Passive and specific targeting of lymph nodes: the influence of the administration route

Abstract: Patients diagnosed with an advanced-stage cancer present a dismal prognosis due to the presence of metastases. From the primary tumor, the cancer cells are disseminated via lymphatic circulation; metastases develop initially in lymph nodes. Therefore, the targeting of lymph nodes needs to be improved in the design of future chemotherapy, and one way to ensure this targeting is by using the subcutaneous (SC) route. Using lipid nanocapsules (LNCs) (40 nm and fluorescently-labeled with DiD) as nanocarriers, a correlation between the SC injection site (behind the neck, the right and left flanks, and above the tail) for LNC administration and specific lymph node accumulation (left and right cervical, axillary and inguinal lymph nodes) was achieved for Sprague-Dawley rats. The pharmacokinetic and biodistribution profiles confirmed the absence of LNCs in systemic circulation after SC administration due to the optimal size of the LNCs. With appropriate SC administration, LNCs can accumulate in specific lymph nodes, whereas IV administration led to a weak accumulation of LNCs in all lymph nodes. Specific accumulation followed the lymph flow: bottom-up from the lower to upper limbs and top down from the head, with two lymph circulation partitions: right upper limb and the rest. Administration above the tail presented high inguinal and axillary lymph node accumulation whereas weak accumulation was observed after administration behind the neck. LNCs administered in the left flank only accumulated in the left inguinal and axillary lymph nodes, whereas left and right inguinal and axillary lymph nodes presented accumulation after administration in the right flank. Cervical lymph nodes, in the opposite direction of lymph flow, were never targeted after SC administration, whatever the injection site.

Keywords: lipid nanocapsules; lymph-node targeting; subcutaneous administration.

Introduction

In the USA, in 2014, cancer was the leading cause of death and was responsible for the death of 1600 people per day (1). The main explanation is poor treatment efficacy, in spite of recent improvements [the 5-year relative survival rate for all cancer types increased from 49% to 68% between 1975 and 2009 (1)], because the early detection of cancer is very difficult to achieve. For example, pancreatic cancer usually develops without any symptoms in the early stages, and only 9% of pancreatic cancer is diagnosed as non-metastatic cancer (1). Breast, colon, lung and prostate cancers generally include some lymph-node metastases, and these four types of cancer are themselves responsible for almost half (47%) of all cancer deaths (1). The presence of metastases in lymph nodes is always a dismal prognosis for patients. Metastasis formation results in the migration of cancer cells from the primary tumor. Two ways are considered for cancer cell dissemination: via the blood circulation and the lymphatic system. Although systemic circulation is often more readily accessible to tumor cells, the lymphatic system gives a better chance for metastasis formation. Firstly, the smallest lymphatic vessels are broader than blood capillaries and the lymph moves slowly. Secondly, lymphatic fluid and interstitial fluid have an almost identical composition, so the lymph encourages tumor cell viability and proliferation. Finally, lymph nodes can be compared to cell incubators: lymph nodes enable the stagnation of cancer cells and exchanges with blood (2, 3).

Currently, the treatment of lymph node metastases is the conventional surgical resection of the lymph nodes, often leading to complications such as pain, infection, and lymphedema (1, 4). Moreover, the role of lymph nodes in immune response against cancer is not well-known, and their suppression can prove to be an obstacle to patient
recovery. When tumors spread beyond the sentinel nodes, patients are no longer surgical candidates because metastasis resection often becomes impractical and inefficient. In these cases, standard treatment is by systemic chemotherapy (1, 4). However, the lymphatic system is not easily accessible by conventional intravenous chemotherapy, which limits treatment efficacy (5). Additionally, chemotherapy is responsible for many side effects and poor compliance. For all these reasons, the targeting of metastases in lymph nodes can play a key role to improve cancer treatment. Over the last 2 years, there have been various studies focusing on the targeting of lymph nodes and the lymphatic system. Kono and collaborators developed specific antigen-loaded liposomes to activate the immune response system against tumor cells. These liposomes had their surface modified with a pH-sensitive polymer, with the objective of promoting antigen release in the endosomes of dendritic cells, and to encourage cross presentation for T cell activation (6, 7). Others studies used nanoparticles to release luciferin and allowed the in vivo imaging of lymph-node metastases (8), and the release of immunostimulating agents for vaccination (9) or against autoimmune diseases (10). The subcutaneous (SC) administration of the treatment was the common administration route for lymph-node targeting, and the injections were performed in the vicinity of the target.

The goal of this study was to provide a correlation between SC sites for nanocarrier administration and specific lymph-node accumulation. The choice of the nanocarrier was lipid nanocapsules (LNCs). This nanocarrier was obtained using a solvent-free phase-inversion process and was composed of a lipid core and a PEGylated surfactant shell. Numerous studies have reported the use of LNCs with intravenous (IV) or local brain administration (11, 12), but no data have been published using SC administration. Pharmacokinetic and biodistribution profiles were established after SC administration, using four various injection sites: behind the neck, the right and left flanks, and above the tail. IV administration was used as a control. Right and left inguinal, axillary and cervical lymph nodes were chosen as organs of interest. For this purpose, fluorescence labeling, using DiD, was performed to monitor LNC distribution.

Materials and methods

Materials

Labrafac® WL 1349 (caprylic–capric acid triglycerides) was provided by Gattefosse® S.A. (Saint-Priest, France). Kolliphor® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) was supplied by BASF (Ludwigshafen, Germany). Sodium chloride and acetone were purchased from VWR (Fontenay-sous-Bois, France), Span® 80 (sorbitane monooleate) was purchased from Sigma (St. Quentin Fallavier, France), 1,1′-Dioctadecyl-3,3,3′,3′-tetrakis(methylene) dimethyloctadecylcarboxyamine 4-chlorobenzenesulfonate (DiD) was provided by Life Technologies (Saint-Aubin, France). Water was obtained from a MilliQ filtration system (Millipore, Paris, France).

Preparation of the formulations and characterization

The LNC technology was prepared following the phase-inversion temperature (PIT) process (13). Briefly, 750 μL of DiD dissolved in acetone (1 mg/mL) and acetone was evaporated before the addition of Labrafac® WL 1349 (0.75 g), Kolliphor® HS15 (1.25 g), Span® 80 (0.25 g), NaCl (0.045 g) and water (1.02 g). All were then mixed and heated to 75°C under magnetic stirring followed by cooling to 45°C (rate of 5°C/min). This cycle was repeated three times, and during the final cooling at about 60°C, a sudden dilution with 2 mL room-temperature water was added to dilute the emulsion and form DiD-loaded LNCs. The resulting formulation was filtered (0.22 μm filter) and syringes with a 28-Gauge needle were filled with the LNC suspension. A non-loaded LNC suspension was also prepared as reported previously without the first DiD addition step.

The hydrodynamic diameter (D-average), the polydispersity index (PDI) and the zeta potential of DiD-loaded LNCs were determined by dynamic light scattering using a Zetasizer Nano series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). LNC-based formulations were diluted 1:60 (v/v) in deionized water. The curve fittings of the correlation functions were performed using an exponential fit (Cumulant approach) for D-average and PDI determinations for LNC suspensions. Smoluchowski’s model was used to obtain the zeta potential value. Each measurement was done in triplicate at 25°C. Fluorescence measurements were assessed to control the DiD loading in LNCs, using a microplate reader Fluoroscan Ascent® (Labsystems SA, Cergy-Pontoise, France). Filter pairs with adequate excitation and emission wavelength couples (λex–λem) were used, i.e., 646–678 nm, respectively. Titration curves with DiD dissolved in Labrafac® WL 1349 was performed to evaluate the encapsulation rate.

Animals

For in vivo studies, female Sprague-Dawley rats (Janvier Labs, Le Genest-Saint-Ise, France) (n=120) were housed and maintained at the university animal facility (SCAHU) in disposable plastic cages (4 ad libitum). The cages were isolated from each other, and the animals were maintained in an air-conditioned room with a 12-h-light-12-h-dark cycle. All the animal experiments were carried out in accordance with EU Directive 2010/63/EU and with the agreement of the “Comité d’Ethique pour l’Expérimentation Animale des Pays de la Loire” (authorization CEEA; 2012/37 and 2012/73).

Pharmacokinetic and biodistribution studies

Rats (9 weeks old, n=24 for each group) were anesthetized (isoflurane) and 500 μL of DiD-loaded LNC suspensions were injected...
intravenously into the tail vein or subcutaneously behind the neck, in left and right flanks or above the tail. From 30 min. to 24 h, about 300 μL of blood was removed from the lateral tail vein into a collection tube containing Li-heparin (Tube Micro from Sarstedt, Marnay, France). In addition, 1, 3, 7, 14, 21 and 28 days post administration, 4 rats per group were sacrificed by carbon dioxide asphyxia. Blood, organs (i.e., liver and spleen) and lymph nodes (i.e., left and right inguinal, axillary and cervical lymph nodes) were removed. The lymph node removal was performed as described by Tilney et al. (14).

For pharmacokinetic studies, each blood sample was diluted by a factor of 2 with deionized water. The DiD concentrations (loaded inside LNCs) in blood over time were determined using the microplate reader at the excitation and emission wavelengths previously described, using extrapolation from the linear titration curve ($r^2=0.988$). The titration curve was established by mixing the DiD-loaded LNC suspension (250 μL) and fresh blood from rats (250 μL), with final DiD concentrations ranging from 0.05 to 2 μg/mL. The DiD concentration in blood was normalized in function of animal weight, assuming blood represents 64 mL/kg of rat body weight (15), and was reported as the percentage of the injected dose. The trapezoidal method was used to determine the area under the curve (AUC) during the whole experimental period.

For the biodistribution study, all organs and lymph nodes were analyzed by a fluorescence CRI Maestro™ imaging system (Woburn, USA). Semi-quantitative data were obtained by using a time exposure of 10 ms between 630 and 800 nm. The software Maestro 2.10 (Woburn, USA) was used to calculate the average signal expressed in photons/cm²/s.

Results

LNC-based formulation

LNCs were obtained with the phase-inversion temperature method (13) and are composed of an oily core (Labrafac® WL 1349) surrounded by an organized assembly made of sorbitan oleate (Span® 80) and PEGylated surfactants (Kolliphor® HS15) with the PEG chains oriented towards the aqueous phase. The DiD-loaded LNC diameter was 38±6 nm (Z-average). The PdI was 0.12±0.05, i.e., a monomodal and monodispersed distribution. The zeta potential of the nano-objects was slightly negative (−4±1 mV). The wave length of Zetasizer® Nano Series DTS 1060 laser beam used for the physico-chemical parameter analysis is close to the DiD wave length excitation, and the auto-correlation function is not optimal to determine the LNC size using the Cumulant approach or others methods. To confirm the DiD-loaded LNC parameters, non-loaded LNCs were prepared and the Z-Ave, PdI and zeta potential were found to be similar to those obtained with DiD-loaded LNCs. Fluorescence analysis confirmed the total encapsulation of DiD inside LNCs as previously reported for others lipophilic carbocyanine dyes (16).

LNC pharmacokinetics vs. injection sites and administration routes in healthy rats

The pharmacokinetic profiles of fluorescent-labeled DiD-loaded LNCs was examined after intravenous (IV) and subcutaneous (SC) administration in Sprague-Dawley rats (Figure 1). Four sites were chosen for SC injections: behind the neck, in the right and the left flanks, and above the tail, whereas the tail vein was selected for IV injection. IV pharmacokinetic profile was standard, with a first distribution phase followed by the elimination step. The percentages of injected dose of 1, 0.25 and <0.15% were recovered 1, 3 and 7-days post administration, respectively. It corresponded to an AUC of 11 μg.h/mL. After SC administration, the blood exposition of DiD-loaded LNC was much lower. The percentages of the injected dose were always lower than about 0.4% whatever the post-administration time and the injection site. It corresponded to an AUC of 0.008, 0.008, 0.005 and 0.007 μg.h/mL when the formulation was subcutaneously injected behind the neck, in the right flank, in the left flank and above the tail, respectively, i.e., a decrease by a mean factor of about 1600.

LNC biodistribution vs. injection sites and administration routes in healthy rats

To complete the pharmacokinetic study, the biodistribution of DiD-loaded LNCs was assessed after IV and SC administrations in Sprague-Dawley rats. Semi-quantitative
fluorescence signals for liver and spleen extracted after 1, 3, 7, 14, 21 and 28 days are reported in Figure 2. One day after IV administration, high DiD accumulation in the liver and spleen were visible, followed by a continuous decrease in the average fluorescence signal until 28 days. After SC administration in the right flank and above the tail, a very low accumulation was observed in the liver and spleen one day after administration. Similar results were observed after SC administration in the left flank and behind the neck of the animal (data not shown). The high LNC accumulation in the liver and spleen after IV administration were consistent with the numerous published data using LNC (17, 18). In addition, the differences in the LNC biodistribution profiles after IV and SC administration were correlated to the pharmacokinetic profiles, since a very low fraction of LNCs reached systemic circulation after SC administration whereas the total injected dose was in the blood after IV administration (Figure 1).

Left and right cervical, axillary and inguinal lymph nodes were also extracted and the fluorescence signal was analyzed after IV (Figure 3A) and SC (above the tail, in the right flank, in the left flank, and behind the neck in Figure 3B, 3C, 3D and 3E, respectively) administrations.

After IV administration, the DiD accumulation was distributed in a homogeneous fashion in all lymph nodes studied, with a fairly limited fluorescence signal, whereas SC administration produced specific accumulation depending on the injection site. Cervical lymph nodes were never targeted by LNCs regardless the site of SC administration. After administration behind the neck, only the axillary lymph nodes (right and left) were preferentially targeted by LNC. However this accumulation presented low fluorescence values compared to the other injection sites. The rats that had received an injection above the tail, presented a highly homogenous fluorescence in inguinal and axillary lymph nodes on both sides of the animal. LNCs subcutaneously delivered in the left flank accumulated exclusively in the left side lymph nodes, and mostly in the axillary lymph node. Finally, after right flank administration, LNCs were spread over both sides of the animal.

**Discussion**

This study was carried in order to understand LNC circulation in the animal body, focusing on the specific targeting of lymph nodes after SC administration from various injection sites. The monitoring of LNCs was possible to assess the pharmacokinetic and biodistribution profiles and to make comparisons with IV administration, by using fluorescent-labeled LNCs. For this purpose, DiD was encapsulated in LNCs because the fluorescence and physico-chemical properties of DiD supported the choice of this fluorescence labeling. It is highly fluorescent in lipophilic nanocarriers and weakly fluorescent in aqueous media (19); the auto-fluorescence wavelength emitted by animals is limited because it is a near-infrared fluorophore (20, 21), and there is a strong fluorescence labeling stability of the nanocarrier (no DiD release even in favorable media) (16).

The SC administration of nanocarriers to deliver anticancer treatment is a promising alternative to conventional IV administration for lymph-node targeting. Indeed, nanoparticles are small enough to travel through the aqueous channels of the interstitial tissue, but large enough to prevent their absorption into the blood. Size is a fundamental parameter to reach the lymphatic circulation system. With diameter <10 nm, nanoparticles are mainly absorbed into the blood stream. When larger than 100 nm, they do not spread through the lymphatic system, and remain trapped in interstitial tissue (2, 3). Moreover, it has been observed that lipid compositions for nanocarriers improve lymphatic uptake efficiency, and that PEGylated surface modification can improve lymph node targeting (2, 3). Based on such observations, the LNCs used for this
Figure 3: Biodistribution profiles of DiD-loaded lipid nanocapsules (LNCs) in healthy Sprague-Dawley rats, after (A) intravenous (IV) administration in the tail vein and subcutaneous (SC) administrations (B) above the tail, (C) in the right flank, (D) in the left flank and (E) behind the neck. Semi-quantification of the fluorescence signals of the removed right (R) and left (L) inguinal, axillary and cervical lymph nodes (LN) using fluorescence images at various times post LNC administration (1, 3, 7, 14, 21 and 28 days) (n=4, mean±SD).

Two behaviors were observed after IV and SC administrations. The distribution of LNCs after IV injection was not specific and related to all the body as all the lymph nodes were targeted (Figures 3A and 4A). The accumulation in all lymph nodes was weak in comparison to the accumulation observed after SC administration. SC administrations enabled sequestration in the lymphatic system and specific lymph node targeting (Figure 3B to 3E), without absorption in the blood stream, preventing the accumulation of LNCs in the liver and spleen (Figures 1 and 2).

study have been identified as good candidates to target the lymphatic system after SC administration: i) the size of the LNCs was about 40 nm, which is in the optimal size range; ii) the LNC core and shell were composed of triglyceride and PEGylated surfactant.

To determine a correlation between the SC administration sites and the targeted lymph nodes, the formulations were administered to rats in five different ways: IV administration in the tail vein, and SC administration behind the neck, on the right and left flanks, and above the tail.
In addition to lymph-node targeting efficacy, SC administration was a promising alternative to conventional IV administration to avoid severe side effects due to too much systemic exposure to chemotherapeutics. Using gemcitabine-loaded LNCs, recent results have shown that SC administration reduced the myelosuppression that is observed after IV administration (22), at the same dose.

Differences observed for LNC biodistribution after the four SC administrations can be explained by the anatomy of the lymphatic system. The lymphatic system is separated into two parts: a right part which drains only the head and the right upper limb, and a left part for the other upper limb and lower limbs (grey arrows in Figure 4A to 4E). The lymph flow is bottom-up from lower to upper limbs and top down from the head, since the lymph returns in the blood via the subclavian vein (14, 23, 24). The distribution of LNCs after SC administration followed the direction of lymph circulation. When LNCs were administered behind the neck, cervical lymph nodes, even close to the administration site, were not targeted (in the opposite direction of the lymph flow) even with passive spreading. Similar results were obtained for the other SC administration sites. Nevertheless, a low accumulation was observed in the right and left axillary lymph nodes, due to partial passive spreading in the opposite direction of the lymph flow. No accumulation was observed in the two inguinal
lymph nodes, that were too far from the injection site for passive spreading (Figure 4E). When administered above the tail, a high direct distribution of LNCs was observed in the left and right inguinal lymph nodes, followed by a direct distribution to the left axillary lymph nodes (lymph flow in the direction of the lymph node). In addition, high passive spreading was also observed in the right axillary lymph node (Figure 4B). When LNCs were administered in the left flank, a high direct distribution of LNCs was observed in the left axillary lymph node, and passive spreading in the opposite direction of the lymph flow was observed in the left inguinal lymph node. No LNCs were distributed in the right inguinal and axillary lymph nodes, since they were too far from the injection site for passive spreading, and it corresponds to the other lymphatic circulation sites (for the right axillary lymph node) (Figure 4D). Administered on the right side, a high, direct distribution was observed in the right axillary lymph node, and passive spreading was observed in the right inguinal lymph node. The injection site was located at the frontier of the two lymphatic circulation sites. As the inguinal right lymph node was targeted, it follows that direct accumulation was observed in the left inguinal lymph node followed by accumulation in the left axillary lymph node (Figure 4C). When LNCs were administered in the left flank, they were sequestered in the left lymphatic system and in the left side of the animal. After administration in the right flank, LNCs were found between the two sides since they can circulate in both flanks.

**Conclusion**

To improve chemotherapy protocols, a promising way to deliver drugs in lymph nodes should be the SC administration of drug-loaded nanocarriers. Using LNCs as nanocarriers, passive and specific targeting of lymph nodes was observed, depending on the SC administration site. With appropriate SC administration, LNCs can accumulate in specific lymph nodes. Therefore a personalized therapeutic scheme for patients could be considered when specific lymph node targeting is needed. In addition, LNCs were sequestered in the lymphatic system, without returning to the blood circulation. The limitation of side effects, often due to excessive drug dosage to overcome liver metabolism, could be expected.

**Acknowledgments:** This work has been carried out within the research program NICHE, financially supported by EuroNanoMed2 ERA-NET. SCAHU at Angers University is also acknowledged for animal facility.

**References**


Bionotes

Marion Pitorre, 25 years old, is a PhD student and she performs her research works in the INSERM 1066 laboratory (Angers, France), under the co-direction of Pr Jean-Pierre Benoit and Dr Guillaume Bastiat. She obtained her Pharm D at the University Paul Sabatier (Toulouse, France) in 2013, with a Master of University of Angers, based on the innovation in pharmaceutical technologies, with an internship position in Pierre Fabre group in Toulouse. During her PhD, Marion PITORRE is interested about new nanocarriers for the targeting of lymph nodes to stop the dissemination of metastases.

Guillaume Bastiat, 38 years old, is an associate professor at the Faculty of Pharmacy, University of Angers (France). After his PhD in the field of polymer physico-chemistry (University of Pau, France) in 2003, he joined the University of Montreal (Canada) for post-doctoral positions in the Center for Self-Assembled Chemical Structures and in the Canadian Research Chair in Drug Control Release, focusing in self-assembled vesicle structures and organogel for drug delivery. He follows his research works in the INSERM 1066 laboratory and develops new pharmaceutical technologies for parenteral administration based on lipid nanocapsules, gels and non-phospholipidic vesicles. During his scientific carrier, he wrote 28 publications and was the author of more than 40 communications in national and international conferences.

Elodie Marie dit Chatel, 22 years old, obtained her Bachelor’s degree in analytic biology and micro-organism experimental design, with a specialization in animal model (University of Angers, France). She obtained an internship position in the INSERM 1066 laboratory (Angers, France), under the co-direction of Dr Guillaume Bastiat and Marion Pitorre. During her trainee, she was in charge of the pharmacokinetic and biodistribution experimental protocols after subcutaneous administration of nanocarriers in murine model.

Jean-Pierre Benoit, trained in pharmacy, teaches Pharmaceutical Technology at the Faculty of Pharmacy, Angers University. He acquired a solid foundation in the domain of micro- and nanoencapsulation. He has developed a highly competitive research group at an international level. Jean-Pierre Benoit is currently the director of a 70 members strong research team, recognised and financed by INSERM. His research activity has resulted in the publication of over 260 original refereed scientific articles and reviews, and 25 patents. In 2010 Benoit received the PSWC Research Achievement Award in New Orleans from the FIP, and in 2011 the Prize of Notoriety from the French National Academy of Pharmacy.