Sphingolipid Metabolism Determines the Therapeutic Efficacy of Nanoliposomal Ceramide in Acute Myeloid Leukemia

SUPPLEMENTARY METHODS

Patient samples and clinical data

AML sub-classification is not static as additional subtypes are periodically recognized in disease classification systems and by authors studying large series of patients. In 2008, the WHO defined the AML-MRC subtype with criteria including morphological dysplasia, a history of MDS or MDS/MPN, and/or various cytogenetic findings. In the most recent WHO classification, this disease subtype has been retained, but modified. AML-MRC, in general, occurs in older patients and carry worse prognoses than other subtypes. Recently, a report by Papaemmanuil et al. highlighted the association of specific mutations with cytogenetic and clinical classes of AML, as well as with clinical outcomes. While the molecular properties of AML-MRC has not been formally defined, the Papaemmanuil study defined 14 karyotypic and molecular classes of AML, of which two (the chromatin-spliceosome and TP53-aneuploidy classes) share poor prognoses and mutational profiles also seen in poor prognosis MDS, and thus must be substantially congruent with AML-MRC.

For this study, samples were prepared from peripheral blood, bone marrow, or apheresis material collected from patients diagnosed with AML-MRC or DN-AML (Supplementary Table 1). All clinical samples and clinical information were collected at the Penn State College of Medicine under Institutional Review Board-approved informed consent (#2000-186). Mononuclear cells were prepared using Ficoll-PaquePLUS (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient centrifugation. For this project, patient cases were classified as AML-MRC according to WHO criteria or as DN-AML if they did not have or MDS-related changes or an antecedent hematologic disorder or dysplasia (Supplementary Table 1). MDS-related changes were noted if
there was a history of prior MDS or cytogenetic abnormalities associated with MDS as defined by the Vardiman group in an update to the WHO classification.\textsuperscript{3} It is noteworthy that many of the AML-MRC cases that are used in this study had poor risk and very complex cytogenetic abnormalities. We also performed next-generation mutational profiling on some cases as previously described.\textsuperscript{4,15} With the use of clinical, karyotypic, and these molecular data, we subclassified many cases per Papaemmanuil \textit{et al.}\textsuperscript{1} Here we refer to AMLs in the chromatin-spliceosome or TP53-aneuploidy groups as having “MDS-Mutations”. Cases used and clinical, karyotypic, and molecular details are listed in Supplementary Table 2. For this study, Lip-C6 was prepared as previously described (Supplementary Table 3).\textsuperscript{14-17} Freshly prepared patient samples were used for apoptosis assays as previously described,\textsuperscript{16} while cryopreserved samples were thawed for colony-forming assays as previously described as previously described.\textsuperscript{16} The use of patient materials, and associated clinical data, was approved by the Penn State College of Medicine, University of Virginia, Memorial Sloan-Kettering Cancer Center, and University of New Hampshire Institutional Review Boards.

\textbf{Cell culture}

Human MOLM-13, and OCI-AML-3 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Murine C1498 cells and patient AML samples were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Human Kasumi-1, ME-1, and SKM-1 cells were maintained in RPMI-1640 supplemented with 20% FBS. Human MV4-11-LUC-YFP (MV4-11) cells were maintained in Isocove’s Modified Dulbecco’s Medium supplemented with 10% FBS and 1% penicillin/streptomycin. Human KG-1 cells were maintained in Isocove’s Modified Dulbecco’s Medium supplemented with 20% FBS. Human SKNO-1 cells were maintained in RPMI-1640 supplemented with 10% FBS and 10 ng/ml granulocyte-macrophage colony-stimulating factor. All cultures were incubated at 37°C and 5% CO$_2$. ME-1 and OCI-AML-3 cells were gifts from Dr. Jacqueline Cloos of the VU University Medical Center.
Amsterdam, and Dr. Xiaorong Gu of the Cleveland Clinic, respectively. Cell lines were authenticated by short tandem repeat profiling (Genetica DNA Laboratories, Burlington, NC).

**Animal studies**

The Institutional Animal Care and Use Committees of the University of New Hampshire and of the Penn State College of Medicine approved of the studies and procedures utilizing laboratory animals. The following is an elaboration on specific animal study methodology:

1) Bone marrow cells were isolated for colony-forming assays, at the University of New Hampshire, from the femurs and tibias of 4-8 month-old (male and female) C57BL/6-Tg(Vav1-NUP98/HOXD13)G2Apla/J (Nup98-HoxD13), B6.129-Flt3\textsuperscript{tm1Dgg}/J (Flt3\textsuperscript{ITD}), and STOCK Kmt2a\textsuperscript{tm2(MLLT3)Thr}/KsyJ (MLL-AF9) transgenic mice and their respective wild-type littermates. Mice were submitted to the New Hampshire Veterinary Diagnostic Laboratory (NHVDL) at the University of New Hampshire for histopathological evaluation. Leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. The Nup98-HoxD13 transgenic mouse is a model of MDS, fully recapitulates the cellular features of MDS, and predictably evolves to AML.\textsuperscript{18} Therefore, we used the Nup98-HoxD13 transgenic mouse as a model of AML-MRC. In contrast, we used the MLL-AF9 and Flt3\textsuperscript{ITD} transgenic mice as models of DN-AML.

2) Therapeutic efficacy of Lip-C6 as a standalone treatment was evaluated using transgenic mouse models of AML-MRC (Nup98-HoxD13) and DN-AML (Flt3\textsuperscript{ITD}). 4-8 month-old (male and female) mice were injected with Lip-C6 (11.6 mg/kg) or Lip-Ghost (volume-matched) daily for 10 days. One day after the final injection, mice were euthanized and bone marrow was collected from femurs and tibias, spleens were removed and measured, and blood was collected by cardiac puncture for blood smears. There was n=4 mice/group for bone marrow
evaluations due to poor recovery in some mice in the treatment groups. Mice were submitted to the New Hampshire Veterinary Diagnostic Laboratory (NHVDL) at the University of New Hampshire for histopathological evaluation. Leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. Isolated bone marrow cells were prepared and stained with Mouse Fc Block, anti-mouse Gr-1 (APC-conjugated) (clone RB6-8C5), anti-mouse Ly-6C (PE-conjugated) (clone AL-21), and anti-mouse CD11b (V450-conjugated) (clone M1/70) monoclonal antibodies from BD Biosciences (San Jose, CA). Flow cytometry was performed at the University of New Hampshire’s University Instrumentation Center using a Sony SH800Z sorting flow cytometer. Gr-1+ CD11b+ cells were evaluated and quantified as representative of leukemia burden.

3) $2.5 \times 10^6$ C1498 cells were engrafted by retro-orbital intravenous injection into 6-8-week-old C57BL/6J mice at the University of New Hampshire, similar to as previously described.\(^{20}\) Beginning on day 5 (post-engraftment), mice received intravenous injections of either Lip-C6 (14.6 mg/kg), anionic liposomal vinblastine (5.34 mg/kg), a combination of Lip-C6 with anionic liposomal vinblastine (dose-matched to stand-alone treatments), or anionic Lip-Ghost control (volume-matched), three times per week for the duration of the trial. Mice were sacrificed once they were determined to be moribund by the investigative team in consultation with the University of New Hampshire’s Animal Resource Office, and were submitted to the NHVDL for histopathological evaluation. Notably, this model used an anionic liposomal formulation of vinblastine, which allowed for co-delivery of both C6-ceramide and vinblastine in the combinatorial group.

4) $2.5 \times 10^6$ MV4-11-LUC-YFP (luciferase- and YFP-expressing derivation of MV4-11) cells were engrafted by intravenous injection into ~8-week old NOD.Cg-Prkdc\(^{scid}\)Il2rg\(^{m1Wjl}\)SzJ (NSG) mice at the Penn State College of Medicine, similar as previously described.\(^{15}\) Beginning on day 16
(post-engraftment), mice received intravenous injections of Lip-C6 (14.6 mg/kg), vinblastine (not formulated in a liposome) (1.24 mg/kg), or a combination of both, three times per week for the first three weeks and then two times per week for three additional weeks. Control mice received equal volume intravenous injections of Lip-Ghost. To track leukemia burden, bioluminescence was quantified following luciferin injection using an IVIS Lumina Series III Imaging System (PerkinElmer, Waltham, MA) at the Penn State College of Medicine Imaging Core Facility.

**Liposome generation**

Proprietary Lip-C6 (nanoliposomal C6-ceramide), Lip-Ghost (control nanoliposomal formulation with no C6-ceramide), and anionic nanoliposomal variants (used to load vinblastine without the use of acid-trapping strategies), were prepared as previously described. Briefly, lipids dissolved in chloroform, were combined in specific molar ratios (Supplementary Table 3), dried to a film under nitrogen, and then hydrated by addition of 0.9% NaCl. Solutions were sealed, heated at 60°C for 60 minutes, subjected to vortex mixing, and sonicated until light no longer diffracted through the suspensions. Lipid vesicle-containing solutions were next extruded at 60°C by passing the solutions 10 times through 100 nm polycarbonate filters using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Size and charge were validated using a Malvern Zetasizer Nano ZS at 25°C, and C6-ceramide and vinblastine encapsulation into anionic formulations was quantified by mass spectroscopy (Supplementary Table 2). Liposomal suspensions were stored at room temperature until use.

**Apoptosis assays**

Freshly isolated patient samples were treated with 20 µM Lip-C6, 5 nM vinblastine, the combination of both, or controls, for 48 hours prior to apoptosis detection. These standardized doses were selected based on the IC$_{50}$ values observed in cellular viability assays using HL-60/VCR cells (data not shown). Apoptosis was detected by Annexin V and 7-AAD staining
according to the manufacturer’s protocol (BD Biosciences, San Diego CA) and as previously described. Samples were also stained with anti-CD34 and anti-CD38 antibodies to evaluate apoptosis within leukemia progenitor populations (CD34+CD38-). All antibodies and apoptosis detection reagents were obtained from BD Biosciences (San Jose, CA). Samples were evaluated at the Penn State College of Medicine Flow Cytometry Core using an LSR II flow cytometer and BD FACS Diva software.

**Colony-forming assays**

Cryopreserved human AML patient samples were thawed, then cell suspensions were washed with a 10X volume of RPMI-1640 supplemented with 2% FBS. Patient samples were cultured in triplicate in 12-well plates at a density of 0.1 x 10^5 to 2 x 10^5 cells per well in Human Methylcellulose Complete Media (#HSC003) (R&D Systems, Minneapolis, MN) as previously described. Plating densities were selected for each case to yield colony out-growth of ~20-200 colonies per well in the absence of drug treatment. Sterile water was added into empty wells, and space between wells, to maintain optimal humidity necessary for colony growth. During assay set-up, patient cells were added simultaneously to the culture media with Lip-C6 or controls. The cultures were mixed vigorously and then dispensed to multi-well plates and incubated for 10-14 days. Blinded investigators manually counted blast colonies (>20 cells).

Murine bone marrow mononuclear cells were isolated and prepared from transgenic mice and their wild-type counterparts and cleaned of debris using Ficoll-Paque PLUS (GE Healthcare Life Sciences) density gradient centrifugation. Prior to colony-forming assays, leukemia was confirmed by the investigative team and the NHVDL in transgenic mice based on spleen or bone marrow histopathology, and/or peripheral blood smear evaluation. Murine samples were cultured in triplicate in 12-well plates at a density of 2.5 x 10^4 cells per well in Murine MethoCult GF (#M3434) (StemCell Technologies, Vancouver, BC). The plating density was selected to yield
typical wildtype or transgenic mouse colony out-growth of ~30-150 colonies per well in the absence of drug treatment. Sterile water was added into empty wells, and space between wells, to maintain optimal humidity necessary for colony growth. During assay set-up, murine bone marrow cells were added simultaneously to the culture media with Lip-C6 or controls. The cultures were mixed vigorously and then dispensed to multi-well plates and incubated for 10-14 days. Blinded investigators manually counted colonies (>20 cells), and total colonies, including blast or miscellaneous colonies, were scored.

In both the mouse and human colony-forming assays, Lip-Ghost (no C6-ceramide, but the same ratio of constituent lipids that compose the base formulation) did not exert any substantial toxicity (Supplementary Figure 3).

**Lipidomics**

To evaluate the metabolism of C6-ceramide (functional lipidomics), AML patient samples or cell lines were cultured with 10 µM Lip-C6, 2.5 nM vinblastine, the combination of both, or controls, for 24 hours prior to lipid extraction. Patient AML samples included those with defined AML-MRC or MDS-Mutations (cases 126, 733, 786, and 796), as well as those classified as DN-AML (cases 665, 719, 801, and 807). The doses were approximately half of the IC₅₀ for HL-60/VCR cells, as determined by cellular viability assay at 48 hours (data not shown) and were selected to avoid cytotoxic effects that otherwise could interfere with sphingolipid metabolism. Biological replicates (n≥3) were performed for all experiments studying the metabolism of C6-ceramide. Following treatments, lipids were extracted using a modified Bligh-Dyer extraction and extracts were subjected to liquid chromatography and electrospray ionization-tandem mass spectroscopy (LC-MS³) to detect sphingolipid metabolites, as previously described. Lipid mass was normalized to total cellular protein, determined using a Pierce BCA protein assay according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA).
The metabolism of C6-ceramide to specific metabolites was quantified by first determining the contribution of Lip-C6 treatment to a given metabolite’s abundance compared with control (Lip-Ghost) liposomal treatment (Adjusted C6 Metabolite) (Equation 1). Next a percent portion of a single lipid metabolite was determined from the sum of the lipid metabolites of interest (sphingosine, total physiological/endogenous ceramides, C6-sphingomyelin, and C6-cerebrosides, as well as C6-ceramide when specifically noted) (Equation 2).

Equation 1: Adjusted C6-Metabolite = Raw C6 Metabolite \(_{\text{Lip-C6}}\) (pmol/mg cellular protein) - Raw C6 Metabolite \(_{\text{Lip-Ghost}}\) (pmol/mg cellular protein)

Equation 2: Percent Metabolism = Adjusted C6 Metabolite (pmol/mg cellular protein) / \(\sum\) All Adjusted C6 Metabolites (pmol/mg cellular protein) x 100

**Statistical analysis**

GraphPad Prism 4 (GraphPad Software, La Jolla, CA) and SAS version 9.4 (SAS Institute, Cary, NC) were used to perform analyses. Comparisons of apoptosis, colony-forming, or lipidomic data were made using Wilcoxon rank sum tests or 1-way ANOVA followed by a Tukey’s multiple comparisons test. Combined lipidomic C6-metabolite (pro-death or neutral/pro-survival categories) comparisons between AML-MRC and DN-AML, as well as leukemia burden in transgenic mouse trials, were made using a two-tailed unpaired t-test with Welch's correction. Combination treatments were evaluated using a mixed-effects ANOVA for factorial design. For dose-response colony-forming assays, a 2-way ANOVA with either a Tukey’s (when comparing mouse samples of AML-MRC to both DN-AML and wild-type) or Sidek’s (when comparing human samples of AML-MRC to DN-AML) post hoc multiple comparisons test was performed. A linear mixed-effects model with repeated measures was used to confirm statistical significance when
comparing human samples in the dose-response colony-forming assays of AML-MRC with DN-AML. For *in vivo* trials using MV4-11-LUC-YFP cells engrafted into NSG mice, a 2-way ANOVA with a Tukey’s post hoc multiple comparisons test was performed. To further verify statistical significance, a linear growth curve model was fit to examine the impact of time and group on bioluminescence measurements (log$_{10}$ scale). Lastly, the Mantel-Cox Logrank test was used to determine survival significance between *in vivo* treatment groups.
Supplementary Table 1. Patient characteristics.

See Excel file.

Supplementary Table 2. Patient AML-MRC is uniquely sensitive to Lip-C6.

<table>
<thead>
<tr>
<th>Pt. Case#</th>
<th>AML-Subgroup</th>
<th>Genomic Classification</th>
<th>Class Defining Mutations</th>
<th>Other Mutations</th>
<th>IC50 of Lip-C6 (µM)</th>
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<td>TP53</td>
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<td>t(3;5)(q25;q35)</td>
<td>FLT3</td>
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<td>NPM1</td>
<td>FLT3</td>
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Colony-forming assay Lip-C6 IC50 values (n≥3) were determined for patient AML samples with defined AML-MRC or with MDS-Mutations (red) or DN-AML patient samples (blue). Molecular profiling was used to determine class-defining and other mutations, as previously described. Class-defining mutations were assigned according to Papaemmanuil et al. Genomic classifications were assigned based on criteria outlined in Table 1 of Papaemmanuil et al.

Classification #2: AML with mutated chromatin, RNA-splicing genes, or both. Classification #3: AML with TP53 mutations, chromosomal aneuploidy, or both. Classification #12: AML with driver mutations but no detected class-defining lesions.
**Supplementary Table 3. Liposome formulations.**

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<td>C6</td>
<td>37.5%</td>
<td>17.5%</td>
<td>7.5%</td>
<td>-</td>
<td>30%</td>
<td>7.5%</td>
</tr>
<tr>
<td>Neutral Ghost</td>
<td>56.6%</td>
<td>28.7%</td>
<td>14.7%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anionic</td>
<td>63%</td>
<td>23%</td>
<td>10%</td>
<td>10%</td>
<td>-</td>
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<table>
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<th>Liposome</th>
<th>Size</th>
<th>Zeta potential</th>
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<td>-7 mV</td>
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Anionic liposomes were incubated with 5 mg/ml vinblastine overnight at 37°C. Unbound or unencapsulated vinblastine was removed by size exclusion chromatography utilizing a CL-4B column. Vinblastine concentration was determined by mass spectrometry, and the loading efficiency was determined to be 25%, yielding concentrations in the range of 1 mM. Encapsulating vinblastine does not change the size or zeta potential of the anionic liposomal formulations.
Supplementary Figure 1. AML-MRC is uniquely sensitive to Lip-C6 (normalized colony count). Colony-forming assays evaluating a dose-response of Lip-C6 were performed using bone marrow samples harvested from wild-type, transgenic AML-MRC, or transgenic DN-AML mice, or using human mononuclear cells obtained from patients with AML-MRC or DN-AML. Depiction of individual sample dose-response curves with normalized colony counts for both mouse (a) and human (b) samples. Error bars represent the standard error of the mean, n=3 biological replicates. Red lines: AML-MRC; blue lines: DN-AML; green lines: wildtype.
Supplementary Figure 2

B

![Graphs showing colony number versus Lip-C6 concentration for different patients](image-url)
Supplementary Figure 2. AML-MRC is uniquely sensitive to Lip-C6 (absolute colony count).

Colony-forming assays evaluating a dose-response of Lip-C6 were performed using bone marrow samples harvested from wild-type, transgenic AML-MRC, or transgenic DN-AML mice, or using human mononuclear cells obtained from patients with AML-MRC or DN-AML. Depictions of individual sample dose-response curves with absolute colony counts for both mouse (a) and human (b) samples. Error bars represent the standard error of the mean, n=3 biological replicates. Red lines: AML-MRC; blue lines: DN-AML; green lines: wildtype.
Supplementary Figure 3

A

DN-AML

AML-MRC

Untreated

Lip-Ghost

B

DN-AML

AML-MRC

Wildtype #74

Wildtype #79

Wildtype #412

FLT3-ITD #126

FLT3-ITD #164

MLL-AF9 #426

MLL-AF9 #478

Nup98-HoxD13 #60

Nup98-HoxD13 #75

Nup98-HoxD13 #212

Nup98-HoxD13 #213

Relative Colony Number

0.00

0.25

0.50

0.75

1.00

1.25

0.00

0.25

0.50

0.75

1.00

1.25

Relative Colony Number

Unlabeled

Lip-Ghost (10 µM equivalent)

Lip-Ghost (40 µM equivalent)
Supplementary Figure 3. Lip-Ghost exerts minimal toxicity. (a) Colony-forming assays were performed by exposing primary human DN-AML patient samples, or AML-MRC, to Lip-Ghost. (b). Colony-forming assays were performed by exposing samples of bone marrow-isolated cells from transgenic mice with DN-AML or AML-MRC manipulations, or their wild-type littermates, to Lip-Ghost. Error bars represent the standard deviation, n≥3.
Supplementary Figure 4. Lip-C6 effectively treats AML-MRC transgenic mice but not DN-AML transgenic mice (representative blood smears). Therapeutic efficacy of Lip-C6 as a standalone treatment was evaluated using transgenic mouse models of AML-MRC (Nup98-HoxD13) and DN-AML (Flt3ITD). Mice were treated for 10 days with daily injections of Lip-C6 (11.6 mg/kg) or Lip-Ghost (volume-matched). Mice were then euthanized and blood was collected by cardiac puncture for blood smear preparation and evaluation. Images are representative of AML-MRC and DN-AML transgenic mice treated with either Lip-Ghost or Lip-C6. The presence of blasts is substantially diminished in the blood smears of AML-MRC transgenic mice treated with Lip-C6, but not the others.
Supplementary Figure 5. Lip-C6 effectively treats AML-MRC transgenic mice but not DN-AML transgenic mice (representative bone marrow flow cytometry). Therapeutic efficacy of Lip-C6 as a standalone treatment was evaluated using transgenic mouse models of AML-MRC (Nup98-HoxD13) and DN-AML (Flt3ITD). Mice were treated for 10 days with daily injections of Lip-C6 (11.6 mg/kg) or Lip-Ghost (volume-matched). Mice were then euthanized and bone marrow was isolated and prepared for flow cytometry where Gr-1+ CD11b+ cells were evaluated and quantified as representative of leukemia burden. Representative flow cytometry histograms depicting a substantially diminished leukemia burden in AML-MRC transgenic mice treated with Lip-C6, but not the others.
Supplementary Figure 6

A

Pro-Death Metabolites

Neutral/Pro-Survival Metabolites

Endogenous Ceramides
Sphingosine
S1P
C6-SM
C6-Cerebrosides

AML-MRC (MDS-Mutations)
DN-AML

B

Pro-Death Metabolites

Neutral/Pro-Survival Metabolites

Endogenous Ceramides
Sphingosine
S1P
C6-SM
C6-Cerebrosides

AML-MRC (MDS-Mutations)
DN-AML
Supplementary Figure 6. AML converts Lip-C6-delivered C6-ceramide to a diversity of sphingolipid metabolites. Patient samples were exposed for 24 hours in vitro to 10 µM nanoliposomal C6-ceramide (Lip-C6), and the metabolism of C6-ceramide was evaluated by lipidomic analysis. (a) Mass of C6-ceramide metabolites. (b) Percent metabolism of C6-ceramide to its metabolites. Percentages are representative of metabolites without C6-ceramide included as a metabolite. There were no significant differences between metabolites from either patient AML samples with defined AML-MRC or with MDS-Mutations or metabolites from DN-AML. Please see Figure 1F for metabolite grouping, which revealed a significant difference between pro-apoptotic and neutral/pro-survival metabolites from patient AML samples with defined AML-MRC or with MDS-Mutations. C6-SM = C6-sphingomyelin. Error bars represent the standard error of the mean, n=4. There are no significant differences in this analysis.
Supplementary Figure 7

E  C6-Sphingomyelin

F  Total Endogenous Sphingomyelins
Supplementary Figure 7

G  C6-Cerebrosides

H  Total Endogenous Cerebrosides
Supplementary Figure 7. Metabolism of C6-ceramide delivered by Lip-C6 +/- co-treatment with vinblastine. Eight AML patient samples were exposed for 24 hours in vitro to 10 µM nanoliposomal C6-ceramide (Lip-C6) alone or in combination with 2.5 nM vinblastine (VB), and the metabolism of C6-ceramide was evaluated by lipidomic analysis. (a) C6-ceramide, (b) total endogenous/physiological ceramides, (c) sphingosine, (d) sphingosine-1-phosphate, (e) C6-sphingomyelin, (f) total endogenous/physiological sphingomyelins, (h) C6-cerebroside, and (i) total endogenous/physiological cerebrosides. Error bars represent the standard error of the mean of at least three independent experiments (1-way ANOVA with Tukey’s post hoc comparison; *p ≤ 0.02 compared with untreated, Lip-Ghost, and VB; **p ≤ 0.0493 compared with untreated, Lip-Ghost, VB, and Lip-C6; ^p ≤ 0.0464 compared with Lip-Ghost and VB; §§p ≤ 0.0035 compared with untreated, Lip-Ghost, VB, and Lip-C6 + VB; ¥p ≤ 0.0355 compared with untreated; §§§p ≤ 0.0431 compared with Lip-Ghost and Lip-C6; %p ≤ 0.0372 compared with untreated and Lip-Ghost; ^p = 0.0445 compared with Lip-Ghost; §§§p < 0.0001 compared with untreated, Lip-Ghost, and VB, while omitting the Lip-C6 category from the 1-way ANOVA because it otherwise would not be significant).
Supplementary Figure 8. AML converts Lip-C6-delivered C6-ceramide to a diversity of sphingolipid metabolites. Patient samples were exposed for 24 hours in vitro to 10 µM nanoliposomal C6-ceramide (Lip-C6), and the metabolism of C6-ceramide was evaluated by lipidomic analysis. Patient DN-AML converts C6-ceramide to various pro-death metabolites (sphingosine and endogenous/physiological ceramides), and neutral/pro-survival metabolites (S1P, C6-sphingomyelin/SM, C6-cerebrosides; 1-way ANOVA with Tukey’s post hoc comparison; *p ≤ 0.008 compared with all other metabolites; **p < 0.0001 compared with all other metabolites; #p ≤ 0.0173 compared with S1P and C6-SM; $p = 0.0277 compared with C6-SM). Error bars represent the standard error of the mean, n≤4.
Supplementary Figure 9. Vinblastine focuses the metabolism of Lip-C6-delivered C6-ceramide to pro-apoptotic metabolites. Seven AML cell lines were exposed for 24 hours in vitro to 10 μM nanoliposomal C6-ceramide (Lip-C6) alone or in combination with 2.5 nM vinblastine (VB), and the metabolism of C6-ceramide was evaluated by lipidomic analysis. Vinblastine co-treatment significantly shifted C6-ceramide metabolism to sphingosine and endogenous/physiological ceramides, both pro-apoptotic metabolites. Unpaired t-test with Welch’s correction; *p ≤ 0.03 (specific values indicated). Data points are averages of biological replicates (n≥3).
Supplementary Figure 10

A

Concentrations: Lip-C6 (14.6 mg/kg)
VB (1.24 mg/kg)

B

Concentrations: Lip-C6 (15.6 mg/kg)
VB (1.24 mg/kg)

C

Study 1 and 2 combined: plot of the estimated smooth growth curve (on log-10 scale) in each group
Supplementary Figure 10. Vinblastine and Lip-C6 exert combinatorial anti-AML in vivo efficacy. Leukemia burden was quantified following bioluminescent imaging of NSG mice engrafted with MV4-11-LUC-YFP AML cells in two separate studies. Mice were treated with intravenous injections of either the Lip-Ghost control, Lip-C6, vinblastine (VB) (not formulated in a liposome), or the combination of both Lip-C6 and VB, for (a) study one (n=3 per group), and (b) study two (n=3 per group). Each mouse is represented by an individual curve. (c) Plot of the estimated bioluminescence growth curves (on log_{10} scale) by combining studies one and two, for the VB treatment and Lip-C6 + VB treatment groups. These treatment groups remain significantly different upon combining these two studies (p<0.0001, n=6 overall). A linear growth curve model was fit to examine the impact of time and group on bioluminescence measurements (log_{10} scale).
Supplementary Figure 11. Leukemia burden in mice treated with vinblastine and Lip-C6.

Leukemia burden was evaluated using bioluminescent imaging of NSG mice engrafted with MV4-11-LUC-YFP AML cells in two separate studies. Mice were treated with intravenous injections of either the Lip-Ghost control, Lip-C6, vinblastine (VB) (not formulated in a liposome), or the combination of both Lip-C6 and VB. Mice were routinely imaged to track leukemia burden. "X" represents a mouse that no longer was alive. See Supplementary Figure 10A-B for the graphical representation of leukemia burden over time for these studies.