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Combinatorial Efficacy of Nanoliposomal Ceramide and the Antioxidant 7,8-Benzoflavone for Acute Myeloid Leukemia

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Abstract

Ceramide-based therapeutics have gained recent attention as anti-neoplastic therapeutics. These include standard of care therapeutics that in part exert efficacy through the generation of ceramide, as well as new therapeutics that seek to specifically deliver or augment ceramide levels in malignant cells. Ceramide is a bioactive sphingolipid involved in apoptotic and stress cellular signaling pathways. It has also been shown to regulate oxidative stress, which may negate its otherwise anti-neoplastic effects by promoting the proliferation of leukemia cells. Metabolism of ceramide to neutral or pro-oncogenic metabolites can serve as a further pathway of therapeutic resistance. In this study, the antioxidant 7,8-benzoflavone (BF) was identified through a natural products chemical library screening process as a compound that can augment the efficacy of nanoliposomal C6-ceramide (Lip-C6) in cellular models of Acute Myeloid Leukemia (AML). This study demonstrates that BF exerts an antioxidant effect in AML, which likely refines the bioactivity of ceramide as an anti-leukemic agent. Intriguingly, BF has been shown to block drug efflux pumps, such as P-glycoprotein, allowing BF to also impede P-glycoprotein-mediated ceramide glycosylation. In this study, BF was further formulated into nanoliposomes for *in vivo* studies using two murine models of AML. Treatment of C3H/HeJ mice engrafted with a FLT3-ITD driven AML with a combinatorial nanoliposomal formulation of BF and Lip-C6 significantly augmented the survival of mice beyond that of nanoliposomal formulations containing either agent alone. This was in contrast to the modest extension of survival of C57BL/6J mice engrafted with C1498 AML cells utilizing either single agent or combinatorial nanoliposomal formulations. Altogether this study demonstrates that the anti-AML efficacy of Lip-C6 as a ceramide-based therapeutic can be augmented for particular types of AML, such as that driven by FLT3-ITD, by combinatorial treatment with the antioxidant BF.

Keywords: Acute myeloid leukemia; Ceramide; 7,8-benzoflavone; Antioxidant; Nanoliposome

Introduction

Flavonoids are a natural plant product ubiquitous in nature whose potential benefits in medicine have been known and extensively studied [1]. 7,8-Benzoflavone (BF) is a synthetic flavonoid that has previously been implicated in aromatase inhibition, breast cancer resistant protein inhibition, and aryl hydrocarbon receptor signaling [1-4]. Flavones have been investigated as chemopreventive agents due to their ability to scavenge reactive oxygen species, either produced de novo or by carcinogens, and have been shown to be antiproliferative *in vitro* [5,6].

Over-production of Reactive Oxygen Species (ROS) can alter the redox environment of the cell and have consequences on growth regulation. In particular, oxidation of a cysteine residue in the catalytic center of protein tyrosine phosphatase prevents removal of phosphate groups on receptor tyrosine kinase (RTK) target molecules [7,8]. This, in turn, removes an important regulatory point and constitutive activation of these molecules, promoting a pro-mitogenic cellular environment [7,8]. Theoretically, alteration of the redox state of cells could prevent unregulated growth of cells. Of particular interest to this study is the balance between ROS accumulation and the propagation and survival of Acute Myeloid Leukemia (AML) cells *in vitro* through modification of tyrosine phosphatases. Several studies have linked increases in ROS to both hematopoietic stem cell proliferation and the proliferation of AML cells [9-12]. AML is a cancer of myeloid precursor cells that results in proliferation of immature myeloblasts and can often follow a rapid clinical course [13-16]. Several cytogenetic abnormalities are responsible for the development of AML and many have been clinically categorized by prognosis [15,16]. While knowledge of the molecular basis of AML has grown substantially in the past 30 years, development and implementation of new therapeutics has been somewhat stagnant [15,16].

Ceramide is a natural sphingolipid that can be produced de novo in cells or formed from a variety of metabolites and is known to be extensively involved with apoptosis [17-22]. Furthermore, altered ceramide metabolism and efflux has been linked to cellular resistance to apoptosis and is frequently found in cancer cells as a mechanism clearly associated with the development of drug resistance [17,19,21,22]. Ceramide and its metabolite, sphingosine-1-phosphate, strike a delicate intracellular balance between ceramide – induced apoptosis and sphingosine-1-phosphate-influenced cell survival and proliferation [18-20]. Another ceramide metabolite, glucosylceramide, has been linked to drug-resistant breast cancer [19-21]. Interestingly, the glycosylation of ceramide species occurs at the Golgi membrane by the coordinated activities of glucosylceramide synthase and P-glycoprotein [19,21]. Importantly, BF may behave as an inhibitor of P-glycoprotein, a drug efflux pump implicated in conferring drug resistance in multiple tumor types [23]. The use of BF has been shown to re-sensitize tumors to chemotherapies [23]. Liposomal formulations of ceramide and ceramide metabolites have been extensively studied in our lab [19,20,24,25]. Due to its apoptosis-inducing properties, cellular ceramide accumulation is an ideal pharmacologic target. The liposomal formulation of ceramide allows for stabilization of the otherwise insoluble ceramide as well as enhanced ceramide delivery to the cells [19]. Recently we demonstrated that blocking ceramide metabolism could induce apoptosis or autophagy in leukemia cells [20].

In the present study, we used a chemical library screen to identify BF as a compound that augments the anti-AML efficacy of nanoliposomal C6-ceramide (Lip-C6). We further showed that BF and Lip-C6 act synergistically to decrease the viability of multiple AML cell lines and that BF exerts an antioxidant effect independent of, and complementary to, the effect of ceramide. Finally, this study demonstrated that the combination of Lip-C6 with BF can significantly increase the survival of mice engrafted with AML driven by FLT3-ITD.

Materials and Methods

Cell culture

Human HL-60, HL-60/VCR, 32D-FLT3-ITD, 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.

Nanoliposome formulation

Nanoliposomes were prepared by the Penn State College of Medicine Drug Discovery Core following previously established methods with minor changes for the nanoliposomal BF (Lip-BF) formulation. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Ghost nanoliposomes (Lip-Ghost) and Lip-C6 were prepared as previously described [20,24,25]. Briefly, lipids dissolved in chloroform, or other organic solvents, were combined in specific molar ratios. For Lip-BF, 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 BF, were made in a 5.66:2.87:1.47:0.05 molar ratio. For Lip-C6 containing BF (Lip-C6/BF) the same molar equivalent of BF in Lip-BF was added to the Lip-C6 formulation. 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 and integrity was determined using a Malvern Zetasizer Nano ZS at 25°C. Nanoliposome formulaitons were stored at room temperature until use.

Cellular viability assays

Human HL-60, HL-60/VCR, 32D-FLT3-ITD, and murine C1498 cells, were plated at 2.5x104 cells per well in 96-well tissue culture plates and treated for 48 h. Following treatment, cellular viability was assessed using a Cell Titer 96 AQ^{ueous} Non-Radioactive Cell Proliferation Assay according to the manufacturer's instructions (Promega, Madison, WI, USA). Viability was determined by measuring absorbance at 490 nm using a microplate reader and normalizing to the viability observed under control conditions. CalcuSyn Software (Biosoft, Cambridge, UK) was used to determine combinatorial effects of treatments [24]. 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.

TimTec natural products chemical library screen

Cellular viability assays evaluating HL-60/VCR cells were used to screen the Tim Tec natural products chemical library (TimTec, Newark, DE, USA) for compounds that augmented the efficacy of Lip-C6. A screen of 480 different compounds was performed (10 μM per compound) in combination with Lip-C6 (10 μM) and compared to the Lip-C6 alone. Hits were determined as those compounds that augmented the anti-AML efficacy of Lip-C6 beyond three standard deviations from the mean according to Z-score analysis (3/480 = 0.625% hit rate).

ROS assay

HL-60/VCR cells were treated for 24 h prior to addition of 2 μM of the redox-sensitive indicator H2-dichlorofluorescein diacetate, which was added directly to the culture media 30 min prior to analysis. Upon oxidation, dichlorofuorescein (DCF) fluorescence was indicative of the generation of ROS, and was evaluated at the Penn State College of Medicine Flow Cytometry Core using a LSR II flow cytometer and BD FACS Diva software.

In Vivo studies

All procedures were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee. C57BL/6J mice were engrafted by retro-orbital injection with 1x10^6 C1498 cells and C3H/HeJ mice were engrafted by retro-orbital injection with 2.5x10^6 32D-FLT3-ITD cells. Mice were treated with Lip-Ghost, Lip-C6, Lip-BF, or Lip-C6/BF (0.1 mL i.p. injections of 25 mg/mL liposomal formulations) three times per week for four weeks or until they became moribund and were euthanized.

Results and Discussion

We screened the TimTec natural products chemical library (480 molecules) to identify compounds that enhance the anti-AML efficacy of Lip-C6. Using the HL-60/VCR human AML cell line three flavone compounds were identified including glabranine, 6,7-dimethoxyflavone, and BF (Figure 1). Overall, there was a positive hit rate of 0.625% with a positive result identified as being three standard deviations beyond the mean in a Z-score analysis. In particular, we observed BF exerting its effects due in part to its ability to act as an antioxidant. Current literature proposes that antioxidants such as BF prevent oxidation of cysteine residues in the catalytic center on tyrosine phosphatases that can lead to unregulated proliferation by constitutive activation of RTK second messengers [7,8]. Additionally, BF may behave as an inhibitor of drug efflux pumps such as P-glycoprotein [1,23]. In addition to traditional roles eliminating cytotoxic agents from cells, drug efflux pumps such as P-glycoprotein have also been shown to participate in the metabolism of ceramide to glucosylceramide by glycosylation at the Golgi membrane [19-21]. Therefore, in addition to an antioxidant role for BF, alteration of the activity of P-glycoprotein may serve to alter the metabolism of ceramide to favor its accumulation. For these reasons, we chose to further evaluate the combination of BF and ceramide as an anti-AML therapeutic strategy.

Following the identification of BF from screening the Tim Tec natural products library as a compound that can augment the anti-AML efficacy of Lip-C6, a more thorough analysis of the combinatorial effects was evaluated using a variety of AML cell lines and cellular viability assays (Figure 2). Profound combinatorial efficacy of BF and Lip-C6 was observed in both 32D-FLT3-ITD and HL-60/VCR cell lines, with synergistic efficacy confirmed for the later (CI=0.177). Parental HL-60 cells (not selected by vincristine resistance) and C1498 cells were sensitive to either agent alone but only combinatorial effects were modest and/or not dose dependent. For the more substantial combinatorial effects observed with 32D-FLT3-ITD and HL-60/VCR cells the combination promotes an anti-AML effect at lower doses than either treatment alone. It is possible that C1498 cells do not show this same combinatorial effect due to differential expression of drug efflux pumps [20], and therefore less sensitivity to BF antagonism of ceramide glycosylation, or because they do not generate and benefit from a profound pro-oxidant state. In further support of the notion that BF exerts its combinatorial anti-AML efficacy with Lip-C6 by acting as an antioxidant, by using an ROS assay we observed that HL-60/VCR cells existed in a substantial pro-oxidant environment that was specifically down regulated by BF (Figure 3). This was noted both with and without the addition of Lip-C6. By reducing the redox state of the cells, BF may prevent cysteine oxidation on protein tyrosine phosphatases which otherwise may lead to unregulated growth and proliferation [7,8]. It is known that ceramide induces oxidative stress within the cell. Therefore, the addition of BF may relieve this pro-oxidant effect which would refine the effect of ceramide to be more specifically directed towards inducing apoptosis of the malignant cells. More so, BF may be a better candidate to pair with Lip-C6 than other antioxidants, such as vitamin E, given BF's potential role in blocking ceramide glycosylation by impeding P-glycoprotein [19-21].

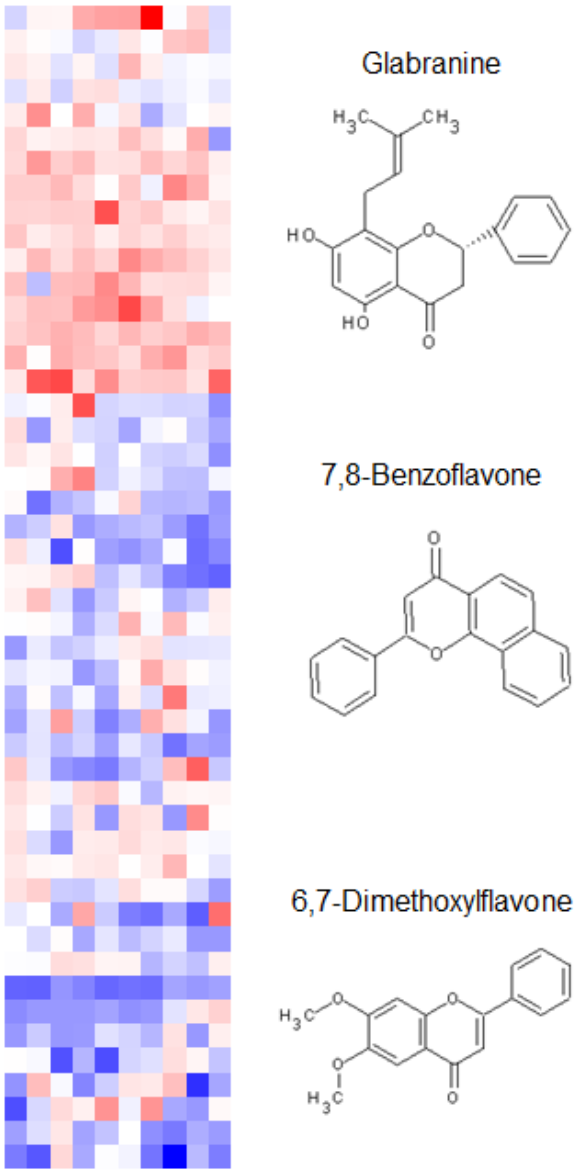

Figure 1: Screen of natural products chemical library evaluating those that augment the anti-AML efficacy of nanoliposomal C6-ceramide. Z-score analysis was used to identify hits as those augmenting therapeutic efficacy at least 3 standard deviations from the mean. 3/480 hits were identified (0.625%).

A murine study further reinforced the in vitro findings. First, to improve its *in vivo* delivery BF was formulated into nanoliposomes with or without C6-ceramide (Figure 4a). The anti-AML efficacy of Lip-BF compared with free BF using the 32D-FLT3-ITD cell lines, and was found to have nearly equivalent efficacy (Figure 4b). C57BL/6J mice engrafted with C1498 cells (Figure 4d), and C3H/HeJ mice engrafted with 32D-FLT3-ITD cells (Figure 4e), were then treated with Lip-Ghost, Lip-C6, Lip-BF, or the combinatorial Lip-C6/BF. Survival curves from mice engrafted with 32D-FLT3-ITD cells demonstrated enhanced survival with the combinatorial liposomal formulation than

compared to control and either treatment alone. However, the mice engrafted with C1498 cells showed no enhanced survival by the combinatorial formulation compared to single agent treatment. These *in vivo* studies confirmed *in vitro* observations, suggesting that the combination of BF with Lip-C6 may only work in the scenarios where the leukemia has a profound pro-oxidant state by offsetting the additional and counterproductive pro-oxidant actions of Lip-C6. This was evidenced in our study by the substantial increase in survival of C3H/HeJ mice engrafted with 32D-FLT3-ITD cells, with 60% of the mice surviving long term when treated with the combinatorial Lip-C6/BF formulation. Overall, this study identified the antioxidant BF as a compound that can effectively be combined with Lip-C6 for the treatment of certain AMLs with enhanced oxidative states such as those harboring mutations of FLT3 [12]. The further development of liposomal formulations of BF, and other antioxidants, may hold a promising future for the treatment of aggressive AML.

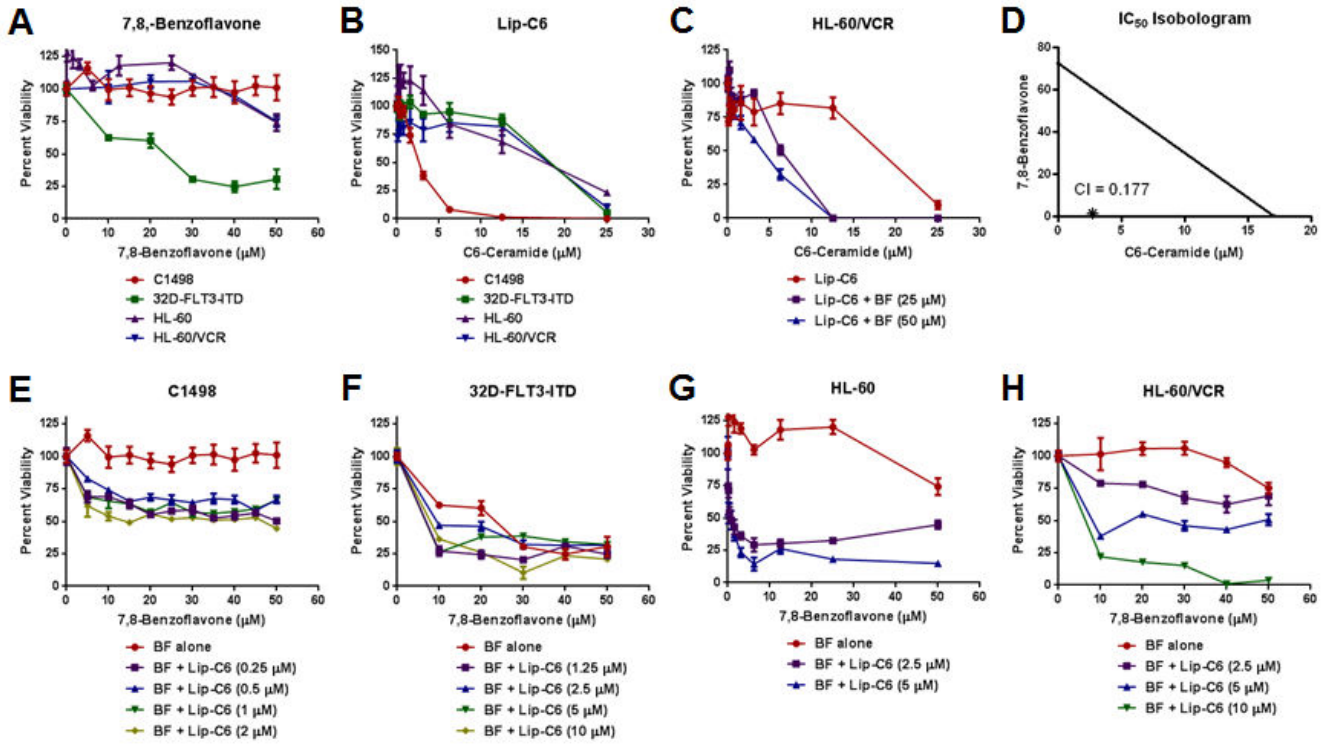

Figure 2: AML cell lines were evaluated for therapeutic sensitivity to nanoliposomal C6-ceramide (Lip-C6) and 7,8-benzoflavone (BF) using cellular viability assays. (A) BF anti-AML efficacy was evaluated using murine C1498 and 32D-FLT3-ITD cells as well as human HL-60 and HL-60/VCR cells. (B) Lip-C6 anti-AML efficacy was evaluated using murine C1498 and 32D-FLT3-ITD cells as well as human HL-60 and HL-60/VCR cells. (C) The combination of Lip-C6 and BF was evaluated using HL-60/VCR cells by evaluating a range of Lip-C6 concentrations while holding BF at constant concentration. (D) Isobologram depicting the synergistic combinatorial index (CI) observed for Lip-C6 and BF using HL-60/VCR cells. The combination of Lip-C6 and BF was evaluated using C1498 (E), 32D-FLT3-ITD (F), HL-60 (G), and HL-60/VCR (H) cells by evaluating a range of BF concentrations while holding Lip-C6 at constant concentration.

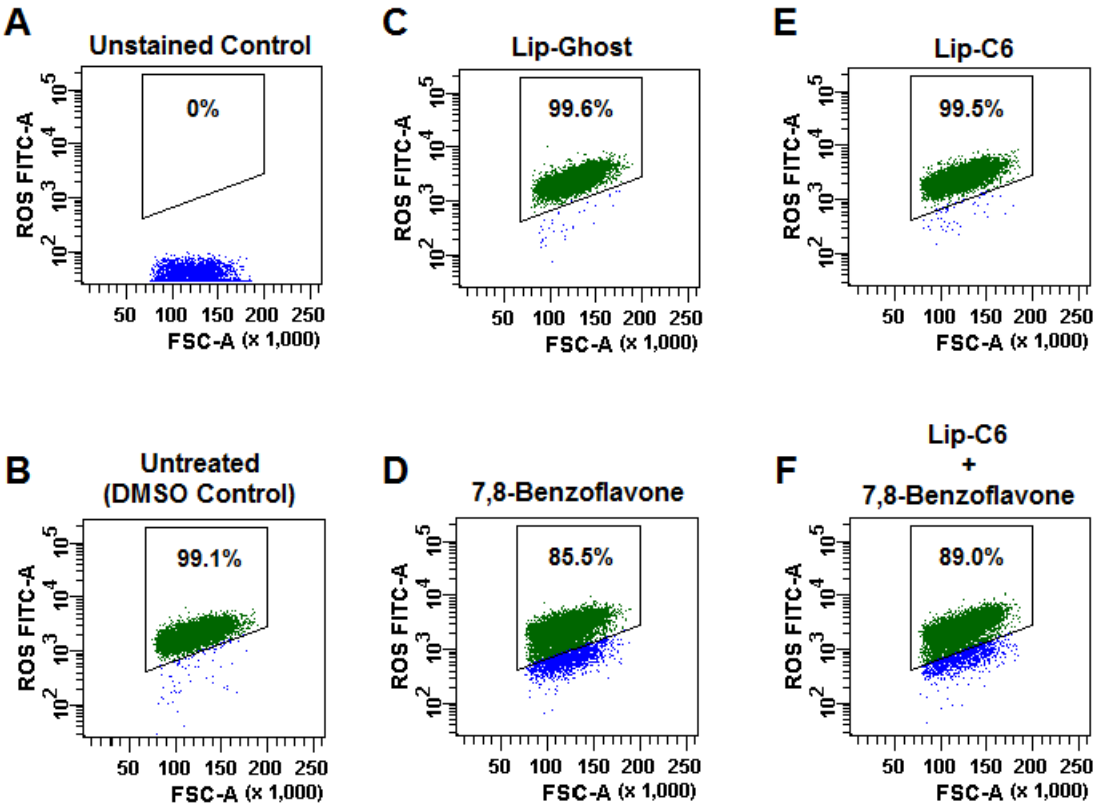

Figure 3: 7,8,-Benzoflavone (BF) exerted an antioxidant effect in HL-60/VCR cells, an effect apparent where nanoliposomal C6-ceramide (Lip-C6) and BF exert synergistic efficacy. Following treatment, cells were loaded with a redox-sensitive indicator and analyzed by flow cytometry. Untreated control (A), DMSO vehicle control (B), empty "ghost" nanoliposomal (Lip-Ghost) control (C), BF treatment (D), Lip-C6 treatment (E), and treatment with the combination of Lip-C6 and BF (F).

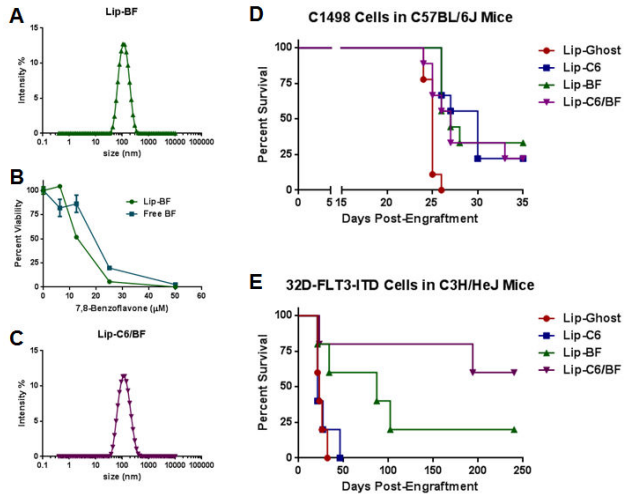

Figure 4: Nanoliposomal formulation of 7,8-benzoflavone (BF) and *in vivo* evaluation of anti-AML efficacy with nanoliposomal ceramide (Lip-C6). (A) Light scatter analysis confirmed nanoliposomal BF (Lip-BF) nanosize and stability. (B) Cellular viability assays confirmed that Lip-BF and free/unencapsulated BF exerted similar therapeutic efficacy using 32D-FLT3-ITD cells. (C) Light scatter analysis confirmed combinatorial nanoliposomal C6-ceramide and BF (Lip-C6/BF) nanosize and stability. (D-E) Survival was evaluated using two murine models treated with liposomal formulations. Combinatorial extension in survival was only observed in 32D-FLT3-ITD-engrafted C3H/HeJ mice, but not C1498-engrafted C57BL/6J mice. This confirmed in vitro studies which showed that 32D-FLT3-ITD cells were synergistically sensitive to Lip-C6 and BF, but C1498 cells were not.

Acknowledgements

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