The Effect of Liposomal KT109 and c6-Ceramide on Reducing Inflammation in the Liver

Authors:

Sydney Henriques

Helena Snyder, PhD

Timothy Ware

Ku-Lung Hsu, PhD

Mark Kester, PhD

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The Effect of Liposomal KT109 and c6-Ceramide on Reducing Inflammation in the Liver

Sydney R. Henriques[a,1], Helena W. Snyder[b], Timothy Ware[c], Ku-Lung Hsu[c], & Mark Kester[d]

[a] Department of Biomedical Engineering, The University of Virginia
[b] Department of Material Science, The University of Virginia
[c] Department of Chemistry, The University of Virginia
[d] Department of Pharmacology, The University of Virginia
[1] Correspondence: srh2eq@virginia.edu, 5404143685

Abstract

This paper reports on a study designed to test the effects of KT109 and c6-ceramide in reducing inflammation in those diagnosed with nonalcoholic steatohepatitis (NASH). NASH involves inflammation of the liver as a result of excess adipose tissue on the liver. This study explored possible mechanisms for reducing inflammation via two drugs, KT109 and c6-Ceramide. Diacylglycerol lipase-beta (DAGLβ) is an enzyme that cleaves diacylglycerol (DAG) to produce numerous endocannabinoids involved in inflammatory signaling. KT109 is a triazole urea inhibitor known to inhibit DAGLβ, therefore inhibiting the production of inflammation inducing endocannabinoids. Both c6-Ceramide and KT109 have been found to activate protein kinases (PKC) zeta and epsilon, respectively, which leads to the upregulation of adenosine monophosphate activated protein kinase (AMPK), which is also known to reduce inflammation. These drugs were attempted to be encapsulated in a combination liposome, with both hydrophobic drugs existing in the phospholipid bilayer. The attempt at encapsulation had mixed results. These liposomal drugs were delivered to mice via injection. While the results were not significant, it was found that in liposomal form, the drugs both inhibited DAGLβ and activated AMPK. It was determined that the drugs were more successful at impacting these markers of inflammation when prepared in separate liposomes and then mixed together before injection, rather than in the combination liposome. Based on our results, our study proposes a potential novel treatment to the disease of NASH via liposomal forms of KT109 with c6-ceramide.

Keywords: Nonalcoholic steatohepatitis, liposome, DAGLβ, AMPK

Introduction

Nonalcoholic Steatohepatitis

The liver, which is the largest gland in the human body, is involved in over 150 functions, including the catabolism of toxins, the storage of glycogen and iron, and playing a major role in the immune system[1]. Therefore, it is essential that the liver's health is maintained in order for organisms to function as a whole. Nonalcoholic fatty liver disease (NAFLD) is a condition that involves the excess of adipose, or fatty tissue, in the liver. Although NAFLD patients do not normally experience intense symptoms, the buildup of fat on the liver can lead to more harmful liver conditions such as nonalcoholic steatohepatitis (NASH), which occurs when the liver tissue becomes inflamed and often causes pain[2]. It is estimated that over a quarter of American adults suffer from the condition and of these, 20% develop a more serious version, non-alcoholic steatohepatitis (NASH)[3]. Prolonged inflammation due to NASH can eventually progress into cirrhosis, a disease characterized by tissue scarring and cell damage (Figure 1). NAFLD also contribute to an increased risk of cardiovascular disease[3]. The risk of being affected by these conditions dramatically increases with being overweight as roughly 50% of obese and diabetic (type-2) people having some form of steatohepatitis[3,4]. Currently, there are no pharmaceutical treatments for NASH on the market.

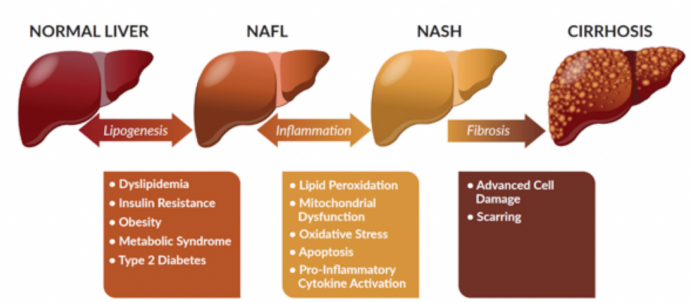

Figure 1. NASH Progression Diagram illustrates the progression from a normal liver to NAFLD to NASH to eventual cirrhosis. https://ivtd.research.vub.be/en/nafld-nash-modelling

Instead, physicians will typically recommend weight loss induced by healthier diets and exercise, dietary supplements such as vitamins D, E, and omega-3, and for patients with diabetes, insulin medication can help[5]. However, these methods are not always effective or able to be adapted by the patient. Therefore, it is vital that an effective medical treatment be developed in order to treat the effects of these diseases. The goal of this study is to investigate the potential roles that liposomal KT109 and c6-Ceramide could play in the reduction of inflammation, which could provide a therapy for NASH.

KT109 and c6-Ceramide

While there are few treatments for NASH, there is growing evidence that the suppression of certain endocannabinoids—lipid metabolites—could aid in reducing inflammation and lipogenesis in affected livers[6]. KT109 is a chemical compound that is a selective inhibitor of diacylglycerol lipase-β (DAGLβ)[7]. DAGLβ is an enzyme that primarily aids in the production of endocannabinoids via the destruction of diacylglycerol (DAG), including arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG)[6,8].

KT109 and c6-Ceramide, our second drug, have both been previously shown to increase the presence of protein kinases (PKC) epsilon and zeta, respectively[9,10]. The increase in these PKCs upregulates the presence of adenosine monophosphate-activated protein kinase (AMPK), which has been found to reduce inflammation[11,12]. It should be noted that c6-Ceramide is typically an apoptotic drug that has been previously used to treat various cancers. The potential toxicity of c6-ceramide will need to be evaluated in order to deem this drug combination safe for human use[13].
Prior to this study, we have tested liposomal KT109 in its ability to reduce DAGLβ in macrophages. It has been found that the 5 micrograms of liposomal KT109 has reduced DAGLβ up to 80% in these macrophages[7].

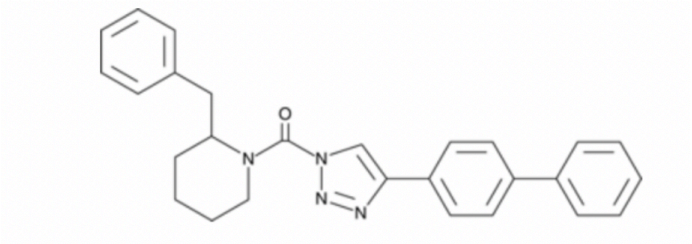

Figure 2. KT109 Diagram of the structure of a KT109 molecule
https://www.caymanchem.com/product/18933/kt109

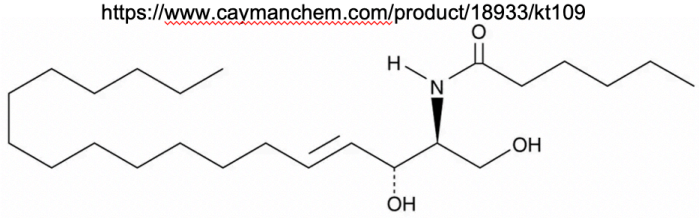

Figure 3. c6-Ceramide Diagram of the structure of a c6-ceramide molecule
https://www.caymanchem.com/product/62525/c6-ceramide-(d18%3A1-6%3A0)

Liposomes

Liposomes are vital to this study as they are the delivery vehicle of the drugs through the body. Liposomes are nanovesicles that are hollow spheres with an outer lipid bilayer[14]. These lipid spheres are a beneficial method of drug delivery, especially compared to injection of free drugs because of liposomes' non-toxic properties and ability to be used for targeted delivery[15,16]. The variety of lipids that can be used to form liposomes allow for them to be heavily altered and tailored for specific drugs. Different methods of preparation can alter the size, charge, and half-life of the liposomes[17]. Compared to other nanoparticles, liposomes are the most extensively researched, can achieve a high payload of drug, and are easily prepared[18]. However, while they have lots of benefits with many drugs, there are some potential disadvantages as compared with free drug injection. Some of these disadvantages include

their high production cost, risk of leakage, and potentially shorter half-life[19].

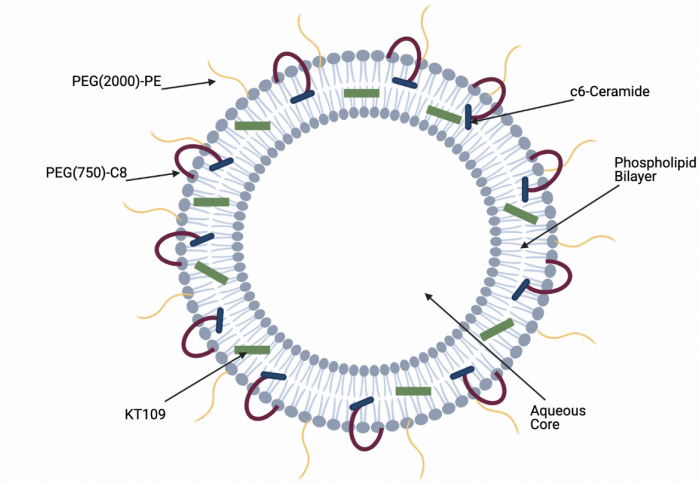

Figure 4. Combination Liposome The diagram illustrates the combination liposome with both both c6-ceramide and KT109. Both hydrophobic drugs exist in the phospholipid bilayer. Created using BioRender

The liposome used in this study, which can be seen in the diagram in Figure 4, is composed of four major components: 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), dioleoyl phosphatidylethanolamine (DOPE), PEG(2000)-PE, and PEG(750)-C8. DPSC is a phospholipid with a high transition temperature, thus promoting a rigid structure within the liposome[20]. DOPE, a neutral phospholipid, stabilizes the liposome and its neutrality helps to increases the hydrophobicity of the lipid bilayer to promote interaction with cell walls[20].

Without anything to inhibit it, liposomes are taken up by the mononuclear phagocyte system and typically have a short half-life. By coating the outside of the liposome with polyethylene glycol, or specifically PEG(2000)-PE. PEG(2000)-PE is a polymer that creates a steric barrier around the liposome and thus increases it's longevity[21]. The final component is another polymer, PEG(750)-C8, which like PEG(2000)-PE also binds to the outer layer of the liposome, and then binds to the c6-ceramide[22]. C6-Ceramide is hydrophobic, so once it binds to the PEG(750)-C8, it folds back into the hydrophobic phospholipid bilayer. KT109 is also hydrophobic, so it exists in the bilayer with the c6-Ceramide, creating a competition for space.

In this study, we aimed to see if we could develop a combination liposome with both KT109 and c6-ceramide in sufficient concentrations and then tested the effects of our liposomal drugs in reducing DAGLβ and activating AMPK. It was hypothesized that the two drugs will work synergistically together to reduce inflammation.

Results

Liposome Efficiency

Several trials were performed to test the efficiency and replicability of the combination liposomes. Table 1 displays the results of these trials. As seen in the table, there is a wide range of encapsulation

Table 1. Liposome Encapsulation The concentrations of KT109 liposomes and KT109+C6 liposomes over five trials. C6-Ceramide binds to PEG (750)-C8 and is produced in standard concentrations each time and therefore not tested. Concentrations were determined using mass spectrometry.

Trial	KT109 liposomes (ug/mL)	Kt109 + C6 liposomes (ug/mL)
Previously collected Kt109 data	19	N/A
Trial 1	N/A	53.8 +/- 1.5
Trial 2	N/A	5.41 +/- 0.04
Trial 3	N/A	19.7 +/- 0.26
Trial 4	29.9 +/- 0.78	19.7 +/- 0.29
Trial 5	24.8 +/- 0.29	90.5 +/- 1.2

concentrations of the KT109 in the combination liposomes, which vary from roughly 5 ug/mL to 90 ug/mL. It should be noted that since c6-Ceramide binds to the PEG(750)-C8, it produces similar concentrations each time and therefore was not tested. Both KT109 and c6-ceramide are hydrophobic drugs that exist in the phospholipid bilayer and therefore, there is a competition for space. The lower concentrations of KT109 in the combination liposome in Trials 2 through 4 were expected, as there would be limited space for the KT109 to exist in the bilayer due to the presence of the c6-ceramide, compared to the liposomes with just KT109. However, the substantial increases in KT109 concentrations in Trials 1 and 5 were unexpected. Lipidomic studies to image the liposome are necessary to see how the KT109 and c6-ceramide interact within the liposome to help us understand why the increase in KT109 concentration occurred. Such a large variability in the KT109 concentrations is a point of concern for the viability of the liposomes as a marketable drug. If the liposomes are unable to be produced at a replicable concentration, then it would be difficult to manufacture them at a large quantity for a marketable drug. Although this was a potential issue with the drug, we tested its effectiveness in reducing DAGLβ and AMPK upregulation in order to evaluate if it had serious potential as an anti-inflammatory.

DAGLβ Inhibition

Mice were injected with the liposomal drugs and after four hours were sacrificed. Their macrophages were harvested from their bone marrow and Western blots were performed to determine if DAGLβ levels were inhibited in the presence of the different treatments. The six treatment groups included a control of DMSO, free KT109 not in a liposome, the c6-ceramide liposome (CNL), liposomal KT109 with no c6-Ceramide, the combination liposome with both KT109 and c6-Ceramide, and finally the CNL mixed with a separately prepared KT109 liposome.

Figure 6 shows the results of the Western that was performed. It can be seen that the mixture of the two separate liposomes had the lowest intensity band, meaning it inhibited DAGLβ the most. Aside from this treatment group, KT109 both as a free drug and in a liposome was also successful in inhibiting DAGLβ, but not as efficiently as the two separate liposomes, whereas the CNL, and combination liposome were not inhibitory. This experiment was repeated, and similar results were found.

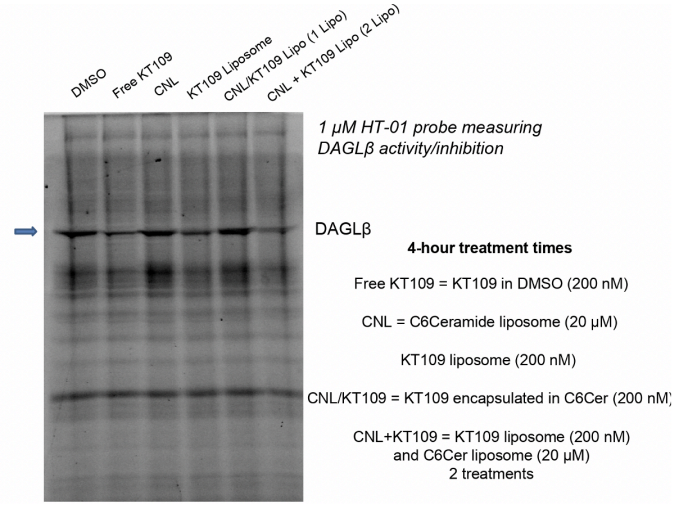

Figure 5. DAGLβ Inhibition Western blot showing DAGLβ inhibition in the presence of six different trials. Intensity of the band shows the amount of DAGLβ expressed. Experiment was repeated and produced similar results.

These results were unexpected, as it was hypothesized that the combination liposome would perform comparable or better than the separately prepared and then mixed liposomes. It is possible that the interaction of the c6-ceramide and Kt109 within the phospholipid bilayer affected how the drugs were released and how they interacted with the cells of the mice. When delivering the liposomes, the concentrations were controlled to the amount of KT109, meaning each mouse received the same amount of KT109 between the combination and separately prepared liposomes. This likely meant the amount of c6-Ceramide was reduced in the treatment group with the combination liposome, which is one possibility as to why it was less effective. However, these results were promising in showing that the c6-Ceramide and KT109 worked synergistically together to inhibit the presence of DAGLβ.

AMPK Activation

Western blots analysis was performed to evaluate the presence of AMPK. The same trial groups were done in this experiment as were done with the DAGLβ trials. Figure 7a shows the actual images of the Western blots done and Figure 7b shows the average values given to the intensities of the bands, with the DMSO acting as a control and given a value of 100. Similar to the DAGLβ trials, the separately prepared and mixed

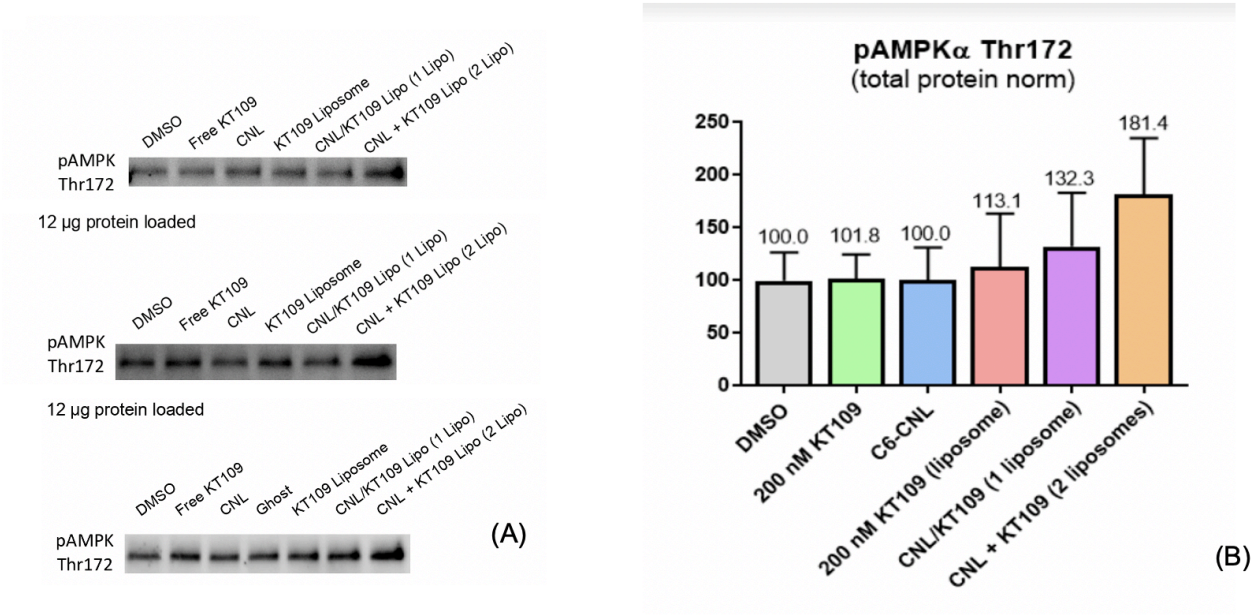

Figure 6. AMPK Upregulation (A) The bands from the three Western blots performed, showing the intensity of the amount of AMPK present. (B) Averaged values associated with the intensities of the bands of the Western blots in (A). A one-factor ANOVA test was run, and no significance was found (n=3).

liposomes were the most successful at activating AMPK. There was almost no activation of the AMPK with the free KT109 or c6-Ceramide liposome, and only slight activation with the KT109 liposome and combination liposome. Again, this is likely due to potential factors listed in the previous section. It was encouraging to see at least a slight increase in performance in the combination liposome compared to the KT109 liposomes and CNLs. A One-Factor ANOVA test was performed, and no significance was found between the treatment groups ($p > 0.05$). Although there was not a significant difference between the groups, the results were still promising to show that the c6-ceramide and KT109 worked synergistically together to activate AMPK.

Discussion

Significance

Proper liver function is vital for the overall health of an organism. When there is excess fat in the liver, there is an increased risk for very serious illnesses such as cardiovascular disease and NASH. Non-alcoholic fatty liver disease is also highly prevalent, affecting approximately 25% of American adults. A major problem of the disease is the lack of treatment that exists today. The two drugs, KT109 and c6-ceramide, have shown both inhibitory effects on DAGLβ and activate AMPK, which have positive implications for inflammation reduction. Overall, the results from the study so far are promising and the liposomal drug forms are strong potentials for NASH treatments. Likewise, the liposome can be altered to treat other areas of inflammation within the body.

Design Constraints and Limitations

While NASH is the main target for this drug, current NASH mice models are not widely accepted within the community. As of now, there are many confounding variables involved with NASH models, hence the reason for their lack of acceptance. For this reason, the testing done in this study focused on the effects of markers of inflammation, rather than treating NASH itself.

COVID-19 caused massive delays in this project. Restrictions put in place by the pandemic resulted in a halt in the study, but as restrictions are being lifted, it is hoped that additional testing will be completed, and a paper will be soon submitted for publication.

Future Testing

Several more experiments will need to be conducted in order to confirm our results. First, lipidomic studies

are required to better understand why the combination liposome was not as successful compared to the separately prepared and mixed liposomes. Lipidomic studies involve the testing of networks and interactions between lipids. Performing lipidomic studies will help evaluate how the drugs exist within the liposomes and their release patterns. This will allow us to determine any major discrepancies between the behavior of the combination liposomes and the separately prepared liposomes. While these lipidomic studies will be used to better understand the liposomes, if the separately prepared and mixed liposomes continue to outperform the combination liposome, it is likely that the study will continue using the two separate liposomes, since they are also easier to produce with replicable results.

Although Western blots were performed to test the activation of AMPK in the presence of the liposomal drugs, Westerns will also need to be performed to evaluate if there is an increase in PKC zeta and epsilon. This is necessary to establish a pathway of action from the drugs to the reduction in inflammation.

The selectivity of the liposomes will also need to be evaluated. To determine this, we will add an additional treatment group to our mice trials. In this group, a fluorophore will be incorporated into the liposomes. When analyzing the mice bodies four hours after injection, we will use the fluorophore to determine where in the body the liposomes aggregated. It is hypothesized that they will aggregate in the liver, carried there by macrophages. If our hypothesis is correct, there is no need to bioconjugate a ligand to aid in targeting the liver. However, if it is found that the liposomes collect in areas of the body to a level that is potentially toxic, a ligand will be bioconjugated to the liposomes to ensure aggregation in the liver.

Materials and Methods

Liposome Preparation

KT109 was measured out and placed into an Eppendorf tube. It was then diluted in chloroform to a concentration of 500 micrograms per milliliter. DSPC, DOPE, PEG(2000)-DSPE, PEG(750)-C8, and c6-ceramide were added to a glass vial at a molar ratio of 3.75:1.75:0.75:0.75:3, respectively. One milliliter of the lipid solution was combined with one milliliter of the KT109-chloroform solution. A KT109 only solution was also prepared. DSPC, DOPE, and PEG(2000)-DSPE were combined at a molar ratio of 5.66:2.87:1.47. Similarly, one milliliter of this solution was added to one milliliter of the KT109 solution.

Nitrogen blower

Each of the samples were placed in the nitrogen blower for roughly two hours. This was in order to ensure all of the chloroform had evaporated from the lipids, leaving a dried down liposome layer at the bottom of the glass vials. The dried down liposome precipitate was then placed in a freezer at 4◦ Celsius until rehydration occurred.

Heater Shaker

After removing the samples from the freezer, one milliliter of 1X phosphate buffer saline was added to each of the samples. The samples were then covered with parafilm to keep any liquid from evaporating. The samples were vortexed and then placed in a heat shaker at 60° Celsius. The samples were kept in the heat shaker for two hours and vortexed every ten to fifteen minutes in order to dissolve the dried down precipitate into the PBS.

Sonicating and Extruding

After being removed from the heat shaker, the samples were placed in the sonication bath, at 60° Celsius, for roughly 10 minutes. After being sonicated, the extruder was set up with a membrane size of 0.2 micrometers.

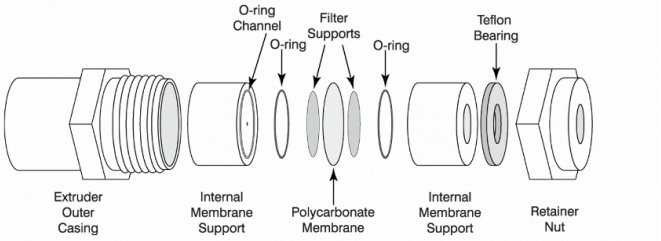

Figure 7. Extruder A schematic of the extruder used to create a uniform size of liposomes. From Avanti Polar Lipids Inc.

The solution was pushed through the extruder 11 times before being removed. A schematic of the extruder used can be seen in Figure 7. The sample was then extruded through a size 0.1 micrometer membrane. It was pushed through 11 times, once again. While 11 is a slightly arbitrary number, it is

essential that the solutions be extruded an odd number of times, as to not have the samples end up in the original syringe with any of the larger liposome remnants. This was done for each of the samples. After being extruded, the samples were run through a size exclusion column that was prepared with seraphose beads and then collected in Eppendorf tubes. This allowed the liposomal drug to separate from the unencapsulated free drug.

Dynamic Light Scattering and Mass Spectrometry

After running the samples through the gel column, Dynamic Light Scattering was run on each of the samples to determine the average size of the liposomes. Mass spectrometry was performed to determine the concentration of KT109 within the liposomes.

Western Blot Analysis

Cell lysates were separated via centrifugation at 100,000 x g for 45 min at 4°C. Soluble fraction (supernatant) was used for subsequent gel-based analyses. Proteins separated by SDS-PAGE (7.5% polyacrylamide, TGX Stain-Free Mini Gel) at 150 V for 55 min. Gel transfers were performed using the Bio-Rad Trans-Blot Turbo RTA Midi Nitrocellulose Transfer Kit with a Bio-Rad Trans-Blot Turbo Transfer System (25V, 10 min). The nitrocellulose blot was then incubated in blocking solution [30 mL, 3% BSA in TBS-T (1.5 M NaCl, 0.25 M Tris pH 7.4 in ddH_2O)] for 1 h at 25°C with gentle shaking. Immediately following this, the blot was transferred to primary antibody solution (1:1,000 anti-AMPKα or 1:1,000 pAMPKα Thr172, Cell Signaling Technology) diluted in 1% BSA in TBS-T and incubated overnight at 4°C with gentle shaking. The blot was then rinsed 5 times for 5 minutes in TBS-T, transferred immediately into secondary antibody solution [1:10,000 goat anti-rabbit DyLight 550 (Thermo Fisher Scientific) in TBS-T)], and incubated for 1 h at 25°C with gentle shaking. The blot was then rinsed 5 times for 5 min in TBS-T, transferred into ddH_2O, and imaged by in-blot fluorescence scanning on a ChemiDoc MP Imaging System.

Mice

Mice used in this study were C57BL/6J mice that ranged from 6 to 12 weeks old. The protocol used for these experiments have all been approved by the ACUC/University of Virginia. The mice were housed according to the ACUC policy on social housing of animals. Both male and female mice were used in this study and all animals were given access to a standard chow diet and water. Experiments were controlled by using same-sex littermates.

Bone Marrow Derived Macrophages (BMDMs) differentiation

Male and female mice were used in the following experiments. To culture BMDMs, bone marrow was extracted from the hind legs of the mice and incubated for 5 minutes with 0.83% ammonium chloride, which cleared erythrocyte progenitors. The bone marrow was cultured with RPMI media supplemented with 10% FBS (Omega Scientific), 5% HEPES (Gibco), 1% antibiotic-antimitotic (penicillin-streptomycin, Gibco), and 10% L929-conditioned media (L929 cells purchased from ATCC). The culture continued for 7 days and the media was changed every 3 to 4 days, after which the media was switched for one without L929-conditioned media. On day 7, the BMDMs were separated from the petri dish using 0.25% (Gibco) and then centrifuged and the media was replaced. Lastly, BMDMs were plated on non-tissue culture treated petri dishes for various treatments and analysis.

End Matter

Author Contributions and Notes

S.R.H, H.W.S, T.W, K.H, and M.K designed research; S.R.H, H.W.S, and T.W. performed research; S.R.H, H.W.S, T.W, K.H, and M.K analyzed data; T.W. aided in writing of methods and S.R.H wrote the paper.
The authors declare no conflict of interest.

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