulating drug resistance in AML.

Material and Methods

Patient samples, cell lines and reagents

AML patient samples were obtained with informed consents signed for sample collection according to a protocol approved by the Institutional Review Board of the Milton S. Hershey Medical Center. All patient samples were collected from untreated individuals at diagnosis (n=18), relapsed (n=5), refractory (n=2) or unknown (n=2) disease stages. Normal cord blood and PBMCs were obtained from the Blood Bank (Milton S. Hershey Medical Center). All primary samples were enriched for PBMCs using the Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) gradient separation method. The HL-60 cell line was purchased from American Type Culture Collection (ATCC). Vincristine-resistant HL-60/VCR cells were generously provided by A.R. Safa and generated as previously described (32). Drug resistant HL-60/ABTR,

KG1/ABTR and KG1a/ABTR cell lines were generated as previously described (33). HL-60 parental and resistant cell lines were maintained in RPMI-1640 supplemented with 10% FBS (Atlanta Biologicals). In addition, HL-60/VCR were maintained in media containing 1 μg/ml vincristine (Selleck Chemicals) and HL-60/ABTR were maintained in media with 5 μM ABT-737 (Selleck Chemicals). KG1/ABTR and KG1a/ABTR were grown in IMDM supplemented with 20% FBS and 1 μM ABT-737. HEK293 T/17 cells were purchased from ATCC and maintained in DMEM supplemented with 10% FBS. All cell lines were grown in a 37°C humidified, 5% CO_2 atmosphere incubator. LCL204 [(1R,2R) 2-(N-tetradecylamino)-1-(4-NO2)-phenyl- 1,3-dihydroxy-propane HCl] was synthesized as previously described (34, 35). Bay-11-7082, JSH-23, zosuquidar, SKI-II, cytarabine, daunorubicin and mitoxantrone were purchased from Selleck Chemicals. SKI-178 was purchased from Cayman Chemicals.

Flow cytometry

To determine P-gp expression, 500,000 cells were washed twice with PBS then incubated with MDR1 antibody (UIC2; Sigma Aldrich) for 30 minutes, followed by FITC-conjugated anti-mouse antibody (Ancell Corporation) for 30 minutes. FITC-tagged normal mouse IgG was used as control. Cells were washed and resuspended with PBS, followed by analysis via flow cytometry. For every sample analyzed, 10,000 events were collected. The FITC-positive population, which is indicative of MDR1 expressing cells, was calculated and reported as a percentage.

P-gp efflux assays utilized the MDR1 efflux kit (EMD Millipore, Billerica, MA) according to the manufacturer's protocol. Briefly, cells were incubated with P-gp substrate DIOC2(3) (3, 3-Diethyloxacarbocyanine iodide) for 15 minutes at 4°C, then transferred to 37°C for 45 minutes to allow efflux to occur. Cells were washed, resuspended in cold efflux buffer, plated in 96-well plates and fluorescence intensity was measured (BioTek Cytation 3 plate reader; excitation: 485 nm, emission: 530 nm). A portion of cells kept at 4°C was used as a negative control. Percentage DIOC2(3) efflux was calculated by subtracting the fluorescence intensities of the 37°C sample from the 4°C sample and dividing the difference by the fluorescence intensity of the 4°C sample.

Overexpression and knockdown of AC via lentiviral transduction

AC-expressing plasmid pLOC-ASAH1 was purchased from Open Biosystems (Thermo Scientific) and pLKO.1-ASAH1 containing AC shRNA and pLKO.1-GFP control were purchased from Mission (Sigma-Aldrich). Plasmids were transfected into HEK293 T/17 cells with lentiviral packaging plasmids (Invitrogen). Viral supernatant was collected after 48 and 72 hours and filtered using a 0.45 μm-filter syringe. Cells were transduced with viral supernatant plus 6 μg/ml polybrene every 12 hours for three days. Transduced cells with pLOC-ASAH1 were selected with 6 μg/ml Blasticidin S for 12 days. Lysates were harvested 72 hours post-transduction for AC knockdown and 72 hours after stable selection for AC overexpression.

***In vitro* viability assay**

All viability assays were measured using CellTiter 96 Aqueous One Solution assay kit (Promega) according to the manufacturer's protocol. The absorbance of the formazan product at 490 nM was determined using a BioTek Cytation 3 plate reader. For cell lines co-treated with LCL204, zosuquidar, daunorubicin and/or mitoxantrone, cells were plated at 25,000 cells/well and treated for 24 hours. Absorbance from treated wells was compared to absorbance from vehicle controls to calculate percentage viable cells.

Western blot analysis

Cells were cultured at 1 million cells/ml for the drug incubation experiments. Following drug treatment, cells were lysed using RIPA buffer containing phosphatase inhibitor cocktail and protease inhibitor P8340 according to the manufacturer's protocol (Sigma), resolved on 4-12% SDS-PAGE gels (Bolt gels, Invitrogen) and transferred onto PVDF membranes (Bio-Rad). Primary antibodies used in this study are as follows: AC (BD Biosciences), MDR1/ABCB1 (#13342, Cell Signaling), NF-κB p65 (#4764; Cell Signaling) and beta-actin (#3700; Cell Signaling). For secondary antibodies, HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (Cell Signaling) antibodies were used and Clarity™ Western ECL blotting substrate (Bio-Rad) was applied to blots according to the manufacturer's protocol. Image Processing and

Analysis in Java (Image J) software or Image Lab (Bio-Rad) were used to measure band density. Band density was normalized to β-actin, and the first lane in each blot was utilized as the reference that was set to 1.

Lipid extraction and analysis by electrospray ionization-tandem mass spectrometry

Lipids were extracted from cell pellets (equivalent to 600 μg to 1mg of protein depending on the experiment) using an azeotrophic mix of isopropanol:water:ethyl acetate (3:1:6; v:v:v). Sphingolipids were analyzed by LC/ESI-MS/MS based on the method previously described (22, 36). All data reported are based on monoisotopic mass and are represented as pmol/mg protein.

Real-time quantitative RT-PCR

For S1P-treated cells, cells were serum-starved and plated in RPMI media at 1 million cells per ml. S1P (100 nM) or methanol vehicle was added to the cells and incubated at 37°C for 24h.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using primers specific for human MDR1 with GAPDH as internal standard in an ABI PRISM 7900 sequence detector. Total RNA was harvested from cells using TRIzoL Reagent (Invitrogen) following the manufacturer's protocols. cDNA was synthesized using random hexamers and MMLV reverse transcription reagent (Invitrogen). Amplification of the cDNA was performed in triplicate using Quantitect SYBR Green PCR kit (Qiagen) following the manufacturer's instructions. Primer sequences are as follows: GAPDH sense 5'-GAAGGTGAAGGTCGGAGTC -3', antisense 5'-GAAGATGGTGATGGGATTTC-3' and MDR1 sense 5'-GGTTTATAGTAGGATTTACACGTGGTTG-3', antisense 5'-AAGATAGTATCTTTGCCCAGACAGC-3'. All primers were supplied by IDTDNA (Coralville, IA).

NF-κB activation assay

Cells were plated at 1 million cells per ml and serum-starved overnight. Then, nuclear extract was isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce; Thermo Scientific). Nuclear

extracts were analyzed for NF-κB activation using NF-κB p50/p65 EZ-TFA Transcription Factor Assay Kit (Millipore) following the manufacturer's protocol.

Statistical analyses and experimental design

Statistical comparisons between two treatment groups utilized Student's *t*-test. Mann-Whitney U-test was used for the P-gp expression screening analysis. For *in vitro* assays and western blot data using cell lines, results were from three independent experiments with three to four replicates, unless otherwise noted in the figure legends. Each graph represents an average of three to four replicates from 3 independent experiments and error bars represent standard error of mean (SEM), unless otherwise noted in the figure legends. Due to the limitation of primary samples, data from primary patient and normal donor samples were from one independent experiment.

Results

P-gp is highly expressed in AML patient samples and drug-resistant cell lines.

To verify the levels of P-gp in AML, we screened 27 human AML patient samples, six normal PBMC and three cord blood samples for P-gp expression using flow cytometry detection of MDR1 (UIC2) cell-surface epitope (**Figure 1A**). Our data showed that P-gp expression did not differ significantly between normal PBMC and cord blood samples. We found that P-gp expression was significantly higher in AML patient samples compared to the nine normal PBMC and cord blood control samples ($p<0.05$, Mann-Whitney test). Collectively, the data validate earlier findings that AML patient samples exhibit elevated P-gp expression (5, 14). For further mechanistic studies of drug resistance in AML, we used two HL-60-derived cell lines with acquired resistance to vincristine (HL-60/VCR) and Bcl-2 family inhibitor ABT-737 (HL-60/ABTR) (32, 33). HL-60/VCR cells are known to exhibit increased MDR1 transcription relative to HL-60 (37). Compared to parental HL-60, both of the cell lines exhibit a drug-resistance phenotype with elevated P-gp expression (**Figure 1B**) and efflux activity as indicated by a P-gp-specific substrate (**Figure 1C**). The drug resistant cell lines also exhibited increased levels of AC (**Figure 1B**), which we previously demonstrated

is elevated in AML patient samples and contributes to AML pathogenesis (22). Therefore, these cell lines were utilized as models to investigated the role of AC in AML drug resistance.

AC inhibitor LCL204 reversed drug resistance and induced AC and P-gp loss in HL-60/VCR.

One of the main issues plaguing AML patients is the lack of response to standard of care chemotherapeutics such as cytarabine (ara-C), daunorubicin and mitoxantrone (38, 39). To determine whether AC contributes to drug resistance in AML, we treated HL-60/VCR cells for 24 hours with each chemotherapeutic agent without or with AC inhibitor LCL204 (7.5 μM), which is known to both inhibit AC and promote its degradation via lysosomal proteases (22, 35). AC inhibition sensitized HL-60/VCR cells to all three chemotherapeutic agents and significantly reversed drug resistance for cytarabine ($p<0.0005$, **Figure 2A**), daunorubicin ($p<0.005$, **Figure 2B**) and mitoxantrone ($p<0.0005$, **Figure 2C**). Consistent with loss of AC enzymatic activity, 24-hour treatment with this dose of LCL204 increased ceramide (2.2-fold) and decreased sphingosine and S1P (2.7-fold and 1.8-fold, respectively) in HL-60/VCR cells (**Figure 2D**). Furthermore, HL-60/VCR cells treated with increasing doses of LCL204 exhibited lower levels of both AC and P-gp (**Figure 2E**). Taken together, these data suggest a functional relationship between AC and P-gp whereby loss of AC activity is associated with reduced P-gp levels.

AC knockdown decreased P-gp protein expression.

Because AC inhibitor treatment decreased P-gp levels in HL-60/VCR cells, we next utilized a genetic approach to knock down AC to determine whether the loss of P-gp expression was related to decreased AC activity or a function of LCL204 acting directly on P-gp. Four different drug resistant cell lines were utilized (HL-60/VCR, HL-60/ABTR, KG1/ABTR and KG1a/ABTR) that all exhibit robust P-gp expression. The cells were transduced with lentiviral particles containing AC shRNA every 12 hours for three days. Lysates were harvested 72 hours post-transduction and probed for AC and P-gp protein expression levels. AC knockdown corresponded with decreasing P-gp expression levels in all four cell lines (**Figure 3A**). The AC and P-gp protein bands were quantified and normalized to β-actin to show the decrease in expression levels

compared to GFP control vector (**Figure 3B**). AC knockdown ranged from 71% to 92% and corresponded to a 45% to 87% decrease in P-gp expression. Hence, loss of AC protein leads to decreased P-gp expression.

AC overexpression increased P-gp levels and induced drug resistance to chemotherapeutic agents.

Previous reports showed that the sphingolipid metabolizing enzyme glucosylceramide synthase (GCS) upregulates P-gp/MDR1 expression (30, 40). However, there are no reports linking AC to P-gp. Given that AC inhibition or knockdown suppressed P-gp expression (**Figures 2 and 3**) and AC inhibition increased ceramide and decreased sphingosine and S1P in HL-60/VCR cells (22), we next evaluated P-gp levels upon AC overexpression. HL-60 cells were transduced with a lentiviral AC expression vector (referred hereon as HL-60/AC) and stably selected for 12 days (22). Consistent with elevated AC activity, we previously demonstrated that HL-60/AC cells exhibit decreased ceramide (2.1-fold) and increased sphingosine and S1P (2-fold and 3.5-fold, respectively) relative to parental HL-60 (22). These AC overexpressing cells exhibited elevated P-gp protein (**Figure 4A**) and higher P-gp-mediated efflux activity (**Figure 4B**) compared to parental HL-60. Furthermore, treatment of HL-60/AC cells with LCL204 decreased AC and P-gp protein levels (**Figure 4C**), as previously observed in HL60/VCR cells (**Figure 2E**). LCL204 treatment increased ceramide (1.3-fold) while decreasing sphingosine and S1P content (1.9-fold and 1.7-fold, respectively) in HL-60/AC cells (**Figure 4D**).

To confirm that the protein expression data translated to functional drug resistance, HL-60 and HL-60/AC cells were treated with daunorubicin (25 μM) or mitoxantrone (5 μM) for 24 hours. HL-60/AC cells (**Figure 4E;** left three bars) were significantly more resistant to both chemotherapeutic agents ($p<0.0005$ for daunorubicin and $p<0.005$ for mitoxantrone, Student's *t* test) compared to parental HL-60 (**Figure 4F**). Co-treatment that added AC inhibitor LCL204 (7.5 μM) to the chemotherapeutic drugs reversed the observed daunorubicin ($p<0.005$, Student's *t* test) and mitoxantrone resistance in HL-60/AC cells, but did not affect the response in drug-sensitive HL-60 cells (**Figures 4E-F**, middle three bars). Finally, we treated HL-60/AC and HL-60 cells with zosuquidar (1 μM) and chemotherapeutic agents to determine whether

chemoresistance in HL-60/AC cells requires P-gp activity. P-gp inhibition significantly sensitized cells to both chemotherapeutic agents in HL-60/AC cells (p<0.0005, Student's t test) but not in drug-sensitive HL-60 cells (**Figures 4E-F**, right three bars). These studies demonstrate that the drug resistance phenotype of HL-60/AC cells is dependent on P-gp. Thus, AC overexpression in HL-60 cells increased resistance to chemotherapeutic agents by increasing P-gp expression and AC inhibition reversed drug resistance by reducing P-gp expression.

AC overexpression increased NF-κB activation and NF-κB inhibition reduced P-gp levels.

We previously showed that AC overexpression increased S1P levels, and S1P has been shown to activate the NF-κB pathway (22, 41, 42), which is known to induce P-gp/MDR1 transcription (43). Hence, we investigated whether the NF-κB pathway represents a mechanistic link between AC and P-gp expression. Nuclear lysates were prepared from HL-60, HL-60/AC and HL-60/VCR cells, which showed that HL-60/AC (p<0.05, Student's t-test) and HL-60/VCR (p<0.005, Student's t-test) cells had significantly higher nuclear levels of NF-κB subunit p65 compared to HL-60 (**Figure 5A**). Both HL-60/AC and HL-60/VCR cells also showed higher MDR1 transcript levels compared to parental HL-60 (Figure 5B). HL-60/AC cells are known to exhibit 3.5-fold higher levels of S1P relative to parental HL-60 (22). Treatment of parental HL-60 cells with exogenous S1P also increased P-gp (MDR1) mRNA levels (**Figure 5C**). To confirm that AC modulates P-gp expression through S1P, HL-60/AC and HL-60/VCR cells were treated with sphingosine kinase inhibitors SKI-II (Figures 5D-E) and SKI-178 (Figures 5F-G). Both inhibitors decreased NF-κB p65 and P-gp levels dose-dependently in HL-60/AC and HL-60/VCR cells. We next investigated whether NF-κB promotes P-gp expression in drug resistant AML cell lines by treating HL-60/AC and HL-60/VCR cells with NF-κB inhibitors (Bay-11-7082 and JSH-23). Treatment with both inhibitors decreased the levels of NF-κB p65 and P-gp dose-dependently in HL-60/AC (**Figure 5H-I**) and HL-60/VCR cells (**Figure 5J-K**). Together with published literature, these data indicate that AC overexpression and the corresponding increase in S1P leads to increased NF-κB pathway activation and transcriptional activity that stimulates P-gp expression in drug resistant AML cells (22, 41-43).

Discussion

Our data suggest that targeting AC leads to P-gp downregulation and reverses drug resistance. This is a novel finding that potentially enhances the functional significance of AC to not only include AML blast survival but also drug resistance (22). AML patients are frequently unresponsive to induction chemotherapy and may exhibit high levels of P-gp, which we confirmed in our patient cohort (5, 12, 44, 45). This results in poor survival rates and adds to the complexity of treating AML patients, especially those with relapsed disease. Novel treatments including hypomethylating agents and targeted therapies (ex. venetoclax, midostaurin and ivosidenib) are currently gaining momentum as AML therapeutics, but these compounds are frequently given in combination with chemotherapy and the issue of multidrug resistance still remains a substantial barrier (46-49). We previously demonstrated that AC is highly expressed in AML patient samples; therefore, targeting AC may represent a novel approach to reverse drug resistance in AML. More studies and larger patient cohorts are needed to more accurately define the AC/P-gp correlation and the role of AC in the many AML subtypes that differ in terms of molecular and cytogenetic alterations (50-52).

We showed that AC overexpression confers drug resistance to AML cells and that AC inhibition sensitizes cells to chemotherapeutic agents. Furthermore, AC inhibition and knockdown both decreased P-gp expression. Therefore, targeting AC may represent a substantially improved method to decrease AML drug resistance as an alternative to pharmacological agents that only inhibit P-gp. These findings are very relevant to the ongoing drug resistance dilemma, as many P-gp inhibitors have not shown significant benefit in clinical trials of AML patients (3, 15, 53). AC inhibition canonically increases pro-apoptotic ceramide and depletes pro-survival S1P. Therefore, combination of AC inhibitors with standard induction chemotherapy may create a multipronged approach to eliminate more AML blasts, increase the odds of achieving complete remission, and provide a new and more effective approach for AML chemotherapeutics.

Mechanistically, our data indicate that the NF-κB transcription factor contributes to AC-mediated regulation of P-gp expression. Previously, we showed that AC overexpression increases S1P by 3.5-fold

(22). As shown by Alvarez *et al*., S1P induces NF-κB by binding to TRAF2 and activating the IκB kinase complex, which releases the NF-κB dimer from IκB thereby freeing it to enter the nucleus and activate transcriptional targets (41). The P-gp/MDR1/ABCB1 promoter region contains a consensus binding sequence for NF-κB (43, 54). Others have also reported that AC deletion downregulated TRAF2 (55). Here, we showed that exogenous S1P increases P-gp/MDR1/ABCB1 transcript levels while sphingosine kinase inhibitors reduce P-gp expression. We also demonstrated that NF-κB is upstream of P-gp in AML cell lines by showing that AC expression increased NF-κB activation and that NF-κB inhibitors reduced P-gp expression.

We previously showed that AC inhibition increased levels of pro-apoptotic ceramide (22). P-gp functions as a flippase for glucosylceramide and ceramide, including short-chain exogenous ceramide species that are cytotoxic towards cancer cells (7, 22, 56-59). Additional study is needed to fully characterize the substrate specificity, universality and physiological relevance of P-gp flippase activity for endogenous ceramide species (60, 61), Nonetheless, AC inhibition may increase ceramide levels through two complimentary mechanisms: blocking the conversion of endogenous ceramide to sphingosine and preventing P-gp-mediated ceramide and glucosylceramide transport into the golgi for further glycosylation and detoxification. The coupling of these two pro-ceramide mechanisms with simultaneous S1P depletion makes AC inhibition a particularly appealing therapeutic strategy. Thus far the contributions of additional sphingolipid metabolizing enzymes, such as ceramide synthases or S1P lyase, to AML pathogenesis or the observed effects of AC inhibitors remain undefined. Further studies will test whether treatment with AC inhibitors in conjunction with exogenous short-chain ceramides may further enhance endogenous ceramide accumulation to synergistically reduce AML viability while simultaneously increasing chemotherapy sensitivity.

Summary

Collectively, our data further highlight AC as an exciting and novel target to reduce cell survival and combat

drug resistance in AML. Aberrant AC expression increased P-gp and conferred drug resistance in our model. Furthermore, combinatorial approaches to inhibit AC along with standard AML chemotherapeutic agents reduced survival in AML cell lines. Hence, further development of AC inhibitors for clinical use will enhance potential synergistic treatments to improve clinical outcomes in AML.

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CONFLICTS

Thomas P. Loughran, Jr. is on the Scientific Advisory Board and has stock options for both Keystone Nano and Bioniz Therapeutics. Mark Kester is the Chief Medical Officer and cofounder of Keystone Nano, Inc. There are no conflicts of interest with the work presented in this manuscript.

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REFERENCES

1. Kuczynski, E. A., D. J. Sargent, A. Grothey, and R. S. Kerbel. 2013. Drug rechallenge and treatment beyond progression-implications for drug resistance. *Nature reviews. Clinical oncology*.

2. Gottesman, M. M., O. Lavi, M. D. Hall, and J. P. Gillet. 2016. Toward a Better Understanding of the Complexity of Cancer Drug Resistance. *Annu Rev Pharmacol Toxicol* **56**: 85-102.

3. Shaffer, B. C., J. P. Gillet, C. Patel, M. R. Baer, S. E. Bates, and M. M. Gottesman. 2012. Drug resistance: still a daunting challenge to the successful treatment of AML. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **15**: 62-69.

4. Burnett, A., M. Wetzler, and B. Lowenberg. 2011. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol* **29**: 487-494.

5. Leith, C. P., K. J. Kopecky, J. Godwin, T. McConnell, M. L. Slovak, I. M. Chen, D. R. Head, F. R. Appelbaum, and C. L. Willman. 1997. Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. *Blood* **89**: 3323-3329.

6. Smyth, M. J., E. Krasovskis, V. R. Sutton, and R. W. Johnstone. 1998. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 7024-7029.

7. Lavie, Y., H. Cao, A. Volner, A. Lucci, T. Y. Han, V. Geffen, A. E. Giuliano, and M. C. Cabot. 1997. Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *The Journal of biological chemistry* **272**: 1682-1687.

8. Taylor, J. C., A. R. Horvath, C. F. Higgins, and G. S. Begley. 2001. The multidrug resistance P-glycoprotein. Oligomeric state and intramolecular interactions. *The Journal of biological chemistry* **276**: 36075-36078.

9. Hodges, L. M., S. M. Markova, L. W. Chinn, J. M. Gow, D. L. Kroetz, T. E. Klein, and R. B. Altman. 2011. Very important pharmacogene summary: ABCB1 (MDR1, P-glycoprotein). *Pharmacogenet Genomics* **21**: 152-161.

10. Goldstein, L. J., H. Galski, A. Fojo, M. Willingham, S. L. Lai, A. Gazdar, R. Pirker, A. Green, W. Crist, G. M. Brodeur, and et al. 1989. Expression of a multidrug resistance gene in human cancers. *Journal of the National Cancer Institute* **81**: 116-124.

11. Lin, S. F., S. M. Huang, T. P. Chen, C. S. Chang, T. C. Liu, S. J. Wang, and H. W. Liu. 1995. MDR1 gene expression in acute myeloid leukemia: clinical correlation. *J Formos Med Assoc* **94**: 111-116.

12. van den Heuvel-Eibrink, M. M., B. van der Holt, A. K. Burnett, W. U. Knauf, M. F. Fey, G. E. Verhoef, E. Vellenga, G. J. Ossenkoppele, B. Lowenberg, and P. Sonneveld. 2007. CD34-related coexpression of MDR1 and BCRP indicates a clinically resistant phenotype in patients with acute myeloid leukemia (AML) of older age. *Annals of hematology* **86**: 329-337.

13. Samdani, A., U. Vijapurkar, M. A. Grimm, C. S. Spier, T. M. Grogan, B. J. Glinsmann-Gibson, and A. F. List. 1996. Cytogenetics and P-glycoprotein (PGP) are independent predictors of treatment outcome in acute myeloid leukemia (AML). *Leukemia research* **20**: 175-180.

14. Pirker, R., J. Wallner, K. Geissler, W. Linkesch, O. A. Haas, P. Bettelheim, M. Hopfner, R. Scherrer, P. Valent, L. Havelec, and et al. 1991. MDR1 gene expression and treatment outcome in acute myeloid leukemia. *J Natl Cancer Inst* **83**: 708-712.

15. Mahadevan, D., and A. F. List. 2004. Targeting the multidrug resistance-1 transporter in AML: molecular regulation and therapeutic strategies. *Blood* **104**: 1940-1951.

16. Ogretmen, B. 2018. Sphingolipid metabolism in cancer signalling and therapy. *Nat Rev Cancer* **18**: 33-50.

17. Taha, T. A., T. D. Mullen, and L. M. Obeid. 2006. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochimica et biophysica acta* **1758**: 2027-2036.

18. Oskouian, B., and J. D. Saba. 2010. Cancer treatment strategies targeting sphingolipid metabolism. *Advances in experimental medicine and biology* **688**: 185-205.

19. Hannun, Y. A., and L. M. Obeid. 2018. Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol* **19**: 175-191.

20. Hengst, J. A., T. E. Dick, A. Sharma, K. Doi, S. Hegde, S. F. Tan, L. M. Geffert, T. E. Fox, A. K. Sharma, D. Desai, S. Amin, M. Kester, T. P. Loughran, R. F. Paulson, D. F. Claxton, H. G. Wang, and J. K. Yun. 2017. SKI-178: A Multitargeted Inhibitor of Sphingosine Kinase and Microtubule Dynamics Demonstrating Therapeutic Efficacy in Acute Myeloid Leukemia Models. *Cancer Transl Med* **3**: 109-121.

21. Powell, J. A., A. C. Lewis, W. Zhu, J. Toubia, M. R. Pitman, C. T. Wallington-Beddoe, P. A. Moretti, D. Iarossi, S. E. Samaraweera, N. Cummings, H. S. Ramshaw, D. Thomas, A. H. Wei, A. F. Lopez, R. J. D'Andrea, I. D. Lewis, and S. M. Pitson. 2017. Targeting sphingosine kinase 1 induces MCL1-dependent cell death in acute myeloid leukemia. *Blood* **129**: 771-782.

22. Tan, S. F., X. Liu, T. E. Fox, B. M. Barth, A. Sharma, S. D. Turner, A. Awwad, A. Dewey, K. Doi, B. Spitzer, M. V. Shah, S. A. Morad, D. Desai, S. Amin, J. Zhu, J. Liao, J. Yun, M. Kester, D. F. Claxton, H. G. Wang, M. C. Cabot, E. H. Schuchman, R. L. Levine, D. J. Feith, and T. P. Loughran, Jr. 2016. Acid ceramidase is upregulated in AML and represents a novel therapeutic target. *Oncotarget* **7**: 83208-83222.

23. Lewis, A. C., C. T. Wallington-Beddoe, J. A. Powell, and S. M. Pitson. 2018. Targeting sphingolipid metabolism as an approach for combination therapies in haematological malignancies. *Cell Death Discov* **4**: 4.

24. Liu, X., S. Elojeimy, L. S. Turner, A. E. Mahdy, Y. H. Zeidan, A. Bielawska, J. Bielawski, J. Y. Dong, A. M. El-Zawahry, G. W. Guo, Y. A. Hannun, D. H. Holman, S. Rubinchik, Z. Szulc, T. E. Keane, M. Tavassoli, and J. S. Norris. 2008. Acid ceramidase inhibition: a novel target for cancer therapy. *Front Biosci* **13**: 2293-2298.

25. Realini, N., F. Palese, D. Pizzirani, S. Pontis, A. Basit, A. Bach, A. Ganesan, and D. Piomelli. 2016. Acid Ceramidase in Melanoma: EXPRESSION, LOCALIZATION, AND EFFECTS OF PHARMACOLOGICAL INHIBITION. *The Journal of biological chemistry* **291**: 2422-2434.

26. Liu, X., J. C. Cheng, L. S. Turner, S. Elojeimy, T. H. Beckham, A. Bielawska, T. E. Keane, Y. A. Hannun, and J. S. Norris. 2009. Acid ceramidase upregulation in prostate cancer: role in tumor development and implications for therapy. *Expert Opin Ther Targets* **13**: 1449-1458.

27. Zeidan, Y. H., R. W. Jenkins, J. B. Korman, X. Liu, L. M. Obeid, J. S. Norris, and Y. A. Hannun. 2008. Molecular targeting of acid ceramidase: implications to cancer therapy. *Curr Drug Targets* **9**: 653-661.

28. Shah, M. V., R. Zhang, R. Irby, R. Kothapalli, X. Liu, T. Arrington, B. Frank, N. H. Lee, and T. P. Loughran, Jr. 2008. Molecular profiling of LGL leukemia reveals role of sphingolipid signaling in survival of cytotoxic lymphocytes. *Blood* **112**: 770-781.

29. Saad, A. F., W. D. Meacham, A. Bai, V. Anelli, S. Elojeimy, A. E. Mahdy, L. S. Turner, J. Cheng, A. Bielawska, J. Bielawski, T. E. Keane, L. M. Obeid, Y. A. Hannun, J. S. Norris, and X. Liu. 2007. The functional effects of acid ceramidase overexpression in prostate cancer progression and resistance to chemotherapy. *Cancer Biol Ther* **6**: 1455-1460.

30. Gouaze, V., Y. Y. Liu, C. S. Prickett, J. Y. Yu, A. E. Giuliano, and M. C. Cabot. 2005. Glucosylceramide synthase blockade down-regulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs. *Cancer research* **65**: 3861-3867.

31. Liu, Y. Y., V. Gupta, G. A. Patwardhan, K. Bhinge, Y. Zhao, J. Bao, H. Mehendale, M. C. Cabot, Y. T. Li, and S. M. Jazwinski. 2010. Glucosylceramide synthase upregulates MDR1 expression in the regulation of cancer drug resistance through cSrc and beta-catenin signaling. *Molecular cancer* **9**: 145.

32. McGrath, T., C. Latoud, S. T. Arnold, A. R. Safa, R. L. Felsted, and M. S. Center. 1989. Mechanisms of multidrug resistance in HL60 cells. Analysis of resistance associated membrane proteins and levels of mdr gene expression. *Biochem Pharmacol* **38**: 3611-3619.

33. Doi, K., Q. Liu, K. Gowda, B. M. Barth, D. Claxton, S. Amin, T. P. Loughran, Jr., and H. G. Wang. 2014. Maritoclax induces apoptosis in acute myeloid leukemia cells with elevated Mcl-1 expression. *Cancer Biol Ther* **15**: 1077-1086.

34. Dagan, A., C. Wang, E. Fibach, and S. Gatt. 2003. Synthetic, non-natural sphingolipid analogs inhibit the biosynthesis of cellular sphingolipids, elevate ceramide and induce apoptotic cell death. *Biochimica et biophysica acta* **1633**: 161-169.

35. Holman, D. H., L. S. Turner, A. El-Zawahry, S. Elojeimy, X. Liu, J. Bielawski, Z. M. Szulc, K. Norris, Y. H. Zeidan, Y. A. Hannun, A. Bielawska, and J. S. Norris. 2008. Lysosomotropic acid ceramidase inhibitor induces apoptosis in prostate cancer cells. *Cancer Chemother Pharmacol* **61**: 231-242.

36. Shaner, R. L., J. C. Allegood, H. Park, E. Wang, S. Kelly, C. A. Haynes, M. C. Sullards, and A. H. Merrill, Jr. 2009. Quantitative analysis of sphingolipids for lipidomics using triple quadrupole and quadrupole linear ion trap mass spectrometers. *J Lipid Res* **50**: 1692-1707.

37. Ogretmen, B., and A. R. Safa. 2000. Identification and characterization of the MDR1 promoter-enhancing factor 1 (MEF1) in the multidrug resistant HL60/VCR human acute myeloid leukemia cell line. *Biochemistry* **39**: 194-204.

38. Lowenberg, B., S. Suciu, E. Archimbaud, H. Haak, P. Stryckmans, R. de Cataldo, A. W. Dekker, Z. N. Berneman, A. Thyss, J. van der Lelie, P. Sonneveld, G. Visani, G. Fillet, M. Hayat, A. Hagemeijer, G. Solbu, and R. Zittoun. 1998. Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy--the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. *J Clin Oncol* **16**: 872-881.

39. Anderson, J. E., K. J. Kopecky, C. L. Willman, D. Head, M. R. O'Donnell, F. W. Luthardt, T. H. Norwood, I. M. Chen, S. P. Balcerzak, D. B. Johnson, and F. R. Appelbaum. 2002. Outcome after induction chemotherapy for older patients with acute myeloid leukemia is not improved with mitoxantrone and etoposide compared to cytarabine and daunorubicin: a Southwest Oncology Group study. *Blood* **100**: 3869-3876.

40. Gouaze, V., J. Y. Yu, R. J. Bleicher, T. Y. Han, Y. Y. Liu, H. Wang, M. M. Gottesman, A. Bitterman, A. E. Giuliano, and M. C. Cabot. 2004. Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy. *Molecular cancer therapeutics* **3**: 633-639.

41. Alvarez, S. E., K. B. Harikumar, N. C. Hait, J. Allegood, G. M. Strub, E. Y. Kim, M. Maceyka, H. Jiang, C. Luo, T. Kordula, S. Milstien, and S. Spiegel. 2010. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature* **465**: 1084-1088.

42. Shatrov, V. A., V. Lehmann, and S. Chouaib. 1997. Sphingosine-1-phosphate mobilizes intracellular calcium and activates transcription factor NF-kappa B in U937 cells. *Biochemical and biophysical research communications* **234**: 121-124.

43. Bentires-Alj, M., V. Barbu, M. Fillet, A. Chariot, B. Relic, N. Jacobs, J. Gielen, M. P. Merville, and V. Bours. 2003. NF-kappaB transcription factor induces drug resistance through MDR1 expression in cancer cells. *Oncogene* **22**: 90-97.

44. Estey, E., and H. Döhner. 2006. Acute myeloid leukaemia. *The Lancet* **368**: 1894-1907.

45. Kim, D. H., J. Y. Park, S. K. Sohn, N. Y. Lee, J. H. Baek, S. B. Jeon, J. G. Kim, J. S. Suh, Y. R. Do, and K. B. Lee. 2006. Multidrug resistance-1 gene polymorphisms associated with treatment outcomes in de novo acute myeloid leukemia. *Int J Cancer* **118**: 2195-2201.

46. Levis, M. 2017. Midostaurin approved for FLT3-mutated AML. *Blood* **129**: 3403-3406.

47. FDA. 2018. FDA approves venetoclax in combination for AML in adults. Retrieved from https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm626499.htm. *In*.

48. FDA. 2018. FDA approves ivosidenib for relapsed or refractory acute myeloid leukemia. Retrieved from https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm614128.htm. *In*.

49. Stein, E. M., and M. S. Tallman. 2016. Emerging therapeutic drugs for AML. *Blood* **127**: 71-78.

50. Dohner, H., E. Estey, D. Grimwade, S. Amadori, F. R. Appelbaum, T. Buchner, H. Dombret, B. L. Ebert, P. Fenaux, R. A. Larson, R. L. Levine, F. Lo-Coco, T. Naoe, D. Niederwieser, G. J. Ossenkoppele, M. Sanz, J. Sierra, M. S. Tallman, H. F. Tien, A. H. Wei, B. Lowenberg, and C. D. Bloomfield. 2017. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **129**: 424-447.

51. Grimwade, D., A. Ivey, and B. J. Huntly. 2016. Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance. *Blood* **127**: 29-41.

52. Tyner, J. W., C. E. Tognon, D. Bottomly, B. Wilmot, S. E. Kurtz, S. L. Savage, N. Long, A. R. Schultz, E. Traer, M. Abel, A. Agarwal, A. Blucher, U. Borate, J. Bryant, R. Burke, A. Carlos, R. Carpenter, J. Carroll, B. H. Chang, C. Coblentz, A. d'Almeida, R. Cook, A. Danilov, K. T. Dao, M. Degnin, D. Devine, J. Dibb, D. K. t. Edwards, C. A. Eide, I. English, J. Glover, R. Henson, H. Ho, A. Jemal, K. Johnson, R. Johnson, B. Junio, A. Kaempf, J. Leonard, C. Lin, S. Q. Liu, P. Lo, M. M. Loriaux, S. Luty, T. Macey, J. MacManiman, J. Martinez, M. Mori, D. Nelson, C. Nichols, J. Peters, J. Ramsdill, A. Rofelty, R. Schuff, R. Searles, E. Segerdell, R. L. Smith, S. E. Spurgeon, T. Sweeney, A. Thapa, C. Visser, J. Wagner, K. Watanabe-Smith, K. Werth, J. Wolf, L. White, A. Yates, H. Zhang, C. R. Cogle, R. H. Collins, D. C. Connolly, M. W. Deininger, L. Drusbosky, C. S. Hourigan, C. T. Jordan, P. Kropf, T. L. Lin, M. E. Martinez, B. C. Medeiros, R. R. Pallapati, D. A. Pollyea, R. T. Swords, J. M. Watts, S. J. Weir, D. L. Wiest, R. M. Winters, S. K. McWeeney, and B. J. Druker. 2018. Functional genomic landscape of acute myeloid leukaemia. *Nature* **562**: 526-531.

53. Cripe, L. D., H. Uno, E. M. Paietta, M. R. Litzow, R. P. Ketterling, J. M. Bennett, J. M. Rowe, H. M. Lazarus, S. Luger, and M. S. Tallman. 2010. Zosuquidar, a novel modulator of P-glycoprotein, does not improve the outcome of older patients with newly diagnosed acute myeloid leukemia: a randomized, placebo-controlled trial of the Eastern Cooperative Oncology Group 3999. *Blood* **116**: 4077-4085.

54. Kuo, M. T., Z. Liu, Y. Wei, Y. C. Lin-Lee, S. Tatebe, G. B. Mills, and H. Unate. 2002. Induction of human MDR1 gene expression by 2-acetylaminofluorene is mediated by effectors of the phosphoinositide 3-kinase pathway that activate NF-kappaB signaling. *Oncogene* **21**: 1945-1954.

55. Lai, M., N. Realini, M. La Ferla, I. Passalacqua, G. Matteoli, A. Ganesan, M. Pistello, C. M. Mazzanti, and D. Piomelli. 2017. Complete Acid Ceramidase ablation prevents cancer-initiating cell formation in melanoma cells. *Sci Rep* **7**: 7411.

56. Eckford, P. D., and F. J. Sharom. 2005. The reconstituted P-glycoprotein multidrug transporter is a flippase for glucosylceramide and other simple glycosphingolipids. *The Biochemical journal* **389**: 517-526.

57. Ryland, L. K., T. E. Fox, X. Liu, T. P. Loughran, and M. Kester. 2011. Dysregulation of sphingolipid metabolism in cancer. *Cancer Biol Ther* **11**: 138-149.

58. Chapman, J. V., V. Gouaze-Andersson, R. Karimi, M. C. Messner, and M. C. Cabot. 2011. P-glycoprotein antagonists confer synergistic sensitivity to short-chain ceramide in human multidrug-resistant cancer cells. *Exp Cell Res*.

59. Senchenkov, A., D. A. Litvak, and M. C. Cabot. 2001. Targeting ceramide metabolism--a strategy for overcoming drug resistance. *J Natl Cancer Inst* **93**: 347-357.

60. Devaux, P. F., A. Herrmann, N. Ohlwein, and M. M. Kozlov. 2008. How lipid flippases can modulate membrane structure. *Biochimica et biophysica acta* **1778**: 1591-1600.

61. Pomorski, T. G., and A. K. Menon. 2016. Lipid somersaults: Uncovering the mechanisms of protein-mediated lipid flipping. *Prog Lipid Res* **64**: 69-84.

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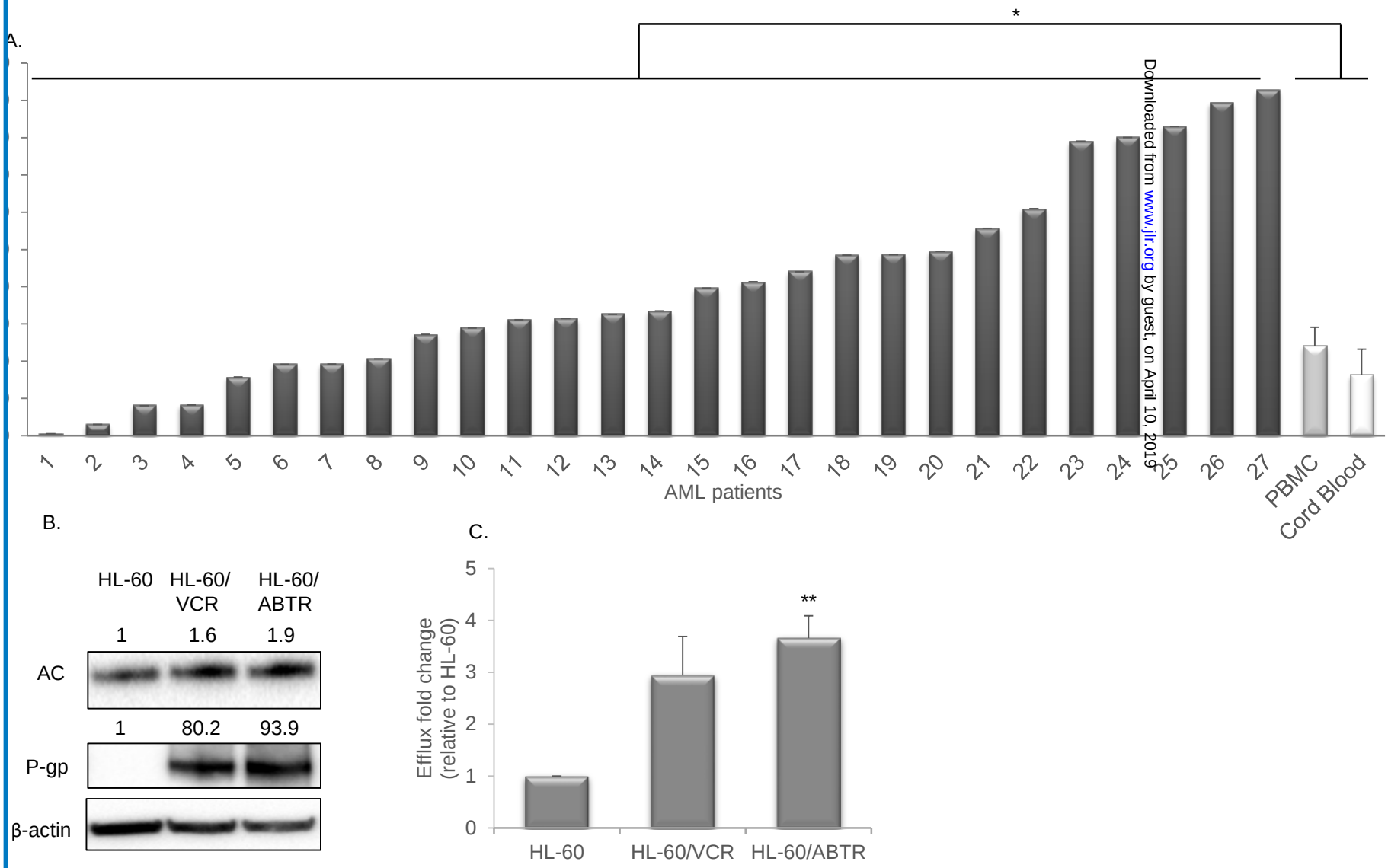
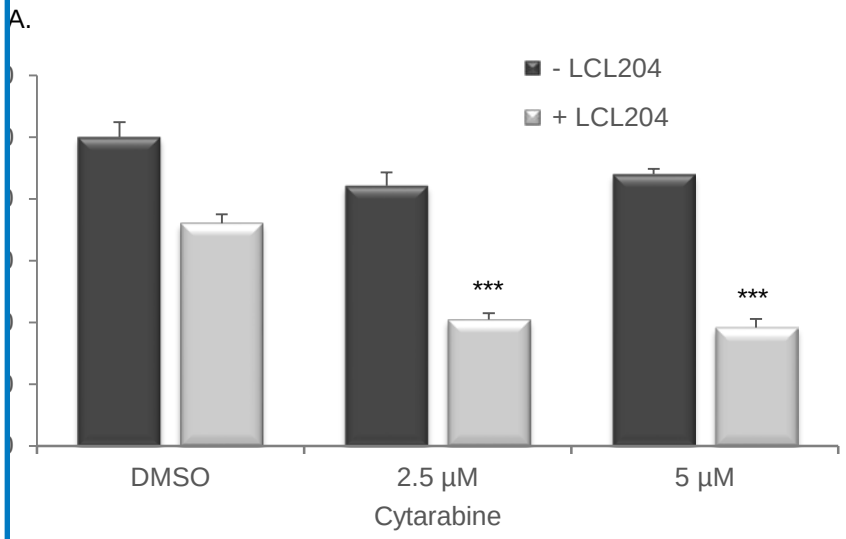
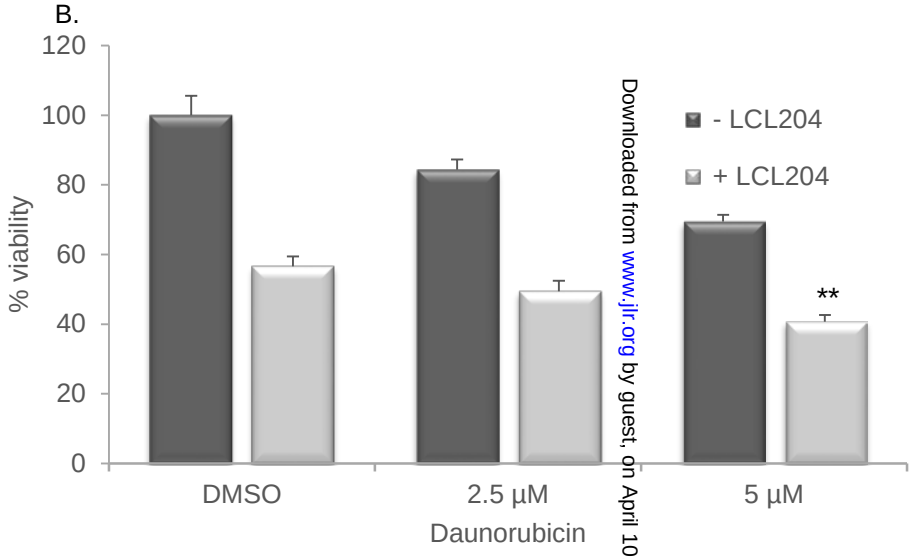
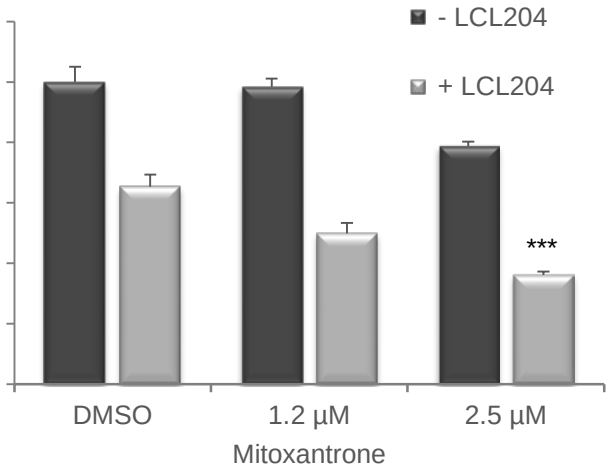
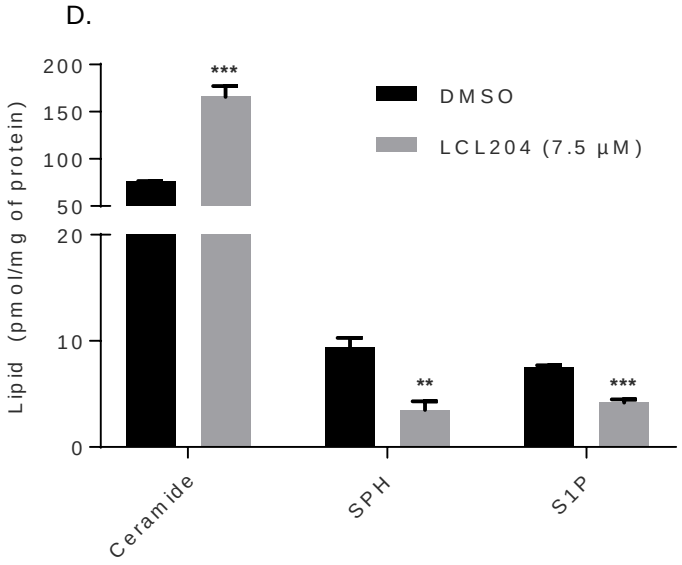


re 1. P-glycoprotein is highly expressed in AML patient samples and drug-resistant cell lines. A) P-glycoprotein cell membrane protein expression of 27 an AML samples, normal PBMC donors (n=6) and cord blood samples (n=3) was determined via flow cytometry using P-gp external-epitope antibody 2. Expression bars represent percentage of gated cells that were positive for UIC2. *, $p<0.05$ compared to normal PBMC and cord blood (Mann-Whitney . B) AC and P-gp are highly expressed in drug-resistant HL-60/VCR and HL-60/ABTR compared to parental HL-60 cells. Western blot showing AC and /MDR1 protein levels. C) P-gp efflux activity was assayed in HL-60, HL-60/VCR and HL-60/ABTR using DIOC2(3) efflux assay. **, $p<0.005$ compared to 60 (Student's *t* test).









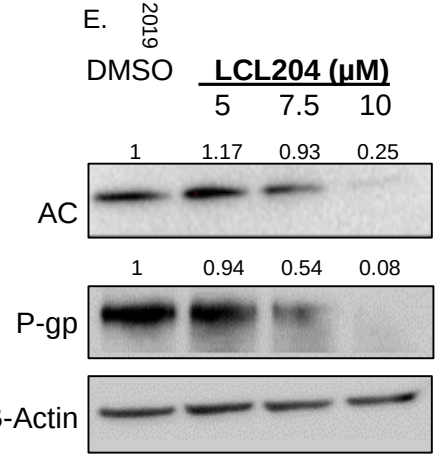
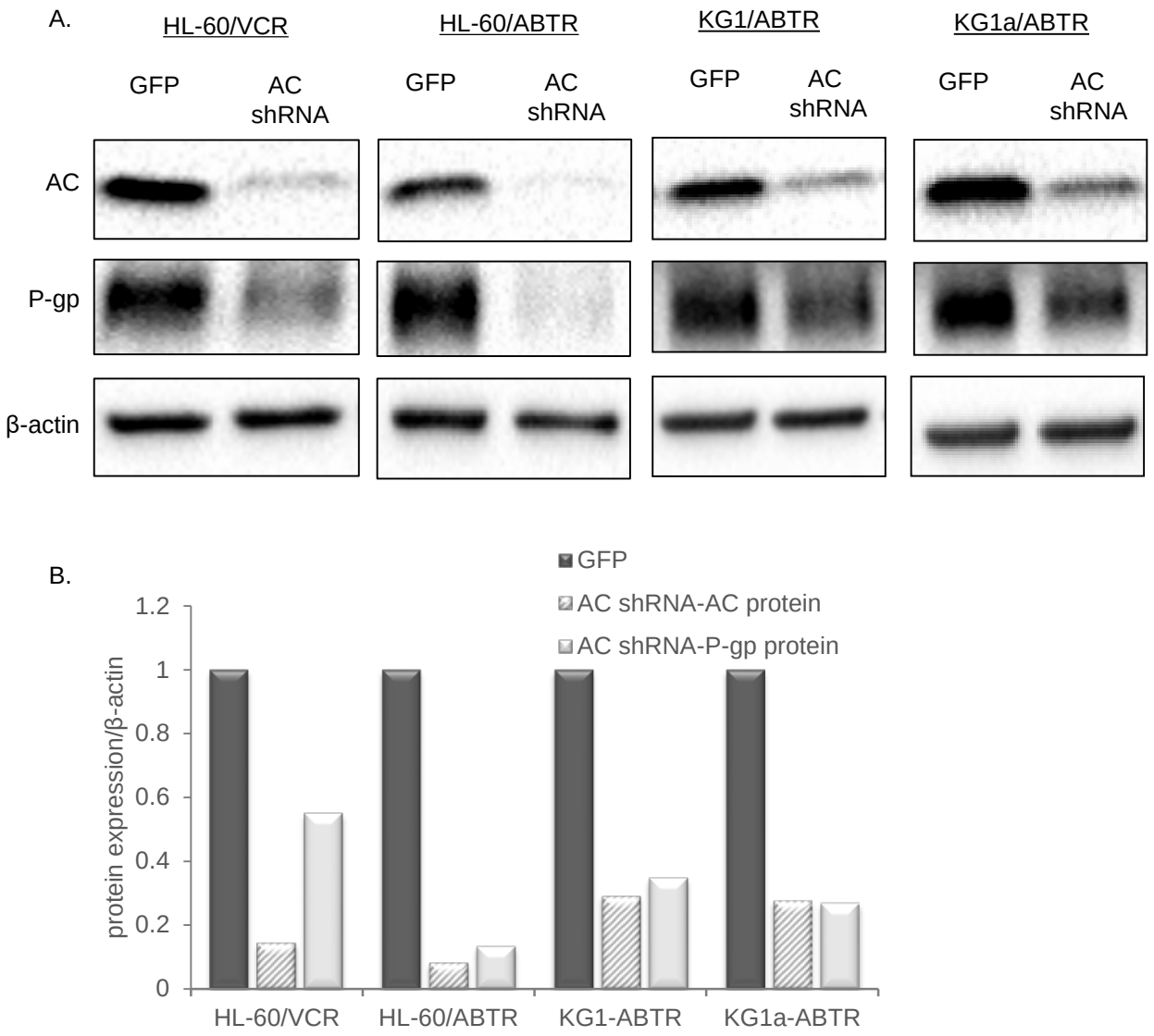
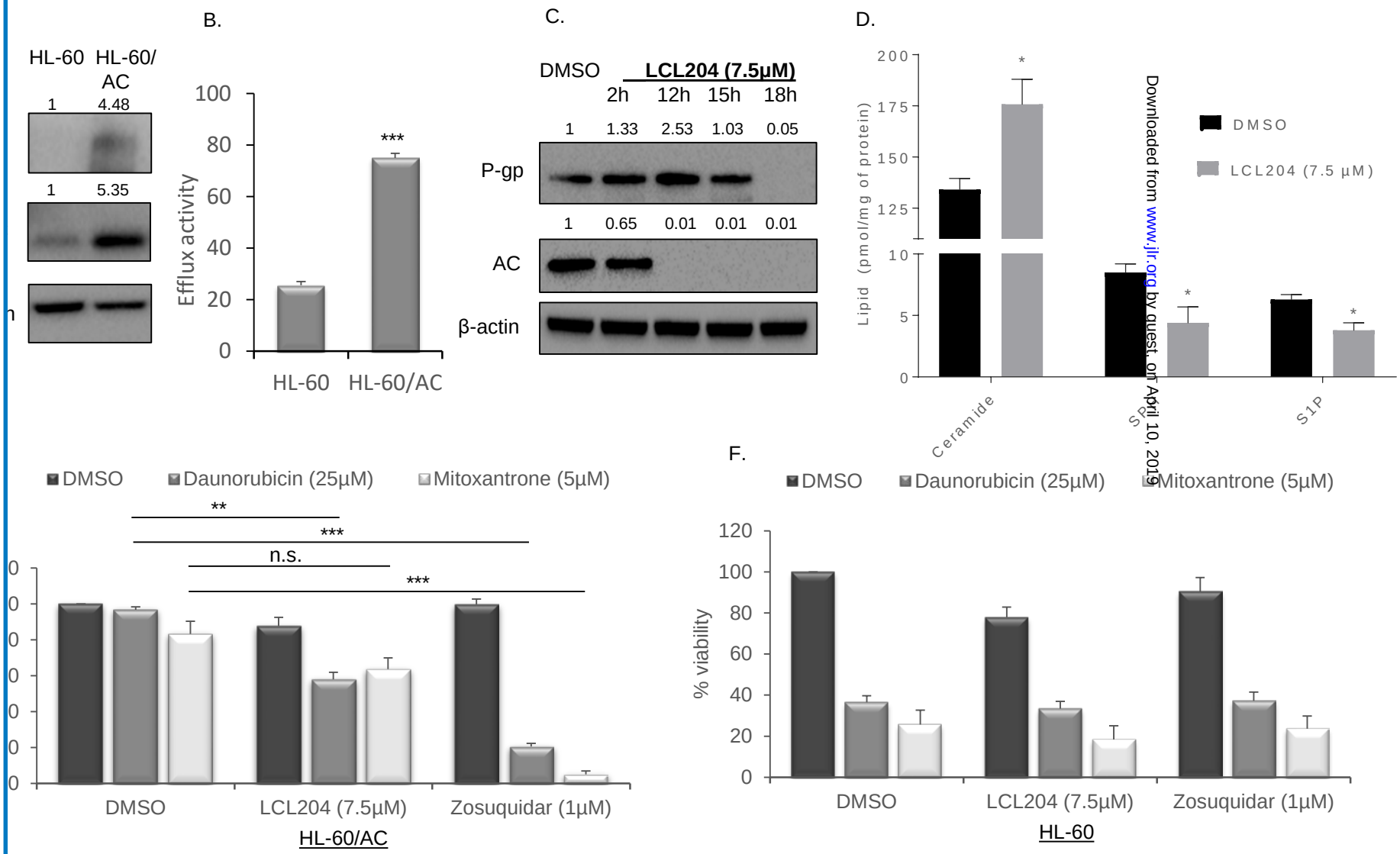


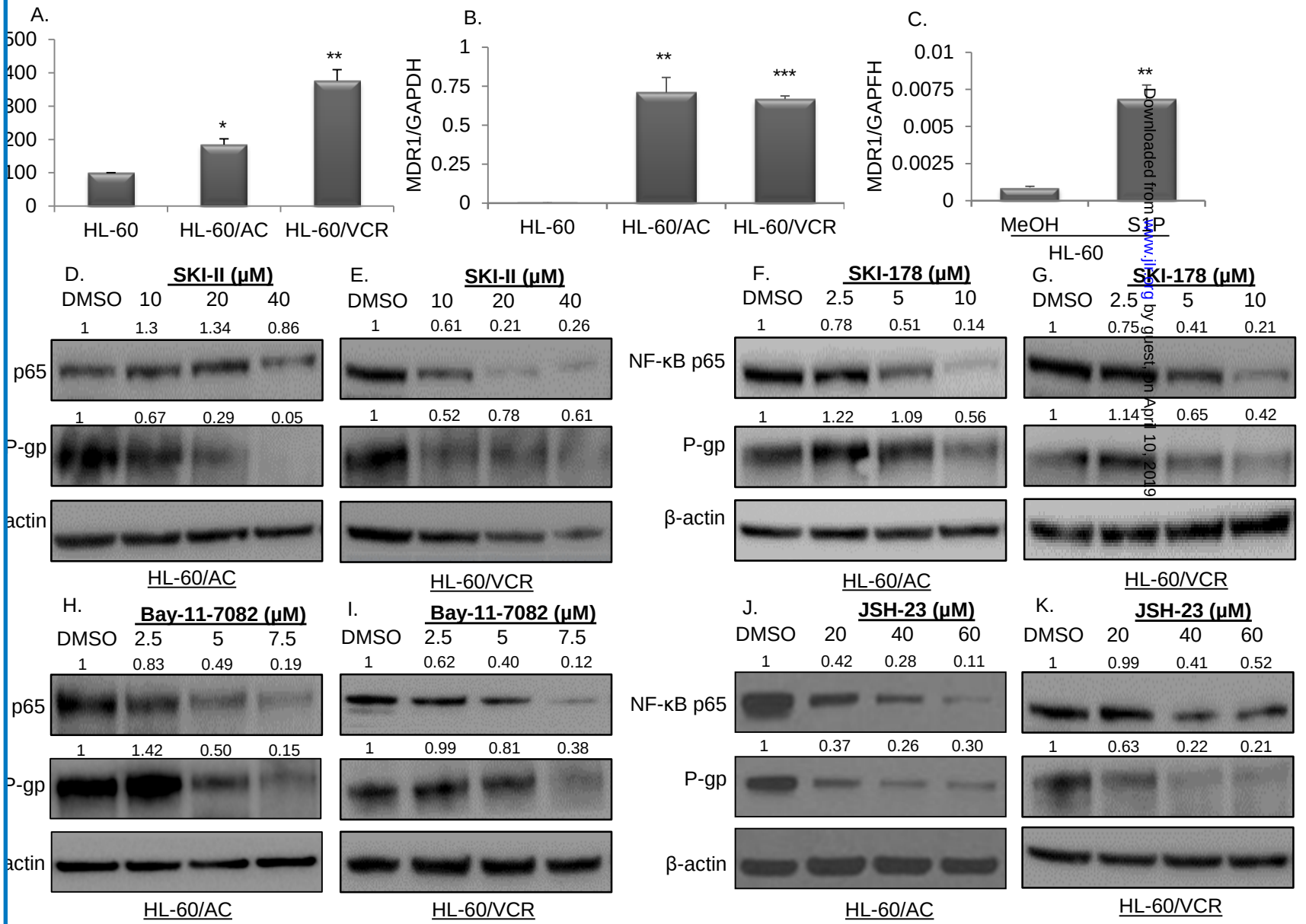
Figure 2. AC inhibitor LCL204 reversed drug resistance and induced AC and P-gp loss in HL-60/VCR. LCL204 (7.5 µM for 24h) co-treatment increased tivity of HL-60/VCR cells to chemotherapeutic agents A) cytarabine, B) daunorubicin and C) mitoxantrone (n=4, 2 independent experiments). **, 005, ***, $p<0.0005$ versus LCL204 and chemotherapeutic single treatments (Student's *t* test). D) Sphingolipid levels from HL-60/VCR cells treated 7.5 µM LCL204 for 24 hours. **, $p<0.005$, ***, $p<0.0005$ versus DMSO controls (Student's *t* test). E) Western Blot of HL-60/VCR cells incubated with dicated doses of LCL204 for 24 hours and probed for AC, P-gp and β–actin protein levels. Quantification above the blots were band intensities alized to β–actin.



ure 3. AC knockdown decreased P-gp protein expression. A) Western blot showing the reduction of AC and P-gp levels upon lentiviral-mediated AC ockdown (72h) in drug-resistant HL-60/VCR, HL-60/ABTR, KG1/ABTR and KG1a/ABTR cell lines compared to GFP control vector. B) AC and P-gp nds were quantified and normalized to β-actin. The blots and graph are representative of two independent experiments.



re 4. AC overexpression increased P-gp expression and induced drug resistance to chemotherapeutic agents. A) Western blot showing AC expression increased P-gp expression in HL-60/AC cells compared to parental HL-60 cells. Quantification above the blots were band intensities alized to β–actin. B) HL-60 and HL-60/AC cells were assayed for P-gp efflux activity using the DIOC2(3) efflux assay. The graph is representative of two pendent experiments (n=3 replicates). C) Western blot showing LCL204 treatment of HL-60/AC cells at different time points decreased AC and P-gp in levels. D) Sphingolipid levels from HL-60/AC cells treated with 7.5 μM LCL204 for 24 hours. E-F) HL-60/AC cells were resistant to AML notherapeutic drugs compared to parental HL-60 and were sensitized to AML chemotherapeutic drugs with LCL204. HL-60/AC (E) and HL-60 (F) cells co-treated for 24h with 7.5 μM LCL204 or 1 μM zosuquidar and 25 μM daunorubicin or 5 μM mitoxantrone. *, $p<0.05$, **, $p<0.005$ and ***, $p<0.0005$ pared to parental HL-60 (B), DMSO control (D) or double treatments compared to single chemotherapy treatments (E,F, Student's *t* test).



e 5. AC overexpression increased NF- κB activation and SPHK and NF-κB inhibition reduced P-gp levels. A) Nuclear NF-κB p65 levels in serum-starved) cells were compared to serum-starved HL-60/AC and HL-60/VCR cells. Calculated percent nuclear NF-κB p65 is the percent relative luminescence +/- B) MDR1 expression of HL-60, HL-60/AC and HL-60/VCR were analyzed by qRT-PCR and normalized to GAPDH. C) Serum-starved HL-60 cells were d with either methanol vehicle or 100 nM S1P for 24h and MDR1 expression was analyzed by qRT-PCR. D-E) HL-60/AC and HL-60/VCR cells were treated phingosine kinase inhibitors SKI-II (24h) and F-G) SKI-178 (48h). SPHK inhibition decreased NF-κB p65 and P-gp expression. H-I) Protein levels of p65 and were reduced in HL-60/AC and HL-60/VCR with NF-κB inhibitors Bay-11-7082 (16h) and J-K) JSH-23 (24h). Quantification above the blots represents ities normalized to β–actin. *, $p<0.05$, **, $p<0.005$, ***, $p<0.0005$ compared to parental HL-60 or methanol control (Student's *t* test).