

CHEMOTHERAPEUTIC AGENT AND COMPOSITIONS***Related Applications***

This present case is related to, and claims the benefit and priority of, PT 119120 filed on 12 December 2023 (12.12.2023) and PT 119899 filed on 10 December 2024 (10.12.2024), the contents of both of which are hereby incorporated by reference in their entirety.

Technical Field

This invention relates to chemotherapeutic agents, including piperlongumine. The invention provides pharmaceutical compositions the chemotherapeutic agents and medical use of the pharmaceutical compositions.

Background

Piperlongumine, which is also called piplartine, is a naturally occurring alkaloid/amine derived from the long pepper plant (*Piper longum*) (Zhang *et al.*). Long pepper is used in Ayurvedic medicine, for example, to treat diseases including tumours. Piperlongumine is also found in a number of other medicinal plants including *Piper tuberculatum*, *Piper arborescens*, *Piper chaba*, *Piper sylvaticum*, *Piper cenocladum*, *Piper alatabaccum* and *Piper puberulum* (Bezerra *et al.*).

Recent studies have demonstrated that piperlongumine exhibits anxiolytic, antiangiogenic, antidepressant, anti-metastatic, anti-tumoral, cytotoxic, genotoxic, antibacterial, antifungal, and anti-diabetic activities (Henrique *et al.*; Möhler *et al.*; Tripathi *et al.*).

Studies have suggested that piperlongumine induces cytotoxicity in cancer cells mainly through the accumulation of intracellular reactive oxygen species and it also interferes with various aberrant mechanisms involved in inflammation, proliferation, angiogenesis and cell survival (Tripathi *et al.*).

Preformulation studies on piperlongumine have shown that this compound has poor water solubility. Piperlongumine was found to be unstable under alkaline conditions, and also to undergo photodegradation when exposed to light (Aodah *et al.*). Degradation products of piperlongumine have been reported to include 3,4,5-trimethoxycinnamic acid and piperlongumine acid.

Despite promising results of piperlongumine in preclinical models, the available pharmacokinetic data are extremely limited and this active ingredient has not yet entered into clinical evaluation stage (Tripathi *et al.*). There is a need to provide stable composition

containing piperlongumine, and there is also a need to provide stable composition for other active agents.

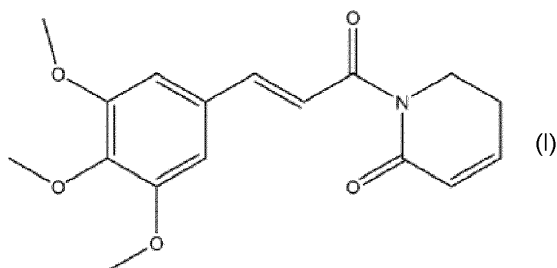
Summary of the Invention

At its most general, the invention relates to pharmaceutical compositions comprising piperlongumine, which is useful for the treatment of cancers. The piperlongumine may be particles of piperlongumine. The particles may have an average particle size of 10 μm or less, such as 5 μm or less. Advantageously, piperlongumine particles can be provided in a composition, such as a suspension or hydrogel, at a higher loading or higher concentration than bulk piperlongumine. Piperlongumine particles are thus particularly suitable for use in pharmaceutical formulations. Moreover, the formulations have the potential for sustained piperlongumine release when administered to a patient. The particles, such as when provided in a suspension or hydrogel, also have good stability.

The invention also relates to a crystalline form of piperlongumine. The crystalline form may be characterized by a powder x-ray diffraction pattern comprising one or more 2θ values selected from $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

The particles and/or crystalline form of piperlongumine may be provided in a pharmaceutical composition.

In a first aspect, the invention provides a pharmaceutical composition comprising a crystalline compound of Formula (I),



optionally together with a pharmaceutically acceptable carrier, diluent, or excipient; wherein the crystalline compound is in particulate form having an average particle size of less than about 5 μm .

A compound of Formula (I) may also be referred to as piperlongumine. A reference to a compound of Formula (I) includes a solvate thereof.

The average particle size is typically the average size of the population of particles of the crystalline compound present within the pharmaceutical composition.

Piperlongumine particles of the dimensions described herein may have improved solubility in excipients compared to bulk piperlongumine, such as excipients suitable for pharmaceutical formulations. Bulk piperlongumine typically has an average particle size that is greater than 10 μm , such as an average particle size of at least 12 μm . Advantageously, the particles described herein may be incorporated into a composition, such as a suspension or hydrogel, at a higher loading or higher concentration than bulk piperlongumine. The particles also have good stability compared to bulk piperlongumine, as well as a good release profile when provided in a pharmaceutical composition. This allows for sustained release of piperlongumine when used as an active pharmaceutical ingredient. Moreover, the particles have good syringeability and are suitable for administration to patients. The particles can also be isolated, such as from a suspension, to improve long-term stability of the particles.

Preferably, the crystalline compound of Formula (I) has an average particle size of less than about 2 μm , such as less than about 1 μm . The average particle size may be from about 100 nm to about 5 μm , such as from about 200 nm to about 2 μm .

In some embodiments, the average particle size is about 2 μm or less, such as about 1 μm or less, such as about 900 nm or less, such as about 800 nm or less, such as about 750 nm or less. The average particle size may be about 200 nm or more, such as about 300 nm or more, such as about 400 nm or more, such as about 450 nm or more. The average particle size may be in a range with upper and lower values as described, such as from about 200 nm to about 2 μm , such as from about 200 nm to about 1 μm , such as from about 400 nm to about 900 nm, preferably from about 450 nm to about 750 nm.

The average particle size of piperlongumine particles may be the median particle size of particles within a population of particles. The median particle size may be represented by a D50 value, which may be calculated using a method described herein, such as by scanning electron microscopy (SEM) or dynamic light scattering (DLS). The crystalline compound may have a median particle size (such as a D50 value) from about 200 nm to about 1,000 nm, such as from about 400 nm to about 900 nm, such as from about 450 nm to about 750 nm. The crystalline compound may have a D10 value from about 200 nm to about 600 nm, such as from about 300 nm to about 500 nm. The crystalline compound may have a D90 value from about 500 nm to about 2,000 nm, such as from about 700 nm to about 1,000 nm. These particles may be produced by a method comprising a step of dissolving piperlongumine into supercritical carbon dioxide, such as by a CESS[®] process as described herein.

In some embodiments, the crystalline compound may have an average particle size, such as a D50 value, from about 1,000 nm to about 2,500 nm, such as from about 1,500 nm to about 2,000 nm. The crystalline compound may have a D10 value from about 200 nm to about 800 nm, such as from about 400 nm to about 700 nm. The crystalline compound may have a D90

value from about 2.0 μm to about 10.0 μm , such as from about 3.0 μm to about 4.0 μm . These particles may be produced by a wet milling process described herein.

The crystalline compound of Formula (I) may be characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$. The crystalline compound may have the following peaks in a powder x-ray diffraction pattern: $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

The crystalline compound may have a powder x-ray diffraction according to the particles shown in Figure 10 (labelled "particles", blue trace having a peak at 14.888°). The crystalline compound may be a compound having a powder x-ray diffraction pattern with peaks according to the particles of Table 30 ("particles" column, with peaks at 12.363° and 14.888° , for example).

An x-ray diffraction pattern may be measured using a Cu anode, such as with a wavelength of 1.54 Å. X-ray powder diffraction may be measured in reflection mode and may be carried out at room temperature, such as at 25°C . X-ray powder diffraction may be carried out with a scanning time of 30 minutes, with a scan range $5^\circ < 2\theta < 40^\circ$ and with a step size of 0.013° .

The crystalline compound of Formula (I) may have an onset melting temperature, $T_{m(\text{onset})}$, from 121°C to 122°C when heated to 134°C at a rate of $5^\circ\text{C}/\text{min}$ during Differential Scanning Calorimetry. The onset melting temperature may be in the range of $121.8 \pm 0.5^\circ\text{C}$, such as $121.8 \pm 0.3^\circ\text{C}$.

The crystalline compound of Formula (I) may have an infrared spectrum comprising a doublet peak at $1,650$ to $1,700\text{ cm}^{-1}$, such as a doublet peak at $1,670$ and $1,690\text{ cm}^{-1}$. Preferably, the doublet peak is at around $1,678\text{ cm}^{-1}$, such as at around $1,678\text{ cm}^{-1} \pm 5\text{ cm}^{-1}$, such as around $1,678\text{ cm}^{-1} \pm 2\text{ cm}^{-1}$, such as around $1,678\text{ cm}^{-1} \pm 1\text{ cm}^{-1}$. The doublet peak may have a mid-point in a range described above, or the doublet peak may be contained within a range described above.

The crystalline compound of Formula (I) may have a peak in an infrared spectrum at $1,460$ to $1,500\text{ cm}^{-1}$ and/or at $1,400$ to $1,440\text{ cm}^{-1}$. Preferably, there is a peak in the infrared spectrum at $1,470$ - 90 cm^{-1} and/or $1,405$ - $1,425\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1}$ and/or around $1,416\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 5\text{ cm}^{-1}$ and/or around $1,416\text{ cm}^{-1} \pm 5\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 2\text{ cm}^{-1}$ and/or around $1,416\text{ cm}^{-1} \pm 2\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 1\text{ cm}^{-1}$ and/or around $1,416\text{ cm}^{-1} \pm 1\text{ cm}^{-1}$.

In the pharmaceutical compositions of the invention, the crystalline compound may be dispersed in a carrier comprising one or more of a surfactant, and a polymer. The carrier may comprise a surfactant and/or a polymer. This advantageously allows for a higher

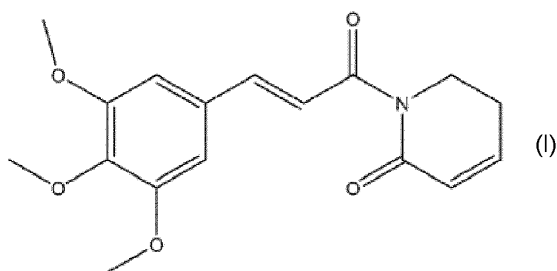
concentration or higher loading of piperlongumine to be achieved, such as compared to (bulk) piperlongumine provided in solution.

Preferably, the crystalline compound is present in the pharmaceutical composition from about 10% to about 40% by weight of the composition, more preferably from about 15% to about 30% by weight of the composition, such as from about 20% to about 30% by weight of the composition. The crystalline compound may be present in the pharmaceutical composition at a concentration of more than 1 mg/mL, such as at least 2 mg/mL, such as at least 3 mg/mL.

The pharmaceutical composition may be a suspension comprising one or more poloxamers. Preferably, the poloxamer(s) are selected from poloxamer 407, poloxamer 338, and poloxamer 188. The poloxamer(s) may be present from about 10% to about 40% by weight of the composition, preferably from about 10% to about 30% by weight of the composition, such as from about 15% to about 25% by weight of the composition, such as wherein poloxamer is poloxamer 407. In this way, piperlongumine can be prepared at a high loading within the composition and is particularly useful for therapeutic applications such as described herein.

The pharmaceutical composition may comprise polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG). Preferably, the PCL-PVAc-PEG is present from about 10% to about 40% by weight of the composition, more preferably about 25% to about 35% by weight of the composition. This allows piperlongumine to be formulated at a high loading and is particularly useful for therapeutic applications such as described herein.

In a second aspect, the invention provides a pharmaceutical composition comprising a crystalline form of a compound of Formula (I),



wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$, optionally together with a pharmaceutically acceptable carrier, diluent, or excipient.

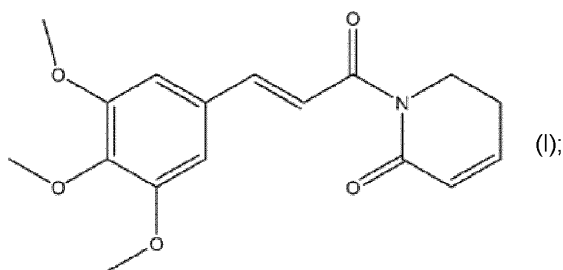
Preferred features of the first aspect apply equally to the second aspect.

The crystalline form of piperlongumine according to the present invention is a polymorph that differs from bulk piperlongumine. Bulk piperlongumine may have a powder x-ray diffraction pattern as shown for the bulk sample analysed at Table 31. Advantageously, smaller particles of the crystalline form of piperlongumine can be conveniently prepared compared to bulk piperlongumine. The crystalline form may have improved solubility or may be provided in a composition at a higher maximum concentration. The crystalline form may also have improved stability. In this way, the crystalline form of piperlongumine is useful as a medicament, such as when provided in a suspension and/or in a pharmaceutical formulation.

The crystalline form of piperlongumine may have the following peaks in a powder x-ray diffraction pattern: $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

The compound of Formula (I) may be in the form of particles as described herein, such as having an average particle size of less than about 5 μm , such as less than about 2 μm , such as less than about 1 μm . The average particle size may be from about 200 nm to about 1 μm . The crystalline compound may have a D10, D50 and/or D90 value as described herein.

In a third aspect, the invention provides a pharmaceutical composition comprising an aqueous suspension of from about 5% to about 40% by weight of a compound of Formula (I),



and:

from about 5% to about 30% by weight of a poloxamer selected from one or more of poloxamer 407, poloxamer 338, and poloxamer 188; or

from about 10% to about 40% by weight of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).

Preferred features of the first and second aspect apply equally to the third aspect.

In the pharmaceutical composition the compound of Formula (I) is preferably present from about 10% to about 40% by weight of the composition, such as from about 15% to about 30% by weight of the composition, including from about 20% to about 30% by weight of the composition.

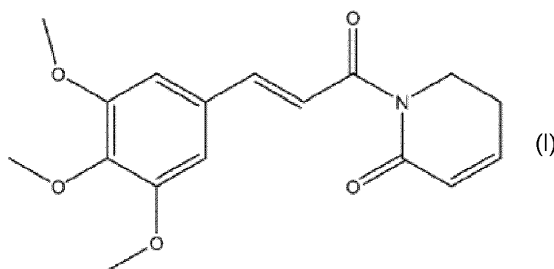
In a fourth aspect, the invention provides a pharmaceutical composition according to the first, second or third aspects, for use in a method of treatment.

The pharmaceutical compositions may be for use in the treatment of cancer, such as brain cancer, such as glioblastoma.

The crystalline compound and particles have good solubility in pharmaceutically acceptable excipients and can also be provided at a desirable concentration in a suspension. The crystalline compound and particles also have a good dissolution profile and are capable of permeating through a membrane. In this way, the crystalline compound and the particles are particularly suitable for medical use.

Preferred features of the first, second and third aspects apply equally to the fourth aspect.

In a fifth aspect, the invention provides a crystalline form of a compound of Formula (I),



wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

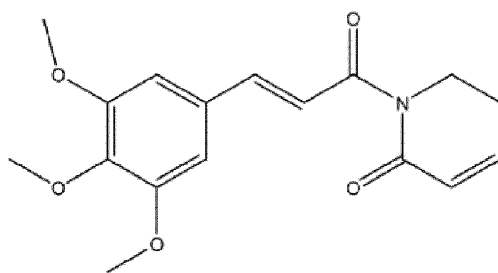
Preferably, the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

Preferred features of the first aspect apply equally to the fifth aspect.

Additionally or alternatively, in a sixth aspect the invention provides a pharmaceutical composition comprising a thermoresponsive hydrogel and a chemotherapeutic agent dispersed in the hydrogel, wherein the pharmaceutical composition comprises:

- one or more poloxamers, wherein the total amount of poloxamers is from about 15% to about 20% by weight of the pharmaceutical composition;
- from about 0.5% to about 2% by weight of polyvinylpyrrolidone;
- from about 18% to about 20% by weight of polyethylene glycol;
- and from about 0.2 to 0.3% by weight of a polysorbate.

The chemotherapeutic agent may be a compound of Formula (I),



such as wherein the pharmaceutical composition comprises up to about 5% by weight of the compound of Formula (I), such as about 2% by weight

The one or more poloxamers may be selected from poloxamer 407 and poloxamer 188. In some embodiments, the pharmaceutical composition comprises poloxamer 407, such as about 15% by weight of poloxamer 407. In some embodiments, the pharmaceutical composition comprises poloxamer 188, such as from about 2.5% to about 5% by weight of poloxamer 188. In some embodiments, the pharmaceutical composition comprises poloxamer 407 and poloxamer 188, such as about 15% by weight of poloxamer 407 and from about 2.5% to about 5% by weight of poloxamer 188.

The pharmaceutical composition may comprise about 1% by weight of polyvinylpyrrolidone.

In some embodiments, the polyethylene glycol has a molecular weight of about 350 to about 450 g/mol. The polyethylene glycol may be polyethylene glycol 400 (PEG400).

The pharmaceutical composition may comprise from about 19% to about 20% of the polyethylene glycol, such as from about 19.5% to about 20% by weight of the polyethylene glycol, such as about 19.8% by weight of the polyethylene glycol.

The polysorbate may be polysorbate 80. The pharmaceutical composition may comprise about 0.3% or about 0.25% of the polysorbate.

In a seventh aspect, the invention provides a pharmaceutical composition according to the sixth aspect, for use in a method of treatment. The method may be a method of treating cancer, such as sarcoma, such as soft-tissue sarcoma. The method may comprise administering the pharmaceutical composition to a subject by intratumoral injection.

Summary of the Figures

The present invention is described with reference to the figures listed below.

Figure 1 shows SEM images of bulk piperlongumine.

Figure 2 shows the particle size distribution (PSD) of bulk piperlongumine.

Figure 3 shows SEM images of particles of piperlongumine according to the present invention prepared by micronisation. Micronisation was carried out by wet milling as described in General Method C.

Figure 4 shows the particle size distribution (PSD) of piperlongumine particles according to the present invention prepared by micronisation.

Figure 5 shows the *in vitro* release profile of bulk piperlongumine (% release) in methylcellulose-based gels for up to 3 weeks.

Figure 6 shows the *in vitro* release profile of bulk piperlongumine (% release) in poloxamer-based gels for up to 3 weeks.

Figure 7 shows the *in vitro* diffusion profile of piperlongumine particles according to the present invention (concentration, mg/mL) formulated in nanosuspension incorporated in hydrogel comprised of poloxamer 407 (22% in acetate buffer) in diffusion cells (Franz cells) over 1 week (top) and 48 h (bottom).

Figure 8 shows the *in vitro* release profile of piperlongumine particles according to the present invention (% release) formulated in nanosuspension incorporated in hydrogel comprised of poloxamer 407 (22% in acetate buffer) in small scale dissolution test.

Figure 9 shows an XRPD diffractogram acquired for bulk piperlongumine. The main reflection peaks are assigned in the graph.

Figure 10 shows an XRPD diffractogram of bulk piperlongumine overlayed with that of piperlongumine particles. The trace for the bulk sample (red) has a peak at 25.902° , and the trace for the particles sample (blue) has a peak at 14.888° .

Figure 11 shows DSC curves for bulk piperlongumine. The first heating cycle is shown in the upper panel and the second heating cycle is shown in the lower panel.

Figure 12 is an SEM image of bulk piperlongumine showing rod-shaped particles in the micrometer range (top and bottom-left panels), and needle-like particles (bottom-right panel).

Figure 13 shows representative SEM images of piperlongumine particles according to the present invention. The D50 value for each sample shown is as follows: 1: 594, 2: 591, 3: 650, 4: 600, 5: 675, 6: 660 nm.

Figure 14 shows an FTIR spectrum of bulk piperlongumine (upper trace, “bulk”) and the crystalline form of piperlongumine according to the present invention (lower trace, “nanoformed”).

Figure 15 shows DSC curves for batches of the crystalline form of piperlongumine according to the present invention. The values on top of each curve corresponds to, respectively, onset melting temperature ($T_{m(\text{onset})}$) and enthalpy of melting (ΔH). The curves correspond to the first heating cycle.

Figure 16 shows the content uniformity of aqueous piperlongumine hydrogel samples according to the present invention ($n = 5$) with nominal active loading indicated in the sample name.

Figure 17 shows the content uniformity of 20% piperlongumine hydrogel samples according to the present invention ($n = 3$).

Figure 18 shows the content uniformity of hydrogel samples according to the present invention before and after UV sterilization ($n = 3$).

Figure 19 shows the chemical stability of 20% piperlongumine hydrogel samples according to the present invention stored at 40 °C with nanoformed material as a reference. All samples are displayed on the upper diagram while citrate buffer-based gels were discarded from the lower diagram. Nano reference was pure nanoformed powder without any excipients. Top panel: from top to bottom at last time point: Nano Reference, Soluplus in citrate pH 6, P407 in citrate pH 6. Lower panel: from top to bottom at t_0 : P407 in tris pH 7, P407 in water, Soluplus in tris pH 7, Soluplus in water/Nano reference.

Figure 20 shows the chemical stability of gels according to the present invention before and after UV irradiation.

Figure 21 shows the dissolution of hydrogel samples according to the present invention (at about 20 hours one of the nano Soluplus samples detached from the weighing boat piece and was broken apart by the magnetic stirrer bar, increasing the contact surface, resulting in faster dissolution. This is believed to explain the increased standard deviation). From top to bottom at 10 h: bulk in 18% poloxamer, nanoformed in 27.5% Soluplus, nanoformed in 18% poloxamer, bulk in 27.5% Soluplus.

Figure 22 shows the macroflux data of hydrogels according to the present invention (note: only positive error values are shown to aid visualization). From top to bottom at 12 h: Bulk P407 Acceptor, Nano P407 Acceptor, Bulk P407 Donor, Bulk Soluplus Acceptor, Nano Soluplus acceptor, Bulk Soluplus Donor, Nano P407 Donor, Nano Soluplus Donor.

Figure 23 shows the SEM images of poloxamer 407 gels stored according to the present invention at 4 °C for 2 days. Images a) and b) show gel with bulk API while gel with nanoformed API is presented on images c)-f).

Figure 24 shows the SEM images of Soluplus gels according to the present invention stored at 4 °C for 1 day. Images a) and b) show gel with bulk API while gel with nanoformed API is presented on images c)-f).

Figure 25 shows the viscosity and temperature variation for each formulation of HA, PVP, PVA and CMC, with 25 °C viscosity values shown on the left-hand side and 37 °C viscosity values shown on the right-hand side for each formulation (mean $\pm$ SD [n = 3]).

Figure 26 shows results from adhesion studies; top panel shows the area under force-time curve results for PVP, PVA, HA and CMC at 1% with P407/P188 15/5% (w/w); and bottom panel shows mucoadhesion studies: area under force-time curve results for PVP, PVA, HA and CMC at 1% with P407/P188 15/5% (w/w) and mucin 5% (right) (mean $\pm$ SD, n = 6).

Figure 27 shows the release profile of piperlongumine from P407/P188 15/5% (w/w) hydrogel with each of HA, PVP, PVA and CMC. (mean $\pm$ SD; n = 4). Quantification was carried out by HPLC.

Figure 28 shows the release profiles of piperlongumine from P407/P188 15/2.5% (w/w) (purple), and P407/P188 15/5% (w/w) (black). Control represents PEG400/Tween80 (98.77/1.23%) + 2% PL (grey) (mean $\pm$ SD; n = 4). Quantification by HPLC.

Detailed Description of the Invention

In a general aspect, the invention relates to pharmaceutical compositions comprising piperlongumine, such as particles of piperlongumine. The particles may have an average particle size of 10 μ m or less, such as 5 μ m or less. The invention also relates to a crystalline form of piperlongumine, which may be characterized by a powder x-ray diffraction pattern comprising one or more 2 θ values selected from $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

Aodah *et al.* report that the solubility of piperlongumine, in its bulk form, is approximately 26 μ g/mL in water. The solubility may be enhanced using 10% polysorbate 80 as a surfactant, whilst the use of co-solvents and cyclodextrins allows concentrations of up to 1 mg/mL to be achieved.

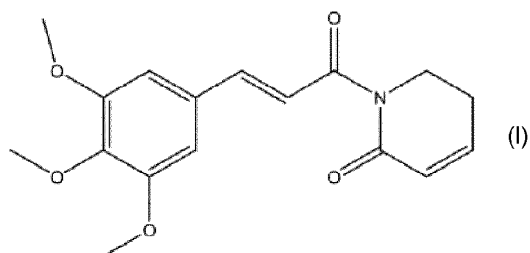
Consistent with these findings, the present inventors have found that bulk piperlongumine has limited solubility in pharmaceutical excipients. A maximum concentration of approximately 4 mg/mL could be achieved in poloxamer-based hydrogel formulations (see results and discussion section below).

The present inventors have found that the crystalline form and/or particles of piperlongumine can advantageously be incorporated into a composition, such as a suspension or hydrogel, at a high concentration. The stability of the particles may be improved compared to bulk piperlongumine. The crystalline form and/or a reduction in particle size is thus found to be beneficial, particularly for pharmaceutical applications of piperlongumine. The crystalline form may be incorporated into a hydrogel that is advantageously able to gel at body temperature. The hydrogels can be easily dispensed through a syringe needle. The hydrogels also exhibit a good release profile that is suitable for use as a pharmaceutical formulation.

Moreover, solid piperlongumine particles can be isolated from the suspensions, such as by freeze-drying. This further improves the long-term stability of the piperlongumine particles described herein. The lyophilisates are advantageously physically and chemically stable for up to 4 weeks at 25 °C, as well as in the temperature range of 2 to 8 °C.

Piperlongumine as described herein refers to 5,6-dihydro-1-[(2*E*)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1*H*)-pyridinone (a compound of formula (I) below).

Piperlongumine is also sometimes referred to as pipartine. The compound has CAS registry number 20069-09-4.



Particles

An aspect of the invention relates to particles of a compound of Formula (I), i.e. piperlongumine. Particles of piperlongumine, such as of the crystalline form described herein, may have an average particle size of up to about 10 µm, preferably up to about 7 µm, more preferably up to about 6 µm, more preferably up to about 5 µm. The particles may be referred to as nanoparticles, such as when the average particle size is up to about 1 µm. These dimensions differ from that measured for bulk piperlongumine, which may typically have an average particle size of that is greater than 10 µm, such as an average particle size of at least 12 µm (see Results and Discussion section below).

“Bulk piperlongumine” as described herein may have a particle size, D10, D50 and/or D90 value as shown in Figure 2. Bulk piperlongumine may be characterized by an x-ray diffraction

pattern as shown in Figure 9 and/or Table 30. Bulk piperlongumine may be characterized by a powder x-ray diffraction pattern comprising the 2θ values selected from $11.3 \pm 0.1^\circ$, $14.5 \pm 0.1^\circ$, and $25.9 \pm 0.1^\circ$, or the 2θ values selected from $11.3 \pm 0.1^\circ$, $14.5 \pm 0.1^\circ$, $21.3 \pm 0.1^\circ$, $25.9 \pm 0.1^\circ$, and $31.9 \pm 0.1^\circ$.

The particles of piperlongumine may be provided in a pharmaceutical composition as described herein, such as together with a pharmaceutically acceptable carrier, diluent, or excipient.

By "particles of piperlongumine" it is meant a population of piperlongumine particles. Within the population, individual particles of piperlongumine may have differing sizes to each other such that there is a distribution of particle sizes present within the population.

The particles may be provided in a composition, such as a pharmaceutical composition.

In an aspect there is provided a pharmaceutical composition comprising a crystalline compound of Formula (I), wherein the crystalline compound has an average particle size of less than about $5 \mu\text{m}$. The pharmaceutical composition may further comprise one or more of a pharmaceutically acceptable carrier, diluent, or excipient.

The average particle size typically refers to the average size of piperlongumine particles that are present in the pharmaceutical composition.

The "average particle size" may refer to the mean value or the median value of particle sizes across a population of particles.

A mean value may refer to the number average or the mass average.

Preferably, the average particle size refers to the median particle size of a population. The population of particles may be a typical amount that can be analysed together, such as by microscopy or by spectroscopic methods. Suitable methods include scanning electron microscopy (SEM) and dynamic light scattering (DLS). The number of particles within a population may be at least a typical amount that can be analysed together, such as at least 50 particles, such as at least 100 particles.

A particle may be characterized by its largest dimension. By "largest dimension", it is meant the maximal diameter in the largest cross-section of the particle, such as the diameter of the particle.

A population of particles may be defined by an average largest dimension. By "average largest dimension", it is meant the mean value or the median value, for example, of the largest dimension of individual particles in a collection of particles, where the largest dimension is as defined above, such as the diameter. The population of particles may be a typical amount that can be analysed together such as by microscopy, such as at least 50 particles, such as at least 100 particles.

The particles may be spherical in shape, or substantially spherical.

The particles may be non-spherical, such as elongate or ellipsoidal.

When a particle is irregular in shape, the particle size may refer to the geometric average of two or more lengths measured across the particle. For example, when a particle is ellipsoidal, the particle size preferably refers to the geometric average of the length of the two main axes that define the ellipsoid.

The crystalline compound of Formula (I) may have an average particle size of less than about 5,000 nm, such as less than about 2,000 nm, such as less than about 1,000 nm.

Particles of piperlongumine, such as when provided in a pharmaceutical composition as described herein, may have an average particle size of about 5.0 μm or less, such as about 4.0 μm or less, such as about 3.0 μm or less, such as about 2.0 μm or less, such as about 1.0 μm or less, such as about 900 nm or less, such as about 800 nm or less, such as about 750 nm or less, such as about 700 nm or less, such as about 600 nm or less. Preferably, the average particle size is about 2.0 μm or less, more preferably about 1.0 μm or less.

The particles may have an average particle size of about 50 nm or more, such as about 100 nm or more, such as about 150 nm or more, such as about 200 nm or more, such as about 250 nm or more, such as about 300 nm or more, such as about 350 nm or more, such as about 400 nm or more, such as about 450 nm or more, such as about 500 nm or more, such as about 600 nm or more, such as about 700 nm or more, such as about 800 nm or more, such as about 900 nm or more, such as about 1.0 μm or more. Preferably, the particles have an average particle size of 50 nm or more, including 100 nm or more, including 200 nm or more, including 250 nm or more.

The particles may have an average particle size in a range with upper and lower values selected from the values as described above, such as from about 100 nm to about 5.0 μm , including from about 200 nm to about 2.0 μm , such as from about 200 nm to about 1.0 μm , such as from about 400 nm to about 900 nm, such as from about 450 nm to about 750 nm. Preferably, the average particle size in a range of from about 200 nm to about 2,000 nm, such as from about 200 nm to about 1,000 nm, including from about 400 nm to about 1,000 nm.

The particles of piperlongumine with dimensions described above may be obtainable by a process involving dissolving piperlongumine, such as bulk piperlongumine, in supercritical carbon dioxide. Examples of these methods are described herein.

Piperlongumine, such as when provided in a pharmaceutical composition, may have a median particle size (D50 value) of up to about 2.0 μm , such as up to about 1.0 μm , such as up to about 900 nm, such as up to about 800 nm, such as up to about 750 nm, such as up to about 700 nm, such as up to about 675 nm. The D50 value may be about 100 nm or more, such as about 200 nm or more, such as about 300 nm or more, such as about 400 nm or more, such as about 450 nm or more, such as about 500 nm or more, such as about 550 nm or more, such as about 575 nm or more. The D50 value may be in a range with upper and lower values selected from the above, such as about 100 nm to about 2.0 μm , including about 200 nm to about 2.0 μm , such as about 400 nm to about 900 nm, such as about 450 nm to about 750 nm. Preferably, the median particle size in a range of from about 200 nm to about 2,000 nm, such as from about 200 nm to about 1,000 nm, including from about 400 nm to about 1,000 nm.

The particles may be defined by a D10 value. The D10 value refers to the 10-percentile mark, wherein 10% of all particles in a population are below the D10 value and 90% of the particles are above the D10 value. The D10 value may be up to about 800 nm, such as up to about 700 nm, such as up to about 600 nm, such as up to about 500 nm, such as up to about 450 nm, such as up to about 400 nm, such as up to about 350 nm, such as up to about 300 nm. The D10 value may be about 50 nm or more, such as about 100 nm or more, such as about 150 nm or more, such as about 200 nm or more, such as about 225 nm or more, such as about 250 nm or more, such as about 275 nm or more. The D10 value may be in a range with upper and lower values selected from the above, such as from about 50 nm to about 800 nm, including from about 200 nm to about 600 nm, such as from about 300 nm to about 500 nm.

The particles may be defined by a D90 value. The D90 value refers to the 90-percentile mark, wherein 90% of all particles in a population are below the D90 value, and 10% of the particles are above the D90 value. The D90 value may be up to about 4.0 μm , such as up to about 2.0 μm , such as up to about 1.5 μm , such as up to about 1.25 μm , such as up to about 1.2 μm , such as up to about 1.1 μm , such as up to about 1.0 μm , such as up to about 900 nm. The D90 value may be about 400 nm or more, such as about 500 nm or more, such as about 600 nm or more, such as about 700 nm or more, such as about 750 nm or more, such as about 800 nm or more. The D90 value may be in a range with upper and lower values selected from the above, such as about 400 nm to about 4.0 μm , including about 500 nm to about 2.0 μm , such as about 700 nm to about 1.0 μm . Preferably, the D90 value is from about 500 nm to about 2,000 nm, more preferably from about 700 nm to about 1,000 nm.

The particles may have D10, D50 and/or D90 values as described above. For example, the particles may have a D10 value in the range of about 300 nm to about 500 nm, a D50 value in the range of about 450 nm to about 750 nm, and a D90 value in the range of about 700 nm to about 1,000 nm.

In other embodiments, particles of the crystalline compound may be obtainable by a wet milling method, such as a method described herein.

Therefore, the particles may have a median particle size (D50 value) of about 3.0 μm or less, such as about 2.5 μm or less, such as about 2.0 μm or less, such as about 1.9 μm or less, such as about 1.8 μm or less, such as about 1.75 μm or less, such as about 1.7 μm or less. The D50 value may be about 0.5 μm or more, such as about 0.75 μm or more, such as about 1.0 μm or more, such as about 1.2 μm or more, such as about 1.3 μm or more, such as about 1.4 μm or more, such as about 1.5 μm or more, such as about 1.6 μm or more, such as about 1.7 μm or more. The D50 value may be in a range with upper and lower values selected from the above, such as about 0.5 μm to about 3.0 μm , including about 1.0 μm to about 2.5 μm , such as about 1.5 μm to about 2.0 μm .

The D10 value of the particles may be about 1.0 μm or less, such as about 900 nm or less, such as about 800 nm or less, such as about 700 nm or less, such as about 600 nm or less, such as about 500 nm or less. The D10 value may be about 100 nm or more, such as about 200 nm or more, such as about 300 nm or more, such as about 400 nm or more, such as about 450 nm or more, such as about 500 nm or more, such as about 550 nm or more. The D10 value may be in a range with upper and lower values selected from the above, such as about 100 nm to about 1.0 μm , including about 200 nm to about 800 nm, such as about 400 nm to about 700 nm.

The D90 value of the particles may be about 10.0 μm or less, such as about 6.0 μm or less, such as about 5.0 μm or less, such as about 4.5 μm or less, such as about 4.0 μm or less, such as about 3.75 μm or less, such as about 3.5 μm or less, such as about 3.0 μm or less. The D90 value may be about 1.0 μm or more, such as about 1.5 μm or more, such as about 2.0 μm or more, such as about 2.5 μm or more, such as about 3.0 μm or more, such as about 3.25 μm or more, such as about 3.5 μm or more, such as about 3.75 μm or more. The D90 value may be in a range with upper and lower values selected from the above, such as about 1.0 μm to about 10.0 μm , including about 2.0 μm to about 10.0 μm , such as about 3.0 μm to about 4.0 μm .

Particles of the crystalline compound may have D10, D50 and/or D90 values as described above. For example, particles may have a D10 value in the range of about 400 nm to about 700 nm, a D50 value in the range of about 1.5 μm to about 2.0 μm , and a D90 value of about 3.0 μm to about 4.0 μm .

The average, D50, D10 and D90 values described above are preferably measured by SEM or by DLS, and more preferably by SEM.

The particles size of the crystalline compound may be defined by a span, such as when measured by SEM. By span it is meant a value that gives a statistic on the width of the particle size distribution. The span may be defined as $(D90 - D10)/D50$, where D10, D50 and D90 are as defined above. The span value may be normalized to the D50 value. The span gives information about how far apart the smallest and largest particles are from each other. The particles may have a size span in the range of about 0.2 to about 2, such as about 0.5 to about 1.5.

Particles of the crystalline compound according to the invention have good syringeability, such as when the particles are incorporated into a suspension and/or a hydrogel. The particles, which may be in the form of a suspension and/or a hydrogel may be injectable through a needle with a gauge size of 14 G or more, such as 16 G or more, such as 18 G or more. The particles may be injectable through a needle with a gauge size of 14 G to 30 G, such as 14 G to 25 G, such as 16 G to 20 G, such as 18 G.

By "syringeability", it is meant the ability to inject the particles, which may be in a suspension and/or a hydrogel, through a needle. The flow of the particles during injection may be drop wise or continuous, and preferably is continuous.

In some embodiments, the crystalline compound is in particulate form and is non-encapsulated. For example, the crystalline compound is not encapsulated within a particle or nanoparticle, such as where the crystalline compound is not encapsulated with a polymer nanoparticle. Preferably, the compositions of the invention are substantially free of polymer nanoparticles in which piperlongumine is encapsulated. In some preferred embodiments, the crystalline compound in the composition is in the form of free piperlongumine, which is optionally dispersed in the composition such as in a hydrogel matrix.

In some embodiments, the composition comprises particles that consist of, or consist essentially of, a crystalline form of piperlongumine. The composition may be substantially free of particles, or nanoparticles, which comprise piperlongumine together with one or more components other than piperlongumine. Thus, a particle that contains piperlongumine may contain no other components. Consequently, a reference to a particle may exclude a nested particle where a first particle that consists of, or consists essentially, of piperlongumine is contained within or together with one or more components in a second particle.

Polymorph

An aspect of the invention relates to a polymorph of piperlongumine. The polymorph is a crystalline form of piperlongumine and may be obtained by micronizing piperlongumine, such

as micronizing bulk piperlongumine, or by preparing particles of piperlongumine, for example using the methods described herein.

The crystalline form may be provided in a pharmaceutical composition as described herein.

In an aspect, the invention provides a pharmaceutical composition comprising a crystalline form of piperlongumine, wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$, optionally together with a pharmaceutically acceptable carrier, diluent, or excipient.

The crystalline form of piperlongumine, which may be provided in a pharmaceutical composition, has an x-ray powder diffraction pattern (wavelength $1.54 \text{ \AA}$) containing peaks at the following 2θ values: 14.9° , 16.7° and 21.6° .

The crystalline form may also have the following additional peaks in an x-ray powder diffraction pattern at the following 2θ values: 18.0° and 28.9° , optionally 12.4° , 25.4° , and 26.6° , further optionally 19.5° and 23.8° .

In some embodiments the crystalline form has an x-ray powder diffraction pattern containing peaks at the following 2θ values: 14.9° , 16.7° , 18.0° , 21.6° and 28.9° .

In some embodiments the crystalline form has an x-ray powder diffraction pattern containing peaks at the following 2θ values: 12.4° , 14.9° , 16.7° , 18.0° , 21.6° , 25.4° , 26.6° and 28.9° .

In some embodiments the crystalline form has an x-ray powder diffraction pattern containing peaks at the following 2θ values: 12.4° , 14.9° , 16.7° , 18.0° , 19.5° , 21.6° , 23.8° , 25.4° , 26.6° and 28.9° .

The 2θ values described herein may be in a range that is $\pm 0.2^\circ$ of the specified value, preferably within $\pm 0.1^\circ$ of the specified values.

The crystalline form may have an x-ray diffraction pattern containing one or more peaks at the following 2θ values: 12.36° , 14.89° , 16.71° , 18.03° , 19.51° , 21.62° , 23.77° , 25.44° , 26.61° , and 28.89° . The 2θ values may be in a range that is $\pm 0.02^\circ$ of the specified value, preferably within $\pm 0.01^\circ$ of the specified values.

The crystalline form may be characterized by any combination of three or more peaks selected from the ten peaks described above, such as any combination of 4 or more, such as any combination of 5 or more.

A representative powder XRPD pattern of the crystalline form of a compound of Formula (I) is shown in Figure 10 (labelled "particles", which is the trace with a peak at 14.888°). Main peaks in the XRPD pattern of the bulk and crystalline form, respectively, are assigned in Table 30.

An x-ray diffraction pattern may be measured using a Cu anode, such as with a wavelength of 1.54 Å. The K-α1 value may be 1.5406 Å, K-α2 may be 1.54443 Å, and K-β may be 1.39225 Å. The K-α2/K-α1 intensity ratio may be 0.5, and optionally with a K-α2 shift of 0. X-ray powder diffraction may be carried out with a K-β filter material of Ni, such as at a filter thickness of 0.02 mm. X-ray powder diffraction may be measured in reflection mode and may be carried out at room temperature, such as at 25 °C. X-ray powder diffraction may be carried out with a scanning time of 30 minutes, with a scan range $5^\circ < 2\theta < 40^\circ$ and with a step size of 0.013° . X-ray powder diffraction may be carried out with a Goniometer Radius of 240 mm.

The crystalline form of a compound of Formula (I) may also be characterized using Differential Scanning Calorimetry (DSC). When subject to DSC, with a heating rate of 5 °C/min to a heat of 134 °C, such as when followed by cooling down to 20 °C at a rate of 17 °C/min, the crystalline form of the present invention may have an onset melting temperature, $T_{m(\text{onset})}$ from 121 °C to 122 °C. The onset melting temperature, $T_{m(\text{onset})}$, is preferably the onset melting temperature during the first cycle.

The onset melting temperature is the temperature corresponding to the beginning of a phase transition detected during DSC. An onset melting temperature of 122 °C, for example, may be in the range of 122.0 ± 0.5 °C.

The onset melting temperature of the crystalline form may be in the range of 121.8 ± 0.5 °C, such as 121.8 ± 0.3 °C.

The crystalline form may also be characterized using Fourier Transform Infrared (FTIR) spectroscopy. Characteristic IR signals include a doublet at around $1,678\text{ cm}^{-1}$. The doublet peak may be in the range of $1,600$ to $1,700\text{ cm}^{-1}$, such as about $1,650$ to $1,700\text{ cm}^{-1}$, such as about $1,670$ to $1,690\text{ cm}^{-1}$, such as about $1,675$ to $1,685\text{ cm}^{-1}$. The doublet peak may around $1,678\text{ cm}^{-1} \pm 10\text{ cm}^{-1}$, such as $1,678\text{ cm}^{-1} \pm 5\text{ cm}^{-1}$, such as $1,678\text{ cm}^{-1} \pm 2\text{ cm}^{-1}$, such as $1,678\text{ cm}^{-1} \pm 1\text{ cm}^{-1}$. The doublet peak may have a mid-point in a range described above, or the doublet peak may be contained within a range described above.

The crystalline form may have a peak in an infrared spectrum at $1,460$ to $1,500\text{ cm}^{-1}$ and/or at $1,400$ to $1,440\text{ cm}^{-1}$. Preferably, there is a peak in the infrared spectrum at $1,470$ - 90 cm^{-1} , such as around $1,482\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 10\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 5\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 2\text{ cm}^{-1}$, such as around $1,482\text{ cm}^{-1} \pm 1\text{ cm}^{-1}$. In addition or alternatively, there may be a peak in the infrared spectrum at $1,405$ - $1,425\text{ cm}^{-1}$, such as around $1,416\text{ cm}^{-1}$, such as around $1,416\text{ cm}^{-1} \pm 10\text{ cm}^{-1}$, such as around $1,416\text{ cm}^{-1} \pm 5\text{ cm}^{-1}$, such as around $1,416\text{ cm}^{-1} \pm 2\text{ cm}^{-1}$, such as around $1,416\text{ cm}^{-1} \pm 1\text{ cm}^{-1}$.

A peak in a FTIR spectrum may be a peak in the transmittance spectrum. FTIR spectroscopy may be carried out using a diamond accessory, such as using a Perkin Elmer Spectrum Two FT-IR spectrometer in Attenuated Total Reflectance (ATR) mode. FTIR spectra may be acquired with scanning from 4,000 to 450 cm^{-1} , such as with 32 scans.

The crystalline form of piperlongumine has good solubility in a range of solvents. The crystalline form of piperlongumine may have good solubility in pharmaceutically acceptable excipients. A high concentration of the particles can be obtained in a suspension or a hydrogel. The crystalline form has a good release profile when provided in a pharmaceutical composition, and also has good stability.

The crystalline form may be provided as particles as described herein.

Salts, Solvates and Other Forms

Piperlongumine as described herein, such as when provided in a pharmaceutical composition, may be a solvate or a salt of piperlongumine.

Examples of salts of piperlongumine include all pharmaceutically acceptable salts, such as, without limitation, acid addition salts of strong mineral acids such as HCl and HBr salts and addition salts of strong organic acids such as a methanesulfonic acid salt. Further examples of salts include sulphates and acetates such as trifluoroacetate or trichloroacetate.

A reference to piperlongumine, i.e. a compound of Formula (I), is also a reference to a solvate of that compound. Examples of solvates include hydrates, such as mono-hydrates or di-hydrates.

A compound of Formula (I) includes a compound where an atom is replaced by a naturally occurring or non-naturally occurring isotope. In one embodiment the isotope is a stable isotope. Thus, a compound described herein includes, for example deuterium containing compounds and the like. For example, H may be in any isotopic form, including ^1H , ^2H (D), and ^3H (T); C may be in any isotopic form, including ^{12}C , ^{13}C , and ^{14}C ; O may be in any isotopic form, including ^{16}O and ^{18}O ; and the like.

Unless otherwise specified, a reference to a particular compound includes all such isomeric forms, including mixtures (e.g., racemic mixtures) thereof. Methods for the preparation (e.g., asymmetric synthesis) and separation (e.g., fractional and chromatographic means) of such isomeric forms are either known in the art or are readily obtained by adapting the methods taught herein, or known methods, in a known manner.

One aspect of the present invention pertains to compounds in substantially purified form and/or in a form substantially free from contaminants.

In one embodiment, the substantially purified form is at least 50% by weight, e.g., at least 60% by weight, e.g., at least 70% by weight, e.g., at least 80% by weight, e.g., at least 90% by weight, e.g., at least 95% by weight, e.g., at least 97% by weight, e.g., at least 98% by weight, e.g., at least 99% by weight.

Unless specified, the substantially purified form refers to the compound in any stereoisomeric or enantiomeric form. For example, in one embodiment, the substantially purified form refers to a mixture of stereoisomers, i.e., purified with respect to other compounds. In one embodiment, the substantially purified form refers to one stereoisomer, e.g., optically pure stereoisomer. In one embodiment, the substantially purified form refers to a mixture of enantiomers. In one embodiment, the substantially purified form refers to an equimolar mixture of enantiomers (i.e., a racemic mixture, a racemate). In one embodiment, the substantially purified form refers to one enantiomer, e.g., optically pure enantiomer.

In one embodiment, the contaminants represent no more than 50% by weight, e.g., no more than 40% by weight, e.g., no more than 30% by weight, e.g., no more than 20% by weight, e.g., no more than 10% by weight, e.g., no more than 5% by weight, e.g., no more than 3% by weight, e.g., no more than 2% by weight, e.g., no more than 1% by weight.

Unless specified, the contaminants refer to other compounds, that is, other than stereoisomers or enantiomers. In one embodiment, the contaminants refer to other compounds and other stereoisomers. In one embodiment, the contaminants refer to other compounds and the other enantiomer.

In one embodiment, the substantially purified form is at least 60% optically pure (i.e., 60% of the compound, on a molar basis, is the desired stereoisomer or enantiomer, and 40% is the undesired stereoisomer or enantiomer), e.g., at least 70% optically pure, e.g., at least 80% optically pure, e.g., at least 90% optically pure, e.g., at least 95% optically pure, e.g., at least 97% optically pure, e.g., at least 98% optically pure, e.g., at least 99% optically pure.

Pharmaceutical Compositions

The particles and/or crystalline form of piperlongumine as described herein may be provided in a pharmaceutical composition. As such, aspects of the invention relate to pharmaceutical compositions comprising the crystalline compound of Formula (I), which may comprise a population of piperlongumine particles as described herein and/or the crystalline form (i.e. polymorph) described herein.

In a further aspect, the invention provides a pharmaceutical composition comprising an aqueous suspension of from about 5% to about 40% by weight of piperlongumine and:

- (i) from about 5% to about 30% by weight of a poloxamer selected from one or more of poloxamer 407, poloxamer 338, and poloxamer 188; or
- (ii) from about 10% to about 40% by weight of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).

Preferably, piperlongumine is present in the pharmaceutical composition in an amount of about 10% or more by weight of the composition, such as about 15% or more, such as about 20% or more, such as about 25% or more. Preferably, piperlongumine is present in an amount of 40% or less, such as 35% or less, such as 30% or less, such as 25% or less. Piperlongumine may be present in the pharmaceutical composition in a range with upper and lower limits described above, such as about from 10% to about 40% by weight of the composition, such as from about 15% to about 40% by weight of the composition, including from about 15% to about 30% by weight of the composition, such as from about 20% to about 30% by weight of the composition.

In some embodiments the pharmaceutical composition is a suspension comprising a poloxamer. The poloxamer is preferably selected from poloxamer 407 and poloxamer 188. Preferably, the composition comprises from about 10% to about 30% by weight of the composition of a poloxamer, preferably from about 15% to about 25% by weight of the composition of a poloxamer, which is preferably poloxamer 407.

In some embodiments the pharmaceutical composition is a suspension comprising PCL-PVAc-PEG. The PCL-PVAc-PEG may be present in an amount of from about 10% to about 40% by weight of the composition, preferably from about 20% to about 40% by weight of the composition, more preferably from about 25% to about 35% by weight of the composition.

The pharmaceutical composition may be substantially free of bulk piperlongumine, which may have an average particle size of 10 μm or more, such as 12 or more, or a particle size distribution as shown in Figure 2, and/or may be characterized by a powder x-ray diffraction pattern described herein for bulk piperlongumine, such as an x-ray diffraction pattern shown in Figure 9 or Table 30.

The pharmaceutical composition may be used as a medicament. The pharmaceutical composition may be administered alone or together with a second agent or composition. It is preferable to present piperlongumine as a pharmaceutical formulation (e.g., composition, preparation, medicament) comprising the particles and/or crystalline form as described herein, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, diluents, excipients, adjuvants, fillers, buffers, preservatives, anti-oxidants, lubricants, stabilisers, solubilisers, surfactants (e.g., wetting agents), masking agents, colouring agents, flavouring

agents, and sweetening agents. The formulation may further comprise other active agents, for example, other therapeutic or prophylactic agents.

Preferably, the pharmaceutical composition comprises, in addition to a compound of Formula (I), one or more of a pharmaceutically acceptable carrier, diluent or excipient, which may be as described herein such as described for the suspension or hydrogel, such as surfactant and/or a polymer.

The pharmaceutical composition may have a pH in the range of about pH 4 to pH 8, such as about pH 5 to pH 7, such as about pH 5, about pH 6 or about pH 7. Preferably, the pharmaceutical composition comprises, in addition to piperlongumine, a buffer to maintain the pH of the composition at a desired level. The buffer may be an acetate, phosphate, HEPES, TRIS, or citrate buffer. The buffer may be suitable for maintaining the pH in a range as described above.

The pharmaceutical composition may be lyophilized.

The present invention provides pharmaceutical compositions, as defined above, and also methods of making a pharmaceutical composition comprising admixing piperlongumine as described herein, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, e.g., carriers, diluents, excipients, etc. If formulated as discrete units (e.g., tablets, etc.), each unit contains a predetermined amount (dosage) of the compound. The composition optionally further comprises the second active agent in a predetermined amount.

The term "pharmaceutically acceptable," as used herein, pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, diluent, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

Suitable carriers, diluents, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990; and Handbook of Pharmaceutical Excipients, 5th edition, 2005.

The formulations may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association particles of piperlongumine or the crystalline form of piperlongumine with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into

association the compound with carriers (e.g., liquid carriers, finely divided solid carrier, etc.), and then shaping the product, if necessary.

Formulations may suitably be in the form of liquids, solutions (e.g., aqueous, non-aqueous), suspensions (e.g., aqueous, non-aqueous, nanosuspensions), gels, hydrogels, pastes, emulsions (e.g., oil-in-water, water-in-oil), elixirs, syrups, electuaries, mouthwashes, drops, tablets (including, e.g., coated tablets), granules, powders, lozenges, pastilles, capsules (including, e.g., hard and soft gelatin capsules), cachets, pills, ampoules, boluses, suppositories, pessaries, tinctures, ointments, creams, lotions, oils, foams, sprays, mists, or aerosols.

Preferably, the formulations are in the form of suspensions or hydrogels.

Formulations may suitably be provided as a patch, adhesive plaster, bandage, dressing, or the like which is impregnated with one or more compounds and optionally one or more other pharmaceutically acceptable ingredients, including, for example, penetration, permeation, and absorption enhancers. Formulations may also suitably be provided in the form of a depot or reservoir.

The crystalline compound of Formula (I) as described herein may be dissolved in, suspended in, or admixed with one or more other pharmaceutically acceptable ingredients.

In some embodiments, the pharmaceutical composition comprises the crystalline compound dispersed in a carrier comprising one or more of a surfactant and a polymer. The carrier may comprise a surfactant and/or a polymer. In some embodiments, the carrier comprises a polymer and optionally a surfactant. In some embodiments, the carrier comprises a one or more of a surfactant, and optionally a polymer. Preferably, the crystalline compound is present in the composition in an amount from about 10% to about 40% by weight of the composition, such as from about 15% to about 30% by weight of the composition, such as from about 20% to about 30% by weight of the composition. The crystalline compound may be present at a concentration of more than 1 mg/mL, such as at least 2 mg/mL, such as at least 3 mg/mL of the composition.

Preferred polymers include copolymers, such as poloxamers and polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer.

The pharmaceutical composition may comprise a poloxamer, which may be selected from poloxamer 407, poloxamer 338, and poloxamer 188. These poloxamer(s) may be present in the pharmaceutical composition from about 10% to about 40% by weight of the composition, or from about 10% to about 30% by weight of the composition, or from about 15% to about 25% by weight of the composition. The poloxamer may be poloxamer 407.

The pharmaceutical composition may comprise polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG). Preferably, PCL-PVAc-PEG is present from about 10% to about 40% by weight of the composition, such as from about 20% to about 40% by weight of the composition, or from about 25% to about 35% by weight of the composition.

A pharmaceutical composition comprising one or more of a poloxamer, or polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG) may be provided as a hydrogel as described herein.

The pharmaceutical composition, which may be in the form of a suspension and/or a hydrogel, may be injectable through a needle with a gauge size of 14 G or more, such as 16 G or more, such as 18 G or more. The particles may be injectable through a needle with a gauge size of 14 G to 30 G, such as 14 G to 25 G, such as 16 G to 20 G, such as 18 G. Preferably, the pharmaceutical composition is a syringeable suspension that is capable of forming a gel at human body temperature.

Formulations suitable for administration to a surgical or resection cavity include liquids, solutions, (e.g., aqueous, non-aqueous), suspensions (e.g., aqueous, non-aqueous, nanosuspensions), gels, hydrogels, pastes, depots, and reservoirs.

Formulations suitable for non-oral transmucosal administration include liquids, solutions (e.g., aqueous, non-aqueous), suspensions (e.g., aqueous, non-aqueous, nanosuspensions), emulsions (e.g., oil-in-water, water-in-oil), suppositories, pessaries, gels, pastes, ointments, creams, lotions, oils, as well as patches, adhesive plasters, depots, and reservoirs.

Formulations suitable for transdermal administration include gels, hydrogels, pastes, ointments, creams, lotions, and oils, as well as patches, adhesive plasters, bandages, dressings, depots, and reservoirs.

Tablets may be made by conventional means, e.g., compression or moulding, optionally with one or more accessory ingredients.

Ointments are typically prepared from the compound and a paraffinic or a water-miscible ointment base.

Emulsions are typically prepared from the compound and an oily phase, which may optionally comprise merely an emulsifier (otherwise known as an emulgent), or it may comprise a mixture of at least one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier which acts as a stabiliser. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabiliser(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat

make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Formulations suitable for intranasal administration, where the carrier is a liquid, include, for example, nasal spray, nasal drops, or by aerosol administration, include aqueous or oily solutions of the compound. As an alternative method of administration, a dry powder delivery may be used as an alternative to aerosols.

Formulations suitable for intranasal administration, where the carrier is a solid, include, for example, those presented as a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose.

Formulations suitable for pulmonary administration (e.g., by inhalation or insufflation therapy) include those presented as an aerosol spray from a pack, with the use of a suitable propellant, such as dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, carbon dioxide, or other suitable gases. Additionally or alternatively, a formulation for pulmonary administration may be formulated for administration from a dry powder inhaler. For example, the formulation may be provided with carriers or liposomes to provide a suitable particle size to reach the appropriate parts of the lung, to aid delivery of an appropriate dose to enhance retention in the lung tissue.

Formulations suitable for parenteral administration (e.g., by injection), include aqueous or non-aqueous, isotonic, pyrogen-free, sterile liquids (e.g., solutions, suspensions), in which the compound is dissolved, suspended, or otherwise provided (e.g., in a liposome or other microparticulate). Such liquids may additionally contain other pharmaceutically acceptable ingredients, such as anti-oxidants, buffers, preservatives, stabilisers, bacteriostats, suspending agents, thickening agents, and solutes which render the formulation isotonic with the blood (or other relevant bodily fluid) of the intended recipient. Examples of excipients include, for example, water, alcohols, polyols, glycerol, vegetable oils, and the like. Examples of suitable isotonic carriers for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

Typically, the concentration of the compound in a liquid, suspension, gel or hydrogel, for example, is from about 1 ng/mL to about 1 mg/mL, for example from about 10 ng/mL to about 500 mg/mL, for example from about 10 mg/mL to about 500 mg/mL. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

Suspensions and Hydrogels

In some aspects, the invention provides pharmaceutical compositions comprising an active agent, in particular piperlongumine. The pharmaceutical composition may comprise, or may be, a suspension or a hydrogel containing the piperlongumine dispersed therein. The chemotherapeutic agent may be a compound of Formula (I), such as particles or the polymorph described herein.

The compound of Formula (I), may be provided in a suspension. A further aspect of the invention thus relates to a pharmaceutical composition comprising a suspension of the particles and/or crystalline form of piperlongumine, which is dispersed in a carrier comprising one or more of a surfactant and a polymer. Preferably, the suspension is an aqueous suspension.

A surfactant is an amphiphilic molecule having a hydrophilic portion and a hydrophobic portion.

A surfactant may be an ionic surfactant or a non-ionic surfactant.

Examples of suitable nonionic surfactants include polymers, such as polyoxyethylated fatty acids, polysorbates and polyethylene oxide derivatives.

Polyoxyethylated fatty acids include polyoxyethylated 12-hydroxystearic acid (e.g. Solutol HS15), and polyoxyethylated capric acid and caprylic acid (e.g. Labrasol).

Polysorbates include polysorbate 20 (Tween 20), polysorbate 60 (Tween 60) and polysorbate 80 (Tween 80).

Polyethylene oxide derivatives include hydrocarbon derivatives of polyethylene oxide, such as Triton X-100 and Triton X-114.

Examples of suitable ionic surfactants include sodium dodecyl sulfate (SDS), ammonium dodecyl sulfate and sodium laureth sulfate.

Suitable polymers in the suspension and/or hydrogel include cellulose and derivatives thereof; vinyl polymers; and alginates.

Derivatives of cellulose include methylcellulose, carboxymethylcellulose, hydroxyethylcellulose (HEC) and hydroxypropyl methylcellulose. Of these, hydroxypropyl methylcellulose (HPMC) may be preferred, such as HPMC 606.

Vinyl polymers include polyvinylpyrrolidone (PVP K25, PVP K30, PVP K60, PVP K90 where the K-value denotes an intrinsic viscosity of the polymer derived from the relative viscosity of

an aqueous solution measured at 25 °C); poly(1-vinylpyrrolidone-co-vinyl acetate) (copovidone), polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (e.g. Soluplus), polyvinyl alcohol; and polyvinyl acetate.

Alginates include alginate salts such as sodium alginate and potassium alginate.

The composition may further comprise a solvent, such as water and/or an alcohol. Preferably, the composition comprises an aqueous solvent. Suitable vehicle compositions include Solutol HS15 in water, such as in an amount of 1 to 10% w/v, such as about 5% w/v.

The composition preferably comprises a surfactant and one or more polymers, for example a surfactant such as SDS or a polysorbate together with one or more polymers as described herein. Preferably, the surfactant is SDS.

In some embodiments the composition comprises HPMC, together with a vinyl polymer such as polyvinylpyrrolidone (PVP) and also SDS as the surfactant. These components of the composition may be provided in water, each independently at a concentration from 0.01% to 5% w/v, such as 0.1% to 1% w/v.

A preferred composition comprises HPMC 606/PVP K30/SDS, more preferably at a concentration of 0.5%/0.5%/0.1% w/v.

A suspension may comprise piperlongumine particles and/or the crystalline form of piperlongumine from about 10% to about 40% by weight of the composition, such as from about 15% to about 30% by weight of the composition, including from about 20% to about 30% by weight of the composition.

A suspension may comprise piperlongumine particles and/or the crystalline form of piperlongumine in an amount of 1 mg/mL or more, such as 2 mg/mL or more, such as 3 mg/mL or more, such as 10 mg/mL or more, such as 25 mg/mL or more, such as 30 mg/mL or more, such as 40 mg/mL or more, such as 50 mg/mL or more.

The suspension preferably has a pH of from about 4 to 8, such as from about 5 to 7.

The polymer in the suspension may be selected from a poloxamer, a vinyl polymer, a protein and a polysaccharide. In these embodiments the pharmaceutical composition may be a hydrogel, wherein the crystalline form and/or particles of piperlongumine is a suspension, such as a nanosuspension within the hydrogel.

Preferably, the composition forms a gel at human body temperature, such as around 35 °C to around 40 °C, such as around 37 °C or 32 °C.

Thus, in a further aspect the invention provides a pharmaceutical composition comprising a hydrogel with the particles and/or crystalline form of piperlongumine as described herein. Additionally or alternatively, the invention provides a pharmaceutical composition comprising a hydrogel with piperlongumine.

Poloxamers include poloxamer 188, poloxamer 237, poloxamer 338 and poloxamer 407. Of these, poloxamer 188, poloxamer 407, and a combination thereof may be preferred. The polymer may display a thermoresponsive behaviour that is suitable for forming thermoreversible gels.

Vinyl polymers include polyvinylpyrrolidone (PVP K25, PVP K30, PVP K60, PVP K90 where the K-value denotes an intrinsic viscosity of the polymer derived from the relative viscosity of an aqueous solution measured at 25 °C); polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (e.g. Soluplus); poly(1-vinylpyrrolidone-co-vinyl acetate) (copovidone); polyvinyl alcohol; and polyvinyl acetate. Of these, polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (e.g. Soluplus) may be preferred.

Suitable proteins include collagen and derivatives thereof, such as gelatin.

Suitable polysaccharides include starch, cellulose, alginate, chitosan, hyaluronic acid, and derivatives thereof. A derivative may be a salt, such as a sodium, potassium or lithium salt.

Cellulose derivatives include methylcellulose (MC), hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC), and carboxymethylcellulose salts such as sodium carboxymethylcellulose. Of these, hydroxypropylmethylcellulose (HPMC) may be preferred.

The composition, which may be a hydrogel, may comprise two or more polymers. For example, the composition may comprise a first polymer that is a poloxamer, and a second polymer such as hyaluronic acid or a cellulose derivative.

Preferably, the pharmaceutical composition comprises piperlongumine, such as the crystalline compound, dispersed in a carrier comprising a polymer.

In some embodiments the composition comprises one or more of poloxamer 188 and poloxamer 407, and optionally further comprises hyaluronic acid. In some preferred embodiments, the composition comprises poloxamer 407, optionally wherein the composition comprises 15-25% w/w of poloxamer 407, such as 18-20% w/w of poloxamer 407, such as about 18% w/w or about 20% w/w of poloxamer 407.

In some embodiments the composition comprises polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (e.g. Soluplus). In some preferred embodiments, the

composition comprises 25-35% w/w of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer, such as 27-30% w/w of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer, such as about 27.5% w/w polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer. In some preferred embodiments, the composition comprises 10% w/w or more of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer, such as 10-30% w/w or more of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer.

The composition may further comprise one or more additives selected from PEG and polyacrylic acids and derivatives thereof.

PEG is polyethylene glycol, which may have a molecular weight in the range of 300 to 10,000 g/mol. Examples include PEG300, PEG400, PEG800 and PEG1000.

The composition may be a hydrogel that further comprises a solvent such as water or an alcohol, preferably wherein the solvent is water.

The hydrogel may comprise a buffer to maintain the pH at a desired level. The buffer may be an acetate, phosphate, HEPES, TRIS, or citrate buffer, and may be suitable for maintaining the pH in the range of about pH 4 to pH 8, such as about pH 5 to pH 7, such as about pH 5, about pH 6 or about pH 7.

The suspensions and/or hydrogels may comprise a crystalline compound, such as of Formula (I), in particulate form as described herein. Preferably the suspensions and/or hydrogels comprise particles consisting of, or consisting essentially of, a crystalline form of piperlongumine. Typically, the suspensions and/or hydrogels are substantially free of particles, or nanoparticles, which comprise piperlongumine together with one or more components other than piperlongumine.

Additionally or alternatively, the invention provides a pharmaceutical composition comprising a thermoresponsive hydrogel and a chemotherapeutic agent dispersed in the hydrogel, wherein the pharmaceutical composition further comprises:

- one or more poloxamers, wherein the total amount of poloxamers is from about 15% to about 20% by weight of the pharmaceutical composition;
- from about 0.5% to about 2% by weight of polyvinylpyrrolidone;
- from about 18% to about 20% by weight of polyethylene glycol;
- and from about 0.2 to 0.3% by weight of a polysorbate.

The pharmaceutical composition may be a thermoresponsive hydrogel.

The chemotherapeutic agent may be a compound of Formula (I). The compound of Formula (I) may be bulk piperlongumine as described herein, or may be particles or a polymorph of

piperlongumine as described herein. In some embodiments, the compound of Formula (I) dispersed in the thermoresponsive hydrogel is bulk piperlongumine.

In some embodiments, the compound of Formula (I) has an average particle size of 10 μm or more, such as 12 μm or more. The compound of Formula (I) may have a D50 value of 10 μm or more, such as 12 μm or more. The compound of Formula (I) may have a particle size distribution as shown in Figure 2. The compound of Formula (I) may be a crystalline form characterized by a powder x-ray diffraction pattern comprising the 2θ values selected from $11.3 \pm 0.1^\circ$, $14.5 \pm 0.1^\circ$, and $25.9 \pm 0.1^\circ$. The crystalline form may be characterized by a powder x-ray diffraction pattern comprising the 2θ values selected from $11.3 \pm 0.1^\circ$, $14.5 \pm 0.1^\circ$, $21.3 \pm 0.1^\circ$, $25.9 \pm 0.1^\circ$, and $31.9 \pm 0.1^\circ$. The crystalline form may be characterized by a powder x-ray diffraction pattern described herein for bulk piperlongumine, such as an x-ray diffraction pattern shown in Figure 9 or Table 30.

In other embodiments, the compound may be a crystalline form of Formula (I) having a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$, or the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$. The compound may have an average particle size of less than about 5 μm , such as less than about 2 μm , such as less than about 1 μm ,

The chemotherapeutic agent is preferably piperlongumine (a compound of Formula (I)).

In some embodiments the pharmaceutical composition comprises up to about 5% by weight of the chemotherapeutic agent, such as up to about 3% by weight of the chemotherapeutic agent, such as about 2% by weight of the chemotherapeutic agent. In some embodiments the pharmaceutical composition comprises 0.5% or more by weight of the chemotherapeutic agent, such as 1% or more by weight, such as 2% or more by weight. In some embodiments the pharmaceutical composition comprises an amount of the chemotherapeutic agent in a range with upper and lower limits as described above, such as from about 0.5% by weight to about 5% by weight, including from about 1% by weight to about 3% by weight, such as about 2% by weight. Preferably, in these embodiments the chemotherapeutic agent is a compound of Formula (I).

The one or more poloxamers may be selected from poloxamer 407 and poloxamer 188. In some embodiments, the pharmaceutical composition comprises poloxamer 407. In some embodiments, the pharmaceutical composition comprises poloxamer 188. In some preferred embodiments, the pharmaceutical composition comprises poloxamer 407 and poloxamer 188, wherein the total amount of poloxamer 407 and poloxamer 188 is from about 15% to about 20% by weight of the pharmaceutical composition, such as from about 17% to 20% by weight, such as from about 17.5% to about 20% by weight of the pharmaceutical composition.

The pharmaceutical composition may comprise from about 10% by weight to about 20% by weight of poloxamer 407. Preferably, the pharmaceutical composition comprises from about 14% by weight to about 16% by weight of poloxamer 407, such as about 15% by weight of poloxamer 407.

The pharmaceutical composition may comprise from about 1% by weight to about 7% by weight of poloxamer 188, such as from about 2.5% by weight to about 5% by weight of poloxamer 188. In some preferred embodiments, the pharmaceutical composition comprises about 2.5% by weight of poloxamer 188, or about 5% by weight of poloxamer 188.

The pharmaceutical composition may comprise about 1% by weight of polyvinylpyrrolidone.

The polyethylene glycol in the pharmaceutical composition may have molecular weight of from about 350 to about 450 g/mol, such as from about 380 to about 420 g/mol. The polyethylene glycol may have an average molecular weight of about 400 g/mol, such as a number average molecular weight. The polyethylene glycol may be polyethylene glycol 400. The pharmaceutical composition may comprise from about 19% to about 20% of the polyethylene glycol, such as from about 19.5% to about 20% by weight of the polyethylene glycol, such as about 19.8% by weight of the polyethylene glycol.

The polysorbate may be polysorbate 80. The pharmaceutical composition may comprise about 0.25% by weight of the polysorbate.

In some preferred embodiments, the pharmaceutical composition comprises:

- 15% by weight of poloxamer 407;
- 2.5% by weight of poloxamer 188;
- 1% by weight of polyvinylpyrrolidone;
- 19.75% by weight of polyethylene glycol;
- 0.25% by weight of a polysorbate, such as polysorbate 80.

In some preferred embodiments, the pharmaceutical composition comprises:

- 15% by weight of poloxamer 407;
- 5% by weight of poloxamer 188;
- 1% by weight of polyvinylpyrrolidone;
- 19.75% by weight of polyethylene glycol;
- 0.25% by weight of a polysorbate, such as polysorbate 80.

In these embodiments, the chemotherapeutic agent may be a compound of Formula (I), such as particles of a compound of Formula (I) with an average particle size of 10 μm or more.

Uses and Methods of Treatment

The crystalline compound of Formula (I), particles, and pharmaceutical composition containing these, are suitable for use in methods of treatment and prophylaxis. The compounds and, particles and formulations may be administered to a subject in need thereof.

Piperlongumine as described herein is suitable for use in a method of treatment of the human or animal body by therapy. In some aspects of the invention, the pharmaceutical composition may be administered to a mammalian subject, such as a human, in order to treat a disease, such as cancer.

Another aspect of the present invention pertains to use of crystalline piperlongumine and/or particles in the manufacture of a medicament for use in treatment. In one embodiment, the medicament comprises the crystalline form and/or particles of piperlongumine.

The compounds of the present case may be useful for the treatment of a proliferative disease, such as cancer.

Preferably, piperlongumine as described herein is for the treatment of brain cancer such as glioblastoma. Additionally or alternative, piperlongumine or a pharmaceutical composition described herein may be for the treatment of solid cancers, including sarcoma, such as soft-tissue sarcoma.

The term "treatment," as used herein in the context of treating a condition, pertains generally to treatment and therapy, whether of a human or an animal (e.g., in veterinary applications), in which some desired therapeutic effect is achieved, for example, the inhibition of the progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress, alleviation of symptoms of the condition, amelioration of the condition, and cure of the condition. Treatment as a prophylactic measure (i.e., prophylaxis) is also included. For example, use with patients who have not yet developed the condition, but who are at risk of developing the condition, is encompassed by the term "treatment."

The term "therapeutically-effective amount," as used herein, pertains to that amount of a compound, or a material, composition or dosage form comprising a compound, which is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio, when administered in accordance with a desired treatment regimen.

The term "treatment" includes combination treatments and therapies, in which two or more treatments or therapies are combined, for example, sequentially or simultaneously.

Routes of Administration

A chemotherapeutic agent, such as piperlongumine, which may be in crystalline and/or particulate form as described herein, may be administered to a subject by any convenient route of administration, whether systemically/peripherally or topically (i.e., at the site of desired action).

Routes of administration include, but are not limited to, oral (e.g., by ingestion); buccal; sublingual; transdermal (including, e.g., by a patch, plaster, etc.); transmucosal (including, e.g., by a patch, plaster, etc.); intranasal (e.g., by nasal spray); ocular (e.g., by eyedrops); pulmonary (e.g., by inhalation or insufflation therapy using, e.g., via an aerosol, e.g., through the mouth or nose); rectal (e.g., by suppository or enema); vaginal (e.g., by pessary); parenteral, for example, by injection, including subcutaneous, intracranial, intradermal, intramuscular, intravenous, intraarterial, intracardiac, intrathecal, intraspinal, intracapsular, subcapsular, intraorbital, intraperitoneal, intratracheal, subcuticular, intraarticular, subarachnoid, and intrasternal; by implant of a depot or reservoir, for example, intracranially, subcutaneously or intramuscularly; or to a resection cavity such as an organ resection cavity or a tumour resection cavity.

Preferably, piperlongumine or the compositions as described herein is administered to a subject by a route selected from intracranial, intravenous and transdermal.

Piperlongumine or a composition as described herein may be administered during surgery, whereby piperlongumine or the composition is applied directly into the surgical cavity, for example after tumour removal. For example, piperlongumine or the composition may be administered intracranially to a tumour resection cavity, such as during open brain surgery. Piperlongumine or the composition may be administered intracranially during surgery to a glioblastoma resection cavity.

In alternative embodiments, piperlongumine or a pharmaceutical composition as described herein may be administered to a tumor site, such as by *in situ*-intratumoral injection. The pharmaceutical composition may be for use in a method that does not include surgery.

Dosage

Generally, the methods of the invention comprise administering to a subject an effective amount of piperlongumine as described herein so as to provide a biological effect.

It will be appreciated by one of skill in the art that appropriate dosages of piperlongumine, and compositions comprising piperlongumine, can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects. The selected dosage level will depend on a variety of

factors including, but not limited to, the activity of piperlongumine particles and/or the crystalline form, the route of administration, the time of administration, the rate of excretion of the compound, the duration of the treatment, other drugs, compounds, and/or materials used in combination, the severity of the condition, and the species, sex, age, weight, condition, general health, and prior medical history of the patient. The amount of compound of piperlongumine and route of administration will ultimately be at the discretion of the physician, veterinarian, or clinician, although generally the dosage will be selected to achieve local concentrations at the site of action which achieve the desired effect without causing substantial harmful or deleterious side-effects.

Administration can be effected in one dose, continuously or intermittently (e.g., in divided doses at appropriate intervals) throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the formulation used for therapy, the purpose of the therapy, the target cell(s) being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician, veterinarian, or clinician.

Subject or Patient

The subject/patient may be a chordate, a vertebrate, a mammal, a placental mammal, a marsupial (e.g., kangaroo, wombat), a rodent (e.g., a guinea pig, a hamster, a rat, a mouse), murine (e.g., a mouse), a lagomorph (e.g., a rabbit), avian (e.g., a bird), canine (e.g., a dog), feline (e.g., a cat), equine (e.g., a horse), porcine (e.g., a pig), ovine (e.g., a sheep), bovine (e.g., a cow), a primate, simian (e.g., a monkey or ape), a monkey (e.g., marmoset, baboon), an ape (e.g., gorilla, chimpanzee, orangutan, gibbon), or a human.

Furthermore, the subject/patient may be any of its forms of development, for example, a fetus. The present inventors have found that the conjugates of the invention may not be taken up by mammalian cells, and this includes rapidly dividing mammalian cells, such as those within the developing fetus.

Thus, the conjugates of the invention may be used to treat pregnant subjects and subjects who are intending to conceive.

In one preferred embodiment, the subject/patient is a human.

It is also envisaged that the invention may be applied on a non-human animal having a microbial infection. A non-human mammal may be a rodent. Rodents include rats, mice, guinea pigs, chinchillas and other similarly-sized small rodents used in laboratory research.

Kits

Disclosed herein is a kit comprising:

- (a) a crystalline form of piperlongumine and/or particles of piperlongumine as described herein, or a pharmaceutical composition comprising the crystalline form and/or particles of piperlongumine; and
- (b) a pharmaceutically acceptable carrier, diluent or excipient.

The pharmaceutically acceptable carrier, diluent or excipient may be selected from those described herein for preparing a suspension or hydrogel comprising piperlongumine.

In some embodiments, the kit is for preparing a suspension of piperlongumine particles, comprising:

- (a) The crystalline form and/or particles of piperlongumine; and
- (b) a surfactant, and optionally one or more of a polymer and a solvent.

Preferences for the surfactant and the polymer are as described herein.

The solvent may be an aqueous solvent, or an alcohol, such as methanol or ethanol. Preferably, the solvent is water.

In some embodiments, the kit is for preparing a hydrogel comprising piperlongumine, the kit comprising:

- (a) the crystalline form of piperlongumine and/or particles of piperlongumine described herein; and
- (b) a polymer, which is optionally selected from a poloxamer, a vinyl polymer, a protein and a polysaccharide.

The kit for preparing a hydrogel may comprise a suspension of piperlongumine particles, or components for preparing a suspension.

A kit is preferably provided in a suitable container and/or with suitable packaging.

A kit may further comprise instructions for use, e.g., written instructions on how to administer the compound or composition.

The written instructions may also include a list of indications for which piperlongumine is a suitable treatment.

In one embodiment, the kit further comprises a second active agent, or a composition comprising the second active agent. Here, the written instructions may also include a list of

indications for which the second active agent, together with piperlongumine particles and/or crystalline form, is suitable for treatment.

Methods of Preparation

The crystalline form of piperlongumine according to the present invention and/or the piperlongumine particles as described herein can be prepared by methods such as wet milling or controlled expansion of supercritical solution (CESS®).

Particles of piperlongumine, which may be in the crystalline form, may be prepared according to the method as generally described in Pessi *et al* or in US2017231914. For example, the method and in particular parameters such as temperature and pressure can be optimized as described in the “*Materials and Methods*” section of Pessi *et al*. to obtain particles of desired dimensions.

Particles of piperlongumine may be prepared by a method involving dissolution of piperlongumine into supercritical CO₂ under controlled pressure and temperature. The solution may then be driven, such as through a nozzle, from the dissolution vessel into a collection vessel under thermodynamic flow control. During the expansion at the nozzle, the pressure and temperature of supercritical CO₂ may be decreased. Solid CO₂ may then be presented as dry ice flakes, which entraps piperlongumine nanoparticles. After the conclusion of the production phase, CO_{2(s)} is let to sublime, leaving in the collection vessel the pure nanoformed piperlongumine, usually in the form of fine powder.

Preferably the pressure is 20,000 kPa to 60,000 kPa, such 40,000 kPa to 60,000 kPa. The temperature is preferably from 20 °C to 65 °C. These parameters result in good homogeneity, uniformity, crystallinity, and throughput and allow particles of piperlongumine to be obtained with preferred particle size whilst avoiding degradation.

The present invention also provides a method of preparing particles of piperlongumine by wet milling, comprising the steps of:

- (i) providing a suspension comprising piperlongumine;
- (ii) contacting the suspension with a plurality of beads; and
- (iii) milling the suspension at a speed of 200 RPM or higher.

Steps (i) to (iii) are preferably performed in order.

The beads are preferably silica beads. By “silica beads” it is meant beads that comprise silica and optionally one or more materials other than silica. The beads may comprise silica and zirconia.

The beads may be about 0.01 to 10 mm in size, such as about 0.05 to 2 mm in size, such as 0.1 to 2 mm in size, such as about 0.1-1 mm in size. The bead size may be the average diameter of a population of beads. The beads may be provided in the sample at a piperlongumine:bead ratio in the range of about 1:5 to 1:100, such as a ratio of about 1:10 to 1:50, such as a ratio of about 1:25 to 1:50, such as a ratio of about 1:45.

The suspension may contain, in addition to piperlongumine, one or more of a solvent or anti-solvent.

An anti-solvent is a solvent in which a substance has little or no solubility. A suitable anti-solvent for use in a wet milling process described here includes hydrocarbons, which may optionally be halogenated. For example, the anti-solvent may be selected from pentane, hexane, cyclohexane, heptane, cycloheptane, octane, cyclooctane, nonane, decane and halogenated derivatives thereof. By "halogenated derivative" of a hydrocarbon it is meant a hydrocarbon where one or more hydrogen atoms are replaced with a halogen atom, such as fluorine, chlorine or bromine, preferably fluorine.

In some embodiments, the anti-solvent is a fluoroalkane or a hydrofluoroalkane. Preferred anti-solvent include perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane, perfluorononane, perfluorodecane, and decafluoropentane. More preferably, the anti-solvent is decafluoropentane, such as 2H,3H-decafluoropentane. In these embodiments, the beads are preferably 1 mm in size. The beads may be zirconia/silica beads that may be used at a piperlongumine:bead ratio of about 1:10.

Suitable solvents may include water and alcohols, such as methanol or ethanol. Preferably, an aqueous solvent is used, which may further include an alcohol such as ethanol and/or an antifoaming agent, such as simethicone.

Preferably, the suspension provided in step (i) may comprise piperlongumine in a vehicle composition as described herein, which comprises one or more of a surfactant and a polymer. For example, preferred vehicle compositions that may be provided in step (i) include HPMC 606/PVP K30/SDS (such as at 0.5%/0.5%/0.1% w/v in aqueous solvent) and Solutol HS15 (such as at 5% w/v in aqueous solvent). In these embodiments, the beads are preferably silica beads that may be 0.1-0.5 mm in size and used at a piperlongumine:bead ratio of about 1:45.

The sample may be mixed in step (iii) at a speed in the range of about 200 RPM to 2,000 RPM, such as about 300 RPM to 1,000 RPM, such as about 400 to 800 RPM.

Step (iii) is carried out until a suspension is obtained. A suitable end-point may be determined by visual inspection of particle size, such as by light microscopy, light scattering such as dynamic light scattering and scanning electron microscopy (SEM). The total mixing time in

step (iii) may be in the range of about 5 minutes to 10 hours, such as about 10 minutes to 5 hours, such as about 15 minutes to 1 hour, such as about 30 minutes.

Other Embodiments

Each and every compatible combination of the embodiments described above is explicitly disclosed herein, as if each and every combination was individually and explicitly recited. Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

“and/or” where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example “A and/or B” is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described above.

Results and Discussion**Materials**

Details of the materials used in the examples below are summarized in Table 1a and Table 1b.

Table 1a: Materials used.

Material/Equipment	Supplier/Manufacturer	Batch number
Piperlongumine	ABCR	1411544 (product code AB148964)
3,4,5 Trimethoxycinnamic acid	Sigma Aldrich	BCCC8678
Sodium Chloride (NaCl)	Emprove® essential	K51096300919
Potassium Chloride (KCl)	Sigma Aldrich	BCBV5162
Disodium phosphate dehydrate (Na ₂ HPO ₄ ·2H ₂ O)	AnalaR Normapur	16H254111
Monopotassium phosphate (KH ₂ PO ₄)	AnalaR Normapur	16F164122
Sodium hydroxide (NaOH)	VWR	18I274117
Boric acid	Sigma Aldrich	BCBZ7336
Sodium acetate trihydrate	Sigma Aldrich	STBG9663
Acetic acid glacial	VWR	20104.298
PEG 300	Sigma-Aldrich	BCBV0194
PEG 400	Sigma-Aldrich	BCBV7845
Solutol HS15	Sigma-Aldrich	BCBV8994
Glycerol	Sigma-Aldrich	18J164108
Propylene glycol (PG)	Sigma-Aldrich	MKCB0613V
Nicotinamide	Sigma-Aldrich	BCCB6184
SR Polysorbate 80	Croda	0001244433
Poloxamer 188	BASF	GNA19221BT
Poloxamer 407	BASF	GND02121B
Hyaluronic acid (pharma grade FCH 80)	Kikkiman Biochemifa	16005A
Methylcellulose (Methocel A15LV)	Sigma Aldrich	BCBZ7204
Polyvinylpyrrolidone K12 (PVP k12)	BASF	18160916K0
DMA	Sigma Aldrich	STBG1600V

<i>Material/Equipment</i>	<i>Supplier/Manufacturer</i>	<i>Batch number</i>
DMSO	VWR	18J094040
NMP	Sigma Aldrich	SZBG1600V
Super Refined Span 20	Croda	0001458710
Span 80	Sigma Aldrich	BCBT7716
Captex 300 EP/NF	Abitec	180228-7
Capmul PG-8 NF	Abitec	170821-P
Labrasol ALF	Gattefossé	175109
Super Refined Sesame Oil	Croda	0001240233
Labrafil M1944CS	Gattefossé	166088
Labrafil lipophile WL1349	Gattefossé	162835
Capryol 90	Gattefossé	165985
Lauroglycol 90	Gattefossé	164334
Miglyol 812	IOI oleochemical	170929
Miglyol 840	IOI oleochemical	170701
Phosal 53 MCT	Lipoid	368201-3180033/916
Phosal 50 PG	Lipoid	368214-3180045/910
Vitamin E TPGS	Sigma Aldrich	BCCC6476
Gelucire 44/14	Gattefossé	175971
Maisine CC	Gattefossé	175006
Capmul MCM C8 EP/NF	Abitec	171203-6
Capmul MCM EP/NF	Abitec	180430-8
Peceol	Gattefossé	176555
Plurol Oleique CC 497	Gattefossé	164033
Labrafil M2130 CS	Gattefossé	171968

Table 1b: Excipients used for hydrogel composition screening

Material	Source
Hydroxypropylmethyl cellulose (HPMC)	Alfa Aesar
Poly-2-ethyl-2-oxazoline, Mw = 500,000 g/mol (PEOX)	Sigma
Copovidone	Sigma
Polyethyleneglycol, Mw = 12 000 g/mol (PEG)	Sigma
Chitosan, low molecular weight	Sigma
Chitosan, medium molecular weight	Sigma
Polyvinylpyrrolidone, mw = 360 000 g/mol (PVP)	Sigma
Gelatine	VWR
Carbopol EDT2020	Lubrizol
Carbopol CP980	Lubrizol
Carbopol CP971P	Lubrizol
D-α-tocopherol-PEG1000-succinate (TPGS)	TCI
Polyvinylalcohol (PVA)	Acros
Sodium carboxymethylcellulose (NaCMC)	EMD Millipore
Hydroxyethylcellulose (HEC)	Sigma
Methylcellulose (MC)	Acros
Poloxamer 188 (P188)	PanReac
Poloxamer 338 (P338)	Sigma
Poloxamer 407 (P407)	Spectrum
Soluplus	BASF
Eudragit E PO	Evonik

Methods

Method A: particle size distribution (PSD)

The particle size distribution of the feed API was confirmed using a Sympatec Helos particle size analyser equipped with a RODOS sampling module and ASPIROS dosing system. Table 2 lists the experimental parameters used.

Table 2: Parameters set for PSD.

<i>Parameter</i>	<i>Conditions</i>
Instrument	Sympatec Helos
Software	Sympatec Windox 5.0
Dispersing Accessory	Aspiros
Sample weight Optical concentration	20 mg of sample into a sample vial 3.56%
Lens	R3
Product details	Density: 1.0000 g/cm ³ Shape factor 1.000 Calculation mode: LD
Trigger Condition	Reference measurement: duration 5 s/single Time base: 1 ms Measurement: standard mode Start 0.000 s opt. concentration $\geq 1.0\%$ Stop after 5 s opt. concentration $\leq 1.0\%$ Trigger time out 60 s
Disperser Condition	Disperser Type: RODOS Injector: 4 mm Primary pressure: 4 bar
Feeder	Aspiros speed 10 mm/s

Method B: scanning electron microscopy (SEM) – bulk piperlongumine

Particle shape and surface topography were examined by scanning electron microscopy (SEM). Approximately 1 mg of powder (feed piperlongumine and micronised) was mounted onto an aluminium stub using double-sided carbon adhesive tape, sputter coated to 10 nm with gold in a Quorum Q150ES sputter coater (Quorum Technologies Ltd UK) and photographed using a Tescan Vega 3 Scanning Electron Microscope (Tescan Bruno, Czech Republic). Magnification details and beam voltages are as indicated with the scanning electron micrographs.

Method C: scanning electron microscopy (SEM) and particle size distribution analysis

Scanning electron microscopy (SEM) images from bulk and nanoformed material were taken with a COXEM EM-30PLUS and a Field Emission ZEISS Sigma 300. Briefly, the sample was placed onto an SEM stub partially covered with carbon tape. The sample

was then sputtered with Pt (typical coating thickness of 5 to 8 nm as measured by a QCM sensor and assuming a density of 21.45 g/cm³ for Pt). Images were taken at various magnifications with acceleration voltages of 10 to 15 kV, for the analyses done in COXEM EM-30PLUS and 10 to 5 kV for analyses done in ZEISS Sigma 300.

For PSD evaluation, the particles were counted from images of appropriate magnification and identified as follows. The pictures were chosen to represent the highest number of individual particles possible that are visually identifiable and classifiable through the attribution of measurable ellipsoid shapes. For nanoformed piperlongumine, the magnifications that allow this classification were between x10.000 and x25.000. Each histogram was based on, at least, 60 randomly chosen particles per image counted. The evaluated statistics for SEM imaging are:

D50: Median particle size, i.e., in the case of ellipsoids, is the geometric average of the length of the two main axes that define the ellipsoid (xy-coordinate). The value is the same as the diameter for a circle that would have the same total area. This value is given in nanometres (nm).

Span: This value gives a statistic on the width of the particle size distribution. The span is defined as $(D90 - D10)/D50$. Here D10 refers to the 10-percentile mark, i.e., 10% of all the particles are smaller than this value (and conversely, 90% of the particles are larger than this value). D90 is defined similarly, i.e., 90% of all particles are smaller than this value. The span value is normalized to the D50 value. The span gives information about how far apart the smallest and largest particles are from each other.

Method D: dynamic light scattering (DLS)

The particle size of the nanosuspensions prepared as described in General Method D was analysed by dynamic light scattering (DLS). The nanosuspensions were diluted 1000 fold in type I water, 500 µL of diluted sample was transferred into a semi-micro disposable cuvette and the particle size measured using a Zetasizer ZS instrument (Malvern Panalytical, Malvern, UK). The measurements were performed in backscattering mode (173°) at 25 °C. An equilibration time of 120 seconds was applied prior analysis. Three replicates were obtained for each sample and the average calculated using the software Zetasizer v 7.11. For samples with a polydispersity index (PDI) less than 0.2, the sample can be considered monodisperse and size can be determined by cumulants analysis (z-average), for samples with a PDI greater than 0.2, the size was determined using the distribution algorithm. The ζ-potential was also calculated using the Zetasizer ZS instrument applying the following procedure. 1 mL of diluted sample (1000 fold in type I water) were transferred into a folded capillary cell (DTS1060, Malvern Panalytical), equilibrated at 25 °C for 120 seconds prior the analysis. Three measurements were performed for each sample and the average was calculated using the software Zetasizer v 7.11. The Smoluchowski model was applied for data calculation.

Conventionally, a ζ -potential of ± 30 mV is considered an indication that the sample has good stability against aggregation. However, considering the presence of polymers in the vehicles which could offer stabilisation by steric hindrance, a ζ -potential of ± 20 mV can be considered acceptable.

Method E: determination of the sol-gel temperature into hydrogels

Hydrogels solutions were prepared in Type I water and acetate buffer and the sol-gel temperature was determined by vial inversion method following the procedure described below. 1 mL of solution was transferred into a 4 mL glass vial. The vial was placed in a water bath and the temperature was increased from RT (~ 22.5 °C) to 39 °C at 2 °C step every 10 minutes. At each step the vials were removed from the water bath and inverted to assess the liquid-gel behaviour. The samples were classified as described in the table below (Table 3). The temperature at which the liquid appears as gel (G) is classified as the sol-gel transition temperature.

Table 3: Classification of sol-gel temperature based on the rheological properties

<i>Rheological properties</i>	<i>Abbreviation</i>	<i>Description</i>
Liquid	L	moving rapidly in the direction of gravity
Viscous liquid	VL	moving slowly down in the direction of gravity
Gel	G (*)	remaining on the bottom of the vial

Method F: X-ray powder diffraction

X-ray powder diffraction (XRPD) was used to evaluate the crystallinity of the material as well as the crystalline structure and possible polymorphisms. Analyses were carried out on a Malvern PANalytical Empyrean powder X-ray diffractometer equipped with a Cu anode (wavelength of 1.54 Å) in reflection mode. The scanning time was 30 min; the scan ranges were between $5^\circ < 2\theta < 40^\circ$ with a step size of 0.013° . The analyte was placed directly in a non-hollow metal holder and covered with Kapton tape. The size of the window was also 5 μm , and the time of scan was 30 min. The background was automatically defined using the background recorded and saved in HighScore software, which was used to obtain the raw data. The diffractograms were plotted using OriginLab 2021 software. Details are provided in Table 4.

Table 4: Details of XRPD method.

<i>Parameter</i>	<i>Conditions</i>
K- α 1 [Å]	1.5406
K- α 2 [Å]	1.54443
K- β [Å]	1.39225
K- α 2/K- α 1 Intensity Ratio	0.5
K- α 2 Shift	0
Temperature [°C]	25
Sample Mode	Reflection
Length linear detector [°2 θ]	3,34727
Tube Current [mA]	40
Tube Tension [kV]	45
K- β Filter Material	Ni
K- β Filter Thickness [mm]	0.02
Anode Material	Copper (Cu)
Goniometer Radius [mm]	240
Dist. Focus to Div. Slit [mm]	144.5

Method G: Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were acquired using a Perkin Elmer Spectrum Two FT-IR spectrometer in Attenuated Total Reflectance (ATR) mode, using a diamond accessory. A minute amount of the sample was directly placed on top of the ATR diamond. The default scan ranged from 4000 to 450 cm⁻¹, and the number of scans was 32. Data were analysed using the Perkin Elmer Spectrum IR software.

Method H: Differential Scanning Calorimetry

DSC measurements were performed using the Q2000 DSC instrument under dry N₂(g) purge. The samples, typically 3 to 5 mg, were placed in a T0 low-mass pan (aluminium), and the pan was closed with a proper lid. The routine used is described as follows: the first cycle - heating up to 134 °C, at a heating rate of 5 °C/min; cooling step down to -20 °C at 17 °C/min; isothermal step of three minutes. The second cycle – heating up to 134 °C with a heating rate of 3 °C/min and temperature modulation of 0.5 °C at every 40 seconds. The thermal events were depicted from the second cycle.

Method I: HPLC method transfer

The details of the method used is listed in Table 5.

Table 5: Details of HPLC method.

<i>Parameter</i>	<i>Conditions</i>
HPLC system	Agilent 1100
HPLC column	Luna C18(2) 150x2.0 mm; 5 pm Part no: 00F-4252-BO
Flow rate	0.3 mL/min
Injection volume	5 µL/min
Temperature	30 °C
Wavelength	328 nm and 228 nm
Detector	DAD
Diluent	ACN:H ₂ O (40:60)
Retention time	5.4 min
Isocratic method	-

Standards solutions of piperlongumine (batch# 1411544) were prepared in diluent (100% ACN) at concentration of 0.2 mg/mL, 4 mg of RV299 were accurately weighed in a 20 mL volumetric flask and the volume adjusted with 100% ACN. The samples were sonicated for 20 minutes until complete dissolution, injected into HPLC for system suitability testing. To assess precision and repeatability, standard A was injected 5 times and standard B was injected twice, the mean main peak area and % relative standard deviation (%RSD) were calculated. The %RSD of the main peak of the API for standard solutions must be <2.0%, according to internal guidelines. Finally, the standard agreement was calculated as described by the following equation:

$$\text{standard agreement} = \frac{\text{Area}_A}{\text{Area}_B} \times \frac{\text{Conc}_B}{\text{Conc}_A} \quad (\text{Equation 1})$$

Where Area_A and Area_B is the area under the peak of standards A and B, respectively and Conc_A and Conc_B is the concentration of the standards A and B, respectively.

Linearity, LOD and LOQ: Piperlongumine solution in diluent at 0.2 mg/mL was injected at several injection volumes, the d column loading was calculated based on the injection volume and initial concentration of the piperlongumine solution. The main peak area was then plotted against the concentration and the correlation coefficient (r) was calculated. LOD and LOQ were calculated using the equations below:

$$LOD = 3.3 \times \frac{SEM}{Slope} \quad \text{Equation 2}$$

$$LOQ = 10 \times \frac{SEM}{Slope} \quad \text{Equation 3}$$

Method J: Filter compatibility

Limited filter compatibility assessment was carried out in selected filters. Piperlongumine solution prepared in diluent (ACN:H₂O 40:60) at 0.2 mg/mL was filtered through filters of different membrane material and pore size as detailed in Table 6, following the protocol described below.

Table 6: List of filters of different membrane material and pore size.

<i>Filter membrane</i>	<i>Pore size (µm)</i>
PTFE	0.2
PVDF	0.2
RC	0.2
SFCA	0.2
PES	0.2
PVDF	0.45
Nylon	0.45

Three aliquots of 1 mL of solution were filtered through the same filter and the filtered solution was collected in different glass vial. The filtered samples were transferred into HPLC vials and analysed using the analytical method described in Method I. An aliquot of the same piperlongumine solution was analysed by HPLC unfiltered for comparison. The % recovery of the piperlongumine solution was calculated using Equation 4.

$$\% \text{ recovery} = \frac{\text{Peak Area Filtered Sample}}{\text{Peak Area Unfiltered Sample}} \quad (\text{Equation 4})$$

Method K: High-performance liquid chromatograph (HPLC) for nanoformed samples

HPLC measurements were performed using a Waters ARC system and applying the method conditions below and in Table 7:

Mobile Phase A: 0.2% OPA

Mobile Phase B: Acetonitrile

Diluent: Acetonitrile and water in the ratio of 40:60.

Standard Preparation: Weigh accurately about 10 mg of STL001 Bulk in 10 mL

volumetric flask, dissolve and dilute up to the mark with diluent.

Sample Preparation: Weigh accurately about 10 mg of sample in 10 mL volumetric flask, dissolve and dilute up to the mark with diluent.

Blank Preparation: Diluent.

Chromatographic System

Column: Synergi 4µm Hydro-RP 80A° 250 mm × 4 mm

Detector: PDA detector

Wavelength: 325 nm

Injection Volume: 5 µL

Flow rate: 1.0 mL/min

Column Temperature: 30 °C

Sample Temperature: 20 °C

Run time: 40 minutes

Table 7: Gradient Program

<i>Time (Minutes)</i>	<i>Mobile Phase A (%)</i>	<i>Mobile Phase B (%)</i>
0	68	32
2	68	32
10	64	36
12	62	38
22	60	40
28	20	80
30	20	80
32	68	32
40	68	32

General Method A: Aqueous, thermal and photostability studies of Piperlongumine

Aqueous solubility of piperlongumine was evaluated in selected buffer/solvent, incubated at 37 °C for up to 14 days. A solution of piperlongumine was prepared in 100% acetonitrile (ACN) at 10 mg/mL. The stock solution in acetonitrile was diluted in buffers to achieve a final API concentration of 1 mg/mL and final ACN concentration of 10%. Precipitation was observed within 10-15 minutes upon addition of the stock solution of piperlongumine to the buffers solution. The suspensions were incubated at 37 °C protected from light. HPLC analysis was performed at fixed time points (t₀, 24 h, 5, 7 and 14 days) on the filtered and unfiltered suspensions, as described below and Method I.

An aliquot (20 µL) of the unfiltered suspension (combination of dissolved and undissolved API) was sampled and diluted 20 fold with diluent (ACN:H₂O 40:60). In order to analyse the degradation only of the dissolved fraction of piperlongumine, an aliquot of approx. 100 µL of

the suspensions were filtered through 0.2 µm PTFE filters. The filtrate was then diluted 20 fold with diluent and analysed by UV-HPLC.

The thermal stability of piperlongumine solid powder was assessed. Approx. 10 mg of piperlongumine was transferred in a 4 mL closed borosilicate glass vial, incubated at 25 and 60 °C, protected from light. At defined time point (24 h, 3 and 14 days), the API was removed from the storage conditions and analysed by HPLC, as described below. Approx. 2 mg of piperlongumine were sampled and transferred into a 20 mL volumetric flask and the volume adjusted with diluent (ACN:H₂O 40:60).

The photostability of a piperlongumine stock solution in acetonitrile (10 mg/mL) was assessed by exposing the API solution to fluorescent light under a fume hood cabinet. The samples were stored in clear glass vials and exposed to continuous light for up to 7 days. At fixed time points (0, 4 and 7 days) aliquots of stock solution in acetonitrile were sampled, diluted 100 fold with diluent (ACN:H₂O 40:60) and analysed by HPLC.

Table 8: Details of the aqueous, thermal and photostability conditions and samples.

<i>Batch ID</i>	<i>Solutions composition</i>	<i>Piperlongumine conc. (mg/mL)</i>	<i>Stability conditions</i>	<i>Incubation temperature (°C)</i>
A	100% ACN	10	Aqueous stability	37
B	10% ACN in 10 mM PBS pH 7.4	1		37
C	10% ACN in 1 M Borate pH 9	1		37
D	100% ACN	10	Photostability	~22 (uncontrolled)
E	Feed piperlongumine	-	Thermal stability	25 and 60

General Method B: Excipient Screening

The solubility of piperlongumine was evaluated in selected excipients as described below. Piperlongumine was incubated at initial concentration of 5 mg/mL in a heater/shaker apparatus at 25 and 50 °C, under agitation at 300 RPM. Once complete dissolution was observed, a further aliquot was added to the solution until saturation was achieved. At this stage evaluation of the solubility was performed by visual observation. The list of excipients were investigated and the incubation temperature is provided in Table 9.

Table 9: List of excipients tested for the solubility screening of piperlongumine and recommended route of administration.

<i>Excipients</i>	<i>Recommended route of administration</i>	<i>Excipients</i>	<i>Recommended route of administration</i>
SEBCD	Parenteral/oral	Sesame oil	Oral
HPBCD	Parenteral/oral	DMSO	Oral
Poloxamer 188	Parenteral/oral	NMP	Parenteral/oral
Poloxamer 407	Oral	DMA	Parenteral/oral
Span 20	Oral	Labrafac lipophile WL 1349	Oral
Span 80	Oral	Phosal 53 MCT	Oral
Captex 300	Oral	Phosal 50 PG	Oral
Capmul PG-8	Oral	Vitamin E TPGS	Oral
Labrafil M 1944 CS	Oral	Gelucire 44/14	Oral
Labrafil M2130 CS	Oral	Maisine CC	Oral
PEG 300	Oral	Capmul MCM C8 EP	Oral
PEG 400	Oral	Capmul MCM EP	Oral
Glycerol	Oral	Solutol	Parenteral/oral
Capryol 90	Oral	Peceol	Oral
Lauroglycol 90	Oral	Plurol Oleique CC 497	Oral
Miglyol 812	Oral	PEG1000	Oral
Miglyol 840	Oral	PEG2000	Oral
Labrasol	Oral	PEG4000	oral

General Method C: production of piperlongumine nanoparticles by wet milling

Micronisation of piperlongumine to achieve a particle size of $\leq 5 \mu\text{m}$ was carried out by wet bead milling using a planetary milling equipment (pulverisette 6, Fritsch) equipped with Automaxion SARL vessel. Approximately 100 mg of piperlongumine was weighted into a 4 mL glass vial to which 1 mm zirconia/silica beads were added in ratio of 1:10 (API to beads). A volume of 1 mL of decafluoropentane was added to vial containing the beads and piperlongumine, and the suspension was micronised for 30 minutes at 530 RPM. The particle size assessment was performed visually using a light microscope. The solid powder was obtained after 24 hours drying at RT and the particle size analysed by light scattering (Method A) and the morphology assessed by SEM (Method B).

General Method D: production of aqueous nanosuspension

Nanosuspensions were prepared at 100 or 50 mg/mL using a wet bead milling technique, in several aqueous vehicles suitable for parenteral administration, according to FDA database: 2.5% and 5% w/v Solutol HS15, tween 80/PVP K12 (0.1%/0.5% w/v). Silica beads of different size (0.5 mm and 0.1 mm) at different API:beads ratio (1:30 and 1:45) was investigated. The screening was widened and aqueous vehicles more suitable for oral administration were also

investigated: 2.5% w/v Vitamin E TPGS and HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1% (w/v)). Aqueous nanosuspensions were prepared at different piperlongumine concentration: 50, 100, 150 and 200 mg/mL in the selected vehicles following the method described below. Briefly, the required amount of piperlongumine was weighed in a 4 mL vial to which 0.5 (or 0.1 mm) silica beads were weighed in the appropriate ratio, 0.5 mL of each vehicle was added to the vials. The samples were milled at 600 RPM and the particle size reduction was monitored through DLS (Method D) at fixed time point.

General Method E: preparation of hydrogels

Poloxamers-based hydrogels

Poloxamer based gels were prepared at different concentrations using the cold method as described below. In a 30 mL vial, the right amount of refrigerated Type I water or acetate buffer pH 5 was weighed. The vial was then placed on a magnetic stirrer in ice bath. To the vial, the right amount of poloxamer (188 or 407) powder was slowly added under stirring. The vial was kept under stirring until complete dissolution of the polymer. The gels were stored in refrigerated conditions. The sol-gel temperature was evaluated following the protocol described in Method E, to identify the optimal concentration able to gelify at approx. body temperature (35-39 °C).

Methylcellulose-based hydrogels

Methylcellulose gels were prepared at different concentrations and were also prepared in combination with Hyaluronic acid. Hydrogels were prepared in type I water or in acetate buffer pH 5. The sol-gel temperature was evaluated following the protocol described in Method E, to identify the optimal concentration able to gelify at approx. body temperature.

Preparation of hydrogels for in vitro testing (Franz cells)

Preparation of Methylcellulose/hyaluronate (6%/0.25% w/w) in Type I water

In a 30 mL vial, 9.4 g of type 1 water was accurately weighed and placed in a hot plate magnetic stirrer pre-heated at approx. 70 °C. Methylcellulose (600 mg) was slowly added to the pre-heated aqueous phase under stirring. Once the methylcellulose was homogeneously dispersed in solution, the temperature of the hot plate was reduced to room temperature and left under stirring until a clear solution was obtained.

In a separate 4 mL glass vial, 2.5 mg of sodium hyaluronate was weighed, to this 997.5 mg of the 6% methylcellulose solution prepared as described above was weighed. The mixture was left under stirring until a clear solution was obtained.

Preparation of hydrogels with piperlongumine in Methylcellulose/PEG400/hyaluronate (6%/10%/0.25% w/w) in Type I water

In a 4 mL glass vial, 2.1 mg of piperlongumine was weighed, to this 50 mg of PEG 400 was added and mixed using a heater/shaker apparatus at 25 °C for 15 minutes. 447.9 mg of Methylcellulose/hyaluronate (6%/0.25% w/w) solution prepared as described above was weighed to the above mixture using a positive displacement pipette and left under stirring until a homogenous suspension was obtained.

Preparation of Methylcellulose/hyaluronate (6%/0.25% w/w) in acetate buffer pH 5

In a 30 mL vial, 9.4 g of acetate buffer pH 5 was accurately weighed and placed in a hot plate magnetic stirrer pre-heated at approx. 70 °C. 600 mg of methylcellulose was slowly added to the pre-heated aqueous phase under stirring. Once the methylcellulose was homogeneously dispersed in solution, the temperature of the hot plate was reduced to room temperature and left under stirring until a clear solution was obtained.

In a separate 4 mL glass vial, 2.5 mg of sodium hyaluronate was weighed, to this 997.5 mg of the 6% methylcellulose solution prepared as described above was weighed. The mixture was left under stirring until a clear solution was obtained.

Preparation of hydrogels with piperlongumine in Methylcellulose/hyaluronate (6%/0.25% w/w) in Type I water and in acetate buffer

In a 4 mL glass vial, 2.1 mg of piperlongumine was weighed, to this 497.9 mg of Methylcellulose/hyaluronate (6%/0.25% w/w) solution in type I water prepared as described above and in acetate buffer prepared as described above was weighed using a positive displacement pipette and left under stirring until a homogenous suspension was obtained.

Preparation of poloxamer 188/hyaluronate hydrogels (35%/0.5%)

In a 30 mL glass vial, 6.5 g of refrigerated acetate buffer, pH 5 was accurately weighed. 3.5 g of poloxamer 188 was slowly added to the above vial under stirring on a magnetic stirrer in ice bath. The vial was kept under stirring refrigerated until a clear solution was obtained.

In a separate 4 mL glass vial, 10 mg of sodium hyaluronate was weighed, to this 1.99 g of the 35% poloxamer gel prepared as described above was weighed. The vial was kept under stirring on a magnetic stirrer in ice bath until a clear solution was obtained.

Preparation of hydrogels with piperlongumine in poloxamer 188/hyaluronate/PEG400 hydrogels (28%/20%/0.5% w/w) in acetate buffer pH 5

In a 4 mL glass vial, 4.2 mg of piperlongumine was weighed, to this 198 mg of PEG 400 was added and left under stirring until a clear solution was obtained. Subsequently 797.8 mg of refrigerated poloxamer188/hyaluronate gel (35%/0.5%) prepared as described below was weighed using a positive displacement pipette. The vial was kept under stirring for 30 minutes until a clear solution was obtained.

Preparation of poloxamer 188/hyaluronate hydrogels (30%/0.5%)

In a 30 mL glass vial, 7 g of refrigerated acetate buffer, pH 5 was accurately weighed. 3 g of poloxamer 188 was slowly added to the above vial under stirring on a magnetic stirrer in ice bath. The vial was kept under stirring refrigerated until a clear solution was obtained.

In a separate 4 mL glass vial, 10 mg of sodium hyaluronate was weighed, to this 1.99 g of the 30% poloxamer gel prepared as described above was weighed. The vial was kept under stirring on a magnetic stirrer in ice bath until a clear solution was obtained.

Preparation of hydrogels with piperlongumine in poloxamer 188/hyaluronate hydrogels (30%/0.5% w/w) in acetate buffer pH 5

In a 4 mL glass vial, 4.2 mg of piperlongumine was weighed, to this 995.8 mg of refrigerated poloxamer188/hyaluronate gel (30%/0.5%) prepared as described was weighed using a positive displacement pipette. The vial was kept under stirring for 30 minutes, a homogenous suspension was obtained.

Preparation of hydrogels with micronised piperlongumine in poloxamer 188/hyaluronate hydrogels (30%/0.5% w/w) in acetate buffer pH 5

In a 4 mL glass vial, 4.2 mg of micronised piperlongumine was weighed, to this 995.8 mg of refrigerated poloxamer188/hyaluronate gel (30%/0.5%) prepared as described was weighed using a positive displacement pipette. The vial was kept under stirring for 30 minutes, a homogenous suspension was obtained.

General Method F: nanosuspension incorporation into hydrogels

The feasibility of incorporating the nanosuspension into selected hydrogels was investigated at different ratio in order to identify the optimal conditions that will still allow the gelification of the sample. The test was performed at 250 µL scale to simulate the volumes that will be administered in *in vivo* studies. The preparation of the samples follows the below procedure. The appropriate amount of nanosuspension was weighed in a 1.5 mL glass vial to which the

amount of hydrogels was added according to the desired ratio (30:70, 40:60; 50:50, nanosuspension:hydrogel). The sample was placed under stirring in ice bath and monitored until visual homogenous sample was obtained. Within 2-3 minutes under mild stirring, a milky suspension was observed. The gelification temperature of the hydrogels with incorporated nanosuspension was assessed following Method E.

General Method G: syringeability assessment of final formulations comprised of nanosuspensions incorporated into hydrogels

The syringeability of the hydrogels with incorporated nanosuspensions was assessed through a needle of suitable gauge (18G, 1x1/2", BD Microlance™ 3). The hydrogels with incorporated nanosuspensions were stored in an ice bath prior the syringeability test for approx. 15 minutes. An aliquot of the formulation (100 µL) was dispensed in a 1.5 mL glass vials incubated at 37 °C and parameters such as pressure/force required for injection and evenness of flow was classified as described in Table 10. The gelification tendency of the dispensed aliquot was also monitored by vial inversion method.

Table 10: Syringeability classification based on the pressure/force required for injection

<i>Category</i>	<i>Injectability</i>	<i>Flow</i>
1	not possible	no flow
2	difficult	drop wise
3	moderate	Continuous
4	easy	continuous

General Method H: in vitro diffusion cells (Franz cells) of bulk piperlongumine hydrogels and nanosuspension incorporated into hydrogels

An *in vitro* diffusion test (Franz cells, Copley) was performed to determine the release profile of piperlongumine from the hydrogels developed (as described in Table 11). The test was performed in sink conditions. 100 µL of piperlongumine-loaded hydrogels (containing 0.4 mg of API) were added to the donor chamber of the Franz cells. 7 mL of PBS pH 7.4 was added to the receptor chamber. The chambers were separated by a membrane of approx. 13 mm diameter (0.45 µm pore size, PVDF). The Franz cells were incubated at 37 °C in an appropriate heating block and under stirring at 450 RPM. At selected time points, 200 µL of sample was withdrawn from the receptor chamber and the same volume was replaced with fresh PBS buffer pre-heated at 37 °C. The samples were centrifuged at 12,500 RPM for 5 minutes, diluted with diluent (1:1) and analysed by HPLC for drug release.

Table 11: List of hydrogels used for in vitro diffusion testing

<i>Formulation ID</i>	<i>Composition</i>	<i>API concentration in the formulations</i>	<i>Observations</i>
20	MC/PEG 400/HA (6%/10%/0.25%)	4 mg/g	VL at RT
21	MC/HA (6%/0.25%) in acetate buffer pH 5	4 mg/g	VL at RT
22	MC/HA (6%/0.25%) in type I water	4 mg/g	VL at RT
23	P188/PEG 400/HA (28/20/0.5 %w/w)	4 mg/g	Clear, VL at RT
24	P188/HA (30%/0.5%)	4 mg/g	Opaque, no particles were observed, VL at RT
25	P188/HA (30%/0.5%)	4 mg/g (micronized API)	Opaque, no particles were observed, VL at RT

For nanosuspensions, in vitro diffusion test (Franz cells) was performed to determine the release profile of piperlongumine from the formulations composed of nanosuspension incorporated into hydrogel poloxamer 407 (22% w/v in acetate buffer, pH 5). The test was performed in sink conditions. In order to increase the API concentration and maintain the sink condition additional excipients were added to PBS pH 7.4 (such as PEG400). Table 12 summarises the experimental parameters applied.

Table 12: Experimental parameters applied for the in vitro diffusion test

<i>Apparatus</i>	<i>Franz cells</i>
Dose	0.4 mg per vessel (100 µL of formulation was added to the donor chamber)
Replicates	n=3
Dissolution Medium	PEG400:PBS media (30:70) 7 mL in the receptor chamber
Paddle Speed	450 RPM
Temperature	37.0 ± 0.5 °C
Sampling Time Points	5, 15, 30, 60, 120, 180, 240, 360 minutes and 24 hours
Sampling and detection	Manual sampling – 200 µL were removed and centrifuged for 10 minutes at 12,500 RPM. The supernatant was diluted 1:1 v/v with diluent (ACN:H ₂ O 40:60) and analysed by UV-HPLC. 200 µL of dissolution medium was replaced at each time point

General Method I: In vitro dissolution test of nanosuspension incorporated into hydrogels

Small scale dissolution test was carried out on the lead formulations comprised of nanosuspensions prepared in 5% Solutol at 50 mg/mL, and in HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1% w/v) at 100 and 200 mg/mL piperlongumine concentration. The scope of the test was to transfer similar parameters used in the in vitro diffusion test (Table 12 in General Method H) to compare the dissolution profile of piperlongumine from the formulations using two different in vitro testing. Table 13 summarises the experimental parameters used for the test.

Table 13: Experimental parameters applied for the small scale dissolution test

Apparatus	Heater/shaker apparatus (RS9000)
Dose	0.4 mg per vessel
Replicates	n=3
Dissolution Medium	PEG400:PBS media (30:70) 7 mL
Paddle Speed	400 RPM
Temperature	37.0 ± 0.5 °C
Sampling Time Points	5, 15, 30, 60, 120, 180, 240, 360 minutes and 24 hours
Sampling and detection	Manual sampling – 200 µL were removed and centrifuged for 10 minutes at 12,500 RPM. The supernatant was diluted 1:1 v/v with diluent (ACN:H ₂ O 40:60) and analysed by UV-HPLC. 200 µL of dissolution medium was replaced at each time point

General Method J: isolation of nanoparticles by freeze-drying

The nanosuspensions in HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1% w/v) prepared as described in General Method F at 100 and 200 mg/mL were freeze-dried following a generic fit-for-purpose lyocycle, the experimental parameters are described in Table 14. The freeze-drying was carried out using Epsilon 2-4 LSC Plus (Martin Christ) freeze-dryer. Approximately 200 µL of nanosuspensions was transferred in a 2 mL vial (Tubular Type I Glass Injection Vials/Freeze Drying vials, SCHOTT Pharmaceutical Packaging). The lyophilisates were reconstituted in type I water to the initial concentration and analysed for size by DLS and assay by UV-HPLC. The scope of the work is to assess the in-use stability of the reconstituted nanosuspension at selected time points (t_0 , 3 h, 6 h, and 24 h). The lyophilisate powder was stored at room temperature (approx. 20-22 °C) and in refrigerated conditions (2-8 °C), and at selected time point (1 and 4 weeks) reconstituted in water and the in-use stability monitored for assay (UV-HPLC) and size (DLS).

Table 14: Experimental parameters applied to the lyocycle used.

<i>Process Steps</i>	<i>Temperature (°C)</i>	<i>Time (min)</i>	<i>Vacuum (mbar)</i>
Freeze	-45	180	-
Main drying (primary drying)	-40	90	0.2
	-35	90	0.2
	-30	90	0.2
	-25	90	0.2
	-20	90	0.2
	-15	90	0.2
	-10	90	0.2
	-5	90	0.2
	0	90	0.2
	5	90	0.2
	10	90	0.2
	15	90	0.2
	20	90	0.2
Final drying (secondary drying)	20	120	0.001

General Method K: reconstitution protocol

The amount of water was calculated based on the measured drug load of the lyophilisate. To calculate the drug load, an aliquot of lyophilisate powder was sampled from one vial, transferred into a volumetric flask and volume adjusted with diluent (ACN:H₂O 40:60). The solution was vortexed mixed and then analysed by UV-HPLC. Based on the calculated drug load the exact amount of water was added to the lyophilized vials to target the initial API concentration of 100 and 200 mg/mL. After the addition of water, the vial was mixed by vortex for approx. 2 minutes until particles were no longer visible at the bottom of the vial and a homogenous milky suspension was obtained.

The reconstituted samples were analysed by DLS for particle size (Method D). The assay of piperlongumine in the reconstituted sample was confirmed by UV-HPLC. The reconstituted samples were stored upright on the bench at RT and an aliquot sampled at selected time points (*t*₀, 3, 6, 24 hours) to confirm the stability upon reconstitution of the nanosuspensions.

General Method L: Production of piperlongumine nanoparticles using the CESS® process

Nanoparticles were produced according to the CESS® process described in Pessi *et al* and US2017231914. First, the bulk API is loaded in a thermostated pressure vessel, where the dissolution of the API into supercritical CO₂ is controlled by pressure and temperature. Next, the solution is driven, through a nozzle, from the dissolution vessel into a collection vessel under thermodynamic flow control. During the expansion at the nozzle, the pressure and

temperature of supercritical CO₂ decrease, leading to the formation of solid CO₂ (CO_{2(s)}) presented as dry ice flakes, which entraps the API nanoparticles. After the conclusion of the production phase, CO_{2(s)} is let to sublime, leaving in the collection vessel the pure nanoformed API, usually in the form of fine powder. The material is then collected into labelled vials and transferred to the Quality Control (QC) laboratory for characterization. The materials presenting photosensitivity were stored in amber vials, and the thermosensitive ones were stored under cool conditions, normally 2 to 8 °C, unless stated otherwise.

General Method M: Hydrogel composition screening

Various excipient solutions (Table 1b) were prepared in water, 0.1 M pH 5 acetate buffer or 0.1 M pH 5 citrate buffer and their flow properties were evaluated after storage at 4 °C, 22 °C and 37 °C. A sample was considered to be a gel (G) if no flow was detected for at least 1 min after turning the vial upside down. In all other cases, even for very viscous samples, the materials were considered to be liquids (L).

General Method N: Hydrogel preparation method using piperlongumine nanoparticles

Piperlongumine was accurately weighed into an amber vial using an analytical balance. Calculated amount of the gel matrix solution was pipetted onto the powder to obtain 10%, 20% or 30% piperlongumine loading (all active concentrations are given as % w/w in the gels). The samples were homogenized by intensive magnetic stirring for 3 min. For P407 based samples the homogenization was performed on ice bath. Dispersion of the Soluplus based samples was run at room temperature and was aided with a spatula at higher active loadings due to the very high viscosity of the systems. Moreover, a subset of Soluplus based samples (the ones for UV irradiation experiments, see later) was prepared by mixing the nano API and the polymer matrix with a spatula for 1 min, without any magnetic stirring.

General Method O: UV irradiation of the gels

Approximately 300 - 500 mg of the P407 and Soluplus based gel samples in water and 0.1 M tris-HCl pH 7 buffer containing 20% piperlongumine were measured onto plastic Petri dishes. The samples were placed into a Biowizard Platinum 130 MF laminar flow box (Kojair Tech Oy, Mänttä-Vilppula, Finland) equipped with Philips UV-C disinfection lamps. UV irradiation was continued for 10 min to simulate sterilization prior administration. Chemical stability and content uniformity of the gels were assessed before and after the procedure with the methods described in General Method S and General Method T, respectively.

General Method P: Blank gel dissolution assessment

500-600 µL of the blank gel samples was pipetted into 100 ml of 37 °C water. 50-75 min⁻¹ magnetic stirring was applied. Dissolution of the gel was monitored visually. Time until complete dissolution was recorded.

General Method Q: Syringeability test

Syringe filling and pushing out of blank aqueous 20% poloxamer 407 and 30% Soluplus gels were tested with a 2.5 mL plastic syringe equipped with an 18 G needle.

General Method R: Quantification of piperlongumine in solution

Piperlongumine was quantified in solution by UV-Vis spectroscopy or HPLC measurement. Pion Rainbow UV probes (Pion Inc UK Ltd, East Sussex, United Kingdom) were used to monitor the concentration of the active ingredient in solution at 325 nm wavelength. Concentrations were determined after obtaining the calibration curve.

Quantification of piperlongumine in solution by HPLC was performed using a Waters Acquity Arc instrument (Waters Ltd, Wilmslow, UK) with a Synergi C18 4 µm Hydro-RP 80Å 250 mm x 4.6 mm column (Phenomenex Inc, Værløse, Denmark). Mobile phase A was 0.2% orthophosphoric acid in water while mobile phase B was acetonitrile. Run time was 40 min with the gradient program shown in Table 15.

Table 15: Gradient program used for HPLC measurements.

<i>Time (min)</i>	<i>Mobile Phase A (%)</i>	<i>Mobile Phase B (%)</i>
0	68	32
2	68	32
10	64	36
12	62	38
22	60	40
28	20	80
30	20	80
32	68	32
40	68	32

Piperlongumine was detected with a PDA detector at 325 nm wavelength. Injection volume was 2 µL and flow rate was 1 mL/min. The temperature of the sample and column were set to 20 °C and 30 °C, respectively. Piperlongumine concentrations were evaluated after calibration.

General Method S: Chemical stability

Chemical stability was assessed using the HPLC method described in General Method R.

General Method T: Content uniformity assessment

Content uniformity of the gel samples was evaluated. From random places of the gel 4 ± 3 mg sample was accurately weighed into an amber HPLC vial using a Mettler Toledo XRS205DU balance (GW Berg&Co AB Oy, Vantaa, Finland). Analytical grade MeOH (1 mL) was added to the gel sample. Dissolution was aided by gentle hand shaking.

Piperlongumine concentration was quantified with the HPLC method described in General Method R. Sampling and concentration measurements were run in at least triplicate. Average piperlongumine concentration and standard deviation were calculated and compared to the nominal value.

General Method U: Dissolution measurement from gels

To assess the release of piperlongumine from the gels approximately 60 mg of the formulations was weighed onto a weighing boat piece. The sample was dropped into 100 mL 0.1 M phosphate buffer pH 7.4 containing 7 mg/mL NaCl supplemented with 10% PEG400 to facilitate the dissolution of piperlongumine. Mild magnetic stirring was applied at 100 min⁻¹ rotation speed. The temperature was set to 37 °C. Piperlongumine concentrations were monitored for up to 4 days with the UV method described in General Method R.

General Method V: Permeability measurement from gels

The dissolution and permeability of 20% w/w piperlongumine loaded gels were measured in a MacroFlux instrument (Pion Inc UK Ltd, East Sussex, United Kingdom). Approximately 75 mg hydrogel was weighed onto a weighing boat piece. The sample was dropped into the donor compartment containing 250 mL 0.1 M phosphate buffer pH 7.4 supplemented with 7 mg/mL NaCl. 55 µL of dodecane based artificial lipid membrane separated the donor and acceptor compartments. The acceptor medium was 15 mL HEPES buffer supplemented with 10 mg/mL SDS. The system temperature was set to 37 °C. 40 min⁻¹ paddle stirring was applied in the donor compartment. Piperlongumine concentrations were monitored in both the donor and acceptor sides with UV probes using the method described in General Method R. Run time was approximately 20 hours. The experiments were conducted in triplicate. It is to be noted that strictly linear relationship between absorbance and concentration was found up to 25 µg/mL and 40 µg/mL, in the donor and acceptor compartments, respectively. However, higher concentrations are also plotted on the charts to have a conceptual understanding and support visualization.

General Method W: SEM analysis of the hydrogels

Imaging of the hydrogels was conducted with a Zeiss Sigma VP scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany). Hydrogels with 20% w/w piperlongumine content were prepared in 18% w/w aqueous poloxamer 407 and 27.5% w/w Soluplus matrices using both the bulk and nanoformed APIs. The poloxamer and Soluplus gels were stored at 4 °C for 2 days and 1 day, respectively. After storage small samples of the gels were smeared onto graphite sample holder stubs and subsequently spin coated with gold. The exact imaging conditions (accelerating voltage, working distance, magnification) are indicated on each picture.

Results

Characterisation of bulk piperlongumine

Physical properties of bulk piperlongumine were characterised by several analytical techniques. Morphology of the crystalline powder was visualised by optical microscope and scanning electron microscope. The particle size distribution was determined by laser diffraction analysers.

Figure 1 shows SEM images of bulk piperlongumine. Acicular particles of heterogeneous size greater than 200 µm were observed by SEM images. PSD analysis (Method D) identified a D90 value of 38.91 µm (Figure 2).

Stability studies on bulk piperlongumine

Aqueous, thermal and photostability of piperlongumine was evaluated in several conditions as described in General Method A. Aqueous stability was evaluated in PBS buffer (pH 7.4) and in borate buffer (pH 9). From information found in the literature, it was found that at higher pH (>7) in aqueous media, degradation is induced in piperlongumine. This was confirmed by experiments performed. In borate buffer approx. 60% degradation was observed after 24 hours of storage. Some degree of degradation was also observed in PBS pH 7.4 (14% degradation after 5 days in the filtered sample (that is the fraction of piperlongumine solubilised in the buffer) and, 30% degradation was observed after 14 days). A control in 100% acetonitrile confirmed the stability of piperlongumine in the organic solvent for at least up to 7 days with 0.5% degradation observed.

Thermal stability of piperlongumine was also assessed to test the in-use stability of the API. An aliquot of solid powder was incubated at 25 and 60 °C and analysed for up to 14 days. Piperlongumine was found to be stable in the tested conditions for up to 14 days as no change in assay and % peak area was observed.

Finally, to assess the in use stability of piperlongumine, an aliquot of piperlongumine solution in acetonitrile (ACN) was exposed to the fluorescent light of a fumehood cabinet for up to 7 days. Approx. 4% degradation was observed after 4 days, which increased to 6% after 7 days. The degradation was more evident at the detection wavelength of 228 nm while wavelength 338 nm was not as sensitive in detecting the degradants.

From the stability tests conducted it can be concluded that piperlongumine as powder is thermally stable for at least 14 days up to 60 °C. Degradation was observed at basic pH, more evident at pH 9, however certain degree of degradation was also observed in PBS pH 7.4 which may pose some concerns for in vitro release tests of the formulation developed. Photostability tests suggest also some degradation may be observed upon exposure to the light which suggest protection from light to be recommended. Finally, as observed from the impurities profile the two wavelengths at 228 and 328 nm will be necessary to perform further studies to monitor the formation of degradants.

Excipient screening

Excipients screening was carried out to investigate the solubilisation of bulk piperlongumine. The excipients tested included those suitable for parenteral administration, as well as those suitable for oral administration including solvents, co-solvents, surfactants, natural oil and lipid-based excipients.

The excipients screened included glycerol, SR polysorbate 80, Solutol HS15 poloxamers (188 10% w/v, 407 10% w/v), PEG (300, 400, 1000, 4000), propylene glycol (PG), oils (safflower, soybean, sesame), oleic acid, transcitol, tetraglycol, Cremophor RH40, DMA, cyclodextrins (HP- β -CD, SEBCD, HP- β -CD, HP- γ -CD), nicotinamide, Span (20, 80), captex 300, Labrafil M 1944 CS, Labrafac lipophile WL 1349, miglyol (812, 840), DMSO, NMP, capmul (PG-8, MCM C8 EP, MCM EP), Labrasol, Capryol 90, lauroglycol 90, phosal 53 MCT, phosal 50 PG, vitamin E TPGE, gelucire 44/14, maisine CC, peceol, plurol oleique CC 497, Labrafil M2130 CS.

Highest solubilisation of bulk piperlongumine was observed in high molecular weight PEG (PEG1000) which was able to solubilise piperlongumine at 60 mg/mL after incubation at 50 °C and in PEG 400 (30 mg/mL). However, considering the limited solubility observed in other excipients the eventuality of obtaining a solution of bulk piperlongumine at 50 mg/mL or above in a suitable vehicle would not be feasible.

Micronisation feasibility of piperlongumine by wet milling

The results of the excipients screening concluded that the development of a solution formulation of piperlongumine at the desired concentration of 50 mg/mL was unfeasible. The feasibility of a suspension of piperlongumine in suitable vehicles was therefore explored.

The feasibility to micronise piperlongumine to target D90 of 3.75 µm was investigated as a mean of controlling the size of the drug and optimise the suspendability in the designed vehicles. Wet milling was carried out according to General Method C.

Micronised sample showed presence of aggregates, possibly due to the small size of the particles which tend to be more cohesive, as observed in the SEM images (Figure 3). Particles size distribution by light scattering (Method D) confirmed the size to be less than 5 µm (Figure 4).

Aqueous nanosuspension feasibility and optimisation

The feasibility of developing aqueous nanosuspensions of piperlongumine was investigated. Several trials were performed by optimising the vehicles and experimental settings to identify the optimal conditions. General Method D was used for the production of the nanosuspensions. A summary of the initial trials and observations is provided in Table 16.

Table 16: Summary of nanosuspension trials detailing vehicle composition, API concentration, silica beads used.

Sample	Silica beads size (mm)	Vehicle composition	API concentration (mg/mL)	Observations	Milling time
01	0.5	2.5% Solutol	100	Extensive foaming	Total of 9 hours, high polydispersity
02	0.5	Tween 80/PVP K12 0.1%/0.5%	100	Highly viscous/paste	-
03	0.5	Tween 80/PVP K12 0.1%/0.5%	50	Not viscous	Total of 9 hours, high polydispersity
04	0.1	2.5% Solutol HS15	100	Foaming	Total of 9 hours of milling, size of ca. 825 nm
05	0.5	2.5% Vitamin E TPGS	100	Extensive foaming	Total of 4 hours of milling, high polydispersity

Based on these initial trials, Nanosuspension Sample 04 (vehicle composition of 2.5% w/v Solutol HS15 with 0.1 mm silica beads) appeared to be more promising as compared to the other vehicles. The particle size observed was approx. 800 nm.

Optimisation to further reduce the particle size was performed by increasing the API:beads ratio (use 0.1 mm ratio), by using micronizing-nanosizing approach. Addition of solvents (i.e. ethanol) or antifoaming agent (30% simethicone emulsion) was also included in the vehicle to reduce the excessive foaming. From the trials performed it could be concluded that Solutol HS15 was the most promising vehicle and the most promising nanosuspension could be obtained with micronised piperlongumine in 2.5% w/v Solutol HS15 using 0.1 mm silica beads in ratio 1:45 (Sample 12, see Table 17).

Table 17: Summary of optimisation trials of nanosuspension in 2.5% Solutol HS15.

Sample ID	API	Vehicle composition	Silica beads size	API:beads ratio	Comments
06	Bulk PL	2.5% Solutol HS15	0.1	1:45	605 nm (PDI 0.405) after 8 h of milling (no improvement after additional milling)
07	Bulk PL	2.5% Solutol HS15+0.1% antifoam	0.5	1:30	High polydispersity
08	Bulk PL	2.5% Solutol HS15+0.1% ethanol	0.5	1:30	High polydispersity
09	Bulk PL	2.5% Solutol HS15	0.1	1:30	High polydispersity
10	Micronised PL	2.5% Solutol HS15	0.5	1:30	High polydispersity
11	Micronised PL (50 mg/mL)	0.1% Tween 80+0.5% PVP k12	0.1	1:45	High polydispersity after 3 h of milling
12	Micronised PL (100 mg/mL)	2.5% Solutol HS15	0.1	1:45	Approx. 600 nm (PDI 0.3) after 1 h of milling (no further improvement after additional milling, 3 h)

Considering the promising results obtained nanomilling piperlongumine with 2.5% (w/v) Solutol, additional trials were performed to optimise the nanosuspension by increasing the level of Solutol HS15 to 5% and investigate at which concentration of piperlongumine a stable nanosuspension could be obtained.

Table 18 summarises the size distribution of the nanosuspensions prepared in 5% (w/v) Solutol HS15 with micronized piperlongumine at different concentration. The samples prepared at 150 mg/mL was found to be monodisperse (PDI approx. 0.25) however it could not be nanosized and size in micrometer range was obtained. The nanosuspension at 100 mg/mL appeared to be monodisperse (PDI = 0.22), however, a size of 778 nm was obtained which slightly increase upon storage at RT over 24 hours. It could be concluded that the maximum concentration of piperlongumine that can be nanomilled with Solutol HS15 is 50 mg/mL, although a slightly increased in size was observed after 7 days of storage at room temperature. HPLC analysis of dissolved fraction in the nanosuspension was 0.48 mg/mL. A

comparison with the preparation of nanosuspension by using bulk piperlongumine (non-micronised) showed that micronisation of piperlongumine allows a decrease of milling time.

Table 18: Summary of DLS analysis on nanosuspension prepared in 5% (w/v) Solutol at different API concentration

Storage time	Micronised Sample 13 (50 mg/mL)			Micronised Sample 14 (100 mg/mL)		Micronised Sample 15 (150 mg/mL)	
	PDI	Peak 1 (nm)	Peak 2 (nm)	PDI	Peak 1 (nm)	PDI	Peak 1 (nm)
0	0.334	580	158	0.222	778	0.255	1093
24 h	NA	NA	NA	0.253	867	NA	NA
7 d	0.465	695	215	NA	NA	NA	NA

Scalability trial of the nanosuspension at 50 mg/mL in 5% Solutol was investigated at 4 mL scale. No stable nanosuspension could be obtained even after 5 hours milling, after which no further milling was performed. Nanosuspension at 100 mg/mL in 5% Solutol was prepared at 2 mL scale and size of 800 nm was obtained at t_0 , after 6 days of storage at RT increase in size in the micron meter range was observed. In conclusion, nanosuspension of approx. 600 nm could be obtained in 5% Solutol using micronized piperlongumine at 50 mg/mL concentration at 1 mL scale.

The initial feasibility trial investigated the use of vehicles suitable for parenteral application, identifying 5% (w/v) Solutol HS15 as the most promising vehicle, however a particle size of approx. 600 nm was achieved and the maximum concentration of piperlongumine able to obtain a stable nanosuspension was 50 mg/mL. However, scalability issues were also identified. In attempt to increase the maximum concentration and identify a more robust nanosuspension, an additional feasibility trial was performed by using an alternative vehicle, suitable for oral administration and comprised of HPMC 606, PVP K30, SDS (0.5%/0.5%/0.1% w/v). The nanosuspension was prepared at 100 mg/mL with micronized and feed API (i.e. non-micronised) and the size distribution is presented in Table 19. Monodisperse nanosuspension (PDI < 0.2) was obtained in both cases with a size of approx. 300 nm.

Table 19: DLS analysis of nanosuspension prepared in HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1%) at 100 mg/mL with non-micronised and micronised piperlongumine measured at selected milling time points.

Milling Time	Sample 16 micronized API			Sample 17 feed API		
	PDI	Peak 1 (nm)	ζ-potential (mV)	PDI	Peak 1 (nm)	ζ-potential (mV)
15 min	0.081	284	-14 mV	Fit error		
45 min	-	-	-	0.158	303	-

Both nanosuspensions were stored at RT for 5 days and the size analysed by DLS (see Table 20). A slight increase in size and PDI was observed in nanosuspension prepared with micronized API (Nanosuspension Sample 16), whereas PDI and size appeared to be more consistent in the nanosuspension prepared using the feed API. This may be due to the longer milling time which helped in the particles size reduction to the optimal level reaching the equilibrium.

Table 20: DLS analysis of nanosuspension prepared in HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1% w/v) at 100 mg/mL with non-micronised and micronised piperlongumine measured at selected stability time points after storage at RT

Storage Time	Sample 16 micronized API			Sample 17 feed API		
	PDI	Peak 1 (nm)	ζ-potential (mV)	PDI	Peak 1 (nm)	ζ-potential (mV)
0	0.081	284	-14	0.158	303	-
24 h	0.139	334	-	-	-	-
5 d	0.157	365	-17.7	0.159	300	-15

Considering the promising results obtained with the vehicle comprised of HPMC 606, PVP K30, SDS (0.5%/0.5%/0.1% w/v), the concentration of piperlongumine in the vehicle was further challenged to 150 and 200 mg/mL. The use of non-micronised piperlongumine was considered sufficient to obtain a stable nanosuspension. As it can be observed in Table 21, a slightly polydisperse system was obtained at higher concentration (PDI approx. 0.3) and the size increased to approx. 500-600 nm.

Table 21: Summary of DLS analysis on nanosuspension prepared in HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1% w/v) at different concentration of piperlongumine

Milling Time	Storage Time	Sample 18 micronized API		Sample 19 feed API		
		PDI	Peak 1 (nm)	PDI	Peak 1 (nm)	Peak 1 (nm)
1 h	-	0.295	420	0.308	599	-
2 h	-	0.228	557	-	-	-
3 h	-	0.282	520	-	-	-
-	24 h	-	-	0.271	605	160

The stability of the nanosuspension at 200 mg/mL was investigated further by DLS and HPLC. The size was maintained stable at approx. 600 nm with comparable PDI (0.3) for at least 7 days while a slight increase was observed after 4 weeks of storage at RT. HPLC analysis did not show any degradation for at least 4 weeks, however at 4 weeks a decrease in assay was observed which could be due to sedimentation of the sample which could not be resuspended. A low assay was also observed at 7 days (88.3%), however an additional test performed confirmed the 100% assay suggesting a potential experimental error at that time point. Considering the data generated a stability of 7 days is recommended for the nanosuspension prepared in HPMC 606/ PVP K30/SDS (0.5%/0.5%/0.1% w/v) at 200 mg/mL.

Nanosuspension incorporation into hydrogels

The feasibility of incorporating the most promising nanosuspensions identified above in 5% Solutol and HPMC/PVP/SDS (0.5%/0.5%/0.1%) was investigated using the protocol described in General Method F. Nanosuspensions in Solutol were taken into consideration for tolerability reasons, as Solutol is an approved excipient for parenteral administration. HPMC 606, PVP K30 and SDS are approved excipients for oral administration.

A nanosuspension at 50 mg/mL piperlongumine concentration in 5% Solutol using micronized API was incorporated into hydrogels at 20 and 50%, respectively.

From an initial investigation of the incorporation of the nanosuspension at 50 mg/mL, the maximum feasible amount of nanosuspension that can be incorporated into poloxamer-based hydrogels (P188/HA 35%/0.5%) was found to be 20%, which corresponds to 10 mg/mL final concentration. Additional screening was performed by incorporating the nanosuspension at different % level in different hydrogels. The most promising results were obtained by incorporating the nanosuspension in P188/HA (40%/0.5%) at 50% corresponding to a final drug load of 25 mg/mL. However, an immediated gelification at 22.5 °C was observed which could potentially pose issues for the syringeability of this sample.

Additional investigation on the incorporation nanosuspension in 5% Solutol into various hydrogels of different composition identified that a maximum of 30% of nanosuspension could be incorporated into hydrogels without compromising the gelification at body temperature. Therefore, considering the limiting amount of nanosuspension that can be incorporated in order to increase the drug load, a nanosuspension at 100 mg/mL in 5% Solutol was also tested.

Promising results were obtained by incorporating the nanosuspension at 100 mg/mL in ratio 30:70 (nanosuspension:gel) in 20% P407 hydrogel and in P188/HA (40%/0.5%) hydrogel in ratio 50:50 (nanosuspension:gel) which allowed a drug load of 30 and 50 mg/mL, respectively in the final formulation and still able to gelify at body temperature (37 °C). The nanosuspensions could be easily incorporated into the gels by simple stirring, 1-2 minutes under stirring was sufficient to obtain a visually homogenous suspension. From the incorporation investigation of the nanosuspensions in 5% Solutol at 100 mg/mL into hydrogels it could be concluded that the above mentioned poloxamer based gel offered improved stability and higher dissolved fractions as compared to methocel-based gels, although no gelation was observed at 37 °C at nanosuspension:hydrogel ratio of 50:50. Incorporation in gel composed of P407 (20% in acetate buffer) in ratio 30:70 (nanosuspension:gel), produced a formulation able to form a gel at 37 °C with theoretical drug load of approx. 30 mg/mL. However, nanosuspension in Solutol HS15 showed some reproducibility and scalability issues.

Incorporation investigation of nanosuspension prepared in HPMC 606/PVP K30/SDS (0.5%/0.5%/0.1% w/v) at 100 and 200 mg/mL was also performed in various hydrogels. Based on the results observed on the nanosuspensions in Solutol HS15, the investigation focused on poloxamer-based hydrogels. The nanosuspension at 100 mg/mL could be incorporated in P188/HA (40%/0.5%) which results in a gel at RT at 50% incorporation corresponding to a theoretical drug load of 50 mg/mL. The nanosuspension at 200 mg/mL could be successfully incorporated in hydrogels comprised of P188 (45%), in P407 (22%) which gelified at 37 °C and in P188/HA (40%/0.5%) which gelified at room temperature.

Overall, HPMC/PVP/SDS (0.5%/0.5%/0.1%) proved to be more robust vehicle to prepare stable nanosuspensions, with reproducible and scalable results. Incorporation trials into hydrogels showed the best gel composition to be poloxamer 407 (22%) and P188/HA (40%/0.5%) in which sample was able to form a gel at RT (approx. 23 °C). Same result could be observed by incorporating nanosuspensions at 100 mg/mL and 200 mg/mL with a final theoretical API concentration of 30 and 60 mg/mL, respectively. Nanosuspension in HPMC/PVP/SDS (0.5%/0.5%/0.1%) at 200 mg/mL could be also incorporated in hydrogel comprised of P407 (22%) which was able to form a gel at 37 °C. The final composition of the samples suggested to be progressed for in vivo study is summarized in Table 22. It was recommended to incorporate the nanosuspension in the hydrogel comprised of poloxamer/hyaluronic acid (40%/0.5%) in acetate buffer. HPLC analysis and dissolved fraction, that is the concentration of piperlongumine solubilised in the final formulations

Table 23: Summary of the HPLC analysis and dissolved fraction of piperlongumine formulated in nanosuspensions incorporated into hydrogels (t_0).

<i>Formulations ID</i>	<i>Vehicle Composition</i>	<i>Theoretical API conc. (mg/mL)</i>	<i>Hydrogel composition in acetate buffer pH 5</i>	<i>pH</i>	<i>API conc. (HPLC Assay)</i>	<i>Dissolved fraction (mg/mL)</i>
A1	HPMC/PVP/SDS (0.5%/0.5%/0.1%)	200	P407 (22%)	5.4	60.82±4.77 mg/mL	Very viscous, not analysed
A2		200	P188/HA (40/0.5%)	NA	55.57±22.15 mg/mL	Very viscous, Not analysed
A3		100	P407 (22%)	5.26	26.40±0.04 mg/mL	0.84
A4	5% Solutol	50	P407 (22%)	5.41	13.53±0.04 mg/mL	0.81

As a control experiment, the gelification was investigated also in placebo vehicles to support the tolerability studies in which the selected formulations summarised in Table 22 and the corresponding placebo vehicles are to be tested. The gelification was carried out following the same procedure used for the nanosuspensions containing piperlongumine. For both vehicles comprised of HPMC/PVP/SDS and 5% Solutol, after incorporation in 22% poloxamer 407 in acetate buffer, gelification was observed in ratio 30:70 (nanosuspension:hydrogel). However, upon incorporation of vehicle comprised of HPMC/PVP/SDS in 45% poloxamer 188 in acetate buffer, gelification was observed upon mixing nanosuspension vehicle in hydrogel in ratio 20:80, slightly different as compared to the nanosuspension containing piperlongumine which gelified upon mixing in the same hydrogel in ratio 25:75.

Syringeability assessment of final formulations comprised of nanosuspensions incorporated into hydrogels

From the investigation of the incorporation of nanosuspension into hydrogels at least four lead formulations were identified and are summarised in Table 22. The feasibility of dispensing the hydrogels with incorporated nanosuspension (from HPMC/PVP/SDS vehicle) was performed as described in General Method G. The results from the test are summarised in Table 24. Both formulations appeared to be easy to dispense through a 18G needle. Immediate

gelification was also observed in the collecting vials which was incubated at 37 °C to simulate in vivo situation. Upon removal of the vial from the incubator, the formulations maintained the gelification status for at least 10 minutes.

Table 24: Syringeability results for the hydrogels with incorporated nanosuspension (in HPMC/PVP/SDS vehicle). Gelification observation and piperlongumine assay by HPLC pre and post syringeability is also reported.

<i>Formulations ID</i>	<i>Formulation composition</i>	<i>Syringeability</i>	<i>Gelification observ.</i>	<i>HPLC assay (%)</i>	
				<i>Pre-syringeability test</i>	<i>Post-syringeability test</i>
A5	Nanosuspension:	4, continuous	Immediate gelification	84.69±6.44	86.41±5.53
	100 mg/mL in HPMC/PVP/SDS Hydrogel: P407				
	22%				
A6	Nanosuspension:	4, continuous	Immediate gelification	107.39±4.23	111.56±5.82
	200 mg/mL in HPMC/PVP/SDS Hydrogel: P407				
	22%				

In vitro diffusion and dissolution tests

In vitro diffusion testing according to General Method H was carried out on the most promising hydrogels poloxamers and methylcellulose based selected in the development phase based on their gelation properties. This was carried out for bulk piperlongumine and nanosuspensions.

Bulk and micronized piperlongumine hydrogels

In vitro diffusion studies using Franz cells were conducted in hydrogels by suspending piperlongumine in the gels or pre-solubilising in PEG 400 at 4 mg/mL concentration which was the suitable concentration to guarantee the sink conditions in the in vitro studies. The hydrogels tested were methylcellulose/hyaluronic acid and poloxamer188/hyaluronate. The methylcellulose-based gel gelled at 37-39 °C and gels were prepared in type I water and acetate buffer pH 5 and methylcellulose/hyaluronic acid/PEG 400 (6%/0.25%/10% w/w).

For the poloxamer-based gels, 20% PEG 400 was identified as the optimal concentration that can be incorporated into the gels. Details of the gels prepared for in vitro testing are provided in Table 25 and Table 26.

Table 25: Details of methylcellulose-based gels prepared for in vitro release testing. MC: methylcellulose; HA: hyaluronic acid.

<i>Formulation ID</i>	<i>Composition</i>	<i>API concentration in the formulations</i>	<i>Observations</i>	<i>pH in the cells (after 3 weeks)</i>
20	MC/PEG 400/HA (6%/10%/0.25%) in type I water	4 mg/g	VL at RT	7.32
21	MC/HA (6%/0.25%) In acetate buffer pH 5	4 mg/g	VL at RT	7.31
22	MC/HA (6%/0.25%) in type I water	4 mg/g	VL at RT	7.30

Table 26: Details and visual observations of poloxamer 188-based gels prepared for in vitro release testing. P188: poloxamer 188; HA: hyaluronic acid

<i>Formulation ID</i>	<i>Composition</i>	<i>API concentration in the formulations</i>	<i>Observations (t0)</i>	<i>Observations (7days)</i>
23	P188/PEG 400/HA (28/20/0.5% w/w)	4 mg/g	Clear (solution), VL at RT	Particles in suspension, appear opaque (stored at 2-8 °C)
24	P188/HA (30%/0.5% w/w)	4 mg/g	Opaque, no particles were observed, VL at RT	Particles settled at the bottom, particles suspended not homogeneously upon stirring (stored at 2-8 °C)
25	P188/HA (30%/0.5% w/w)	4 mg/g (micronised API)	Opaque, no particles were observed, VL at RT	No difference observed by visual inspection after 7 days of storage at RT

Piperlongumine was loaded into the hydrogels at the concentration of 4 mg/g, as according to the solubility screening in PBS pH 7.4 (identified as the optimal concentration to maintain in sink conditions). The assay and dissolved fractions of the hydrogels were determined by UV-HPLC after 7 days, stored at room temperature.

The formulations were found to be chemically stable for up to 7 days, and approx. 1% piperlongumine (corresponding to 0.04 mg/mL) was found to be dissolved in the vehicle for formulations 21 and 22, due to the high viscosity, the dissolved fraction could not be analysed in formulation 20. Formulation 20 (MC/PEG400/HA) appeared to be physically stable for up to

7 days, whereas sediments were observed in formulations 21 (MC/HA in acetate buffer pH 5) and 22 (MC/HA in water), the sediments could be re-suspended by stirring in formulation 21 but could not be homogeneously re-suspended in formulation 22.

Figure 5 summarizes the release profile of bulk piperlongumine from the methylcellulose-based gels. After 7 days, approximately 36% of piperlongumine was released from formulations 20 (MC/PEG400/HA) and 21, while 47% was released from 22.

In vitro release profile of piperlongumine in the poloxamer-based gels are shown in Figure 6. A slower release was observed in the first 6 hours in the P188/HA gel (Formulation 24) as compared to P188 gel containing PEG 400 (Formulation 23) and an overall slower release as compared to the MC based gels (7 times less after 24 hours). Formulation 25 was prepared in P188/HA (30%/0.5%) using micronized API, higher release was observed after 24 hours compared to the equivalent formulation prepared with unmicronised piperlongumine (Formulation 24). This suggests that a reduced particle size would lead to a faster initial release. However, a drop in piperlongumine concentration was observed at 1 week time point.

Formulation 23 comprised of P188/PEG400/HA (28%/20%/0.5%) and formulation 24 comprised of P188/HA (30%/0.5%) showed slower release after 2 weeks (approx. 10%), which appeared to be maintained constant up to 3 weeks. Degradant at RRT 0.27 was observed in both formulations at 2 and 3 week time point. The degradation observed could explain the decreased release of piperlongumine. Both formulations appeared to be physically unstable after 1 week storage at 2-8 °C as particles were visually observed. However, no chemical degradation was observed by HPLC. Formulation 25 exhibited the highest release of piperlongumine almost 30% achieved within 6 hours and maintained at approx. 20% in subsequent time points, except for 1 week time point which could have been an outlier or due to experimental issues (evaporation of solvent which precluded the contact between buffer and membrane and therefore did not allow the correct transfer through the membrane). The formulation 25 was stored at room temperature and it appeared to be physically and chemically stable for at least 1 week. It can be concluded that micronized piperlongumine improved the release profile through the gels and potentially the stability of the gel. The storage condition may also have an impact in improving the physical stability since at room temperature high viscous gel was obtained which could prevent the precipitation of piperlongumine powder suspended, while in refrigerated condition the poloxamer-based gel exist in liquid form and therefore precipitation of the suspended API may occurred.

In summary, a higher release profile was observed in methylcellulose-based gels for up to 1 week, followed by a decrease after 2 and 3 weeks. A slower release was observed in poloxamers based gels which was maintained constant for up to 3 weeks. An increased release profile was observed upon incorporation of micronized piperlongumine, suggesting an improved solubility may be obtained with particle size reduction.

Nanosuspension hydrogels

In vitro diffusion (Franz cells) and dissolution testing were conducted to compare the release profile of piperlongumine from selected formulations. The compositions of the formulations are summarised in Table 27. Table 28 summarises the details of the formulations (composition and piperlongumine concentration) prepared for in vitro tests. The particle size distribution of the nanosuspensions stock used for the preparation of the final formulation over 6 days of storage at room temperature is provided in Table 29. The particle size of nanosuspension in 5% Solutol HS15 is in the micron meter range at t0 and no changes were observed after 6 days. For the nanosuspensions in HPMC/PVP/SDS vehicle at 100 and 200 mg/mL a size of approx. 400 and 700 nm was observed; however an increase in size was observed after 6 days of storage, confirming some, but limited stability of the nanosuspensions.

Table 27: Composition of the lead formulations tested by in vitro

<i>Nanosuspension vehicle (N)</i>	<i>Piperlongumine concentration in the nanosuspension (mg/mL)</i>	<i>Hydrogel vehicle (H)</i>	<i>N:H ratio</i>	<i>Final piperlongumine concentration (mg/mL)</i>
HPMC/PVP/SDS (0.5%/0.5%/0.1%)	200	22% Poloxamer 407	30:70	60
	200	Poloxamer 188/hyaluronic acid (40%/0.5%)	30:70	60
	100	22% Poloxamer 407	30:70	30
5% Solutol HS15	50	22% Poloxamer 407	30:70	15

Table 28: Final formulation composition tested in vitro diffusion and dissolution test. The final concentration of piperlongumine in the formulations was calculated by UV-HPLC.

<i>Formulation ID</i>	<i>Nano suspension stock</i>	<i>Vehicle Composition</i>	<i>Theoretical API conc (mg/mL)</i>	<i>Hydrogel composition</i>	<i>Nano suspension: hydrogel ratio</i>	<i>Final API conc upon dilution into hydrogel (30:70) (%assay)</i>
26	29	HPMC/PVP/SDS (0.5%/0.5%/0.1%)	200	P407 (22%) in acetate buffer pH 5	30:70	4.75±0.14
27	30		100			4.01±0.01
28	31	5% Solutol HS15	50			4.22±0.00

Table 29: DLS analysis of the stock nanosuspensions prepared to support the in vitro diffusion and dissolution tests

Nanosuspensions ID	Vehicle Composition	Theoretical API concentration (mg/mL)	Time Point (days)	DLS Analysis		
				PDI	Size (nm)	ζ-potential (mV)
29	HPMC/PVP/SDS	200	0	0.255	709	NA
			6	0.187	1102	-19.5
30		100	0	0.236	424	NA
			6	0.309	723	NA
31	5% Solutol HS15	50	0	0.373	1275	NA
			6	0.345	1205	-7.21

Nanosuspensions incorporated into the most promising hydrogel (22% poloxamer 407) were prepared at API concentration of approx. 4 mg/g and the release profile analysed using diffusion cells (Franz cells) and a small scale dissolution (General Method I). Nanosuspensions incorporated into gels showed a pH of 5 (no changes as compared to the pH of the hydrogel). Physicochemical analysis on the nanosuspensions in HPMC/PVP/SDS and Solutol HS15 incorporated into gels showed similar dissolved fraction (0.8 mg/mL).

In vitro release profile from diffusion test (Franz cells) is provided in Figure 7. The parameters, i.e. diffusion media, filter membrane and piperlongumine concentration were defined after an

optimisation stage. Figure 7 overlays the diffusion profiles of piperlongumine from the different nanosuspensions incorporated into the hydrogel for up to 1 week. A similar profile was observed in all cases, showing a decrease after 48 hours. A zoom in of the first 48 hours is provided in the lower panel of Figure 7.

A small-scale dissolution test was conducted to replicate the same experimental conditions applied in the diffusion cells without the constraints of the membrane. Figure 8 shows the overlay of the release profiles. In diffusion cell system, a slower release was observed as compared to the small scale dissolution: 50% of release was observed after 6 hours, whereas in the small scale dissolution, 100% release was observed within 15 minutes.

Isolation of nanosuspension by freeze-drying

Further improved long-term stability of the nanosuspensions was investigated in this study by isolating the nanoparticles by freeze-drying. This can also further improve the scalability and reproducibility of the nanosuspension in excipients such as Solutol.

Considering the good tolerability observed in the nanosuspension in HPMC/PVP/SDS, it was decided to progress the additional *in vivo* studies only on the nanosuspension prepared in HPMC/PVP/SDS to be incorporated into the hydrogel in poloxamer 407 (22% w/v). Therefore, the feasibility of isolating the particle for the nanosuspension to improve the stability focused only on the nanosuspensions prepared in HPMC/PVP/SDS at 100 and 200 mg/mL. Both concentrations were considered because of the different particles size obtained to understand the effect of the particle size on the *in vivo* performance. For the feasibility work, a fit-for-purpose lyocycle was used as described in General Method J.

The nanosuspensions were prepared following the protocol developed through the optimisation stage (General Method D), both nanosuspensions showed acceptable size and polydispersity index (PDI) at both concentrations (100 and 200 mg/g) after 45 minutes of milling.

The concentration (quantified by UV-HPLC), size and PDI of the lyophilisates were assessed at t₀, post-reconstitution with type I water (General Method K). The amount of water to be added was calculated based on the measured drug load after the freeze-drying process as described in General Method J.

The size and PDI of the reconstituted freeze-dried nano-suspensions at both concentrations are comparable at all time points up to 24 hours. The concentration of a lyophilisate at 100 mg/g remained consistent for up to 24 hours, with no decrease in the percentage area of the API peak in the chromatograms, indicating acceptable physical and chemical stability. The concentration of a lyophilisate at 200 mg/g decreased with time after 3 hours up to 24 hours, suggesting some physical instability as no changes in the percentage area of the API peak

was observed nor appearance of additional peaks in the chromatograms, indicating chemical stability.

The concentration (quantified by UV-HPLC) and particle size (by DLS) of the lyophilisates 1 week and 4 weeks post-freeze-drying was assessed post-reconstitution with water. This was to assess the physical and chemical stability of the lyophilisates upon storage at 25 °C.

The results from the 1-week stability assessment were all comparable with the findings from t_0 .

The results from the 4-week stability assessment are all comparable with the findings from t_0 , no difference was observed at different storage temperature.

Discussion on micronization, nanosuspensions and hydrogels

The purpose of the present work was to develop hydrogels formulation for intracranial administration able to provide an extended release of piperlongumine. Investigation in excipients able to solubilise bulk piperlongumine proved not satisfactory and a maximum concentration of approx. 4 mg/mL could be achieved. Suitable hydrogels were identified comprised of thermoresponsive polymers: methylcellulose/hyaluronate 6%/0.25% and poloxamer 188/hyaluronate 35%/0.5% which are liquid/viscous liquid at temperature ≤ 25 °C but able to gelify at temperature close to 37 °C.

Preliminary in vitro diffusion studies with one of the most promising hydrogel (poloxamer 188/hyaluronate/PEG400 28%/0.5%/20%) identified an improved release profile using micronized piperlongumine, suggesting a beneficial effect in particle size reduction. Therefore, due to the challenges in developing a solution at target concentration of bulk piperlongumine, the development of nanosuspension formulations incorporated in the most promising hydrogel were investigated.

Additional investigation confirmed that nanosuspensions at 100 and 200 mg/g were successfully developed in vehicle comprised in HPMC/PVP/SDS (0.5%/0.5%/0.1% w/v) which could be incorporated in hydrogel comprised of poloxamer 407 (22% w/v in acetate buffer pH 5) able to gelify at body temperature. The final formulation could be easily dispensed through a syringe using a 18G gauge needle. To improve long term stability of the nanosuspensions, the solid was successfully isolated by freeze-drying. The lyophilisates powder are considered to be physically and chemically stable for up to 4 weeks at 25 and 2-8 °C, with an in-use stability of 3 and 24 hours for the nanosuspensions at 200 and 100 mg/mL, respectively.

Production of piperlongumine nanoparticles

Nanoparticles of piperlongumine were produced by an alternative process according to General Method L.

The produced material was sampled, and data was collected for process control and particle characterization purposes. The bulk, as well as the produced nanoparticles, were kept under cooling conditions (2 to 8 °C) during the entire period of the project.

Experiments were planned and executed to optimize throughput and quality, i.e., particle size and distribution, crystallinity, and uniformity of the batches. Temperature and pressure were tuned to optimize the throughput. Optimal parameters to obtain uniform-sized crystalline nanoparticles in homogeneous batches were assessed.

The pressure ranged from 200 to 500 bar and the temperature ranged from 5 °C to 80 °C. Homogenous and uniform materials were produced in multiple batches across different process parameters. Four batches of piperlongumine particles were produced (batch numbers 1-4), with D50 values of 520 nm, 630 nm, 480 nm and 680 nm, respectively. In general, a lower temperature in the range tested was found to be particularly suitable for ensuring homogeneity, uniformity, crystallinity and throughput of piperlongumine. SEM images (Method C) show individual and regularly shaped nanoparticles were formed.

Due to environmental conditions, meaning high ambient humidity, piperlongumine was exposed to water during CO_{2(s)} sublimation. Nanoformed piperlongumine presented variations in morphology and particle size after being exposed to high humidity. Samples were therefore placed under vacuum to produce piperlongumine with good uniformity and homogeneity.

X-Ray Powder Diffraction (XRPD) analysis

The bulk piperlongumine (97% purity, ABCR batch no. 1446971) was analysed together with the nanoformed material using X-ray powder diffraction (XRPD).

The results for bulk piperlongumine are shown in Figure 9, and the main reflection peaks for are assigned in the graph: 12.8°, 21.3°, 25.9°.

The XRPD diffractograms of the bulk and nanoformed samples (Figure 10) revealed that the nanoformed piperlongumine has a different crystalline structure than bulk piperlongumine. This result was observed in all diffractograms acquired for the nanoparticles. Since only one known polymorph of piperlongumine was found in the literature (Bezerra *et al.*), it can be stated that a new polymorph of piperlongumine was obtained through the CESS® process.

The XRPD main peaks in the bulk and nanoparticle samples are shown in Table 30.

Table 30: XRPD main peaks for bulk and nanoparticle piperlongumine.

<i>Bulk (2θ degree)</i>	<i>Particles (2θ degree)</i>
7.080	12.363
11.261	14.888
12.565	16.713
12.864	18.028
14.467	19.508
18.216	21.616
21.340	23.766
22.645	25.439
24.488	26.607
25.902	28.890
31.874	

Comparison of bulk piperlongumine and crystalline form of piperlongumine

Piperlongumine was successfully nanoformed to produce crystalline nanoparticles within the D50 of 400 to 788 nm.

The nanoformed material, together with bulk piperlongumine (97% purity, ABCR batch no. 1446971), was analysed using scanning electron microscopy (SEM), differential scanning calorimetry (DSC), high performance liquid chromatography (HPLC), and Fourier transform infrared spectroscopy, using attenuated total reflectance (FTIR-ATR). This array of techniques provided an overview of the material properties, including particle morphology, size, chemical structure, thermal behavior, crystallinity, and impurity profile. The bulk API characteristics served as the reference for evaluating the properties of the nanoformed API. The results indicated high-quality, stable crystalline material suitable for formulation development and preclinical *in vivo* studies.

Bulk Piperlongumine

The bulk piperlongumine DSC curve (collected using Method H) showed a single melting event at 122.7 °C (peak temperature) and melting enthalpy of 115.0 J/g in the first heating cycle (Figure 11). In the second heating cycle it was possible to observe a glass transition (T_g) at 10.1 °C, and a broad crystallization event at 79.5 °C.

Some differences were detected in the DSC thermographic profiles between separate samples of bulk piperlongumine. For a separate sample, $T_{m(onset)}$ was found to be 121.3 °C, $T_{m(peak)}$ was 122.7 °C, ΔH was 118.1 J/g (all first cycle). The morphology between the samples also varied, with one showing rod-like morphology and another presented needle-like habit. These

samples of bulk piperlongumine had been purified by different methods. This could explain the differences observed.

HPLC data (collected using Method K) was used to evaluate the impurity profile of bulk piperlongumine. The results comply with the ones found in the literature.

SEM images of the bulk material (collected using Method C) revealed either rod-shaped particles in the micrometer range (Figure 12, top and bottom left panels) or needle-shaped particles (Figure 12, bottom right panel).

Crystalline form (nanoformed) Piperlongumine

The data obtained from DSC analyses of batches 1-1 to 1-6 of piperlongumine particles are summarized in Table 31. The melting temperature (T_m) observed for nanoformed piperlongumine (average of 121.81 °C across six batches) is in accordance with the melting temperature found for the bulk (122.7 °C), as is also the glass transition temperature (T_g). Moreover, the melting enthalpy values suggest the presence of stable crystals.

Table 31: Thermal characteristics of representative batches of nanoparticles analysed by DSC. T_m and ΔH values were depicted from the first heating cycle.

Batch	$T_{m(onset)}$ (°C)	T_c (°C)	T_g (°C)	ΔH (J g⁻¹)
1-1	121.1	105.2	8.9	104.1
1-2	121.0	60.8	9.2	68.2
1-3	122.4	110.0	9.2	102.5
1-4	122.6	108.0	9.8	110.9
1-5	121.3	98.0, 84.8, 78.8, 71.1	9.7	84.6
1-6	122.5	-	9.7	111.7

The purity of the nanoformed material was assessed by HPLC using (Method K). The impurity profile obtained for the nanoformed piperlongumine did not show modifications compared to the bulk, and the purity obtained was over 98% for all batches. Thus, the material remained chemically stable after the CESS® process.

SEM images of the nanoparticle samples (batches 1-1 to 1-6) produced show uniformity and homogeneity of the samples (Figure 13).

FTIR spectrum were collected (using Method G) for bulk piperlongumine and the new crystalline form (nanoformed piperlongumine). The bulk FTIR spectrum serves as a reference for nanoformed piperlongumine, and some key peaks are set out in Table 32.

Characteristic bands attributed to the carbonyl stretching ($\nu\text{C=O}$) from the amide band I is observed at 1668 cm^{-1} . The amide band II corresponding to the N-H deformation ($\nu\text{N-H}$) is seen at 1585 cm^{-1} . The sharp peak at 2830 and the 1618 cm^{-1} , correspond respectively to the C-H stretching and deformation from the unsaturated C-C bond (C=C-H).

Table 32: Selected peaks in FTIR spectrum of bulk piperlongumine and new crystalline form (nanoformed piperlongumine).

<i>Wavenumber (cm^{-1})</i>	<i>Compound Form</i>	<i>Attribution</i>
2943 2831	Bulk	-CH ₂ - stretching asymmetric, stretching symmetric
1678 (doublet)	New crystalline form	-C=O stretching (Amide I)
1678 (singlet)	Bulk	-C=O stretching (Amide I)
1482, 1416	New crystalline form	-CH ₂ - deformations (bending)
1187	New crystalline form	-CH ₂ - deformations
3013	Bulk	C=CH- stretching

FTIR spectra of sampled batches showed no degradation or chemical modifications after the nanoforming process. The HPLC results (Method K) corroborate this result. Nevertheless, some new bands were found in the FTIR spectra of the nanoformed piperlongumine. As the degradation hypothesis was excluded, and after visiting the literature (Kuhn et al., 2010), it was concluded that those bands are attributed to different conformations of the piperlongumine molecule, which occur due to the process. An important feature that must be highlighted concerns the carbonyl (C=O) stretching, seen at around 1679 cm^{-1} as a singlet in the bulk spectra, which is observed as a doublet in the nanoformed piperlongumine. The appearance of a doublet in the spectra of nanoformed material confirms that one (or both) carbonyl group(s) is interacting in a different way than observed for the bulk, either with some other part of the same piperlongumine molecule, or with another piperlongumine molecule. This changes the unit cell and, therefore, the crystal lattice producing the new polymorph.

A representative FTIR spectrum of the bulk and nanoparticles (batch 1-2) of piperlongumine is shown in Figure 14.

A second production was carried out using varying process conditions, and four batches in this production were blended and homogenized accordingly. The particles size (D50) ranged from 571-640 nm. The average onset melting temperature ($T_{m(\text{onset})}$) by DSC was $121.7 \pm 0.3\text{ }^{\circ}\text{C}$. FTIR spectra were compatible with the chemical structure, not showing signs of degradation. XRPD profile corresponds to the polymorph obtained in the first production. The purity assessed by HPLC of the combined final batch is 98.00%.

Five further batches in the production were blended and homogenized accordingly. The particles size (D50) ranged from 487 to 705 nm and the characterization matched those in the previous productions.

The results from particle size distribution analysis of representative batches are shown in Table 33. These results show that piperlongumine particles are reliably produced by the method.

Table 33: PSD parameters and results

<i>Batch</i>	<i>No images</i>	<i>No. particles</i>	<i>D10 (nm)</i>	<i>D50 (nm)</i>	<i>D90 (nm)</i>
1-2	3	152	434	591	801
2-1	3	107	309	487	918
2-3	4	91	492	705	942
2-4	5	166	396	586	806
2-5	4	188	420	561	745
2-6	3	113	443	592	762

DSC curves for piperlongumine particles (batches 2-1 and 2-3 to 2-5) are shown at Figure 15. The curves present one main endothermic event attributed to the melting of the nanoformed and bulk piperlongumine, respectively during a first heating cycle.

The DSC curves shown at Figure 15 also present a minor exothermic event, which can be attributed to crystallization. The values on top of each curve correspond to, respectively, onset melting temperature (T_m) and enthalpy of melting (ΔH). The T_m is comparable with the bulk (122.7 °C) but slightly lower, whilst the ΔH (78.77 J.g⁻¹) suggests that the crystalline form of nanoformed particles is more stable compared with bulk.

A three-month stability study was conducted for batches of nanoformed piperlongumine. The objective of the study was to evaluate the chemical and morphological stability of the nanoformed Piperlongumine.

Briefly, the material, which had been stored in refrigerator, was allowed to equilibrate at room temperature. The amber flasks containing the material were then packaged in aluminum pouches and placed in a stability chamber at 25 °C and 60% RH. Aliquots of the samples were collected in three time points, at 1 week (T_1), 1 month (T_2) and 3 months (T_3).

The D50 obtained for the starting material (T_0) was 360 nm, which is relatively smaller than the D50s described above. A plausible hypothesis for that is the humidity visually detected in the sample. The water/API interaction could provoke the molecule to contract, which is reflected in the size of the particles.

The particles showed a growth after one week (T_1). Growth was also observed from one week to one month (T_2) and, after three months (T_3), there was practically no

changes in particle size. Morphology changes were observed during the study, being the most preeminent the one observed between T_0 and T_1 . It has been observed that drier samples presented a more spherical morphology, whereas samples containing certain humidity showed a more polygonal morphology. This behaviour was verified in the stability study from T_0 to T_1 , as the original sample was noticeable humid. The purity of the material at the end of the study assessed by HPLC was 98.84% meaning that there was no decomposition of the material during the stability test at 25 °C and 60% RT.

No major differences were found in the thermal behaviour through DSC. A slightly decrease was observed in the enthalpy of fusion, suggesting a minor decrease in the crystal stability.

The material stored in refrigerator was also subject of the stability study. The timepoint zero was the same as shown in the previous room temperature condition. However, the first time point (T_1) corresponds to three months stored sample, and the time point two (T_2) corresponds to 4.5 months.

The results showed that after three months, the D50 of the particles increased. After 4.5 months, the D50 of the particles decreased. However, the span, which gives information about the size distribution was higher in this last case (0.56, 0.88 and 1.03, respectively). The morphology observed through SEM suggests a merging of the particles forming a larger particle, but it shows also single particles in the nm range. This can explain the higher number for the span and smaller number for D50.

DSC curves showed no major differences between the thermal behaviour of T_0 and T_1 . T_3 showed a decrease in the enthalpy that may indicate that the stability of the crystals decreased during the study. The crystallization peak present at about 109 °C confirms the presence of amorphous material since the beginning of the test. The purity assessed through HPLC was 98.87%, confirming that there was no degradation of the molecule also in cool conditions, during the period tested.

Conclusion

In conclusion, piperlongumine was successfully nanoformed to produce crystalline nanoparticles within the D50 of around 400 - 800 nm. The material was analysed by SEM, HPLC, XRPD, DSC, and FTIR, and the results indicated high-quality, stable crystalline material suitable for formulation development and preclinical in vivo studies.

Incorporating piperlongumine nanoparticles into hydrogels

Potential blank hydrogel compositions were screened to obtain thermoreversible gels exhibiting gel transition at room temperature or body temperature. Based on the experiments, the most promising gel matrices could be narrowed down to poloxamers and Soluplus. These

include poloxamer 407 (P407) at 16%, 18%, or 20% w/w, and Soluplus at 27.5% or 30% w/w, each in 0.1 M pH 5 acetate buffer.

The polymers P407 and Soluplus were selected for further experiments with their gel transition in acetate buffer. It is to be noted, that similar results were obtained when the medium was switched to water. Poloxamer 338, poloxamer 188 or mixtures of various poloxamer grades could also be viable alternatives to the poloxamer 407 samples.

The cold P407 solution could be drawn into the syringe barrel easily. Even after transition into gel at room temperature the material could still be pushed out of the syringe. The Soluplus sample was much more viscous at both 4 °C and room temperature compared to the P407 based one. Nevertheless, the syringe could be filled slowly with the material. Pushing out the Soluplus solution from the syringe barrel was effortless. For the end-user filling of the Soluplus sample into the syringe barrel could be easier without a needle, especially considering the further increased viscosity of the nano piperlongumine loaded gels.

Hydrogels were prepared according to General Method N with an API loading of 10-30% in the media as shown in Table 34.

In general, the addition of nanoformed piperlongumine prepared as described above into the blank gel formulations did not fundamentally alter gel transition properties, the same patterns were observed for the blank hydrogels, and the gels exhibited gel transitions within the desired temperature range.

In conclusion, poloxamer 407 and Soluplus based gel compositions were successfully developed using piperlongumine nanoparticles.

Table 34: Piperlongumine loaded gel compositions.

<i>Gel matrix</i>	<i>C_{polymer} (% w/w)</i>	<i>Medium</i>
Poloxamer 407	16-20	Water
Poloxamer 407	16-20	0.1 M acetate buffer pH 5
Poloxamer 407	16-20	0.1 M citrate buffer pH 6
Poloxamer 407	16-20	0.1 M tris buffer pH 7
Soluplus	27.5-30	Water
Soluplus	25-30	0.1 M acetate buffer pH 5
Soluplus	15-25	0.1 M citrate buffer pH 6
Soluplus	27.5-30	0.1 M tris buffer pH 7

Content Uniformity of Piperlongumine gels

Preliminary content uniformity analysis was performed on aqueous poloxamer and Soluplus based gels with 10% and 20% piperlongumine loading (General Method T). Measured active content values are shown in Figure 16 and were in agreement with nominal concentrations justifying the viability of the sample preparation method.

The content uniformity assays were repeated on 20% API loading samples prepared in various media with P407 and Soluplus gel matrices (Figure 17). The tests confirmed the previous findings: the measured active loadings at the initial time point were practically identical to the nominal values with very low standard deviations with the exception of citrate buffer based gels. During storage a statistically significant increase in active loading could be observed for each gel excluding the citrate buffer-based gels. Most probably this could be explained by the loss of some water (evaporation) from the gel samples that increased the active concentration. For the citrate buffer-based gels the steady active loading might be the result of gradual chemical decomposition which is compensated by the drying of the gels.

A third set of samples used for sterilization studies further confirmed excellent content uniformity (Figure 18). In this case the Soluplus-based samples were prepared even without magnetic stirring but mixing manually with spatula. Therefore, homogenization of the components with a spatula could be a viable preparation method as well. Again, a very slight increase in active loading after sterilization was observed which could be explained by the evaporation of water from the samples on open Petri dishes.

Chemical Stability of Gels During Storage

Chemical stability of piperlongumine gels was monitored for 14 days with HPLC during storage at 40 °C (General Method S). The results are shown on Figure 19. Aqueous and tris buffer-based gels exhibited excellent chemical stability together with the nanoformed material with practically no decomposition detected.

There might be chemical incompatibility on the long run between aqueous poloxamer gel matrix and the API (ANOVA, $p = 0.029$), however, two tailed t-tests between the various time points and 14 days yielded $p > 0.05$ in every case. Nevertheless, the differences in peak purity were extremely low, therefore, it should not impact *in vivo* experiments.

Citrate buffer samples yielded high levels of impurities immediately after production. The peak purity decreased steadily indicating gradual decomposition of piperlongumine during storage (see light green and orange curves on the upper diagram on Figure 19).

Based on the stability assessment it could be refuted that piperlongumine decomposed rapidly at neutral pH. No decomposition products were found in pH 7 tris-HCl buffers regardless of the gel matrix for at least 14 days at accelerated conditions.

Chemical Stability of UV Irradiated Gels

The chemical stability of the gels was monitored before and after UV irradiation (General Method S). The results are shown on Figure 20. A very slight decrease in main piperlongumine peak% could be observed throughout the samples. However, this should not impact in vivo performance, especially considering the variability of the measurement (see the lower diagram on Figure 5) and the fact that the differences are not statistically significant for the aqueous samples. The following values were obtained with two tailed t-tests: $p = 0.18$ for P407 in water, $p = 0.001$ for P407 in tris, $p = 0.26$ for Soluplus in water and $p = 0.02$ for Soluplus in tris.

Piperlongumine Dissolution from Hydrogels

Piperlongumine release data from the gel samples are plotted on Figure 21 (General Method U). Interestingly bulk poloxamer samples dissolved much faster compared to the nano poloxamer gels. This might be explained by secondary interactions between the piperlongumine nanoparticles and the PEO-PPO-PEO micelle scaffold. The hypothesis was also supported by the observation that bulk API samples are suspensions at 20 °C while nanoformed API samples form gels at the same temperature. The rest of the samples had comparable dissolution rates. It is to be noted that contact surface area between the gels and the medium and agitation rate had profound effect of dissolution rates (data not shown).

Permeability Measurements

Results of the permeability measurements are shown on Figure 22 (General Method V). The data are in agreement with the dissolution results showing fastest dissolution rate for the bulk poloxamer gel. It can also be concluded that piperlongumine readily permeated through artificial lipid membranes with permeated amounts correlating well with dissolved amounts in the donor side. Based on the results one would not expect issues with the uptake of the API (assuming negligible efflux transport and metabolism). It is to be noted again, that API release rate and thus flux into the acceptor compartment depended strongly on agitation and the contact surface between the gel and the dissolution medium (data not shown).

No change was observed in the UV spectra throughout the measurement indicating no piperlongumine decomposition.

SEM Analysis of the Hydrogels

SEM images are shown on Figure 23 and Figure 24 for the poloxamer and Soluplus based gels, respectively (General Method W). No large aggregates or crystals could be observed. The particle sizes are in the range of the unformulated nanoformed material. These preliminary data suggest that the gels can be stored safely in cold conditions without significant aggregation or particle size growth.

Summary

Chemically stable poloxamer 407 and Soluplus based gel compositions were successfully developed using piperlongumine nanoparticles. Piperlongumine nanoparticles remained stable in the hydrogel formulations prepared over a 14-day period. An active loading of 30% can be achieved in the final gel formulation, representing a substantial improvement over solutions of bulk piperlongumine.

It was also shown that the gel compositions withstood UV sterilization with piperlongumine purity remaining stable at 98+%, prior and post UV irradiation. Overall, the following gel samples are the most preferred for *in vivo* use:

- 10 - 30% nano piperlongumine in 18 - 20% poloxamer 407 in water
- 10 - 30% nano piperlongumine in 18 - 20% poloxamer 407 in 0.1 M pH 7 tris buffer
- 10 - 30% nano piperlongumine in 27.5 - 30% Soluplus in water
- 10 - 30% nano piperlongumine in 27.5 - 30% Soluplus in 0.1 M pH 7 tris buffer

The bulk API and API particles in poloxamer 407 gel matrix had markedly different dissolution times, interestingly bulk gels dissolved faster. Soluplus gels offered comparable or slightly slower dissolution to the nanoparticle poloxamer samples. Piperlongumine permeated readily through artificial lipid membranes indicating no issues expected with the uptake of the compound.

Additional Examples

Materials

Kolliphor® P188 Geismar (P188), Kolliphor® P407 Sigma-Aldrich (P407), Kollidon® 17PF Polyvinylpyrrolidone (PVP), and Kollisolv® PEG400, Polyglycol (PEG400) were purchased from BASF (Germany). Polyvinyl alcohol (PVA) was purchased from Sigma-Aldrich (USA). Carboxymethylcellulose Sodium Salt high viscosity (CMC) and Polysorbate 80, Tween 80 (Tween®80) were purchased from Panreac (Spain). High MW Sodium hyaluronate (HA) was purchased from Inquiaroma (Spain). Mucin from porcine stomach Type II (Mucin) was purchased from Sigma-Aldrich (USA). Piperlongumine (PL) was purchased from Indofine

Chemical Company (USA). Acetonitrile (ACN) and Trifluoroacetic acid (TFA) HPLC Grade were purchased from Honeywell (USA). Ultrapure water (18.2 MΩ cm) was obtained with a MilliQ apparatus by Millipore (Milford, MA, USA).

Results and Discussion

The examples below are aimed at developing a novel therapeutic approach for the treatment of diseases including soft tissue sarcomas (STS) by formulating a thermoresponsive hydrogel loaded with piperlongumine for *in situ*-intratumoral injection. The results establish a localized delivery system capable of targeted drug release within STS tumors.

Solubility Studies

In a first study, four different PEGs (PEG 1500, PEG 2000, PEG 4000, and PEG 6000) were evaluated alongside PEG 400 in a 40:60 (w/w) ratio. Solubility tests were conducted using various mixtures of polyethylene glycols (PEGs) as described herein. Each mixture was prepared at 40% PEGs to 60% (w/w) PEG 400 and heated to 50 °C. Subsequently, the higher molecular weight PEG was added to PEG 400, and the solution was stirred until a transparent and homogeneous mixture was achieved. In an initial phase, the combination of PEG 4000 and PEG 400 was promptly discarded due to rapid solidification even before PL was added. For the remaining mixtures, PL was added at a 20 mg/mL concentration and stirred adequately. Solubility evaluation was performed through visual observation techniques.

In a second phase, the solubility of PL was assessed in PEG 400 with Tween 80 at varying concentrations: Tween 80/PEG 400 (0.46/99.54% [w/w]), Tween 80/PEG 400 (1.23/98.77% [w/w]) and Tween 80/PEG 400 (5/95% [w/w]). Solutions were prepared at room temperature with agitation set at 300 rpm. After complete dissolution, PL was introduced into the solution at a 20 mg/mL concentration. Following overnight agitation, the samples underwent centrifugation at 12,300 g for 5 minutes. Subsequently, the supernatant was analyzed using the HPLC method outlined below.

Visual analysis indicated that the solutions containing 20 mg/mL of PL exhibited a solid white appearance, indicating poor solubility, which suggests that higher molecular weight PEGs do not improve PL solubility.

The impact of adding Tween 80 to PEG 400 at different ratios (0.46/99.54%, 1.23/98.77%, and 5/95%) was explored next. Visual analysis revealed that higher concentrations of Tween 80 correlated with enhanced PL solubility, suggesting that Tween 80 can improve solubilization of PL within the PEG 400 matrix. HPLC analysis also suggested that Tween 80 plays a crucial role in enhancing the solubility of PL compared to PEG400 alone.

Overall, the results show that a Tween 80 concentration of 1.23% provides a balance between effective solubilization of PL and practical considerations for formulation development.

Preparation of Poloxamer 407 hydrogels

P407-based control gels were prepared at a concentration of 15, 16, 16.5 and 17 % (w/w). In a 30 mL vial, the stated amount of refrigerated deionized water was weighed. Vials were then placed on a magnetic stirrer. The stated amount of poloxamer powder was added to the vials under stirring. The vials were kept stirring until complete dissolution of the polymer. The hydrogels were stored in refrigerated conditions (4 °C).

P407-based test gels were prepared at a concentration of 15% (w/w). In a 30 mL vial, the stated amount of refrigerated MilliQ water was weighed. Vials were then placed on a magnetic stirrer. The stated amount of poloxamer powder was added to the vial under stirring. The vial was kept stirring until complete dissolution of the polymer. The hydrogel was stored in refrigerated conditions (4 °C). In a separate glass vial, 20% (w/w) of Tween 80 and PEG400 (1.23/98.77% (w/w)) were added to another glass vial and left to stir under magnetic stirring until complete homogenization. PL was added (2% (w/w)) and left to stir overnight. Then the Tween 80, PEG400, and PL solution were added to the P407 solution and left to stir for 4 h. Finally, 1% (w/w) of bioadhesive polymer (PVA, PVP, CMC or HA) was weighed and added to the poloxamer hydrogel prepared and left to stir until a homogeneous solution was obtained.

P407/P188-based gels were prepared at a concentration of 15/5% (w/w). In a 30 mL vial, the stated amount of refrigerated deionized water was weighed. Vials were then placed in a magnetic stir plate. The stated amount of P407 and P188 powders was added to the vial under stirring. The vial was kept stirring until complete dissolution of the polymers. The hydrogel was stored in refrigerated conditions (4 °C). In a separate glass vial, 20% (w/w) of Tween 80 and PEG400 (1.23/98.77% (w/w)) were added to another glass vial and left to stir under magnetic stirring until complete homogenization. PL was added (2%, w/w) and left to stir overnight. Then the Tween 80, PEG400, and PL solution were added to the P407 solution and left to stir for 4 h. Finally, 1% (w/w) of bioadhesive polymer (PVA, PVP, CMC or HA) was weighed and added to the poloxamer hydrogel prepared and left to stir until a homogeneous solution was obtained.

Hydrogel Analysis

Four hydrogel formulations were prepared with varying P407 concentrations (15%, 16%, 16.5%, and 17% w/w) as described above. Rheological characterization was performed to

determine the T_{sol-gel} of each formulation; PL was added to the hydrogel formulation, and its effect on T_{sol-gel} was evaluated.

Rheological analyses were performed using a rheometer (Malvern Kinexus Lab + Rheometer from Malvern Instruments in Malvern, UK) in a cone-and-plate geometry with a diameter of 50 mm (cone angle 1°). Samples were kept at 5 °C ± 3 °C until measurement. A solvent trap was used to minimize evaporation and keep a solvent-saturated atmosphere surrounding the sample. Measurements were performed in at least triplicate.

Viscoelastic properties were determined by oscillatory rheometry. To determine the linear-viscoelastic region (LVER), the amplitude sweep conditions used were shear strain between 0.01 % and 100 %, with 1 Hz frequency. The LVER was at shear strain of 1 %. From these results, the temperature sweep tests with oscillatory shear were performed by subjecting the sample, initially loaded at 4 or 25 °C, to a 5 °C/min heating rate. These tests were carried out at a frequency of 1 Hz and a shear strain of 1%. Throughout the process of heating from 4 or 25 °C to 50 °C, the G' and G'' moduli were measured. Oscillatory rheology provides the most sensitive measurement of the main parameters T_{sol-gel}, G' and G'' moduli and the loss factor tangent δ ($\tan\delta = G''/G'$). The gelation point, the crossover transition temperature, was identified when G' equaled G''.

The results show that increasing P407 concentration led to a decrease in T_{sol-gel}, with transition temperatures ranging from 28.9 ± 0.7 °C for 15% P407 to 26.7 ± 0.1 °C for 17% P407. The addition of PL significantly reduced the T_{sol-gel} to 21.5 ± 0.1 °C. These results show that both P407 concentration and PL presence influence hydrogel T_{sol-gel}. The significant reduction in T_{sol-gel} upon PL addition suggests potential interactions between PL and P407, altering the hydrogel's rheological properties. The results indicate that 15% P407 concentration offers an advantage since even with the reduction in T_{sol-gel} due to the presence of PL, the final temperature is higher than the other formulations tested. Additionally, maintaining a lower concentration of P407 can have additional benefits such as cost reduction and minimizing potential adverse effects associated with higher polymer concentrations. Therefore, 15% P407 was selected for further experiments.

A combination of P407 with Poloxamer 188 (P188), a more hydrophilic poloxamer with a lower molecular weight was also investigated. Three formulations were tested: 15/5 % (w/w) P407/P188, 20/1 % (w/w) P407/P188, and 20/5 % (w/w) P407/P188.

T_{sol-gel} was chosen as the temperature at which both moduli, G' and G'', were equal, reflecting similar elastic and viscous properties (G' = G'' crossover). The 15/5% composition exhibited the highest T_{sol-gel} of 34.5 °C. The 20/1% and 20/5% compositions resulted in lower T_{sol-gel} values of 23 °C and 25 °C, respectively. Adding 5% w/w of P188 to P407 also resulted in lower G' modulus values than using 15% w/w P407 alone. The values of the loss

factor, $\tan\delta$, were also lower than 1.0 for the P407/P188 at 37 °C and better than 1.0 at 25 °C, indicating a G' higher than G'' at 37 °C.

The observed dependence of Tsol-gel with the ratio of P407 and P188 suggested that the balance between these two polymers is important in determining the gelation temperature. The results suggest that a higher content of P407 might lead to faster gelation due to its thermosensitive properties, while a lower content of P188 may not provide sufficient structural support, resulting in a lower Tsol-gel.

P407/P188 15/5% w/w is made of uncrosslinked copolymers and behaves like a liquid below the Tsol-gel. However, above this point, it turns into a gel and provides an elastic semi-solid material at 37 °C. Thus, it appears to be the most suitable for the intended use, as it achieves a higher Tsol-gel and results in an optimal gelation temperature for use in e.g. STS treatment.

Effect of bioadhesive polymers on hydrogel properties.

In this example, four bioadhesive polymers - Polyvinylpyrrolidone (PVP), Polyvinyl alcohol (PVA), Carboxymethyl cellulose (CMC), and Hyaluronic acid (HA) at 1% (w/w) - were evaluated in combination with the most suitable hydrogel identified above, containing P407/P188 15/5% (w/w), PEG400/Tween80 (98.77/1.23%) 20%, and PL 2%. The four polymers were also tested in conjunction with 15% (w/w) P407 as an alternative formulation.

The evaluation of adhesive strength and mucoadhesion was conducted with a plate-on-plate geometry by setting a velocity of 0.1 mm/s and a final gap of 5 mm; the adhesive value represented the force required to displace the sample from the probe. To assess mucoadhesion, the aforementioned adhesive force protocol involved combining each sample with a 10 % (w/w) mucin solution at a 1:1 ratio. The mucin solution, prepared by gentle stirring until complete dissolution, was hydrated with water, resulting in a 10% dispersion (w/w). The mucoadhesive strength of both individual samples and mucin was measured in triplicate.

Viscoelastic properties were comprehensively evaluated through oscillatory studies. HA-based hydrogels demonstrated a Tsol-gel of 34.6 ± 0.5 °C, indicating a moderate gelation temperature compared to other formulations. PVP-based hydrogels exhibited a slightly higher sol-gel transition temperature of 35.2 ± 0.3 °C. PVA-based hydrogels displayed a sol-gel transition temperature of 32.7 ± 0.1 °C, falling within the range of the other formulations. Overall, the results show that hydrogel samples containing HA and PVP exhibit similar viscoelastic behaviour. At 25 °C, these samples demonstrate $\tan\delta > 1.0$, with G'' surpassing G' , indicating a weak hydrogel state. However, at 37 °C, their behaviour shifts, displaying $\tan\delta < 1.0$, characteristic of strong hydrogels. In contrast, the PVA and CMC hydrogels maintain strong hydrogel behavior at both 25 °C and 37 °C, with $\tan\delta < 1.0$. This suggests distinct viscoelastic properties compared to the other formulations. Furthermore, PVP exhibited the highest sol-gel transition temperature, while PVA demonstrates the lowest.

Despite these differences, all samples exhibit sol-gel transition temperatures within the range of 28 °C to 35 °C, making them suitable candidates for thermosensitive hydrogels intended for in situ administration.

The impact of the bioadhesive polymers on hydrogel viscosity was also determined. The hydrogels were found to exhibit thermoresponsive behaviour, as evidenced by notable changes in viscosity at different temperatures. The rise in temperature led to an increase in viscosity, indicating that the incorporation of bioadhesives did not affect the phase transition of the poloxamer hydrogel. Moreover, below the $T_{sol-gel}$, the hydrogel behaved like a low viscous liquid ($\eta < 1 \text{ Pa.s}$), while above it, it transformed into a medium viscous gel system at 37 °C ($100 \leq \eta \leq 1 \times 10^3 \text{ Pa.s}$). The viscosity measurements were taken at constant temperatures of 25 °C and 37 °C, representing the SOL and GEL states, respectively. Figure 25 displays the viscosity results for the different formulations at these two temperatures.

Overall, the results indicate that PVP is a promising candidate due to its balanced viscosity profile and thermoresponsive behaviour.

Next, solutions containing a 1% concentration of PVA, PVP, CMC, or HA combined with 15/5% (w/w) P407/P188 were utilized to assess adhesiveness, alongside a control solution comprising 15/5% (w/w) P407/P188 without any bioadhesive polymer. For this analysis, solutions with a 1% concentration of PVA, PVP, CMC, or HA combined with 15/5% (w/w) P407/P188 were conjugated with 10% Mucin Type II (1:1). Additionally, a control solution of 10% Mucin Type II with H₂O (1:1) without any bioadhesive polymer was included. Mucin is a major component of mucus secretions, which can be used as a substrate to assess mucoadhesivity. The results are presented in Figure 26.

When combined with mucin, PVP demonstrated the highest performance with an average area under the force-time curve of $0.56 \pm 0.08 \text{ N.s}$. CMC and HA demonstrated moderate mucoadhesion, with average values of $0.39 \pm 0.05 \text{ N.s}$ and $0.36 \pm 0.06 \text{ N.s}$, respectively. PVA showed the lowest mucoadhesion among the tested polymers, with an average value of $0.31 \pm 0.04 \text{ N.s}$. adhesive strength and mucoadhesive properties highlight the importance of considering multiple factors in material selection.

These findings demonstrate that PVP surpasses the other bioadhesive polymers evaluated, including HA, PVA, and CMC, in terms of mucoadhesive performance. Thus, PVP was identified as the most preferred polymer for incorporation into hydrogel formulations intended for biomedical applications. These superior mucoadhesive properties suggest that PVP-based hydrogels will exhibit enhanced adhesion and retention on mucosal surfaces.

In vitro release studies

The release profiles of PL from the P407/P188 hydrogel with different bioadhesive polymers (HA, PVP, PVA, and CMC) were analyzed over a 30-hour period and quantified by HPLC. The results are shown in Figure 27.

Release of PL from the different formulated hydrogels was measured in infinite dose conditions using Supor® 0.45 µm 23 mm PES 100/pk membrane disc filters. The membranes were washed and equilibrated with the receptor phase for 30 min and then set between the donor and receiver compartments of the Franz diffusion cells. The receptor phase was PEG400/Ethanol/Water (40/10/50% (v/v)). The system was maintained at 37 ± 2 °C for 30 min before the experiment started. The samples were then applied (0.3 g) evenly on the surface of the membrane in the donor compartment and immediately sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) to prevent water evaporation. The Franz cells were incubated at 37 °C in an appropriate heating block and under stirring at 220 rpm. Samples of the receptor phase (200 µL) were collected at predefined times. After sampling, the same volume was replaced with fresh receptor phase, kept at the same temperature. These release tests were performed using three Franz cells per formulation. The amount of permeated drug was assayed using HPLC.

At 30 hours, PVA exhibited the highest release percentage (45 ± 5 %), followed by PVP (40 ± 5 %), HA (17 ± 7 %), and CMC (15 ± 1 %). PVA and PVP are therefore beneficial by having a more sustained release of PL. By contrast, HA and CMC exhibited relatively lower release percentages at 30 hours. While CMC had the lowest release percentage among all additives at this time point, it still contributed to the overall release profile of PL from the hydrogel. The sustained release observed for all formulations suggests the stability of the hydrogel and its potential for maintaining therapeutic concentrations of PL over an extended period.

Based on all the performed assays, PVP was identified as the most suitable additive. The formulation has a favourable release profile, coupled with potential advantages observed in other assays, suggesting that PVP is a promising additive for enhancing the performance of the P407/P188 hydrogel in drug delivery applications.

Further formulation optimization

Lastly, the optimal concentrations for each component of the hydrogel formulation were determined to be 2.5% P188, 15% P407, and 1% PVP.

Subsequently, rheological characterization studies and extrudability tests were conducted on hydrogel formulations containing 2.5% and 5% P188 to assess their suitability for the intended application. The sol-gel transition temperatures of both formulations fell within the desired

range of 26 °C to 36 °C, with the 2.5% P188 formulation exhibiting a slightly higher transition temperature of 35.3 ± 0.04 °C compared to 31.5 ± 0.09 °C for the 5% P188 formulation.

Viscosity measurements at different temperatures revealed that at 25 °C, both formulations displayed relatively low viscosity, indicative of fluid solutions suitable for injection. Both formulations exhibited significant increase in viscosity at 37 °C, but remained injectable when combined with PL due to their low viscosity. Furthermore, analysis of shear rate dependency underscored the shear-thinning behavior of both formulations at 37 °C, facilitating smooth injection through a syringe. Conversely, at 25 °C, viscosity remained consistent regardless of shear rate, indicating stability and uniform flow behavior.

Adhesion and mucoadhesion studies revealed subtle differences between the two formulations, with the 2.5% P188 formulation demonstrating marginally higher adhesion and mucoadhesion values compared to the 5% P188 formulation.

In conclusion, rheological characterization elucidated some small differences between the 2.5% and 5% P188 formulations. Both formulations exhibit favorable properties for intratumoral injection, and each of these more be most suitable for specific application requirements.

The release profiles of PL from the P407/P188 15/2.5% (w/w) and 15/5% (w/w) hydrogels were analyzed over a 50-hour period and quantified by HPLC. The data is represented in Figure 28. Both of the formulations tested exhibited higher initial release rates compared to the control group, which was tested using PEG400/Tween80 (98.77/1.23%) + 2% PL (grey). These results show enhanced initial PL release facilitated by the hydrogel formulations.

The 15/2.5% formulation demonstrated a slightly higher initial release rate than the 15/5% formulation. However, the latter exhibited a more sustained release profile over time. Throughout the duration of the study, both P407/P188 formulations demonstrated sustained PL release, with a gradual increase observed up to 120 hours. Conversely, the control group exhibited an initial burst release followed by a slower release phase, although with lower overall PL release compared to the hydrogel formulations.

These findings underscore the potential of P407/P188 hydrogel formulations for controlled drug delivery applications. The choice of P407/P188 ratio appears to influence both the initial release rate and sustained release behavior of PL. While the 15/2.5% formulation shows a higher initial release rate, the 15/5% formulation offers a more sustained release profile over an extended period.

Hydrogel Formulations - Summary

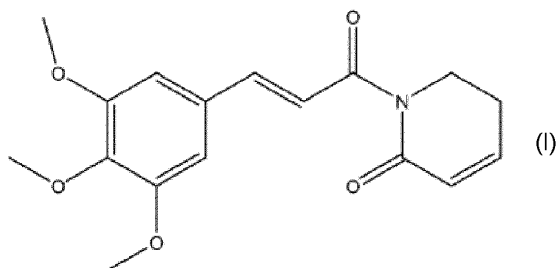
In the above examples, a thermoresponsive hydrogel containing P407/P188/PVP/PEG400 + Tween80/PL 15/2.5/1/20/2% w/w was developed. A second formulation additionally containing 5% P188 was also developed. Both formulations demonstrate increased resistance to extrusion compared to controls. Additionally, the 2.5% P188 formulation exhibits a more favorable extrusion profile with lower peak force values, indicating easier extrusion. The hydrogels were shown to have excellent viscoelastic and physicochemical properties, which is suitable for preclinical efficacy.

These results show that the formulations have potential applications in drug delivery systems, which are particularly useful in the treatment of cancers such as STS.

Further Aspects and Embodiments

Further aspects and embodiments of the invention are listed in the numbered paragraphs below.

A1. A pharmaceutical composition comprising an effective amount of a crystalline compound of Formula (I),



optionally together with a pharmaceutically acceptable carrier, diluent, or excipient; wherein the crystalline piperlongumine has an average particle size of less than about 5 μm , such as less than about 2 μm .

A2. The pharmaceutical composition of embodiment A1, wherein the crystalline compound has an average particle size of from about 200 nm to about 1 μm .

A3. The pharmaceutical composition of embodiment A1, wherein the crystalline compound has an average particle size and/or D50 value of from about 400 nm to about 900 nm, or from about 450 nm to about 750 nm.

A4. The pharmaceutical composition of any one of embodiments A1 to A3, wherein the crystalline compound has a D10 value of from about 200 nm to about 600 μm , or from about 300 nm to about 500 nm.

A5. The pharmaceutical composition of any one of embodiments A1 to A4, wherein the crystalline compound has a D90 value of from about 500 nm to about 2 μm , or from about 700 nm to about 1 μm .

A6. The pharmaceutical composition of any one of embodiments A1 to A5, wherein the crystalline compound is prepared by a method comprising dissolving piperlongumine into supercritical carbon dioxide.

A7. The pharmaceutical composition of any one of embodiments A1 to A6, wherein the crystalline compound is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

A8. The pharmaceutical composition of embodiment A7, wherein the crystalline compound is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

A9. The pharmaceutical composition of embodiment A7 or A8, wherein the crystalline compound has the x-ray diffraction spectrum substantially according to the particles shown in Figure 4.

A10. The pharmaceutical composition of any one of embodiments A1 to A9, wherein the crystalline compound has an onset melting temperature ($T_{m(\text{onset})}$), from 121 $^\circ\text{C}$ to 122 $^\circ\text{C}$ when heated to 134 $^\circ\text{C}$ at a rate of 5 $^\circ\text{C}/\text{min}$ during Differential Scanning Calorimetry.

A11. The pharmaceutical composition of any one of embodiments A1 to A10, wherein the crystalline compound has a doublet peak at 1650 to 1700 cm^{-1} in an infrared spectrum, optionally wherein the doublet peak is at 1670 and 1690 cm^{-1} .

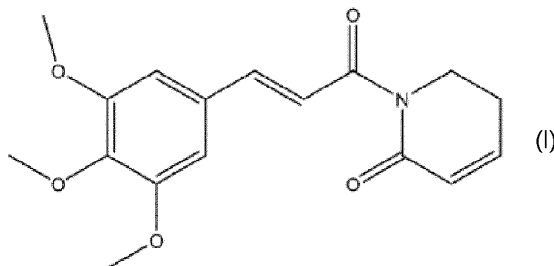
A12. The pharmaceutical composition of any one of embodiments A1 to A11, wherein the crystalline compound has a peak infrared spectrum at 1460 to 1500 cm^{-1} and/or at 1400 to 1440 cm^{-1} .

A13. The pharmaceutical composition of any one of embodiments A1 to A12, wherein the crystalline compound is dispersed in a carrier comprising one or more of a surfactant and a polymer.

A14. The pharmaceutical composition of any one of embodiments A1 to A13, wherein the composition is lyophilized.

- A15. The pharmaceutical composition of embodiment A13, wherein the crystalline compound is present from about 10% to about 40% by weight of the composition, and optionally from about 15% to about 30% by weight of the composition, or from about 20% to about 30% by weight of the composition.
- A16. The pharmaceutical composition of embodiment A13, wherein the crystalline compound is present at a concentration of more than 1 mg/mL, optionally at a concentration of at least 2 mg/mL, and optionally at a concentration of at least 3 mg/mL.
- A17. The pharmaceutical composition of embodiment A15 or A16, wherein the composition is an aqueous suspension having a pH of from about 5 to about 7, and is optionally buffered.
- A18. The pharmaceutical composition of any one of embodiments A15 to A17, wherein the composition is a suspension comprising one or more poloxamers.
- A19. The pharmaceutical composition of embodiment A18, wherein the poloxamer(s) are selected from poloxamer 407, poloxamer 338, and poloxamer 188.
- A20. The pharmaceutical composition of embodiment A18 or A19, wherein the poloxamer(s) are present from about 10% to about 40% by weight of the composition, or from about 10% to about 30% by weight of the composition, or from about 15% to about 25% by weight of the composition, and wherein the poloxamer is optionally poloxamer 407.
- A21. The pharmaceutical composition of any one of embodiments A15 to A17, wherein the composition comprises polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).
- A22. The pharmaceutical composition of embodiment A21, wherein the PCL-PVAc-PEG is present from about 20% to about 40% by weight of the composition, or from about 25% to about 35% by weight of the composition.
- A23. The pharmaceutical composition of any one of embodiments A15 to A22, wherein the composition is a syringeable suspension that forms a gel at human body temperature.

B1. A pharmaceutical composition comprising an effective amount of a crystalline form of a compound of Formula (I),



wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$, optionally together with a pharmaceutically acceptable carrier, diluent, or excipient.

B2. The pharmaceutical composition of embodiment B1, wherein the crystalline compound is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

B3. The pharmaceutical composition of embodiment B1 or B2, wherein the crystalline compound is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

B4. The pharmaceutical composition of any one of embodiments B1 to B3, wherein the crystalline compound has the x-ray diffraction spectrum substantially according to the particles of Figure 4.

B5. The pharmaceutical composition of any one of embodiments B1 to B4, wherein the crystalline compound has an onset melting temperature ($T_{m(\text{onset})}$) from 121°C to 122°C when heated to 134°C at a rate of $5^\circ\text{C}/\text{min}$ during Differential Scanning Calorimetry.

B6. The pharmaceutical composition of any one of embodiments B1 to B5, wherein the crystalline compound has a doublet peak at 1650 to 1700 cm^{-1} in an infrared spectrum, optionally wherein the doublet peak is at 1670 and 1690 cm^{-1} .

B7. The pharmaceutical composition of any one of embodiments B1 to B6, wherein the crystalline compound has a peak infrared spectrum at 1460 to 1500 cm^{-1} and/or at 1400 to 1440 cm^{-1} .

B8. The pharmaceutical composition of any one of embodiments B1 to B7, wherein the crystalline compound has an average particle size of less than about $5\text{ }\mu\text{m}$, or less than about $2\text{ }\mu\text{m}$, or less than about $1\text{ }\mu\text{m}$.

- B9. The pharmaceutical composition of any one of embodiments B1 to B8, wherein the crystalline compound has an average particle size of from about 200 nm to about 1 μ m.
- B10. The pharmaceutical composition of any one of embodiments B1 to B9, wherein the crystalline compound has an average particle size and/or D50 value of from about 400 nm to about 900 nm, or from about 450 nm to about 750 nm.
- B11. The pharmaceutical composition of any one embodiment B1 to B10, wherein the crystalline compound has a D10 value of from about 200 nm to about 600 μ m, or from about 300 nm to about 500 nm.
- B12. The pharmaceutical composition of any one of embodiments B1 to B11, wherein the crystalline compound has a D90 value of from about 500 nm to about 2 μ m, or from about 700 nm to about 1 μ m.
- B13. The pharmaceutical composition of any one of embodiments B1 to B12, wherein the crystalline compound is prepared by a method comprising dissolving piperlongumine into supercritical carbon dioxide.
- B14. The pharmaceutical composition of any one of embodiments B1 to B13, wherein the crystalline compound is dispersed in a carrier comprising one or more of a surfactant and a polymer.
- B15. The pharmaceutical composition of any one of embodiments B1 to B14, wherein the composition is lyophilized.
- B16. The pharmaceutical composition of embodiment B14, wherein the crystalline compound is present from about 10% to about 40% by weight of the composition, and optionally from about 15% to about 30% by weight of the composition, or from about 20% to about 30% by weight of the composition.
- B17. The pharmaceutical composition of embodiment B14, wherein the crystalline compound is present at a concentration of more than 1 mg/mL, optionally at a concentration of at least 2 mg/mL, and optionally at a concentration of at least 3 mg/mL.
- B18. The pharmaceutical composition of embodiment B16 or B17, wherein the composition is an aqueous suspension having a pH of from about 5 to about 7, and is optionally buffered.
- B19. The pharmaceutical composition of any one of embodiments B16 to B18, wherein the composition is a suspension comprising one or more poloxamers.

B20. The pharmaceutical composition of embodiment B19, wherein the poloxamer(s) are selected from poloxamer 407, poloxamer 338, and poloxamer 188.

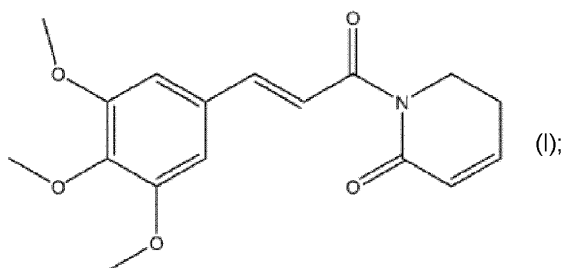
B21. The pharmaceutical composition of embodiment B19 or B20, wherein the poloxamer(s) are present from about 10% to about 40% by weight of the composition, or from about 10% to about 30% by weight of the composition, or from about 15% to about 25% by weight of the composition, and wherein the poloxamer is optionally poloxamer 407.

B22. The pharmaceutical composition of any one of embodiments B16 to B18, wherein the composition comprises polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).

B23. The pharmaceutical composition of embodiment B22, wherein the PCL-PVAc-PEG is present from about 20% to about 40% by weight of the composition, or from about 25% to about 35% by weight of the composition.

B24. The pharmaceutical composition of any one of embodiments B16 to B23, wherein the composition is a syringeable suspension that forms a gel at human body temperature.

C1. A pharmaceutical composition comprising an aqueous suspension of from about 5% to about 40% by weight of a compound of Formula (I),



and

from about 5% to about 30% by weight of a poloxamer selected from one or more of poloxamer 407, poloxamer 338, and poloxamer 188; or

from about 10% to about 40% by weight of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).

C2. The pharmaceutical composition of embodiment C1, wherein the compound of Formula (I) is present from about 10% to about 40% by weight of the composition, and optionally from about 15% to about 30% by weight of the composition, or from about 20% to about 30% by weight of the composition.

C3. The pharmaceutical composition of embodiment C1, wherein the compound of Formula (I) is present at a concentration of more than 1 mg/mL, optionally at a concentration of at least 2 mg/mL, and optionally at a concentration of at least 3 mg/mL.

C4. The pharmaceutical composition of any one of embodiments C1 to C3, wherein the composition is an aqueous suspension and having a pH of from about 5 to about 7, and is optionally buffered.

C5. The pharmaceutical composition of any one of embodiments C1 to C4, wherein the composition is a suspension comprising poloxamer 407.

C6. The pharmaceutical composition of embodiment C5, wherein poloxamer 407 is present from about 10% to about 30% by weight of the composition, and optionally from about 15% to about 25% by weight of the composition.

C7. The pharmaceutical composition of any one of embodiments C1 to C4, wherein the composition comprises polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).

C8. The pharmaceutical composition of embodiment C7, wherein the PCL-PVAc-PEG is present from about 20% to about 40% by weight of the composition, or from about 25% to about 35% by weight of the composition.

C9. The pharmaceutical composition of any one of embodiments C1 to C8, wherein the composition is a syringeable suspension that forms a gel at human body temperature.

C10. The pharmaceutical composition of any one of embodiments C1 to C9, wherein the compound of Formula (I) incorporated into the composition is a crystalline compound having an average particle size of less than about 5 μm , or less than about 2 μm , or less than about 1 μm .

C11. The pharmaceutical composition of embodiment C10, wherein the crystalline compound has an average particle size of from about 200 nm to about 1 μm .

C12. The pharmaceutical composition of embodiment C10 or C11, wherein the crystalline compound has an average particle size and/or D50 value of from about 400 nm to about 900 nm, or from about 450 nm to about 750 nm.

C13. The pharmaceutical composition of any one of embodiments C10 to C12, wherein the crystalline compound has a D10 value of from about 200 nm to about 600 μm , or from about 300 nm to about 500 nm.

C14. The pharmaceutical composition of any one of embodiments C10 to C13, wherein the crystalline compound has a D90 value of from about 500 nm to about 2 μm , or from about 700 nm to about 1 μm .

C15. The pharmaceutical composition of any one of embodiments C1 to C14, wherein the crystalline compound is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

C16. The pharmaceutical composition of embodiment C15, wherein the crystalline compound is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

C17. The pharmaceutical composition of embodiment C15 or C16, wherein the crystalline compound has the x-ray diffraction spectrum substantially according to the particles of Figure 4.

C18. The pharmaceutical composition of any one of embodiments C10 to C17, wherein the crystalline piperlongumine is prepared by a method comprising dissolving piperlongumine into supercritical carbon dioxide.

C19. The pharmaceutical composition of any one of embodiments C10 to C18, wherein the crystalline compound has an onset melting temperature ($T_{m(\text{onset})}$), from 121 $^\circ\text{C}$ to 122 $^\circ\text{C}$ when heated to 134 $^\circ\text{C}$ at a rate of 5 $^\circ\text{C}/\text{min}$ during Differential Scanning Calorimetry.

C20. The pharmaceutical composition of any one of embodiments C10 to C19, wherein the crystalline compound has a doublet peak at 1650 to 1700 cm^{-1} in an infrared spectrum, optionally wherein the doublet peak is at 1670 and 1690 cm^{-1} .

C21. The pharmaceutical composition of embodiments C10 to C20, wherein the crystalline compound incorporated has a peak infrared spectrum at 1460 to 1500 cm^{-1} and/or at 1400 to 1440 cm^{-1} .

D1. Use of the pharmaceutical composition of any one of embodiments A1 to C21 for treatment of a proliferative disease in a subject.

D2. The use of embodiment D1, wherein the proliferative disease is cancer.

D3. The use of embodiment D2, wherein the cancer is brain cancer, and optionally glioblastoma.

D4. The use of any one of embodiments D1 to D3, wherein the subject is a mammal, and optionally a human subject.

D5. The use of any one of embodiments D1 to D4, wherein the composition is administered to a tumor resection cavity, and optionally administered intracranially to a glioblastoma resection cavity during surgery.

E1. A method for treating a proliferative disease in a subject, comprising administering the pharmaceutical composition of any one of embodiments A1 to C21 to a subject in need thereof.

E2. The method of embodiment E1, wherein the proliferative disease is cancer.

E3. The method of embodiment E2, wherein the cancer is brain cancer, and optionally glioblastoma.

E4. The method of any one of embodiments E1 to E3, wherein the subject is a mammal, and optionally a human subject.

E5. The method of any one of embodiments E1 to E4, wherein the composition is administered to a tumor resection cavity, and optionally administered intracranially to a glioblastoma resection cavity during surgery.

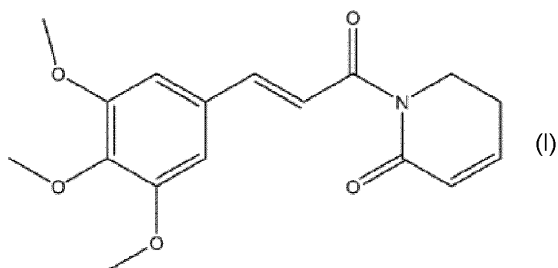
References

A number of publications are cited above in order to more fully describe and disclose the invention and the state of the art to which the invention pertains. Full citations for these references are provided below. The entirety of each of these references is incorporated herein.

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Zhang *et al.* *Front. Pharmacol.* 2019, 10

Claims:

1. A pharmaceutical composition comprising a crystalline compound of Formula (I),



optionally together with a pharmaceutically acceptable carrier, diluent, or excipient; wherein the crystalline compound is in particulate form having an average particle size of less than about 5 μm .

2. The pharmaceutical composition of claim 1, wherein the crystalline compound has an average particle size of less than about 2 μm , or less than about 1 μm .
3. The pharmaceutical composition of claim 1 or claim 2, wherein the crystalline compound has an average particle size and/or D50 value of from about 400 nm to about 900 nm, or from about 450 nm to about 750 nm.
4. The pharmaceutical composition of any one of claims 1 to 3, wherein the crystalline compound has a D10 value of from about 200 nm to about 600 μm , or from about 300 nm to about 500 nm.
5. The pharmaceutical composition of any one of claims 1 to 4, wherein the crystalline compound has a D90 value of from about 500 nm to about 2,000 nm, or from about 700 nm to about 1,000 nm.
6. The pharmaceutical composition of any one of claims 1 to 5, wherein the crystalline compound has an onset melting temperature ($T_{\text{m(onset)}}$), from 121 $^{\circ}\text{C}$ to 122 $^{\circ}\text{C}$ when heated to 134 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C}/\text{min}$ during Differential Scanning Calorimetry.
7. The pharmaceutical composition of any one of claims 1 to 6, wherein the crystalline compound has a doublet peak in the range 1,650 to 1,700 cm^{-1} in an infrared spectrum, such as wherein the doublet peak is in the range 1,670 and 1,690 cm^{-1} .
8. The pharmaceutical composition of any one of claims 1 to 7, wherein the pharmaceutical composition comprises a thermoresponsive hydrogel, wherein the crystalline

compound of formula (I) is dispersed in the hydrogel, and the pharmaceutical composition further comprises:

one or more poloxamers, wherein the total amount of poloxamers is from about 15% to about 20% by weight of the pharmaceutical composition;
from about 0.5% to about 2% by weight of polyvinylpyrrolidone;
from about 18% to about 20% by weight of polyethylene glycol;
and from about 0.2 to 0.3% by weight of a polysorbate.

9. The pharmaceutical composition of claim 8, wherein the crystalline compound is present from about 1% to about 5% by weight of the pharmaceutical composition, such as about 2% by weight of the pharmaceutical composition.

10. The pharmaceutical composition of any one of claims 1 to 7, wherein the crystalline compound is dispersed in a carrier comprising one or more of a surfactant and a polymer.

11. The pharmaceutical composition of claim 10, wherein the crystalline compound is present from about 10% to about 40% by weight of the composition, and optionally from about 15% to about 30% by weight of the composition, or from about 20% to about 30% by weight of the composition.

12. The pharmaceutical composition of claim 10 or claim 11, wherein the crystalline compound is present at a concentration of at least 1 mg/mL, optionally at a concentration of at least 2 mg/mL, and optionally at a concentration of at least 3 mg/mL.

13. The pharmaceutical composition of any one of claims 10 to 12, wherein the composition is a suspension comprising one or more poloxamers, optionally wherein the poloxamer(s) are selected from poloxamer 407, poloxamer 338, and poloxamer 188.

14. The pharmaceutical composition of claim 13, wherein the poloxamer(s) are present from about 10% to about 40% by weight of the composition, or from about 10% to about 30% by weight of the composition, or from about 15% to about 25% by weight of the composition, and wherein the poloxamer is optionally poloxamer 407.

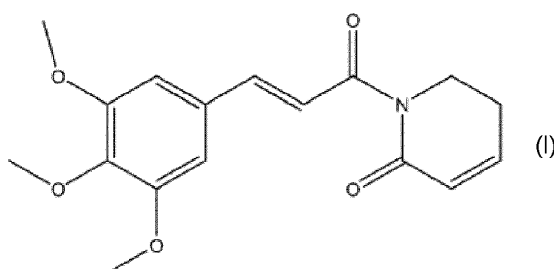
15. The pharmaceutical composition of any one of claims 10 to 12, wherein the composition comprises polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG), and optionally wherein the PCL-PVAc-PEG is present from about 10% to about 40% by weight of the composition, or from about 25% to about 35% by weight of the composition.

16. The pharmaceutical composition of any one of claims 1 to 15, wherein the crystalline compound is non-encapsulated.

17. The pharmaceutical composition of any one of claims 1 to 16, wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$, optionally together with a pharmaceutically acceptable carrier, diluent, or excipient.

18. The pharmaceutical composition of claim 17, wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

19. A pharmaceutical composition comprising a crystalline form of a compound of Formula (I),



wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$, optionally together with a pharmaceutically acceptable carrier, diluent, or excipient.

20. The pharmaceutical composition of claim 19, wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.

21. The pharmaceutical composition of claim 19 or claim 20, wherein the compound of Formula (I) has an average particle size of less than about $5 \mu\text{m}$, or less than about $2 \mu\text{m}$, or less than about $1 \mu\text{m}$.

22. The pharmaceutical composition of any one of claims 19 to 21, wherein the compound of Formula (I) has an average particle size of from about 200 nm to about 1,000 nm.

23. The pharmaceutical composition of any one of claims 19 to 22, wherein the compound of Formula (I):

(i) has an average particle size and/or D50 value of from about 400 nm to about 900 nm, or from about 450 nm to about 750 nm;

(ii) has a D10 value of from about 200 nm to about 600 μ m, or from about 300 nm to about 500 nm; and/or

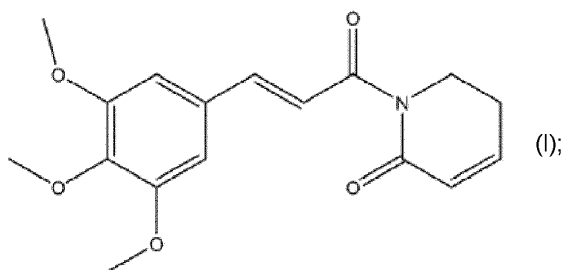
(iv) has a D90 value of from about 500 nm to about 2,000 nm, or from about 700 nm to about 1,000 nm.

24. The pharmaceutical composition of any one of claims 19 to 23, wherein the pharmaceutical composition comprises a thermoresponsive hydrogel, wherein the crystalline compound of Formula (I) is dispersed in the hydrogel, and the pharmaceutical composition further comprises:

one or more poloxamers, wherein the total amount of poloxamers is from about 15% to about 20% by weight of the pharmaceutical composition;
from about 0.5% to about 2% by weight of polyvinylpyrrolidone;
from about 18% to about 20% by weight of polyethylene glycol;
and from about 0.2 to 0.3% by weight of a polysorbate.

25. The pharmaceutical composition of claim 24, wherein the crystalline compound is present from about 1% to about 5% by weight of the pharmaceutical composition, such as about 2% by weight of the pharmaceutical composition.

26. A pharmaceutical composition comprising an aqueous suspension of from about 5% to about 40% by weight of a compound of Formula (I),

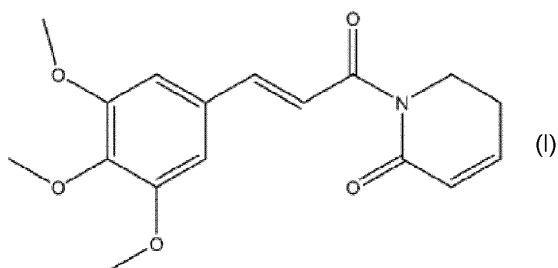


and:

from about 5% to about 30% by weight of a poloxamer selected from one or more of poloxamer 407, poloxamer 338, and poloxamer 188; or
from about 10% to about 40% by weight of polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG).

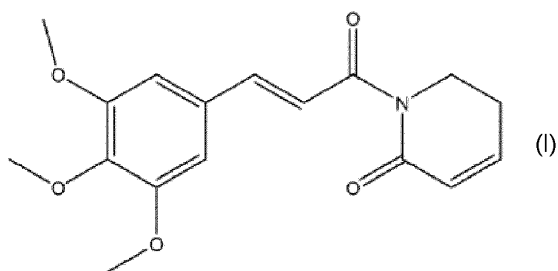
27. The pharmaceutical composition of claim 26, wherein the compound of Formula (I) is present from about 10% to about 40% by weight of the composition, and optionally from about 15% to about 30% by weight of the composition, or from about 20% to about 30% by weight of the composition.

28. A pharmaceutical composition of any one of claims 1 to 27 for use in a method of treatment.
29. A pharmaceutical composition of any one of claims 1 to 28 for use in a method of treating cancer.
30. A pharmaceutical composition for use according to claim 29, wherein the cancer is brain cancer, and optionally glioblastoma.
31. A pharmaceutical composition for use according to claim 29, wherein the cancer is sarcoma, such as soft-tissue sarcoma.
32. A crystalline form of a compound of Formula (I),



wherein the crystalline form is characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$ and $21.6 \pm 0.1^\circ$.

33. The crystalline form of a compound of Formula (I) of claim 32, characterized by a powder x-ray diffraction pattern comprising the 2θ values $14.9 \pm 0.1^\circ$, $16.7 \pm 0.1^\circ$, $18.0 \pm 0.1^\circ$, $21.6 \pm 0.1^\circ$ and $28.9 \pm 0.1^\circ$.
34. A pharmaceutical composition comprising a thermoresponsive hydrogel and a chemotherapeutic agent dispersed in the hydrogel, and the composition further comprising:
one or more poloxamers, wherein the total amount of poloxamers is from about 15% to about 20% by weight of the pharmaceutical composition;
from about 0.5% to about 2% by weight of polyvinylpyrrolidone;
from about 18% to about 20% by weight of polyethylene glycol;
and from about 0.2 to 0.3% by weight of a polysorbate.
35. The pharmaceutical composition of claim 34, wherein the chemotherapeutic agent is a compound of Formula (I),



36. The pharmaceutical composition of claim 35, comprising up to about 5% by weight of a compound of Formula (I).
37. The pharmaceutical composition of claim 34 or claim 35, comprising about 2% by weight of a compound of Formula (I).
38. The pharmaceutical composition of any of claims 34 to 37, wherein the one or more poloxamers is selected from poloxamer 407 and poloxamer 188.
39. The pharmaceutical composition of any of claims 34 to 38, comprising poloxamer 407.
40. The pharmaceutical composition of any of claims 34 to 39, comprising about 15% by weight of poloxamer 407.
41. The pharmaceutical composition of any of claims 34 to 40, comprising poloxamer 188.
42. The pharmaceutical composition of any of claims 34 to 41, comprising from about 2.5% to about 5% by weight of poloxamer 188.
43. The pharmaceutical composition of any of claims 34 to 42, comprising about 1% by weight of polyvinylpyrrolidone.
44. The pharmaceutical composition of any of claims 34 to 43, wherein the polyethylene glycol has a molecular weight of about 350 to about 450 g/mol.
45. The pharmaceutical composition of any of claims 34 to 44, wherein the polyethylene glycol is polyethylene glycol 400.
46. The pharmaceutical composition of any of claims 34 to 45, comprising from about 19% to about 20% by weight of the polyethylene glycol.

47. The pharmaceutical composition of any of claims 34 to 46, comprising from about 19.5% to about 20% by weight of the polyethylene glycol.
48. The pharmaceutical composition of any of claims 34 to 47, comprising about 19.8% by weight of the polyethylene glycol.
49. The pharmaceutical composition of any of claims 34 to 48, wherein the polysorbate is polysorbate 80.
50. The pharmaceutical composition of any of claims 34 to 49, comprising about 0.25% of the polysorbate.
51. A pharmaceutical composition of any one of claims 34 to 50 for use in a method of treatment.
52. A pharmaceutical composition of any one of claims 34 to 50 for use in a method of treating cancer.
53. A pharmaceutical composition for use according to claim 52, wherein the cancer is sarcoma, such as soft-tissue sarcoma.