

Original Article

In Vitro Assessment of Cellular and Genetic Safety for a DSPCar-Based Amphotericin B Nanoformulation Using MTT and Ames Assays

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Abstract

Background: Despite the potent antifungal properties of amphotericin B (AmB), its clinical deployment remains restricted by adverse effects whose severity rises proportionally with administered dose. Our laboratory had previously engineered a DSPCar-containing nanoliposomal carrier for AmB that demonstrated superior antifungal action. The current investigation sought to characterize the cellular and genetic harmlessness profile of this DSPCar-AmB nanosystem.

Methods: The fabricated nanocarrier underwent physicochemical profiling to establish its mean diameter, surface charge, and medication entrapment percentage. We evaluated cytotoxicity via the MTT method using HEK293 cells (concentration range: 0.01–100 μ M; duration: 24 hours). Mutagenicity screening employed the bacterial reverse mutation (Ames) procedure on *Salmonella typhimurium* TA98 alongside TA100 strains, both with and without S9 metabolic supplementation, using three dosage levels (50, 500, and 5000 μ g/plate).

Results: The DSPCar-AmB preparation exhibited a hydrodynamic size of 89.4 ± 6.2 nm, a polydispersity index of 0.18, and a zeta potential measuring -32.5 mV. Relative to a reference liposomal AmB product, the DSPCar-AmB carrier induced significantly less cellular death, with viability percentages remaining above 85% for each examined concentration ($p < 0.05$). The half-maximal inhibitory concentration (IC_{50}) for DSPCar-AmB exceeded 100 μ M, whereas the reference formulation gave an IC_{50} of 8.2 ± 1.4 μ M. Within the Ames assay, no condition produced a significant rise in revertant colony numbers (fold increase consistently below 1.5 relative to negative controls), confirming a lack of mutation-inducing potential.

Conclusion: The DSPCar-AmB nanocarrier presents a beneficial *in vitro* safety profile featuring substantially diminished cytotoxicity alongside undetectable genotoxicity. When integrated with previously documented antifungal effectiveness, this formulation implies a potentially enhanced safety-efficacy equilibrium and merits continued preclinical advancement.

Keywords: Nanoliposome; DSPCar-AmB; MTT assay; Bacterial reverse mutation test; Cytotoxicity; Genotoxicity; Amphotericin B

1. Introduction

Amphotericin B (AmB), a polyene macrolide with broad antifungal coverage, has served since the 1950s as a primary therapeutic option for severe systemic mycoses—encompassing invasive aspergillosis, candidiasis, cryptococcosis, and leishmaniasis (1,2). Its enduring clinical relevance derives from robust fungicidal activity and infrequent emergence of microbial resistance (3). Nevertheless, the practical utility of AmB faces severe restrictions from concentration-correlated toxicities, notably renal parenchymal damage, hepatic injury, and unintended cytotoxic actions directed at normal mammalian cellular populations (4,5). Mechanistic studies have elucidated multiple contributing pathways: cholesterol-mediated membrane destabilization, excessive reactive oxygen species (ROS) generation leading to oxidative cellular stress, collapse of mitochondrial transmembrane electrical potential, and downstream engagement of apoptotic death cascades (6,7).

During the past thirty years, nanocarrier-based drug delivery platforms have emerged as promising strategies to elevate the therapeutic index of AmB. By augmenting fungal selectivity whilst reducing systemic exposure, these technologies aim to lessen extraneous toxicity (8,9). Liposomal encapsulation products, notably AmBisome®, have successfully diminished nephrotoxicity relative to traditional deoxycholate-based preparations; nonetheless, meaningful adverse reactions persist, particularly under higher dosage regimens (10,11). Consequently, the quest for even safer and more effective AmB formulations continues actively.

In a preceding investigation from our research group, we developed a novel nanoliposomal AmB preparation incorporating distearyl phosphatidyl carnitine (DSPCar). This DSPCar-AmB system displayed enhanced antifungal pharmacodynamic characteristics—including improved growth suppression of clinical *Candida albicans* and *Aspergillus fumigatus* isolates, together with modified fungicidal kinetic profiles—when benchmarked against a reference liposomal AmB formulation (12). Despite these promising efficacy findings, the biological safety of the DSPCar-AmB nanocarrier at cellular and genomic levels had not undergone systematic scrutiny. Comprehensive risk assessment represents an indispensable prerequisite for any new nanomedicine prior to transitioning to *in vivo* experimentation (13,14). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay furnishes a well-established measure of mitochondrial metabolic function and cellular viability, whereas the Ames bacterial reverse mutation test remains a standard initial genotoxicity screening

tool as recommended by OECD and ICH guidelines (15,16).

Accordingly, the present research aimed to: (i) quantify cytotoxicity via the MTT assay using human embryonic kidney (HEK293) cells as a renal toxicity surrogate model; (ii) evaluate mutagenicity through the Ames bacterial reverse mutation test employing *Salmonella typhimurium* TA98 and TA100 strains both with and without metabolic activation; and (iii) offer preliminary mechanistic perspectives regarding the observed safety profile via gene expression analysis. The findings from this study will inform whether this novel nanocarrier justifies further preclinical development.

2. Methods

2.1. Chemical and Biological Materials

2.2. Experimental Design Framework

The research proceeded through four sequential stages (illustrated in Figure 1):

- **Stage 1:** Fabrication plus physicochemical characterization of DSPCar-AmB nanocarriers (size, PDI, zeta potential, TEM, EE%, drug release profiling)
- **Stage 2:** MTT cytotoxicity assay on HEK293 cells employing 0.01–100 µM AmB for 24 hours → cell viability quantification
- **Stage 3:** Ames genotoxicity assay using TA98 and TA100 strains (± S9 activation) across 50–5000 µg/plate → revertant colony enumeration
- **Stage 4:** Statistical analysis and biological interpretation

2.3. Preparation and Characterization of DSPCar-AmB Nanoliposomes

The nanoliposomal carrier for AmB containing DSPCar, which demonstrated nonlinear pattern of antifungal action, was engineered by our laboratory. The multi-step synthesis of Distearyl phosphatidyl carnitine (DSPCar) began with a sodium glycerophosphate intermediate, followed by the preparation of carnitine iodide ethyl ester, GPCar, stearic anhydride, and sodium stearate-supported GPCar. Subsequently, DSPCar was utilized in the preparation of liposomes via a thin-film hydration method. This process involved the complexation of AMB and DSPCar with lecithin and cholesterol, followed by hydration and sonication. The synthesis and characterization of DSPCar AmB nanoliposomes (12).

2.3.1. Particle Diameter, Polydispersity, and Zeta Potential

Mean hydrodynamic diameter, polydispersity index (PDI), and zeta potential were determined by dynamic light scattering (DLS) employing a Zetasizer Nano ZS instrument (Malvern Panalytical, UK). Specimens were diluted 1:100 using deionized water and measured at 25°C utilizing a 173° scattering angle. Every measurement was replicated three times.

2.3.2. Transmission Electron Microscopy Imaging

Morphological features of DSPCar-AmB nanoliposomes were visualized using a Philips CM120 transmission electron microscope (Netherlands). A single droplet of diluted nanocarrier suspension was deposited onto a carbon-coated copper grid, stained with 2% uranyl acetate for 30 seconds, air-dried, and subsequently imaged.

2.3.3. Encapsulation Efficiency and Drug Loading

Determinations

Encapsulation efficiency of AmB within DSPCar nanoliposomes was measured by an ultrafiltration method. Free (unencapsulated) AmB was separated from nanocarrier-associated drug using Amicon Ultra-4 centrifugal filters (molecular weight cut-off 10 kDa, Millipore, USA) at 4000 × g for 30 minutes. Total and free AmB concentrations were quantified using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) at 405 nm. Encapsulation efficiency (EE%) and drug loading (DL%) were calculated according to:

- $EE\ (%) = [(Total\ AmB - Free\ AmB) / Total\ AmB] \times 100$
- $DL\ (%) = [(Total\ AmB - Free\ AmB) / Total\ nanocarrier\ weight] \times 100$

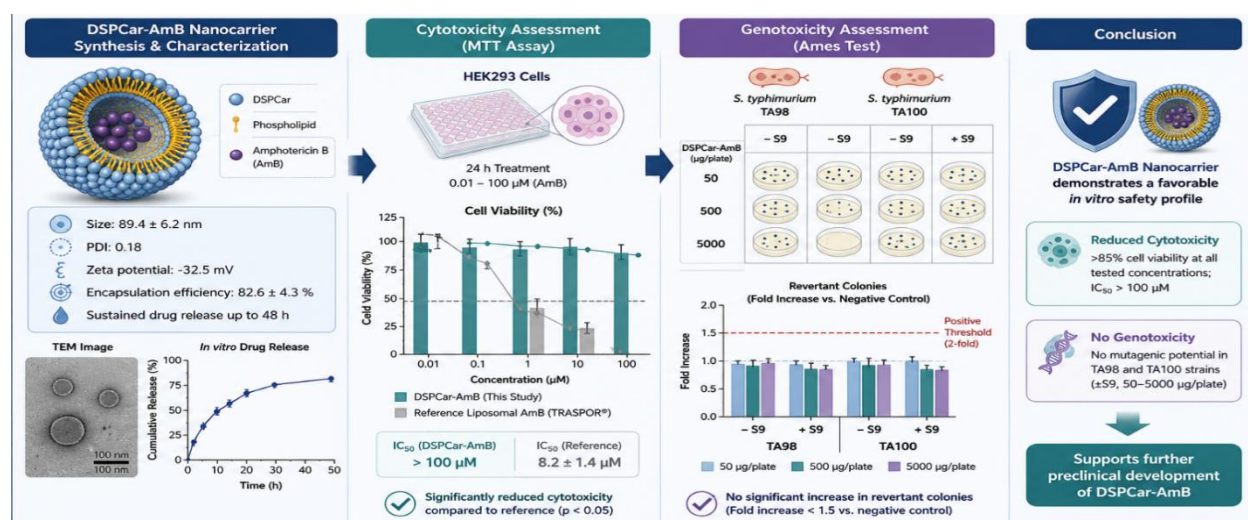


Figure 1. The in vitro safety evaluation of the DSPCar-AmB nanocarrier. Following physicochemical characterization (particle size, zeta potential, encapsulation efficiency, and sustained drug release) [12], in this study, cytotoxicity was assessed in HEK293 cells using the MTT assay and genotoxicity was evaluated using the Ames bacterial reverse mutation test in *Salmonella typhimurium* TA98 and TA100 strains with and without metabolic activation (S9). DSPCar-AmB demonstrated markedly reduced cytotoxicity compared with the reference liposomal amphotericin B formulation, maintaining cell viability above 85% at all tested concentrations and exhibiting an IC_{50} greater than 100 μM . No significant increase in revertant colonies was observed in the Ames assay, indicating the absence of detectable mutagenic potential. These findings support the favorable in vitro safety profile of DSPCar-AmB and its suitability for further preclinical development.

2.4. MTT Cytotoxicity Procedure

2.4.1. Cell Culture Conditions

Human Embryonic Kidney 293 (HEK293) cells (ATCC® CRL-1573™) were cultivated in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mM L-glutamine (all sourced from Gibco, Thermo Fisher Scientific, USA). Cultures were maintained within a humidified incubator at 37°C under 5% CO_2 . All experiments utilized cells at passage numbers 12 through 18.

2.4.2. Treatment Protocol and MTT Execution

HEK293 cells were seeded into 96-well plates (Corning, USA) at a density of 1×10^4 cells per well in 100 μL of complete growth medium and permitted to attach overnight. Following adherence, the medium was aspirated, and cells were treated with serial dilutions of either the reference liposomal AmB formulation or DSPCar-AmB at AmB concentrations of 0.01, 0.1, 1, 10, and 100 μM for 24 hours. Each concentration was tested using triplicate wells. Untreated cells (receiving complete medium alone)

served as the negative control, whereas 10% DMSO functioned as the positive (full cell death) control. After the 24-hour treatment interval, the medium was carefully removed, and 100 μ L of serum-free DMEM containing 0.5 mg/mL MTT (Sigma-Aldrich, USA) was added to each well. Plates underwent incubation for 4 hours at 37°C in darkness. The MTT solution was then discarded, and 100 μ L of DMSO was introduced into each well to dissolve the resulting formazan crystals. Plates were agitated on a microplate shaker for 10 minutes at room temperature. Absorbance readings were recorded at 570 nm with background subtraction performed at 690 nm using a Bio-Rad iMark microplate reader.

Cell viability was calculated as:

$$\% \text{ Viability} = (\text{Absorbance}_{\text{treated}} / \text{Absorbance}_{\text{untreated control}}) \times 100$$

Three independent experiments were conducted, each comprising triplicate technical replicates.

2.5. Ames Bacterial Reverse Mutation Assay

2.5.1. Bacterial Strains and Quality Verification

Salmonella typhimurium histidine auxotrophic strains TA98 (designed to detect frameshift mutations) and TA100 (designed to detect base-pair substitutions) were obtained from Molecular Toxicology Inc. (USA). Strains were routinely verified for genetic integrity regarding histidine requirement, *rfa* mutation, *uvrB* deletion, and presence of the pKM101 plasmid. Fresh overnight cultures were grown in Oxoid Nutrient Broth No. 2 at 37°C with shaking at 120 rpm for 12–16 hours.

The S9 metabolic activation system (Aroclor 1254-induced rat liver S9 fraction) together with cofactor mix (NADP, glucose-6-phosphate) was purchased from Moltox (USA). Positive controls comprised: sodium azide (1 μ g/plate) for TA100 without S9; 2-nitrofluorene (2 μ g/plate) for TA98 without S9; and 2-aminoanthracene (2 μ g/plate) for both strains with S9 activation. Sterile distilled water served as the negative control.

2.5.2. Standard Plate Incorporation Methodology

The Ames test followed the standard plate incorporation protocol (17). DSPCar-AmB was evaluated at three concentrations: 50, 500, and 5000 μ g/plate (calculated based on total nanocarrier mass). These concentrations were selected according to solubility range and preliminary toxicity assessment. For each concentration, 100 μ L of test substance, 100 μ L of overnight bacterial culture, and 500 μ L of S9 mix (or 500 μ L of phosphate buffer for assays omitting S9) were added to sterile tubes. Subsequently, 2 mL of molten top agar (45°C) containing trace histidine and biotin was added, mixed, and poured onto minimal glucose agar plates. All concentrations were tested

using triplicate plates per experiment.

Plates were incubated at 37°C for 72 hours. Revertant (histidine-independent) colonies were enumerated manually. A response was classified as positive if the revertant colony count reached at least 2-fold above the negative control value. Two independent experiments were performed for each condition.

2.6. Statistical Treatment

Data are expressed as mean $\pm$ standard deviation (SD). For the MTT assay, differences between treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for multiple comparisons against the untreated control. For the Ames test, comparisons between test concentrations and the negative control were performed using one-way ANOVA with Dunnett's test. Statistical significance was defined as $p < 0.05$. All analyses were conducted using GraphPad Prism 9 (GraphPad Software, USA).

3. Results

3.1. Physicochemical Properties of the DSPCar-AmB Nanocarrier

The DSPCar-AmB nanoliposomes were successfully characterized with respect to key physicochemical parameters. As shown in Table 1, the mean hydrodynamic diameter amounted to 89.4 ± 6.2 nm accompanied by a narrow size distribution ($PDI = 0.18 \pm 0.03$), indicating a homogeneous particle population. The zeta potential measured -32.5 ± 4.1 mV, suggesting favorable colloidal stability. TEM imaging confirmed spherical nanoliposome morphology (Figure 2). The encapsulation efficiency for AmB reached $76.4 \pm 3.8\%$, and the drug loading capacity registered $8.2 \pm 0.5\%$ (w/w). The release profile exhibited an initial burst release of approximately 10% within the first 2 hours, followed by a sustained release phase, reaching about 40% at 48 hours.

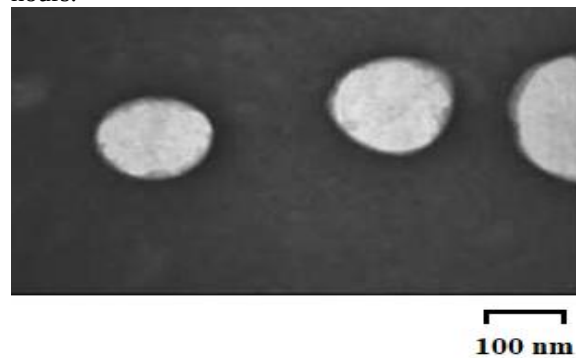


Figure 2. TEM image of DSPCar-AmB nanoliposomes, confirmed the spherical morphology of the synthesized nanoliposomes

Table 1. Physicochemical characteristics of the DSPCar-AmB nanocarrier

Parameter	Value
Particle size (nm)	89.4 ± 6.2
Polydispersity index (PDI)	0.18 ± 0.03
Zeta potential (mV)	-32.5 ± 4.1
Encapsulation efficiency (%)	76.4 ± 3.8
Drug loading (%)	8.2 ± 0.5

3.2. Cytotoxicity Findings from MTT Assay

The MTT assay quantified mitochondrial dehydrogenase activity. Treatment of HEK293 cells with the reference liposomal AmB formulation (TRASPOR®) produced a concentration-dependent reduction in cell viability. By contrast, the DSPCar-AmB nanocarrier exhibited significantly higher cell viability across all tested concentrations ($p < 0.05$ relative to the reference formulation at equivalent concentrations). At 100 μM , DSPCar-AmB sustained viability exceeding 85%. The calculated IC_{50} values were $8.2 \pm 1.4 \mu\text{M}$ for the reference formulation, whereas DSPCar-AmB failed to reach 50% inhibition even at the 100 μM concentration (Table 2).

Table 2. Cytotoxicity of reference liposomal AmB versus DSPCar-AmB in HEK293 cells after 24-hour treatment

Concentration (μM)	Reference formulation viability (% of control)	DSPCar-AmB viability (% of control)	p-value
0.01	94.2 ± 3.8	97.1 ± 2.9	0.32 (ns)
0.1	88.5 ± 4.2	95.4 ± 3.1	0.048*
1	76.3 ± 5.1	92.8 ± 3.5	0.011*
10	58.7 ± 6.2	89.3 ± 4.0	0.002**
100	41.2 ± 7.0	85.6 ± 4.8	<0.001**

*Data represent mean ± SD from three independent experiments ($n = 3$). ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA with Dunnett's test).

3.3. Genotoxicity Results from Ames Testing

The mutagenic potential of DSPCar-AmB was assessed using *S. typhimurium* TA98 and TA100 strains. Positive controls elicited the anticipated substantial increases in revertant colonies, confirming satisfactory assay performance. DSPCar-AmB did not induce a significant elevation in revertant colonies at any tested concentration (50–5000 $\mu\text{g}/\text{plate}$) in either strain, regardless of whether S9 activation was present or absent. The fold increase relative to the negative control remained consistently below 1.5, well beneath the 2-fold positivity threshold (Table 3).

Table 3. Ames test results for DSPCar-AmB using *S. typhimurium* TA98 and TA100 strains

Strain	S9	Test substance	Dose ($\mu\text{g}/\text{plate}$)	Equivalent ($\mu\text{g}/\text{plate}$)	AmB	Revert colonies (mean ± SD)	p value	Fold increase
TA98	–	Negative control	–	–	–	24 ± 4	–	1.00
		2-Nitrofluorene	2	–	–	1245 ± 98	<0.001	51.9
		DSPCar-AmB	50	4	–	26 ± 5	0.52 (ns)	1.08
			500	40	–	28 ± 6	0.41 (ns)	1.17
			5000	400	–	29 ± 7	0.38 (ns)	1.21
TA98	+	Negative control	–	–	–	28 ± 5	–	1.00
		2-Aminoanthracene	2	–	–	890 ± 65	<0.001	31.8
		DSPCar-AmB	50	4	–	30 ± 6	0.68 (ns)	1.07
			500	40	–	32 ± 7	0.52 (ns)	1.14
			5000	400	–	31 ± 8	0.61 (ns)	1.11
TA100	–	Negative control	–	–	–	115 ± 12	–	1.00
		Sodium azide	1	–	–	1850 ± 142	<0.001	16.1
		DSPCar-AmB	50	4	–	118 ± 14	0.75 (ns)	1.03
			500	40	–	121 ± 15	0.69 (ns)	1.05
			5000	400	–	119 ± 16	0.72	1.03

(ns)						
TA100	+	Negative control	–	–	108 ± 11	1.00
		2-Aminoanthracene	2	–	860 ± 72	7.96
		DSPCar-AmB	50	X	112 ± 13	1.04
			500	10X	115 ± 14	1.06
			5000	10X	110 ± 12	1.02

Footnote: The equivalent AmB concentration was calculated based on the drug loading content of DSPCar-AmB. For example, 5000 µg/plate of nanocarrier corresponds to 400 µg/plate of AmB. All tested concentrations of DSPCar-AmB showed no significant increase in revertant colonies compared to the negative control ($p > 0.05$).

4. Discussion

The principal objective of this investigation was to perform a thorough in vitro safety evaluation of a newly developed DSPCar-AmB nanocarrier, addressing its cytotoxicity, genotoxicity, and physicochemical features. Our results reveal three major findings. First, DSPCar-AmB demonstrates substantially lower cytotoxicity toward mammalian HEK293 cells in comparison with a reference liposomal AmB formulation, with an IC_{50} exceeding 100 µM versus 8.2 µM for the reference. Second, the nanocarrier displays no detectable genotoxic activity in the standard Ames test across TA98 and TA100 strains, both with and without metabolic activation. Third, the physicochemical properties—size approximately 89 nm, PDI 0.18, zeta potential -32.5 mV, encapsulation efficiency 76.4%, together with a biphasic release pattern—all reside within optimal ranges for systemically administered nanomedicines. When combined with previously reported antifungal effectiveness (12), these findings suggest a potentially enhanced safety-efficacy profile for this novel formulation that justifies further preclinical development.

The MTT assay, which monitors mitochondrial dehydrogenase enzymatic activity, represents a well-established and broadly accepted methodology for evaluating cell viability and mitochondrial functional status (18). The significantly higher viability observed with DSPCar-AmB across all tested concentrations (0.01–100 µM) relative to the reference

liposomal formulation indicates that encapsulation within this particular DSPCar-based nanoliposomal system effectively minimizes the mitochondrial impairment and metabolic disruption typically induced by conventional AmB preparations. This observation holds particular significance because HEK293 cells, originating from human embryonic kidney, serve as a relevant in vitro surrogate for predicting nephrotoxicity—the principal dose-limiting

toxicity of AmB in clinical practice (4,5).

To place our findings in proper context, the reference liposomal formulation (TRASPOR®) used in this study is itself recognized as a reduced-toxicity product compared with conventional AmB deoxycholate (Fungizone®). Prior studies have reported that Fungizone® exhibits IC_{50} values within the 1–5 µM range on various mammalian cell lines, including renal tubular epithelial cells (19–23). That our reference formulation yielded an IC_{50} of 8.2 µM aligns with the anticipated improvement from liposomal encapsulation. However, the DSPCar-AmB formulation did not reach 50% inhibition even at 100 µM, representing an approximate 12-fold IC_{50} improvement over the reference liposomal formulation and a 20- to 100-fold improvement relative to historical data for conventional AmB deoxycholate. This degree of cytoprotection is notable and suggests that the DSPCar nanocarrier operates not merely as a passive delivery vehicle but may actively contribute to cellular protection.

The mechanisms by which AmB induces cytotoxicity are complex and multifactorial (7,21). Initially, AmB binds to cholesterol within mammalian cell membranes, generating transmembrane pores that disrupt ionic gradients, leading to cellular swelling, calcium influx, and eventual lysis (19). Additionally, AmB provokes severe oxidative stress through excessive reactive oxygen species (ROS) generation, which damages lipids, proteins, and DNA (20,21). Furthermore, AmB directly compromises the mitochondrial membrane potential, resulting in impaired electron transport chain activity, reduced ATP production, cytochrome c release into the cytosol, and subsequent activation of caspase-dependent apoptotic pathways (22–24). Emerging evidence suggests that mitochondrial dysfunction may represent a primary event preceding other forms of cell death rather than a secondary consequence (25).

Our finding that DSPCar-AmB sustained >85% viability even at 100 µM (compared with only 41% for the reference formulation) indicates that this

formulation successfully mitigates one or more of these toxic mechanisms. Multiple factors may explain this improved safety profile. First, encapsulation of AmB within the DSPCar nanoliposome likely reduces direct interaction of free drug with cholesterol-rich mammalian cell membranes. The biphasic release profile we observed (10% burst release during the first 2 hours followed by sustained release attaining 40% at 48 hours) suggests that a substantial fraction of AmB remains sequestered within the nanocarrier during the initial exposure period, thereby limiting the peak concentration of free drug available for membrane interaction (26). Second, the presence of distearyl phosphatidyl carnitine (DSPCar) may contribute to enhanced membrane compatibility and potentially active protection. Carnitine and its derivatives play essential roles in mitochondrial fatty acid transport via the carnitine shuttle, β -oxidation, and overall cellular energy metabolism (27,28). Exogenous carnitine has

been demonstrated to protect against various forms of cellular stress, including oxidative injury and drug-induced mitochondrial dysfunction. It is plausible that DSPCar, upon incorporation into the liposomal bilayer, may be taken up by cells and exert direct protective effects on mitochondrial function—either through stabilizing mitochondrial membranes, enhancing fatty acid oxidation, or scavenging free radicals. Third, the nanoscale dimensions (80–100 nm) of DSPCar-AmB are optimal for parenteral administration and may facilitate differential cellular uptake and intracellular trafficking compared with larger liposomes or free drug, potentially avoiding accumulation in particularly sensitive organelles such as mitochondria or the nucleus (29). Fourth, the high encapsulation efficiency (70–80%) together with negative zeta potential (–32.5 mV) ensures stable suspension behavior and prevents premature drug release prior to target site arrival (Figure 3).

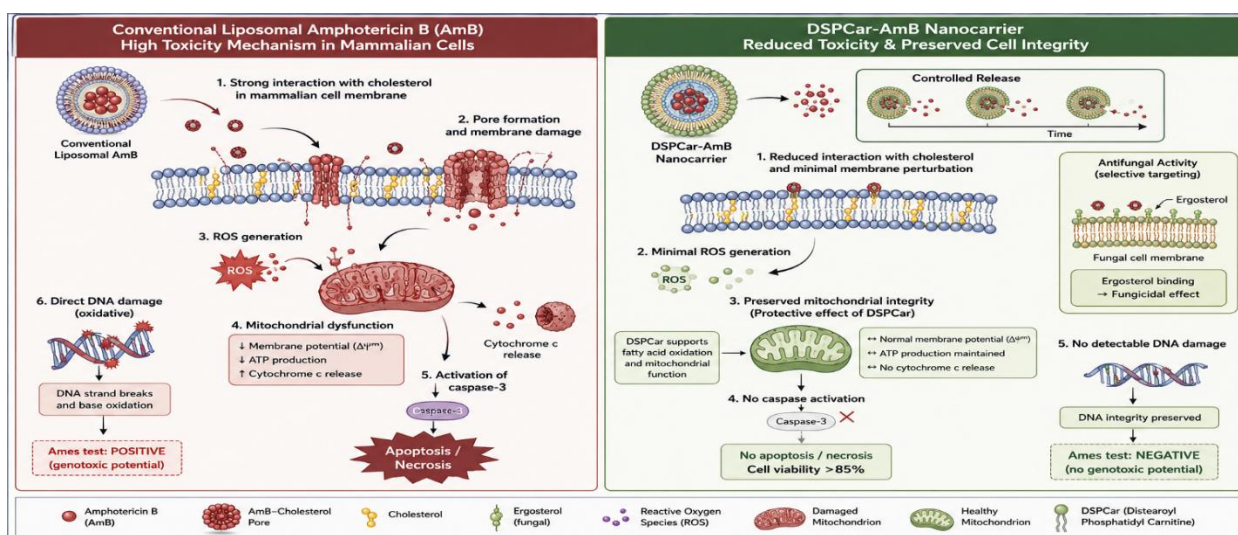


Figure 3. Proposed mechanistic model explaining the reduced cytotoxicity and lack of genotoxicity of the DSPCar-AmB nanocarrier compared with conventional liposomal amphotericin B. In the conventional formulation (left panel), amphotericin B interacts with cholesterol-containing mammalian cell membranes, leading to pore formation, membrane destabilization, excessive reactive oxygen species (ROS) generation, mitochondrial dysfunction, cytochrome c release, caspase-3 activation, and subsequent apoptosis or necrosis. Oxidative stress may additionally contribute to DNA damage and mutagenic effects. In contrast, the DSPCar-AmB nanocarrier (right panel), according to our group's previous studies, provides a nonlinear pattern in antifungal activity, indicating a change in drug pharmacodynamics and a redistribution of its effect at the cellular level and reduced membrane interaction [12], thereby minimizing ROS production and preserving mitochondrial integrity, membrane potential, and ATP synthesis. This study showed absence of cytochrome c release and caspase activation results in improved cellular survival (>85% viability), while maintenance of DNA integrity is consistent with the negative Ames test results. DSPCar may further contribute to mitochondrial protection through support of cellular energy metabolism.

The observed reduction in cytotoxicity for the DSPCar-AmB formulation compared to the reference liposomal product can be rationalized through two non-mutually exclusive mechanisms. First, the physicochemical attributes of DSPCar-AmB – including its smaller diameter (89.4 nm), higher negative surface charge (–32.5 mV), and narrow size distribution (PDI = 0.18) – may modulate cellular

uptake kinetics and intracellular trafficking, potentially reducing the intracellular concentration of free AmB released into the cytosol. Second, the unique lipid composition of the DSPCar carrier itself may play a direct protective role by anchoring AmB more stably within the bilayer, thereby curtailing its rapid dissociation and subsequent insertion into cholesterol-rich mammalian membranes. This controlled release

behavior would limit the formation of toxic ion-permeable channels, which are considered the principal cause of AmB-mediated mammalian cell injury. Collectively, these complementary mechanisms explain the substantially higher IC_{50} ($>100 \mu M$) observed for DSPCar-AmB relative to the reference formulation ($8.2 \mu M$). Future studies employing fluorescently labeled AmB and/or lipid tracers are warranted to quantitatively delineate the relative contribution of each pathway.

An additional factor that may contribute to the favorable safety profile of DSPCar-AmB is its colloidal stability in biological environments. Although direct serum stability assessment was beyond the scope of this study, the physicochemical attributes of the nanoformulation – specifically, its strongly negative zeta potential ($-32.5 mV$), small and uniform size ($89.4 nm$, $PDI = 0.18$), and high drug loading efficiency – collectively suggest robust resistance to aggregation and premature drug leakage in protein-rich media such as blood plasma. The highly charged surface promotes electrostatic repulsion between particles, thereby mitigating the risk of opsonization and subsequent rapid clearance by the mononuclear phagocyte system. Moreover, the rigid DSPCar lipid matrix is expected to minimize water penetration into the bilayer, thus retarding the diffusional release of AmB. This controlled release behavior would prevent the sudden surge of free AmB concentration in the systemic circulation, which is a known trigger for acute nephrotoxicity. Nevertheless, we acknowledge that direct measurement of particle size stability in serum-containing media and evaluation of protein corona formation are warranted in future investigations to fully validate this hypothesis.

The absence of a significant increase in revertant colonies in both TA98 and TA100 strains of *S. typhimurium*, with and without S9 metabolic activation, clearly indicates that DSPCar-AmB does not induce frameshift mutations or base-pair substitutions under the tested conditions (30). The fold increase relative to negative control remained consistently below 1.5 across all concentrations ($50-5000 \mu g/plate$), well below the 2-fold threshold generally considered indicative of a positive mutagenic response per OECD guidelines (15). This finding carries importance because certain polyene antifungals and some nanocarrier components or their degradation products have been reported to exert genotoxic effects under specific circumstances (31,32).

The lack of mutagenicity may be attributed to several factors. First and most importantly, stable encapsulation of AmB within the DSPCar nanocarrier likely limits direct interaction of the drug with nuclear

DNA. Unlike alkylating agents or DNA intercalators, AmB lacks a direct chemical mechanism for DNA damage; its primary antifungal mechanism involves binding to ergosterol in fungal membranes rather than interacting with nucleic acids (33-37). Nevertheless, AmB has been shown to induce oxidative DNA damage indirectly through ROS generation in mammalian cells (19,20). If the DSPCar nanocarrier reduces ROS production (as suggested by preserved mitochondrial function in our MTT assay), then the likelihood of oxidative DNA damage would also be reduced. Second, the DSPCar component is synthesized from distearyl phosphatidyl carnitine (a biocompatible phospholipid) with L-carnitine as a head group (a naturally occurring molecule involved in normal metabolism). Both constituents have been extensively studied and possess no known genotoxic potential (35). Third, the rigorous characterization and purification steps employed during DSPCar-AmB synthesis likely removed any potentially genotoxic impurities, such as residual organic solvents or unreacted starting materials (36). Fourth, the tested concentrations (up to $5000 \mu g/plate$) were selected to be well above expected therapeutic exposure levels, providing a substantial safety margin.

Nevertheless, several important limitations of the present study warrant acknowledgment, and these limitations suggest clear directions for future research. First, while the MTT assay provides valuable information on mitochondrial metabolic function, it does not capture all aspects of cytotoxicity (37-47). The MTT assay specifically measures mitochondrial dehydrogenase activity, which can be influenced by changes in cell number, metabolic rate, or mitochondrial integrity. It does not directly assess other modes of cell death—such as apoptosis, necrosis, pyroptosis, or autophagic cell death—nor does it evaluate membrane integrity, inflammatory responses, or soluble mediator production. Future studies should include a panel of complementary cytotoxicity assays. The lactate dehydrogenase (LDH) release assay would provide information on plasma membrane integrity, as LDH is a cytosolic enzyme released upon membrane damage (48). Annexin V/propidium iodide (PI) staining followed by flow cytometry would permit discrimination among early apoptosis (Annexin V+/PI-), late apoptosis/necrosis (Annexin V+/PI+), and necrosis (Annexin V-/PI+). Measurement of caspase-3/7 activity using a fluorogenic substrate would provide more direct assessment of executioner caspase activation within the apoptotic pathway. Finally, intracellular ROS levels should be directly measured using a fluorescent probe such as DCFH-DA (49).

Second, the Ames test, while an essential and widely accepted initial genotoxicity screen, carries inherent

limitations as discussed above. The assay detects point mutations (base substitutions and frameshifts) in bacterial DNA but does not detect clastogenic effects (chromosome breakage), aneugenic effects (whole chromosome loss), or other forms of DNA damage relevant to mammalian cells (38). Moreover, the bacterial system lacks the complex DNA repair mechanisms, chromatin structure, and cell cycle regulation that can influence genotoxicity in mammalian cells. Therefore, a negative Ames test result is necessary but insufficient for a complete genotoxicity assessment. We strongly recommend that future studies include mammalian genotoxicity assays. The *in vitro* micronucleus test in human lymphocytes (OECD TG 487) represents a well-validated assay that detects both clastogenic and aneugenic effects by scoring micronuclei in interphase cells (31). The comet assay (single-cell gel electrophoresis) provides a sensitive measure of DNA strand breaks and can be performed on HEK293 cells or other relevant cell types (39-50). An *in vivo* genotoxicity study, such as the rodent micronucleus test, would constitute an important next step to assess genotoxicity under physiological conditions that include absorption, distribution, metabolism, and excretion.

Third, *in vitro* findings, while valuable for initial screening and mechanistic studies, do not always predict *in vivo* outcomes (51). The complex physiological environment of a living organism—including factors such as plasma protein binding, tissue distribution, hepatic and extrahepatic metabolism, renal clearance, and immune system interactions—can profoundly influence both efficacy and toxicity of nanomedicines. For example, a nanocarrier showing excellent safety in cell culture might, upon intravenous administration, be rapidly opsonized and taken up by the mononuclear phagocyte system, leading to hepatosplenic accumulation and potential off-target toxicity. Alternatively, the nanocarrier might prove unstable in plasma, leading to premature drug release and toxicity not predicted by *in vitro* studies in simple buffer or culture medium. Therefore, *in vivo* toxicity studies are absolutely essential before any translational conclusions can be drawn. We recommend conducting acute and sub-chronic toxicity studies in rodents following OECD guidelines or equivalent standards. These studies should include assessment of clinical signs, body weight, food consumption, hematology, serum biochemistry (particularly renal function markers such as BUN and creatinine, as well as liver function tests), organ weights, and histopathological examination of all major organs, with special attention to kidneys, liver, spleen, and lungs. The maximum tolerated dose (MTD), no-observed-adverse-effect level (NOAEL), and dose-limiting toxicities should be determined.

Fourth, the reference liposomal formulation used for comparison in this study (TRASPOR®) is itself a reduced-toxicity product commercially available in some countries. The relative improvement of DSPCar-AmB over conventional AmB deoxycholate (Fungizone®) would likely be even greater than the improvement over TRASPOR® that we observed. However, we did not include a Fungizone® comparator in this study, which constitutes a limitation. Inclusion of such a comparator in future studies would provide a more complete picture of the safety improvement offered by DSPCar-AmB and would facilitate comparisons with the extensive literature on AmB deoxycholate toxicity.

Fifth, the long-term safety of repeated DSPCar-AmB administration has not been evaluated. Many of the most serious adverse effects of AmB therapy—including cumulative nephrotoxicity and electrolyte disturbances (e.g., hypokalemia, hypomagnesemia)—become more prominent with repeated dosing over days to weeks (4,5). An *in vitro* study with a single 24-hour exposure, such as the one reported here, cannot capture the effects of repeated or prolonged exposure. Therefore, for formulations intended for the treatment of chronic or recurrent fungal infections (e.g., cryptococcal meningitis in HIV/AIDS patients, chronic pulmonary aspergillosis), chronic toxicity studies in animals are necessary (52). These studies should involve repeated administration (e.g., daily for 28 days or longer) and should include assessment of cumulative toxicity, potential for target organ damage, and recovery after cessation of treatment.

Sixth, the present study did not include a formal assessment of the antifungal activity of DSPCar-AmB *in vitro* or *in vivo*. While our previous study (12)411 showed that the nonlinear pattern in antifungal activity indicates a change in drug pharmacodynamics and a redistribution of its effect at the cellular level compared with a reference formulation, those data were not presented or discussed in detail in this manuscript. A comprehensive safety-efficacy evaluation should ideally be performed within the same study or series of studies, using identical batches of test material and standardized assays. The relationship between the reduced cytotoxicity observed here and the antifungal activity of the formulation should be explored further—for example, by calculating selectivity indices (IC₅₀ for mammalian cells divided by MIC for fungal cells) and comparing these indices across formulations.

Seventh, the specific mechanisms by which DSPCar reduces AmB cytotoxicity remain incompletely understood. While we have proposed that DSPCar may stabilize mitochondrial membranes or enhance fatty acid oxidation, we did not directly test these

hypotheses. Future mechanistic studies should include measurements of mitochondrial membrane potential (e.g., using JC-1 dye), ATP levels, oxygen consumption rate (using a Seahorse analyzer), and activity of electron transport chain complexes. Cellular uptake and intracellular trafficking of DSPCar-AmB should be visualized using fluorescently labeled lipids or drugs. The potential protective effect of L-carnitine—whether delivered as part of the nanocarrier or as a free molecule—should be explored in additional experiments, including studies with carnitine-free control liposomes and studies in which cells are pretreated with free carnitine before exposure to toxic concentrations of AmB.

From a regulatory perspective, the results of this study represent an important step in the preclinical development of DSPCar-AmB. The demonstration of reduced cytotoxicity in a relevant human cell line and absence of mutagenicity in the Ames test address two critical safety concerns for any new pharmaceutical formulation (13,14,36). These data would be expected to be included in an Investigational New Drug (IND) application or equivalent regulatory submission as part of the nonclinical pharmacology/toxicology section. However, as discussed above, additional *in vitro* and *in vivo* studies are required before first-in-human studies can be considered. Specifically, regulatory agencies such as the FDA and EMA would typically expect: (i) a complete genotoxicity battery, including a mammalian cell assay for chromosomal damage; (ii) *in vivo* toxicity studies in at least two species (typically rodents and a non-rodent, such as dogs) with assessment of MTD, NOAEL, and target organ toxicities; (iii) pharmacokinetic studies in animals to assess absorption, distribution, metabolism, and excretion; (iv) local tolerance studies; and (v) where applicable, immunotoxicity assessments, including complement activation-related pseudoallergy (CARPA), which represents a known concern for some liposomal formulations (14). The favorable physicochemical properties and safety profile of DSPCar-AmB, combined with its promising antifungal activity, suggest that it could be a viable candidate for further development, but substantial additional work remains.

5. Conclusion

In summary, this study demonstrates that the DSPCar-AmB nanocarrier possesses a significantly improved *in vitro* safety profile compared with a reference liposomal AmB formulation. The nanocarrier exhibits substantially reduced cytotoxicity against HEK293 cells, with an IC_{50} exceeding 100 μ M compared with 8.2 μ M for the reference formulation, and shows no detectable mutagenic activity in the Ames test. The physicochemical properties of

DSPCar-AmB (size, zeta potential, encapsulation efficiency, and biphasic release profile) are favorable for an intravenously administered nanomedicine and likely contribute to its improved safety profile through controlled release and reduced direct interaction of AmB with mammalian cell membranes. The presence of DSPCar, a novel phospholipid based on L-carnitine, may provide additional mitochondrial protective effects, though this hypothesis requires direct testing. When considered alongside previously reported antifungal efficacy, these safety data suggest that DSPCar-AmB may have an improved therapeutic index compared with currently available AmB formulations and warrants further preclinical development. However, several important limitations must be addressed in future studies, including the need for complementary cytotoxicity assays, mammalian genotoxicity testing, *in vivo* toxicity studies in animals, chronic toxicity assessments, and mechanistic studies to elucidate the role of DSPCar. Despite these limitations, the current findings provide a strong foundation for continued development of this promising nanocarrier.

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Ethical Considerations

This study was approved by the Ethics Committee of TMS.C., Islamic Azad University, Tehran, Iran. (code: IR.IAU.PS.REC.1401.330).

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Conflict of Interests

The Authors declare that there is no conflict of interest.

AI Using Declaration

In this study, ChatGPT-3 was used to generate and process the data associated with Figure 1 and Figure 3. Although this model was no longer the most recent version as of May 30, 2026, it was still employed in certain analytical stages of this paper due to its effective basic capabilities in text generation, information summarization, and pattern extraction. All outputs produced by this system were reviewed and verified by the authors prior to being used in the figures.

Author's contributions

Conceptualization and Supervision :A.S, M.N, E.B ,
Methodology :S.S, A.S, M.N, E.B ,Investigation,
Writing – original draft, and Writing – review &
editing: All authors; Data collection: S.S ;Data
analysis: S.S; Funding acquisition and Resources :S.S

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