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Liposome encapsulated berberine treatment attenuates cardiac dysfunction after myocardial infarction

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ABSTRACT

Inflammation is a known mediator of adverse ventricular remodeling after myocardial infarction (MI) that may lead to reduction of ejection fraction and subsequent heart failure. Berberine is a isoquinoline quarternary alkaloid from plants that has been associated with anti-inflammatory, anti-oxidative, and cardioprotective properties. Its poor solubility in aqueous buffers and its short half-life in the circulation upon injection, however, have been hampering the extensive usage of this natural product. We hypothesized that encapsulation of berberine into long circulating liposomes could improve its therapeutic availability and efficacy by protecting cardiac function against MI *in vivo*. Berberine-loaded liposomes were prepared by ethanol injection and characterized. They contained 0.3 mg/mL of the drug and were 0.11 μm in diameter. Subsequently they were tested for IL-6 secretion inhibition in RAW 264.7 macrophages and for cardiac function protection against adverse remodeling after MI in C57BL/6J mice. *In vitro*, free berberine significantly inhibited IL-6 secretion (IC_{50} = 10.4 μM), whereas encapsulated berberine did not as it was not released from the formulation in the time frame of the *in vitro* study. *In vivo*, berberine-loaded liposomes significantly preserved the cardiac ejection fraction at day 28 after MI by 64% as compared to control liposomes and free berberine. In conclusion, liposomal encapsulation enhanced the solubility of berberine in buffer and preserves ejection fraction after MI. This shows that delivery of berberine-loaded liposomes significantly improves its therapeutic availability and identifies berberine-loaded liposomes as potential treatment of adverse remodeling after MI.

1. Introduction

Acute myocardial infarction (MI) after coronary occlusion leads to scarring of the left ventricle that can induce adverse left ventricular remodeling, which may lead to congestive heart failure (CHF) [1]. CHF is a rapid growing pathology in which the heart cannot supply enough blood to the body resulting in severe fatigue, breathlessness and ultimately death [2]. Despite advances in the treatment of CHF over the last decades, mortality and readmission remains high [3]. Left ventricular remodeling is a complex inflammatory process involving the innate immune system with infiltration of immune cells, in particular macrophages, matrix degradation and scarring [4,5]. Although it is generally

Abbreviations: BB, berberine chloride; BB-lip, berberine liposomes; CAD, charged aerosol detector; CHF, congestive heart failure; DLS, dynamic light scattering; HBS, HEPES buffered saline; HF, heart failures; LPS, lipopolysaccharide; LVEF, left ventricular ejection fraction; MI, myocardial infarction; PBS, phosphate buffered saline; RT, room temperature.

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accepted that inflammation plays crucial roles in adverse left ventricular remodeling and subsequently CHF [6], there is no evidence-based therapy focused on reducing inflammation yet.

Berberis spp. are widely distributed plants from the Berberidaceae family [7,8], from which berberine, a small fluorescent isoquinoline quarternary alkaloid [9,10] (Fig. 1), can be isolated [8,10]. Both berberine and the Berberis plants have a long history in Middle Eastern [11], Ayurvedic [10], Chinese [7] and Native American [9] traditional medicine. Berberine has been indexed in the Medical Subject Headings (MeSH) since 1975 [12] and nowadays has gained considerable attention from the Western scientific community [10]. Beneficial properties such as anti-inflammatory, anti-microbial, anti-diarrheal [9], anti-oxidative [9,13], vasorelaxant [9,11] and cholesterol lowering [11] effects have been ascribed to this natural product.

Importantly, long term and high dose berberine treatment has been reported to improve cardiac function [3,7] and to exert cardioprotective effects [14] in murine heart failure models. Furthermore, berberine was shown to improve survival in patients with CHF when given orally or intraperitoneally [15]. Clinical use of berberine, however, has been greatly impeded by its limited bioavailability due to the low absorption rate in the intestine (<5%) and excretion by P-glycoprotein and multidrug resistance associated protein-1 [16].

In this study, we investigated the use of berberine to reduce adverse remodeling and protect heart function in a mouse MI model. To overcome the low bioavailability of free berberine (BB) in the affected heart, berberine was loaded in long circulating liposomes (BB-lip) and administered intravenously. Due to locally enhanced vascular permeability in the affected regions of the heart [17], liposomes are expected to passively target the inflammatory site and subsequently release the drug after uptake by macrophages present in the region. This process would improve local drug delivery that may improve the therapeutic effect and reduce potential adverse effects by decreasing systemic exposure to berberine,

2. Materials and methods

2.1. Chemicals

Berberine chloride, lipopolysaccharide from *Escherichia coli* 055:B5, cholesterol and penicillin/streptomycin, sulfuric acid, triethylamine, methanol LC-MS grade (Fluka Analytical) and resazurin sodium salt were purchased from Sigma-Aldrich Chemie BV, The Netherlands and used without further purification. Albumin bovine fraction V, sodium chloride and 1-step ultra TMB ELISA substrate were obtained from Fisher Chemical, Thermo Scientific, BV, The Netherlands. IL-6 cytoset was acquired from Life Technologies BV, The Netherlands. Prednisolone disodium phosphate was obtained from Fagron BV, The Netherlands. Tween 20 and HEPES were purchased from Acros Organics, Belgium. RPMI-1640 without L-glutamine and L-glutamine 200 mM from PAA Laboratories GmbH, Germany. Fetal bovine serum (FBS) was purchased from Lonza, Belgium, dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphoethanolamine-poly ethylene glycol 2000 (DSPE-PEG_{2000}) were obtained from Lipoid GmbH, Germany. Ethanol absolute, ACS, ISO, Reag. Ph Eur was purchased from Merck Millipore B.V., Amsterdam, The Netherlands. MilliQ water was obtained from a Merck Millipore Q-POD. Medetomidine and atipamezole were purchased from Pfizer Animal Health, Exton, PA, USA. Midazolam was purchased from sciencelab.com, Texas, USA, fentanyl from Pfizer Pharmaceuticals Group, New York, USA, flumazenil from Sagent Pharmaceuticals, Illinois, USA, buprenorphine from Hospira Inc., Illinois, USA, and isoflurane from Baxter, Singapore.

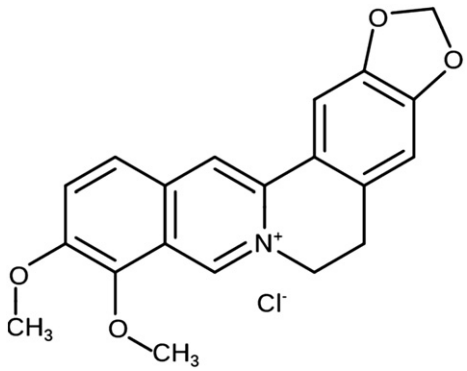

Fig. 1. Molecular structure of the isoquinoline quarternary alkaloid berberine chloride.

2.2. BB-lip preparation

Liposomes were prepared by the 'ethanol injection method' [18]. Briefly, 734 mg DPPC, 226 mg DSPE-PEG_{2000} and 209 mg cholesterol (molar ratio: 1:0.08:0.28) were dissolved in 1 mL ethanol absolute while stirring on a hot plate. BB, with a final concentration of 3 mg/mL, was dissolved in 9 mL HEPES buffered saline (HBS, 10 mM HEPES with 150 mM NaCl) at pH 7.4 while stirring on a hot plate. Once both solutions were clear, the lipids were rapidly injected into the BB solution using a preheated 5 mL syringe (BD Plastipak, Ireland) through a 18 gauge needle (BD Microlance 3, Ireland). The BB-lipid solution was extruded multiple times at 70 °C through a final membrane pore size of 0.1 μm (Nuclepore Track-Etch Membrane, Whatman) using a thermostat pump (Polystat 36, Fisher Scientific, The Netherlands) coupled to an extruder (Lipofast LF-50, Avastin, Germany). Subsequently, the BB-lip solution was dialyzed against HBS buffer at pH 7.4 for two days, changing buffer four times, to remove ethanol and free BB. To remove the final free BB, BB-lips were cleaned using a PD10 Sephadex G-25M column (GE Healthcare, UK). BB-lips were stored at 4 °C until further usage. Control liposomes (control-lip) without BB were prepared in the same way. Cyanine 5.5 liposomes (Cy5.5-lip) were prepared as described by Lobatto et al. [19].

2.3. Size determination of BB-lip

The diameter of the BB-lip was determined using dynamic light scattering (DLS, Malvern Instruments). Samples were diluted 100 times until the solution was only slightly yellow and measured at 20 °C using a 173° scattering angle. Intensity results were selected and the diameter and polydispersity index (pdi) of BB-lip was calculated using the Zetasizer Software (version 7.02, Malvern).

2.4. BB concentration in BB-lip and release in plasma

BB concentration in BB-lip was determined using the UV-VIS program of a nanodrop (NanoDrop ND-1000 spectrophotometer, Fisher Scientific, The Netherlands). A standard calibration curve of 10, 25. 50, 75 and 100 μg/mL of BB in water was used to determine the concentration in BB-lip. BB-lip solution was diluted 1:10 and 1:20 in methanol to dissolve the lipids. For lipid background controls, control liposomes were diluted in methanol (1:10, 1:20) and control liposomes were spiked diluted with methanol, with a final concentration of 25 μg/mL BB. Furthermore, solvent controls included water (blank), HBS and methanol. Absorption was measured at 346 nm and the concentration of BB in BB-lip was deduced from the standard curve.

For release experiments, BB-lip 100 μg/mL final concentration were placed in a dialysis bag with a molecular weight cut-off of 300 kDa in a 20× larger volume of human plasma for 24 h at 37 °C. Released BB was determined in the plasma compartment from a standard curve also prepared in plasma that was diluted in water as described above.

2.5. Liposome composition of BB-lip

The lipid composition of BB-lip was measured using a charged aerosol detector (CAD, Corona Ultra eso, Thermo Fisher/Dionex) coupled to an UPLC device (Ultimate 3000, Thermo Fisher/Dionex), using a BEH C18 1.7 μm 2.1 × 50 mm column (Acquity, Waters, Ireland). A standard lipid curve containing DPPC, DSPE-PEG_{2000} and cholesterol was prepared with the following concentrations (DPPC:DSPE-PEG_{2000}:cholesterol in mg/mL): 0.015:0.009:0.0045, 0.150:0.09:0.045, 0.375:0.23:0.11, 0.600:0.360:0.180, 0.750:0.450:0.220 and 0.900:0.540:0.270. A gradient run was used with mobile phase A = 80% MeOH and 0.1% TEA in milliQ water and mobile phase B = 100% MeOH and 0.1% TEA. Mobile phases were degassed using a water bath sonicator (Branson 2510) for 5 min. The UPLC had a runtime of 12 min/sample at a flow rate of 0.200 mL/min. The gradient was set as follows: starting with 100% A, followed by 100% B at 2 min and back at 100% A at 8 min. Injection volume was 4 μL and column temperature was set to 50 °C. Lipids were detected with peaks at ~7.52 min for DSPE-PEG_{2000}, ~8.45 min for cholesterol and ~9.1 min for DPPC using the CAD. Lipid concentrations in the liposomes were deduced from the standard curve. Concentrations were calculated using the accompanying software (Chromeleon Chromatography Data System version 7.1.2.1713).

2.6. In vitro performance of BB-lip

RAW 264.7 macrophages were cultured in RPMI-1640 medium substituted with 10% FBS, 2 mM penicillin/streptomycin and 2 mM L-glutamine. Cells were split twice a week until maximum passage number 32. Cells were seeded into 96-wells cell culture plates at a concentration of $5 * 10^5$ cells/mL and left to settle at 37 °C in a humidified incubator containing 5% CO_2. After 6–8 h, the medium was taken off and 5, 10 and 50 μM of BB-lip or 3.9, 15.6, 62.5 and 250 μM of free BB in HBS were added twice in triplicate per concentration to the cells and incubated for 2 h. Medium only and control liposomes (control-lip) were used as negative control. After 2 h, the medium was taken off and cells were incubated for 12 h with medium only (control) or medium containing 250 ng/mL LPS to induce an inflammatory response. After LPS stimulations, supernatant was used for cytokine expression determination using an ELISA assay.

For cell viability measurements a 10% Alamar blue (440 μM resazurin salt in PBS) solution in medium (v/v) was added to all wells and incubated for ~3 h at 37 °C in a humidified incubator containing 5% CO_2. Fluorescence was measured at 560/590 nm in a plate reader (Wallac 1420 Victor^3, Perkin Elmer).

2.7. IL-6 secretion determination by ELISA

IL-6 ELISA was performed as per manufacturer's instructions with minor modifications. Briefly, immuno Maxisorp plates (Nunc art no. 439454) were coated with coating antibody in phosphate buffered saline (PBS), sealed and left to incubate over night at room temperature (RT). Between each step, wells were washed using 0.05% Tween 20 in PBS. After coating, cells were blocked for at least 1.5 h using block buffer (0.5% BSA in PBS). First incubation: wells were either incubated with standards (0–1000 pg/mL) in 1:2 serial dilutions in assay diluent (0.5% BSA and 5% FBS in PBS) in duplicates or with samples (the supernatant of the *in vitro* incubation assay) in triplicates for 1 h at RT in the dark. Second incubation: wells were incubated with detection antibody in block buffer for 1 h at RT in the dark. Third incubation: wells were incubated with streptavidin-HRP solution in block buffer for 1 h at RT in the dark. Plates were developed by adding 1-step ULTRA TMB ELISA substrate and the reaction was stopped after all concentrations of the standard showed coloration, with the same volume of Stop Solution (1.8 M H_2SO_4). Optical density was measured at 450 nm using a platereader (Multiskan GO, Thermo Fisher).

2.8. Animals

10–12 weeks old male C57BL/6J mice (InVivos, Singapore) with a body weight between 20 and 25 g were used for all experiments. The mice received a standard diet and water *ad libitum*. All the procedures involving animal handling were performed with prior approval and in accordance with the protocols and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore.

2.9. Surgical procedures and cardiac function assessment

MI was induced in mice as previously described [4]. Briefly, mice were anesthetized with a mixture of 0.5 mg/kg medetomidine, 5.0 mg/kg midazolam and 0.05 mg/kg fentanyl and subjected to permanent ligation of the left anterior descending artery. Mice were recovered by a subcutaneous injection of 0.5 mg/kg atipamezole and 5 mg/kg flumazenil followed by 0.1 mg/kg buprenorphine. 100 μL/10 g body weight of control-lip, 1.5 mg/kg BB or 1.5 mg/kg BB-lip was injected *via* the tail vein immediately after chest closure. Additional drug injections were given on day 3 and 6 after MI. The surgeon was blinded for drug delivery.

Cardiac function was assessed with a high frequency ultrasound system Vevo® 2100 (Visualsonics) and analyzed with Vevo® 2100 software, version 1.7.0. Echocardiography was performed on mice under general anesthesia (1–1.5% isoflurane) at baseline, day 7 and day 28 after MI. Body temperature was monitored with a rectal probe and maintained at 36–37 °C. Volumes and functional parameters were measured in parasternal long-axis view (LV trace mode) and analyzed by a blinded researcher.

2.10. Imaging of the heart

The mice were injected with liposomes containing Cy5.5 dye (Cy5.5-lip) *via* the tail vain immediately after MI surgery. Hearts were harvested at day 1, 3 and 7 after surgery, and snap frozen or fixed in 4% paraformaldehyde followed by paraffin embedding, for analysis. Liposomes, accumulated in the heart were visualized with an IVIS spectrum-imaging system (PerkinElmer). The frozen left ventricles were embedded with FCS 22 Frozen Section Media (Leica Biosystems) for cryosectioning. Cryosectioning of heart tissue (6 μm) were directly imaged for Cy5.5-lip using a confocal microscope (FLUOVIEW FV10i Olympus) or used for histological analysis. Cryosections were stained with rat anti-mouse MAC3 monoclonal antibody (clone M#/84, BD Biosciences) followed by horseradish peroxidase (HRP)-conjugated goat anti-rat secondary antibody (Life Technologies). To investigate the co-localization with macrophages, the cryosections were stained with polyclonal rat anti-mouse CD68 antibody followed by a secondary antibody goat anti-rat antibody labeled with Cy3 fluoresce dye. Images were analyzed with a confocal microscope (FLUOVIEW FV10i Olympus). Sections were developed in ImmPACT NovaRED Peroxidase (HRP) Substrate (Vector Laboratories) and counter stained with haematoxylin. The paraffin embedded heart sections (5 μm) were stained with a Masson's Trichrome Staining Kit (Polyscience). The staining was analyzed with a Nikon Eclipse Ti light microscope (Nikon Instruments Inc.).

2.11. Scientific calculations and statistical analysis

IC_{50}, maximum effect (E_{max}), and curve fit (R^2) of IL-6 secretion inhibition by free BB, were calculated in GraphPad Prism (GraphPad Prism version 5.0 for Windows) using the 'log(inhibitor) *vs.* response' of the 'non-linear regression of single data' analysis function. The curve was fit following the 'least squares (ordinary) fit' and the top of the curve was set to 1.0 (to which the data was normalized). Each replicate of Y was considered as an individual point and the curve was fit with a maximum of 1000 iterations.

Comparisons between the groups of mice in time were performed using a two-way ANOVA with Bonferroni *post hoc* analysis using SPSS software (IBM® SPSS® Statistics version 22.0). Values were reported as mean ± SEM and a *p*-value ≤0.05 was considered statistically significant.

3. Results and discussion

3.1. Size of the liposomes

DLS measurements on BB-lip showed an average diameter of 0.11 μm with a polydispersity index of 0.048, which indicates a homogeneous particle size distribution. The control liposomes were with a diameter of 0.11 μm and a polydispersity index of 0.043, very comparable in both size and distribution (Table 1). When kept at 4 °C over a period of two years, the diameter of the BB-lip did not significantly change, demonstrating the stable nature of the liposomes (Fig. 2).

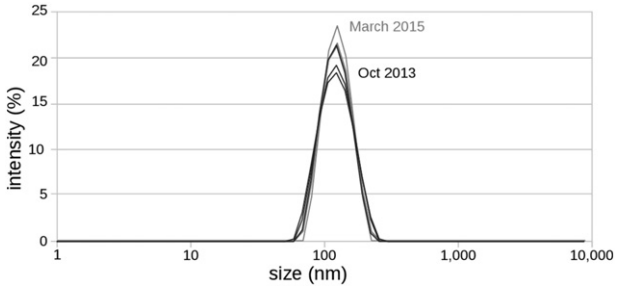

Fig. 2. Particle size distribution of BB-lip. Measurements performed in October 2013 compared to measurements in March 2015 on the same samples.

3.2. BB concentration in liposomes is higher than the saturated concentration of free BB in buffer

A BB content of 0.3 mg/mL for the BB-lip was determined using UV-VIS measurements. Of the initial 2.9 mg/mL berberine solution in HBS, only 10.4% was incorporated. The remainder was lost during extrusion and/or purification of the liposomes. BB could only be entirely dissolved at a concentration of 2.9 mg/mL in buffer at 70 °C, the temperature at which the extrusion took place. As soon as the solution cooled down, BB started precipitating.

At a concentration of 0.3 mg/mL BB was not completely dissolved at RT. In liposomes, the concentration of 0.3 mg/mL BB in was maintained even at 4 °C. The fact that BB poorly dissolves in buffer solutions underlines the importance of the introduction of a nanocarrier like BB-lip for this natural product. BB could be dissolved at a concentration of 0.3 mg/mL by warming to 37 °C. This is of importance for the *in vivo* experiments performed in this study as this allowed to inject equal doses with equal injection volumes.

3.3. Lipid composition of BB-lip

The lipid composition of BB-lip was determined using UPLC-CAD and a lipid mixture calibration standard containing the same lipids as used in the preparation of BB-lip (Fig. 3). In BB-lip we recovered 57.8, 18.01 and 15.2 mg/mL for DPPC, DSPE-PEG$_{2000}$ and cholesterol respectively, which is a molar ratio of 1:0.08:0.50. This is similar to what we recovered in the blank liposomes (Table 1), making these liposomes a suitable control. The molar ratio of the lipids in the liposomes shows that the liposomes become relatively enriched with cholesterol as compared to the feed ratio.

3.4. BB does but BB-lip does not inhibit pro-inflammatory cytokine IL-6 secretion in vitro

As we have previously shown, free berberine inhibits IL-6 cytokine expression in RAW 246.7 macrophages (IC_{50} = 10.4 μM, E_{max} = 44% and R^2 = 0.77) [20]. Inhibition of IL-6 by BB-lip, following the same protocol, showed no concentration dependent inhibition (Fig. 4). In contrast they might even appear to increase IL-6 concentration, although the relationship is not dose-dependent and shows substantial variation. These results show that *in vitro* the liposomal formulation does not increase the effectiveness of BB. This is likely due to the fact that long circulating PEGylated liposomes are designed to have a prolonged circulation time *in vivo* by avoiding cell interaction. This is beneficial *in vivo* since it enhances the accumulation of drug-loaded liposomes at the desired tissue. However, the reduced cellular uptake in *in vitro* settings limit efficacy. It also underlines that *in vitro* leakage is minimal in the cell culture medium containing 10% serum. Also in 100% plasma minimal leakage (7 ± 3%) was observed after 24 h at 37C.

Table 1
Liposome characteristics. The diameters including the pdi and the concentrations of the lipids and BB with molar ratio to DPPC are given. Both liposomes are very similar in both composition and size.

Characteristics	BB-lip	Ratio	Control-lip	Ratio	Feed ratio
Diameter (μm)	0.11		0.11		
pdi	0.048		0.043		
DPPC (mg/mL)	57.8	1	60.2	1	1
DSPE-PEG$_{2000}$ (mg/mL)	18.1	0.08	17.7	0.08	0.08
Cholesterol (mg/mL)	15.2	0.50	15.7	0.50	0.28
BB (mg/mL)	0.3	0.01	–		

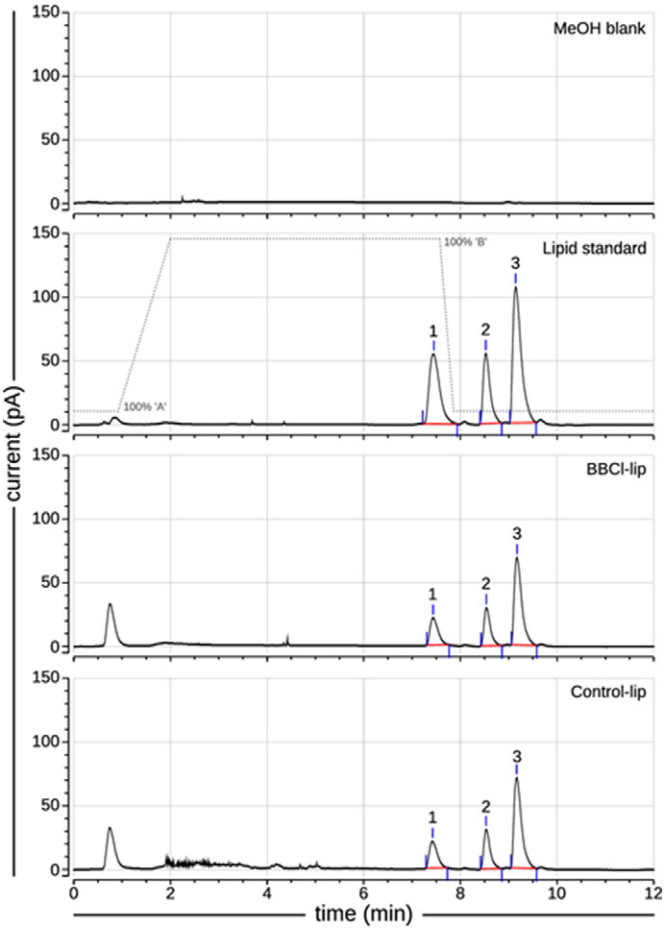

Fig. 3. Lipid profile of BB-lip. Lipid composition of BB-lip was determined using a lipid mixture calibration standard (highest standard shown) containing 1) DSPE-PEG$_{2000}$, 2) cholesterol and 3) DPPC. The solvent gradient was included in the chromatogram of the lipid standard with 'A' = 80% MeOH with 0.1% TEA and 'B' = 100% MeOH with 0.1% TEA.

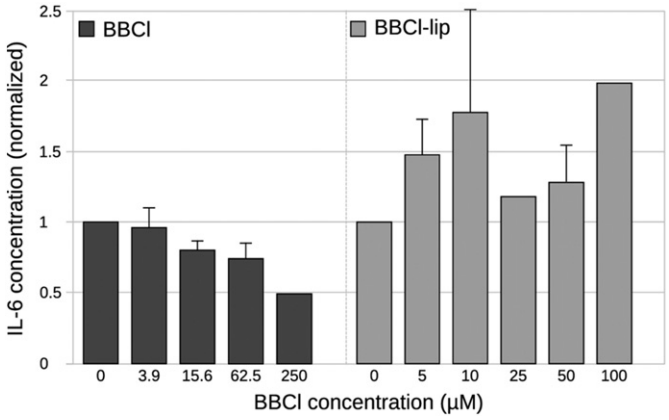

Fig. 4. *In vitro* IL-6 expression levels after incubation with BB or BB-lip in RAW 264.7 macrophages. Incubation with BB showed a dose dependent response with an E_{max} of 44%, a curve fit of R^2 of 0.77 ($p < 0.01$) and an IC_{50} of 10.4 μM. Incubation with BB-lip did not result in a concentration dependent inhibition ($n = 3$) ($p = 0.6$). Liposomes without drug, added at an equivalent concentration to the highest BB-lip concentration induced a normalized IL-6 release of 1.48 ± 0.34. This was not significantly different from liposomes with drug. Bars represent means ± SEM.

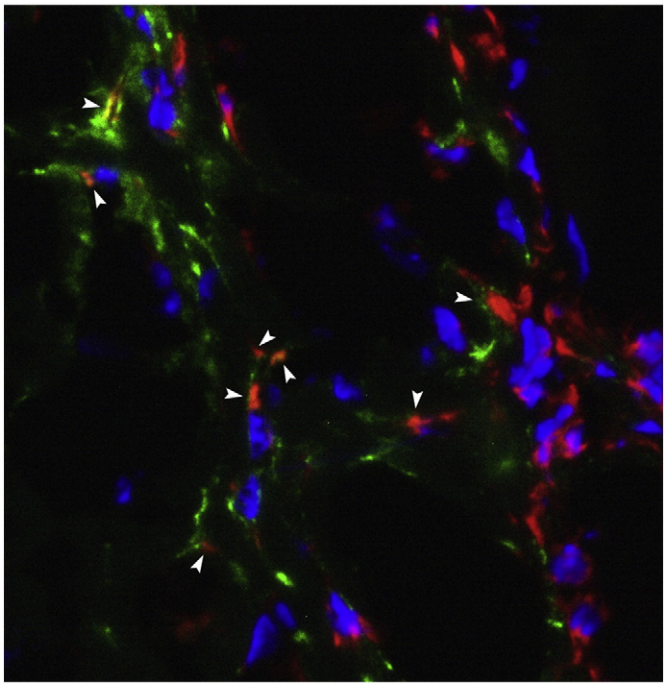

Fig. 6. Co-localization of liposomes (green) and macrophages (CD68 staining in red) in infarct area in the heart indicated by white arrowheads as observed by confocal microscopy. Nuclear staining was performed with DAPI (blue).

3.5. Cy5.5-lip accumulates in myocardial infarction

Macrophages are known to infiltrate into the infarcted heart tissue from circulation and constitute a hallmark of inflammation (Fig. 5A–B) [4], in addition to increased capillary permeability, we hypothesized that encapsulation of berberine into liposomes would enrich the berberine in the infarcted heart tissue that would be able to extravasate by virtue of the locally enhanced permeability and thereby improve its local delivery and treatment efficacy. To prove this concept, we directly visualized the distribution of liposomes in the heart by intravenously injecting liposomes containing Cy5.5 fluorescent dye in mice. As expected, the liposomes accumulated specifically in the infarcted heart tissue (3 days after MI) (Fig. 5C–E). Liposomes were also observed in the

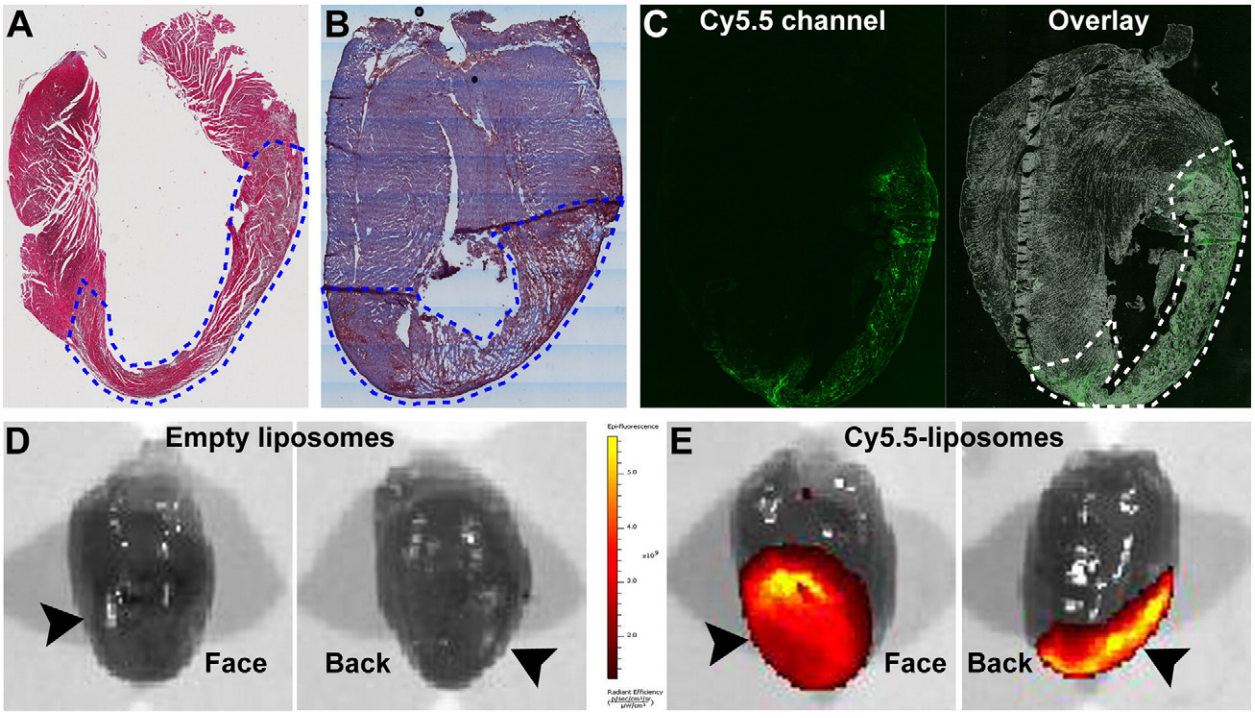

Fig. 5. Infiltration of macrophages and accumulation of liposomes in the infarcted heart tissue. Left ventricles were isolated 3 days after MI and analyzed for liposomes and macrophages. A) Masson's Trichrome stain to indicate the infarcted area. B) Infiltration of macrophages into the infarcted heart tissue. MAC3 staining is shown in brown. C) Liposomes visualized with confocal microscopy. Cy5.5 is shown in green and merged with heart tissue obtained under bright field. D) Accumulation of control liposomes in the infarcted tissue visualized with IVIS spectrum imaging system. E) Accumulation of Cy5.5-liposomes in the infarcted tissue visualized with IVIS spectrum imaging system. Infarct region is indicated by dashed line (A–C) or arrowhead (D–E).

Table 2
Cardiac function.

	Control-liposome		BBCL		BBCL-liposome	
	Baseline	MI	Baseline	MI	Baseline	MI
HR, bpm	402.90 ± 7.12	462.20 ± 21.83	434.33 ± 14.50	448.83 ± 9.76	407.70 ± 10.36	411.20 ± 12.60
ESV, μL	38.97 ± 1.89	126.47 ± 10.97*	43.21 ± 4.80	144.53 ± 32.06*	41.21 ± 2.40	74.39 ± 3.77
EDV, μL	71.62 ± 1.77	152.53 ± 9.90*	73.80 ± 4.53	171.21 ± 31.03*	71.83 ± 2.15	104.99 ± 3.15
SV, μL	32.65 ± 1.08	26.06 ± 2.98	30.60 ± 1.06	26.67 ± 2.63	30.63 ± 1.41	30.60 ± 1.41
EF, %	45.77 ± 1.74	18.01 ± 2.59**	42.29 ± 3.28	18.24 ± 3.21*	42.91 ± 2.10	29.53 ± 1.91
FS, %	24.20 ± 0.93	7.21 ± 71**	23.15 ± 2.73	7.97 ± 1.85**	23.89 ± 1.20	13.79 ± 0.78

Cardiac function parameters of mice treated with control-liposomes, free beberine (BBCL) and liposome-encapsulated beberine (BBCL-liposome) measured before induction of MI (baseline) or 28 days after MI (MI). HR indicates heart rate; bpm, beat per minute; ESV, end systolic volume; EDV, end diastolic volume; SV, stroke volume; EF, left ventricular ejection fraction; FS, fraction shortening. Mice received control-liposomes, n = 10; BBCL, n = 6; BBCL-liposome, n = 10. One-way ANOVA with Bonferroni *Post Hoc* Test, $^{*}p < 0.05$, $^{**}p < 0.01$, compared to BBCL-liposome.

heart 1 day and 7 days after MI (data not shown). These results show that liposomes are able to facilitate local delivery of anti-inflammatory drugs such as berberine into the insulted heart tissue. Further analysis demonstrated that part of liposomes were up taken by the macrophages infiltrated into the infarcted heart tissue (Fig. 6), suggesting that the macrophages may facilitate release of the preloaded berberine and are targeted by liposomes improving the anti-inflammatory efficacy of berberine. Nevertheless, liposomes can also be observed outside macrophages indicating that alternative mechanisms for release are possible.

3.6. BB-lip protects heart function against myocardial infarction in vivo

As expected, MI led to cardiac adverse remodeling including increases in end-diastolic and end-systolic volumes resulting in cardiac dysfunction (Table 2). Mouse heart function was evaluated by cardiac left ventricular ejection fraction (LVEF). At day 28 after MI, BB-lip significantly ($p < 0.05$ compared to control lip and free BB) preserved LVEF, while free BB did not show any effect compared to control-lip (29.5 ± 1.9 for BB-lip, 18.2 ± 3.2% for BB and 18.0 ± 3.1% for control-lip). Similarly, BB-lip ameliorated the loss of cardiac contractility by preservation of fractional shortening following MI (Table 2). Furthermore, hypertrophy of the left ventricle was less severe, though not statistically significant, in mice treated with BB-lip (Fig. 7). These results indicate that the delivery of BB by long circulating liposomes *via* intravenous injection efficiently reduced cardiac adverse remodeling and hence attenuated heart dysfunction in mice subjected to MI.

Berberine has shown therapeutic efficacy in CHF patients [15] and in animal models of MI [14] or ischemic reperfusion injury [3,21]. In these studies, berberine was given orally or intraperitoneally in long term studies using high concentrations (3–66 times higher than our dosage). In these animal studies, berberine has mostly been given before MI induction, however, in practice, patients are treated after the diagnosis of MI. To mimic the clinical situation, we have chosen to treat our mice after the onset of MI.

In our mouse MI model, the first week is dominated by the inflammatory response, including the production of reactive oxygen species, inflammatory cell infiltration (like macrophages) in the heart and subsequently cytokine expression [6,22,23]. To reduce the inflammatory damage after MI and hence protect heart function, we ensured delivery of BB to the local inflammatory environment *via* long-circulating liposomes. Since the blood circulation half-life of PEGylated liposomes is around 16–24 h in mice [24–27], additional injections on day 3 and day 6 after MI ensured continued presence of BB during this first week. The therapeutic effects in these conditions resulted in a significant improvement 28 days after MI compared to the delivery of control-lip and BB. In this manner, we have dramatically augmented the treatment efficacy of berberine and improved cardiac function by 64% compared to the same dose of free BB (Fig. 7). This is a promising first step for translation of this formulation to the clinic.

4. Conclusion

Encapsulation of berberine into liposomes is crucial to allow for intravenous administration and enhances the therapeutic availability of this compound. Intravenous administration *via* liposomes significantly

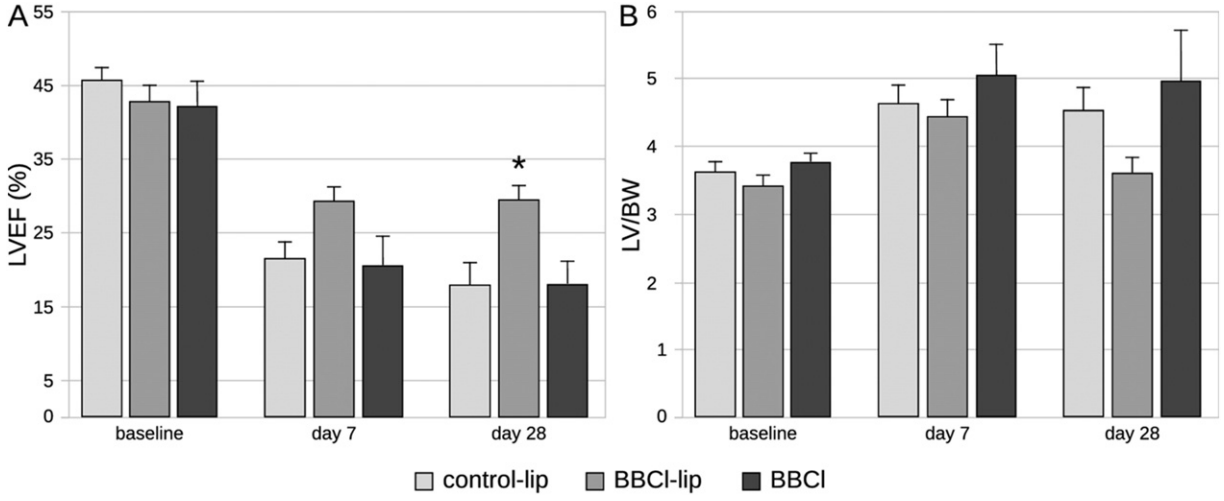

Fig. 7. Cardiac function and remodeling after MI. Mice are treated with control liposomes (control-lip, n = 10), liposome encapsulated berberine (BB-lip, n = 10) or free berberine (BB, n = 6). A) Cardiac LVEF was determined by echocardiography at baseline, 7 days and 28 days after MI surgery, $^{*}p < 0.05$ for BB-lip, free BB did not preserve the ejection fraction compared to control-lip. B) Left ventricular mass estimated by echocardiography at baseline, 7 days and 28 days after MI surgery. Two-way ANOVA with Bonferroni *post hoc* correction was performed for multiple comparisons. Bars represent mean + SEM. LVEF = left ventricle ejection fraction, LV/BW = left ventricle weight per body weight.

improved the treatment efficacy of berberine to protect cardiac function in an animal model of MI.

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