Hijacking the intrinsic Vitamin B_{12} pathway for oral delivery of nanoparticles ensuing an enhanced *in vivo* anti-leishmanial activity

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Supporting information

Methodology

Nuclear changes and live dead assay

The nucleus is an essential organelle involved in the growth and proliferation of a cell. Chromosomal damage resulting from prolonged exposure was assessed by nuclear change

assay [S1–3]. Because a significant amount of nuclear internalization of VBS-AmB-SLN was observed in cellular uptake studies, we hypothesized that nuclear accumulation at massively high treatment concentrations could interrupt machinery within the nucleus and subsequently impede cellular metabolic processes. To further validate the above hypothesis, we evaluated the maximum concentration (100–1000 µg/mL) of VBS-AmB-SLN required to induce nuclear changes in macrophage cells after an exposure period of 48 h. Using DAPI as a marker dye for the nucleus, we assessed the cytotoxic effects of VBS-AmB-SLN reflected by changes in cell nuclei morphology.

The cellular viability of macrophage cells was qualitatively assessed by live/dead assay. The subsequent confocal microscopy images were analyzed to evaluate the percentage of the population undergoing cell death after VBS-AmB-SLN exposure. Correspondingly, nanoparticle efficacy was estimated in a visualized manner, where macrophage cells were differentiated by co-staining with fluorescein diacetate (FDA) dye (5 mg/mL) for 30 min, wherein non-fluorescent FDA is metabolized into green fluorescent fluorescein by live cells. In comparison, dead cells with compromised membrane integrity were stained red with propidium iodide (PI) dye (2 mg/mL), incubated for 15 min, and afterward visualized under a confocal laser scanning microscope.

Plasma membrane integrity assay

Plasma membrane damage is an important marker for assessing mechanical damage and subsequent oxidative stress and cell death. Principally, membrane damage results in membrane permeability triggering cell death [S4–6]. Macrophage cells were plated at 10^5 cells/well in a 6-well plate. After 12 h of incubation, membrane integrity analysis following a 48 h VBS-AmB-SLN exposure (200 µg/mL) was performed by adding 10 µL propidium

iodide solution (50 µg/mL). An alteration in membrane integrity leads to intensified red fluorescence intensity as observed through a confocal laser microscope.

Results and discussion

Nuclear changes and live dead assay

The cytotoxic effects on macrophage cells were investigated by changes in the morphology of the nucleus after long-term exposure to VBS-AmB-SLN. Nuclear colocalization of VBS-AmB-SLN for a longer duration might induce DNA damage leading to swelling and fragmentation of cell nuclei [S7]. Therefore, the DAPI-stained nucleus of macrophage cells was evaluated using confocal microscopy. Microscopic images of macrophage cells in Fig. S1 showed that the cell nuclei were normal and indistinguishable in cellular morphology even when the cells were exposed to 400 µg/mL. However, cell nuclei swelled and fragmented when cells were exposed to increased concentrations of 800 µg/mL and 1000 µg/mL with a slight decrease in cell density.

Live dead assay confirmed that VBS-AmB-SLN did not negatively influence cell viability. The results in Fig. S2 showed no visible effect on cell viability, as evidenced by microscopic images of macrophage cells on exposure to 400 µg/mL of VBS-AmB-SLN. However, after incubation at a concentration above 800 µg/mL, many dead adherent cells were evident, indicating cells undergoing cell death. Furthermore, the nuclear staining images were consistent with the live dead assay.

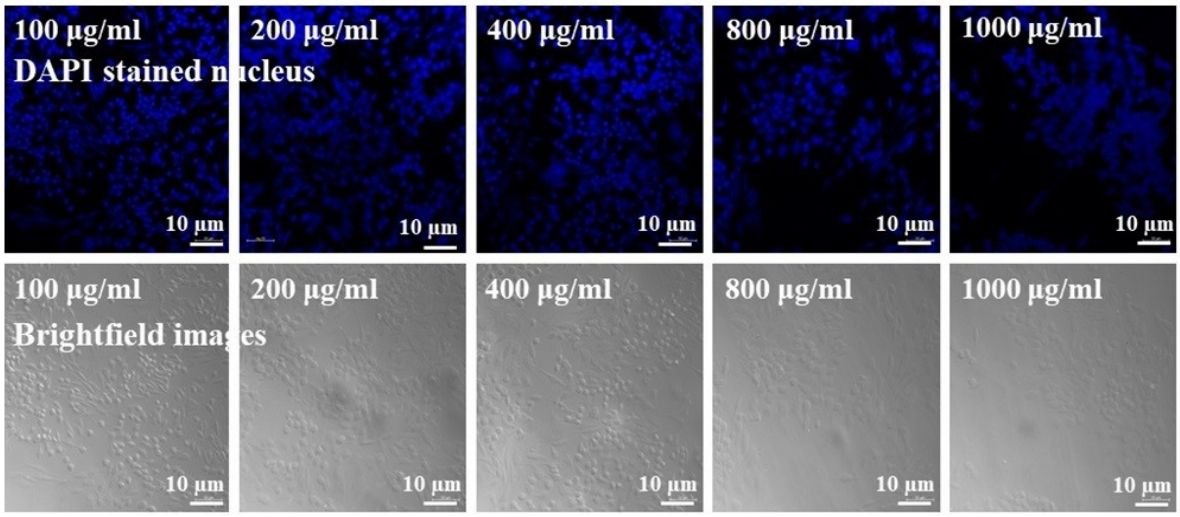

Fig. S1 CLSM images of DAPI (λ_{ex}: 405 nm) stained & bright-field images of nucleus of macrophage cells after 48 h exposure of VBS-AmB-SLN; 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, 1000 µg/mL.

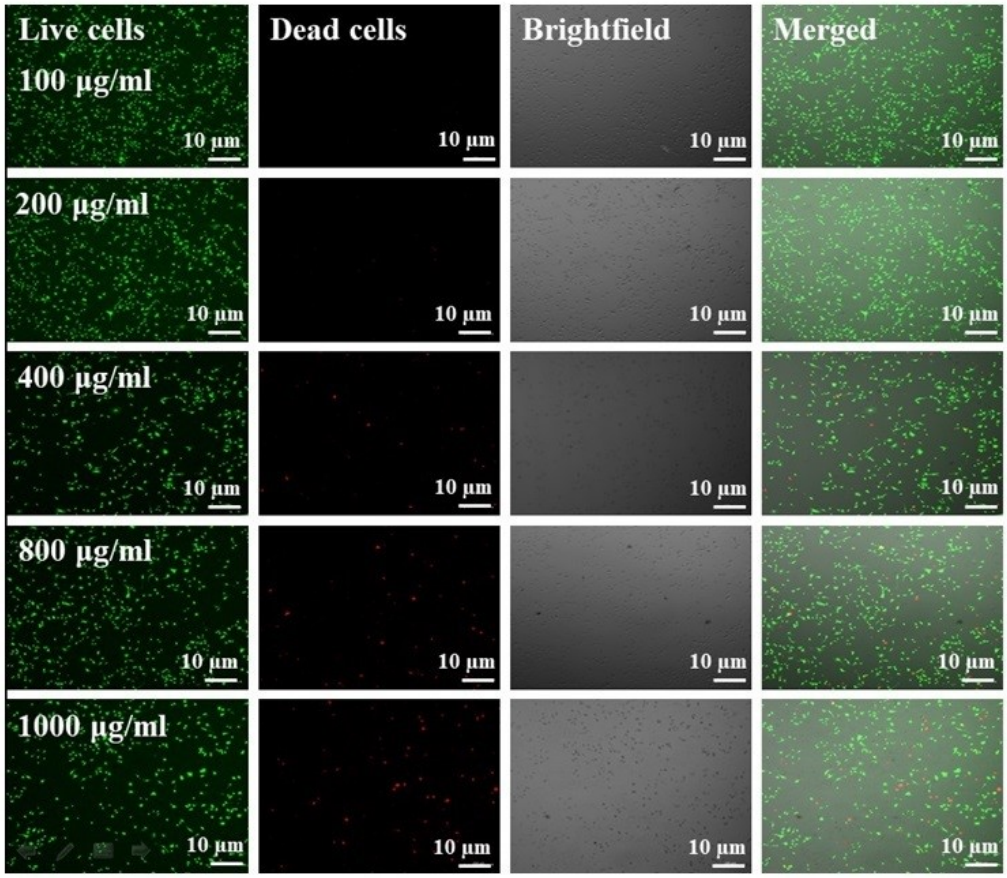

Fig. S2 CLSM images of fluorescein diacetate stained live macrophage cells after treatment with VBS-AmB-SLN 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, and 1000 µg/mL for

48 h, and propidium iodide stained dead macrophage cells, bright-field images, and merged images.

Plasma membrane integrity assay

To further substantiate the above results and delineate the effect of VBS-AmB-SLN on plasma membrane integrity, macrophage cells were incubated with FITC labeled VBS-AmB-SLN for 48 h followed by treatment with propidium iodide (PI). Upon VBS-AmB-SLN treatment, macrophage cells with plasma membrane damage allow PI to passively diffuse into the cytoplasm and bind with intracellular nucleic acids [7]. Therefore, by estimating PI-positive cells, one could assess the percentage of cells undergoing plasma membrane damage in the total population. The results showed that membrane integrity was confirmed for macrophage cells, and no passive diffusion was observed, as displayed in Fig. S3. The results further demonstrated that VBS-AmB-SLN (200 µg/mL) did not compromise the integrity of the plasma membrane even after 48 h of exposure.

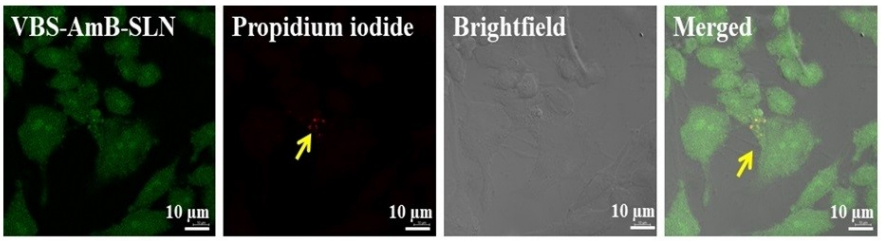

Fig. S3 CLSM images of colocalization of FITC (λ_{ex}: 488 nm) labeled VBS-AmB-SLN (200 µg/mL) after 48 h of treatment, PI (λ_{ex}: 493 nm) stained (50 µg/mL) macrophage cells, bright-field images, and merged images.

References

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FTIR spectrum of AmB, VB_{12}, stearic acid, and VBS-AmB-SLN

The characteristic peaks of AmB, Fig. 3B at 2914 cm^{-1}, represent the C–H stretching vibrations of the alkane and alkene groups. The peak at 1469 cm^{-1} can be assigned to N–H stretching vibration of amine groups and C=C of alkene groups, while the peak at 1700 cm^{-1} represents C=O stretching vibrations of carboxylic groups. The peak at 1096 cm^{-1} is associated with the C–OH bond present in AmB. The FTIR spectra of VB_{12} show a characteristic peak at 3346 cm^{-1}, which is attributed to the O–H stretching of hydroxyl groups, and a peak at 1660 cm^{-1} is accredited to the C=O stretching vibration of the propionamide chain of VB_{12}. The peak at around 1577 cm^{-1} is ascribed to the C=C double bond. The peak at 1490 cm^{-1} is allocated to the C=C bonds of the corrin ring. The FTIR spectrum of stearic acid shows a carbonyl peak at 1700 cm^{-1}. The peaks at 2845 cm^{-1} and 2913 cm^{-1} are attributed to CH_2 symmetric stretch and CH_2 anti-symmetric stretch. The characteristic peak of VBS at 1742 cm^{-1} is ascribed to the formation of a new band representing the esterification of carbonyl carbon of stearic acid with primary alcohol. It is discerned that characteristic peaks of AmB and VBS did not change after subjecting the

formulation to FTIR analysis. Hence it can be concluded that the drug is in the free state, and there is no interaction between the drug and the excipients used.

Supplementary Figures

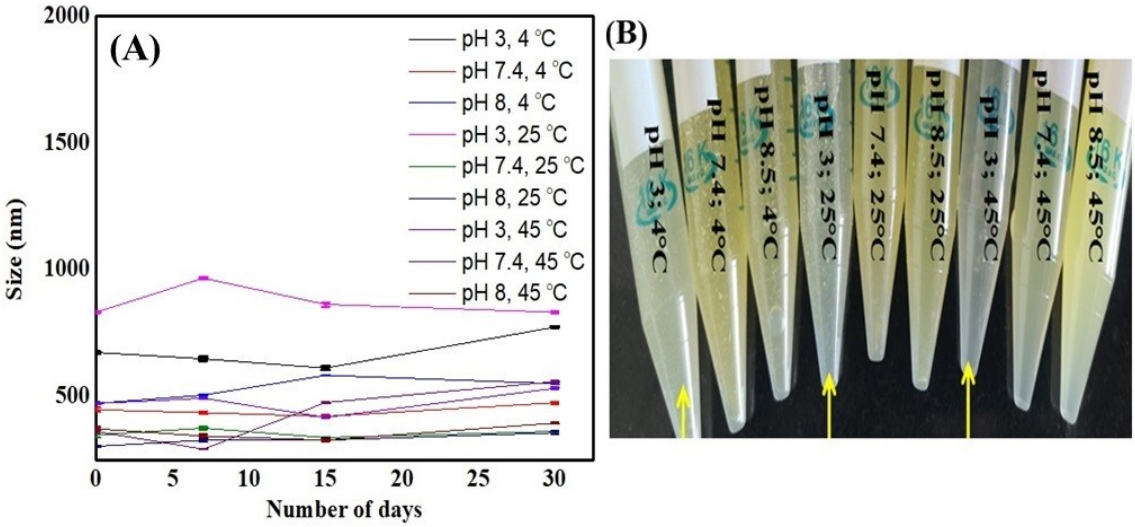

Fig. S4 (A) Average diameter and (B) release of lyophilized VBS-AmB-SLN when reconstituted with PBS buffer of different pH and temperature conditions (n = 3).

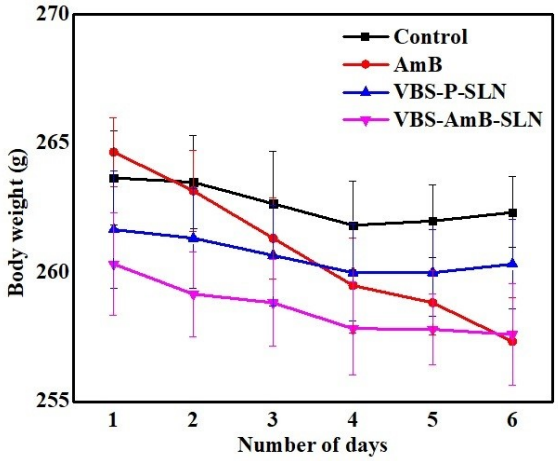

Fig. S5 Mean body weight of control rats and rats exposed to 1 mg/kg of AmB, VBS-P-SLN, and VBS-AmB-SLN. Data are represented as mean ± SD (n = 6).

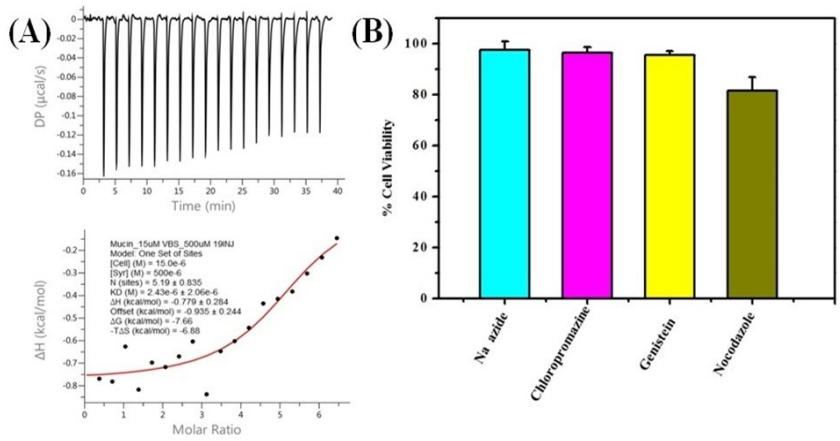

Fig. S6 (A) Isothermal titration calorimetry (ITC) analysis of the interaction between vitamin B_{12} stearate (VBS) and mucin: changes in heat flow over time when 500 µM VBS was injected into 15 µM mucin suspension. (B) Cell viability percentage of macrophage cells treated with sodium azide (100 µg/mL), chlorpromazine (100 µg/mL), genistein (100 µg/mL) and nocodazole (100 µg/mL) for 24 h (n = 3).

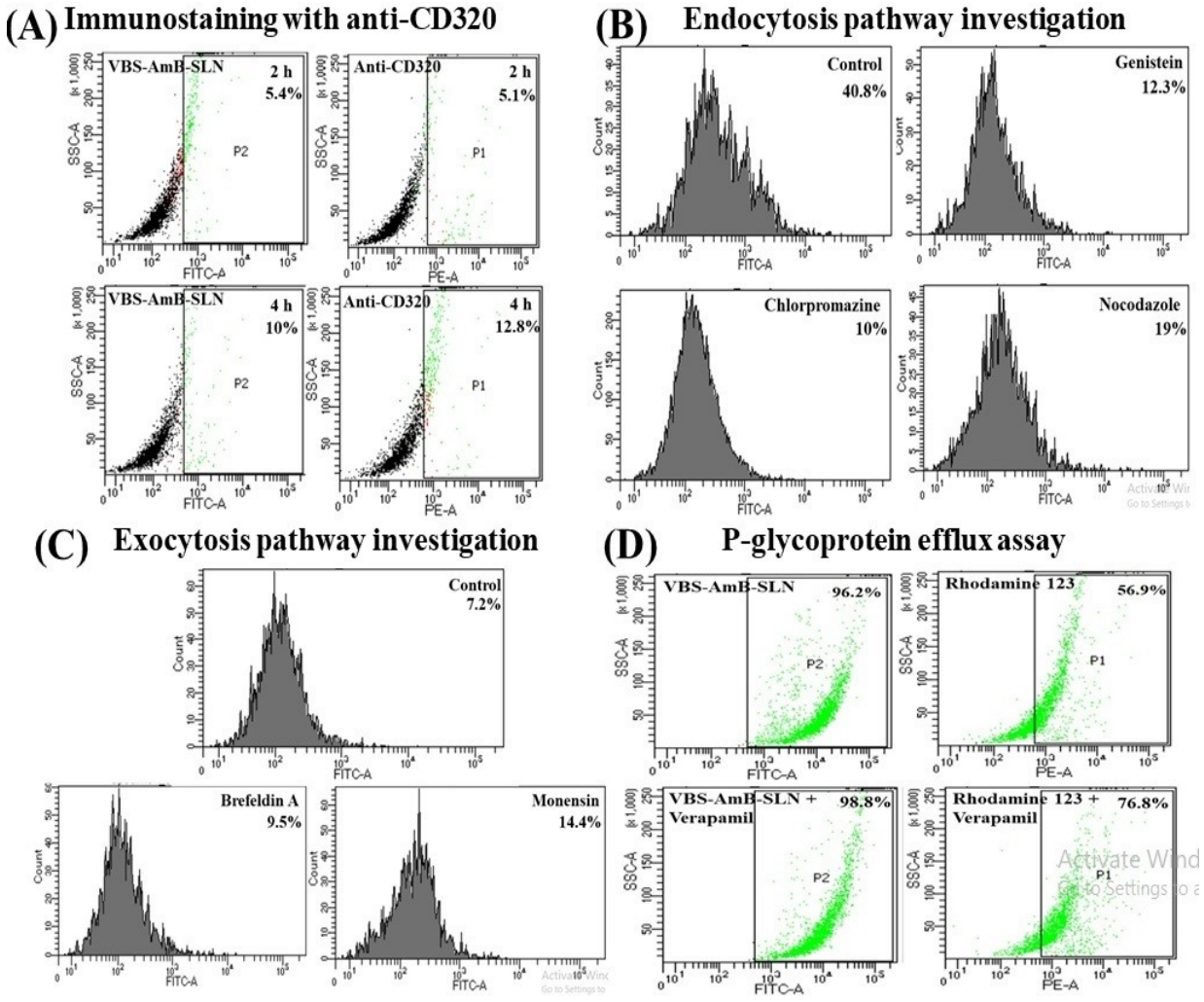

Fig. S7 (A) Dot plot FACS analysis of internalized FITC labeled VBS-AmB-SLN in caco-2 cells after 48 h, and colocalization of rhodamine-labeled anti-CD320 antibody. (B) Histogram FACS analysis of internalized FITC labeled VBS-AmB-SLN in caco-2 cells after treatment with endocytosis inhibitors. (C) Histogram FACS analysis of internalized FITC labeled VBS-AmB-SLN in caco-2 cells after treatment with exocytosis inhibitors. (D) Dot plot analysis of internalized rhodium 123 and FITC labeled VBS-AmB-SLN in caco-2 cells as control and after treatment with verapamil.

Supplementary videos

Supplementary video 1:  Supplementary video 1.mp4

Supplementary video 2:  Supplementary video 2.mp4

Supplementary video 3:  Supplementary video 3.mp4

Supplementary video 4:  Supplementary video 4.mp4