

Unlocking the Unreachable: Cationic ThermoSomes for Next-Generation Posaconazole Therapy

Ankita N. Yawalkar,[#] Kshitija M. Phatak,[#] Sushant S. Sole, and Pradeep R. Vavia*



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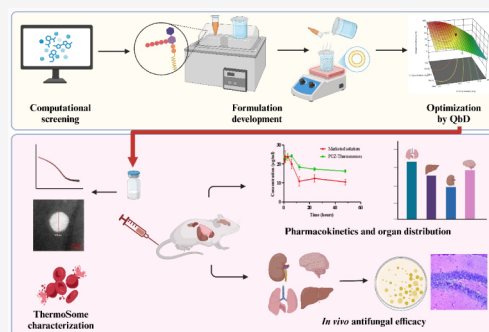
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ABSTRACT: Invasive fungal infections (IFIs) represent a serious global health threat due to rising incidences and drug resistance. Traditional antifungal therapies face limitations, including toxicity, rapid clearance, and resistance, demanding nanotechnology-driven solutions. While lipid-based formulations, such as AmBisome, have achieved clinical success, scalability is restricted by high costs and complex manufacturing. This study advances the solvent-free approach by developing “ThermoSomes”, bilayered lipid vesicles encapsulating the FDA-approved antifungal Posaconazole (PCZ) for IFI treatment. The formulation was optimized through *in silico* drug–excipient interaction studies, validated *in vitro*, and optimized using Quality-by-Design (QbD). A 6-fold improvement was observed in fungal cell internalization of cationic ThermoSomes compared to anionic and neutral counterparts, owing to ionic interactions with the fungal cell wall. Pharmacokinetic studies showed improvements in parameters like $AUC_{0-48\text{ h}}$ by 4.68-fold and $t_{1/2}$ by 1.37-fold. *In vivo* studies demonstrated that intravenous PCZ-ThermoSomes outperformed the conventional marketed solution (Noxafil) in terms of therapeutic efficacy, exhibiting approximately 2-fold greater reduction in fungal burden across all critical organs in a murine candidiasis model. Here, we have broadened the therapeutic framework of PCZ by targeting critical organs and maintaining therapeutic concentrations, which are difficult to achieve by conventional formulations. Thus, PCZ-ThermoSomes serve as a safe, stable, nontoxic, efficacious, scalable, and cost-effective alternative to existing treatment modalities for IFIs.

KEYWORDS: invasive fungal infections, posaconazole, bilayered lipid vesicles, materials studio, thermoSomes



1. INTRODUCTION

Invasive fungal infections (IFIs) represent a grave clinical challenge, arising from the systemic dissemination of pathogenic fungi into the bloodstream and deep-seated tissues. These infections are predominantly driven by fungal species such as *Candida*, *Aspergillus*, *Cryptococcus*, *Pneumocystis*, and members of the Mucorales order.¹ High-risk populations include critically ill patients, especially immunocompromised individuals, and those suffering from malignancies, HIV/AIDS, organ transplant recipients, and individuals with chronic respiratory diseases or tuberculosis infection.² A significant surge in the global incidence of fungal infections has been documented, with an estimated 1.9 million cases occurring annually and nearly 1.6 million associated fatalities worldwide.³

Current therapeutic interventions for IFIs are limited to three main classes: ergosterol inhibitors (azoles, polyenes), glucan synthase inhibitors (echinocandins), and agents targeting RNA/DNA metabolism (flucytosine).⁴ Despite their clinical use, these agents face significant challenges such as resistance (azoles, echinocandins), toxicity (polyenes), and the need for combination therapy (flucytosine).^{5–8,9} All classes suffer from poor solubility, limited bioavailability, suboptimal pharmacokinetics, and toxicity, particularly at higher doses, complicating treatment

in vulnerable patients.^{8,9} These inherent limitations highlight the pressing need for advanced nanotechnology-based formulations, such as nanoparticles, liposomes, and nanoemulsions, which present a promising alternative in the management of IFIs. These nanoscale delivery systems offer distinct advantages by enhancing drug solubility and bioavailability, facilitating targeted drug delivery, enabling sustained drug release, reducing systemic toxicity, circumventing drug resistance, and optimizing pharmacokinetic (PK) profiles. Liposomal Amphotericin B formulation represents one of the best examples of such a nanotechnology-based approach available commercially.^{10,11,12}

Posaconazole (PCZ) is a broad-spectrum triazole antifungal approved by the FDA for the treatment and prophylaxis of infections caused by *Candida*, *Aspergillus*, and Mucorales, including mucormycosis. Its broad-spectrum efficacy, favorable PK properties, low toxicity, and effectiveness against resistant

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fungal strains render PCZ a superior choice for managing IFIs compared to other antifungal agents.¹³ Despite these advantages, oral PCZ is characterized by variable bioavailability due to its erratic, food-dependent absorption profile.^{11,12} On the other hand, intravenous formulations of PCZ utilize a cyclodextrin vehicle, sulfobutyl ether β -cyclodextrin (SBECD), to enhance solubility. Although SBECD is generally well-tolerated, its accumulation in patients with impaired renal function can result in nephrotoxicity, particularly in those with pre-existing kidney conditions or those receiving other nephrotoxic agents.¹¹ These limitations associated with conventional formulations underscore the need for improved drug delivery systems. Therefore, to overcome these challenges, we, for the first time, report formulating PCZ into a lipid bilayered nanovesicular system, having structural resemblance to that of liposomes, hereafter referred as ThermoSomes, for intravenous administration. We anticipate that the nanostructured ThermoSomes would not only serve as a safer alternative but also facilitate enhanced delivery to pharmacologically and anatomically challenging infection sites, especially organs, such as the brain, lungs, and kidneys, which are often poorly accessible due to biological barriers. This ability of ThermoSomes to achieve higher drug concentrations and maintain therapeutic levels for a significantly longer duration in these difficult-to-access tissues expands the therapeutic reach of hydrophobic drugs like PCZ.

Our work utilizes a novel heat-mix method for the formulation of ThermoSomes, which facilitates the spontaneous formation of liposome-like lipid bilayered nanovesicular structures using a greener approach. This method utilizes a solubilizer as a vehicle for the non-aqueous components of the system, instead of traditionally used volatile organic solvents. Furthermore, the process is less time-consuming and does not require sophisticated equipment, offering a notable advantage over conventional techniques, such as thin-film hydration and solvent injection. We propose that this innovative method represents a viable alternative for the formulation and scale-up of liposome-like structures with comparable structural integrity and activity profiles. Further, our work focuses on utilizing *in silico* tools for preformulation analysis, encapsulating PCZ in ThermoSomes, optimizing it using the Quality by Design (QbD) approach, and evaluating its performance both *in vitro* and *in vivo*. We have identified the ability of the optimized PCZ-ThermoSomes to significantly lower the fungal burden by performing *in vitro* studies in *Candida albicans* MCC 1794 cells and pharmacodynamic studies in a murine systemic candidiasis model. Thus, PCZ-ThermoSomes highlight their potential as a promising therapeutic approach for the treatment of IFIs.

2. MATERIALS AND METHODS

2.1. Materials. The drug PCZ was obtained as a gift sample from Bajaj Healthcare Ltd., Thane, India. Gattefosse, Mumbai, India, provided Transcutol HP as a gift sample. Soya Phosphatidylcholine (SPC), Egg Phosphatidylcholine (EPC), Hydrogenated Soya Phosphatidylcholine (HSPC), and Hydrogenated Egg Phosphatidylcholine (HEPC) were obtained as gift samples from VAV Lipids, Mumbai, India. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine (DPPE), Distearoylphosphatidylcholine (DSPC), Dimyristoylphosphatidylcholine (DMPC), Dipalmitoylphosphatidylcholine (DPPC), and Sphingomyelin from egg (SM) were obtained as gift samples from Lipoid, Germany. Dicyetyl Phosphate (DCP) and Sodium Lauryl Sulfate (SLS) were obtained from Sigma-Aldrich, Mumbai, India. 2-Pyrrolidone was procured from BASF,

Mumbai, India. Polyethylene Glycol 400 (PEG), Propylene Glycol (PG), Cholesterol, and Dimethyl Sulfoxide (DMSO) were purchased from SDFCL, India. Stearylamine was purchased from SRL Laboratories. Lactose, dextrose, sucrose, mannitol, and trehalose were obtained from Signet Chemical Corporation, India. Sabouraud Dextrose Agar (SDA) media and broth were purchased from Hi-Media, India. Millipore HPLC-grade water was used throughout the study. HPLC-grade acetonitrile was purchased from Merck, India. All other solvents and reagents used were of analytical grade.

2.2. Preformulation Studies. The preformulation studies were carried out using *in silico* and experimental methods. The *in silico* drug-excipient interaction studies were performed on some selected solubilizers and phospholipids using Materials Studio software (version 2017, Accelrys, Inc., San Diego, CA, USA), employing the Forcite and Amorphous Cell modules.^{14,15} The interaction study was particularly based on the calculation of the miscibility of the drug with the excipients, based on the Cohesive Energy Densities (CEDs), Hildebrand solubility parameter (δ), and the Flory–Huggins interaction parameter (χ).¹⁶ The 2D structures of the respective solubilizers, phospholipids, and PCZ were imported from the PubChem database and were energy-minimized, followed by geometry optimization using the Forcite module employing the COMPASS II force field. The Amorphous Cell module of Materials Studio was used to build 3D models of the drug and the selected excipients. Ten molecules of both the drug and excipient were individually assembled within a 3D cubic lattice, with periodic boundary conditions applied to ensure continuity across the model. Each Amorphous Cell (AC) construct underwent geometry optimization using the Forcite module with the COMPASS II force field, followed by an equilibration phase involving 10 simulated annealing cycles under an NVT ensemble (constant volume and temperature) with an Anderson thermostat. Subsequent equilibration employed an NPT ensemble (constant pressure and temperature) managed by a Berendsen barostat, and density and pressure convergence were assessed over a 1 fs simulation time.

Post-equilibration, molecular dynamics (MD) simulations were conducted sequentially using the NVT and NPT ensembles, extending to 100 fs, followed by 5,000 fs in an NHL ensemble (constant energy and volume) to stabilize the system. The resulting trajectory data facilitated the calculation of cohesive energy density (CED) and the derivation of both the Hildebrand solubility parameter and the Flory–Huggins interaction parameter, quantifying the interaction potential between drug and excipient molecules. CED represents the average energy required to separate molecules to infinite distance, calculated as per the formula:

$$E_{coh} = -(E_{inter}) = (E_{intra}) - (E_{total}) \quad (1)$$

where E_{inter} represents the energy between all molecules, E_{total} represents the total energy of molecules in the system, and E_{intra} represents the intramolecular energy. The CED values, as computed by the software, can be used to derive the value of δ , which can be calculated as,

$$\delta = \sqrt{CED} \quad (2)$$

Further, the value of χ can be calculated from the solubility parameters between the drugs and the excipients using the following formula:

$$\chi = V \times (\delta A - \delta B)^2 / RT \quad (3)$$

Table 1. Levels for the Study of Independent Variables

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Drug loading	mg	Numeric	3.50	21.00	-1 ↔ 3.50	+1 ↔ 21.00	12.25	7.29
B	Cholesterol	mg	Numeric	19.00	38.60	-1 ↔ 19.00	+1 ↔ 38.60	28.80	8.16
C	Soya lecithin	mg	Numeric	64.40	193.00	-1 ↔ 64.40	+1 ↔ 193.00	128.70	53.56
D	Solubilizer	mg	Numeric	200.00	335.50	-1 ↔ 200.00	+1 ↔ 335.50	267.75	56.44
E	Sonication cycles	Number	Numeric	1.0000	12.00	-1 ↔ 1.00	+1 ↔ 12.00	6.50	4.58
F	Stabilizer	mg	Numeric	2.70	13.50	-1 ↔ 2.70	+1 ↔ 13.50	8.10	4.50

where V is the volume of the respective segments, δA and δB are the Hildebrand solubility parameters, R is the gas constant, and T is the temperature.

The smaller values of χ and the difference in the value of δ between the drug and excipients indicate the miscibility and compatibility of the components studied.^{17,18}

The results obtained from *in silico* interaction studies were validated by screening excipients experimentally. For this, the saturation solubility of PCZ in several solubilizers, such as Transcutol HP, 2-pyrrolidone, PG, and PEG, was assessed. Also, various natural phospholipids, namely SPC, EPC, and SM, semisynthetic phospholipids, namely HSPC and HEPC, and synthetic phospholipids, namely DPPE, DMPC, DSPC, and DPPC, differing in their chemical composition, were systematically screened to assess the encapsulation efficiency of PCZ within ThermoSomes. The ThermoSomes were formulated using a consistent concentration of 50 mM phospholipid, 50 mM cholesterol, and an excess amount of PCZ. Post fabrication, untrapped PCZ was separated from the ThermoSomes, and the drug loading efficiency was determined using the method outlined in Section 2.5.2.

Subsequent selection of the optimal phospholipid for ThermoSomes preparation was conducted using the Design of Experiments (DOE) methodology. This approach considered both the lipid's capacity to encapsulate the drug and the associated cost, ensuring an efficient and economically viable formulation process.

2.3. Fabrication of PCZ-Thermosomes. The lipid bilayer vesicles were fabricated by using a novel heat mixing method. Briefly, PCZ was solubilized in Transcutol HP as a solubilizer at 70 to 80 °C with gentle shaking. SPC was then incorporated into the drug solution, followed by thorough homogenization. Cholesterol was added as a rigidity-enhancing agent, and stearylamine was included as a stabilizing charge modifier, leading to the formation of a non-aqueous phase mixture. Separately, double-distilled water was heated to the same temperature to create the aqueous phase. The aqueous and non-aqueous phases were rapidly combined with vigorous shaking, then stirred on a magnetic stirrer at 500 rpm for 15 min to promote the spontaneous formation of bilayer lipid vesicles. The resulting ThermoSomes were further subjected to probe sonication to reduce particle size and lyophilized after the addition of cryoprotectants.

2.4. Optimization of PCZ-Thermosomes. The optimization of PCZ-ThermoSomes was conducted utilizing a Quality by Design (QbD) framework employing Design Expert software, version 11. Response Surface Methodology (RSM) was adopted to elucidate the interfactorial interactions among selected independent product and process variables, namely, the quantities of phospholipid, cholesterol, stabilizer, solubilizer, and the number of sonication cycles. The cumulative effects of these variables were assessed on dependent outcomes, such as particle size, polydispersity index (PDI), and entrapment

efficiency. Selection of these factors was based on literature surveys^{19–21} and preliminary experimental trials. The study implemented a Face-Centered type Central Composite Design (CCD), culminating in 50 experimental runs with 6 center points. The higher and lower levels of independent variables were set as shown in Table 1 (refer Table S1 for experimental runs designed by the software). The resulting data were subjected to statistical interpretation using Analysis of Variance (ANOVA) and appropriate statistical models, and the best-fit models were selected based on the significance in p value, regression coefficient, the difference between predicted and actual r^2 values, and the F value (refer Tables S3–S5 for F value and lack-of-fit evaluation). Both numerical and graphical optimizations were performed, after which the software proposed optimal solutions based on predefined criteria for the dependent variables. The validity of the suggested statistical model and the optimized formulation was confirmed through the calculation of % bias.^{22,23} This optimized formulation, having a net positive charge, was then utilized for characterization and evaluation. Additionally, ThermoSomes with neutral and negative charges were prepared and used exclusively in fungal cell uptake studies.

2.5. Evaluation of PCZ-Thermosomes. **2.5.1. Particle Size, Surface Charge, and Morphology.** The particle size, distribution profile, and surface charge of the PCZ-ThermoSomes were meticulously characterized via Dynamic Light Scattering (DLS) using a Zetasizer Nano ZSP (Malvern, USA). The samples were diluted 10 times with water and analyzed for particle size at a 90° angle at 25 °C. Similarly, the samples were diluted and placed in electrophoretic cells for the measurement of zeta potential.²⁴ To gain deeper insights into the morphological attributes of the formulation, Transmission Electron Microscopy (TEM) (Tecnai 12 G2, Philips, Netherlands) was utilized, enabling high-resolution visualization of the nanoparticle size and shape.²⁵ Furthermore, Small-Angle Neutron Scattering (SANS) was employed using a SANS diffractometer at the Guide Tube Laboratory, Dhruva Reactor, Bhabha Atomic Research Centre, Mumbai, India, to confirm the bilayer morphology of the ThermoSomes, providing a comprehensive understanding of the vesicular architecture and validating the structural integrity of the lipid bilayer.²⁶

2.5.2. Purification and Entrapment Efficiency. The purification of the PCZ-ThermoSome formulation was achieved via gel filtration using a Sephadex G-50 column.²⁷ Presoaked Sephadex G-50 was employed to prepare a minicolumn, through which the formulation was passed. The eluted fractions containing purified PCZ-ThermoSomes were collected until the eluent was clear. The pooled fractions were then subjected to ultracentrifugation at 40,000 rpm for 30 min at 10 °C. The resulting pellet was analyzed using a validated HPLC method to quantify the entrapped drug. The HPLC analysis was carried out on a Waters Alliance System (Waters, USA), equipped with a UV detector at a wavelength of 254 nm, using an Inertsil C18

ODS-2 column (4.5 mm × 15 cm, 5 μm packing). Water:–Acetonitrile (40:60) was used as the mobile phase at a flow rate of 1 mL/min. Entrapment efficiency was subsequently calculated by using the appropriate equation.

Entrapment efficiency (%)

$$= \text{Experimental drug content} / \text{Total drug content} \times 100 \quad (4)$$

Further confirmation of drug encapsulation within the ThermoSomes and its interaction with excipients was conducted using Fourier Transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), and X-ray Diffraction (XRD) techniques.²⁸

2.5.3. In Vitro Drug Release. The *in vitro* drug release study was performed using the sample-and-separate method with a USP-2 apparatus.²⁹ PCZ-ThermoSomes, equivalent to 9 mg of PCZ, were added to 900 mL of release medium containing 0.3% SLS in water. The drug release was monitored over a 48 h period at 50 rpm and 37 °C. Samples (1 mL aliquots) were taken at predetermined intervals (0.5, 1, 2, 4, 8, 12, 24, and 48 h) and subjected to ultracentrifugation at 40,000 rpm for 30 min at 10 °C. The supernatant was analyzed for dissolved PCZ content using the HPLC method described in Section 2.5.2. The pellet was returned to the dissolution medium to prevent sample loss, and the release medium was replenished with an equal volume of fresh medium to maintain sink conditions.

2.5.4. In Vitro Time–Kill Assay. The *in vitro* antifungal time–kill assay was performed as per the reported method with slight modification.^{30,31} A fresh suspension of *Candida albicans* MCC 1794 cells was prepared by inoculating colonies grown overnight in RPMI-1640 medium, buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) to a pH of 7.0. A 9.5 mL aliquot of this suspension, containing approximately 1×10^5 CFU/mL, was transferred into sterile conical flasks. To each flask, 0.5 mL of the test sample was added. The test samples included PCZ-ThermoSomes (equivalent to 1.5 μg/mL of PCZ), placebo ThermoSomes, a marketed PCZ solution (Noxafil, equivalent to 1.5 μg/mL of PCZ), and sterile PBS (negative control). All test samples were prepared in a 5% w/v dextrose infusion solution. The cultures were incubated at 35 °C for 24 h. Following incubation, a 100 μL aliquot from each flask was removed and washed with physiological saline (PS) to eliminate any remaining test samples, and the resulting pellet was resuspended and serially diluted in PS. A 20 μL aliquot from each dilution was then plated onto SDA plates and incubated at 35 °C for 48 h. The colonies formed on each plate were subsequently counted. This assay was performed in triplicate to ensure accuracy and reproducibility.

2.5.5. In Vitro Fungal Cell Uptake Analysis. *In vitro* uptake of the developed ThermoSomes by fungal cells was evaluated to understand the potential of ThermoSomes as carriers for PCZ. For this, Rhodamine B-loaded ThermoSomes were formulated to quantitatively understand the uptake of vesicles by fungal cells. Rhodamine B, equivalent to 1 mg, was added to the non-aqueous phase, and ThermoSomes were prepared as per the method described in Section 2.3. Further, to assess the impact of surface charges on the extent of uptake by fungal cells, here, we included uncharged (neutral), positively charged (cationic), and negatively charged (anionic) ThermoSomes. Formulations were prepared by adding charge-imparting agents such as stearyl amine and DCP to yield positively charged and negatively charged particles, respectively. A *Candida albicans* MCC 1794

suspension (1 mL) consisting of 10^6 CFU/mL was centrifuged at 3000 rpm for 5 min to give a fungal cell pellet. This pellet was then redispersed and incubated with the above-mentioned formulations for a period of 30 min. Post incubation, the samples were centrifuged to get a fungal cell pellet, which was washed with saline to remove any traces of free Rhodamine B. The obtained cell pellet was then redispersed in saline and observed using a confocal microscope (Leica Microsystems CMS, Germany). The intensities of uncharged, anionic, and cationic ThermoSomes were quantified using ImageJ software and compared with the intensity of the cell suspension treated with Rhodamine B in DMSO.

2.5.6. In Vitro Plasma Stability. The colloidal stability of the optimized PCZ-loaded ThermoSomes was evaluated to assess potential structural alterations, destabilization, or degradation of the formulation when exposed to plasma proteins or biological fluids, as well as its effect on particle size and size distribution. The formulations were incubated with an equal volume of 50% rat plasma at 37 °C and agitated at 30 rpm. Samples were withdrawn at pre-determined intervals up to 48 h (1, 2, 4, 8, 12, 24, and 48 h), and particle size was analyzed using a Zetasizer Nano ZSP. The particle size of the incubated formulation was then compared with that of the unincubated sample (0 h).³²

2.5.7. In Vitro Hemolysis Analysis. The *in vitro* hemolysis assay was performed using human blood. Briefly, Red Blood Cells (RBCs) were isolated by centrifugation at 3000 rpm for 3 min. The isolated RBCs were then washed and resuspended in PBS (pH 7.4) to match the original blood volume, achieving normal hematocrit levels (40%). A 500 μL aliquot of this RBC suspension was combined with 400 μL of PBS. Subsequently, 100 μL of either the marketed PCZ solution or PCZ-ThermoSomes, prepared at concentrations equivalent to 400, 800, 1200, and 1600 μg/mL of PCZ in 5% w/v dextrose infusion solution, was added. This resulted in final concentrations of 40, 80, 120, and 160 μg/mL of PCZ. An RBC suspension treated with 0.1% Triton X-100 (prepared in PBS, pH 7.4) served as a positive control, and PBS was used as a negative control. All tubes were incubated for 30 min at 37 °C, followed by centrifugation at 3000 rpm for 5 min. The supernatants (400 μL) were then diluted with 600 μL of PBS and transferred to cuvettes. Hemoglobin release, indicative of hemolysis, was quantified by UV spectrophotometry (Jasco, Japan) at 550 nm. The percentage of hemolysis was calculated using the appropriate formula as depicted in eq 5.³³

$$\begin{aligned} \text{\%Haemolysis} &= \frac{(\text{Absorbance of test} - \text{Absorbance of negative control})}{(\text{Absorbance of positive control} - \text{Absorbance of negative control})} \times 100 \quad (5) \end{aligned}$$

2.5.8. Stability. The lyophilized formulations were filled into glass vials, sealed, and their stability was evaluated over a period of 6 months under two storage conditions (2–8 °C and 25 °C/65%RH) as per ICH guidelines.³⁴ The samples were removed at intervals of 1, 3, and 6 months, analyzed for % entrapment and particle size, and compared with the initial samples (0 month) to determine the stability of the developed PCZ-ThermoSomes.

2.5.9. Acute Toxicity. Acute toxicity of the developed PCZ-ThermoSomes was assessed in Swiss albino mice with a uniform body weight of around 20–25 g. This study was conducted in accordance with the CCSEA guidelines and with approval from the Institutional Animal Ethics Committee of the Institute of Chemical Technology, Mumbai (ICT/IAEC/2024/10). The

mice were divided into 3 groups, each containing 10 animals (5 males and 5 females). Before the start of the experiment, the animals were quarantined for a period of 7 days and were maintained on a standard diet. After acclimatization, rats from group I received PCZ-ThermoSomes (equivalent to PCZ 0.8 mg/kg), group II received placebo ThermoSomes, whereas group III served as a control and received normal saline via tail vein injection. After a period of 14 days, blood from each animal was withdrawn from the retro-orbital plexus and subjected to hematological and biochemical examination. Following this, all the animals were humanely sacrificed by excess carbon dioxide, and the vital organs, including lungs, liver, kidneys, spleen, heart, and brain, were surgically removed. The isolated organs were fixed in formalin (10%) and subjected to histopathological examination to understand the effects on tissue morphology.^{35,36}

2.5.10. Pharmacokinetics and In Vivo Biodistribution. The PK performance of the formulation was evaluated in a murine model, following the protocol in accordance with the CCSEA guidelines and approved by the Institutional Animal Ethics Committee of the Institute of Chemical Technology, Mumbai (ICT/IAEC/2024/09). The study was conducted in Swiss albino mice, each weighing around 20–25 g. The animals were divided into 4 groups, each containing 12 animals. Each group received either normal saline, placebo ThermoSome formulation, PCZ-ThermoSome formulation (equivalent to a PCZ dose of 8 mg/kg), or marketed PCZ solution (equivalent to a PCZ dose of 8 mg/kg) via tail vein injection. Blood samples were collected from the retro-orbital plexus at specific time intervals to analyze the *in vivo* behavior of the administered samples. Blood samples from two animals per group were pooled ($n = 6$), and drug content was determined by HPLC using a liquid–liquid extraction method, following the HPLC parameters outlined in Section 2.5.2 and additionally incorporating voriconazole (10 $\mu\text{g/mL}$) as an internal standard during the development of the bioanalytical method. Following a seven-day washout period, the animals were dosed again in a similar manner. At each time point, two animals from each group were sacrificed, and the biodistribution (BD) of the drug in organs, such as the kidneys, brain, liver, lungs, heart, and spleen, was assessed using the bioanalytical HPLC method.

2.5.11. In Vivo Efficacy. The *in vivo* efficacy study was conducted following the protocol in accordance with the CCSEA guidelines and was approved by the Institutional Animal Ethics Committee (ICT/IAEC/2024/08). The study involved Swiss albino mice, each weighing between 20 and 25 g. A systemic candidiasis model was developed based on established literature protocols.^{37–39,40} Fresh *Candida albicans* MCC 1794 colonies, cultured for 24 h, were harvested, washed, and resuspended in normal saline to create a suspension containing mid-log-phase organisms.

The study protocol consisted of two parts: a pilot experiment and a principal experiment. The pilot experiment aimed to optimize the fungal inoculum concentration and determine the drug dosage required to reduce the fungal burden in critical organs by 50% following infection. Before initiating the experiment, all mice were immunocompromised with cyclophosphamide (200 mg/kg) administered via intraperitoneal injection for two consecutive days. For inoculum optimization, the mice received tail vein injections of three different fungal inoculum concentrations: 10^3 , 10^6 , and 10^9 CFU/mL. After 48 h, the mice were sacrificed, and the kidneys, lungs, liver, and brain were harvested. The organs were washed, homogenized

using a tissue homogenizer, and plated on SDA plates. The fungal colonies were counted after 48 h of incubation. The optimal fungal inoculum concentration was determined based on mortality rates using Kaplan–Meier analysis and the fungal counts in the vital organs.

Following this, the optimized fungal inoculum concentration was administered to six mice, which were then treated with four different concentrations of PCZ solutions (0.4, 0.8, 1.2, and 1.6 mg/kg) for two consecutive days starting the day after inoculation. After 48 h, the mice were sacrificed, and their vital organs were isolated, homogenized, and plated on SDA plates to assess fungal burden. The PCZ concentration that resulted in a 50% reduction in the fungal burden was selected for the principal study.

In the principal experiment, the efficacy of the developed PCZ-ThermoSome formulation was compared to that of a marketed PCZ solution. Here, 24 mice were divided into 4 groups, acclimatized, and immunocompromised as in the pilot study before receiving an injection of the fungal inoculum (10^6 CFU/mL). The mice were then given the treatment for 2 consecutive days postinfection. The first group received normal saline, the second group received placebo ThermoSomes, the third group was treated with PCZ-ThermoSomes (equivalent to 0.8 mg/kg), and the fourth group received the marketed PCZ solution (equivalent to 0.8 mg/kg). After 48 h, all mice were sacrificed, and their vital organs were harvested and plated on SDA plates to evaluate fungal growth post 48 h of incubation. Additionally, Kaplan–Meier analysis was conducted to assess the post-treatment survival rates.

3. STATISTICAL ANALYSIS

Data were presented as mean $\pm$ standard deviation (SD). Statistical comparisons across groups were evaluated using one-way analysis of variance (ANOVA), followed by an unpaired *t*-test to assess pairwise differences. A *p*-value of less than 0.05 was set as the threshold for statistical significance. Analyses were conducted using GraphPad Prism software (version 7, GraphPad Inc., San Diego, USA).

4. RESULTS AND DISCUSSION

4.1. Preformulation. The screening of phospholipids and solubilizers for PCZ formulations was conducted through a comprehensive approach that integrated both dry lab and wet lab methodologies. Utilizing Materials Studio, we performed computational simulations to assess the interactions between PCZ and various solubilizers and phospholipids, employing the Forcite and Amorphous Cell modules to evaluate these interactions via δ and χ_{ij} values. It is well-established in the literature that smaller differences in solubility parameters correspond to lower energy requirements for mixing, indicating greater compatibility between components. Additionally, lower χ_{ij} values signify more favorable molecular interactions.^{17,18} Our computational analysis demonstrated that the solubility parameter difference was the smallest for the PCZ-Transcutol HP and PCZ-SPC pairs. Furthermore, PCZ showed the lowest χ_{ij} values with Transcutol HP, followed by 2-pyrrolidone, PG, and PEG, suggesting strong interactions with Transcutol HP. In the case of phospholipids, the lowest χ_{ij} values were observed with SPC, indicating optimal interactions, followed by other semisynthetic and synthetic phospholipids.

Experimental validation of these *in silico* predictions demonstrated an excellent correlation between computational

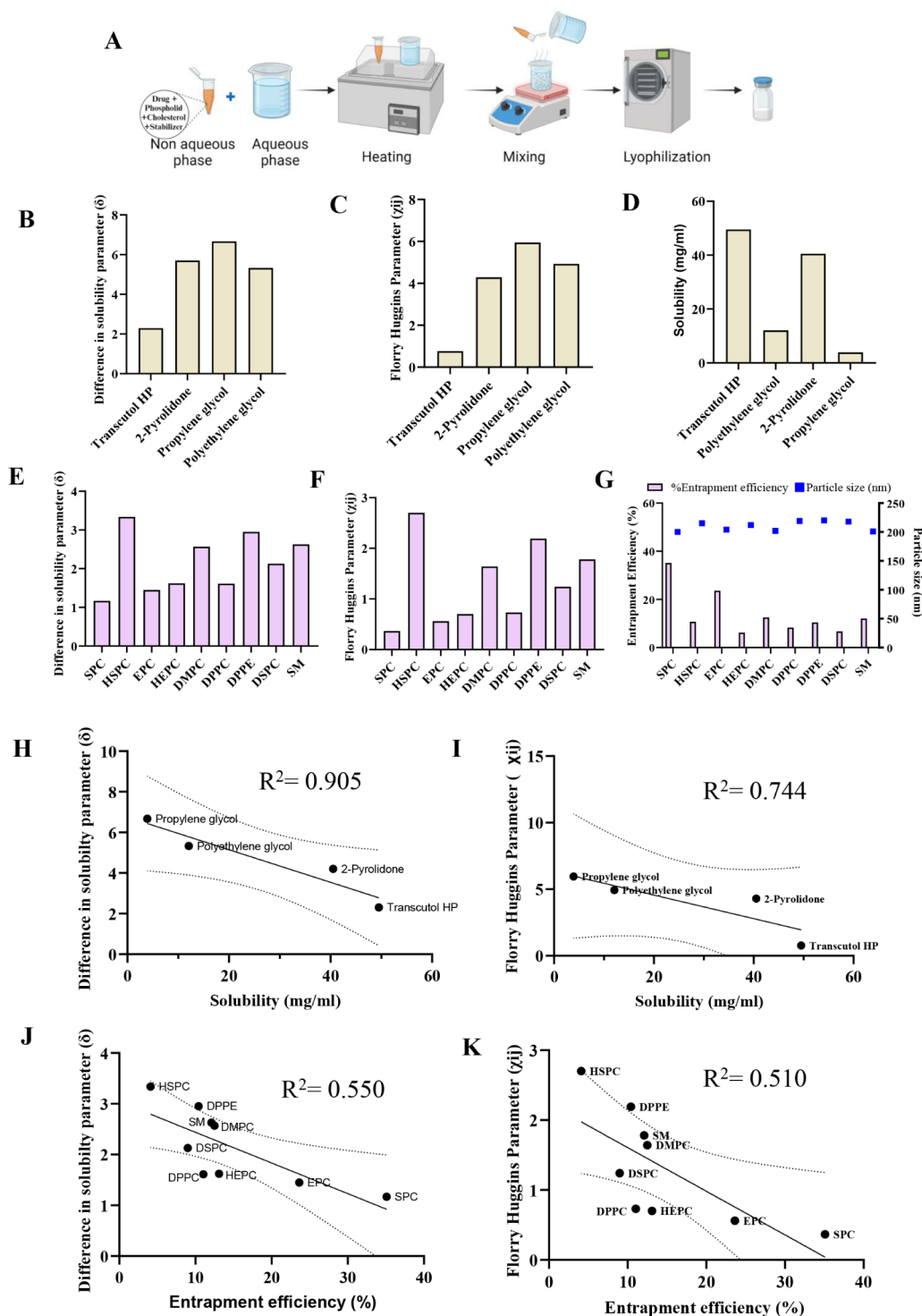


Figure 1. Method of preparation and screening of excipients. (A) Fabrication of PCZ-ThermoSomes. (B) Difference in solubility parameters of solubilizers. (C) Flory–Huggins parameter of solubilizers. (D) Experimental solubility of PCZ in solubilizers. (E) Difference in solubility parameters of phospholipids. (F) Flory–Huggins parameter of phospholipids. (G) Experimental entrapment efficiency and particle size in phospholipids. (H) Statistical correlation between difference in solubility parameters and experimental solubility of PCZ in solubilizers ($R^2 = 0.905$). (I) Statistical correlation between the Flory–Huggins parameter and experimental solubility of PCZ in solubilizers ($R^2 = 0.744$). (J) Statistical correlation between the difference in solubility parameters and experimental entrapment efficiency of PCZ in surfactants ($R^2 = 0.550$). (K) Statistical correlation between the Flory–Huggins parameter and experimental entrapment efficiency of PCZ in surfactants ($R^2 = 0.510$). Data were obtained as a result of statistical correlation calculated using the Pearson correlation coefficient, expressed as R^2 value.

trends and the experimental solubility of PCZ in the solubilizers. Experimentally, the solubility of PCZ was highest in Transcutol HP (48.5 mg/mL), followed by 2-pyrrolidone, PG, and PEG (Figure 1D). Furthermore, SPC exhibited the highest entrap-

ment efficiency for PCZ (35.65%) among the screened phospholipids, followed by EPC and other semisynthetic and synthetic phospholipids (Figure 1G). The superior entrapment capacity of SPC can be attributed to its structural characteristics,

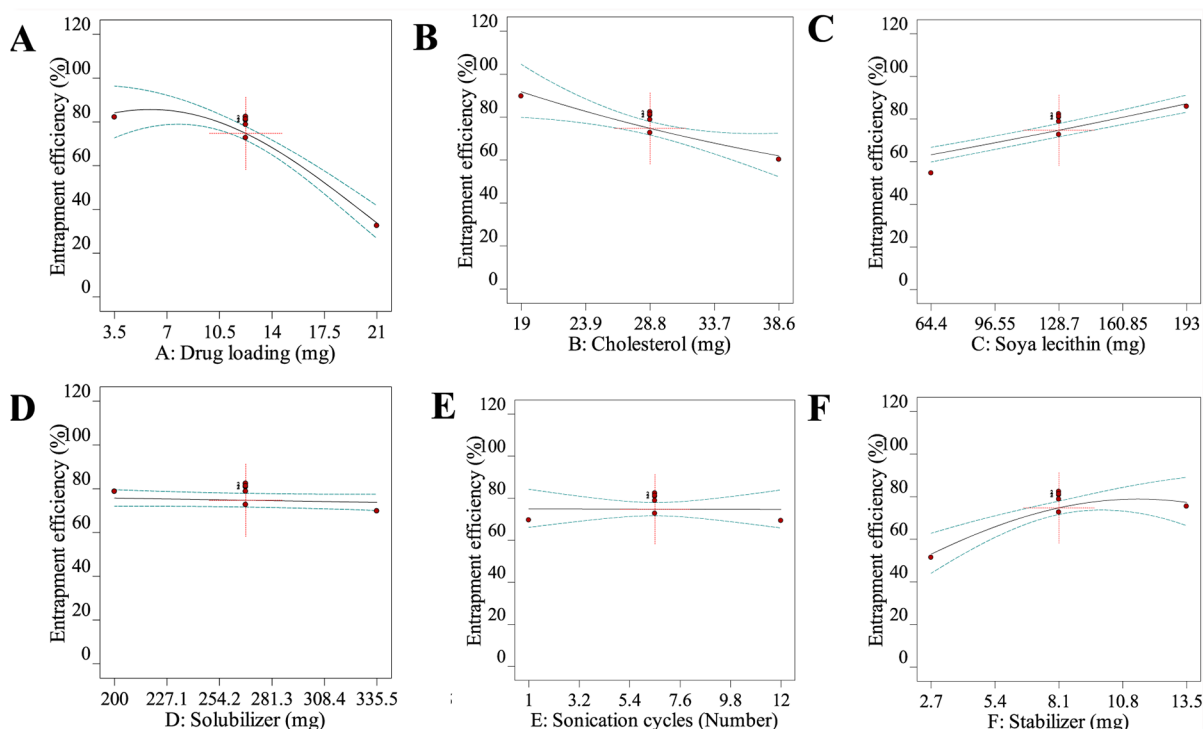


Figure 2. 2D plots depicting the effects of different independent variables on entrapment efficiency (refer Figure S1 for 3D response surface plots).

specifically the presence of unsaturated bonds and extended hydrocarbon chains. The two double bonds in SPC introduce kinks in the ThermoSomes' lipid bilayer, thereby creating additional spatial accommodation for hydrophobic drugs. Additionally, the elongated hydrocarbon chains enhance hydrophobic interactions, leading to improved loading efficiency.^{41,42} EPC and SM, which contain a single double bond, displayed intermediate entrapment efficiencies, surpassing their semisynthetic and synthetic counterparts. These observations highlight the impact of lipid unsaturation on drug loading capacity.

Herein, we correlated *in silico* δ and χ_{ij} values to experimentally determined entrapment efficiency. A strong statistical correlation¹⁸ was found between the difference in δ values of PCZ with various solubilizers and the experimental solubility data ($R^2 = 0.905$), and between χ_{ij} values and solubility data ($R^2 = 0.744$) for solubilizers. While *in silico* predictions provided valuable insights into solubilizer behavior, some discrepancies were noted when predicting lipid entrapment efficiencies. The correlation coefficient for the difference in δ values of PCZ with various phospholipids and the experimental entrapment data ($R^2 = 0.55$), and for χ_{ij} values with entrapment data ($R^2 = 0.51$), indicated reasonable but not perfect alignment. This inconsistency highlights the complexity of lipid structures, indicating a need for more sophisticated computational models. Improved modeling techniques could enhance our ability to predict complex molecular interactions, thereby facilitating more accurate correlations between theoretical predictions and experimental outcomes in future studies. Nevertheless, this study underscores the utility of combining *in silico* tools with experimental validation to streamline excipient selection and optimize drug formulation strategies.

4.2. Fabrication of PCZ-Thermosomes. In this study, we present the fabrication of PCZ-Thermosomes using a novel heat-mixing method that capitalizes on the innate propensity of

lipid molecules to spontaneously form vesicles when exposed to energy, such as heat or agitation. Lipid molecules, due to their amphiphilic nature, have hydrophilic heads and hydrophobic tails. In aqueous environments, these molecules spontaneously arrange themselves to minimize free energy, often resulting in the formation of bilayer structures.

Traditional methods for creating lipid vesicles typically involve the use of organic solvents to form a non-aqueous phase, which facilitates the assembly of lipid bilayers. In contrast, our innovative approach eliminates the need for organic solvents by using a solubilizer to generate the non-aqueous phase. When this non-aqueous phase is mixed with an aqueous phase at the same temperature, it leads to the spontaneous formation of lipid vesicles, encapsulating PCZ in the process. This spontaneous formation of lipid vesicles in this system is primarily driven by a significant reduction in interfacial and free energies, which promotes the self-assembly of lipid molecules into vesicular structures. The introduction of heat plays a crucial role in this process, not only by providing the necessary energy to overcome any potential barriers to self-assembly but also by creating a thermodynamically favorable environment for vesicle formation. This thermodynamic favorability is characterized by an increase in both entropy and enthalpy, which together drive the self-organization of lipids into stable vesicles.^{42,43,44}

This method represents a significant advancement by simplifying the vesicle formation process and avoiding the complexities and potential toxicity associated with organic solvents. Moreover, it leverages the inherent thermodynamic properties of lipids to facilitate efficient drug encapsulation and vesicle formation, making it a promising approach for the development of lipid-nanoparticle-based drug delivery systems.

4.3. Optimization of PCZ-Thermosomes. The PCZ-Thermosomes were optimized with respect to their particle size, zeta potential, and entrapment efficiency using Design Expert software, version 11. Statistical analysis of the identified

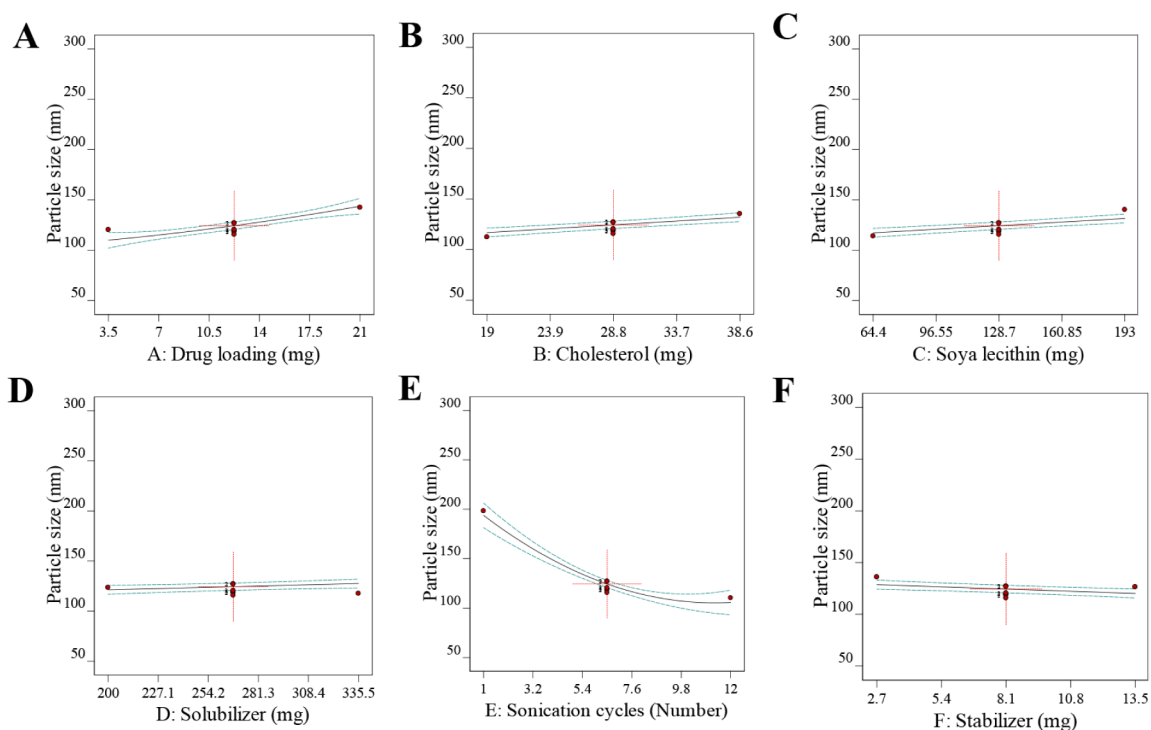


Figure 3. 2D plots depicting the effects of different independent variables on particle size (refer Figure S2 for 3D response surface plots).

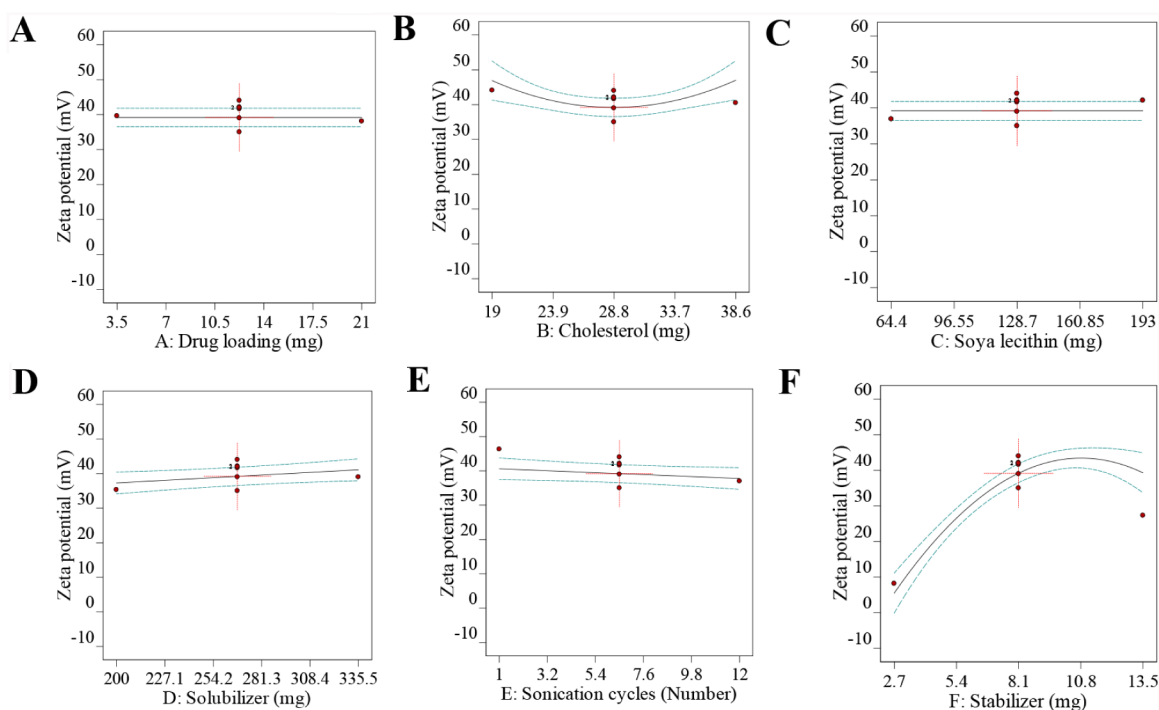


Figure 4. 2D plots depicting the effects of different independent variables on particle size (refer Figure S3 for 3D response surface plots).

dependent variables revealed that the reduced cubic model was found to be significant in predicting the response of encapsulation efficiency and particle size, while the reduced quadratic model was found to be significant for zeta potential. The key variables significantly influencing the vesicles' drug entrapment capacity included drug loading, cholesterol content, phospholipid concentration, and the amount of stearylamine ($p < 0.05$). The effects of the individual independent factors on the entrapment efficiency are represented in Figure 2A–F.

According to the plots obtained and observed, an increase in the SPC concentration led to a marked improvement in the entrapment efficiency (Figure 2C). This is attributed to the enhanced availability of the hydrophobic bilayer region, which provides a more accommodating environment for lipophilic molecules within the bilayer leaflet. Conversely, a linear decrease in entrapment efficiency was observed when the cholesterol concentration was increased from 19 to 36 mg (Figure 2B), or when the drug concentration exceeded 10.5 mg (Figure 2A).

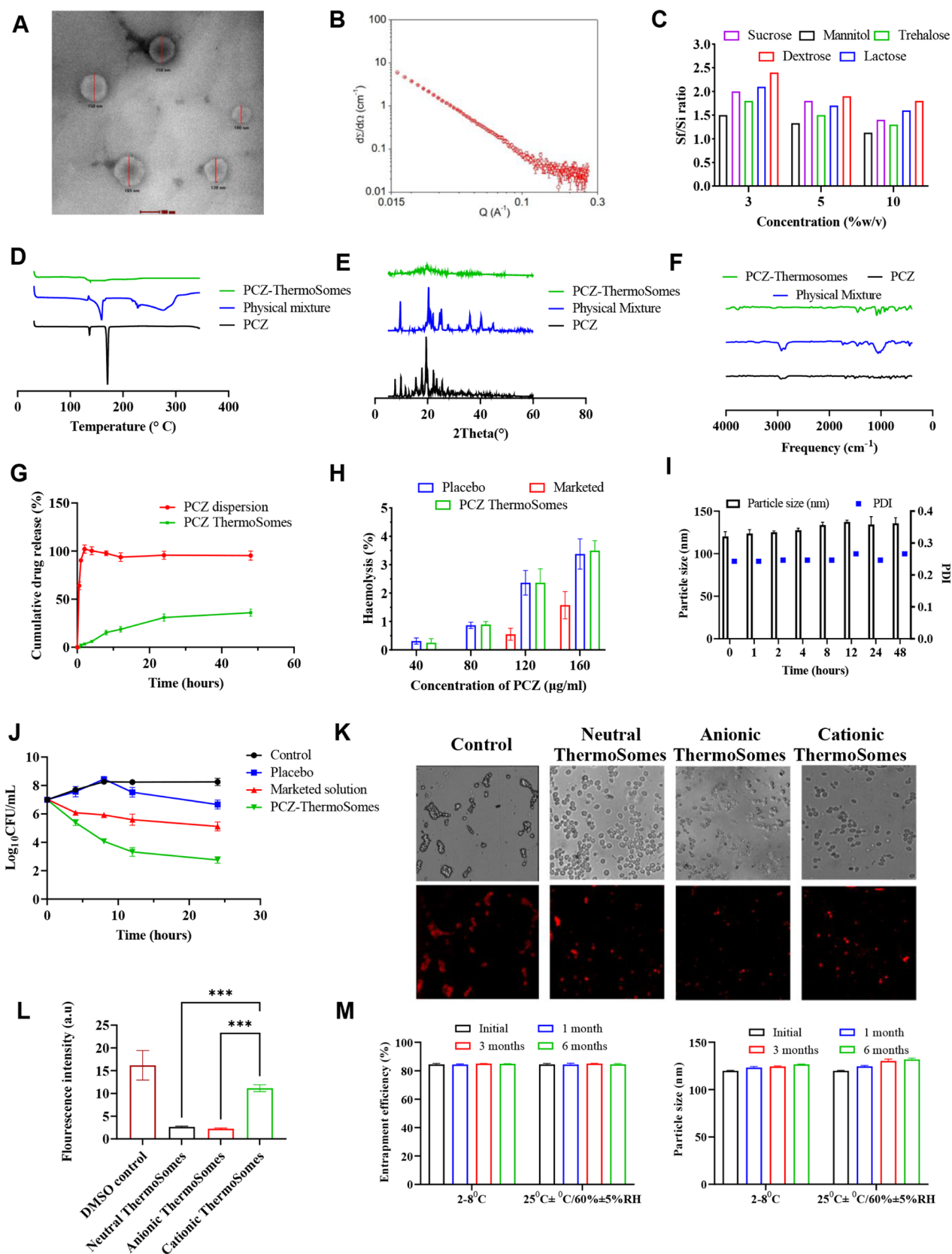


Figure 5. Characterization of PCZ-ThermoSomes. (A) Representative TEM image depicting the spherical morphology and particle size of PCZ-ThermoSomes (scale bar: 100 nm). (B) SANS analysis confirming the vesicular structure of PCZ-ThermoSomes. (C) Cryoprotectant screening. (D–F) DSC, XRD, and FT-IR plots confirming encapsulation of PCZ in ThermoSomes. (G) *In vitro* drug release over a period of 48 h (mean $\pm$ SD, $n = 3$). (H) *In vitro* hemolysis analysis of PCZ-ThermoSomes, placebo ThermoSomes, and the marketed solution (mean $\pm$ SD, $n = 3$). (I) Stability analysis of PCZ-ThermoSomes in rat plasma in terms of particle size and PDI over a period of 5 h. (J) *In vitro* time-kill assay against *Candida albicans* MCC 1794 (mean $\pm$ SD, $n = 3$). (K) Representative CLSM images of *Candida albicans* stained with Rhodamine B-tagged ThermoSomes to indicate uptake of

Figure 5. continued

ThermoSomes by fungal cells. (L) Fluorescence intensity of uptaken ThermoSomes quantified using ImageJ (mean $\pm$ SD, $n = 3$). (M) Stability of lyophilized PCZ-ThermoSomes with respect to particle size and entrapment efficiency over a period of 6 months as per ICH guidelines (mean $\pm$ SD, $n = 3$).

This decline likely results from competition between these two hydrophobic entities (PCZ and cholesterol) for limited space within the lipid bilayer's hydrophobic compartment.²¹ Interestingly, a rise in the entrapment efficiency was noted with the increased addition of stearylamine, a positively charged stabilizer (Figure 2F). This suggests that positive surface charges contribute to enhanced membrane stabilization.⁴⁵ The stearylamine likely binds to the slight anionic charges present on the drug molecules, thereby minimizing drug leakage from the vesicle and reinforcing the structural integrity.

The particle size was found to be influenced by all the selected factors, with the extent of influence being highest with respect to the number of sonication cycles, as depicted by the response curves ($p < 0.05$). The effects of the individual independent factors on the particle size are represented in Figure 3A–F. It is evident that an increase in the number of sonication cycles initially resulted in a decrease in particle size as a result of the applied sonication energy, whereas a further increase in the number of sonication cycles was found to slightly increase the particle size because of the nucleation effect of the finer particles (Figure 3E).²⁰ Also, an increase in drug, cholesterol, and phospholipid content was found to increase the overall particle size when studied as a single factor or even in combination (Figure 3A–C).⁴⁶ Also, it is worth noting that the solubilizer was found to decrease the particle size to a very slight extent, suggesting that the role of the solubilizer was just to solubilize the API and serve as a medium in the nonaqueous phase to facilitate spontaneous vesicle formation (Figure 3D).

Next, the effect of independent variables was studied on the zeta potential (Figure 4A–F). The effect of stearylamine was significant on the zeta potential (Figure 4F), whereas only a very slight effect was also observed with respect to cholesterol (Figure 4B). The zeta potential of liposomes increases with the incorporation of stearylamine due to its intrinsic cationic nature. At physiological pH, stearylamine, an amine-bearing lipid, becomes positively charged and integrates into the lipid bilayer of the liposomes. This integration enhances the surface charge density, as the positively charged amine groups accumulate on the liposome surface. As the zeta potential is directly related to the surface charge, this increase in positive charge results in a higher zeta potential.⁴⁷ Furthermore, stearylamine neutralizes any negatively charged phospholipids of the liposomes, thereby shifting the overall charge toward a more positive value. This reduces any residual negative zeta potential and progressively increases the positive zeta potential. Additionally, as the positive charge on the liposome surface increases with a higher stearylamine content, the resulting stronger electrostatic repulsion between particles enhances colloidal stability, preventing aggregation.

Based on these interactions, the software suggested 100 possible formulation solutions that would yield the desired results. The optimized formula obtained consisted of drug (13.1 mg), SPC (122.4 mg), Transcutol HP (335 mg), and stearylamine (7.2 mg). Also, it was found that a total of 7 sonication cycles were required to achieve the desired entrapment, particle size, and zeta potential. The suggested solution was validated experimentally, and a 4.9% bias was

obtained when compared to the software-predicted results, confirming the optimization model's reliability.^{48,49} The optimized formulation was converted into a free-flowing powder by a freeze-drying process. Various cryoprotectants, namely sucrose, mannitol, trehalose, dextrose, and lactose, were screened at different concentrations (Figure 5C), after which mannitol (5%) was selected owing to the lowest S_0/S_1 ratio and was added to the formulation before lyophilization.

4.4. Evaluation of PCZ-Thermosomes. **4.4.1. Particle Size, Surface Charge, and Morphology.** The optimized PCZ-ThermoSomes exhibited an average particle size of 120 nm with a PDI of 0.25, as determined by DLS. The zeta potential of the PCZ-ThermoSomes was found to be +43.2 mV, indicating good ThermoSome stability and a low tendency for aggregation. TEM images confirmed the vesicles' circular shape, with the observed particle size aligning well with the DLS measurements, further validating the consistency of the vesicle size (Figure 5A).

Given that the ThermoSomes were synthesized using a novel method involving solubilizers, it was crucial to verify the formation of bilayered lipid vesicles and ensure the absence of mixed systems or aggregates. SANS was employed to assess the shape, size, and internal structure of the particles. The SANS data fit a model characteristic of vesicular systems, consistent with findings by Aswal et al.,²⁶ and the scattering curve (Figure 5B) followed Q^{-2} behavior in the low- Q range, as exhibited by typical vesicular systems, confirming the presence of large unilamellar vesicles with a bilayer thickness of 40.1 Å.

4.4.2. Purification and Entrapment. Following purification as mentioned in Section 2.5.2, the PCZ-ThermoSomes showed an entrapment efficiency of 83.69%, demonstrating the vesicles' high capacity to encapsulate PCZ. The DSC analysis of PCZ-ThermoSomes showed a thermogram without the characteristic endothermic peak of PCZ (at 169 °C), confirming successful drug encapsulation (Figure 5D). FT-IR spectroscopy of the PCZ-ThermoSomes showed the absence of the characteristic peaks (at 2800 cm^{-1}) observed in the spectra of pure PCZ and its physical mixture, suggesting complete drug entrapment (Figure 5F). The XRD analysis, which reflects the crystallinity of a substance, further supported this by showing no crystalline peaks of PCZ, confirming its amorphous state (Figure 5E). These comprehensive characterization studies conclusively demonstrated that PCZ was fully encapsulated within the ThermoSomes' formulation, with no free or unbound drug detectable, confirming the effectiveness of the encapsulation process.

4.4.3. In Vitro Drug Release. The drug release study was conducted over a 48 h period to assess the stability of the PCZ-ThermoSomes. Notably, there was no observed drug precipitation or formation of PCZ crystals in the selected release media, indicating a stable dispersion. The results demonstrated that approximately 38.15% of the drug was released from the PCZ-ThermoSomes over the 48-h duration, in contrast to the PCZ dispersion, which exhibited a rapid release and complete release of the drug within just 2 h (Figure 5G). This slower release profile suggests that the PCZ remained effectively associated with the ThermoSomes under the conditions tested, highlighting the formulation's ability to maintain stability.

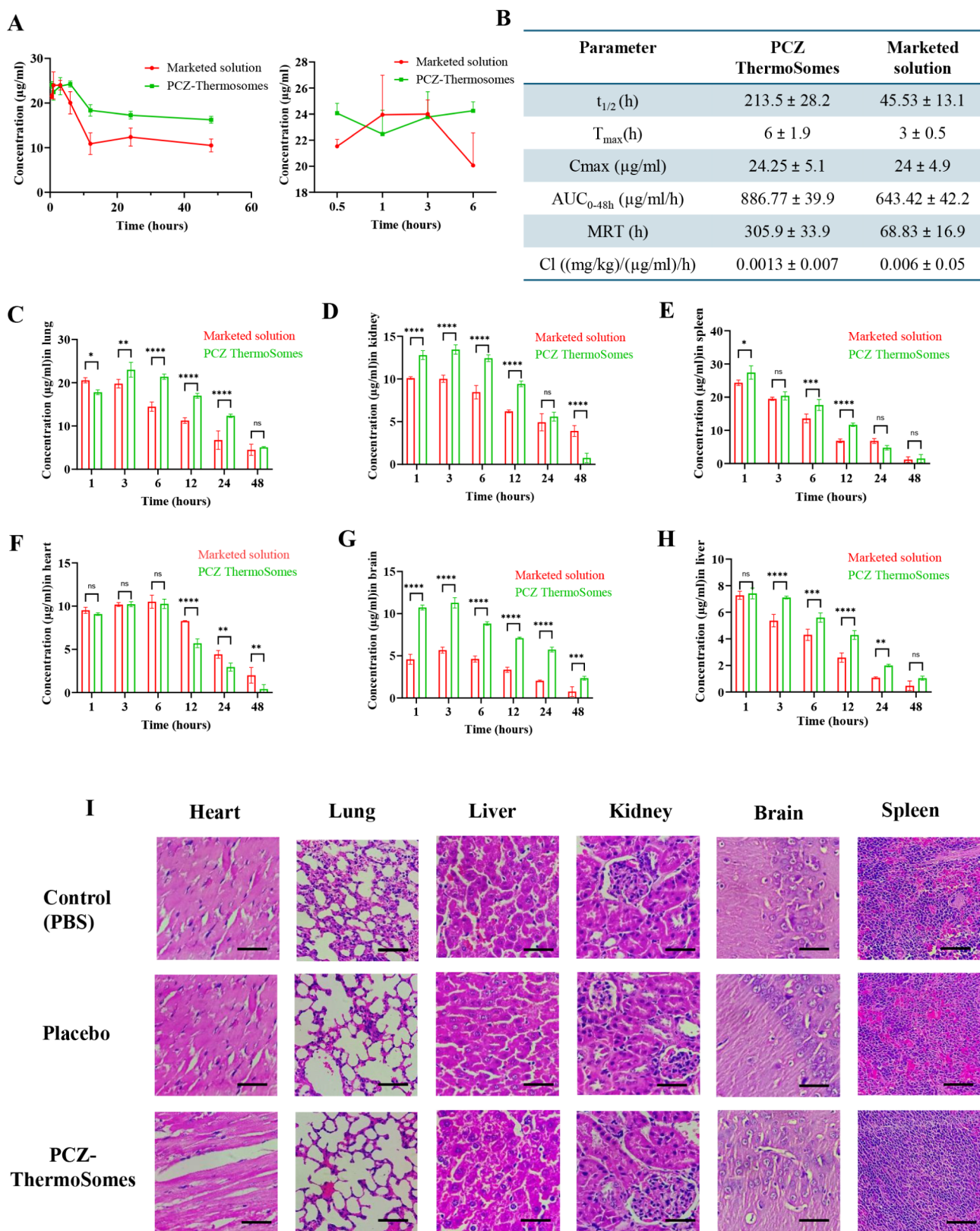


Figure 6. Pharmacokinetics, biodistribution, and acute toxicity of PCZ-ThermoSomes. (A) Pharmacokinetics of PCZ-ThermoSomes and marketed solution over a period of 48 h in Swiss albino mice. (B) Comparative study of pharmacokinetic parameters of optimized PCZ-ThermoSomes and PCZ-ThermoSomes (following intravenous administration, dose: 8 mg/kg). (C–H) Biodistribution profile of PCZ-solution and PCZ-ThermoSomes (8 mg/kg) in the lungs, kidneys, spleen, heart, brain, and liver of Swiss albino mice, respectively, up to 48 h following intravenous administration. (I) Histology profile of vital organs (heart, lung, liver, kidney, brain, spleen) after a 14-day acute toxicity study in groups administered with PBS (control), placebo ThermoSomes, and PCZ-ThermoSomes (left to right).

Furthermore, the findings imply that ThermoSomes exhibit stability even when subjected to a dilution factor of up to 100-fold, emphasizing their potential applicability in clinical settings where dilution upon administration is common. It is important to note that the primary objective of this drug release study was not to establish an *in vitro*–*in vivo* correlation but rather to evaluate the stability of the formulation under the specified conditions.

4.4.4. In Vitro Time–Kill Assay. The time–kill assay was conducted to evaluate the time-dependent fungicidal efficacy of PCZ- ThermoSomes. The results showed that both the marketed PCZ formulation and PCZ-ThermoSomes significantly reduced fungal burden, while the placebo exhibited no significant effect compared to the control group ($p < 0.05$). The fungal load reduction by PCZ-ThermoSomes and the marketed formulation was equivalent at initial time points. However, at later time points, PCZ-ThermoSomes demonstrated markedly superior fungicidal activity (Figure 5K). This enhanced efficacy is likely due to the nanoscale size of PCZ-ThermoSomes, facilitating greater fungal cell uptake and boosting antifungal action.⁵⁰ Furthermore, by the end of the 24-h incubation period, PCZ-ThermoSomes achieved a 2-fold greater reduction in fungal burden compared with the marketed formulation, highlighting its superior internalization and therapeutic potential.

4.4.5. In Vitro Fungal Uptake Analysis. The uptake of negatively charged, positively charged, and uncharged PCZ-ThermoSomes was evaluated by measuring the fluorescence intensity. As shown in Figure 5L, the positively charged ThermoSomes exhibited 5 times higher fluorescence intensity than the uncharged and negatively charged ThermoSomes, indicating higher uptake of cationic ThermoSomes by fungal cells. The enhanced uptake of the cationic particles was likely due to ionic interactions between the positively charged vesicle surface and the negatively charged fungal cell wall, which facilitates the entry of positively charged ThermoSomes, increasing the therapeutic efficiency of cationic ThermoSomes.^{50,51} Further, the enhanced uptake was confirmed by the confocal images (Figure 5K).

4.4.6. In Vitro Plasma Stability Studies. The stability of the developed nanoformulation in the presence of plasma proteins, as illustrated in Figure 5I, was thoroughly evaluated by incubating PCZ-ThermoSomes with separated rat plasma. The analysis revealed that both the particle size and PDI of the ThermoSomes remained consistent throughout the incubation period of 48 h, indicating a lack of significant interaction between the PCZ-ThermoSomes and the plasma proteins.⁵² The nanoformulation maintains its colloidal integrity upon exposure to biological fluids, which is essential for ensuring predictable PK and BD upon *in vivo* administration, where stability in the bloodstream is paramount.

4.4.7. In Vitro Hemolysis Analysis. The *in vitro* hemolysis study was conducted to evaluate the impact of the developed nanoparticles on RBCs under physiological conditions. Both the formulation and the marketed solution were tested at working concentrations ranging from 40 $\mu\text{g/mL}$ to 160 $\mu\text{g/mL}$. The results indicated that both formulations were safe, with hemolysis remaining below the acceptable threshold of 10% (Figure 5H).⁵³ Notably, the developed nanoformulation exhibited slightly higher hemolysis compared to the marketed solution, likely due to the presence of positively charged surface stabilizers. Nevertheless, hemolysis levels below 10% are

deemed safe for intravenous administration, indicating that the formulation is suitable for clinical use.

4.4.8. Stability. There was no significant change ($p < 0.05$) in the particle size and entrapment efficiency upon exposing the formulations to the mentioned stability conditions, suggesting that the developed PCZ-ThermoSomes were stable for a period of 6 months under the conditions studied (Figure 5M).

4.4.9. Acute Toxicity Studies. We tested the toxicity and tolerability of the developed PCZ-ThermoSomes formulation *in vivo* in mice. There were no signs of abnormalities detected in the groups studied, indicating the safety and tolerability of the formulation. No abnormalities or noticeable damage were observed in the histopathological sections of the vital organs studied (Figure 6I). The hematological and biochemical analyses suggested that all vital functions, including kidney and liver functions, were unaffected by the administration of PCZ- ThermoSomes (refer to Figure S4).

4.4.10. Pharmacokinetics and Biodistribution. The *in vivo* PK performance of the developed PCZ-ThermoSomes was analyzed in comparison to the PCZ marketed solution. The plasma concentration of PCZ versus time profile is shown in Figure 6A. The mean PK parameters were calculated using PK Solver, version 2.0, and are listed in Figure 6B. The results demonstrated a significant difference ($p < 0.05$) in the main PK parameters, namely $t_{1/2}$, $\text{AUC}_{0-48\text{ h}}$, MRT, and clearance. It was found that the $\text{AUC}_{0-48\text{ h}}$ of PCZ-ThermoSomes increased by 1.37-fold compared to the marketed solution, with a subsequent decrease in clearance (4.43-fold). Furthermore, the $t_{1/2}$ and MRT of the PCZ-ThermoSomes were found to increase by 4.69 and 4.44 times, respectively. These results may be attributed to the slow release of PCZ from the ThermoSomes vesicles, leading to slower elimination and a higher AUC. Also, the presence of positive surface charges imparted stability to the vesicles, leading to an extended circulation time.⁵⁴

The BD study, conducted over a 48 h period, aimed to assess the partitioning of both the marketed PCZ solution and PCZ-ThermoSomes into vital organs. The marketed solution demonstrated rapid distribution, with higher drug concentrations observed in highly perfused organs, such as the spleen, lungs, and liver, followed by the kidney, heart, and brain, as expected for a hydrophobic drug. However, the inherently poor blood–brain barrier (BBB) permeability of PCZ resulted in low brain tissue concentrations. Conversely, PCZ-ThermoSomes exhibited superior tissue concentrations compared to the marketed solution across nearly all assessed organs, except for the liver (at 1 h) and heart tissues (up to 6 h), where the concentrations of the marketed solution and PCZ-ThermoSomes were found comparable (Figure 6C–H). The cationic nature of the nanoformulation dramatically altered the BD compared to that of the conventional IV solution. Due to the positive surface charge, the cationic nanoparticles adhered to negatively charged cellular membranes, resulting in enhanced cellular uptake in various organs. This resulted in greater accumulation in the lungs, spleen, and liver, similar to the intravenous solution but with more efficient intracellular delivery due to nanoparticle-mediated transport.⁵⁵ A pivotal finding of the study is the interaction between the positively charged ThermoSomes and the negatively charged endothelial cells within the brain vasculature, which might facilitate enhanced accumulation in brain tissue. This observation is particularly significant for PCZ and similar molecules, as improved brain permeability is crucial for the treatment of

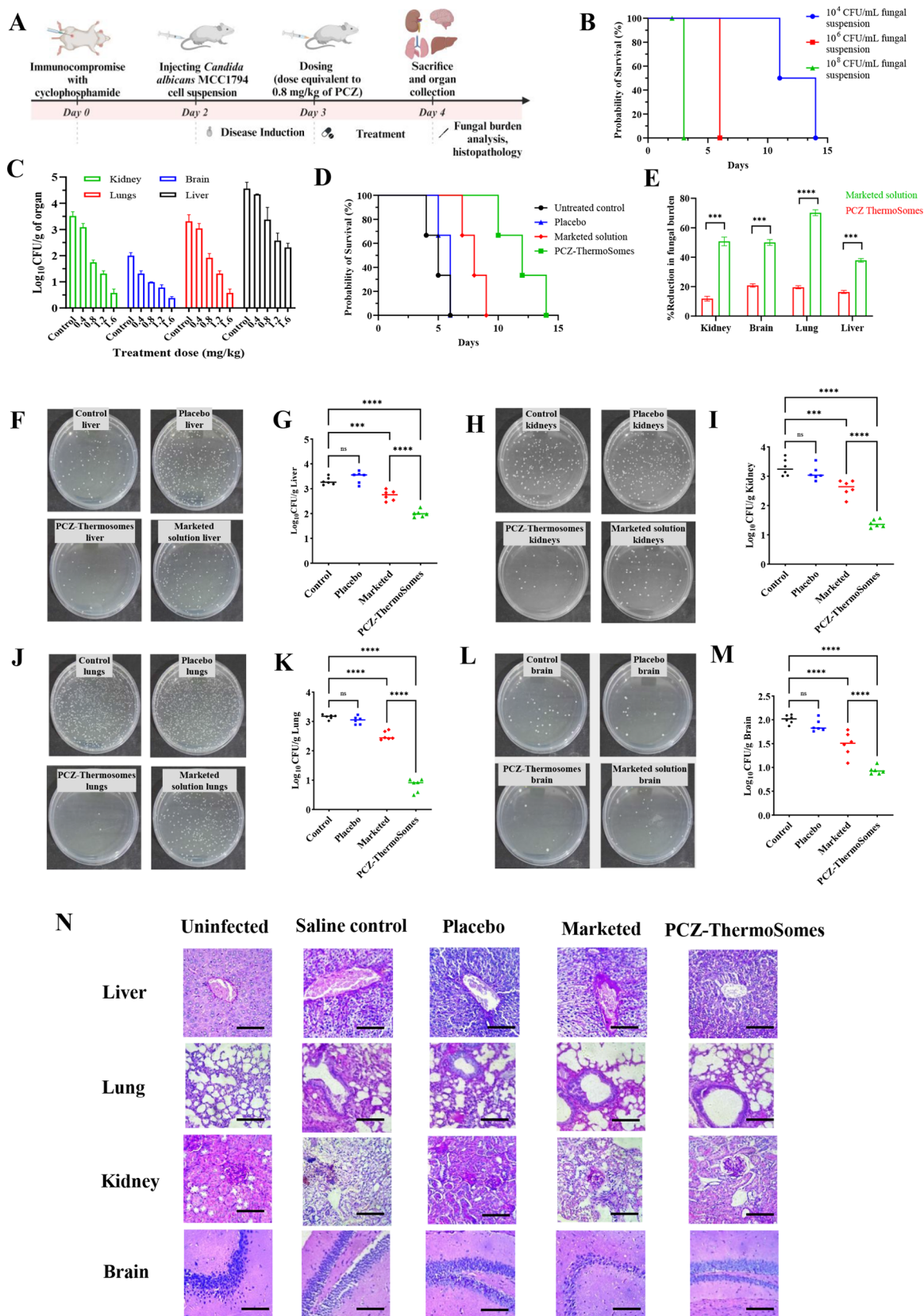


Figure 7. *In vivo* pharmacodynamics study in Swiss albino mice for systemic fungal infections. (A) Diagrammatic representation showing induction of systemic candidiasis in murine model followed by its treatment. (B) Survival plot for selection of optimal fungal inoculum concentration. (C) PCZ dose determination to obtain 50% reduction in fungal burden in infection-prone organs (kidneys, lungs, brain, and liver). (D) Survival plot post treatment in groups (saline-treated control, placebo ThermoSomes, marketed solution, and PCZ-ThermoSomes). (E) Graph representing % reduction in fungal burden in key organs by PCZ-ThermoSomes in comparison with the marketed solution. (F, H, J, L) Representative SDA plates to depict the fungal burden post treatment in groups administered with control saline (top left), placebo ThermoSomes (top right), PCZ marketed solution (bottom left),

Figure 7. continued

and PCZ-ThermoSomes (bottom right) in liver, kidneys, lungs, and brain, respectively. (G, I, K, M) Graphs representing the fungal burden per gram of liver, kidney, lungs, and brain tissues, respectively, in treatment groups (mean $\pm$ SD, $n = 6$). (N) Histopathological sections of uninfected mice, untreated mice, placebo-treated mice, PCZ-marketed solution-treated mice, and PCZ-Thermosomes-treated mice in liver, lungs, kidney, and brain. Data are represented as mean $\pm$ SD ($n = 6$).

cerebral fungal infections, where conventional formulations fail to penetrate the BBB effectively.

4.4.11. In Vivo Efficacy. The *in vivo* efficacy of PCZ-ThermoSomes was systematically evaluated by analyzing the survival rates and reductions in fungal burden across key organs, as per the protocol (Figure 7A). A preliminary study established that an inoculum of at least 10^6 CFU/mL was necessary to induce a detectable infection in mice, while a higher inoculum of 10^8 CFU/mL led to significant mortality within 5 days, as shown by Kaplan–Meier survival analysis (Figure 7B). Based on these findings, a fungal inoculum of 1×10^6 CFU/mL was selected for subsequent studies to balance infection establishment with survival.

Infected mice were treated with varying concentrations of PCZ, ranging from 0.4 to 1.2 mg/kg, and it was determined that a dose of 0.8 mg/kg was necessary to achieve a 50% reduction in fungal burden in critical organs, including the kidneys, liver, lungs, and brain (Figure 7C). This dose was therefore chosen for the principal efficacy study.

In the principal study, the impact of PCZ-ThermoSomes at a dose equivalent to 0.8 mg/kg was examined in comparison to the marketed PCZ solution, the control group (receiving normal saline), and placebo ThermoSomes. The results demonstrated a significant reduction in fungal burden across all isolated organs, namely the kidneys (2.6-fold), lungs (3.4-fold), liver (1.7-fold), and brain (2.2-fold) in mice treated with PCZ-ThermoSomes with respect to the control group (Figure 7F–M). Although the PK study revealed that the BD of the PCZ solution and PCZ-ThermoSomes was comparable in some organs, the dynamics study demonstrated superior antifungal activity of PCZ-ThermoSomes. This superior efficacy of PCZ-ThermoSomes is due to the nanosize and positive surface charge of the formulation, as demonstrated by *in vitro* experiments, which enhance penetration across the negatively charged fungal cell walls.⁵⁶ The PCZ-ThermoSomes outperformed the marketed solution by demonstrating a higher fungal load reduction in each vital organ: kidneys (2.1-fold), lungs (2.6-fold), liver (1.4-fold), and brain (1.9-fold). Notably, the highest reduction in fungal burden was observed in the lungs, followed by the kidneys, brain, and liver, where the fungus is usually highly concentrated in IFIs (Figure 7E). This highlights the potential of PCZ-ThermoSomes in organ-specific targeting for the desired therapeutic response. The results were supplemented by histopathological examinations (Figure 7N), which also revealed the improved efficacy of PCZ-ThermoSomes over the marketed formulation. Additionally, the Kaplan–Meier survival analysis indicated improved survival after treatment in the group treated with PCZ-ThermoSomes (Figure 7D).

5. CONCLUSION

Considering the drawbacks of current therapeutic interventions, we have reported the formulation of a PCZ-loaded lipid-based vesicular system, ThermoSomes, for the first time for intravenous administration. We introduced a novel method for the formulation of liposome-like systems as a greener alternative to conventional methods. We demonstrated the successful

encapsulation of a hydrophobic drug PCZ, introducing the method as a platform technology for the formulation of lipid-based vesicular systems hereafter. We validated the use of *in silico* tools for preformulation studies and confirmed the findings with *in vitro* experiments. The QbD approach enabled precise identification of key factors and optimization of the formulation based on desired constraints. The PK and BD studies highlighted extended systemic circulation and enhanced organ-specific distribution, surpassing the marketed formulation in targeting otherwise difficult-to-reach tissues. An excellent extrapolation was established between *in vitro* antifungal activity and *in vivo* efficacy results in the murine candidiasis model, demonstrating reduced fungal burden and thus enhanced antifungal activity with cationic ThermoSomes. Overall, PCZ-ThermoSomes offer a safe, nontoxic, and effective alternative for treating IFIs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.5c00395>.

Details of optimization experiment designs (Tables S1 and S2), DOE optimization analysis (Tables S3, S4 and S5), 3D response surface plots (Figure S1, S2 and S3), Haematological and biochemical analysis after acute toxicity studies (Figure S4) and particle size data obtained from DLS (Figure S6)(PDF)

■ AUTHOR INFORMATION

Corresponding Author

Pradeep R. Vavia — Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology Elite Status and Center of Excellence — Govt. of Maharashtra, University under Section 3 of UGC Act — 1956, Mumbai, Maharashtra 400019, India; orcid.org/0000-0001-8568-7857; Phone: +91 22 3361 2220; Email: pr.vavia@ictmumbai.edu.in; Fax: +91 22 2414 5614

Authors

Ankita N. Yawalkar — Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology Elite Status and Center of Excellence — Govt. of Maharashtra, University under Section 3 of UGC Act — 1956, Mumbai, Maharashtra 400019, India

Kshitija M. Phatak — Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology Elite Status and Center of Excellence — Govt. of Maharashtra, University under Section 3 of UGC Act — 1956, Mumbai, Maharashtra 400019, India

Sushant S. Sole — Department of Veterinary Pharmacology and Toxicology, Mumbai Veterinary College, Mumbai, Maharashtra 400012, India

Complete contact information is available at: <https://pubs.acs.org/10.1021/acs.molpharmaceut.5c00395>

Author Contributions

[#]A.N.Y. and K.M.P. both authors contributed equally.

Notes

The authors declare no competing financial interest.

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