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Liposomal Hypocrellin B as a Potential Photosensitizer for Age-related Macular Degeneration: Pharmacokinetics, Photodynamic Efficacy, and Skin Phototoxicity *in vivo*

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Photodynamic therapy (PDT) has been successfully implemented as a treatment for wet age-related macular degeneration (AMD), but very few photosensitizers have been developed for clinical use. Herein, we describe a novel formulation of liposomal hypocrellin B (LHB) that was prepared by high-pressure homogenization. The encapsulation efficiency and PDT efficacy *in vitro* of this new preparation were found to remain nearly constant over 1 year. Moreover, LHB is rapidly cleared from the blood, with a half-life of 2.319±0.462 h and very low serum concentration at 24 h after injection. Testing in a rat model of choroidal neovascularization (CNV) showed that leakage of blood vessels in CNV lesions was significantly reduced when LHB PDT was given at a dose of 1 mg/kg along with yellow laser irradiation; the damage to the collateral retina and retinal pigment epithelium was minimal. Skin phototoxicity assays showed that only two of the 200 mice given a 4 mg/kg dose of LHB experienced an inflammatory reaction in the auricle irradiated at 24 h after dosing. These data collectively indicate that LHB may be a safe and effective photosensitizer for vascular-targeted PDT of AMD.

Introduction

Age-related macular degeneration (AMD) has emerged as the primary cause of blindness in the elderly.[1] The two main types of AMD are dry (atrophic) and wet (neovascular) forms, with the latter accounting for up to 90% of AMD cases with severe vision loss.[2] In neovascular AMD, choroidal neovascularization (CNV) develops under the retinal pigment epithelium (RPE) or extends through Bruch's membrane into the subretinal space between the RPE and photoreceptors,[3] leading to a disrupted macula and subsequent vision loss. Several therapeutic treatments have been implemented in clinical practice for treating wet AMD, such as laser coagulation, photodynamic therapy (PDT), radiation, surgery and anti-vascular endothelial growth factor (VEGF) therapy.[4] Currently, the anti-VEGF therapy has been the standard treatment for neovascular AMD; however, while it has been demonstrated as significantly effective for maintaining and improving vision,[5,6] it has also been shown as impotent against reversing pre-existing neovessels. The currently available anti-angiogenesis drugs are therefore considered refractory to the advanced wet form of AMD;[7] moreover, they offer limited convenience because they require monthly or bimonthly intravitreal injections.[6,8] Long-term inhibition of VEGF may also lead to an increased risk of cardiovascular diseases, and cases of patients treated with anti-VEGF drugs suffering from non-ocular hemorrhage, hemorrhagic stroke and myocardial infarction have been reported.[9-11] Thus, systemic cardiovascular risk assessments of these drugs will be of clinical importance for patient safety and prognosis.

Alternatively, several clinical studies have demonstrated that PDT is an effective treatment for neovascular AMD patients with classic CNV subfoveal and juxtafoveal lesions.[12-14] The efficacy of PDT is due to a photosensitizer's ability to generate reactive oxygen species (ROS) following light irradiation, which in turn leads to platelet activation, thrombosis and selective occlusion of the CNV within the treated area.[15,16] In theory, if PDT is selective for CNV, it should be the optimal treatment for neovascular AMD, producing minimal damage to the retina and choroidal tissues. Currently, only one photosensitizer, verteporfin, has been approved for clinical treatment of neovascularization in AMD. Verteporfin efficiently absorbs light at 686 nm[17] and is clinically administered with the irradiation of a red laser for no more than 83 s. Even though activation by light in the red region of the spectrum allows deeper tissue penetration (>1 mm),[18] it is also associated with increased risk for damage to normal tissues. In fact, several recent studies have confirmed that repeated usage of verteporfin for PDT of wet AMD causes great damage to the collateral RPE and photoreceptors.[19-21] Therefore, a photosensitizer that can selectively target the CNV lesion is urgently needed to improve the safety of PDT for AMD.

Hypocrellin B (HB), a photosensitizer isolated from natural

fungus sacs of *Hypocrella bambuase*, is capable of generating ROS at a high rate, has a high metabolic rate *in vivo*, and a low dark toxicity.[22-25] Thus HB is considered a promising photosensitizer for PDT. Moreover, since HB mainly absorbs light within the range of 450 nm to 600 nm, and the depth of tissue penetration is <1 mm at this wavelength,[26,27] HB is considered especially suitable for PDT of superficial neovascular diseases, including port wine stain (PWS) and neovascular AMD. However, HB is a hydrophobic drug and its solubility must be improved in order to maximize its potential bioavailability. One strategy to improve HB solubility is chemical modification,[28-30] but most of the HB derivatives generated to date have exhibited less PDT efficacy than their parent.[31-33] This solubility conundrum could be addressed by use of an alternative pharmaceutical-based strategy. Liposomes are one of the most well studied and widely applied lipophilic drug delivery systems, and verteporfin is prepared using this safe and biocompatible drug delivery approach. Loading HB into liposomes using a thin film method has been shown to improve its delivery into tissues and increase its photodynamic efficacy.[34,35] Moreover, this formulation was demonstrated as an effective preparation for PDT of PWS in longhorn cocks,[36] and to effectively induce vascular thrombosis in choriocapillaries of normal rabbits without causing damage to the retina or deeper tissues.[37] The drawbacks of this preparation are its poor stability (having a storage period of no more than three months) and the inclusion of Tween-80 and of organic solvents in its formulation (none of which are recommended by pharmacopoeias).

Herein, we describe a newly developed formulation of liposomal HB (LHB), which is prepared using high-pressure homogenization and requires no surfactant tween or organic solvents. The LHB morphology, size, stability and *in vitro* PDT efficacy over a 1-year period were evaluated. In addition, we also examined the pharmacokinetics and skin phototoxicity of LHB *in vivo* and investigated its PDT efficacy and safety using a rat model of CNV, the results of which represent useful preclinical data for LHB treatment of AMD.

Experimental

Materials

Hypocrellin B was obtained by the method previously reported.[38] All the following materials were purchased from Sigma-Aldrich: egg lecithin, cholesterol, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium fluorescein, hematoxylin, eosin, verteporfin, xylene and DMSO (high-performance liquid chromatographic grade). All other agents of analytical grade were purchased from the Beijing Chemical Plant.

Preparation of LHB

The LHB was prepared by high-pressure homogenization, as described by Brandl *et al*.[39] In a typical experiment, HB (100 mg), egg lecithin (4 g) and cholesterol (240 mg) were added to 100 mL water and blended vigorously to obtain multilamellar vesicles (MLVs). The MLVs were then homogenized 3 - 5 times under high pressure (180 Mpa) until the solution became clear, after which sucrose (4 g) was added as a cryoprotectant agent to enhance stability. The resultant liposome was immediately sterilized by passing through a 0.22 μm ultrafiltration membrane (Millipore, Bedford, MA, USA) and rapidly frozen at -80 ºC for 2 – 4 h. For lyophilization, the samples were placed in a K4 Modulyo freeze dryer (Edwards, Crawley, UK) at -55 ºC and 0.03 mbar for 24 h. Prior to use, the liposome lyophilizates were stored at 4 ºC in the dark and rehydrated in sterile saline for experimental use.

Characterization

The entrapment efficiency of LHB was determined by standard extraction method. Briefly, 1 mL of LHB solution was extracted by 1 mL petroleum ether, which allows for collection of free HB from the LHB preparation. The LHB:petroleum ether mixture was centrifuged for 1 min at 4000 rpm, and the resulting supernatant was evaporated and dissolved in 10 mL dichloromethane. After repeating the above steps a few times, the residue was collected and its absorbance at 470 nm at room temperature was measured on a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan). The concentration of free drug (E_1) was measured according to the standard curve made with an HB concentration range of 0.5 to 10 μM. The encapsulation efficiency (E) of LHB after 1-year of storage was estimated by the following equation: $E = (1- E_1/E_2) \times 100\%$, where E_2 is the total amount of drug added.

Transmission electron microscopy (TEM) images were measured with a JEM-100CX transmission electron microscope (JEOL, Tokyo, Japan) at an operational voltage of 80 kV. The liposomes' mean diameter and size distribution were determined by a phase analysis light scattering technique (Nano ZS; Malvern Instruments Ltd, Worcestershire, UK).

Cell survival studies

Primary mouse pulmonary microvascular endothelial cells (RMVECs) used in this study were provided by the National Aerospace Laboratory of Beijing, China. RMVECs were cultured in low-sugar DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37ºC under a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2-3 days until the cells had reached subconfluency. Then, the cells were seeded (8.0×10^4 cells/mL) into a 96-well plate and cultured in DMEM for 6 h. The medium from each well was replaced with DMEM (FBS-free) containing photosensitizers in a series of concentrations and the cells were incubated for an additional 4 h. The above medium with photosensitizers was then removed before adding fresh DMEM (FBS-free) medium. The cells were immediately exposed to a copper vapor laser (Institute of Electronics, Chinese Academy of Sciences, Beijing, China) for 1000 s at 578.2 nm wavelength, with fluence of 20 J/cm^2. After the light irradiation, the cells were placed back into the incubator and cultured for an additional 24 h, and then cell viability was examined by MTT assay.

The dark cytotoxicity of photosensitizers was evaluated separately by using the above-described phototoxicity assay without laser irradiation.

Animals

All the experimental procedures involving animals adhered to the protocol approved by the Institutional Animal Care and Use

Committee of the General Hospital of the People's Liberation Army. Kunming mice were provided by the Experimental Animal Center of the Academy of Military Medical Sciences. Brown Norway (BN) rats were obtained from the Animal Care Center of the General Hospital of the People's Liberation Army. Animals were housed with *ad libitum* access to standard chow and water. At the end of each experiment, the animals were sacrificed by a lethal intraperitoneal injection of chloral hydrate.

Pharmacokinetics

Kunming mice received an intravenous tail vein injection of 2 mg/kg LHB. The eyes were enucleated, and blood samples were collected at a series of times after administration of LHB. The blood samples were stabilized for 60 min followed by centrifugation at 4,000 rpm for 10 min. Serum samples (0.2 mL) were collected and the fluorescence of each sample was recorded at 610 nm by a Varioskan Flash micro-plate reader (Thermo-Scientific, Waltham, MA, USA). The LHB concentration was determined from standard curves. The pharmacokinetic parameters were calculated using the Drug and Statistics for Windows (DAS) software package. At least six animals were used for evaluation of LHB concentration at each time point.

Krypton laser-induced CNV in rats

The BN rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg) and administered tropicamide eye drops to dilate both pupils. CNV was induced in one eye of each animal; the other eye was used as a control. One-percent methyl cellulose eye drops were applied to the experimental eye, and a glass coverslip was placed in contact with the lens. The retina was viewed through a slit lamp microscope. Six laser burns were created between the major retinal vessels around the optic nerve head using a Novus 2000 Krypton laser system (Coherent Inc., Santa Clara, CA, USA). The laser power was 260 mW for 20 ms, and the spot diameter was 50 μm. The morphological end point of the laser injury was a photocoagulation spot with the appearance of a bubble with mild bleeding, which is associated with the disruption of Bruch's membrane. The formation of CNV was confirmed by fundus fluorescein angiography (FFA) at day 21 after the photocoagulation, at which time the rats were intraperitoneally injected with 1 mg/g of 10% sodium fluorescein and FFA was performed 500 - 600 s later using a CF-60UD fundus camera (Canon, Tokyo, Japan).

Photodynamic therapy

At 21 days after induction of CNV, anesthetized rats were immobilized on a stereotactic frame and the photosensitizer was injected intravenously at dose of 1 mg/kg or 2 mg/kg. PDT was performed immediately after the injection, using a copper vapor laser at a wavelength of 578.2 nm or a KDH-B red light treatment instrument (Institute of Electronics, Academia Sinica, Beijing, China) at a wavelength ranging from 600 to 700 nm. The irradiation conditions for the two lights were fluence of 36 J/cm^2, spot size of 1000 μm, and exposure time of 90 s. The control group underwent the same treatment but in the absence of a photosensitizer. FFA was used to quantitate CNV occlusion at 24 h and 7 days after the PDT. A total of 60 BN rats were used to evaluate the closure rate of CNV.

Histopathology

The rats were euthanatized with an overdose of chloral hydrate following FFA. The eyes were enucleated and fixed overnight in 10% formalin at 4°C. Tissue samples were dehydrated, cleared, and embedded in paraffin. Five-micron thick tissue sections were obtained from paraffin-embedded tissue blocks. The sections were then washed in xylene to remove the paraffin, rehydrated with serial dilutions of alcohol, and then washed in distilled water. The sections were then stained with hematoxylin and eosin (HE), and examined under a light microscope (Olympus, Tokyo, Japan). Twelve treated eyes and 3 untreated eyes were prepared for HE staining on day 1 or 7 after the LHB PDT.

The histopathological findings at 24 h after LHB PDT were classified into 5 grades based on the extent of retinal and choroidal injury:[40] grade 1, injury to RPE, mild injury to photoreceptors, little or no pyknosis in the outer nuclear layer, and/or occlusion of choroid capillaries; grade 2, injury to RPE and photoreceptors, occlusion of choroid capillaries, and 10-20% pyknosis in the outer nuclear layer; grade 3, injury to RPE and photoreceptors, occlusion of choroid capillaries, and 20-50% pyknosis in the outer nuclear layer; grade 4, injury to RPE and photoreceptors, occlusion of choroid capillaries, and >50% pyknosis in the outer nuclear layer; and grade 5, injury to large retinal and choroid vessels, damage to the inner nuclear layer, and >50% pyknosis in the outer nuclear layer.

Skin phototoxic effect analysis

A total of 200 Kunming mice (100 males and 100 females; 18 - 22 g body weight) were used to investigate the skin phototoxic effects of LHB. The mice were assigned randomly but sex-matched to one of the following four groups: control (without LHB treatment; n = 50), 1 mg/kg LHB treatment (n = 50), 2 mg/kg LHB treatment (n = 50), and 4 mg/kg LHB treatment (n = 50).

Before dosing, the fur of each mouse was removed by shaving from a 2 × 2 cm area of the back. The mice were then administered a single dose of saline (control) or 1, 2 or 4 mg/kg LHB *via* the tail vein. After the single intravenous injection, the exposed skin was then subjected to 60 min of light irradiation (40 mW/cm^2) at room temperature using a TRM-PD solar simulator equipped with a 3 kw xenon long-arc lamp (Jinzhou Sunshine Technology Ltd., Liaoning, China) over a period of 1, 3, 5, 7 or 14 days. Within 1 h after light irradiation, each animal's physical conditions (including the body weight, food consumption, heart rate, respiration rate, and feces and urine volume) were recorded. The symptoms, onset time and duration of the phototoxic reaction were also recorded. Ten mice (5 males and 5 females) from each group were sacrificed 1 h after receipt of light irradiation. The skin phototoxic effect of LHB was investigated by measuring the weight of auricular tissue samples and examining the histological characteristics of auricular and exposed skin tissue samples. Briefly, auricular tissue samples were taken from both ears using a 6-mm biopsy punch, and then weighed. Auricular and exposed skin samples were also obtained from respective organs, fixed in 10% formalin, embedded in paraffin wax, sectioned, and stained with HE for microscopic examination.

Statistical analysis

All statistical analyses were performed using statistical product and service solutions (SPSS) for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined using one-way analysis of variance (ANOVA). A p-value less than 0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Liposomal characterization

As shown by the TEM image in Fig. 1a and the single unilamellar vesicles (SUVs) shown, the LHB had typical spherical morphology and good dispersion. Moreover, the encapsulation efficiency and liposomal size were similar between fresh LHB samples and 6-month and 1-year samples (Table 1), although the polydispersity was considered higher for the 6-month and 1-year samples than for the fresh LHB. The maintenance of encapsulation efficiency and liposomal size may be ascribed to the high-pressure homogenization and lyophilization procedures used to prepare the LHB. The fact that this LHB formulation was stable for at least one year suggests its potential for fulfilling the basic requirements of clinical administration.

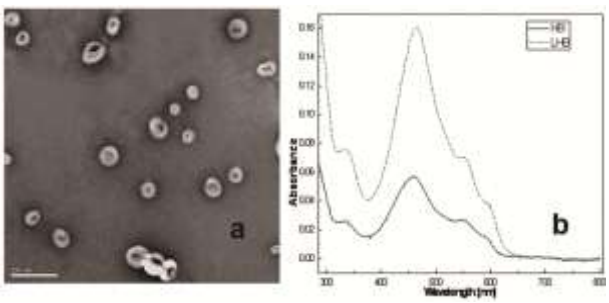


Fig. 1 (a) TEM image of LHB. (b) Absorption spectra of HB and LHB (8 μM) in phosphate buffer saline (pH 7.4).

Table 1 Liposomal size, polydispersity index and encapsulation efficiency of fresh LHB, and LHB after storage for 6 months or 1 year. Data are presented as mean ± SEM of three independent experiments

LHB storage time	Size (nm)	Polydispersity index	Encapsulation efficiency (%)
Fresh	93±16	0.13±0.01	92±8
6 months	104±28	0.21±0.02	88±10
1 year	121±35	0.26±0.03	87±10

The absorption spectra of LHB and HB in phosphate buffered saline are shown in Fig. 1b. LHB showed a remarkably stronger absorption between 400 - 800 nm, compared to free HB. This finding is likely due to the fact that the liposomes provide a much more hydrophobic microenvironment to the HB; in addition, this finding provides further evidence of the high encapsulation efficiency of this formulation.

In vitro photosensitizing efficacy

The cytotoxic effect of LHB was tested on primary mouse pulmonary microvascular endothelial cells treated or untreated by a copper laser at 20 J/cm^2. As shown in Fig. 2a, the IC_{50} (half inhibitory concentration) for LHB was 333 μM under dark conditions. After the light irradiation, the IC_{50} decreased by approximately 3,297-fold (101 nM), suggesting that LHB exerted a photo-dependent cytotoxicity on the RMVECs. In a previously study of cyclohexane-1,2-diamino-substituted HB derivative, the IC_{50} value under dark conditions was reported as only about 80 times larger than that under light conditions.[41] In the current study, the IC_{50} of LHB under light conditions was 101 nM, which was slightly higher than that of the parent HB (80 nM, as shown in the inset of Fig. 2c); nonetheless, the LHB exhibited approximately 88-fold less cytotoxicity than the free HB under dark conditions (Fig. 2c). Thus, these results indicate that LHB is a safer and more effective photosensitizer for PDT than hypocrellins or hypocrellin derivatives. Fig. 2d shows the survival rates of RMVECs under light conditions with treatment using 170 nM LHB that had been stored for 1-year. The rate of cell survival following exposure to LHB after 1-year of storage was not remarkably different from that of fresh LHB under the same irradiation conditions ($p > 0.05$). Therefore, this preparation was determined to have similar PDT efficacy against microvascular endothelial cells after 1-year of storage, suggesting that LHB may be an ideal candidate for PDT of AMD.

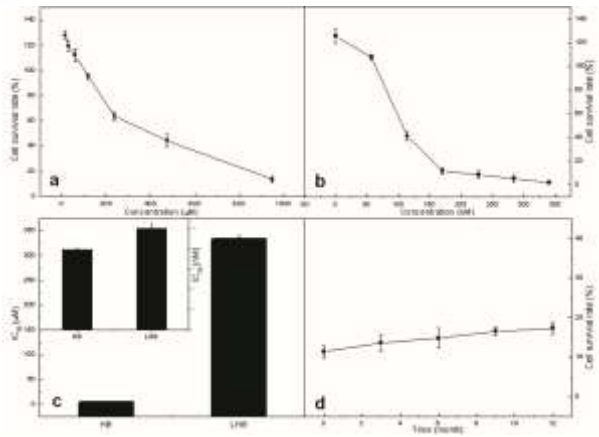


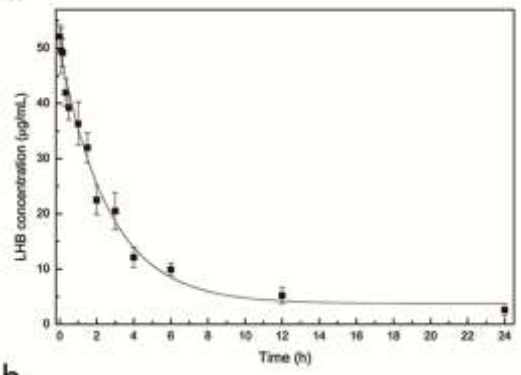
Fig. 2 Cytotoxicity of LHB under (a) dark and (b) light conditions using RMVECs treated with various concentrations of LHB for 4 h and cell viability was measured using MTT assay. (c) IC_{50} of LHB and free HB under dark conditions. Inset: IC_{50} of LHB and free HB following copper laser (578.2 nm) irradiation. (d) RMVECs cell survival rates under light conditions with treatment using 170 nM LHB stored for 1-year. Data are presented as mean ± standard error of three independent experiments.

Pharmacokinetics

A single dose of LHB (2 mg/kg) was administered and mean serum concentration was plotted versus time (Fig. 3a). The serum LHB levels reached a peak instantaneously after LHB administration and thereafter declined, suggesting that laser irradiation should be administered with dispatch after intravenous injection in order to achieve the maximum PDT efficiency for LHB treatment of vascular diseases. The serum concentration declined by 50% approximately 2 h after drug administration and only 19% of the drug was still detectable in serum at 6 h after drug administration, with barely detectable concentrations found at 24 h after injection. Main pharmacokinetic parameters of LHB were shown in Fig. 3b by fitting the concentration-time curve according to the three-compartment model (weight = 1) and these data indicate that the LHB was distributed mainly in the plasma and rapidly removed from the serum. Due to the short half-life of LHB and barely detectable LHB at 24 h after injection, the period of avoiding light exposure to the skin or eyes should be much shorter than that required for verteporfin (recommended at least 5

days), a feature which is an essential precondition for an optimal photosensitizer.

a



b

Pharmacokinetic parameter	Value
Half-life	2.319±0.462 h
Area under concentration-time curve	132.504±5.128 μg*h/mL
Apparent volume of distribution	0.042±0.005 L/kg

Fig. 3 (a) Blood concentration-time curve for LHB (2mg/kg intravenous administration) in Kunming mice. Data are presented as mean ± SEM of six independent experiments. (b) Main plasma pharmacokinetic parameters of LHB (2 mg/kg) in blood measured according to the three – compartment model.

Photodynamic activity *in vivo*

HB can efficiently absorb light in the blue, green and yellow range, which have tissue penetration in accordance with the depth of the lesions in AMD (<1 mm). Since blue and green lights are well absorbed by the macular pigment lutein, which precludes their suitability for PDT of macular diseases such as AMD, yellow light may be the best light source for HB PDT.[42,43] Yellow light not only penetrates to a modest tissue depth but it also exhibits negligible absorption in macular pigments and is more sensitive to new vessels than red light.[44,45] Considering these features, the LHB PDT efficacy in a rat model of CNV under yellow light (578.2 nm) was determined, compared with that under red light (600 - 700 nm) irradiation. Besides, the resultant PDT efficacy of LHB under yellow light irradiation was also compared with that of clinical verteporfin.

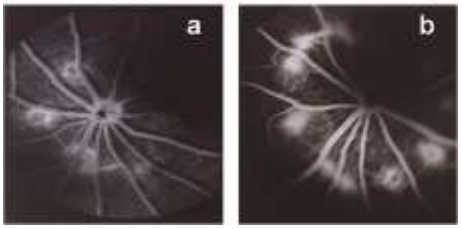


Fig. 4 FFA of the control CNV lesion (a) before and (b) 7 days after treatment with yellow or red light only. Fluence of 36 J/cm^2 was used.

PDT (fluence of 36 J/cm^2) with 1 mg/kg or 2 mg/kg LHB, 1 mg/kg verteporfin or control (without photosensitizers) was performed at 21 days after krypton laser photocoagulation. A 578.2 nm copper laser or a red light (600 - 700 nm) was applied instantaneously after the photosensitizer injection, and the outcomes were evaluated at 24 h and at 7 days after PDT using fundus photography and FFA. Fig. 4 shows the FFA results of a control eye before and 7 days after treatment with control laser only, with no decrease in fluorescein leakage observed. Fig. 5 represents the results of an eye treated with yellow or red light irradiation. It is shown that effective CNV occlusion was observed using yellow light irradiation at 1 or 7 days after injection of 1 or 2 mg/kg LHB (Fig. 5b, 5d and 5f). However, Fig. 5h or 5j shows hypofluorescence was observed at 7 days after intravenous injection of 1 mg/kg LHB using red light irradiation or verteporfin using yellow irradiation. Thus, LHB PDT using red light or verteporfin PDT using yellow light was not as efficacious as LHB PDT using yellow light.

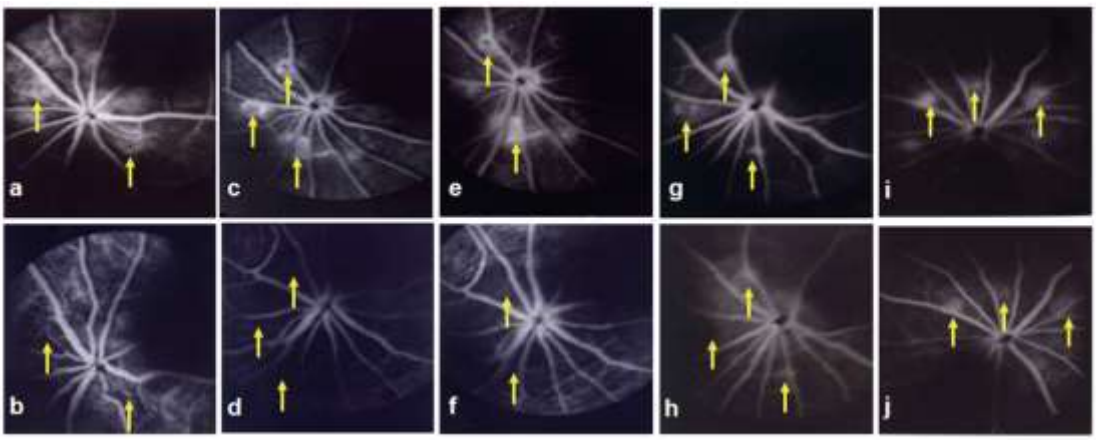


Fig. 5 Standard PDT for a CNV lesion in a rat model using yellow or red light irradiation (fluence of 36 J/cm^2). Representative FFA of a BN rat treated with intravenous injection of 1 mg/kg LHB (a) before and (b) 24 h after PDT using yellow light irradiation. FFA of CNV with intravenous injection of 1 mg/kg LHB (c) before and (d) 7 days after PDT using yellow light irradiation. FFA of CNV with intravenous administration of 2 mg/kg LHB (e) before and (f) 7 days after PDT using yellow light irradiation. FFA of CNV with intravenous injection of 1 mg/kg LHB (g) before and (h) 7 days after PDT using red light irradiation. FFA of CNV with intravenous injection of 1 mg/kg verteporfin (i) before and (j) 7 days after PDT using yellow light irradiation. Arrows indicate the photocoagulation spots. All of the spots after LHB PDT under yellow light irradiation showed no or little fluorescein leakage. Hypofluorescent lesions were observed in the photocoagulation spots after LHB PDT using red light irradiation and verteporfin PDT using yellow light

irradiation.

The CNV occlusion rates at 24 h or 7 days after PDT are summarized in Table 2. Significant differences among the CNV occlusion rates were not observed between the groups treated with 1 and 2 mg/kg LHB using yellow light irradiation ($p > 0.05$). Thus, 1 mg/kg LHB can be effectively used for CNV treatment, and increasing the dose only marginally improves efficacy. The CNV occlusion rate at 24 h after LHB PDT was nearly the same as that at 7 days after LHB PDT using yellow light irradiation. It is known that PDT-induced hypoxia and damage to the retina can induce neovascularization by promoting production of VEGF expression and macrophage aggregation,[46,47] leading to CNV recurrence. However, the above results clearly show that CNV did not recur when the treatment was performed after LHB PDT using yellow light irradiation. While after LHB PDT under red light irradiation, the closure rate observed at 7 days after PDT was less than that at 24 h after PDT (40.0% vs. 65.3%). Moreover, the closure rate treated with 1 mg/kg LHB at 24 h or 7 days after PDT using yellow light irradiation was remarkably more than that treated with 1 mg/kg verteporfin ($p < 0.01$), suggesting that LHB PDT with yellow light is superior to verteporfin as LHB has better efficacy of PDT for the treatment of CNV.

Table 2 Closure rate in CNV lesions at 24 h and 7 days after LHB or verteporfin PDT under yellow or red light irradiation

Photodynamic Agent	Intravenous dose (mg/kg) / Light source	Closure rate (lesions closed at 24 h after PDT/total lesions induced)	Closure rate (lesions closed at 7 days after PDT/total lesions induced)
LHB	1/Yellow light	80.7% (21/26)	88.0% (22/25)
LHB	2/Yellow light	80.7% (21/26)	88.9% (24/27)
LHB	1/Red light	65.3% (16/26)	40.0% (10/25)
Verteporfin	1/Yellow light	34.8% (8/23)	4.2% (1/24)

Histologic analysis of the CNV lesions

Fig. 6 shows HE stained sections from a representative untreated CNV lesion, and from representative CNV lesions at 24 h and 7 days after LHB PDT using the copper vapor laser (400 mW/cm^2, 36 J/cm^2). The control eyes without PDT showed extensive blood vessel formation and destruction of the outer nuclear layer, Bruch's membrane and RPE along with infiltration of macrophages (Fig. 6a). However, the treated CNVs at 24 h after 1 mg/kg LHB PDT showed closing of choroid capillaries, only slight injury to the RPE and photoreceptors, approximately 10-20% pyknosis in the outer nuclear layer (Fig. 6b), which led to a histologic classification of the lesions as grade 2. Treated CNVs at day 7 after administration of 1 mg/kg LHB showed fibrous plaques composed of fibroblasts and RPE (Fig. 6c), further demonstrating that LHB PDT caused damage to the CNV. No new vessel formation was detected in the fibrous plaques, supporting the above findings of no CNV recurrence in LHB PDT treated animals using yellow laser irradiation. Except for the inner and outer nuclear layers, the inner retina, the large retinal and choroid vessels were intact in all CNVs examined. However, animals administered the higher dose of LHB (2 mg/kg) showed extensive injury to collateral retina, RPE and choroid at 24 h after the PDT (Fig. 6d). The CNVs in LHB PDT treated eyes exhibited grade 5 histologic damage, which included gross damage to RPE and photoreceptors, damage to large choroid vessels and inner nuclear layer, and more than 50% pyknosis in the outer nuclear layer. On day 7 after 2 mg/kg LHB PDT, fibrous plaques had formed in the CNV lesions, and the inner and outer nuclear layers were destroyed along with closure of choroidal vessels.

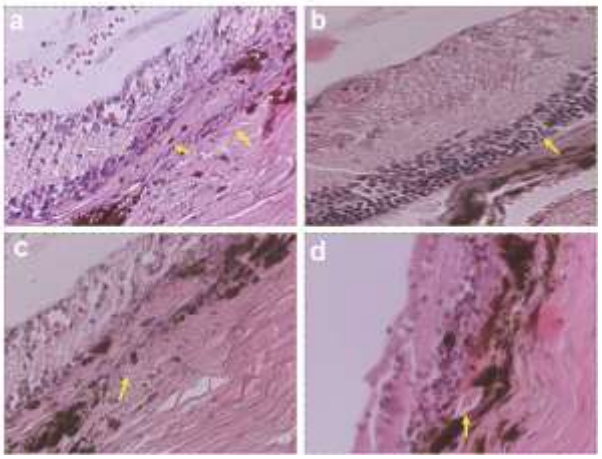


Fig. 6 Light micrograph of CNVs in rat eyes. (a) HE stained CNV lesion from a representative untreated rat containing many new vessels (arrows). (b) HE stained CNV membrane at 24 h after PDT with 1 mg/kg LHB. Occasional pyknosis are present in the outer nuclear layer (arrow). (c) HE stained CNV lesion at 7 days after PDT with 1 mg/kg LHB. Fibrous plaques composed of fibroblasts and RPE are present in the CNV membrane (arrow). (d) HE stained CNV lesion at 24 h after PDT with 2 mg/kg LHB. Damaged choroidal vessels are present (arrow). PDT was performed using the copper vapor laser irradiation with fluence of 36 J/cm^2. All images are shown at 200X magnification.

Accordingly, doubling the LHB dose to 2 mg/kg exhibited increased damage to collateral retina and choroid, which suggested lower dose of LHB should be safer for PDT of AMD. While a similar dose-dependent damage risk has been reported for verteporfin,[40] LHB PDT with the dose of 1 mg/kg exhibited a much higher CNV occlusion rate (88.0% vs. 4.2% at 7 days after PDT) by comparison in our experiment, and less damage to the retina and the choroid (histologic grade 2 vs. grade>4[40,48]) than the previous reports of verteporfin given the similar dosage. These findings suggest that LHB should be more effective and safer than verteporfin for reducing damage to the collateral retina and choroid.

Skin phototoxicity

Kunming mice were exposed to irradiation of simulated noon-time sunlight (40 mW/cm^2)[49] at 1, 3, 5, 7 and 14 days after intravenous injection of vehicle or 1, 2 or 4 mg/kg LHB. No effects on body weight, food consumption, heart rate, respiration rate, or feces and urine volume were observed when comparing the controls and experimental mice given 1, 2 or 4 mg/kg LHB after light irradiation (data not shown). In addition, there was no significant difference in ear weight found between any of the mice given the different doses after light irradiation ($p > 0.05$, Table 3). Although light irradiation induced redness and edema in the auricle of two female mice treated with 4 mg/kg LHB for 1 day, none of the other mice (regardless of dosage or length of time) showed any other symptoms. Fig. 7 shows representative HE-stained micrographs of the auricles and the exposed skin on

the backs of mice after light irradiation. Dermal inflammation, characterized by congestion and infiltration of inflammatory cells, was observed in the auricle of two mice that had received 4 mg/kg LHB after dosing for 1 day (Fig. 7a). No pathological changes were found in the auricles of any other mice given any doses after dosing for 3, 5, 7 and 14 days (Fig. 7b). In addition, the exposed skin on the backs of mice given any of the doses did not reveal any pathological changes after dosing for 1, 3, 5, 7 and 14 days (Fig. 7c).

Table 3 Ear weight of mice after exposure to light irradiation at 1, 3, 5, 7 and 14 days after single intravenous injection of vehicle or 1, 2 or 4 mg/kg LHB

Group	Ear weight, mg				
	1 day	3 days	5 days	7 days	14 days
Control	31.6±2.4	31.2±2.1	31.1±2.6	31.5±2.2	31.2±1.9
1 mg/kg	32.3±1.4[a]	31.3±1.5[b]	31.4±1.5[c]	32.1±2.2[d]	31.2±2.1[e]
2 mg/kg	32.2±2.2[a]	31.5±2.1[b]	31.8±1.2[c]	32.1±2.8[d]	32.1±2.7[e]
4 mg/kg	32.3±2.3[a]	32.1±1.8[b]	32.0±2.1[c]	32.3±1.7[d]	32.3±2.9[e]

[a, b, c, d, e] $p > 0.05$ compared with control.

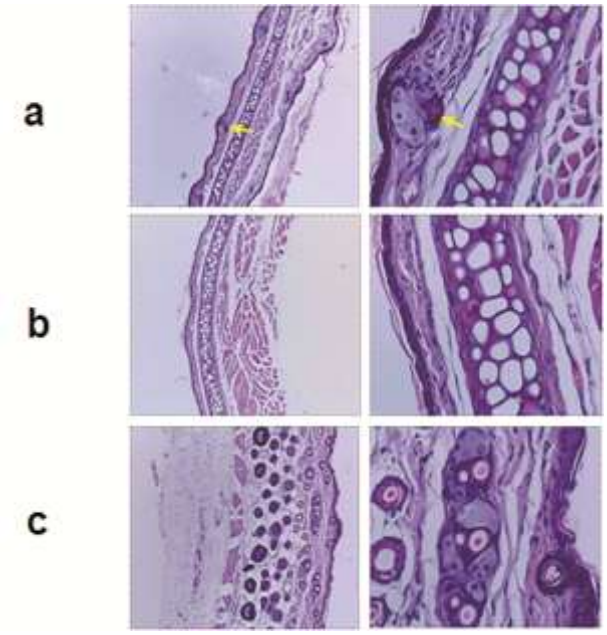


Fig. 7 Representative HE micrographs of the auricles and the exposed skin on the backs of mice observed after light irradiation. (a) Dermal inflammation was present in the auricle of two mice (representative image of one is shown) that had been given 4 mg/kg LHB and observed after dosing for 24 h. (b) No pathological changes were observed on the auricle of any other mice given vehicle or 1, 2 or 4 mg/kg LHB after dosing for 3, 5, 7 or 14 days. (c) The skin of the backs of mice given vehicle or 1, 2 or 4 mg/kg LHB did not show any pathological difference after dosing for 1, 3, 5, 7 or 14 days. Arrows indicate the inflammatory cells. Magnification: left-hand column, 40X; right-hand column, 200X.

Phototoxicity of the skin is one of the clinically significant side effects in patients receiving PDT. The clinically-approved PDT drugs, such as porfimer sodium and verteporfin, are applied with the requirement that patients avoid light exposure for several days or even weeks after the intravenous injection.[50,51] This restriction represents a substantial inconvenience to the patients but also can result in significant pain if ignored. Thus, newly developed PDT drugs should have the property of low skin photosensitivity. In the current study, we found that LHB produced mild skin photosensitivity: only two mice that received the highest dose of LHB (4 mg/kg) had an inflammatory reaction in the auricle irradiated at 24 h after dosing. The low skin phototoxicity of LHB is most probably related to its short half-life (2.319 ± 0.462 h), with near-complete metabolism at 24 h after drug administration. Although the skin photosensitivity of LHB within 24 h after drug administration has not been determined in this study, the findings from previous research studies have demonstrated that photosensitizing agents tend to cause more serious skin phototoxicity during the period of metabolism.[52,53] Besides, LHB exhibits long-term (1-year) stability, and is effectively promoted CNV occlusion in a laser-induced rat model of CNV, resulting in little collateral damage to the retina or RPE when administered at the low dose of 1 mg/kg. These findings suggest that LHB is a safe and effective photosensitizer of PDT for the treatment of AMD. Further extensive evaluations of the PDT efficacy, selectivity on the treated CNVs and skin phototoxicity with a series of LHB doses, irradiation doses and various intervals before the light illumination procedure, and comparisons of them with the use of verteporfin PDT, are underway in our laboratory; we expect the results from these additional studies to provide more specific data to support the potential of LHB for clinical application in targeted PDT of AMD.

Conclusions

Herein, we describe a new formulation of liposomal HB with high encapsulation efficiency prepared by means of a high-pressure homogenization method. This preparation is narrowly dispersed and can withstand storage for one year without loss of pharmaceutical effect *in vitro*. The pharmacokinetic properties of this LHB include a short half-life (2.319±0.462 h) and near-complete metabolism by 24 h after injection. When LHB was intravenously delivered at a low dose (1 mg/kg) to a rat model of CNV, it effectively led to CNV occlusion with minor damage to the collateral retina or RPE under yellow light irradiation. In addition, the LHB-treated mice exhibited low skin phototoxicity to simulated sunlight irradiation at 24 h after dosing. Collectively, our results suggest that LHB has remarkable potential for vascular-targeted photodynamic treatment of AMD.

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References

1. T. Y. Wong, U. Chakravarthy, R. Klein, P. Mitchell, G. Zlateva, R. Buggage, K. Fahrbach, C. Probst and I. Sledge, The natural history and prognosis of neovascular age-related macular degeneration: A systematic review of the literature and meta-analysis, *Ophthalmology*, 2008, **115**, 116-126.
2. J. Ambati, B. K. Ambati, S. H. Yoo, S. Lanchulev and A. P. Adamis, Age-related maculardegeneration: etiology, pathogenesis, and therapeutic strategies, *Surv. Ophthalmol.*, 2003, **48**, 257-293.
3. H. E. Grossniklaus and W. R. Green, Choroidal neovascularization, *Am. J. Ophthalmol.*, 2004, **137**, 496-503.
4. H. R. Coleman, C. C. Chan, F. L. Ferris and E. Y. Chew, Age-related macular degeneration, *Lancet*, 2008, **372**, 1835-1845.
5. D. M. Brown, P. K. Kaiser, M. Michels, G. Soubrane, J. S. Heier, R. Y. Kim, J. P. Sy, S. Schneider and ANCHOR Study Group, Ranibizumab versus verteporfin for neovascular age-related macular degeneration, *New Engl. J. Med.*, 2006, **355**, 1432-1444.
6. D. F. Martin, M. G. Maguire, G. S. Ying, J. E. Grunwald, S. L. Fine, G. J. Jaffe and CATT Research Group, Ranibizumab and bevacizumab for neovascular age-related macular degeneration, *New Engl. J. Med.*, 2011, **364**, 1897-1908.
7. N. Jo, C. Mailhos, M. H. Ju, E. Cheung, J. Bradley, K. Nishijima, G. S. Robinson, A. P. Adamis and D. T. Shima, Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular enclothelial growth factor therapy in multiple models of ocular neovascularization, *Am. J. Pathol.*, 2006, **168**, 2036-2053.
8. D. J. Browning, P. K. Kaiser, P. J. Rosenfeld and M. W. Stewart, Aflibercept for age-related macular degeneration: A game-changer or quiet addition? *Am. J. Ophthalmol.*, 2012, **154**, 222-226.
9. T. Y. Wong, G. Tikellis, C. Sun, R. Klein, D. J. Couper and A. R. Sharrett, Age-related macular degeneration and risk of coronary heart disease - the atherosclerosis risk in communities study, *Ophthalmology*, 2007, **114**, 86-91.
10. J. Tunon, J. M. Ruiz-Moreno, J. L. Martin-Ventura, L. M. Blanco-Colio, O. Lorenzo and J. Egido, Cardiovascular risk and antiangiogenic therapy for age-related macular degeneration, *Surv. Ophthalmol.*, 2009, **54**, 339-3488.
11. L. H. Curtis, B. G. Hammill, K. A. Schulman and S. W. Cousins, Risks of mortality, myocardial Infarction, bleeding, and stroke associated with therapies for age-related macular degeneration, *Arch. Ophthalmol.*, 2010, **128**, 1273-1279.
12. N. M. Bressler and Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP) Study Group, Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials-TAP report, *Arch. Ophthalmol.*, 1999, **117**, 1329-1345.
13. N. M. Bressler and Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP) Study Group, Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials-tap report 2, *Arch. Ophthalmol.*, 2001, **119**, 198-207.
14. M. Varano, P. Iacono, P. Giorno, A. Chiaravalloti and M. Parravano, Photodynamic therapy in subfoveal and juxafoveal myopic choroidal neovascularization: a 10-year retrospective analysis, *Ophthalmologica*, 2014, **231**, 204-210.
15. S. Yano, S. Hirohara, M. Obata, Y. Hagiya, S. Ogura, A. Ikeda, H. Kataoka, M. Tanaka and T. Joh, Current states and future views in photodynamic therapy, *J. Photochem. Photobiol. C*, 2011, **12**, 46-67.
16. N. Thomas, L. Tirand, E. Chatelut, F. Plenat, C. Frochot, M. Dodeller, F. Guillemin and M. Barberi-Heyob, Tissue distribution and pharmacokinetics of an ATWLPPR-conjugated chlorin-type photosensitizer targeting neuropilin-1 in glioma-bearing nude mice, *Photochem. Photobiol. Sci.*, 2008, **7**, 433-441.
17. B. Aveline, T. Hasan and R. W. Redmond, Photophysical and photosensitizing properties of benzoporphyrin derivative monoacid ring-a (Bpd-Ma), *Photochem. Photobiol*., 1994, 59, 328-335.
18. S. Stolik, J. A. Delgado, A. Perez and L. Anasagasti, Measurement of the penetration depths of red and near infrared light in human "ex vivo" tissues, *J. Photochem. Photobiol. B*, 2000, **57**, 90-93.
19. J. J. Arnold, K. J. Blinder, M. Bressler, S. B. Bressler, A. Burdan, L. Haynes, J. I. Lim, J. W. Miller, M. J. Potter, A. Reaves, P. J. Rosenfeld, M. Sickenberg, J. S. Slakter, G. Soubrane, H. A. Strong, M. Stur, Treatment of Age-related Macular Degeneration with Photodynamic Therapy Study Group and Verteporfin in Photodynamic Therapy Study Group, Acute severe visual acuity decrease after photodynamic therapy with verteporfin: Case reports from randomized clinical trials - TAP and VIP report no. 3, *Am. J. Ophthalmol.*, 2004, **137**, 683-696.
20. R. Tzekov, T. Lin, K. M. Zhang, B. Jackson, A. Oyejide, W. Orilla, A. D. Kulkarni, B. D. Kuppermann, L. Wheeler and J. Burke, Ocular changes after photodynamic therapy, *Invest. Ophth. Vis. Sci.*, 2006, **47**, 377-385.
21. H. Ozdemir, S. A. Karacorlu and M. Karacorlu, Early optical coherence tomography changes after photodynamic therapy in patients with age-related macular degeneration, *Am. J. Ophthalmol.*, 2006, **141**, 574-576.
22. E. P. Estey, K. Brown, Z. J. Diwu, J. X. Lin, J. W. Lown, G. G. Miller, R. B. Moore, J. Tulip and M. S. McPhee, Hypocrellins as photosensitizers for photodynamic therapy: A screening evaluation and pharmacokinetic study, *Cancer Chemoth. Pharm.*, 1996, **37**, 343-350.
23. B. Khoobehi, R. Grinstead and E. Passos, Experimental photodynamic effects of hypocrellin A on the choriocapillaris, *Ophthalmic. Surg. Las.*, 2002, **33**, 207-213.
24. L. J. Jiang and Y. Y. He, Photophysics, photochemistry and photobiology of hypocrellin photosensitizers, *Chinese Sci. Bull.*, 2001, **46**, 6-16.
25. G. G. Miller, K. Brown, A. M. Ballangrud, O. Barajs, Z. Xiao, J. Tulip, J. W. Lown, J. M. Leithoff, M. J. AllalunisTurner, R. D. Mehta and R. B. Moore, Preclinical assessment of hypocrellin B and hypocrellin B derivatives as sensitizers for photodynamic therapy of cancer: Progress update, *Photochem. Photobiol.*, 1997, **65**, 714-722.
26. S. H. Barsky, S. Rosen, D. E. Geer and J. M. Noe, Nature and evolution of port wine stains - computer-assisted study, *J. Invest. Dermatol.*, 1980, **74**, 154-157.
27. C. K. Leung, W. M. Chan, K. K. Chong, K. C. Chan, W. H. Yung, M. K. Tsang, R. K. Tse and D. S. Lam, Alignment artifacts in optical coherence tomography analyzed images, *Ophthalmology*, 2007, **114**, 263-270.
28. J. Q. Zhao, H. Deng, J. Xie, X. Liu, Y. Zhang, N. Y. Huang and Y. Gu, Towards characteristics of photodynamic drugs specifically aimed at microvascular diseases, *Mini-Rev. Med. Chem.*, 2010, **10**, 332-341.
29. H. Deng, X. Liu, J. Xie, R. Yin, N. Y. Huang, Y. Gu and J. Q. Zhao, Quantitative and site-directed chemical modification of hypocrellins toward direct dug delivery and effective photodynamic activity, *J. Med. Chem.*, 2012, **55**, 1910-1919.
30. S. J. Xu, X. X. Zhang, S. Chen, M. H. Zhang, T. Shen and Z. P.Wang, Novel phototherapeutic agents: Investigation and progress of hypocrellin derivatives, *Chinese Sci. Bull*., 2003, **48**, 1775-1785.
31. S. Q. Xia, J. H. Zhou, J. R. Chen, X. S. Wang and B. W. Zhang, A tyrosine-modified hypocrellin B with affinity for and photodamaging ability towards calf thymus DNA, *Chem. Commun.*, 2003, **(23)**, 2900-2901.
32. Z. H. Zeng, R. Qiao, J. H. Zhou, S. Q. Xia, Y. Zhang,Y. Y. Liu, J. R. Chen, X. S. Wang and B. W.Zhang, Photodynamic properties of dipeptide-modified hypocrellin B derivatives: The role of tyrosine and tryptophan groups, *J. Phys. Chem. B*, 2007, **111**, 3742-3749.
33. Y. Y. Liu, Q. X. Zhou, Z. H. Zeng, R. Qiao, X. S. Wang and B. W. Zhang, Photodynamic properties of a bispyrrolecarboxamide-modified hypocrellin B: The role of affinity and ascorbic acid, *J. Phys. Chem. B*, 2008, **112**, 9959-9965.
34. Z. X. Zhou, L. N. Zhang, H. M. Liu, Q. Zhang, B. Y. Liu, P. Ai, H. T. Ma, W. Liu, Z. L. Li, W. Sheng, Y. Zeng and R. G. Zhong, Photocytotoxicity of hypocrellin B (HB) was enhanced by liposomalization in vitro, *Int. J. Toxicol.*, 2011, **30**, 174-180.

35. C. L. Yu, S. Chen, M. H. Zhang and T. Shen, Spectroscopic studies and photodynamic actions of hypocrellin B in liposomes, *Photochem. Photobiol*., 2001, **3**, 482-488.
36. H. X. Chen, Z. F. Yang, X. B. Zou, J. G. Zhu, H. Deng, J. Q. Zhao, and Y. Gu, Photodynamic efficacy of liposome-delivered hypocrellin B in microvascular endothelial cells in vitro and chicken combs in vivo: a potential photosensitizer for port wine stain, *Laser Phys.*, 2013, **23**, 025605.
37. F. G. Liu, L. N. Han, Y. Gu, L. Zhang and H. X. Chen, Biological features of the choriocapillaries after hypocrellin B photodynamic therapy, *Chin. J. Clin. Rehab.*, 2005, **9**, 254-256.
38. K. H. Zhao and L. J. Jiang, Conversion of hypocrellin A in alkaline and neutral media, *Chin. J. Org. Chem.*, 1989,**9**, 252-254.
39. M. Brandl, D. Bachmann, M. Drechsler and K. H. Bauer, Liposome preparation by a new high-Pressure homogenizer gaulin micron lab-40, *Drug. Dev. Ind. Pharm.*,1990, **16**, 2167-2191.
40. D. N. Zacks, E. Ezra, Y. Terada, N. Michaud, E. Connolly, E. S. Gragouda and J. W. Miller,Verteporfin photodynamic therapy in the rat model of choroidal neovascularization: angiographic and histologic characterization, *Invest. Ophthalmol. Vis. Sci.*, 2002, **43**, 2384-2391.
41. B. T. Paul, M. S. Babu, T. R. Santhoshkumar, D. Karunagaran, G. S. Selvam, K. Brown, T. Woo, S. Sharma, S. Naicker and R. Murugesan, Biophysical evaluation of two red-shifted hypocrellin B derivatives as novel PDT agents, *J. Photochem. Photobiol. B*, 2009, **94**, 38-44.
42. M. A. Mainster, Wavelength selection in macular photocoagulation - tissue optics, thermal Effects, and laser systems, *Ophthalmology*, 1986, **93**, 952-958.
43. B. Katz, J. McBean and J. S. Cheung, The new laser liposuction for men, *Dermatol. Ther*., 2007, **20**, 448-451.
44. J. H. Lock and K. C. Fong, An update on retinal laser therapy, *Clin. Exp. Optom.*, 2011, **94**, 43-51.
45. H. Deng, T. H. Li, J. Xie, N. Y. Huang, Y. Gu and J. Q. Zhao, Synthesis and bio-evaluation of novel hypocrellin derivatives: Potential photosensitizers for photodynamic therapy of age-related macular degeneration, *Dyes Pigm.*, 2013, **99**, 930-939.
46. C. Sunderkotter, K. Steinbrink, M. Goebeler, R. Bhardwaj and C. Sorg, Macrophages and Angiogenesis, *J. Leukocyte. Biol.*, 1994, **55**, 410-422.
47. S. Piermarocchi, M. Sartore, G. Lo Giudice, G. Monterosso, E. Pilotto and T. Segato, Is there any relationship between photodynamic therapy for exudative age-related macular degeneration and choroidal neovascularization recurrence? A rationale for combined treatments, *Eur. J. Ophthalmol.*, 2006, **16**, 686-694.
48. M. Berdugo, R. A. Bejjani, F. Valamanesh, M. Savoldelli, J. C. Jeanny, D. Blanc, H. Ficheux, A. Scherz, Y. Salomon, D. BenEzra, and F. Behar-Cohen, Evaluation of the new photosensitizer stakel (WST-11) for photodynamic choroidal vessel occlusion in rabbit and rat eyes, *Invest. Ophthalmol. Vis. Sci.*, 2008, **49**, 1633-1644.
49. X. D. Huang, D. G. Dixon and B. M. Greenberg, Increased polycyclic aromatic hydrocarbon toxicity following their photomodification in natural sunlight - impacts on the duckweed lemna-gibba L G-3, *Ecotox. Environ. Safe.*, 1995, **32**, 194-200.
50. M. D. Bilgin, A. E. Elcin and Y. M. Elcin, Topical use of liposomal copper palmitate formulation blocks porphyrin-induced photosensitivity in rats, *J. Photochem. Photobiol. B*, 2005, **80**, 107-114.
51. T. C. Zhu and J. C. Finlay, The role of photodynamic therapy (PDT) physics, *Med. Phys.*, 2008, **35**, 3127-3136.
52. S. T. Wolford, D. L. Novicki and B. Kelly, Comparative skin phototoxicity in mice with 2 photosensitizing drugs - benzoporphyrin derivative monoacid ring-a and porfimer sodium (Photofrin), *Fund. Appl. Toxicol.*, 1995, **24**, 52-56.
53. D. A. Bellnier, W. R. Greco, H. Nava, G. M. Loewen, A. R. Oseroff, and T. J. Dougherty, Mild skin photosensitivity in cancer patients following injection of Photochlor (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a; HPPH) for photodynamic therapy, *Cancer Chemoth. Pharm.*, 2006, **57**, 40-45.