HO-3867 Induces ROS-Dependent Stress Response and Apoptotic Cell Death in *Leishmania donovani*

Amrita Das, Mohd. Kamran and Nahid Ali*

Infectious Diseases and Immunology Division, Council of Scientific and Industrial Research (CSIR)-Indian Institute of Chemical Biology, Kolkata, India

Lack of vaccine and increasing chemotherapeutic toxicities currently necessitate the development of effective and safe drugs against various forms of leishmaniases. We characterized the cellular stress induced by a novel curcumin analogue, HO-3867, encapsulated within the phosphatidylcholine-stearylamine (PC-SA) liposome for the first time against *Leishmania*. The liposomal formulation of HO-3867 (i.e., PC-SA/HO-3867) initiated oxidative stress-induced apoptosis in *L. donovani*, revealed by altered cell morphology, phosphatidylserine externalization, mitochondrial depolarization, intracellular lipid accumulation, and cell cycle arrest in promastigotes. Liposomal HO-3867 was observed to be a strong apoptosis inducer in *L. donovani* and *L. major* in a dose-dependent manner, yet completely safe for normal murine macrophages. Moreover, PC-SA/HO-3867 treatment induced *L. donovani* metacaspase and PARP1 activation along with downregulation of the Sir2 gene. PC-SA/HO-3867 arrested intracellular *L. donovani* amastigote burden in vitro, with reactive oxygen species (ROS) and nitric oxide (NO)-mediated parasite killing. These data suggest that liposomal HO-3867 represents a highly promising and non-toxic nanoparticle-based therapeutic platform against leishmaniasis inspiring further preclinical developments.

**Keywords:** liposome, stress, *Leishmania donovani*, HO-3867, apoptosis

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is one of the deadly systemic infections caused by *Leishmania donovani/Leishmania infantum* with estimated 200,000–400,000 new cases and more than 20,000–30,000 deaths per year [http://www.who.int/mediacentre/factsheets/fs375/en/]. Emergence of resistant parasites, drug-related toxicities, and HIV co-infection has further complicated the scenario of VL treatment in endemic regions (Singh et al., 2016; Alves et al., 2018). Thus, there is an unmet challenge to develop safe and alternative drugs to treat this fatal infection. HO-3867, a curcumin analogue belonging to class diarylidenylpiperidone (DAP), is a robust STAT3 inhibitor inducing reactive oxygen species (ROS), caspase-3, and PARP1-mediated apoptosis in cancer cells (Selvendiran et al., 2010; Tierney et al., 2012; Rath et al., 2014; Madan et al., 2018; Mast et al., 2019; Li et al., 2020). However, its potency has never been evaluated against any kinetoplastid parasites so far. Despite their multiple benefits, curcuminoids have limited clinical applications due to low bioavailability (Mohammed et al., 2004; Anand et al., 2007; Sinjari et al., 2019).
PC-SA liposomes reportedly target surface exposed anionic phosphatidylserine (PS) abundant on majority of cancer cells (De et al., 2018) and Leishmania (Banerjee et al., 2008; Wanderley et al., 2020) owing to the direct interaction of this anionic lipid with SA. Herein, we envisioned the potential targeting of Leishmania parasites by PC-SA liposomes encapsulating HO-3867 for effective therapy. The enhanced ROS accumulation in PC-SA/HO-3867-treated parasites leads to cell cycle arrest and concurrent depolarization of mitochondrial membrane potential (Zhao et al., 2004) possibly causing release of cytochrome c in the cytoplasm and DNA damage. This indicates involvement of the mitochondria-dependent intrinsic pathway (Kroemer et al., 2007) of cell death in L. donovani after PC-SA/HO-3867 incubation, which in turn activates caspase and PARP-1 as precursors of apoptosis. The results are further supported by ROS and NO-mediated inhibition of intracellular amastigote multiplication in murine peritoneal MΦ after treatment, which needs further in vivo evaluations.

**METHOD**

**Animals and Parasites**

Healthy BALB/c mice bred at the animal house facility of the CSIR-Indian Institute of Chemical Biology [approved by the Committee for the purpose of Control and Supervision on Animal Experiments (CPCSEA), Govt. of India, and the Animal Ethics Committee (147/1999/CPSCSEA) of CSIR-IICB] were used. L. donovani strain AG83 (ATCC® PRA413™) were cultured at 22°C in M199 (Sigma-Aldrich, St. Louis, MO) medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma-Aldrich). Stationary-phase parasites are periodically subcultured to maintain an average density of 2 × 10⁶ cells/ml in M199 medium. L. major promastigotes strain 5ASKH (a kind gift from Dr. Subrata Adak, CSIR-IICB) were cultured at 26°C in M199 medium with 10% heat-inactivated FCS, 200 μM adenine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 40 mM HEPES (Dolai et al., 2008).

**Preparation and Characterization of PC-SA/HO-3867 Liposomes**

Cationic liposomes were prepared with 20 mg of phosphatidylcholine (PC) (Sigma-Aldrich) with stearylamine (SA) (Fluka) at a 7:2 molar ratio by thin-film rehydration method (De et al., 2018). For drug encapsulation, a methanolic solution of HO-3867 (Cayman Chemicals) (1 mg/ml) was added to the lipid film, dried overnight, and finally dispersed in 1 ml of 0.02 M PBS (pH = 7.4) to form a stock solution of 20 mg/ml with respect to PC. Rhodamine B (RhB) (red)-labeled PC-SA liposomes were prepared by adding 0.1 mg/ml of RhB (Sigma) in organic phase together with PC and SA followed by lipid film dispersion in 0.02 M PBS containing 5-carboxyfluorescein (0.1 mg/ml).

Particle sizes of PC-SA and PC-SA/HO-3867 were determined by DLS (dynamic light scattering) using Zetasizer Nano ZS (Malvern Instruments) as described previously (De et al., 2018). The morphology of liposomes was examined by atomic forced microscopy (AFM) following standard procedures after placing the sample on a freshly cleaved mica grid (Das et al., 2018).

Measurement of encapsulation efficiency was based on the absorbance at different concentrations of HO-3867 measured at 328 nm using a UV-visible spectrophotometer, calculated from the amount of free HO-3867 present in the unentrapped fraction after ultracentrifugation (100,000× g for 1 h, at 4°C) using the formula:

\[
\text{Percent encapsulated} = \frac{\text{Total HO-3867} - \text{Free HO-3867}}{\text{Total HO-3867}} \times 100\%
\]

**Cellular Uptake**

Peritoneal macrophages (MΦ) isolated from naïve BALB/c mice are cultured overnight on coverslips (18 mm², 10⁵ MΦ/coverslip) at 37°C with 5% CO₂ in RPMI medium (Sigma-Aldrich) (Das et al., 2018). Resident MΦs were infected with freshly transformed L. donovani promastigotes at a ratio of 1:10 for 4 h at 37°C with 5% CO₂. To assess the uptake efficacy and intracellular docking of free liposomes, infected and uninfected MΦs were treated with Rhodamine B (RhB) (red)-labeled PC-SA liposomes loaded with 5-carboxyfluorescein dye (green) for 2 h. The cells were next fixed with 4% paraformaldehyde (pH 7.4) and counterstained with DAPI (Invitrogen). The fluorescent signals were captured by a TCS-SP8 (Leica Microsystems, Germany) microscope equipped with Leica LAS X live cell imaging software. The lasers used were 405, 488, and 577 argon lasers for DAPI, 5-carboxyfluorescein, and RhB, respectively.

**Promastigote and Amastigote Inhibitory Assays**

To evaluate the effects of HO-3867 on promastigotes, 2 × 10⁵ parasites/ml were treated with graded concentrations of PC-SA liposomes and free and liposomal HO-3867 for 2 h, at 22°C. The untreated and treated parasites were further incubated with 2 mg/ml of MTT [3-((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Affymetrix) solution for 2 and 4 h at 37°C as detailed previously (Shadab et al., 2017). The reduced formazan crystals were dissolved in DMSO, and absorbance was measured at 550 nm in a spectrophotometer.

For evaluating inhibitory effects of free and liposomal HO-3867 on intracellular amastigotes, RAW 264.7 cells (2 × 10⁵ cells/coverslip) were infected with log-phase promastigotes of L. donovani (in 1:10 ratio) for 4 h in RPMI 1640 medium supplemented with 10% FCS. Following the removal of free promastigotes by vigorous washing with 0.02 M PBS thrice, infected MΦs were incubated without drug for an additional 24 h at 37°C in 5% CO₂. Next, the infected MΦs were treated with free or liposomal HO-3867 (2, 5, 10, 30, 50, and 100 μg/ml) diluted in fresh medium for 2 h. After the removal of liposomes and drug, the infected MΦs were further kept for 72 h at 37°C in a CO₂ incubator. After 72 h, the coverslips were washed with 0.02 M PBS and fixed with methanol (Merck) followed by Giemsa staining (1:20 dilution with deionized water, kept for 5–10 min).
min). The number of amastigotes was counted under light microscope for 200 Mφs per sample and expressed as means of three independent experiments. The 50% inhibitory concentration (IC_{50}) of free and liposomal drug was calculated for L. donovani promastigotes and intracellular amastigotes via a non-linear curve.

**Determination of Cell Morphology**

L. donovani promastigotes treated with IC_{50} concentration of free and liposomal HO-3867 and empty PC-SA liposomes for 2 h at 22°C were harvested, washed in 0.02 M PBS, fixed in 4% paraformaldehyde on 18-mm² glass coverslips, and air dried. Cells were sometimes washed in 0.05 ml Milli-Q water to remove the salt depositions and air dried as mentioned. Contact mode AFM was done by PicoPlus 5500 AFM using a piezoscanner in a maximum range of 100 μm and processed using PicoView 1.8 Advanced Software.

**PS Exposure and TUNEL Assay**

For Annexin-V binding, differently treated and untreated 1 × 10⁶ L. donovani promastigotes were suspended in 1 ml of 1× Annexin-V binding buffer (BD Biosciences) and incubated for 5 min in the dark, at room temperature. Following addition of 4 μl of propidium iodide (PI) (BD Biosciences), cells were analyzed by flow cytometry. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed using specific instructions. Brieﬂy, 1 × 10⁶ cells were fixed in 4% paraformaldehyde (pH = 7.4) for 15 min and permeabilized using 100 μl of permeabilization buffer for 5 min on ice. The cells were resuspended in 50 μl of TUNEL labeling reaction mixture (TdT enzyme + labeling solution) and incubated in a dark-humidiﬁed chamber at 37°C for 60 min. Cells treated only with 50 μl of labeling solution (without enzyme) which served as negative control. The reaction was ﬁnally terminated by washing with 0.02 M PBS. The green ﬂuorescence of apoptotic cells was analyzed by confocal microscopy at 488 nm.

**Measurement of Mitochondrial Membrane Potential**

Briefly, treated and untreated promastigotes (1 × 10⁶ cells) were resuspended in 500 μl of 2 μM JC-1 (Molecular Probes) and incubated for 15 min at 37°C in the dark (De et al., 2018). Subsequently, the cells were washed in 0.02 M PBS and subjected to confocal microscopy following nuclear staining with Hoechst 33342 blue (Molecular Probes) for 5 min. The live cell confocal images were captured with 405 for Hoechst 33342, and 470 (for non-polar/neutral lipids) and 546 (for polar lipids) argon lasers for Nile Red (Rosa et al., 2015).

**Real-Time PCR**

Real-time PCR analysis for L. donovani metacaspase, PARP-1 genes, and SIR2 genes was done with gene-specific primers (Purkait et al., 2012; Shadab et al., 2017). Primers for amplifying metacaspase, SIR2, and PARP1 cDNAs were as follows: LdMetacaspase forward, 5′ AAA CGG GTC GAC ATT AAT GC 3′ and LdMetacaspase reverse 5′ CGA CGA TGA GGA AAA GAT CA3′, LdSir2 forward, 5′ GGTCTACATCATGCGGACAT 3′ and LdSir2 reverse 5′ TCTACAGGA AGCGGAAGAG 3′, LdPARP1 forward, 5′ TGGCGGAAGGGCGCTATTTC 3′ and LdPARP1 reverse, 5′ CGCGTGCCGTTGCGCATACC 3′, GAPDH forward, 5′ GAGCTACAGGTGGAGCTGCT 3′, GAPDH reverse, 5′ CGCTGATACGACCTTCTTTC 3′. L. donovani cDNAs were ampliﬁed using SYBR Green Real-Time Master Mix (Roche). Gene expression levels were determined from Cq values followed by normalization of values of each gene (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) with expression levels of GAPDH by 2^−ΔΔCt. The fold expression was calculated as:

\[
\text{Fold expression} = 2^{-\Delta\Delta Ct}
\]

**DNA Fragmentation and Cell Cycle Analysis in L. donovani**

L. donovani promastigotes were treated with 10 μg/ml of free and liposomal HO-3867 or empty PC-SA liposomes for 2 h, and genomic DNA was isolated using the Suicide Track™ DNA Ladder Isolation Kit (Calbiochem) using the manufacturer’s instructions (Shadab et al., 2017). After washing in 70% ethanol, the genomic DNA pellets obtained were ﬁnally dispersed in resuspension buffer (10 Mm Tris–HCl, pH = 7.5, 1 Mm EDTA) and visualized with 0.5 μg/ml ethidium bromide (EtBr) staining, under a Bio-Rad Gel Documentation system.

For cell cycle analysis, after for 2 and 6 h of drug treatment, L. donovani promastigotes were harvested, washed in 0.02 M PBS, and ﬁxed in 70% ethanol (constituted in PBS) overnight.
Cells were stained with FxCycle™ PI/RNase Staining Solution (Molecular Probes) for 30 min at room temperature. The percentage of cells in different cell cycle phases, i.e., G0, G0/G1, S, and G2/M, was gated using BD LSRFortessa and analyzed by BD FACS Scan Software.

**Evaluation of In Vitro Macrophage (MΦ) Infection**

Peritoneal MΦs collected from naïve BALB/c mice were plated overnight in RPMI 1640 medium on 18-mm² glass coverslips as detailed previously (Das et al., 2018). MΦs were infected in *vitro* with *L. donovani* promastigotes at a 1:10 ratio for 4 h at 37°C in a CO₂ incubator. After removing free promastigotes by three successive washings with 0.02 M PBS, infected MΦs were incubated without drug for 24 h at 37°C in 5% CO₂. Next, the infected MΦs were treated with free or liposomal HO-3867 (10 μg/ml) diluted in fresh medium for 2 h. Excess liposomes and drug were removed after 2 h, and the infected MΦs were kept for an additional 48 and 72 h at 37°C in a CO₂ incubator. Finally, the coverslips were washed with 0.02 M PBS and fixed with methanol (Merck) followed by Giemsa staining. The number of amastigotes was counted under a light microscope for 200 MΦs per sample and expressed as means of three independent experiments. Nitric oxide (NO) production was quantified in culture supernatants of HO-3867 and PC-SA/HO-3867 (10 μg/ml) treated MΦs by Griess Reagent (absorbance 540 nm) (Das et al., 2018). ROS generation in the infected MΦs was assessed by 10 μM H₂DCFDA using a Synergy H1 (Biotek) microplate reader (excitation/emission = 485 nm/528 nm) (Das et al., 2018).

**Statistical Analysis**

GraphPad prism version 6.0 (GraphPad Software) was used for statistical analyses. One-way analysis of variance (ANOVA) and Tukey–Kramer multiple-comparison test were used for comparing between groups. *p* < 0.05 was considered as statistically significant difference for all the experiments.

**RESULTS AND DISCUSSION**

**Characterization of PC-SA/HO-3867**

HO-3867-entrapped PC-SA liposomes were developed by thin-film hydration method (De et al., 2018). Stearylamine (SA) was incorporated to impart high *Leishmania* targeting ability of these cationic liposomes (Banerjee et al., 2008). The mean particle size, zeta potential, and drug encapsulation efficacy of PC-SA/HO-3867 were 164 ± 15 nm, 46 ± 8.5 mV, and 88.4 ± 5.2%, respectively. The morphology of PC-SA/HO-3867 liposomes as characterized by AFM at 150–300 kHz frequency (Zhao et al., 2004) depicted their smooth spherical structures without visible aggregation (Figures 1A, B).

**PC-SA/HO-3867 Induced Cell-Death in *L. donovani* Is Chiefly ROS-Mediated**

MTT (Invitrogen) colorimetric assay reveals the significant dose-dependent cytotoxicity of free and liposomal HO-3867 (1–200 μg/ml) against promastigotes (Figure 1C) and intracellular amastigotes (Supplementary Table 1) of *L. donovani* without affecting normal mammalian cells, even after treatment with 100 μg/ml of PC-SA/HO-3867 (equivalent to PC-SA/HO-3867 containing 215.26 μM of HO-3867 in liposomes). IC₅₀ values of free HO-3867 against *L. donovani* and *L. major* were 30 and 33.5 μg/ml, respectively. Much lower IC₅₀ values of 10 and 12.5 μg/ml were obtained after exposure to PC-SA/HO-3867 in *L. donovani* and *L. major*, respectively.

Accumulation of excess cellular lipid has long been associated with stress-induced apoptosis (Boren and Brindle, 2012; Rosa et al., 2015). To address the changes in the ratio of polar and non-polar lipids after treatment, lipophilic marker Nile Red (0.1 μg/ml) was used, which shows the characteristic shift from red to green emission in the presence of polar and non-polar lipids, respectively (Greenspan et al., 1985; Rosa et al., 2015). Intense yellowish green (neutral) fluorescent lipid droplets are seen to be accumulated in drug-treated promastigotes compared to prominent orange red punctuations (polar) of membrane lipids seen in control (Figure 1D). Also, quantitative analysis of whole cells (ex 514 ± 20/em 550 ± 40 for non-polar or neutral lipids; ex 560 ± 20/em 620 ± 50 for polar lipids) revealed significant accumulation of neutral lipids in drug-treated parasites over controls. The red/green ratio changed from 2.2 ± 0.1 in controls to 1.6 ± 0.14 in HO-3867 and 1.4 ± 0.12 in PC-SA/HO-3867-treated parasites, confirming the accumulation of non-polar/neutral lipids after 2 h (Figure 1E). Similar to earlier reports (Kathuria et al., 2014; Garcia et al., 2017), our study shows leishmanicidal activity of HO-3867 with surplus accumulation neutral lipid bodies in the cytoplasm indicating faulty lipid metabolism. We also performed DNA laddering assay (Figure 1F), which shows marked genomic DNA fragmentation in HO-3867, PC-SA, and HO-3867/PC-SA-treated *L. donovani* promastigotes compared to untreated controls (showing no such oligonucleosome-sized fragments).

Although sublethal ROS production sustains cellular homeostasis, a drastic ROS upsurge is associated with apoptosis (Basmaciyen et al., 2018). Fluorescent labeling of cells with H₂DCFDA showed an almost fourfold increase in intracellular ROS (Figure 1G) in promastigotes compared to free drugs indicative of imbalanced cellular redox homeostasis and programmed cell death (PCD) in parasites after 2 h. As a key regulator of PCD, the presence of caspase-like proteases was evaluated in untreated and treated *L. donovani* promastigotes (Shadab et al., 2017). A significantly high caspase-like activity of empty PC-SA liposomes and PC-SA/HO-3867 was observed compared to free HO-3867 (Figure 1H). To confirm the assay specificity, promastigotes were preincubated for 1 h with z-VAD-fmk, a pan-caspase inhibitor, followed by PC-SA/HO-3867 treatment which showed significant reduction (*p* < 0.01) in absorbance. Increased caspase-like activity in promastigotes by PC-SA/HO-3867 treatment is possibly due to PC-SA liposomes, a known activator of caspase-mediated cell death (De et al., 2018). We also observed involvement of caspase-independent cell death in free HO-3867-treated *L. donovani* promastigotes but caspase-mediated apoptosis after PC-SA and PC-SA/HO-
3867 treatment. This is probably due to the synergistic antileishmanial activity of PC-SA liposomes and HO-3867.

**PC-SA/HO3867 Causes Altered Cell Morphology, DNA Fragmentation, and Cell Cycle Arrest in Promastigotes**

Marked cytoskeletal alterations like rounded cell shrinkage, decrease in flagellar length, or loss of flagella is frequently reported during PCD in *Leishmania* (Ambit et al., 2011). AFM studies revealed that both free and liposomal HO-3867-treated parasites exhibited cell shrinkage after 2 h, with maximum cytoplasmic condensation with PC-SA/HO3867 treatment (Figures 2A, B). Compared to the typical smooth, elongated shape of normal promastigotes, PC-SA/HO3867 treatment resulted in irregular cell morphology, membrane blebbing, and flagellar distortion (Figures 2A–C) in *L. donovani*. Similar morphological changes have earlier been associated with apoptosis-like cell death in *L. donovani* by clerodane diterpene (Kathuria et al., 2014) and Kalsome (Shadab et al., 2017) and curcumin in *L. infantum* (Eaton et al., 2014).

Fragmentation of nuclear DNA is an essential hallmark of apoptosis in eukaryotes. Quantification of nuclear DNA nicking in PC-SA/HO3867-treated parasites was determined by TUNEL assay based on binding of terminal deoxynucleotidyl transferase (TdT) enzyme to the 3′-OH end of the fragmented DNA. Hence, the FITC-fluorescence obtained is directly proportional to the fragmented...
DNA inside cells. Promastigotes treated with 10 μg/ml of liposomal HO-3867 for 2 h showed increased DNA nicking as evidenced from enhanced green fluorescence compared to the untreated controls, observed under a confocal microscope (Figure 2D).

Targeting the cell proliferation in vitro can provide mechanistic insight into novel antileishmanial drugs. The effect of liposomal and free HO-3867 on cell cycle progression was examined by flow cytometry by staining the permeabilized cells with PI. PC-SA/HO-3867 induced a significant increase (~3.4-fold) in the subG0 cell cycle compared to the untreated controls after 2 and 6 h (Figures 3A, B). After 2 h, the percentage of cells in the subG0 phase was around 8% in the untreated promastigotes, which increased to 20% and 26%, in the L. donovani promastigotes treated with free and liposomal HO-3867, respectively. These data clearly demonstrate the growth-inhibitory effect of free and liposomal HO-3867 on L. donovani, with cell cycle arrest in the subG0 phase, observed till 6 h post-treatment.

PC-SA/HO-3867 Induces PS-Externalization and L. donovani Metacaspase and PARP1 Overexpression

Cellular death has been broadly categorized into apoptotic and necrotic pathways. Apoptotic cell death in unicellular protozoan parasites is almost always associated with translocation of PS from the inner to outer leaflet of the cell membrane (Nikoletopoulou et al., 2013). Flow cytometry analysis based on Annexin V-FITC/PI dual staining was used to differentiate between apoptotic cells after treatment. Our results showed late apoptosis (Annexin V⁺PI⁺) in nearly 30% of PC-SA/HO-3867-treated cells compared to only 8.5% and 6.2% cells in free HO-3867-treated and controls, respectively (Figures 3C, D), without any increase in necrotic population (Annexin V⁻PI⁻). The results strongly indicate that liposomal HO-3867 treatment resulted in apoptosis of L. donovani largely due to increased externalization of some anionic membrane lipids like phosphatidylglycerol, phosphatidylethanolamine,
and phosphatidic acid, along with a small amount of PS in the promastigote outer membrane binding Annexin V (Imbert et al., 2012; Weingärtner et al., 2012). Although Leishmania promastigotes possess less outer-membrane PS compared to amastigotes (Bouazizi-Ben Messaoud et al., 2017), this little amount of PS along with other anionic membrane lipids on Leishmania promastigotes seems to be adequate for interacting with cationic PC-SA liposomes for successful targeting of the parasites.

The viability and virulence of Leishmania parasites depend upon some key enzymes like metacaspase and PARP-1 (poly [ADP]-ribose polymerase-1) genes, compared to SIR2 (silent information regulator)-related proteins (Sengupta et al., 2011; Mittal et al., 2017; Basmaciyan and Casanova, 2019). Stress-induced overexpression of PARP1 can lead to suppression of sirtuins (SIR2) via depletion of intracellular nicotine adenine dinucleotide (NAD) levels resulting in ATP depletion, DNA damage, and cell death in protozoan parasites (Mittal et al., 2017). Real-time PCR analysis with gene-specific primers and L. donovani GAPDH (Purkait et al., 2012; Shadab et al., 2017) showed a 3.5- and 1.6-fold increase of L. donovani metacaspase and PARP1 respectively in liposomal HO-3867-treated parasites over free drugs (Figure 3E). Also, an almost 1.5-fold reduction in SIR2 gene expression was observed in PC-SA/HO-3867-treated parasites compared to free drugs, after 6 h. Thus, liposomal HO-3867-mediated parasite killing is associated with overexpression of L. donovani metacaspase, PARP1 with concomitant downregulation of SIR2 in promastigotes.

**PC-SA/HO-3867 Causes Depolarization of Mitochondrial Membrane**

Mitochondria are vital cell organelles for proper cellular function and viability. Disruption of mitochondrial integrity and loss of mitochondrial membrane potential are striking features of apoptosis (Lee et al., 2002; Kroemer et al., 2007). Changes in mitochondrial membrane potential in treated cells were measured by confocal microscopy and flow cytometry gating using the MitoProbe JC-1 Assay Kit (Reers et al., 1995; Shadab et al., 2017; Sivandzade et al., 2019). As shown in Figures 4A–C, the aggregated JC-1 (red) is released from parasite mitochondria to the cytoplasm as monomers (green) following treatment with free and liposomal HO-3867 (10 μg/ml), indicating significant mitochondrial damage after 2 h. However, in controls, intense JC-1 red fluorescence is indicative of healthy mitochondria (Figure 4A). The relative mitochondrial depolarization (red/green JC-1 ratio) (Figure 4C) showed 9.5-fold lower red/green ratios in liposomal HO-3867-treated promastigotes compared to untreated controls (Figure 4C).

**Internalization of PC-SA Liposomes by L. donovani-Infected МΦ**

Macrophage (МΦ)-targeted drug delivery has been of specific interest in leishmaniasis due to the versatility of macrophages to act as host cells as well as central APCs in the processing of liposomal antigens (Kelly et al., 2011; Bogdan, 2020). A profound effect of particle size on its efficacy and biodistribution has been reported, with medium-sized liposomes of size ~150–200 nm
having the longest circulation time and better efficacy (Sercombe et al., 2015; Bogdan, 2020). Confocal microscopic analysis of intracellular fates of PC-SA liposomes was carried out by labeling the vesicles with Rhodamine B (red) entrapping 5-carboxyfluorescein dye (green) (Das et al., 2018). Although no initial difference in uptake between infected and non-infected MΦs was observed after 10 min (data not shown) of incubation, more accumulation of intact PC-SA liposomes (yellow) was seen inside infected MΦs (nuclei labeled with DAPI) close to intracellular amastigotes (small blue dots) (Figure 4D, i and ii) compared to non-infected cells after a brief incubation time of 2 h. Interestingly, in accordance with some previous studies (Sercombe et al., 2015; Bogdan, 2020), the fluorescent PC-SA liposomes (Figure 4D) were seen to be preferentially internalized by the *L. donovani*-infected MΦs compared to uninfected MΦs, ensuring better antileishmanial effect of the drug *in vivo*. This is possibly due to altered phagocytosis by parasitized APCs (Noronha et al., 2000; Borborema et al., 2011). This suggests that PC-SA liposomes more effectively target parasitized MΦs than the non-infected ones, thereby increasing the efficacy of the drug.

**PC-SA/HO-3867 Activates NO and ROS Mediated Killing of *L. donovani* Amastigotes**

MΦs are the main phagocytic cells playing a dual role in *Leishmania* infection acting as chief host cells sustaining amastigote multiplication as well as helping in parasite clearance (Tomiotto-Pellissier et al., 2018). To investigate...
whether the antileishmanial effect of PC-SA/HO-3867 observed in L. donovani promastigotes extended to intracellular amastigotes, in vitro infected murine peritoneal MΦs were treated with an IC₅₀ concentration of free and PC-SA/HO-3867 (10 μg/ml) for 48 and 72 h. As shown in Figure 4E, a significantly higher time-dependent suppression of intracellular amastigote multiplication was noted upon PC-SA/HO-3867 treatment compared to controls and free HO-3867 at 48 h (p < 0.001) and 72 h (p < 0.0001) post-treatment in peritoneal MΦs. We further investigated the amount of secreted NO and ROS in culture supernatants of infected and drug-treated MΦs after 48 and 72 h. Compared to free HO-3867, PC-SA/HO-3867 treatment induced 1.5- and 2.6-fold higher NO (Figure 4E) and ROS (Figure 4F) generation, respectively (p < 0.0001), after 72 h. One plausible explanation for this enhanced efficacy of liposomal HO-3867 is the synergistic leishmanicidal effect of cationic PC-SA liposomes with HO-3867 in a synergistic manner. Collectively, our results emphasize the antileishmanial potency of liposomal HO-3867 as a strong and safe drug candidate for VL, which merit future preclinical evaluation.

DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT
The animal study was reviewed and approved by the Committee for the purpose of Control and Supervision on Animal Experiments (NPCSEA), Govt. of India, and Animal Ethics Committee (147/1999/PCPSEA) of CSIR-IICB.

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AD and MK performed experiments. AD and NA equally contributed to perception and intellectual conceptualization of the work. All authors contributed to the article and approved the submitted version.

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