Immunomodulatory effects of cholesterol oxidation products and their implications for liposomal drug delivery

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Background / Significant

Liposome-encapsulated chemotherapies have improved pharmacokinetics and tolerability compared to conventional drugs, yet their impact on anticancer efficacy remains uncertain. Emerging evidence suggests that liposomal components, particularly cholesterol, may influence tumor progression by modulating the immune response. Cholesterol within liposomes can be oxidized in the liver, spleen, and tumor microenvironment, generating oxysterols that act as immunoregulatory molecules.

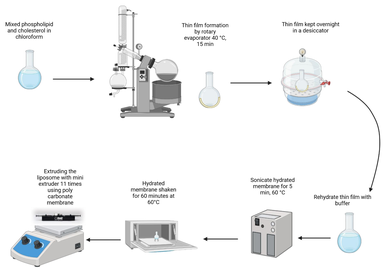
We hypothesized that these liposome-associated oxysterols could alter macrophage function and polarization, thereby affecting antitumor immunity.

This study demonstrates that specific oxysterols incorporated into liposomes distinctly modulate pro- and anti-inflammatory marker gene expression in polarized and non-polarized macrophages, either promoting or suppressing inflammatory responses and diminishing the survival and potentially affecting the tumor microenvironment. These findings highlight a previously overlooked immunomodulatory role of liposomal cholesterol and underscore the importance of engineering liposomal formulations that are safe and do not compromise the therapeutic efficacy of the drug payload.

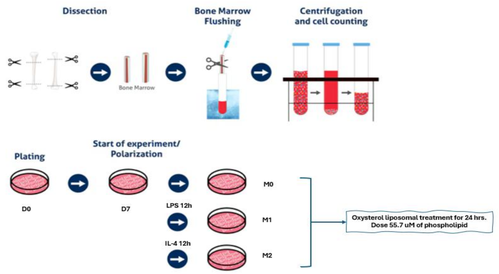
Methods

A. Formulation of different oxysterol liposomes

Liposomes with HSPC, mPEG 2000, and 5,6β-epoxy cholesterol (5,6β-EC), 7-ketocholesterol (7KC), or 24(S)-hydroxycholesterol (24-OHC) (55/5/40 molar ratio) were prepared via the thin film method. The particle size, concentration, and polydispersity index (PDI) were determined by the dynamic light scattering technique using Zetasizer Ultra (Malvern, UK), diluting 5 µl of liposomes to 995 µl of deionized water. The same sample was then transferred to a folded capillary cell to determine the zeta potential of the formulation. Phospholipid content was quantified via a colorimetric Rouser Assay, with results based on a standard curve and validated by quality controls.



B. In Vitro Experiment Procedure: Macrophage Polarization and Treatment



C. In vivo survival study on Oxysterol liposomes in TC-1 C57BL/6 mouse model.

Female C57BL/6 mice (6–7 weeks old) were obtained from Jackson Laboratory and subcutaneously implanted with 0.5 × 10^6 TC-1 cells. Treatment was administered intravenously on days 1, 5, 9, and 13. The study endpoint was a tumor volume of 1000 mm^3, with the experiment lasting a total of 52 days and four total doses given (two per week). The IV dosing was based on an equivalent of 8 mg/kg doxorubicin, as used in Doxil®, with the corresponding phospholipid concentration estimated at approximately 47 nmol PL/g. This dosing strategy was aligned with the formulation described by Sabnani et al. (2015).

Results

1. Characterization of Oxysterol-Loaded Liposomes

Formulation	Size (nm)	PDI	Particle concentration (/mL)	Zeta Potential (mV)	Phospholipids concentration (mM)
7ketocholesterol Liposome	101.7	0.027	1.29 x 10^{12}	-30.40	8.16
5,6 B Epoxy cholesterol Liposome	85.24	0.01	5.80E+13	-32.82	20.45
24-Hydroxy Cholesterol Liposome	94.04	0.132	8.04E+13	-36.12	20.33

Table 1. Physicochemical Characterization of Oxysterol-Containing Liposomes. Particle size, polydispersity index (PDI), particle concentration, and zeta potential were measured by dynamic light scattering (DLS) using the Zetasizer Ultra (Malvern, UK) after diluting 5 µl of liposomes in 995 µl of deionized water. Phospholipid content was quantified using the colorimetric Rouser Assay, with results validated against a standard curve and quality controls.

2. Oxysterols have a heterogeneous immune modulatory effect

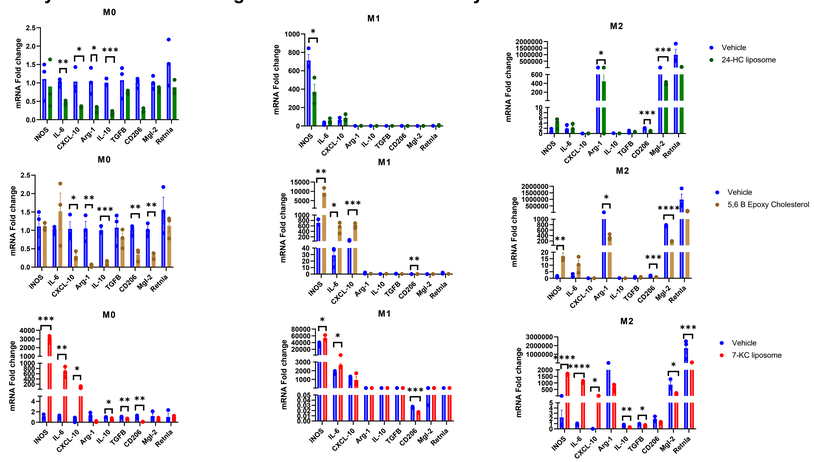


Figure 1. Liposomal oxysterols modulate inflammatory gene expression in polarized and unpolarized bone marrow-derived macrophages (BMDMs). Expression of proinflammatory markers iNOS, IL-6, and CXCL-10, as well as anti-inflammatory markers IL-10, TGF-β, CD206, MGI2, and Retnla, was assessed in unpolarized M0, M1-polarized, and M2-polarized macrophages by RT-qPCR. Gene expression was normalized to GAPDH and expressed as fold change relative to vehicle-treated control cells. Vehicle: 0.9% saline; treatment: liposomes suspended in saline at 55.7 µM total phospholipid concentration. Data represent mean ± SD (n = 3 per polarization/treatment group). Statistical analysis was performed using unpaired t-tests; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. Oxysterol-Loaded Liposomes Diminish Survival compared to cholesterol liposomes

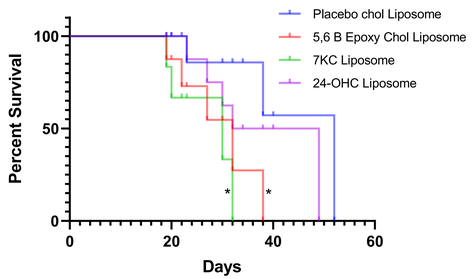
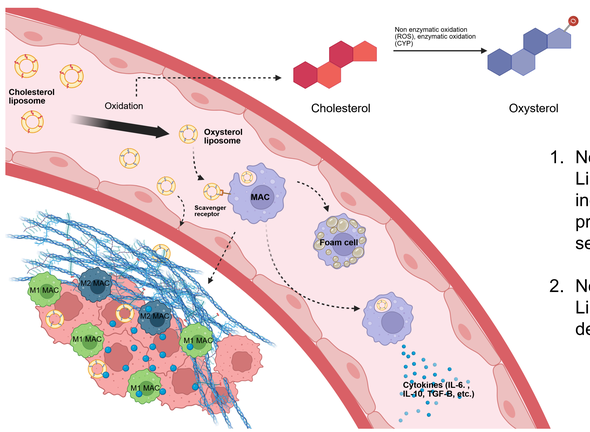


Figure 2. Effect of oxysterol liposomes on overall survival in a TC-1 tumor model. C57BL/6 Mice were implanted with TC-1 cells and then randomized (n = 7 mice/group) to treatments that were given on days 1, 5, 9, and 13. Mice were treated with liposomes containing cholesterol, 5,6β-epoxy cholesterol (5,6β-EC), 7-ketocholesterol (7KC), or 24(S)-hydroxycholesterol (24-OHC) at a dose of 47 nmol total phospholipid per gram of body weight. A total of four doses were administered (two per week) over a 52-day study period (endpoint: tumor volume of 1000 mm^3).Kaplan–Meier survival curves demonstrate that treatment with 5,6β-EC and 7KC liposomes significantly reduced overall survival compared to the cholesterol liposome control group (*p < 0.05). Statistical analysis: Log-rank test.

Summary



1. Non-enzymatically produced Liposomal oxysterols tend to increase the expression of proinflammatory markers in vitro setting.
2. Non-enzymatically produced Liposomal oxysterol significantly decreased the overall survival.

Figure 3. Summary of the Effects of Oxysterol-Containing Liposomes on Immune Components and the Tumor Microenvironment. Cholesterol-containing liposomes can undergo oxidative modification, forming oxysterols that are taken up by macrophages. These oxidized cholesterol molecules modulate macrophage biology through changes in metabolism, inflammatory signaling, and cell fate. Depending on the oxysterol species and context, they can promote pro-inflammatory M1 polarization or anti-inflammatory M2 polarization, shaping both innate and adaptive immunity. These immune-modulatory effects of oxysterols impact the tumor microenvironment by promoting either inflammation and antitumor activity or immunosuppression and pro-antitumor activity.

Conclusion

Non-enzymatically oxidized liposomal oxysterols significantly decreased overall survival and increased the expression of proinflammatory M1 markers in vitro. These findings suggest that cholesterol oxidation negatively impacts the safety profile of liposomal formulations, potentially inducing harmful immune activation and limiting therapeutic efficacy.

Selected References

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