

Orally Bioavailable and Effective Buparvaquone Lipid Based Nanomedicines for Visceral Leishmaniasis

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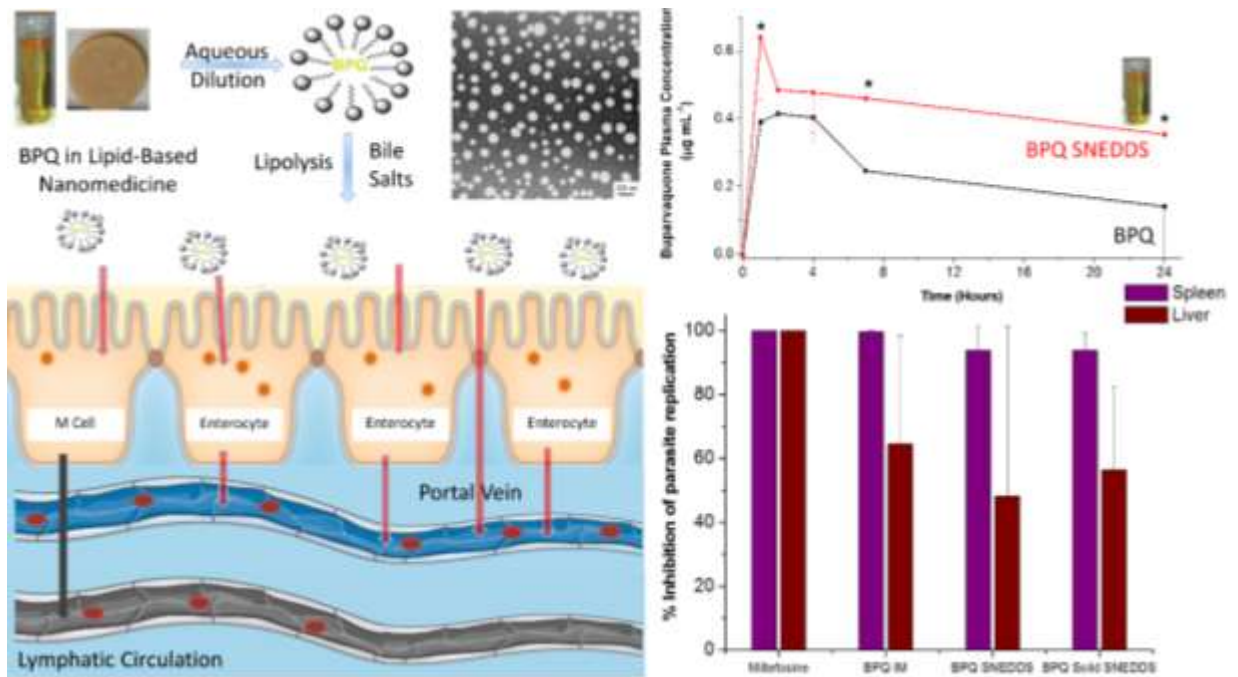
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Graphical Abstract



Abstract

Nano-enabled lipid based drug delivery systems offer a platform to overcome challenges encountered with current failed leads in the treatment of parasitic and infectious diseases. When prepared with FDA or EMA approved excipients, they can be readily translated without the need for further toxicological studies, while they remain affordable and amenable to scale-up. Buparvaquone (BPQ), a hydroxynapthoquinone with *in vitro* activity in the nanomolar range, failed to clinically translate as a viable treatment for visceral leishmaniasis due to its poor oral bioavailability limited by its poor aqueous solubility (BCS Class II drug). Here we describe a self-nanoemulsifying system (SNEDDS) with high loading and thermal stability up to 6 months in tropical conditions able to enhance the solubilisation capacity of BPQ in gastrointestinal media as demonstrated by flow-through cell and dynamic *in vitro* lipolysis studies. BPQ SNEDDS demonstrated an enhanced oral bioavailability compared to aqueous BPQ dispersions (probe – sonicated) resulting in an increased plasma AUC₀₋₂₄ by 55% that is four fold higher than any previous reported values for BPQ formulations. BPQ SNEDDS can be adsorbed on low molecular glycol chitosan polymers forming solid dispersions that when compressed into tablets allow the complete dissolution of BPQ in gastrointestinal media. BPQ SNEDDS and BPQ solid SNEDDS demonstrated potent *in vitro* efficacy in the nanomolar range (<37 nM) and were able to near completely inhibit parasite replication in the spleen and 48 ± 48 and 56 ± 23% inhibition of the parasite replication in the liver respectively compared to oral miltefosine after daily administration over 10 days. The proposed platform technology can be used to elicit a range of cost-effective and orally bioavailable non-invasive formulations for a range of antiparasitic and infectious disease drugs that are needed for closing the global health innovation gap.

Key Words

Neglected parasitic diseases, Visceral Leishmaniasis, Buparvaquone, Oral Delivery, Self-nanoemulsifying drug delivery systems (SNEDDS), Solid Nanomedicines.

Introduction

Advances in biotechnology and drug delivery have transformed medicine in the industrialised world, but these innovations have yet to reach developing countries. More than 10 million people in the developing countries die of infectious and parasitic diseases such as malaria, leishmaniasis, HIV/AIDS, tuberculosis, and diarrheal diseases annually, while morbidity related to these diseases devastates economic development ¹. More importantly, the burden of infectious and parasitic illness falls more heavily on children and pregnant women ².

Many of these diseases have earned the label of “neglected”, because health-care markets in the affected countries are insufficient to attract pharmaceutical industry funding in research and development. These involve African Trypanosomiasis, Chagas diseases, dengue, leishmaniasis, leprosy, lymphatic filariasis, malaria, onchocerciasis, schistosomiasis and tuberculosis ³. Over the past decade, public-private, not-for-profit, product development partnerships (PDPs) have arisen to tackle the development of new drugs, vaccines, diagnostics for developing-world diseases, while governments and foundations have increased their investment in neglected diseases research and development. The challenge remains to augment these public and private sector efforts by providing to those partnerships access to advanced technologies and expertise for discovering, delivering and developing new medicines ⁴.

Leishmaniasis presents as cutaneous, mucosal and visceral forms caused by protozoa of the genus *Leishmania* that spread in humans through the bite of a phlebotomine sandfly ⁵. The cutaneous and mucosal forms cause severe disfigurement and disability resulting in gender inequality, stigmatisation and reduction of economic productivity ⁶, while the visceral (VL) form is fatal if left untreated (with a

100% fatality rate within 2 years). An estimated 12 million people are infected with *Leishmania* with 50,000 deaths annually ⁷, while 1.3 million new cases are reported every year putting in risk 310 million people ⁶ over a wider geographical distribution today due to urbanisation, migration and deforestation, making leishmaniasis a growing public health concern ⁸. To complicate things further, up to twelve percent of all VL cases are associated with HIV/AIDS, while highest prevalence is reported in sub-Saharan countries (20-40% of cases) ⁹⁻¹¹.

First line treatment for VL relies on pentavalent antimonials [sodium stibogluconate (SSG) and N-methylglucamine antimoniate (meglumine antimoniate)] ¹². As resistance to antimonials is increasing limiting their use, emerging second-line treatments involve pentamidine and Amphotericin B (AmB)^{13, 14}. AmB is highly effective in VL when administered intravenously. Available parenteral formulations involve an affordable micellar formulation (Fungizone[®]), which is highly nephrotoxic limiting the dose able to be administered, and a liposomal formulation (Ambisome[®]) with the latter used first line in the developed world. Other parenteral formulations involve lipidic complexes (Abelcet[®]), but these are less likely to be used due to their higher cost compared to Ambisome[®] ¹³. As AmB parenteral formulations are thermally unstable, cost of therapy is raised further to account for the need for cold transport and refrigeration. To overcome this issue, the clinical use of a heated Fungizone[®] has been attempted in India. This involved a 10-fold aqueous dilution of Fungizone[®] and heating to 70 °C for 20 minutes ¹⁵, which elicits larger AmB aggregates that are not renally cleared reducing observed nephrotoxicity ¹⁶. Pentamidine, on the other hand, has been shown to cause insulin dependent diabetes which is irreversible and potentially fatal ¹⁷. Miltefosine, the only oral VL licensed treatment (licensed in 2003 in India), has a long elimination half-life (7 days) and a narrow therapeutic index. These pharmacokinetic

characteristics limit the administered dose which can result in subtherapeutic levels over several weeks encouraging the emergence of resistance ¹⁸. Indeed, resistance has been reported in India (a county that alone accounts for 50% of the VL worldwide burden) and France ^{13, 19}. Thus, available medicines for VL are outdated, impractical (non-oral, requiring hospitalisation and access to healthcare professionals over long treatment periods), insufficiently efficacious, or subject to resistance and unacceptable toxicities ^{4, 20-22}. Based on this, an ideal treatment for VL would possess greater than 95% efficacy, be orally administered, be stable in a tropical environment, be affordable and require less than 11 days treatment with an excellent side-effect profile ²³.

Buparvaquone (BPQ), a hydroxynaphthoquinone licensed for theileriosis as an intramuscular injection, possesses promising activity in the nanomolar range against *Leishmania* spp. amastigotes (ED₅₀: 0.005µM against *L. donovani*) ²⁴. Although *in vitro* experiments have provided promising results in the 90s, *in vivo* activity up to oral doses of 100 mg kg⁻¹ has been low, due to its poor solubility in physiological fluids (<300 ng mL⁻¹, Log D: 7.02 at pH 3.0) and low oral bioavailability ^{24, 25}, which hindered its translation into a viable therapy. Attempts to improve BPQ's aqueous solubility and bioavailability have included the generation of water soluble BPQ phosphate prodrugs ²⁶⁻²⁸, the use of nebulised nanosuspensions ²⁹ BPQ nanosuspensions in mucoadhesive hydrogels ³⁰, oral suspensions and cyclodextrin complexes with limited success³¹. The most promising results towards an oral bioavailable BPQ formulations involved the use of self-emulsifying drug delivery system (SEDDES) ²⁵. However, the oral efficacy of the BPQ SEDDS have not been demonstrated. Here we present, a novel BPQ loaded self-nanoemulsifying drug delivery system (BPQ-SNEDDS) with potent *in vitro* and *in vivo* activity along with oral pharmacokinetic and pharmacodynamic studies. Additionally, we present a facile easily scalable method to

convert these BPQ-SNEDDS into solid nanomedicines for the treatment of VL. The proposed formulations are cost-effective, thermally stable and prepared from generally regarded as safe excipients (GRAS) that can enable their facile clinical translation, while they can be used for other poorly antiparasitic and chemotherapeutic drugs for the treatment of parasitic and infectious diseases.

Methods

Materials

Buparvaquone (>95% HPLC) was obtained from Toronto Research Chemicals (Toronto, Canada). Capryol 90 (propylene glycol monocaprylate 90%), Labrasol (Labrasol ALF, Caprylocaproyl macragol-8 glycerides), Labrafil M 1944 CS (Oleoylocapryoyl macragol-6 glycerides) were kindly provided by Gattefosse (Berkshire, UK) and soya phosphatidylcholine (Lipoid S100) by Lipoid GmbH (Rein, Germany). Pancreatin (porcine, P1625, ≥ 3 USP), bile extract (porcine) and all other chemicals, solvents, and acids, unless otherwise stated, were of ACS grade or above and were obtained from Sigma-Aldrich (Dorset, UK) and used without further purification. Cell culture media were bought from Sigma-Aldrich (Madrid, Spain).

Preparation of BPQ loaded SNEDDS (BPQ SNEDDS)

BPQ loaded SNEDDS (BPQ SNEDDS) were prepared by combining BPQ, Labrafil 1944CS (oleoyl macragol-6 glycerides), Capryol 90 (propylene glycol monocaprylate) and Labrasol (caprylocaproyl macragol-8 glycerides) (0.01:3:1:5.99 w/w) under stirring at 600 rpm. This ratio was identified using pseudo-ternary phase diagram studies constructed with varying concentrations of oily solubilisers (Labrafil M 1944 CS) and oil soluble surfactants (Labrasol) and co-surfactants (Capryol 90) and double-distilled water using the water titration method at room temperature²⁵ (Figure SI-1). The

samples were placed into a water bath at $37 \pm 0.5^{\circ}\text{C}$ for 24 hours under gentle shaking (Kotterman D1365, Hänigsen, Germany). Mixtures were then observed for turbidity when fresh and at 24 hours and particle size was measured when fresh and after 24 hours as described below. Turbidity is indicative of the formation of a coarse emulsion, whereas a clear isotropic solution would indicate a microemulsion (type II).

All formulations containing BPQ were protected from light. BPQ SNEDDS (10, 20 and 50 mg mL^{-1}) were prepared in triplicate as described above to determine the maximum BPQ loading within the SNEDDS. After 24h, the samples were taken out from the waterbath and centrifuged at 10,000 rpm for 10 minutes (Jouan B4i centrifuge, Thermo Scientific, USA) and the supernatant was isolated and analysed using HPLC (after a 1 in 250 dilution with methanol) as described below.

Photon Correlation Spectroscopy (particle size & zeta potential measurements)

SNEDDS were diluted with water at 1 in 200 v/v and 1 in 100 v/v (pH 6.5) prior to particle size and zeta-potential measurements respectively (Malvern Zetasizer Nano-Zs, Malvern Instruments, Worcestershire, UK) at 25°C and at a wavelength of 633 nm. The data were analysed using the *Contin* method of data analysis. Diluted suspensions were left for 15 minutes at room temperature (25°C) before particle size was measured. The accuracy of the instrument was assessed periodically using a drop of latex beads (polystyrene, mean size: $0.1 \mu\text{m}$) in 50 mM sodium chloride (dispersed phase). All measurements ($n=13$) were performed in triplicate and the mean ($\pm\text{SD}$) was reported.

Zeta potential (Malvern Nano-Zs, Malvern Instruments, UK) was measured for the diluted formulations utilizing the Doppler electrophoresis technique. Analysis of the Doppler shift (Fourier transformed) was done by using a mixed-mode measurement phase analysis light scattering (M3-Pals). The viscosity of the sample was

hypothesized to be the viscosity of water at 25°C. All measurements (n=100) were performed in triplicate and the mean ($\pm$ SD) was reported.

HPLC analysis

A modified reverse phase HPLC (RP-HPLC-UV) method based on the optimized and validated method published by Venkatesh et al ³² was used. A Phenomenex Onyx Monolithic C18 column (10+100mm x 4.6mm, 5 μ m) was maintained at 30°C (Varian Prostar column heater) for analysis and the analytical wavelength was set to 251 nm and 342 nm (Varian Prostar, Agilent technologies Inc, USA). The mobile phase consisted of 1% acetic acid in water, acetonitrile and methanol in the ratio 30:60:10 (v/v/v) and was filtered (Millipore nylon filter 0.45 μ m) and degassed using bath sonication (175W, Jencons scientific Ltd, Bedfordshire, UK). The injection volume was set at 40 μ L per sample using Varian Prostar autosampler (model 410, Agilent technologies Inc, USA) and a flow rate of 1.5 mL min⁻¹ was achieved using a Varian Prostar solvent delivery module (Agilent technologies Inc, USA). The retention time was 5.54 minutes for BPQ and the lowest detection limit was set at 0.05 μ g mL⁻¹. A linear calibration curve in a range of 0.05 to 100 μ g mL⁻¹ was used for extrapolation of the concentrations.

Flow-through cell dissolution studies for BPQ SNEDDS

The dissolution of BPQ from BPQ-SNEDDs (10 mg mL⁻¹, 0.3mL) loaded capsules (NP CapsTM, pullulan, size 2, Capsugel) was studied in the flow through cell (USP, Apparatus IV) at various pH levels [USP 2013 simulated gastric fluid without enzyme (1.2), acetate buffer (4.5) and phosphate buffer (6.8), flow rate 6 mL min⁻¹] as previously described ¹⁴.

***In vitro* lipolysis**

In vitro lipolysis experiments were performed as previously described ³³. The lipolysis medium contained bile salts (5 mM) and phosphatidylcholine (1 mM) which simulate fasted conditions in the gastrointestinal tract (GIT), while the digestion buffer (300 mL) was composed of sodium chloride (150 mM) and Trizma maleate (2 mM) at pH 6.5 ± 0.05 ³³. A low buffer concentration was chosen to ensure that ionized fatty acids are able to change pH, while a buffer with low capacity was selected to allow the released fatty acids (FA) to reduce the pH and thus trigger the addition of sodium hydroxide ³³. The lipase suspension was prepared by suspending pancreatin (16.6 g) in 110 mL of Millipore water at 37°C and thoroughly mixing prior centrifugation (4,000 rpm, 7 minutes at 37°C). The pH of the supernatant was adjusted to 6.5 using 1 M sodium hydroxide and 100 mL was used to initiate the lipolysis. To avoid denaturation, preparation of the pancreatin suspension never exceeded 15 minutes. For the *in vitro* digestion study, the lipolysis medium (300 mL) were pre-warmed at 37°C, prior to the addition of BPQ loaded SNEDDS (3g) and adjusting the pH to 6.5 ± 0.01 with 1 M sodium hydroxide. Lipolysis was initiated by adding the 100 mL of the freshly prepared lipase suspension. Ca²⁺ solution (0.5 M) was added at a dispensing rate of 0.045 mM (90 µL) per min (NE-1000 Programmable single syringe pumps, World Precision Instruments Ltd., Hertfordshire, UK). Throughout the study, the pH was kept constant at 6.5 ± 0.01 (Accumet AB200 equipped with an accuTupH rugged bulb, Fisher Scientific, Loughborough, UK) by addition of 1 M sodium hydroxide. In this respect, the number of OH⁻ ions present in the volume of the titrant can be equated with the FA liberation caused by lipolysis. Samples (30 mL) were withdrawn at time 0, 5, 15, 30, 60, and 90 minutes after the addition of the lipase suspension. Samples were frozen using dry ice prior ultracentrifugation at 40,000 rpm at 4°C (CP100NX, Hitachi Koki Co. Ltd, Milton Keynes, UK). The amount of BPQ present in the supernatant and in

the pellet was quantified after their appropriate dilution with methanol using HPLC as described above.

Stability studies of BPQ SNEDDS

Blank SNEDDS and BPQ SNEDDS (10mg mL⁻¹, 0.5 mL, n=3) were divided into amber HPLC vials and stored under 75% relative humidity in amber dessicant jars containing a saturated sodium chloride solution (20 g of sodium chloride in 10 mL of de-ionised water) at 40°C (Raven Incubator, LTE Scientific, Oldham, UK). Samples (10 µL) were diluted with water (1 in 200 v/v, pH 6.5) and analysed for particle size and zeta-potential, while samples (10 µg) were diluted with methanol and analysed by HPLC as described above at weeks 0, 2, 3, 4, 6, 10, 12, 16, 20, 24. Morphology of the diluted aqueous dispersions of the stability samples was assessed by transmission electron microscopy at week 0 and week 6 as described below.

Preparation of BPQ loaded solid SNEDDS (BPQ solid SNEDDS)

Low molecular weight glycol chitosan polymers were produced from 2 g of glycol chitosan that were added to freshly prepared 4M hydrochloric acid (152 mL), and stirred (500 rpm, IKA Werke RTC basic, IKA laboratories, Staufen, Germany) until homogenous (25 min) as previously described³⁴. The solution was then placed in a 50°C waterbath under vigorous shaking (50 rpm) for a total of 24 hours (Kotterman D1365, Hänigsen, Germany). The polymer solution was dialysed with six changes (5 L) of de-ionised water over 24 hours (Dialysis membrane Molecular weight cut-off for Cytochrome C: 12-14 kDa, Medicell Ltd, London, UK). The recovered degraded glycol chitosan (GC 14 kDa) was snap-frozen using liquid nitrogen and lyophilised (Edwards Modulyo freeze drier [Crawley, UK] connected to CIT Alcatel vacuum pump [Pfeiffer, Asslar, Germany]) over 72 hours at -50°C.

Blank SNEDDS (3mL) and BPQ SNEDDS (3mL, 10 mg mL⁻¹) were adsorbed onto 400 mg of degraded glycol chitosan (14 kDa). Croscarmellose sodium (295 mg) was added followed by the addition of lactose BP (5.2 g, BP: British Pharmacopoeia) and mixed in a mortar and pestle. The contents of the mortar were transferred into a round-bottom flask and 60-70mls of de-ionised water were added and stirred until a homogenous viscous yellow suspension was prepared. The resulting suspension was snap-frozen with liquid nitrogen and lyophilised (Edwards Modulyo freeze drier [Crawley, UK] connected to CIT Alcatel vacuum pump [Pfeiffer, Asstar, Germany]) over 24 hours at -50°C. The resulting lyophilised product (15mg) was diluted with 15 mL methanol, vortexed for 1 minute (Topmix vortex, Fischer Scientific, Leicestershire, UK), and centrifuged at 10,000 rpm (Jouan B4i centrifuge, Thermo Scientific, USA) for 10 minutes before the supernatant was analysed using HPLC for drug loading as described below. The solid powders were also characterised using DSC, TGA, PXRD, FT-IR, SEM, and particle size as described below. Physical mixtures of lyophilised BPQ and blank solid SNEDDS (1:10 and 1:100 w/w) were also analysed via DSC and PXRD.

Morphological characterisation

A drop of the diluted SNEDDS or Solid SNEDDS (1 in 200 v/v or 1 in 200 w/v) was placed on Formvar[®]/Carbon Coated Grid (F196/100 3.05 mm, Mesh 300, Tab Labs Ltd, England) and stained with 1% aqueous Uranyl Acetate before TEM analysis as previously described (FEI CM120 BioTwin Transmission Electron Microscope) ³⁵.

Dry powders left to air dry was mounted on a glass slide that was mounted on a standard SEM sample holder and fixed on a brass/aluminium stub using double sided carbon impregnated adhesive discs. The sample was then sputtered coated with a conducting gold-palladium (10 nm, 60% gold-palladium) coating using a SEM coating

system for 2 minutes at 30 mA (Quorum Q 15ORES, Quorum technologies Ltd, Lewes, UK, Deposition range: 0-80 mA, Deposition rate: 0- 25 nm min⁻¹, Sputter timer: 0-60 min, Vacuum Pump: 50 L min⁻¹, room temperature) before being viewed and photographed under a range of magnifications under high vacuum using JEOL JSM-6060LV (Jeol, Welwyn Garden City, UK) scanning electron microscope.

Powder X-Ray Diffraction (PXRD)

PXRD analysis was performed with a Rigaku MiniflexII desktop X-ray diffractometer (Rigaku, Tokyo, Japan) attached to a Haskris cooling unit (Grove Village, IL, USA). The PXRD patterns were recorded from 5° to 40° on the 2 theta at a step scan rate of 0.05° per second as previously described (n=3)³⁶. X-ray powder patterns of single crystal structures were calculated using Mercury® (version 3.9., Cambridge Crystallographic Data Centre, Cambridge, UK) and Miller's indices of various facets of the crystal were identified (SI-3)³⁷. Mercury® was also used to predict the Bravais, Friedel, Donnay and Harker (BFDH) morphology to provide an insight into the molecular arrangement of different crystal facets of BPQ crystals (SI-3)³⁷.

Thermal analysis

Differential Scanning Calorimetry (DSC) experiments were performed using a Mettler Toledo DSC 821e (Greifensee, Switzerland) with a refrigerated cooling system (LabPlant RP-100, Filey, UK) as previously described³⁶ with nitrogen as the purge gas. Aluminum sample holders (40 µL) were sealed with a lid and pierced to provide three vent holes. Sample weight (3-6 mg) was sufficient to provide proper contact between the powder and the bottom of the pan. DSC measurements were carried out at a heating/cooling rate of 10°C min⁻¹. The unit was calibrated with indium standards. The DSC system was controlled by Mettler Toledo STARe software (version 6.10)

working on a Windows NT operating system. Temperatures of melting and crystallisation events refer to onset temperatures. Details for thermogravimetric analysis (TGA) is provided in supplementary information (SI-4).

Fourier-Transformed Infra-red (FT-IR) spectroscopy

The infrared absorption spectra were obtained using a PerkinElmer Spectrum 1 FTIR Spectrometer (PerkinElmer, Massachusetts, USA) equipped with a Universal Attenuated Total Reflectance (UATR) accessory and a diamond/zinc selenide (4000 to 650 cm⁻¹) crystal accessory ³⁶. Baseline correction and data normalization were performed using Spekwin32 version 1.71.6.1 (maximum resolution is 1 cm⁻¹).

Hausner ratio and compressibility index of BPQ solid SNEDDS

BPQ solid SNEDDS (1 g) was placed in a 5 mL measuring cylinder and the volume was recorded. Tapped density was calculated by tapping cylinder into a hard surface 10, 20, 40, 60, 80, 100, 120, and 150 times, recording the volume after each set of taps. The Hausner ratio and compressibility index were determined as described by the equations below:

$$\text{Compressibility Index} = 100 \frac{V_o - V_f}{V_o} \quad (\text{Equation 1})$$

$$\text{Hausner Ratio} = \frac{V_o}{V_f} \quad (\text{Equation 2})$$

where V_o is the unsettled apparent volume and V_f is the final tapped volume.

Preparation and characterisation of BPQ solid SNEDDS tablets

Lyophilised BPQ solid SNEDDS (300 mg) or blank solid SNEDDS (300 mg) were compressed for 10 seconds under 1 bar of pressure using a flat-faced 12mm die (Specac manually operated hydraulic press, Analytical accessories Ltd, Kent, UK).

Disintegration was tested as specified in the British Pharmacopoeia 2018 ³⁸. Tablets were placed in a basket rack assembly, and raised/lowered 30 times per minute at a distance of 55 ± 2 mm in 1L of water heated to $37.0 \pm 0.5^\circ\text{C}$ (Erweka disintegration tester ZT500 series, Erweka GmbH, Heusenstamm, Germany).

Friability studies were conducted using 10 BPQ solid SNEDDS tablets (300 mg) according to British Pharmacopoeia 2018 method for uncoated tablets. Friability was expressed as the percentage of weight loss that was calculated by weighing the tablets after 100 rotations of the drum in 4 min. Tablets were dusted and accurately weighted. Six BPQ solid SNEDDS tablets (300 mg) were crushed using a mortar and pestle and the powder was extracted with 50 mL of methanol, filtered using Whatman No 1 filter paper and the filtrate was made up to 100 mL with methanol in a volumetric flask. The concentration in the final solution was measured using HPLC as described above to identify the uniformity of content. The dissolution of six BPQ solid SNEDDS tablets (300 mg) were tested using the basket method (Appendix I, BP 2018) using a GB Caleva Ltd Ascott model 657 dissolution apparatus, with a Tecam TE7 Tempette heating system. Tablets were immersed in 500 mL of pH 1.2 ± 0.1 for the first hour, after which 400 mL of pH 6.8 buffer were added and the final pH was adjusted to 6.8 using 5 M sodium hydroxide (between 4 - 4.5 mL). The first media was prepared by mixing sodium chloride (2 M, 250 mL) and hydrochloric acid (2 M, 425 mL) and making them up to 1L with deionised water (pH 1.2). The second media contained 250 mL potassium dihydrogen phosphate (2 M, 250 mL) and sodium hydroxide (2 M, 112 mL) that were made up to 1L with deionised water (pH 6.8). Samples (5 mL) were removed at 2, 4, 8, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 145, 150, 165 and 180 minutes and replaced with appropriate volume of the required buffer. Samples were then diluted 1:1 with methanol, vortexed for 30 seconds (Topmix vortex, Fischer

Scientific, Leicestershire, UK), centrifuged at 10,000 rpm for 10 minutes (Jouan B4i centrifuge, Thermo Scientific, USA) before BPQ concentration was quantified by HPLC.

***In vitro* leishmanicidal assays**

L. Infantum (MCAN/ES/92/BCN83) obtained from an asymptomatic dog from the Priorat region (Catalonia, Spain) were kindly donated by Prof Montserrat Portus (Universitat de Barcelona)³⁹. Promastigotes were cultured in Schneider's Insect Medium at 26 °C supplemented with 20% heat-inactivated foetal bovine serum (FBS) and 100 U mL⁻¹ of penicillin plus 100 µg mL⁻¹ of streptomycin ³⁹.

Promastigote drug susceptibility was determined by using the sensitive fluorimetric resazurin model (Sigma-Aldrich®) as previously described by our group ⁴⁰. Briefly, cultured log phase promastigotes (2.5 x10⁵ parasites per well) were plated in 96-well flat-bottomed microtiter plates (200 µL/well final volume), in the presence of different concentrations of the studied drug or formulations. Finally, the relative fluorescence units (Excitation: 535 nm, Emission: 590 nm) was measured (Infinite 200, Tecan i-Control, Männedorf, Switzerland) to determine the growth inhibition rate and then the IC₅₀ calculated by Probit analysis and the statistic parameters were computed using SPSS Statistics v17.0 Software.

Amastigote assays were performed using a fluorimetric assay based on resazurin validated by our group ⁴¹. Macrophages were plated (5 x 10⁴ cells/well cultured in RPMI medium) in a microtiter plates with a flat bottom. Macrophages were then infected with 5 x 10⁵ promastigotes. Plates were incubated at 33°C for 1 day and then at 37°C for another day. After incubation, the culture medium was removed and washed with buffered RPMI-HEPES (pH 7.2) to eliminate any non-internalised

parasites, after which tempered media RPMI (100 μ L) was added to each well along with another 100 μ L of media containing different concentration of drugs or formulations were added. Stock solutions for Miltefosine and BPQ (20 mg mL⁻¹) were prepared initially in DMSO and then diluted to 40 μ g mL⁻¹ with RPMI-HEPES media (pH 6.7), while blank or BPQ SNEDDS and blank or BPQ solid SNEDDS were diluted with RPMI-HEPES media (pH 6.7) at a final concentration of 40 μ g mL⁻¹. Further dilutions of the formulations using the same media were performed and were added up to a final volume of 200 μ L resulting in a final concentration of 40, 20, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 μ g mL⁻¹ per well. The final solvent (DMSO) concentrations never exceeded 0.5% (v/v) warranting no effect on parasites proliferation or morphology ⁴¹. Sixteen wells were used as positive untreated control and eight wells as negative uninfected control were adjusted also to a final volume of 200 μ L with fresh RPMI media and incubated at 37 °C for 48 hours. The culture medium at different drug concentrations was removed by centrifugation at 3,500 rpm for 5 minutes (Eppendorf 5403 Centrifuge, Eppendorf Iberica, Madrid, Spain) and replaced with lysis solution (0.02% sodium dodecyl sulphate in RPMI-HEPES buffer). After 20 minutes, the lysis solution was removed by centrifugation (3,500 rpm, 5 minutes, 4°C). The supernatants were replaced by fresh Schneider's culture media (200 μ L). The plates were then incubated at 26°C for another 3 days to allow the transformation of viable amastigotes into promastigotes and their proliferation. Finally, resazurin salt (2.5mM, 20 μ L/well) were added followed by a 3 hour incubation at room temperature and relative fluorescence units were measured as described below. All *in vitro* experiments were carried out for three times and by triplicate. The IC₅₀ was calculated by Probit analysis and the statistic parameters were computed using SPSS Statistics v17.0 Software.

Cytotoxicity studies on murine macrophages

J774 murine macrophages were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (30 min at 56 °C), penicillin G (100 U mL⁻¹) and streptomycin (100 µg mL⁻¹) as previously described by our group ^{40,53}. Cells in the pre-confluence phase were harvested with 0.03% EDTA-0.05% Trypsin in PBS for 20 minutes and maintained at 37 °C in a humidified environment with 5% CO₂ (Hucoa-Erlöss, Forma Scientific, Ohio, USA). Cytotoxicity studies were conducted using a resazurin assay as described previously ⁴² by determining the reduction of resazurin by measuring the Relative Fluorescence Units (RFU, 535 nm excitation wavelength and 590 nm emission wavelength, Infinite 200, Tecan i-Control, Männedorf, Switzerland) as in the promastigotes assay. Growth inhibition (%) was calculated using Equation 3:

$$\text{Growth inhibition (\%)} = 100 - \frac{\text{RFU treated wells} - \text{RFU signal to noise}}{\text{RFU untreated} - \text{RFU signal to noise}} \times 100 \quad (\text{Equation 3})$$

Every concentration was assayed three times and controls (medium and drug) were used in each test as blanks and the background was subtracted. Cytotoxicity of the tested drugs/formulations was defined as the 50% reduction of cell viability of treated culture cells with respect to untreated cells (CC₅₀). The selectivity index (SI) was calculated as expressed in Equation 4 ⁴²:

$$\text{SI} = \frac{\text{CC}_{50}}{\text{IC}_{50}} \quad (\text{Equation 4})$$

In vivo pharmacokinetic studies of oral BPQ SNEDDS

All experiments were approved and performed in accordance with the local ethics committee rules (Complutense University of Madrid Institutional Animal Care and Ethics Committee, Madrid, Spain). Male CD-1 outbred mice (6 weeks old) were

purchased from Harlan Iberica S.A. (Barcelona, Spain) and were housed in groups of 5 in plastic cages in controlled laboratory conditions with ambient temperature and humidity maintained at ~22 °C and 60% respectively with a 12 h light and dark cycle (lights on at 07:00 and off at 19:00). Food and water were available ad libitum and the animals acclimatized for 5–7 days prior to experiments, within the animal house.

CD-1 mice were randomly split into groups ($n = 3$), fasted overnight and dosed with BPQ aqueous suspensions prepared by probe sonication (50% output, 200 watt for 10 minutes) (11 mg mL^{-1}) or BPQ SNEDDS (11 mg mL^{-1}) at a BPQ dose of 6 mg kg^{-1} were administered (as liquid suspensions via oral gavage). Mice were sacrificed at different time points (1, 2, 4, 7, and 24 h), and blood was harvested. Plasma was separated by centrifugation (2000 rpm, 15 min, 4 °C, Hermle Z323 K centrifuge, VWR, Poole, U.K.), and all tissues were stored at -50 °C until analyses could be performed on them.

Plasma samples (100 μL) were extracted three times with acetonitrile (100 μL). After every extraction the mixture was vortexed (Fisher TopMix Vortex Mixer FB15024, Leicestershire, England) for 5 minutes and the samples were then centrifuged (8,000 rpm for 10 minutes) (SciSpin Micro Centrifuge, Shropshire, England). The supernatants were pooled and evaporated to dryness in a concentrator (Savant Integrated SpeedVac System ISS110, Fisher Scientific, Loughborough, UK). Extracted plasma calibration curves were constructed by spiking (5 μL) of BPQ stocks in DMSO in CD-1 blank plasma to produce a range of $0.1\text{--}50 \text{ }\mu\text{g mL}^{-1}$ and were extracted and dried in a similar manner to *in vivo* plasma samples. Extracted standards and samples were reconstituted with mobile phase (100 μL) and were analysed in the HPLC as described above.

Efficacy in a systemic murine model of Visceral Leishmaniasis

All experiments were approved and performed in accordance with local ethics committee rules (Complutense University of Madrid Institutional Animal Care and Ethics Committee, Madrid, Spain). BALB/c male mice (20–25 g, 6-7 weeks) were purchased from Harlan Iberica S.A. (Barcelona, Spain) and were housed in groups of 5 in plastic cages under controlled laboratory conditions with ambient temperature and humidity maintained at ~22 °C and 60% respectively with a 12 h light and dark cycle (lights on at 07:00 and off at 19:00). Food and water were available ad libitum and the animals acclimatized for 5–7 days prior to experiments, within the animal house.

The preparation of the parasites and the experimental infection were performed as previously described¹⁴. Each BALB/c mouse was infected with 10^7 promastigotes by intracardiac route using a syringe with 26 G needle.

Mice were randomly split into groups ($n = 8$). Treatment was started on day 21 post-infection. A negative control (distilled water) and positive control (miltefosine in distilled water 12mg kg^{-1})^{43, 44} were dosed by oral gavage once daily for 10 consecutive days. Oral BPQ SNEDDS and BPQ Solid SNEDDS (6 mg kg^{-1}) groups were also dosed once daily for 10 consecutive days by oral gavage. Formulations were dosed as liquid suspensions of SNEDDS or solid SNEDDS (powders prior being compressed into tablets) via oral gavage. The final group received a BPQ in DMSO intramuscular dose (25 mg kg^{-1} , $50\mu\text{L}$) in the hind leg at day 21 and 26. Animals were killed on day 35 post-infection. Spleen and liver from each animal were aseptically removed and weighted to quantify the parasite burdens. The parasite burden was quantified by the limit dilution assay as previously described¹⁴ and the percentage suppression of parasite replication (PS) was calculated using the following equation

$$PS = 100 \frac{(PC-PT)}{PC} \quad \text{(Equation 5)}$$

where PC is the number of parasites in the control group per tissue weight (g) and PT is the number of parasites after treatment per tissue weight (g) ⁴⁵.

Statistical Analysis

Microsoft Office Excel 2015 was used to analyse all data and graphs were plotted using Microcal Origin 6.0 or GraphPad Prism 7.0. Student t-test or one-way analysis of variance (ANOVA) with Tukey's post-hoc test were performed at a 5% level of significance using IBM SPSS Statistics 21.

Results

The optimal microemulsion region was achieved for SNEDDS prepared with BPQ: Labrafil C1944CS: Labrasol: Capryol 90 of 0.01:3:1:5.99 w/w. Drug loading studies with exceeding BPQ amounts (10 mg g⁻¹, 20 mg g⁻¹ and 50 mg g⁻¹) demonstrated that the maximum loading was 16.7 ± 2.7 mg g⁻¹, which is comparable to previous published reports of high loading for BPQ (Figure 1A) ²⁵. Dispersions of BPQ SNEDDS illustrated a quasispherical particle size well below 300 nm (206 ± 15 nm) with low polydispersity (0.316 ± 0.014) and colloidal stability (-19.2 ± 0.6 mV) (Figure 1B-E). BPQ content, ζ-potential and particle size remained unaltered over 6 months stored at 40 ± 2°C and 75 ± 5% RH (One Way Anova, p<0.05) (Figure SI-2, 1F).

Near complete release (89.3±1.9%) was observed in flow-through cell studies (BP Apparatus IV) with BPQ SNEDDs filled immediate release pullulan capsules within 30 minutes (pH1.2) (Figure 2A). BPQ remained solubilised and available for absorption within all different pH media tested and complete release was achieved.

The *in vitro* dynamic lipid digestion model offers information regarding drug partitioning during hydrolysis of triacylglycerides and quantifying the amount of solubilised drug in the intestinal fluids. Lipolysis was initiated by the addition of the lipase suspension after which the lipolytic process was monitored as a function of time. Calcium chloride solution was added continuously to control the accumulation of fatty acids in the medium by forming insoluble calcium FA soaps, which precipitate thus removing FA from the system and preventing accumulation^{33, 46}. At 30 minutes, 70% of BPQ remained solubilised and un-precipitated, and thus available for absorption, while more than 10% (10.4+/-1.25%) was available for absorption even after 90 minutes, which is almost double previous reports available in literature for orally bioavailable self-emulsifying drug delivery systems (Figure 2B) ⁴⁷.

Solid SNEDDS were prepared by adsorption of BPQ loaded SNEDDS on low molecular glycol chitosan polymers that were suspended in water and lyophilised to yield BPQ loaded solid SNEDDS. (BPQ solid SNEDDS). PXRD studies illustrated that BPQ exhibits Bragg peaks characteristic of crystalline material even after lyophilisation (Figure 3A, 3B) that are characterised by a strong endothermic peak at 183°C corresponding to the melting point of the drug in DSC studies (Figure 4A-a, 4A-b). BPQ's PXRD spectra is similar to previous reported values ⁴⁸ but the difference in Bragg peak intensities is indicative of narrower BPQ plate crystals [Plane 001 (2θ : 6.55) and 002 (2θ : 12.95), Figure 3 and SI-3]. Attempts to amorphise BPQ by melt

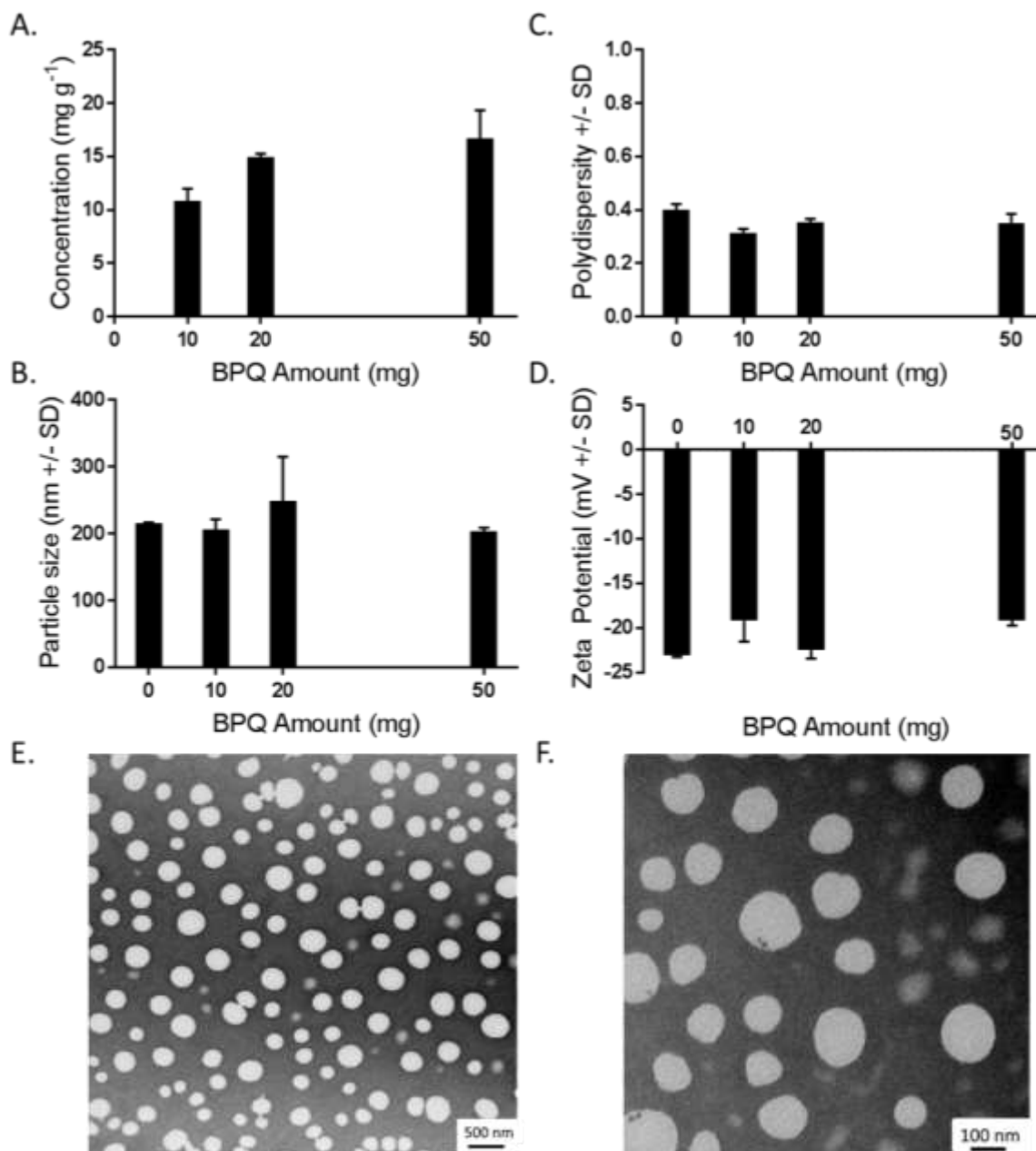


Figure 1. BPQ SNEDDS characteristics; A: Drug Entrapment Studies, B-D: Particle size, polydispersity and zeta-potential of blank and BPQ SNEDDS (n=3) respectively, E-F: TEM of BPQ SNEDDS (1:200 v/v aqueous dilutions) fresh and after 6 weeks storage at 40°C and 75% relative humidity (Bar: 500 and 100 nm respectively).

quenching technique (heating up to 200°C and fast cooling by immersion in liquid nitrogen) was not successful with characteristic BPQ Bragg peaks diminished in intensity but not abolished (Figure 3C). BPQ solid SNEDDS resulted in fewer Bragg peaks which were broader and of lower intensity which are related to other excipients (such as lactose and croscarmellose sodium) (Figure 3D-H). The absence of the

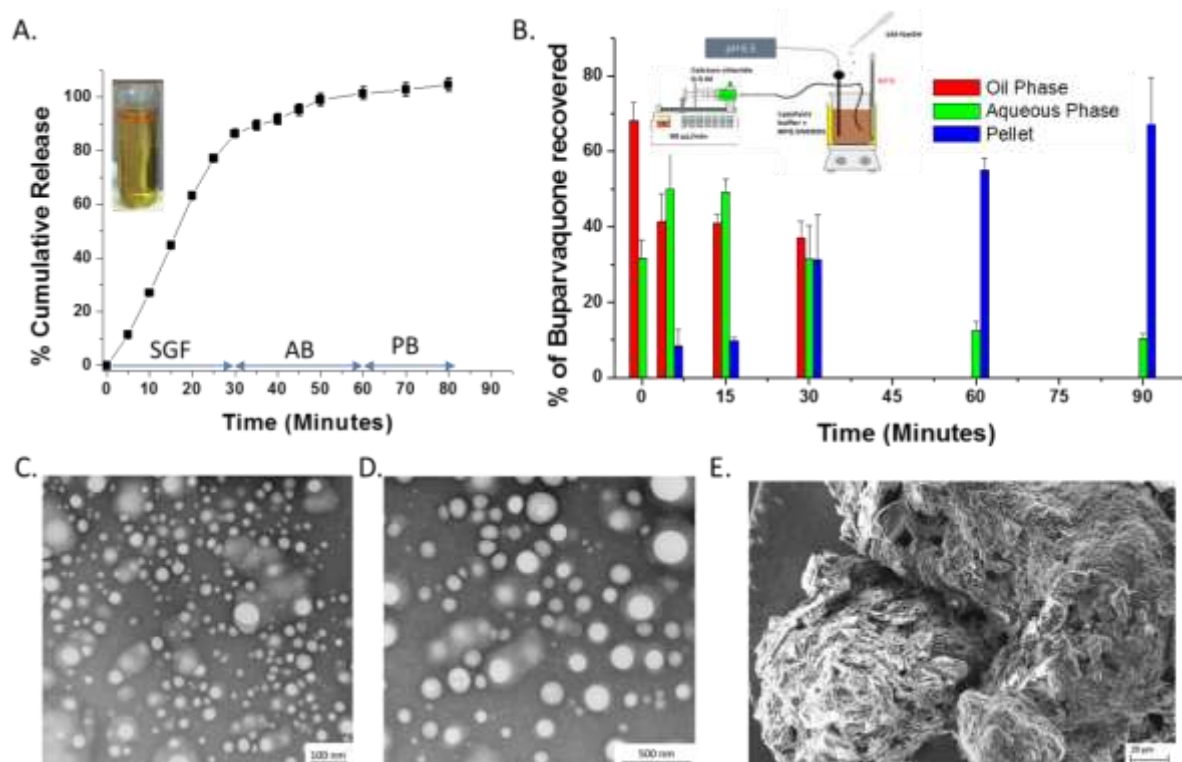


Figure 2. A. Cumulative BPQ release from BPQ SNEDDs pullulan capsules in flow-through cell studies (Simulated Gastric Fluid (SGF) pH 1.2: 0-30min, Acetate Buffer (AB) pH 4.5: 30-60 min, Phosphate Buffer (PB) pH 6.8: 60-80 min) (n=3), B: *In vitro* lipolysis of BPQ – SNEDDs with red, green and blue bars illustrating the % of BPQ recovered in the oil phase, aqueous supernatant and pellet respectively (n=3). C-D: TEM images of BPQ solid SNEDDs aqueous dispersions (1mg mL⁻¹, Bar: 100nm and 500 nm respectively). E: SEM images of BPQ solid SNEDDS (Magnification: 500x, 5.00 KV, Bar 20 μ m).

characteristic peaks of BPQ (2θ : 6.55 and 12.95) points towards the amorphisation of BPQ (Figure 3H). Additionally, DSC analysis of BPQ solid SNEDDS illustrated no peak at 183°C, BPQ's characteristic melting endotherm, suggesting also complete amorphisation of BPQ (Figure 4A, Table SI-1). This is further supported by studies of physical mixtures of lyophilised BPQ and blank SNEDDS adsorbed on low molecular weight glycol chitosan (1:10 w/w), which indicated a peak at 183°C and the characteristic BPQ Bragg peaks of crystalline BPQ (Figure 3I, 3J and 4H).

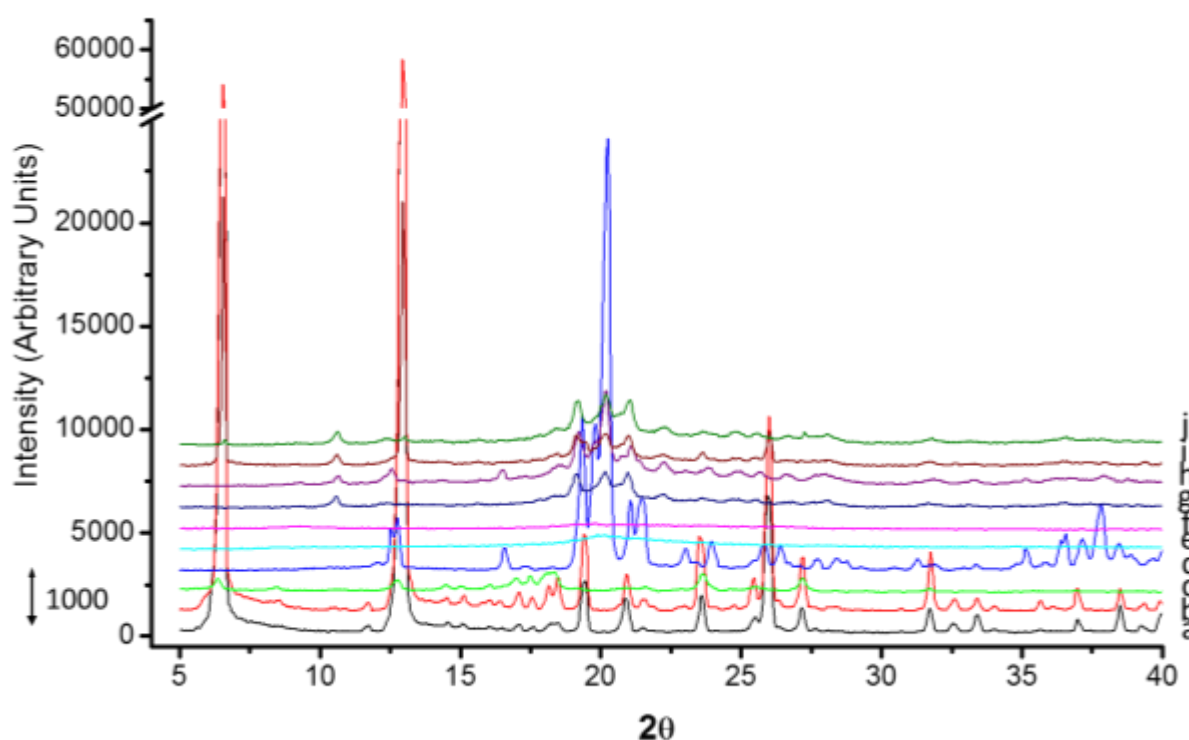


Figure 3. PXRD analysis of prepared blank and BPQ solid SNEDDS along with physical mixtures of BPQ with blank solid SNEDDS; BPQ (a), BPQ lyophilised (b), BPQ amorphised (c), Lactose (d), Croscarmellose Sodium (e), Glycol chitosan (14 – 24 kDa) (f), Blank solid SNEDDS (g), BPQ solid SNEDDS (h), Physical mixture of crystalline BPQ with blank solid SNEDDS (1:10 w/w) (i), Physical mixture of crystalline BPQ with blank solid SNEDDS (1:100 w/w) (j).

FT-IR analysis illustrated that BPQ exhibits two characteristics peaks, a sharp peak at 3300 cm^{-1} attributed to intramolecular hydrogen bonds between the hydroxyl group of BPQ and a peak at 1694 cm^{-1} belonging to the quinine carbonyl group (aromatic group with 2 double bonded oxygens) (Figure 4B). Physical mixtures of BPQ and blank SNEDDS adsorbed on low molecular weight glycol chitosan shows a peak at 1639 cm^{-1} indicative of crystalline BPQ. However, the absence of these peaks for BPQ solid SNEDDS points towards the formation of a solid dispersion (Figure 4B), due to hydrogen bonding between hydroxyl groups of glycol chitosan with the quinine carbonyl groups of BPQ and can further explain the enhanced oral bioavailability of BPQ ^{26, 27}.

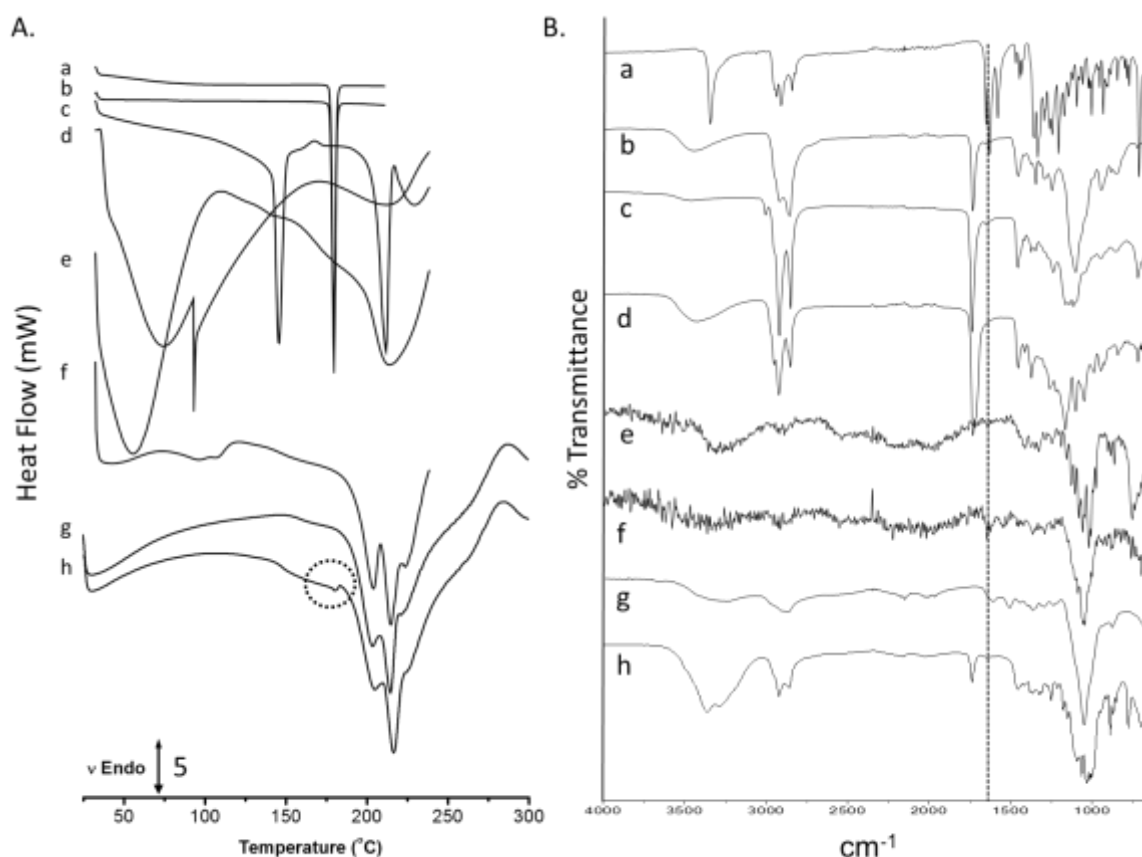


Figure 4. A; DSC thermographs of BPQ (a), BPQ lyophilised (b), Lactose (c), Croscarmellose sodium (d), Glycol chitosan (GC 14-24kDa) (e), BPQ solid SNEDDS (f), Blank solid SNEDDS (g), Physical mixture of BPQ (10% w/w) and blank solid SNEDDS (h). B; FT-IR spectra of BPQ (a), Labrasol (b), Labrafil 1944 CS (c), Capryol 90 (d), Lactose (e), Glycol chitosan (GC 14-24 kDa) (f), BPQ solid SNEDDS (GC 14-24kDa) (g), Physical mixture of BPQ 10% w/w and blank solid SNEDDS (GC14) (h). Vertical dotted line is drawn at 1639 cm⁻¹.

Adsorption of SNEDDs onto low molecular weight glycol chitosan (GC) polymers resulted in the formation of porous solids. (Figure 2E). Glycol chitosan is a biocompatible carbohydrate polymer with good solubility in both acidic and neutral pH. Apart from being known to act as a mucoadhesive and oral bioavailability enhancer^{35, 49, 50}, chitosans can be used as a disintegrant through swelling and formation of a hydrogel with increasing water content and decreasing hydrogel content from the core to the surface⁵¹. Formation of a porous matrix aids disintegration and can speed up the dissolution process. Upon reconstitution of the pre-tabletting lyophilised BPQ

SNEDDS adsorbed on glycol chitosan mix (BPQ solid SNEDDS), a nanosuspension was formed with a particle size similar to the original BPQ SNEDDS in terms of size, polydispersity and zeta potential (284 ± 4.6 nm, 0.372 ± 0.006 , -28.5 ± 0.611 mV). Thus, release of SNEDDS is possible after adsorption on solid carriers (Figure 2C, 2D).

BPQ SNEDDS adsorbed on low molecular weight glycol chitosan and croscarmellose sodium resulted in a pre-tabletting mix with passable flow characteristics (Hausner ratio and the compressibility index was calculated to be 1.34 ± 0.03 and $25.2 \pm 1.52\%$ respectively)⁵². Friability tests of BPQ solid SNEDDS tablets demonstrated losses of 0.89 ± 0.03 %, which are not greater than 1.0 per cent and are acceptable according to BP standards. BPQ solid SNEDDS tablets illustrated a uniformity of content of 87.6 ± 3.6 % which is on the lower end of BP limits (85-115%) with none of the six tested tablets being below 75%. Compressed tablets (275 ± 10.79 mg, 1 bar, 10 sec) were able to disintegrate completely in less than 8 minutes. Premixing the croscarmellose sodium with the glycol chitosan prior to adsorbing the drug loaded SNEDDS is critical in passing pharmacopoeia disintegration tests as simply adding it in the end resulted in times longer than 15 minutes. Additionally, the molecular weight of the carrier polymer (glycol chitosan) is important; using high molecular weight glycol chitosan polymers (~ 150 kDa) resulted in disintegration times well above 15 minutes irrespectively of the inclusion of a superdisintegrant and in the formation of viscous gels on the surface of the tablets that did not allow water penetration within the core of the tablet and the release of the SNEDDS from the solid carrier posing as a diffusion barrier (data not shown). Dissolution studies illustrated that $47.6 \pm 14.7\%$ BPQ was released within 60 minutes in simulated gastric fluids in the absence of pepsin (SGF) and complete release was achieved within 30 minutes after the pH was raised to 6.8

(Figure 5). Thus, the complete release of BPQ from solid dosage forms of BPQ nanomedicines and avoiding its precipitation in the absorptive part of the gastrointestinal tract are likely indicators of enhance oral bioavailability of BCS Class II drugs such as BPQ.

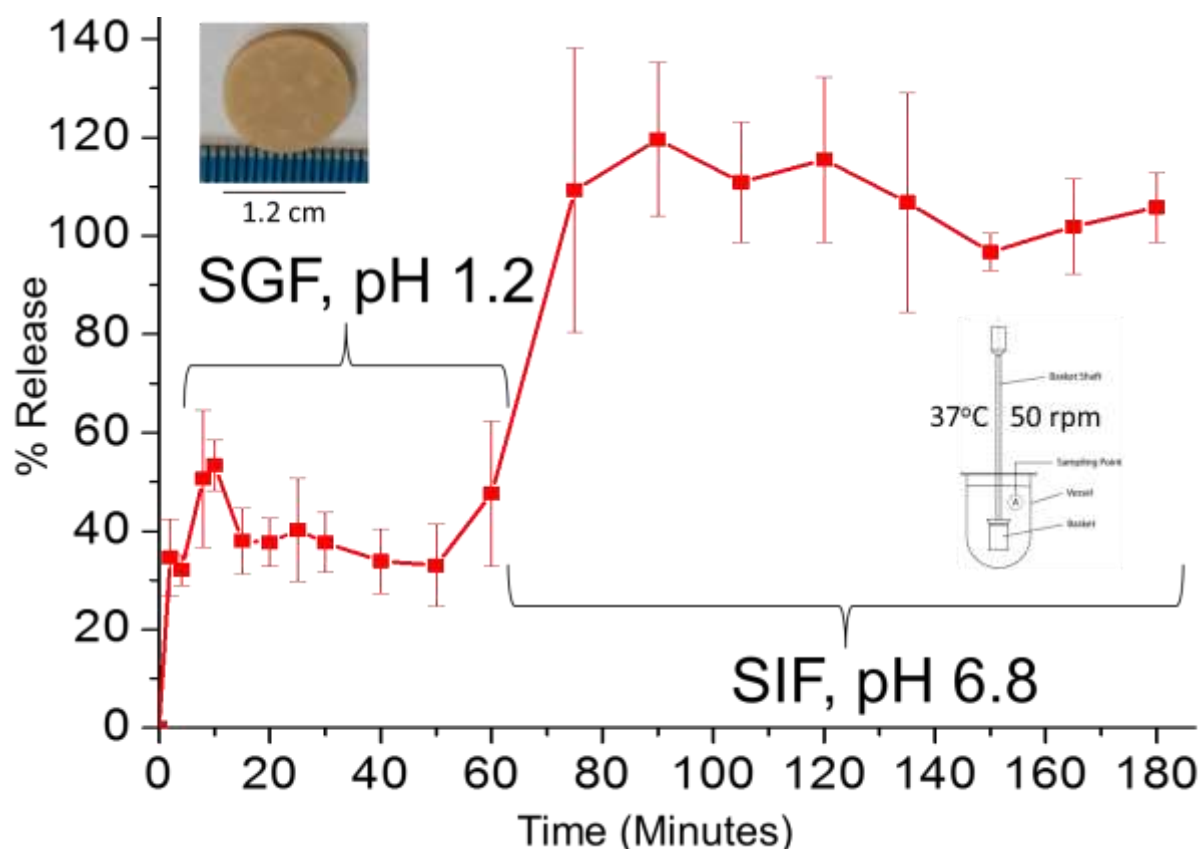


Figure 5. Dissolution of BPQ solid SNEDDS tablets in simulated gastrointestinal tract fluids (SGF: simulated gastric fluid with no pepsin, and SIF: simulated intestinal fluid with no pancreatin).

BPQ SNEDDS and BPQ solid SNEDDS showed potent *in vitro* efficacy in the nanomolar range against *L. Infantum* amastigotes similar to BPQ in DMSO (IC_{50} for all <37 nM), while having negligible cytotoxicity against murine macrophages (Table 1). The activity of the blank SNEDDS can be attributed to inclusion of Labrasol. We have recently reported that Labrasol (a mixed ester of capric and caprylic acid) possesses high antileishmanial activity in the micromolar range with acceptable cytotoxicity against murine macrophages⁵³. Labrafil on the other hand did not illustrate activity

against *Leishmania* parasites similarly to low molecular weight glycol chitosan polymers⁵³. Both BPQ and BPQ SNEDDS illustrated a high selectivity index that are well above DNDi (Drug for Neglected Diseases initiative) accepted hit selection levels (<10 μ M, SI>10)⁵⁴.

Table 1. *In vitro* activity in *L. Infantum* amastigotes

Drug/Formulation	<i>L. Infantum</i> Promastigotes (IC ₅₀ μ g mL ⁻¹)	SI	<i>L. Infantum</i> Amastigotes (IC ₅₀ μ g mL ⁻¹)	SI	J774 CC ₅₀ (μ g mL ⁻¹)
Buparvaquone (in DMSO)	<0.012	>2000	<0.005	5000	>25
Miltefosine (in DMSO)	7.19 $\pm$ 0.6	7.7	23.7 $\pm$ 1.78	2.3	55.4 $\pm$ 4.19
Blank SNEDDS	1.26 $\pm$ 0.10	14.2	9.5 $\pm$ 1.9	1.9	18.18 $\pm$ 1.04
BPQ SNEDDS	3.3 $\pm$ 0.09	7.6	0.093 $\pm$ 0.053	268.8	>25
BPQ Solid SNEDDS	<0.012	NS	<0.005	NS	NS
Key; J774: murine macrophages, NS: Not studied, SI: selectivity index (CC ₅₀ /IC ₅₀)					

Based on this encouraging *in vitro* results, we decided to conduct an *in vivo* pharmacokinetic study in healthy mice and efficacy studies in *L. infantum* infected mice. There are only a few published reports regarding the efficacy or the pharmacokinetic studies of BPQ administered orally. Early reports conducted in a BALB/c mouse model illustrated that oral treatment of BPQ formulated in 0.25% Cellacol (Methylcellulose) at a dose of 100 mg/kg/day over five days resulted in 43%

suppression of liver amastigotes ²⁴, while treatment of infected beagles with (5mg kg⁻¹ day⁻¹ for 5 days intramuscularly (IM)) failed to halt the progression of the disease⁵⁵. Pharmacokinetic parameters after oral administration have only been published in a recent study conducted in rabbits that received orally a dose of 3.0 mg kg⁻¹ of BPQ entrapped in SMEDDS prepared from Capryol 90: Cremophor EL: Labrasol 9.82:70.72:17.68 w/w) (Cmax: 102 ng ml⁻¹, Tmax: 3.17 hours, t_{1/2}: 26.44 hours, AUC_{0-60h}: 2.43 µg h ml⁻¹) ²⁵. We decided to utilise a dose of 6mg kg⁻¹ for a single dose oral pharmacokinetic studies in mice and to utilise this dose over 10 days for our efficacy studies according to the DNDi Target Product Profile for Visceral Leishmaniasis ²³. BPQ SNEDDS resulted in AUC_{0-24h} of 10.12 µg h ml⁻¹ and a Cmax of 0.639 µg mL⁻¹ which are at least four fold higher than previous published reports for self-emulsifying systems²⁵, while also significantly enhancing the bioavailability of BPQ compared to probe sonicated aqueous dispersions of BPQ after oral administration (55% increase in plasma AUC₀₋₂₄) (Figure 6). The latter is difficult to compare as the oral bioavailability of BPQ alone has not been previously published. Additionally, BPQ SNEDDS illustrated an enhanced half-life compared to BPQ probe sonicated aqueous suspensions (46.374 versus 14.415 hours). In a systemic murine model of visceral leishmaniasis, BPQ SNEDDS and BPQ solid SNEDDS (orally, 6 mg kg⁻¹ over 10 daily doses) and BPQ in DMSO (IM, 25 mg kg⁻¹, one dose on day 1 of treatment and one dose at day 5 of treatment) showed comparable efficacy to miltefosine (orally, 12 mg kg⁻¹ over 10 daily doses) in inhibiting parasite replication in the spleen at a dose that is shown to be effective in mice ⁴³ (Figure 7, SI-7). However, BPQ formulations were more variable in inhibiting parasite replication in the liver.

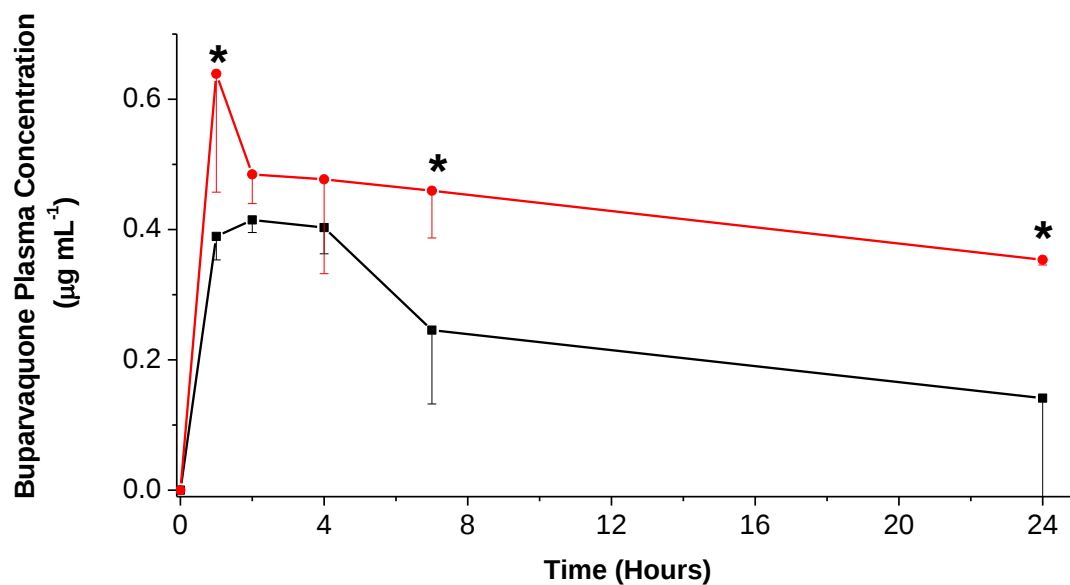


Figure 6. Buparvaquone (BPQ) plasma concentration after oral administration of BPQ (aqueous suspension – probe sonicated, black squares) and BPQ SNEDDS (red circles) formulations (11 mg g⁻¹) to CD-1 mice (n=3). Student t-test: * p<0.05; Dose: 6 mg kg⁻¹ of BPQ.

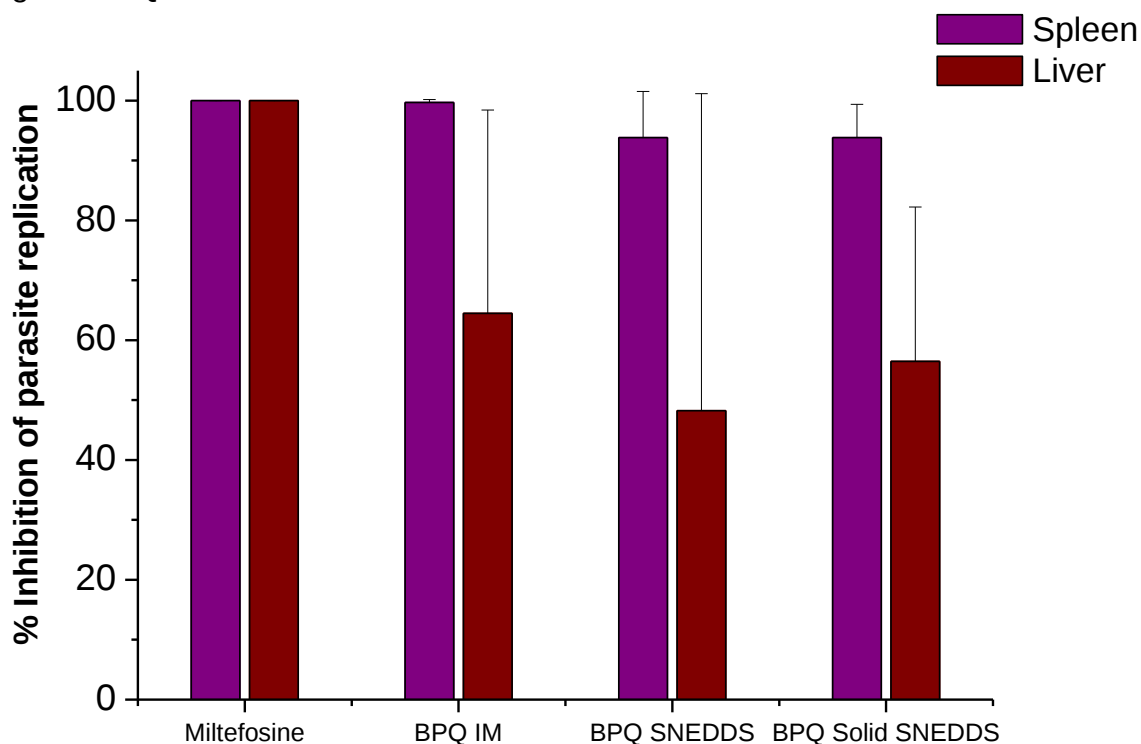


Figure 7. Inhibition of parasite replication after administration of oral miltefosine (12 mg kg⁻¹ day⁻¹, oral, 10 doses), BPQ intramuscularly (25 mg kg⁻¹, IM, 2 doses) and oral BPQ SNEDDS and BPQ solid SNEDDS (6 mg kg⁻¹ day⁻¹, oral, 10 doses) to BALB/c mice (n=6-8). Parasite burden in liver and spleen is described in SI-7.

Discussion

Napthoquinones and its derivatives such as BPQ are widely distributed in nature and are known for their antibacterial, antifungal, antiviral, antitumour and antiparasitic effects ⁵⁶. Napthoquinones interact with biological targets by forming covalent bonds or via their ability to undergo reversible oxidation-reduction reactions by generation of ROS (reactive oxygen species) by the redox cycle under aerobic conditions, by the inhibition of electron transport. The electron transport chain (ETC) entails the sequential transfer of electrons via multiprotein complexes (complex I to IV) present in the inner mitochondrial membrane with electron transfer between several complexes being mediated by ubiquinone ⁵⁷. Compounds that are analogs of ubiquinone that can act as competitive inhibitors of cytochrome bc1 by complexing to the Qi ubiquinone binding site (one of two ubiquinone binding sites on cytochrome bc1 and the one that is oriented toward the luminal side of the inner mitochondrial membrane, as distinct from the Qo site, which is oriented toward the external surface of that membrane) ⁵⁷. Studies on promastigotes and amastigotes of several species of *Leishmania* indicated that ETC is critical for the survival of these pathogens as application of antimycin A, an inhibitor of cytochrome bc1, or cyanide, an inhibitor of complex IV, resulted in death of promastigotes or amastigotes⁵⁷⁻⁵⁹. BPQ has been recently shown to be a potent inhibitor of cytochrome bc1 activity and the ETC, ATP production and intracellular amastigote growth at nanomolar concentrations^{24, 57}.

The activity of BPQ against *Leishmania donovani* was first reported in 1992 ²⁴ while BPQ has antiprotozoal activity against a number of parasite including *Theileria parva*, *Leishmania spp.*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Plasmodium falciparum*, *Eimeria tenella*⁶⁰. BPQ is currently used only in veterinary medicine as antitheilerial to treat East Coast fever in cattle in Eastern and Southern Africa (Butalex ©) formulated

as an IM injection in DMSO (2.5 mg kg^{-1})⁶⁰. BPQ is characterised by a long plasma half-life of at least 7 days and an oral LD_{50} greater than 8 g kg^{-1} in rats⁶⁰ and efficacy against *L. Donovanii* amastigotes in the nanomolar range (ED_{50} : $0.005 \text{ }\mu\text{M}$)²⁴. The methylene group linking the cyclohexyl moiety to the quinone nucleus is particularly important for antileishmanial activity as is the presence of a lipophilic substituent at the 4'-position of the cyclohexyl ring²⁴. *In vivo* BPQ is efficacious against *L. donovani* in a BALB/c mouse model and following 100 mg kg^{-1} for 5 days after oral administration resulted in 43.4% suppression of liver amastigotes (BPQ suspended in 0.25% cellacol). The disappointing *in vivo* data, when compared to *in vitro* potency was attributed to low plasma levels in the experimental animals partly due to the limited solubility of BPQ ($<30 \text{ ng/mL}$ over a pH range of 3.0-7.4) and high lipophilicity²⁶. Permeability studies using in-situ perfused rat intestine classified BPQ as highly permeable with apparent permeability value (P_{app}) of $0.912 \times 10^{-4} \text{ cm sec}^{-1}$ compared to propranolol which is a high permeability marker with P_{app} value of $0.716 \times 10^{-4} \text{ cm sec}^{-1}$ ^{61, 62}. Thus BPQ permeability is limited mostly by its aqueous solubility and precipitation in the gastrointestinal tracts typical of BCS Class II drugs and strategies to improve its dissolution are likely to have a positive effect in increasing its oral bioavailability.

Although several strategies have been developed to increase BPQ's bioavailability, only self-emulsifying drug delivery systems (SEDDS) have shown some promise towards orally BPQ formulations²⁵. Lipid-based nano-enabled delivery systems are receiving increasingly interest in the delivery of poorly soluble antiparasitic drugs because they possess no or limited biotoxicity, they are able to enhance the solubilisation capacity of the poorly soluble in the gastrointestinal fluids (due to their small size and large surface area to mass ratio) and are able to enhance transcellular

and lymphatic oral absorption ⁴⁷. Liposomal formulations are naturally taken up by macrophages via phagocytosis potentially delivering the antiparasitic drugs to their target site. However, liposomes are usually poorly loaded and are unstable in the gastrointestinal tract. SEDDS are able to overcome these problems by ensuring high drug loading and stability in gastrointestinal media, enhancing drug dissolution and increase the rate and extent of absorption, whilst they are easily scalable and amenable to low cost industrial manufacture. Excipients used in SEDDS are usually GRAS without the need for further toxicology to be converted from a colloidal into a solid dosage form via melt granulation, lyophilisation, spray-drying, physical adsorption onto solid carriers or simply by being filled into capsules ⁶³. Here we presented a lipid-based formulation of BPQ that possesses good loading, stability at tropical temperatures and simulated gastrointestinal media, ability to release BPQ avoiding precipitation in the aqueous environment of the GIT, enhanced oral bioavailability while being efficacious in reducing parasite load in the spleen similar to miltefosine (only orally VL licensed drug) and near 50% inhibition of liver parasite load in murine models after 10 days of oral administration. Adsorption of this lipid based formulation on solid carriers and lyophilisation resulted in a solid-dosage form that can be readily translatable with immediate release and enhanced dissolution in the GIT. BPQ SNEDDS were isotropic mixtures of Labrasol: Capryol 90 and Labrafil 1944CS that yield stable nanoemulsions upon dilution in aqueous media with a particle size well below 300 nm of quasispherical shape and good colloidal stability (Figure 1). These excipients were selected based on their HLB values to ensure the production of a clear microemulsion type II but also the solubility of BPQ in them (Labrasol: 17 ± 0.28 , Labrafil: 12.39 ± 0.35 , Capryol 90: 15.58 ± 0.57 at 37°C) ²⁵. Additionally, we have recently shown that Labrasol, a mixed ester of capric and caprylic acid, was shown to

possess high antileishmanial activity in the nanomolar range ⁵³. Inclusion of non-ionic surfactants such as Labrafil 1944CS has also been shown to enable their diffusion through mucous layers ⁶⁴.

The near complete release of BPQ from BPQ SNEDDS loaded in pullulan capsules in flow-through cell studies (USP Apparatus IV) supports that BPQ remains solubilised and available for absorption within all different pH media tested (Figure 2A). The *in vitro* lipolysis model was used to simulate the *in vivo* digestion process of lipid-based formulations of BPQ. The pH was maintained at 6.5 to simulate the fasted conditions of the intestine ⁶⁵ and to ensure the activity of pancreatic lipase ⁶⁶. Lipid digestion was started by the addition of the lipase enzyme while the liberation of free FA that causes a drop in pH was counteracted by addition of sodium hydroxide. As a dynamic lipolysis model was used, the continuous addition of calcium chloride solution controlled the accumulation of FA on the surface of the emulsions particles by forming insoluble calcium FA soaps, which precipitate and are removed from the system ³³. Although lipid glycerides with medium chain fatty acids are subjected to faster lipolysis ⁴⁷, 70% of BPQ remained solubilised avoiding precipitation in the GI tract available for absorption after 30 minutes and more than 10% was available for absorption even after 90 minutes which is double previous published reports for orally bioavailable SEDDS (Figure 2B) ⁴⁷. This is important to note, as our nanoemulsions were well below 300 nm and it is known that reducing the size of the emulsion increases the specific surface area of the lipids and thus the action of pancreatic lipases ⁶⁷. The presence of BPQ as an oil layer for 30 minutes can allow BPQ to be absorbed at a later stage enhancing the gastric residence time ⁶⁸.

The Target Product (treatment) Profile (TPP) for VL is based around the development of a safe, effective, oral, short-course (11 days maximum) VL drug formulations to

replace current treatments. Ideally a solid dosage form with enhanced stability in tropical temperatures is likely to be more cost-effective. BPQ-SNEDDS illustrated stability up to 6 months in tropical conditions (40°C, 75% relative humidity) in terms of drug content, particle size, colloidal stability and morphology (Figure SI-2). In an attempt to reduce the cost of a liquid filled capsule, we adsorbed BPQ SNEDDS on solid carriers to produce conventional solid dosage forms such as tablets. Aqueous dispersions of BPQ solid SNEDDS resulted in nanoemulsions of similar size and morphology (Figure 2C, 2D). Although the flow properties of BPQ solid SNEDDS could be improved by the addition of a glidant or use of micronized lactose, tablets were produced that passed pharmacopoeia disintegration and dissolution tests for immediate release tablets. Dissolution studies illustrated a pH mechanism of release with an initial burst release of BPQ from tablets in SGF that remained constant over 60 minutes that was followed by a complete release of the drug when the pH was raised to 6.8 (Figure 6). Low molecular weight glycol chitosan polymers are biocompatible carbohydrate polymers with good solubility in both acidic and neutral pH^{69, 70}. Chitosans are known to swell when exposed to aqueous media and the inclusion of a super-disintegrant enables the formation of pores to allow water penetration within the polymer gel structure. However, superdisintegrants are known to behave differently in acidic and neutral media⁷¹. Croscarmellose sodium (Ac-Di-Sol ©) has been shown to promote faster dissolution in neutral media than acidic⁷² which can explain dissolution behaviour observed and favouring of BPQ release in simulated intestinal media.

Solid state characterisation of BPQ solid SNEDDS is critical to understand the interaction of BPQ with the solid carriers at the molecular level and assess whether the formation of a BPQ solid dispersion or BPQ amorphisation was possible.

Lyophilisation of BPQ in a method used for the production of solid SNEDDS did not result in amorphous BPQ (Figure 3b, 4b). Similarly, attempts to amorphise BPQ by melt quenching which has been previously reported as a method to amorphise BCS Class II drugs were unsuccessful (Figure 3c). This can be explained as PXRD analysis indicated a prominent Bragg peak at $6.55\ 2\theta^\circ$ which correlates with a hydrophobic surface formed by aromatic rings of BPQ (Plane 001) (Figure SI-3). Additionally, the intensity of the Bragg peak at $12.95\ 2\theta^\circ$ corresponding to plane 002 (location of carbonyl groups) was also high, which indicates that h-bonding is likely to occur between the drug and surrounding hydrogen group donors such as glycol chitosan. PXRD, DSC and FT-IR studies indicate the formation of an amorphous BPQ solid dispersion (a product formed by the conversion of a fluid drug-carrier combination to the solid state ⁷³) which is important towards improved oral bioavailability. FT-IR analysis also pointed to the formation of hydrogen bonding between the hydroxyl groups of glycol chitosan polymer chains and the quinine carbonyl group of BPQ as indicated by the abolishment of the 1639 cm^{-1} peak within the BPQ solid SNEDDS powders.

Entrapment of BPQ within SNEDDS or solid SNEDDS did not affect its *in vitro* efficacy as BPQ SNEDDS and BPQ solid SNEDDS showed potent *in vitro* efficacy in the nanomolar range against *L. Infantum* amastigotes similar to BPQ in DMSO (IC_{50} for all $<37\text{ nM}$) (Table 1). Both BPQ and BPQ SNEDDS showed excellent selectivity for parasite versus mammalian cells with a high selectivity index that are well above DNDi (Drug for Neglected Diseases initiative) accepted hit selection levels ⁵⁴ and that of miltefosine. The high selectivity of BPQ is expected as there is a high degree of sequence divergence between the *Leishmania* and human cytochrome b proteins ⁵⁷.

In vivo pharmacokinetic studies demonstrated an earlier onset of action for BPQ SNEDDS compared to BPQ which supports the immediate release and enhanced solubilisation of BPQ in gastrointestinal media resulting in AUC₀₋₂₄ that are four fold previous reported values for BPQ SNEDDS after a single oral administration ²⁵. Although BPQ SNEDDS and BPQ solid SNEDDS were as efficacious as miltefosine in inhibiting parasite replication in the spleen, they were not able to control parasite replication in the liver. Interestingly, oral BPQ SNEDDS and BPQ solid SNEDDS (6 mg kg⁻¹, 10 oral doses over 10 days) were as efficacious as intramuscular doses of BPQ (25mg kg⁻¹, one dose at day 1 and one dose at day 5), indicating that the reasons resulting in limited inhibition of parasite growth in the liver was not related to the oral bioavailability of BPQ and amount of BPQ reaching the liver, but possibly due to metabolism of BPQ in the liver. BPQ has been reported to be metabolised by mouse and rat liver microsomes into an inactive red-coloured metabolic product with a half-life of less than 10 minutes being a substrate for CYP2C9 (half-life 6 minutes) and CYP2C19 half-life (4 minutes), while it was also metabolised by CYP3A4 (half-life 50 minutes) ^{28, 74, 75}. To overcome this issue and ensure orally bioavailable and efficacious BPQ formulations, further studies of combinations of BPQ with fluconazole, a potent CYP2C9, CYP2C19 and CYP3A4 inhibitor shown to be efficacious in the treatment of visceral leishmaniasis ^{76, 77} can be prepared and formulated easily with described SNEDDS platform technology into patient compliant solid dosage forms (SNEDDS filled capsules or tablets). Additionally, preparing SNEDDS with a lower amount of long-chain triglycerides (i.e. Labrafil 1944CS ~30%w/w of final formulation) and a higher short and medium-chain triglycerides (e.g. Capryol 90 and Labrasol) can potentially decrease the accumulation of the formulation in the liver to reduce BPQ metabolism.

Conclusions

Nanotechnology-enabled drug delivery systems offer a tool for overcoming the challenges encountered with current drugs, failed leads such as BPQ and antigens by improving their efficacy (and thus duration of treatment), safety, bioavailability, half-life, while also by enabling controlled release over tailored period of times and specific site-targeted delivery of therapeutics. When made with FDA or EMA approved scalable excipients they can result in medicine that can be taken readily to phase III human studies providing therapeutic solutions to neglected tropical diseases. Our proposed nano-enabled platform technology prepared from GRAS excipients is cost-effective while it possesses excellent loading, stability up to 6 months in tropical conditions, *in vitro* efficacy in the nanomolar range maintaining the solubilisation capacity of BPQ in gastrointestinal media and ensuring high oral bioavailability for BPQ able to inhibit parasite replication in the spleen and in the liver. Proposed BPQ SNEDDS and solid SNEDDS are cost-effective, patient compliant and readily translated platform technologies towards a nano-enabled safe and efficacious treatment for parasitic and infectious diseases.

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