

Therapeutic Combination of Nanoliposomal Safingol and Nanoliposomal Ceramide for Acute Myeloid Leukemia

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Abstract

Novel approaches to enhancing the efficacy of ceramide-based therapeutics are of interest for the treatment of acute myeloid leukemia (AML). Ceramide is a bioactive sphingolipid that has long been established as an inducer of apoptosis. We have pioneered the effort to develop nanoliposomal C6-ceramide (Lip-C6) as an anticancer therapeutic and recently have engaged in efforts to enhance its therapeutic efficacy. Ceramide catabolism by acid ceramidase and subsequently sphingosine kinase 1 yields the metabolite oncogenic sphingosine-1-phosphate (S1P). Therefore, in the present study we hypothesized that targeting of this metabolic pathway with the sphingosine kinase 1 inhibitor safingol would augment the anti-AML efficacy of Lip-C6. We generated and evaluated nanoliposomes encapsulating safingol (Lip-Saf), in combination with Lip-C6 using AML cell lines and primary AML patient samples. This combination exerted synergistic therapeutic efficacy using HL-60 and KG-1 cells, and additive efficacy using HL-60/VCR cells. In contrast, the combination of Lip-C6 and Lip-Saf yielded an antagonistic effect using the murine AML cell line C1498 and this effect was correlated with an increase in autophagy as a potent leukemia survival mechanism. Intriguingly, by using an inhibitor of ceramide glycosylation, nanoliposomal tamoxifen, we observed synergistic anti-AML efficacy using the C1498 cell line demonstrating that unique manipulations of ceramide metabolism may be prevalent in different AMLs. Lastly, we evaluated both favorable prognosis and poor prognosis primary patient AML samples and observed combinatorial efficacy of Lip-C6 and Lip-Saf. This was reflected by a decrease in autophagy and concomitant increase in apoptosis, as well as the ability to block colony forming capacity. Altogether, these results demonstrated that the efficacy of Lip-C6 as an experimental anti-AML therapy can be dramatically enhanced through combination with inhibitors of ceramide metabolism.

Keywords: Nanoliposome; Ceramide; Safingol; Tamoxifen; Autophagy; Apoptosis; Acute myeloid leukemia; Sphingosine kinase; Glucosylceramide synthase; P-Glycoprotein

Introduction

Acute myeloid leukemia (AML) is a growing public health problem in the United States with approximately 12,000 new cases of AML and nearly 9,000 deaths occurring yearly. In spite of substantial efforts, therapeutic progress has been limited. AML is a highly heterogeneous disease and the 2008 WHO classification, which incorporates known karyotypic data of prognostic importance, has been accepted for its clinical classification [1-3]. New drugs are urgently needed as a large majority of patients with AML have an unfavourable outlook illustrated by relapse and multidrug resistance.

One promising avenue of therapeutic development has been liposomal encapsulation of drugs. This approach has augmented the efficacy of several chemotherapeutics, while simultaneously decreasing toxicity [4,5]. In addition to protecting therapeutics from degradation and augmenting systemic retention, liposomal formulations also offer the prospect of delivering highly insoluble molecules such as lipids [4,5]. Recently, our group has developed nanoliposomal C6-ceramide (Lip-C6) for the treatment of both solid tumor malignancies and hematological malignancies [4,5]. Ceramide is a naturally occurring bioactive sphingolipid that has been well-established as an inducer of apoptosis and regulator of cell stress [4-10]. Ceramide has also been shown to exert therapeutic efficacy in a variety of *in vitro* and *in vivo* models of cancer, and endogenous ceramides have also been documented to increase in response to chemotherapy [5,7,10]. As a very insoluble lipid, the utility of ceramide as a standalone therapeutic

was limited until its formulation into nanoliposomes [4,5]. A significant obstacle to effective ceramide-based therapy is the inherent ability of some malignant cells to detoxify ceramide by metabolism that often yields oncogenic metabolites [6,7]. In one metabolic pathway, ceramide can be converted to sphingosine-1-phosphate (S1P) by the action of acid ceramidase and sphingosine kinase (SphK) [8-10]. S1P is a potent bioactive sphingolipid that can bind to specific S1P receptors which mediate its vast array of mitogenic and oncogenic effects [11,12]. Alternatively, ceramide can be metabolized to glucosylceramide by the coordinated action of glucosylceramide synthase (GCS) and P-glycoprotein (P-gp) [8-10]. In this pathway, the drug efflux pump P-gp is located at the Golgi where it is responsible for moving newly synthesized glucosylceramide into the Golgi thereby promoting further metabolism and preventing product inhibition of GCS [8-10].

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As both the SphK and GCS/P-gp pathways are upregulated in many malignancies, these enzymes have gained substantial attention as targets for the development of cancer therapeutics [10]. Furthermore, second generation development of ceramide as a therapeutic has included strategies to block either the SphK or GCS/P-gp pathways [10].

Recently, a liposomal formulation of the SphK inhibitor Safingol was described and tested in a model of leukemia [13]. Safingol, also known as L-threo-dihydrosphingosine, competitively inhibits SphK1 and has been shown to induce autophagy in pancreatic, colon, and breast cancer cell lines by the generation of reactive oxygen species [14,15]. Autophagy is a cellular self-eating process where intracellular vacuoles sequester organelles and other cellular components. These vacuoles fuse with lysosomes to form autophagosomes that ultimately consume these organelles for the production of energy [16,17]. It was originally suggested that autophagy was a cellular mechanism to avoid starvation during times of nutrient deprivation, but recently autophagy is also implicated as a mechanism of inducing cell death in a caspase-independent fashion [15-17]. Research has suggested that autophagy not only can be a process by which malignant cells overcome nutrient starvation but also a mechanism of therapy resistance [18,19]. Accordingly, studies have demonstrated that inhibition of autophagy can be used to sensitize malignant cells to chemotherapy [18-20]. In contrast, recent studies have also demonstrated conflicting evidence that autophagy may play a role in apoptosis of tumor cells [19,21]. Therefore, it is important to recognize that pharmacological blockade of autophagy remains a questionable proposition without further mechanistic understanding of the roles of autophagy in cancer. Comparatively, pharmacological inhibition of sphingolipid metabolism provides a reasonable therapeutic target because the generation of bioactive sphingolipid metabolites, such as S1P, is readily linked to oncogenesis [4-15].

In the present study, we designed nanoliposomal safingol (Lip-Saf) to specifically combine with Lip-C6 for the treatment of AML. In particular, we utilized Lip-Saf as a means to augment the efficacy of Lip-C6 in AML cell lines and AML patient samples that otherwise were less sensitive to Lip-C6 alone. Furthermore, we evaluated autophagy and apoptosis and demonstrated that efficacy of combining Lip-Saf with Lip-C6 is lost when autophagy is upregulated. However, in this case the combinatorial efficacy of Lip-C6 can be achieved by instead blocking the GCS/P-gp pathway with nanoliposomal tamoxifen (Lip-Tam). Altogether, this study demonstrated the anti-AML efficacy of combining Lip-C6 with nanoliposomal formulations that block its metabolism. This study is highly significant because safingol has recently entered clinical trials for the treatment of solid tumor malignancies [22].

Materials and Methods

Cell culture

Human HL-60, HL-60/VCR, and murine C1498 cells, were maintained at 37°C, and 5% CO₂, in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Human KG-1 likewise maintained, in IMDM supplemented with 20% FBS and 1% penicillin/streptomycin. Patient AML samples were prepared from peripheral blood obtained under informed consent and approved by the Penn State College of Medicine Institutional Review Board. These samples were maintained in RPMI-1640 supplemented with 10% FBS.

Nanoliposomal preparation and characterization

Nanoliposomes were prepared by the Penn State College of

Medicine Drug Discovery Core following previously established methods with minor changes for the new Lip-Saf formulation. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Ghost nanoliposomes (Lip-Ghost), Lip-C6, and Lip-Tam, were prepared as previously described [7,23,24]. Briefly, lipids dissolved in chloroform, or other organic solvents, were combined in specific molar ratios. For Lip-

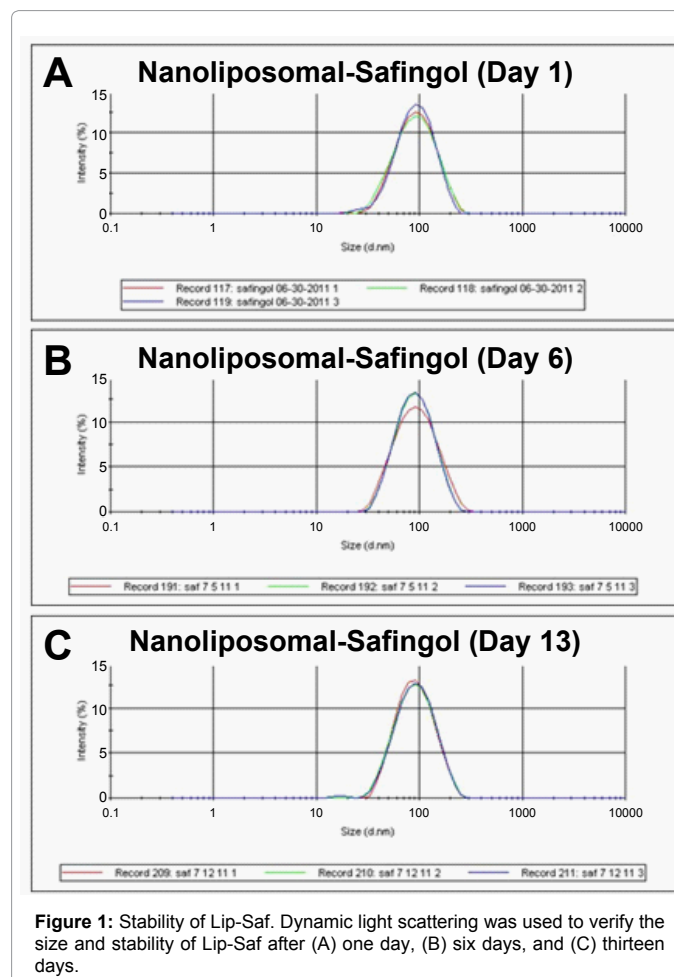


Figure 1: Stability of Lip-Saf. Dynamic light scattering was used to verify the size and stability of Lip-Saf after (A) one day, (B) six days, and (C) thirteen days.

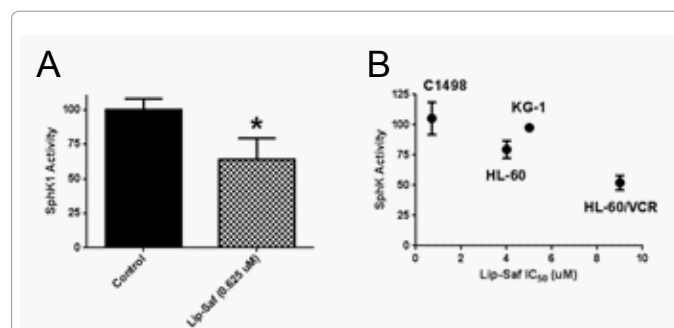


Figure 2: SphK activity. (A) Lip-Saf inhibited recombinant SphK1 activity in a cell-free inhibitor screening assay (t-test, *p<0.05, n=5). Error bars represent standard deviation from the mean. (B) SphK activity was determined and compared for the C1498, KG-1, HL-60, and HL-60/VCR cell lines. Activity was determined by dividing fluorescence (SphK activity) by the total amount of protein assayed for a given sample. Activity was plotted versus the Lip-Saf IC₅₀ for each cell line. The Pearson correlation coefficient was -0.89, but was not significant as p = 0.11. Error bars represent standard deviation from the mean.

Saf, aliquots of DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DSPE-PEG2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]), and safingol, were made in a 4.66:2.37:1.47:1.5 molar ratio. Solutions were dried to a film under a stream of nitrogen, and then hydrated by addition of 0.9% NaCl. Solutions were sealed, heated at 60°C (60 min), and subjected to vortex mixing and sonicated until light no longer diffracted through the suspension. The lipid vesicle-containing solution was quickly extruded at 60°C by passing the solution 10 times through 100 nm polycarbonate filters in an Avanti Mini-Extruder. Nanoliposomal size was determined using a Malvern Zetasizer Nano ZS at 25°C. Nanoliposome solutions were stored at room temperature until use. Lip-Saf was stable for at least two weeks (Figure 1).

Cell viability assay

Cells were plated at 2.5×10^4 cells per well in 96-well tissue culture plates and treated for 48 hours. Following treatment, cellular viability was assessed using a Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay according to the manufacturer's instructions (Promega, Madison, WI). Viability was determined by measuring

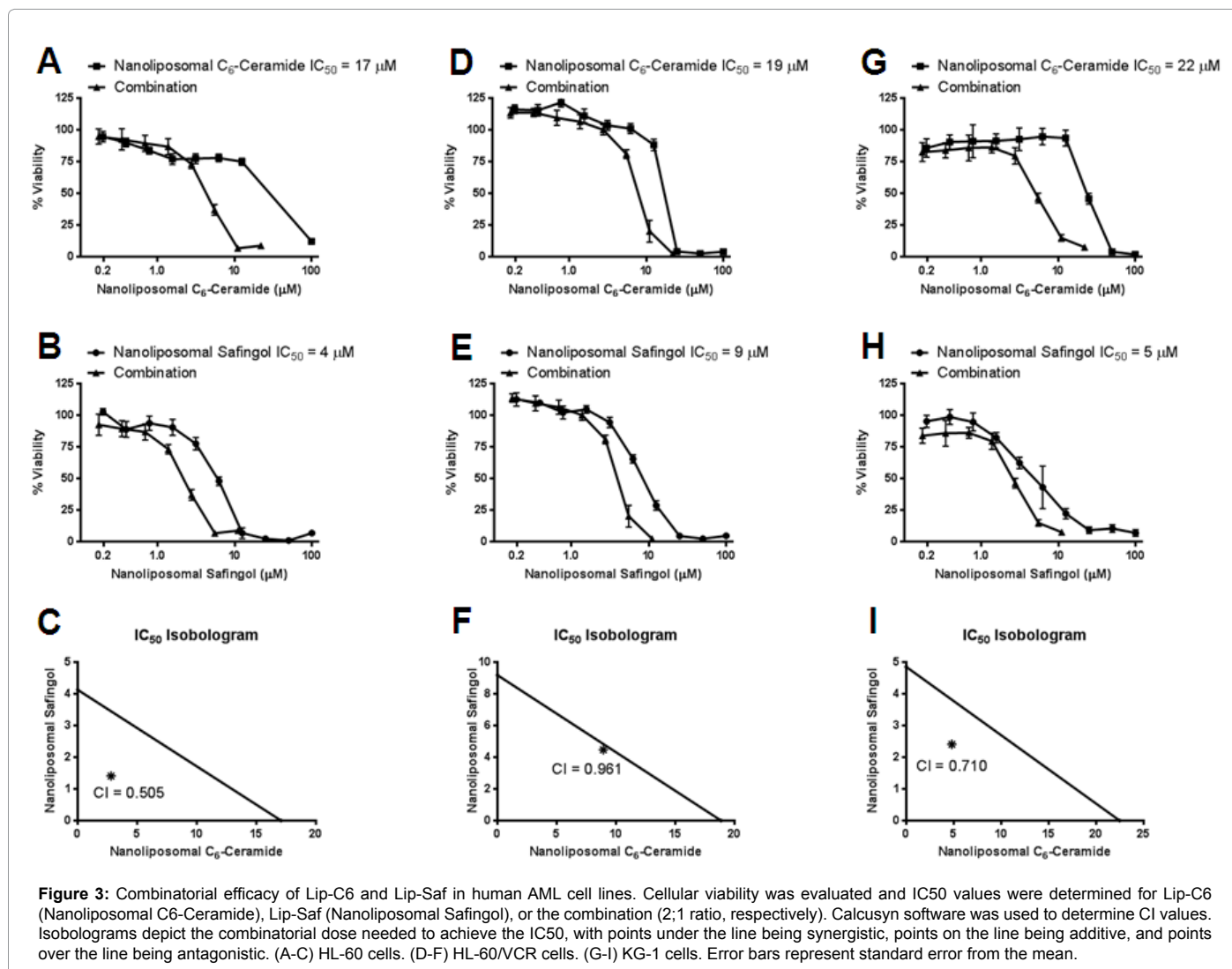
absorbance at 490 nm using a microplate reader and normalizing to the viability observed under control conditions.

Flow cytometry detection of autophagy and apoptosis

Cells were treated for 48 hours prior to autophagy or apoptosis detection. Autophagy was detected using the Cyto-ID Autophagy Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol [24]. Apoptosis was detected by Annexin V and 7-AAD staining. Patient AML samples were also stained with anti-CD34 and anti-CD38 antibodies to evaluate autophagy or 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 a LSR II flow cytometer and BD FACS Diva software.

Colony forming assay

Patient AML was prepared in MethoCult H4434 according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada). Colonies were propagated for two weeks, and blast colonies were counted in a blinded manner using a light microscope.



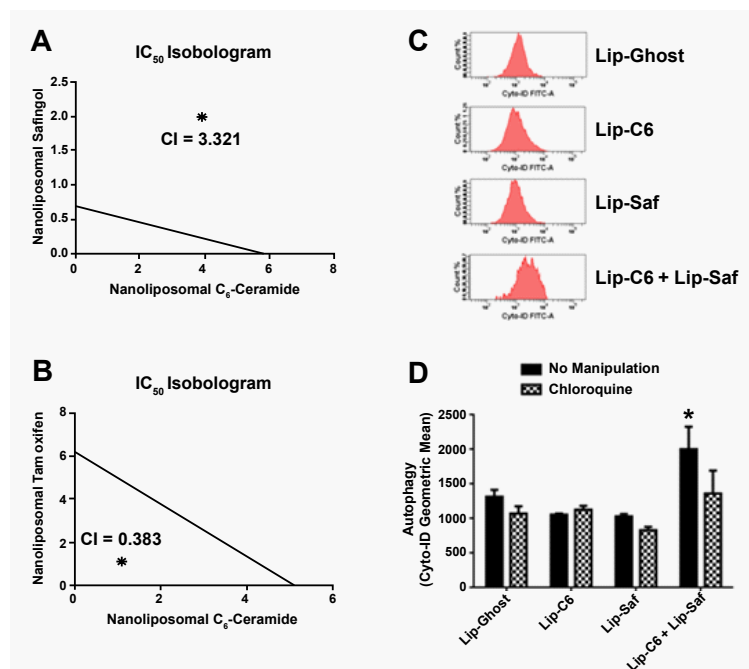


Figure 4: Increased autophagy mediates resistance of murine AML cells to the combination of Lip-C6 and Lip-Saf. The combination of Lip-C6 and Lip-Saf was evaluated using the murine AML cell line C1498. (A) Isobologram analysis revealed that the combination of Lip-C6 and Lip-Saf was antagonistic despite individual treatments being effective. (B) Isobologram analysis showed that the combination of Lip-C6 with Lip-Tam was highly synergistic using C1498 cells. (C) The Cyto-ID Autophagy Detection Kit was used to visualize autophagy by flow cytometry using C1498 cells. The combination of Lip-C6 (4 μ M) and Lip-Saf (0.75 μ M) augmented the Cyto-ID signal, indicating an upregulation of autophagy. (D) Quantification of Cyto-ID autophagy analysis. Alternatively, the autophagy blocker chloroquine was added thirty minutes prior to Cyto-ID addition to verify that the signal observed was due to an increase in autophagy. 1-way ANOVA, * $p < 0.05$ compared to all treatments. Error bars represent standard deviation from the mean.

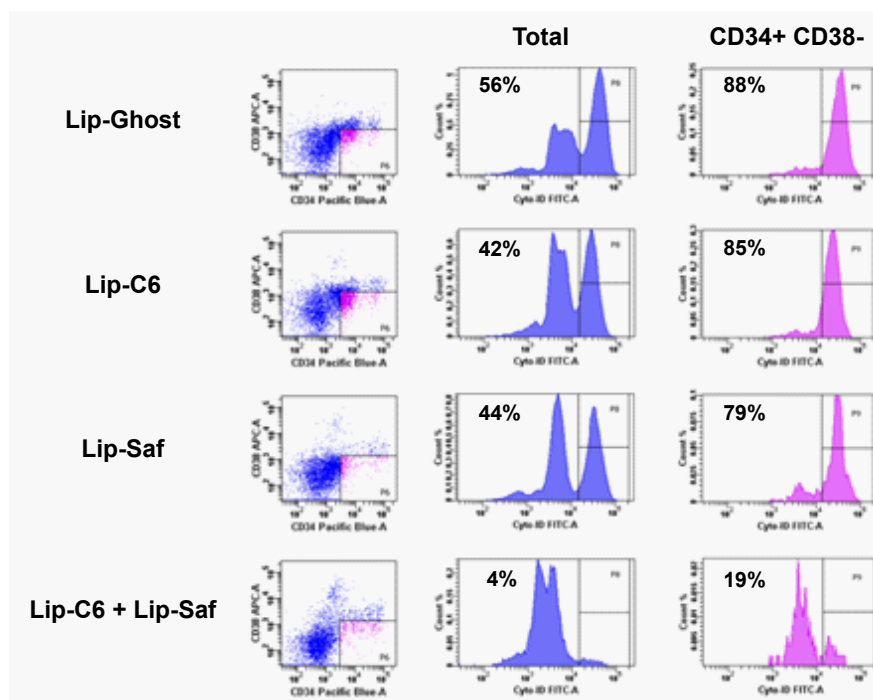


Figure 5: Therapeutic efficacy of Lip-C6 and Lip-Saf using human patient AML is reflected by a decrease of autophagy. The Cyto-ID Autophagy Detection Kit was used to visualize autophagy by flow cytometry using a favorable-prognosis human AML patient sample characterized as having translocation 8;21 (patient 632). Cells were also stained to analyze the leukemia progenitor population (CD34+CD38-). The combination of Lip-C6 (10 μ M) and Lip-Saf (5 μ M) dramatically decreased autophagy. Percentages displayed are for cells undergoing the highest level of autophagy. The intermediate stained population, not gated as positive, corresponds to normal lymphocytes.

Sphingosine kinase assay

The Sphingosine Kinase 1 Inhibitor Screening Assay kit was used according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). To determine SphK activity of cell lines, 10 µl of cells lysed in 1% Triton X-100 was substituted for 10 µl of recombinant SphK1 in the normal kit procedure. Activity was standardized based on total protein content of the lysates determined by BCA assay (Thermo Fisher Scientific, Waltham, MA).

Combinatorial analysis

CalcuSyn Software (Biosoft, Cambridge, UK) was used to determine combinatorial effects of treatments [7]. Cellular viability data was used

for this analysis, and a combination index (CI) less than or equal to 0.9 was considered synergistic. CI values greater than or equal to 1.1 were considered antagonistic, whereas CI values between 0.9 and 1.1 were considered additive.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistically significant differences between treatments ($p < 0.05$). At least three independent experiments were performed for each condition. Post hoc comparisons of specific treatments were performed using either Tukey's or Bonferroni test. For the SphK inhibitor assay a student's t-test was used to determine significance. All statistical analyses were carried out using GraphPad Prism 4 software (La Jolla, CA).

Sample	Karyotype	NPM1	FLT3-ITD	Prognosis	Combination % Apoptosis	Combination % Autophagy
329	inv3, -7	ND	ND	Unfavorable	ND	ND
632	t8;21	ND	ND	Favorable	62	4
663	Normal (prior MDS)	ND	ND	Unfavorable	46	12
664	Complex	ND	ND	Unfavorable	ND	77
667	inv16	Neg	Neg	Favorable	60	54
668	Complex	Neg	Neg	Unfavorable	96	3
669	Normal	Pos	Pos	Unfavorable	22	86

Table 1: Individual information for human clinical AML samples used to evaluate the combinatorial efficacy of Lip-C6 and Lip-Saf in apoptosis, autophagy, and colony forming assays. ND: Not determined.

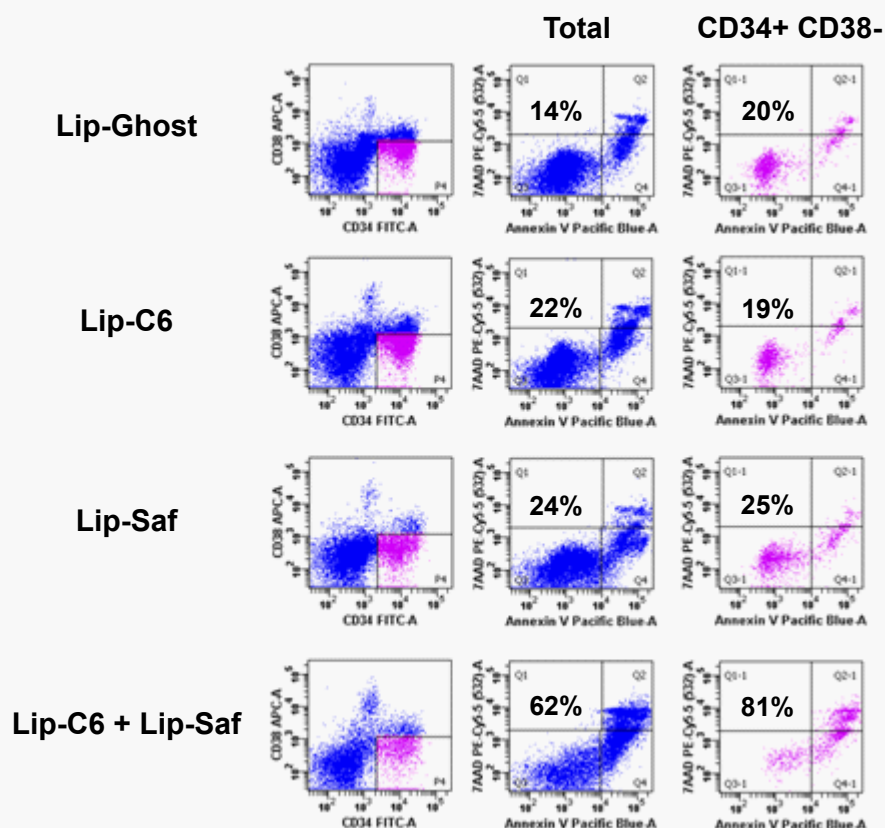


Figure 6: Therapeutic efficacy of Lip-C6 and Lip-Saf using human patient AML is reflected by an increase in apoptosis. Annexin V and 7-AAD staining were used to visualize apoptosis by flow cytometry using a favorable-prognosis human AML patient sample characterized as having translocation 8;21 (patient 632). Cells were also stained to analyze the leukemia progenitor population (CD34+CD38-). The combination of Lip-C6 (10 µM) and Lip-Saf (5 µM) dramatically augmented apoptosis. Percentages displayed are for Annexin V+ cells (upper and lower right quadrants combined).

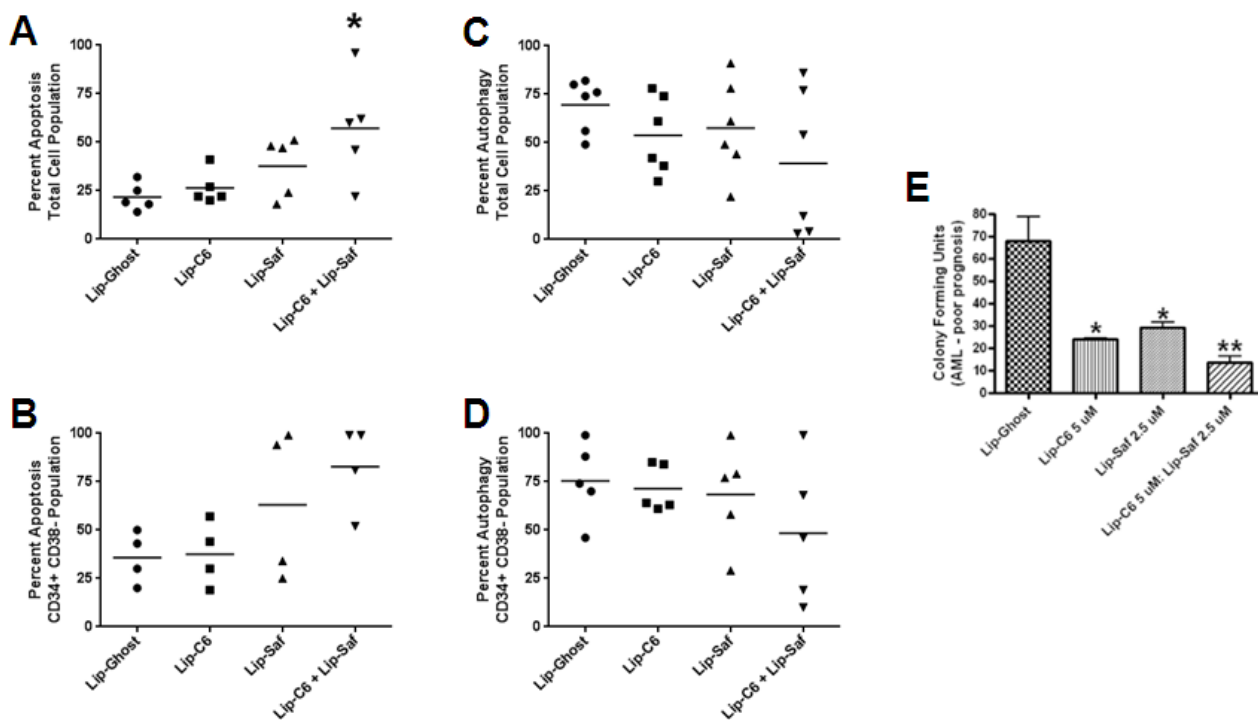


Figure 7: The combination of Lip-C6 and Lip-Saf induce cell death and decrease autophagy of human patient AML. Annexin V and 7-AAD staining were used to determine apoptosis of primary AML samples exposed to the control (Lip-Ghost), Lip-C6, Lip-Saf, or the combination. Alternatively, the Cyto-ID Autophagy Detection Kit was used to visualize autophagy. (A, C) Total cellular population. 1-way ANOVA, * $p < 0.05$ compared to Lip-Ghost. (B, D) CD34+ CD38- LSC fraction. (E) Colony forming capacity was evaluated using a poor-prognosis human AML patient sample characterized as having inversion 3 and monosomy 7 (patient 329). Lip-C6, Lip-Saf, or the combination of both, was shown to block colony forming capacity after a two week growth period. 1-way ANOVA, * $p < 0.05$ compared to Lip-Ghost, ** $p < 0.05$ compared to all treatments. Error bars represent standard error from the mean.

Results and Discussion

Our group is engaged in an effort to augment the efficacy of Lip-C6 for the treatment of cancer. In a previous study evaluating pancreatic cancer, we generated a nanoliposomal formulation that combined C6-ceramide with an inhibitor of GCS to prevent its metabolism to C6-glucosylceramide [7]. In the current study, we generated Lip-Saf to block the catabolism of C6-ceramide to S1P by SphK1, and validated these nanoliposomes using *in vitro* models of AML. Using a SphK1 inhibitor screening assay, we confirmed that Lip-Saf inhibited SphK1 at 0.625 μM (Figure 2A). Next, we adapted this same assay to determine the SphK activity in four AML cell lines. The AML cell lines that we studied, C1498, KG-1 HL-60, and HL-60/VCR (vincristine-resistant variant), were readily stratified from highest to lowest SphK activity, respectively (Figure 2B). We next expanded our evaluation of the three human cell lines, which are noteworthy of being representative of drug-resistant and poor prognosis AML, to evaluate specific sensitivity to Lip-C6 and Lip-Saf (Figure 3). In general, these three cell lines were more resistant to Lip-C6 than others we have previously evaluated (Lip-C6 IC50: HL-60=17 μM , HL-60/VCR=19 μM , KG-1=22 μM) [10]. These AML cell lines were collectively more sensitive to Lip-Saf than Lip-C6 (Lip-Saf IC50: HL-60=4 μM , HL-60/VCR=9 μM , KG-1=5 μM). Most notably, the combination of Lip-C6 and Lip-Saf resulted in synergistic efficacy using HL-60 and KG-1 cell lines, and additive efficacy using the HL-60/VCR cell line. These results demonstrated that the blockade of SphK1 using Lip-Saf can enhance the anti-AML efficacy of Lip-C6.

In contrast, while the murine AML cell line C1498 is highly sensitive to either Lip-C6 (5.8 μM) or Lip-Saf (0.7 μM) individually, the combination of these two experimental therapeutics did not result in a synergistic effect (Figure 4A). However, it is important to note that while the combinatorial effect was not synergistic the IC50 of the combination was achieved with Lip-C6 (3.9 μM) and Lip-Saf (2 μM) concentrations that were still very low. This is important because this demonstrates that the C1498 cell line is much more sensitive to individual treatments of Lip-C6 or Lip-Saf than HL-60, HL-60/VCR, or KG-1 cells. To further explore the antagonistic effects of Lip-C6 and Lip-Saf, and to offer a therapeutic alternative, we first investigated if blockade of another ceramide metabolic pathway other than SphK1 would be beneficial for the treatment of C1498 cells. Indeed, the combination of Lip-C6 with Lip-Tam induced a highly synergistic effect against C1498 cells (Figure 4B). The individual IC50 for Lip-Tam was 6.2 μM . Tamoxifen is an inhibitor of P-gp and therefore blocks the glycosylation of ceramide [6,23,25-27]. We have separately evaluated the sphingolipidome of C1498 cells and have observed a strong propensity to convert C6-ceramide to C6-glucosylceramide (data not shown). This corroborates our current findings that the combination of Lip-C6 and Lip-Tam can exert synergistic efficacy against C1498 cells. We further evaluated a mechanistic reason why the combination of Lip-C6 and Lip-Saf yielded an antagonistic effect using C1498 cells. Previous reports suggested that safingol could induce autophagy in some cancer models [14,15]. Furthermore, it has been suggested that autophagy is a mechanism by which cells can overcome stress conditions including chemotherapy [16,17,28]. For these two reasons, we evaluated autophagy as a possible reason why the combination of

Lip-C6 and Lip-Saf induced an antagonistic effect in C1498 cells. We observed that the combination of Lip-C6 and Lip-Saf, but not individual treatments, induced autophagy in C1498 cells (Figures 4C and 4D). Altogether, these results suggest that up-regulation of autophagy is a mechanism that some AML cells may use to overcome the combination of Lip-C6 and Lip-Saf. Moreover, alternative approaches blocking the metabolism of C6-ceramide, such as blockade of the GCS/P-gp pathway, can effectively be used for anti-AML therapy in these cases.

We lastly evaluated the combination of Lip-Saf and Lip-C6 using primary human patient AML samples. We evaluated several cases of AML spanning from favorable to unfavourable prognosis outlooks (Table 1). As a model case, we demonstrated using a case of favorable prognosis AML (translocation 8;21), that the combination of Lip-C6 and Lip-Saf blocked autophagy in both the total AML population as well as the CD34+CD38- AML progenitor population (Figure 5). We next showed that the combination augmented apoptosis using the same case (Figure 6, indicating that apoptosis is induced concomitantly as autophagy is down-regulated. For the entire cohort of samples evaluated, the combination of Lip-C6 with Lip-Saf significantly stimulated apoptosis in the total AML population (Figure 7A; Table 1). Apoptosis was also induced by the combination of Lip-C6 and Lip-Saf in many of the primary sample CD34+ CD38- LSC fractions (Figure 7B), and this was often associated with a decrease in autophagy in either the total or LSC fractions (Figures 7C and 7D; Table 1). We also observed a similar result using a colony forming assay to evaluate the combination of Lip-C6 and Lip-Saf when evaluating a poor prognosis case of AML (inversion 3, monosomy 7). In this situation, we observed efficacy for the individual treatments, but a significantly more efficacious anti-AML effect with the combination (Figure 7E). Collectively, these results demonstrate that the combination of Lip-C6 and Lip-Saf can exert combinatorial anti-AML efficacy using primary clinical samples.

Finally, it is noteworthy that sensitivity to ceramide-based therapeutics is not necessarily reflective of the standard risk assessment for human AML. It is hypothesized that sensitivity to ceramide-based therapeutics such as Lip-C6, Lip-Saf, Lip-Tam, or combinations of these therapeutics, is directly related to the ability of a given AML to metabolize ceramide and specifically by what pathways. In-depth sphingolipid metabolic analysis would fully reveal the utility of ceramide-based therapeutics for the treatment of AML by identifying sensitivity to specific therapeutics. More so, ceramide metabolism in AML could be further explored as a means to enhance risk stratification. The further advancement of the preclinical development of ceramide-based therapeutics has the potential to ultimately improve the outcomes of AML patients that otherwise may have unfavourable outcomes with standard chemotherapy.

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