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## Salvianolic acid B and its liposomal formulations: Anti-hyperalgesic activity in the treatment of neuropathic pain

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### ABSTRACT

Salvianolic acid B (SalB) represents the most characteristic constituent of *Salvia miltiorrhiza* Bge. with a strong free radicals scavenger activity. This property may be useful in the treatment of some severe chronic diseases, where there is an imbalance of reactive oxygen species formation and where intracellular reactive oxygen and nitrogen species level can cause severe cell damage and even cell death. In particular, SalB can protect against the oxidative stress as well as the antioxidant superoxide dismutase and reduced activity of glutathione, important determinants of neuropathological and behavioural consequences in neuropathic pain. This is a chronic disease defined by the WHO as an untreatable illness because therapeutics are unsatisfactory in many cases and there is an urgent need to discover and develop novel active drugs. In the present work, SalB has been extracted and purified with an efficient and rapid method from the roots and rhizome of *S. miltiorrhiza* Bge. It was firstly submitted to pharmacological studies using the paw-pressure test, in an animal model of neuropathic pain where a peripheral mono neuropathy was produced by a chronic constriction injury of the sciatic nerve. SalB was effective against mechanical hyperalgesia when administered intraperitoneally at the dose of 100 mg/kg, 15 min after administration. Due to the poor chemical stability and bioavailability of SalB, liposomes were developed as drug carriers for parental administration. SalB-loaded liposomes were characterised in terms of particle size, polydispersity index, encapsulation efficacy and morphology. According to the *in vivo* studies, encapsulation, especially into PEGylated liposomes, increased and prolonged the antihyperalgesic activity 30 min after i.p. administration and the effect was still significant at 45 min.

Thus, PEGylated formulation ameliorated the performance of drug delaying, increasing and prolonging in time its antihyperalgesic effect.

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### 1. Introduction

Salvianolic acid B (SalB, Fig. 1) represents the most abundant constituent among the water-soluble compounds of roots and rhizome of Danshen (*Salvia miltiorrhiza* Bge.), a medicinal plant widely used in Asia for the treatment of a variety of diseases, in particular cardiovascular diseases such as angina pectoris, myocardial infarction and stroke (Cheng, 2006; Zhou et al., 2005; The State Pharmacopoeia Commission of the People's Republic of China, 2005). Ampuls of Danshen for parental administration are already present in the Asian market and they are primarily used to treat coronary heart disease, angina pectoris, and other heart and stomach diseases caused by *qi* stagnancy and blood stasis (Wang et al., 1997; Weng et al., 1980; Shi et al., 1981).

SalB and related constituents have strong antioxidant and free radicals scavenger activity (higher than vitamin C) due to their phenol hydroxyl groups (Jiang et al., 2009; Zhao et al., 2008).

Indeed, some severe chronic diseases, such as arthritis, cancer, diabetes, cardiovascular diseases, inflammations and neurological disorders are related to the imbalance of reactive oxygen species (ROS) formation and elimination (Halliwell, 1996). Antioxidants can protect against these complex diseases through scavenging free radicals and reducing hydrogen peroxide (Shahidi and Wanasundara, 1992). Cell damage is induced by reactive oxygen and nitrogen species (RONS), which under normal conditions, are cleared from cells by the action of superoxide dismutase, catalase or glutathione, as well as antioxidant molecules but in pathological conditions, intracellular RONS level can cause severe cell damage and even cell death.

Neuropathic pain is a chronic disease defined by the WHO as an untreatable illness because therapeutics are unsatisfactory in

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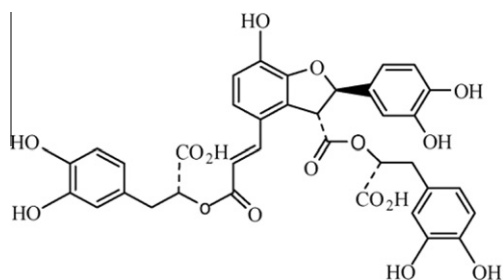


Fig. 1. Chemical structure of SalB.

many cases and there is an urgent need to discover and develop novel active drugs (Syndrup and Jensen, 1999). Among the murine models of neuropathic pain, the chronic constriction injury of the sciatic nerve (CCI, Bennett and Xie, 1988) was selected for our studies. This model is a chronic pain syndrome characterised by allodynia (pain elicited by a non-noxious stimulus) and hyperalgesia (increased pain response to a noxious stimulus) which persists for a long time after the initial injury is resolved (Woolf and Mannion, 1999).

Recently, in the CCI model of neuropathic pain, it has been shown that the oxidative stress as well as the antioxidant superoxide dismutase and reduced activity of glutathione are important determinants of neuropathological and behavioural consequences (Tan et al., 2009). For this reason SalB was selected in this study for testing its properties in reverting hyperalgesia and, in addition, it was investigated for its anti-inflammatory properties using the paw-pressure test in rats (Leighton et al., 1988). The intraperitoneal administration was chosen in order to reproduce the parental administration of the commercial ampuls of Danshen already existing on the Asian market (Wang et al., 1997; Weng et al., 1980; Shi et al., 1981).

Besides the interesting biological activities of SalB, it suffers from specific shortcomings which have limited its use as a drug, namely an instability under UV irradiation and in aqueous solutions, a low oral bioavailability; SalB was absorbed quickly and eliminated rapidly from plasma after oral administration to rats (Zhang et al., 2004). The bioavailability of SalB was found to be extremely low in rats (Wu et al., 2006). Furthermore, studies on rats have proven that SalB has an extensive metabolism with a very short half-life (about 90% of the acid is eliminated within 8 h after oral administration) and a strong plasma protein binding (between 80% and 90%) (Zhang et al., 2004; Kim et al., 2005; Yang et al., 2008).

The use of delivery systems may help overcome both chemical and biopharmaceutical issues and in particular liposomes have been extensively studied for such purposes. Liposomes are very versatile carriers able to formulate both hydrophilic and hydrophobic molecules, increasing drug stability and bioavailability, widely used for parental administration (Shoji and Nakashima, 2004). Consequently, this study focussed on three aims: (i) evaluate the anti-hyperalgesic and anti-inflammatory activities, (ii) formulate and optimise SalB-loaded liposomes to increase its therapeutical efficacy, and (iii) estimate the *in vivo* performance of liposomes in comparison with the free drug.

## 2. Materials and methods

### 2.1. Plant material and standard

*S. miltiorrhiza* Bge. (Lamiaceae) roots and rhizome (500 g) were purchased by Shenzhou Medical Center, import–export s.r.l., Rome, Italy. SalB was extracted as described in Section 2.4.1.

### 2.2. Substances

SalB standard CRS was kindly provided by China's National Institute for the Control of Pharmaceutical and Biological Products (NICPBP); (C<sub>36</sub>H<sub>30</sub>O<sub>16</sub>; MW 718.6; quality contamination >98.5%).

Ethanol was of analytical reagent grade from Riedel-de Haen (Seelze, Germany). All solvents used were of HPLC grade; CH<sub>3</sub>CN and MeOH were purchased from Merck (Darmstadt, Germany). HCOOH (85%) was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q<sub>plus</sub> system from Millipore (Milford, MA, USA). Carboxymethylcellulose sodium salt (CMC) was purchased from Fluka Chemie GmbH, Steinheim, Germany and solution NaCl 0.9% w/v from Fresenius Kabi, Italy. Egg phosphatidylcholine (Phospholipon® 90, P90) was kindly supplied by Natterman Phospholipids, GmbH (Cologne, Germany). Cholesterol (CHOL) was purchased from Aldrich (Milan, Italy). PEG 2000 (18:0/18:0) was purchased from Spectra2000 s.r.l. (Rome, Italy). All other products and reagents were of analytical grade: ketorolac (≥99%) from Sigma, St. Louis, USA; morphine hydrochloride (>99%) from S.A.L.A.R. Pregabalin (>99%) was purchased from Chem Pacific, Baltimore.

### 2.3. Animal experiments

Male Sprague–Dawley albino rats (180–200 g) from Harlan (S. Piero al Nativone, Italy) were used. Four rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatisation. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1 °C with a 12-h light/dark cycle, light at 7 AM. A total of 5–6 rats per group were tested. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care. All efforts were made to minimise the number of animals used and their suffering.

### 2.4. Methods

#### 2.4.1. Isolation of SalB

The isolation procedure was carried out according to the literature (Wang et al., 2007), slightly modified. The dried roots of *S. miltiorrhiza* Bunge were ground to powder and extracted three times with 2 l of 80% aqueous ethanol. The extract was filtered and the filtrate was concentrated in a rotavapor to eliminate ethanol and subsequently extracted with *n*-hexane (500 ml, three times), in order to remove completely the lipophilic constituents, tanshinones. The aqueous phase was then partitioned with dichloromethane (300 ml, twice) to remove non polar constituents, and successively acidified to a final pH 2 (10% hydrochloric acid) and re-extracted with ethyl acetate (300 ml, three times). The organic phase concentrated to give a residue of approximately 12.5 g. Part of the extract (~2 g) was redissolved in 50% MeOH and subjected to column chromatography over Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) using EtOH 20%, to give a salvianolic B enriched fraction. Repeated chromatographic separations of this fraction over Sephadex LH-20 columns using the same elution system afforded pure SalB. All fractionations were monitored by TLC and HPLC-UV-DAD. TLC analyses were carried out on silica gel 60 plates (F254, Merck, Darmstadt Germany) using the solvent system ethylacetate:CH<sub>3</sub>COOH:H<sub>2</sub>O 90:5:5 and vanillin reagent (5% vanillin in MeOH:5% H<sub>2</sub>SO<sub>4</sub> in EtOH, 1:1). Purity of isolated SalB (96%) was assessed by <sup>1</sup>H NMR analysis and HPLC-DAD–ESI-MS analysis using SalB reference standard (purity >98.5% by HPLC-DAD–ESI-MS, GC, NMR).

#### 2.4.2. Analytical HPLC-DAD characterisation

The HPLC system consisted of a HP 1100 L instrument with a Diode Array Detector and managed by a HP 9000 workstation

(Agilent Technologies, Palo Alto, CA, USA). The column was a Phenomenex Luna RP C18 (150 mm × 3 mm i.d., 5 μm) maintained at 26 °C. The eluents were H<sub>2</sub>O at pH 3.2 by formic acid (A) and acetonitrile (B). Two different linear gradients were applied. Linear gradient (1) was used for the monitoring of the fractionation and evaluation of purity of SalB. The following multi-step linear gradient was applied: from 90% A to 85% A in 10 min, in 7 min to 22% B, with a plateau of 8 min; 2 min to 25% and then 13 min to 66% of B. Total time of analysis was 40 min, equilibration time was 10 min, flow rate was 0.4 ml min<sup>-1</sup>, injection volume was 2 μl and oven temperature 26 °C. The UV–Vis spectra were recorded between 220 and 500 nm and the chromatographic profiles were registered at 254, 290, 330 and 350 nm. Linear gradient (2) was used for the calculation of SalB in plasma. The following multi-step linear gradient was applied: from 85% A to 70% A in 10 min, from 70% A to 48% A in 13 min and then 5 min to 85% of A. Total time of analysis was 28 min, equilibration time was 5 min, flow rate was 0.4 ml min<sup>-1</sup>, injection volume was 2 μl and oven temperature 26 °C. The UV–Vis spectra were recorded between 220 and 500 nm and the chromatographic profiles were registered at 254, 290, 330 and 350 nm.

#### 2.4.3. Analytical HPLC–ESI-MS

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebuliser with respect to the capillary inlet, allowed use of analytical conditions similar to those used for HPLC–DAD analysis in order to achieve the maximum sensitivity of ESI values. The same column, time period and flow rate were used during the HPLC–ESI-MS analyses. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionisation mode, scan spectra from *m/z* 100 to 800, was used with a gas temperature of 350 °C, nitrogen flow rate of 10 l min<sup>-1</sup>, nebuliser pressure 30 psi, quadrupole temperature 30 °C, capillary voltage 3500 V. The applied fragmentors were in the range 80–180 V. Identification of constituents was carried out by HPLC–DAD and HPLC–ESI-MS analyses, and/or by comparison and combination of their retention times, UV–Vis and mass spectra of the peaks with those of authentic standards when possible, or isolated compounds or characterised extracts as well as based on literature data.

#### 2.4.4. Linearity, LOD, LOQ, precision

The linearity range of responses of the standard SalB in MeOH and SalB in plasma was determined on five concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amounts ranging from  $6.6 \times 10^{-3}$  to 3.3 μg (SalB in MeOH) and from  $57.2 \times 10^{-3}$  to 0.86 μg (SalB in plasma): stock solutions of the standards (LOQ included) were prepared at different concentrations ranging from  $3.3 \times 10^{-3}$  to 0.33 mg/ml (SalB in MeOH) and from  $28.6 \times 10^{-3}$  to 0.286 mg/ml (SalB in plasma) and injected into HPLC (injection volumes varying from 2 to 10 μl). The limit of detection (LOD) and quantification (LOQ) under the chromatographic conditions were determined by injecting a series of the standard solutions until the signal-to-noise (S/N) ratio for each compound was 3 for LOD and 10 for LOQ. For the intra-day variability test, freshly prepared standard samples (SalB in MeOH and SalB in plasma) in the range of the calibration curve were analysed in six replicates within 1 day. For the inter-day variability test, the standard solutions, the isotonic saline solutions and the liposomal formulations were examined in triplicates for three consecutive days. The contents of SalB in each case were evaluated by HPLC–DAD (for SalB in MeOH or in isotonic saline solution) and HPLC–ESI-MS (for SalB in plasma) to calculate the relative standard deviation (%RSD).

#### 2.4.5. Production of conventional and PEGylated SalB-loaded liposomes

Conventional and PEGylated SalB-loaded liposomes (SalB-CL and SalB-PL respectively) were prepared according to the film hydration method (Bangham et al., 1965). Conventional liposomes were prepared using P90G, cholesterol and SalB and optimised using the OVAT technique (One-Variable-at-a-Time). P90G and cholesterol were firstly dissolved in dichloromethane and the organic solvent was evaporated under vacuum. The dry lipid film was hydrated by the addition of SalB physiological solution (NaCl 0.9% w/v). The dispersion was stirred with a mechanical stirrer for 30 min in a water-bath at the constant temperature of 38 °C. In order to reduce the dimensions of the vesicles from MLVs to LUVs, a high pressure homogenizer Emulsiflex C3® (AVESTIN Europe GmbH, Mannheim, Germany) was used at the applied pressure of 150,000 kPa for 60 s. Alternatively, vesicle dimensions were reduced and their homogeneity was increased by the extrusion technique. Liposomes were extruded 21-times through polycarbonate filters with pore size 400, 200 and 100 nm sequentially using a Liposofast basic (AVESTIN Europe GmbH, Mannheim, Germany). Also the PEGylated liposomes containing 18:0/18:0 PEG2000, P90G, cholesterol and SalB were optimised using the OVAT technique. 18:0/18:0 PEG 2000 was weighted together with P90G and cholesterol and dissolved in dichloromethane using the same method preparation as described for conventional liposomes.

#### 2.4.6. Particle characterisation of liposomes

Particle size was measured by a Dynamic Light Scattering (DLS), Zetasizer Nano series ZS90 (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He–Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water-bath) set at 25 °C. Time correlation functions were analysed to obtain the hydrodynamic diameter of the particles ( $Z_h$ ) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analysed by the cumulants method (fitting a single exponential to the correlation function to obtain the mean size ( $Z_{ave}$ ) and polydispersity index (PDI) or by CONTIN (fitting a multiple exponential to the correlation function to obtain particle size distributions). Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle, diluting the different samples 100-folds in physiological solution NaCl 0.9% w/v.

#### 2.4.7. Electrophoretic mobility

Zeta potentials (ζ-potentials) of the liposome systems were measured using the Malvern Instruments Zetasizer Nano series ZS90. For all samples, an average of three measurements at a stationary level was taken. The cell used was a 5 × 2 mm rectangular quartz capillary. The temperature was kept constant at 25 °C by a Haake temperature controller. The zeta potential was calculated from the electrophoretic mobility,  $\mu_E$ , using the Henry correction to Smoluchowski's equation.

#### 2.4.8. Transmission electron microscopy

Liposome dispersions were analysed by TEM analysis with the negative stain technique in terms of morphology and mean diameter. A drop (10 μl) of vesicle dispersion diluted 10-times was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film specimen, which was stained with a phosphotungstic acid solution 1% w/v in sterile water. The samples were dried for 3 min and then were examined under a JEOL 1010 electron microscope and photographed at an accelerating voltage of 64 kV.

#### 2.4.9. Encapsulation efficacy (EE%)

Free SalB was removed from the surface of liposomes by gel permeation chromatography on Sephadex G-50. Liposomal dispersion (200  $\mu$ l) was loaded in a minicolumn containing a dry matrix (G-50 gel bed). The not entrapped material was removed by centrifugation at 1000g for 3 min at a controlled temperature (+4 °C). Purified liposomes were collected and were disrupted using the dilution method with an organic solvent, then were submitted to ultrasonication in a bath for 30 min. After centrifugation at 12,000 rpm for 5 min, the content of encapsulated SalB in liposomes was quantified by HPLC–ESI–MS analysis, using SalB as external standards. The entrapment efficiency (EE) is reported as the percentage of SalB entrapped in the vesicles:  $EE (\%) = \{(\text{amount of SalB entrapped in the vesicles}) / (\text{total amount of SalB used in the preparation})\} \times 100$ .

#### 2.4.10. Stability studies

Stability both of SalB isotonic saline solutions used in the *in vivo* tests as well as liposomal formulations containing SalB at the concentration of 20 mg/ml was assessed during one week using the developed HPLC–DAD analytical method.

#### 2.4.11. In vivo experiments

**2.4.11.1. Application of test substances.** Drugs were dissolved in isotonic (NaCl 0.9% w/v) saline solution or dispersed in carboxymethylcellulose sodium 1% solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 10 ml  $\text{kg}^{-1}$  by i.p. injection.

**2.4.11.2. Chronic constriction injury (CCI).** A peripheral mono neuropathy was produced in adult rats by placing loosely constrictive ligatures around the common sciatic nerve according to the method described by Bennett and Xie (1988). Rats were anaesthetised with chloral hydrate. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to sciatica's trifurcation, about 1 cm of the nerve was freed of the adhering tissue and four ligatures (3/0 silk thread) were tied loosely around it with about 1 mm spacing. The length of the nerve thus affected was 4–5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted when viewed with 40 $\times$  magnification. In every animal, an identical dissection was performed on the opposite side except that the sciatic nerve was not ligated. The left paw was untouched.

**2.4.11.3. Paw-pressure test.** The instrument exerts a force which is applied at a constant rate (32 g/s) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold is given by the force which induces the first struggling from the rat. An arbitrary cut off value of 250 g was adopted. Those mice scoring less than 40 g or over 75 g in the pretest were rejected (25%). The pain threshold was measured before (pretest) and 15, 30 and 45 min after the beginning of the test.

**2.4.11.4. Plasmatic concentration of SalB.** Fifteen male Sprague–Dawley albino rats (180–200 g) were randomly separated into three groups. One group received free SalB dissolved in physiological solution NaCl 0.9% w/v at the dosage of 100 mg/kg intraperitoneally, and the other two groups were given SalB-loaded conventional liposomes (SalB-CL) and SalB-loaded PEGylated liposomes (SalB-PL) at the same dosage of SalB. Blood samples were collected from the tail vein into heparinized tubes (Multivette<sup>®</sup>, Sarsted, Italy) corresponding to the maximum of antihyperalgesic activity in each case: 15 min after administration, in the case of free SalB as well as SalB-CL, and 30 min after administration in the case

of SalB-PL. Blood samples were immediately centrifuged at 2000 rpm for 20 min at 17 °C to separate plasma from other blood elements. The obtained plasma was stored at –80 °C prior to analysis. A pre-treatment procedure is often needed to remove blood protein and potential interferences prior to HPLC–DAD–ESI–MS analysis. Protein precipitation is commonly used for fast sample clean-up and disrupting of protein–drug binding. A calibration curve ( $r^2 = 0.9999$ ) was determined by the constant volume of blank rat plasma with increasing concentration of SalB (ranging from 0 to 100  $\mu$ M as the final concentration of SalB). Each sample, including each point of the calibration curve, was diluted seven times with acetonitrile in order to precipitate plasma proteins. Samples were vortexed for 15 s and centrifuged at 10,000 rpm for 2.5 min. Each sample (200  $\mu$ l) was transferred in micro-vials.

**2.4.11.5. Statistics.** All experimental results are given as the mean  $\pm$  SEM. An analysis of variance ANOVA, followed by Fisher's Protected Least Significant Difference procedure for post hoc comparison, were used to verify significance between two means. Data were analysed with the StatView software for the Macintosh. *p* values of less than 0.05 were considered significant.

### 3. Results and discussion

#### 3.1. Isolation of SalB

A simple and fast isolation method was set up: the dried crude hydro-alcoholic extract of Danshen was firstly submitted to liquid–liquid extraction obtaining an extract deprived from tanshinones and other lipophilic constituents present in the plant.

Then Sephadex LH-20 using hydro-alcoholic solutions was used for a rapid separation and refinement of SalB, taking advantage of the difference in the molecular size of SalB (tetramer) compared to other monomers, dimers and trimers of caffeic acid present in the extract. The combination of both techniques represented a safe and convenient separation scheme yielding almost pure SalB (96%). The presence of SalB in the extract was assessed by a comparison of its  $t_R$  with the authentic sample SalB international standard and it was confirmed by UV and NMR data.

#### 3.2. Linearity, LOD, LOQ, precision

All calibration curves showed good linearity. The following  $r^2$  values were obtained: SalB  $r^2 = 0.9996$  (regression curve:  $y = 0.0004x - 0.0591$ ); SalB in plasma  $r^2 = 0.9999$  (regression curve:  $y = 1.598 \times 10^{-7} - 0.0341$ ).

LOD for SalB was 1.89 ng (0.94  $\mu$ g/ml, 2  $\mu$ l of injection) and LOQ was 6.6 ng (3.3  $\mu$ g/ml, 2  $\mu$ l of injection). For SalB in plasma, LOD was calculated as 28.6 ng (5.72  $\mu$ g/ml, 5  $\mu$ l of injection) and LOQ was 57.2 ng (28.6  $\mu$ g/ml, 2  $\mu$ l of injection).

The overall intra- and inter-day time variations of the standards were less than 10.64% and 2.34% for SalB and less than 2.13% and 3.44% for SalB in plasma, respectively.

In addition, the amount of SalB in isotonic saline solutions remained stable (not less than 93% residual percentage) during the three days of inter-day variability tests.

#### 3.3. Anti-hyperalgesic activity of SalB

The antihyperalgesic activity of pure SalB was investigated in an animal model of neuropathic pain (CCI). Paw withdrawal threshold was measured using Randall and Selitto apparatus exerting a force that increases at a constant rate (32 g/s). Stimulus at which rats withdrew the paw was recorded before treatment and after drug administration at different times (15, 30, 45 and 60 min). The

results represent means  $\pm$  SEM of the mechanical threshold expressed as grams. The positive controls used as reference compounds are reported in Fig. 2.

SalB was effective against mechanical hyperalgesia when intraperitoneally administered. Several dosages of pure SalB, dissolved in physiological solution, were tested and the dose–response curve was assessed to find the lowest active dosage. SalB reverted the mechanical hyperalgesia, evaluated in the paw-pressure test, in CCI rats when administered at the dose of 100 mg/kg, 15 min after administration ( $41.1 \pm 1.3$  vs.  $60.3 \pm 2.9$ ,  $p < 0.05$ ) (Fig. 3). The dose of 300 mg/kg resulted to be less active

than 100 mg/kg and we might hypothesise the presence of a bell-shaped trend, common feature with natural compounds.

No advantages occurred when administering SalB intraperitoneally for a 7 days period (prolonged administration): hyperalgesia was reverted only in correspondence to a single time point for each dosage. At the dosage of 100 mg/kg, SalB was active with a value statistically acceptable only 15 min after administration ( $38.6 \pm 2.8$  vs.  $58.4 \pm 3.6$ ,  $p < 0.05$ ) and at the dosage of 30 mg/kg with a value statistically acceptable only 30 min after administration ( $34.9 \pm 0.8$  vs.  $54.6 \pm 4.4$ ,  $p < 0.05$ ) (Fig. 4).

The safety of SalB was also assessed: it did not modify animals' gross behaviour at the highest effective doses. The Rota-rod test was employed to reveal any alterations of the motor coordination induced by the investigated compound. The number of falls from the rotating rod showed the lack of any impairment in the motor coordination of animals treated with SalB in comparison to the untreated group, ruling out that the results obtained were due to animals' altered viability (data not shown).

#### 3.4. Preparation and characterisation of liposomes

Liposomes were prepared using different ratios of phospholipids, cholesterol and 18:0/18:0 PEG2000, in order to optimise the formulation in terms of size and encapsulation efficacy. The molar ratio between the constituents of the conventional liposomes P90G:cholesterol:SalB were optimised as 2.5:0.5:2.3 using the OVAT technique. The molar ratio between the constituents of the PEGylated liposomes 18:0/18:0 PEG2000, P90G, cholesterol and SalB were optimised as 0.125:2.5:0.5:2.3, using the OVAT as well. The main parameters typically described for summarising the properties of the tested formulation are reported in Table 1.

Briefly, the particle sizes of various liposomes were around 200 nm in the case of SalB-CL and around 150 nm in the case of

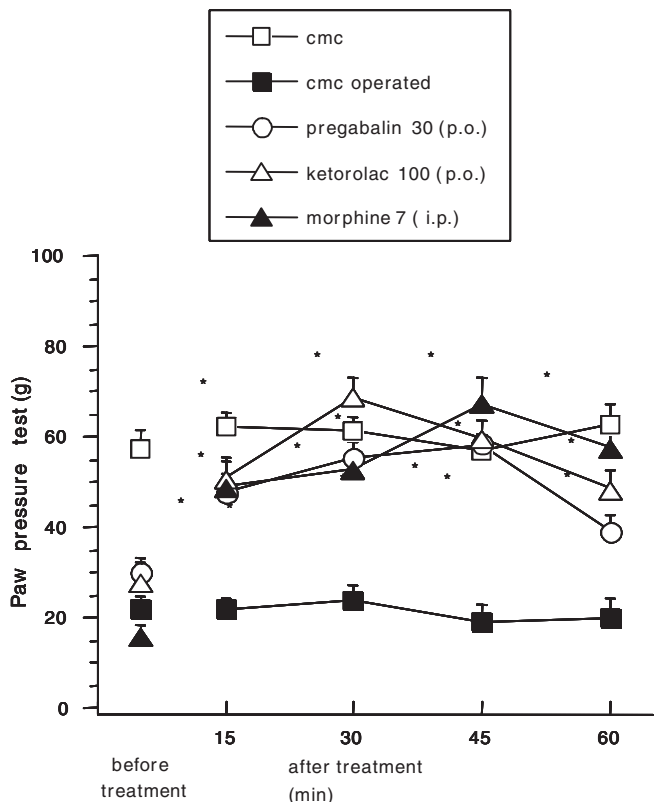


Fig. 2. Positive controls used as reference compounds in a chronic constriction injury of the sciatic nerve (CCI) in the rat paw-pressure test.

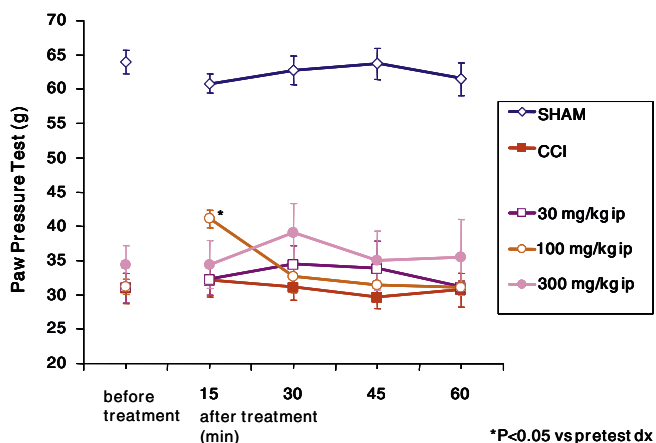


Fig. 3. SalB intraperitoneal administration in a chronic constriction injury of the sciatic nerve (CCI) in the rat paw-pressure test.

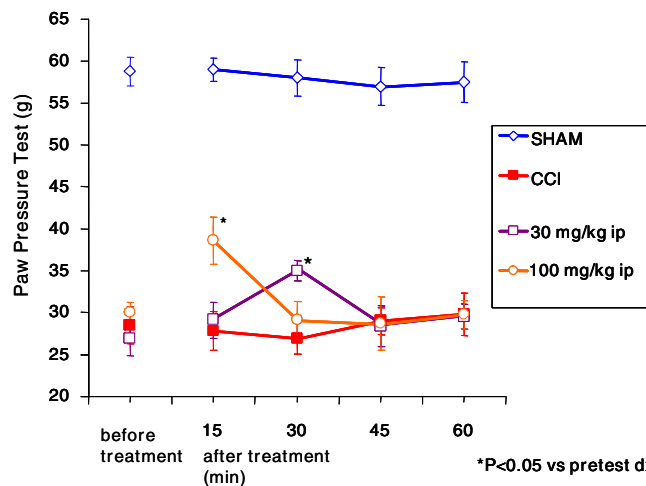


Fig. 4. SalB intraperitoneal administration for 7 days (prolonged administration) in a chronic constriction injury of the sciatic nerve (CCI) in the rat paw-pressure test.

Table 1

Size, polydispersity index (PDI),  $\zeta$ -potential and encapsulation efficacy (EE%) of SalB-loaded conventional (SalB-CL) and PEGylated liposomes (SalB-PL). Data are shown as mean of three samples ( $\pm$ SD).

	Mean diameter (nm)	PDI	$\zeta$ -Potential (mV)	EE (%)
SalB-CL	206.8 $\pm$ 7.3	0.29 $\pm$ 0.01	-11.6 $\pm$ 1.84	24.84 $\pm$ 0.28
SalB-PL	140.0 $\pm$ 6.5	0.23 $\pm$ 0.01	-7.88 $\pm$ 0.52	22.72 $\pm$ 0.88

SalB-PL. Both liposomal formulations had rather narrow size distribution:  $PDI < 0.3$  and  $PDI \approx 0.2$  for SalB-CL and SalB-PL, respectively. In order to reduce the dimensions of the vesicles from MLVs to LUVs, a high pressure homogenizer Emulsiflex C3<sup>®</sup> and an Extruder were used alternatively. The size and the polydispersity index of the samples obtained by these two optimised techniques were comparable. As expected, conventional liposomes are negatively charged ( $\zeta$ -potential is around  $-12$  mV), while the presence of PEG to the liposomal surface provides more efficient charge neutralisation ( $\zeta$ -potential were halved).

The drug loading efficiencies were similar for both conventional and PEGylated liposomes after evaluation by HPLC-DAD analysis. The chemical stability of SalB into the vesicles is maintained constant (not less than 95% residual percentage) at least during the three days of inter-day variability tests. The transmission electron micrograph by negative stain (Figs. 5 and 6) provided important details on the morphology and the dimension of the vesicles. Liposomes were large unilamellar vesicles, sphere-shaped and with a size comparable with the results from DLS analysis. In particular, the SalB-PL showed the emblematic conformational cloud forming by PEG chains (Fig. 6).

### 3.5. In vivo studies of liposomes

SalB-loaded conventional and PEGylated liposomes were tested in the Paw pressure test and compared with the performance of the free drug (Fig. 7). Both for free SalB and

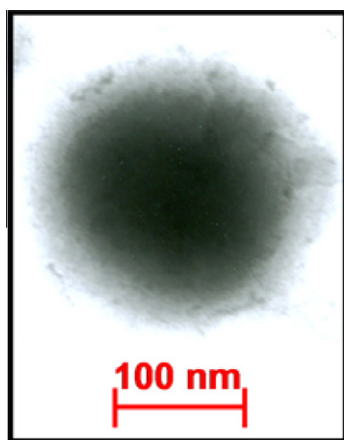


Fig. 5. SalB-loaded conventional liposomes. LUV: large unilamellar vesicles. Transmission electron micrograph by negative stain.

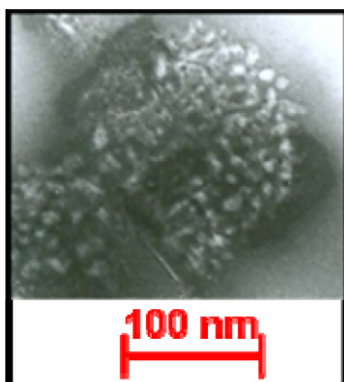


Fig. 6. SalB-loaded PEGylated liposomes. LUV: large unilamellar vesicles. Transmission electron micrograph by negative stain.

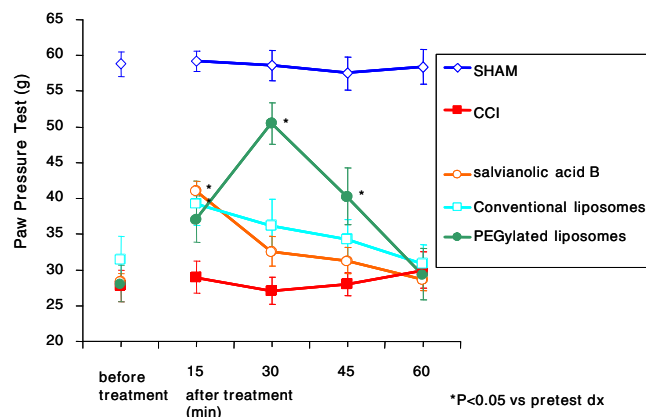


Fig. 7. SalB-loaded liposomes intraperitoneal administration in a chronic constriction injury of the sciatic nerve (CCI) in the rat paw-pressure test.

SalB-loaded conventional liposomes, administered at the active dosage of 100 mg/kg i.p., the antihyperalgesic effect started 15 min after administration ( $39.2 \pm 2.2$  vs.  $59.3 \pm 2.4$ ,  $p < 0.05$ ). On the other hand, SalB-loaded PEGylated liposomes shifted the maximum of antihyperalgesic activity 30 min after administration ( $50.9 \pm 3.8$  vs.  $58.5 \pm 2.7$ ,  $p < 0.05$ ) and the effect was still significant at 45 min ( $40.3 \pm 3.7$  vs.  $58.2 \pm 2.1$ ,  $p < 0.05$ ). This means that only PEGylated formulation ameliorates the performance of the drug by delaying, increasing and prolonging in time its antihyperalgesic effect.

### 3.6. Plasmatic concentration of SalB

In order to strengthen the above pharmacological results, the plasmatic concentrations of SalB in rats were quantified. The blood samples were collected corresponding to the maximum of antihyperalgesic activity in each case: 15 min after administration in the case of free SalB as well as SalB-CL and 30 min after administration in the case of SalB-PL. The three groups of rats received SalB dissolved in physiological solution NaCl 0.9% w/v, SalB-CL and SalB-PL respectively at the same dosage of 100 mg/kg intraperitoneally. The SalB plasmatic concentrations were quantified by HPLC-DAD-ESI-MS analysis, in modality scan and negative fragmentor, monitoring the pseudomolecular ion ( $717$  m/Z). Plasmatic SalB values reported in Table 2, supported the fact that free or drug vehiculated in conventional liposomes had the same blood concentrations at the maximum time of antihyperalgesia, in good agreement with the results obtained in the Paw pressure test having a similar *in vivo* efficacy.

Conversely, SalB-loaded PEGylated liposomes showed a plasmatic concentration more higher than 4-fold in comparison with the other formulations and indeed the *in vivo* efficacy was also increased.

Table 2

Mean SalB plasma concentration in the correspondence of the maximum of antihyperalgesic activity of free SalB, conventional (SalB-CL) and PEGylated (SalB-PL) SalB-loaded liposomes following i.p. administration at the dosage of 100 mg/kg. Values were expressed as mean  $\pm$  SD ( $n = 4$ ).

	Plasmatic concentration ( $\mu$ M)	Time (min)
SalB	$95 \pm 1.7$	15
SalB-CL	$80 \pm 2.0$	15
SalB-PL	$388 \pm 1.3$	30

#### 4. Conclusion

A fast, simple and efficient separation protocol was developed for the purification and isolation of SalB from a crude hydro-alcoholic extract of Danshen; it excludes the use of successive column chromatographies on silica gel and applies instead Liquid–Liquid Extraction and Size Exclusion Chromatography. Both methodologies have distinct advantages for the majority of the natural products: (i) they eliminate irreversible adsorptive loss, (ii) limit the creation of artefacts, (iii) low solvent consumption and (iv) are fast methods and could be easily scaled up at the industrial level.

The anti-hyperalgesic activity of SalB was assessed in an animal model of neuropathic pain (CCI) and to the best of our knowledge, this is the first report. Due to the poor low bioavailability of SalB, conventional and PEGylated liposomes were developed as suitable drug carriers. After optimisation of the vesicles features in terms of particle size, polydispersity index, encapsulation efficacy and morphology, liposomes were administered intraperitoneally to evaluate their antihyperalgesic activity and compare their performance with that of free drug. Drug encapsulation into PEGylated liposomes increased and prolonged the antihyperalgesic activity of SalB. The different *in vivo* performance of conventional and PEGylated liposomes was expected as recently reported in another study by the same authors (Isacchi et al., 2011). It could be related to the incorporation of PEG in the liposomes surface which can modify the pharmacokinetic profile of SalB, its metabolism and/or the plasma protein binding. These effects of the PEGylated liposomes were also supported by a preliminary evaluation of plasmatic concentration of SalB which resulted more than 4-fold in comparison with the other formulations taken at the maximum of the efficacy.

These studies represent the starting point for further and supplementary investigations to understand the potential application in the therapy of SalB-loaded PEGylated liposomes in the treatment of neuropathic pain.

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