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Biodistribution of Stealth and Non-stealth Solid Lipid Nanospheres after Intravenous Administration to Rats

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Abstract

Drug-free stealth and non-stealth solid lipid nanospheres (SLNs) were administered intravenously to rats to evaluate their tissue distribution and their transport across the blood–brain barrier.

Two types of experiments were performed using unlabelled and labelled SLNs. Rats were administered labelled non-stealth or stealth nanospheres (NSSLNs and SSLNs) and their tissue distribution was monitored for 60 min. In another experiment, rats were injected with unlabelled NSSLNs or SSLNs and the cerebrospinal fluid (CSF) was analysed using transmission electron microscopy (TEM) to confirm the presence of the SLNs.

Some differences were found in the biodistribution between labelled NSSLNs and SSLNs. In particular, the radioactivity in the liver and the lung was much lower for SSLNs than for NSSLNs, confirming a difference in their uptake. Both types of SLNs were detected in the brain. TEM analysis showed both types of SLNs in rat CSF.

Many authors have proposed that solid lipid nanospheres (SLNs) be used as drug delivery systems (Westesen et al 1993; Domb 1995; Müller et al 1995; Yang et al 1999). SLNs can be prepared by a variety of methods and for this study we dispersed warm oil-in-water (o/w) microemulsions in cold water; the SLNs are able to carry either lipophilic or hydrophilic drugs (Cavalli et al 1995, 1997; Morel et al 1996). Sterically stabilized SLNs (stealth SLNs) can be prepared using polyethylene glycol 2000 (PEG 2000) lipid conjugates as stealth agents. The surface modification of stealth SLNs can be determined by measuring size, zeta potential and albumin adsorption (Cavalli et al 1999); the albumin adsorption with stealth SLNs (SSLNs) is lower than non-stealth SLNs (NSSLNs). Bocca et al (1998) demonstrated that uptake of SSLNs by macrophages was much lower than that of NSSLNs.

Bargoni et al (1998) examined the uptake and transport of unloaded SLNs in lymph and blood after duodenal administration to rats; transmission electron microscopy (TEM) analysis detected SLNs in the lymph and in blood, and this was confirmed by using labelled SLNs. Fundarò et al (2000) intravenously administered doxorubicin-loaded SLNs (stealth and non-stealth) to rats and studied the plasma pharmacokinetics and tissue distribution. The SSLNs showed a prolonged circulation time compared with NSSLNs. Moreover, doxorubicin was detected in the brain 30 min after the administration of both types of SLNs.

We have studied the pattern of tissue distribution of unloaded SSLNs and NSSLNs, administered intravenously to rats, and have evaluated their transport across the blood–brain barrier. Also, the behaviour of SSLNs and NSSLNs were compared, using γ-counting to analyse the biodistribution of labelled SLNs and TEM to analyse the presence of unlabelled SLNs in rat cerebrospinal fluid (CSF).

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Materials and Methods

Materials

Stearic acid (99·5%) was from Fluka Chemie (Buchs, Switzerland). Epikuron 200 (soya phosphatidylcholine 95%) was a kind gift from Lucas

Meyer (Hamburg, Germany). Taurocholate sodium salt was a kind gift from PCA (Basaluzzo, Italy). Ketamine hydrochloride was from Sigma Chemical (St Louis, MO). 17-[^{131}I]Iodoheptadecanoic acid was a kind gift from Nycomed Amersham Sorin (Saluggia, Italy). Stearic acid–PEG 2000 was prepared as reported by Bocca et al (1998).

Animals

Male albino Wistar rats (380–450 g; Charles-River, Italy) were caged in groups of three and maintained at room temperature. The study complied with the rules set forth in the NIH Guide for the Care and Use of Laboratory Animals. Immediately before the experiment, the animals were fasted for 15 h with free access to water. Animals were anaesthetized with ketamine (75 mg kg^{-1}, i.p.). Eighteen rats were used for the experiments with the radioactive material: nine rats underwent NSSLN administration and nine rats SSLN administration. Eight rats were used for experiments with unlabelled SLNs.

Preparation of unlabelled SLNs

Non-stealth SLNs (NSSLNs). The NSSLNs were prepared from a warm o/w microemulsion containing stearic acid as internal phase (0·70 mmol), Epikuron 200 as surfactant (0·14 mmol), taurocholate sodium salt as cosurfactant (0·66 mmol) and filtered water as continuous phase (111·11 mmol). Epikuron 200 and warm filtered water were added to melted stearic acid at approximately 70°C. The cosurfactant was then added to the warm mixture. A clear system was easily obtained under stirring. NSSLNs were obtained by dispersing the warm o/w microemulsion (approximately 70°C) in filtered cold water (2–3°C) at a ratio of 1:4 (microemulsion–water) (v/v) under mechanical stirring. The nanosphere dispersion was washed three times with equal volumes of distilled water by diaultrafiltration with a TCF2 system (Amicon-Grace, Danvers, MA) and then concentrated to the selected concentration (80 mg mL^{-1}). To determine the concentration of SLNs, a sample of water dispersion was freeze-dried using a Modulyo freeze-dryer (Edwards, Crawley, UK). The dried SLNs were then weighed.

Stealth SLNs (SSLNs). Using the same warm o/w microemulsion as above, 0·15% (w/w) of the stealth agent (stearic acid–PEG 2000) was added to the melted stearic acid and the microemulsion formulation was then prepared as above. The stealth nanospheres were obtained by dispersing the warm o/w microemulsion in cold water, washing and then concentrating the SLN dispersion to the selected concentration (80 mgmL^{-1}).

Preparation of labelled SLNs (NSSLNs and SSLNs)

17-[^{131}I]iodoheptadecanoic acid was used as a radioactive marker. Labelled iodoheptadecanoic acid was prepared by standard procedures for radiopharmaceuticals for human use, with a radiochemical purity of at least 98% at the time of injection. The in-vitro and in-vivo stability of this molecule is widely accepted for human diagnostic use; indeed, 120 min after administration only 0·6% of injected dose was found in the thyroid (unpublished data from Nycomed Amersham Sorin). 17-[^{131}I]iodoheptadecanoic acid activity was 300–350 MBq mg^{-1}; a solution was prepared adding chloroform to achieve approximately 800 MBq mL^{-1}.

Radiolabelled SLNs (NSSLNs and SSLNs) were obtained by adding approximately 100 μL of the chloroform solution, containing approximately 80 MBq 17-[^{131}I]iodoheptadecanoic acid, to the warm microemulsions, the exact volume depending on the number of rats used and the time spent after labelling. After the evaporation of the chloroform, the microemulsion was dispersed in water and SLNs were obtained as described above.

Characterization of unlabelled SLNs

Photon correlation spectroscopy. The sizes of the two types of SLN were determined by photon correlation spectroscopy using an N4-MD Coulter Instrument at a fixed angle of 90° and at a temperature of 25°C. The SLN samples (80 mg mL^{-1}) were diluted 1:40 (v/v) with filtered water before each analysis. Each value was the mean of six measurements. The polydispersity index is a measure of the distribution of the SLN population (Koppel 1972).

Transmission electron microscopy (TEM). TEM analysis was performed using a Philips CM10 instrument. All samples of NSSLNs and SSLNs, either in water dispersion (dilution 1:60, v/v) or in CSF (dilution 1:40, v/v), were diluted with saline solution and stained with a 2% solution of osmium tetraoxide before being sprayed onto copper grids for analysis by TEM.

Zeta potential measurements. The surface charge of the two types of SLN was determined by laser

Doppler anemometry using a Delsa 440 instrument (Coulter, USA). For the determination of the electrophoretic mobility, SLN samples (pH = 5·6) were diluted with KCl 0·1 mM and placed in the electrophoretic cell where an electric field of 15·24 V cm^{-1} was established. Each sample was analysed in triplicate. The zeta potential values were calculated using the Smolochowski equation.

In-vitro release of 17-iodoheptadecanoic acid from solid lipid nanospheres

The in-vitro release experiments were performed using a "side-by-side" glass dialysis cell; a hydrophilic membrane Servapor Dialysis tubing (Serva, Germany) cut-off 12 000 Da was used. The experiments were performed using the 17-iodoheptadecanoic acid-loaded SLNs dispersed at pH 7·4; an equal volume of phosphate buffer (pH 7·4) was placed in the receptor compartment. At fixed times samples of the receptor solution were analysed spectrophotometrically at $\lambda_{max} =$ 254 nm.

Administration of NSSLNs or SSLNs to rats

Labelled NSSLNs and SSLNs. The experiments with radioactive SLNs lasted 60 min. SLN dispersions were administered directly into the tail vein of two groups of nine rats under anaesthesia. Each rat was injected with a fixed volume (0·2 mL) of NSSLN or SSLN dispersion in water (80 mg mL^{-1}); the administered activity was about 3 MBq (approximately 40 mg SLNs kg^{-1}).

Before injection, radioactivity in the syringe was measured with a standard counter (Biogamma, ACN, Italy). After injection, the syringe was measured using the same counter to compute the injected dose.

The CSF was sampled at 10, 30 and 60 min after injection via a cannula inserted in the cisterna magna (De Lange et al 1997). Three rats were sampled at each time point for each group. After CSF collection, the animals were killed and separate samples of 2 mL and 100 μL blood were collected in heparinized tubes. The body was dissected into heart, lungs, spleen, gastrointestinal tract, liver, kidneys, bladder with urine, testicles, brain, head (without brain), tail and carcass (remainder of the body). Each tissue sample was washed with fresh water and the radioactivity measured using the same counter used for the syringe and for the 2-mL blood sample. Where the tail showed a high activity (more than 3% of injected dose), the administration was considered to have failed and data from that animal were excluded from further analysis.

Total blood activity was computed from blood volume of the rat and from the radioactivity of the 2-mL blood sample. The radioactivity of the 100-μL blood sample and of CSF was measured with a γ-counter (Gammadigit, ACN, Italy). From these measurements the CSF-to-blood activity ratio was computed for each animal.

Unlabelled SLNs. The experiments with unlabelled nanospheres lasted 30 min. Rats were administered a fixed volume (0·2 mL) of NSSLN or SSLN dispersions at a concentration of 80 mg mL^{-1}. CSF was obtained via a cannula inserted in the cisterna magna (De Lange et al 1997) and examined by TEM. All TEM samples of CSF were diluted 1:40 with saline solution (v/v) and were stained with a 2% solution of osmium tetraoxide before analysis.

Determination of taurocholate sodium salt

The amount of sodium taurocholate present in the SLNs and in washing waters was determined by HPLC (Nakayama & Nakagami 1980), using a Perkin–Elmer Binary LC Pump 250 liquid chromatograph and BioRad ODS column (25 cm per 4·6 mm). The eluent was acetonitrile–methanol–phosphate buffer, 0·03 M pH 3·4, 15:30:55 (v/v). The analysis was run at a flow rate of 0·6 mL min^{-1} with the UV detector operating at 210 nm.

Results

Labelled SLNs

The injection failed in two rats as evaluated by the radioactivity in the tail. One of these animals had received NSSLNs and the other SSLNs: both had been killed at 10 min. All other animals showed low radioactivity in the tail (2 ± 1% of injected dose (mean ± s.d.)). In two of the remaining rats it was not possible to obtain CSF. One of them had received NSSLNs and the other SSLNs, both had been killed at 30 min. All results and the number of animals from which data were obtained are summarized in Tables 1 (biodistribution data) and 2 (CSF data). Regardless of the type of SLNs administered, activity detected in heart, spleen, kidneys, bladder (with urine) and testicles was negligible in all animals.

NSSLNs. NSSLN blood concentration did not change during the experiment. The liver showed early uptake of NSSLNs with slow clearance and the gastrointestinal tract showed significant uptake. Brain uptake was low whilst lung uptake was

Table 1. Number of animals and radioactivity distribution (% of injected dose) in rats after after non-stealth solid lipid nanosphere and stealth solid lipid nanosphere intravenous injection.

	Non-stealth solid lipid nanospheres			Stealth solid lipid nanospheres		
	10 min	30 min	60 min	10 min	30 min	60 min
Number of animals (n)	2	3	3	2	3	3
Blood (total)	21 ± 3	21 ± 3	20 ± 3	23 ± 2	22 ± 2	17 ± 0
Lungs	14 ± 1	17 ± 2	14 ± 2	1 ± 0	1 ± 0	2 ± 0
Heart	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Spleen	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Gastrointestinal tract	1 ± 0	3 ± 0	3 ± 1	2 ± 0	4 ± 1	3 ± 0
Liver	28 ± 4	19 ± 1	18 ± 3	19 ± 2	14 ± 1	8 ± 1
Kidneys	2 ± 0	2 ± 0	1 ± 0	2 ± 0	2 ± 0	1 ± 0
Bladder and urine	0 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Testis	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Brain	1 ± 0	0 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Head (without brain)	3 ± 1	4 ± 0	4 ± 1	5 ± 1	5 ± 1	6 ± 0
Carcass	25 ± 2	26 ± 1	28 ± 2	37 ± 4	40 ± 2	50 ± 1

Values are mean ± s.d. Tail results are not included in the table.

Table 2. Number of animals, cerebrospinal fluid and blood activity (% of injected dose) and percent cerebrospinal fluid/blood radioactivity ratio in rats after intravenous injection of labelled non-stealth solid lipid nanospheres and stealth solid lipid nanospheres.

	Non-stealth solid lipid nanospheres			Stealth solid lipid nanospheres		
	10 min	30 min	60 min	10 min	30 min	60 min
Number of animals (n)	2	2	3	2	2	3
Cerebrospinal fluid	0·016 ± 0·003	0·022 ± 0·009	0·021 ± 0·008	0·015 ± 0·006	0·020 ± 0·001	0·037 ± 0·023
Blood	0·883 ± 0·065	0·872 ± 0·158	0·668 ± 0·169	0·943 ± 0·091	0·859 ± 0·007	0·713 ± 0·051
Cerebrospinal fluid/blood	1·8 ± 0·5%	2·7 ± 1·5%	3·4 ± 1·7%	1·6 ± 0·5%	2·3 ± 0·1%	5·2 ± 3·6%

Values are mean ± s.d.

significant during the experiment. Head (without brain) uptake was low and carcass uptake increased up to 28% only during the experiment. The radioCSF-to-blood activity ratio showed an increase with time (Table 2).

SSLNs. SSLNs showed a pattern of distribution that differed from that of NSSLNs. The clearance from the blood was slightly faster than with NSSLNs. The liver showed a lower uptake and a faster clearance compared with data obtained with NSSLNs (see Table 1). The gastrointestinal tract uptake was higher than with NSSLNs. Brain uptake was low, the lungs showed low uptake with a marked difference from NSSLNs. The head and the carcass showed a fast uptake that increased up to 50% at the end of the experiment. The CSF-to-blood radioactivity ratio increased with time, as observed with NSSLNs.

Unlabelled SLNs

The size of the two types of unlabelled SLNs before intravenous administration was below 100 nm with a narrow size distribution. NSSLNs, dispersed in water, had a diameter of 80 ± 4 nm and a polydispersity index of 0·20 ± 0·02, while SSLNs had a diameter of 86 ± 2 nm and a polydispersity

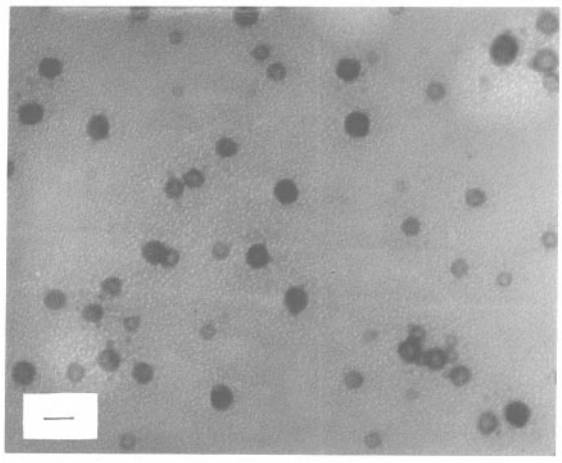

Figure 1. Stealth solid lipid nanospheres before administration. (Bar = 100 nm; magnification × 52 000).

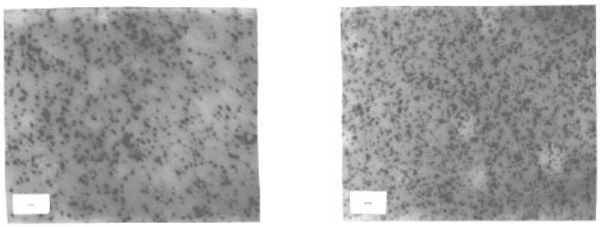

Figure 2. Non-stealth solid lipid nanospheres (left panel) and stealth solid lipid nanospheres (right panel) in cerebrospinal fluid 20 min after intravenous administration of 16 mg solid lipid nanospheres in 0·2 mL of dispersion. (Bar = 100 nm; magnification × 28 500.)

index of 0·20 ± 0·03. Both types of SLNs showed narrow size distribution and spherical shape by TEM analysis. Figure 1 shows a TEM microphotograph of SSLNs dispersed in water before administration.

The zeta potential value of NSSLNs was −47 ± 1 mV and that of SSLNs was −34 ± 1 mV. Approximately 75% of the initial amount of taurocholate added to the microemulsions was eliminated with the washings.

The in-vitro release study showed no detectable amount of 17-iodoheptadecanoic acid from the SLNs at pH 7·4 after 60 min.

Analysis of CSF by TEM after administration of NSSLNs and SSLNs showed that lipid nanospheres were present in the biological fluid 20 min after intravenous administration. They maintained a colloidal size and spherical shape (Figure 2).

Discussion

Biodistribution and the ability to carry drugs, particularly across the blood–brain barrier, has been studied in some lipidic systems such as liquid microspheres (Miganawa et al 1996), liposomes (Siegal et al 1995) or polymeric nanospheres (Illum & Davis 1984; Tröster et al 1990; Kreuter et al 1995; Schroeder et al 1998). Solid lipid nanoparticles containing camptothecin increased significantly the brain area under the concentration–time curve (AUC) of the drug as compared with the camptothecin solution (Yang et al 1999).

We have studied drug-unloaded nanospheres to assess the biodistribution of the particles themselves, rather than that of a carried drug.

The two kinds of SLNs studied were prepared from bioacceptable components and showed an average diameter below 100 nm. For the SSLNs, the zeta potential was lower and the average diameter higher than for the NSSLNs, confirming the presence of the hydrophylic PEG chains of the stealth agent on the surface of the nanospheres.

Labelled SSLNs and NSSLNs were used to evaluate the tissue distribution of the nanospheres after intravenous administration to rats. The marker used was 17-[^{131}I]iodoheptadecanoic acid, a fatty acid with a structure similar to that of stearic acid, the major component of SLNs, and used for the scintigraphic evaluation of cardiac metabolism in man (Railton et al 1987). 17-Iodoheptadecanoic acid and γ-counting has been used to evaluate the transport of SLNs, administered duodenally, in rats (Bargoni et al 1998). The marker is completely incorporated into the SLNs due to its lipophilicity; spectrophotometric analysis of the washing waters of unlabelled SLNs containing 17-iodoheptadecanoic acid did not reveal any iodine compounds (Bargoni et al 1998).

In this study, the in-vitro release experiment did not show any evidence of the release of 17-iodoheptadecanoic acid.

In the experiment with labelled SLNs, the lungs and liver, two reticulo-endothelial system (RES) organs, showed important differences in uptake between NSSLNs and SSLNs. Lung activity with NSSLNs was approximately 10-fold that with SSLNs. Also, liver activity was higher and its decrease was slower with NSSLNs compared with SSLNs; at 60 min, radioactivity in the liver with NSSLNs was more than double that of SSLNs and more than 10-fold in the lungs.

A relevant production of "free" radioactive iodide from the catabolism of the iodinated fatty acid is improbable: iodide is renally excreted (Miller et al 1975) and concentrated in the gastrointestinal tract (Pallardo et al 1979); the activity we found in kidneys and bladder (with urine) was very low. The spleen uptake was low with both types of SLNs, showing that the RES uptake was negligible.

Brain radioactivity was low with both types of SLN; at the same time, the increase of the CSF-to-blood radioactivity ratio was marked, and appeared to be higher with SSLNs than with NSSLNs.

Labelled SLNs were detected in the brain; this is consistent with data obtained after intravenous administration to rats of non-stealth and stealth doxorubicin-loaded SLNs (Fundarò et al 2000). A significant amount of doxorubicin was determined in the brain of rats injected with both kinds of doxorubicin-loaded SLNs; doxorubicin was also detected in the CSF of rats. Thirty minutes after the doxorubicin-loaded SLN administration the highest doxorubicin concentration in brain and CSF was obtained with SSLNs. After the administration of doxorubicin solution no drug was detected in the brain and CSF because it could not cross the blood–brain barrier.

Moreover, in the previous study a prolonged circulation time of doxorubicin in stealth SLNs was observed, in spite of the small amount of the stealth agent added to the microemulsion. Personal data have shown that increasing the amount of stealth agent achieved a marked increase in doxorubicin plasma concentration after SLN administration to rats.

TEM analysis confirmed the results obtained with labelled SLNs. Both kinds of SLN were detected in the CSF; probably their small size and their composition facilitated their passage across the blood–brain barrier, and their physicochemical nature may have made them compatible with blood–brain-barrier transport systems (Aigner et al 1997).

Bargoni et al (1998) used TEM analysis to show that after administration SLNs maintained their spherical shape and size in lymph and plasma. Figure 2 shows that the average diameters of both kinds of SLNs in CSF were quite similar to those measured before their administration (Figure 1). The SLNs also maintained their spherical shape in CSF during the experiment. This may be an advantage for the use of SLNs as a therapeutic system.

In this experiment, the amount of SLNs in the CSF and the brain must be evaluated in light of the very small area of the blood–brain-barrier-transport surface of the rats. CSF samples were taken from the cysterna magna, while the CSF is produced in the two lateral ventricles (Woodbury 1983): thus it is probable that our samples did not reflect fully the content of actual CSF production. Our data relating to CSF support those from the brain: the CSF activity with SLNs was low, but not negligible, and increased over time.

A lipophilic carrier can be transported through a lipophilic barrier (such as the blood–brain barrier) in both directions. Future work will need to examine the incorporation of hydrophylic molecules into SLNs to determine their distribution to the central nervous system after administration by injection to animals.

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