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# *In vivo* imaging of DNA lipid nanocapsules after systemic administration in a melanoma mouse model

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ABSTRACT

The biodistribution of intravenously injected DNA lipid nanocapsules (DNA LNCs), encapsulating pHSV-tk, was analysed by *in vivo* imaging on an orthotopic melanoma mouse model and by a subsequent treatment with ganciclovir (GCV), using the gene-directed enzyme prodrug therapy (GDEPT) approach. Luminescent melanoma cells, implanted subcutaneously in the right flank of the mice, allowed us to follow tumour growth and tumour localisation with *in vivo* bioluminescence imaging (BLI). In parallel, DNA LNCs or PEG DNA LNCs (DNA LNCs recovered with $PEG_{2000}$) encapsulating a fluorescent probe, DiD, allowed us to follow their biodistribution with *in vivo* biofluorescence imaging (BFI). The BF-images confirmed a prolonged circulation-time for PEG DNA LNCs as was previously observed on an ectotopic model of glioma; comparison with BL-images evidenced the colocalisation of PEG DNA LNCs and melanoma cells. After these promising results, treatment with PEG DNA LNCs and GCV on a few animals was performed and the treatment efficacy measured by BLI. The first results showed tumour growth reduction tendency and, once optimised, this therapy strategy could become a new option for melanoma treatment.


## 1. Introduction

In homeostatic conditions, melanocytes produce melanin and thus contribute to the pigmentation of skin and hair, protect the skin from damage by ultraviolet radiation, and prevent skin cancer (Lin and Fisher, 2007). But they are also precursors of melanoma, the most deadly form of skin cancer, following mutations of critical growth regulatory genes, the production of autocrine growth factors, and/or a loss of adhesion receptors (Gray-Schopfer et al., 2007). The primary site of melanoma is the skin, but other pigmented tissues, such as the eyes or the intestine, can also be at the origin of melanoma. The tumour-specific, 10-year survival for melanoma is 75–85% when diagnosed as primary tumours without any evidence of metastasis, but melanoma metastasis dramatically reduces this percentage to 20–70%, this being dependent on the metastasis type (Garbe et al., 2010). If diagnosed early, local surgical resection can cure melanoma in 80% of cases, contrary to metastatic melanoma which is largely refractory to existing therapies (Tawbi and Nimmagadda, 2009).

For these reasons, new therapies need to be developed, and gene therapy, which is an emerging field in cancer treatment, represents a promising option. Gene therapy can be used with the aim of treating the tumour (destructive approach) or with the aim of reverting its malignant phenotype (corrective approach). One tumour-treatment possibility is the 'gene suicide' approach, also called gene-directed enzyme prodrug therapy (GDEPT). This approach is based on the delivery of a gene which codes for an enzyme, and is able to convert a nontoxic prodrug into a cytotoxic metabolite (Gutzmer and Guerry, 1998; Portsmouth et al., 2007). The first proof-of principle of GDEPT was made with the enzyme HSV-tk (Herpes simplex thymidine kinase) and the prodrug ganciclovir (GCV) (Moolten, 1986), which is still widely used in clinical and experimental applications in different tumour models (Portsmouth et al., 2007). The efficacy of this concept was for example shown in 2004 in a randomised controlled study on patients with operable primary or recurrent malignant glioma and allowed a significant increase in patient survival time (71 weeks in the treated group compared to 39 weeks in the control group)

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(Immonen et al., 2004). To transfer a gene effectively, different parameters, such as the administration route, the length of gene expression time, the animal model and/or the need of a delivery vehicle, have to be fixed. Local administration does not necessarily need a delivery vehicle and low doses are usually required; however, the targeted tissue has to be accessible to use this administration route. Systemic administration allows accessibility to the different tissues and has the advantage of rapid action and of allowing repeated administration but it needs a delivery vehicle in order to be effective. Moreover, in clinical applications, many diseases require intravenous (i.v.) or intraperitoneal (i.p.) injection treatment regimens. Delivery vehicles for systemic administration can be classified into two main groups: viral and non-viral delivery systems. Although viral vectors are very effective in terms of gene delivery and expression, the major drawbacks are their potential risk associated with replication-competent viruses, immunogenicity and high cost (Chowdhury, 2009; Collins et al., 2008; Jin and Ye, 2007). Non-viral vectors do not show these drawbacks; they present the advantages of being able to carry large inserts and to be easily formulated (Jin and Ye, 2007; Kreiss et al., 1999; Morille et al., 2008); they can also be adapted to passive or active tumour targeting (Huynh et al., 2010; Kang et al., 2010; Wagner et al., 2004).

In recent literature, nanocarriers (lipid or polymer) used in various treatment strategies, carrying different agents such as plasmids, siRNA, ODN or pharmacological inhibitors, and applied by several different administration routes (intravenous, intratumoural, topical, etc.), have been shown to increase melanoma cell delivery and treatment efficacy (de Campos et al., 2010; Stone et al., 2009; Weiss and Aplin, 2010; Zheng et al., 2009).

Lipid nanocapsules (LNCs) developed in our laboratory (Heurtault et al., 2002) have already been shown to be efficient for *in vitro* and *in vivo* transfection (Morille et al., 2009a, 2010). The formulation process of LNCs requires neither organic solvents nor high energy, and is based on the phase-inversion temperature (PIT) method. It consists of using non-ionic polyethoxylated surfactants to allow the phase inversion of an emulsion (Shinoda and Saito, 1968, 1969) and the application of a temperature-cycle treatment around the PIT to obtain small sizes with a low polydispersity index (PDI) (Anton et al., 2007). A sudden cooling and dilution of the micro-emulsion at the PIT leads to the obtention of LNCs, which consist of a lipid, liquid core of triglycerides, and a rigid shell of lecithin and short chains of poly(ethylene glycol) ($PEG_{660}$) (Heurtault et al., 2002). This formulation process allows fragile molecules such as nucleic acids to be encapsulated. To encapsulate hydrophilic DNA in the lipid core, DNA was complexed with cationic lipids prior to encapsulation (Vonarbourg et al., 2009). As already observed (Morille et al., 2009a; Vonarbourg et al., 2009), the encapsulation of these lipoplexes should provide an efficient loss of toxicity allowing higher doses to be injected *in vivo*. Furthermore, in order to enhance stealth properties of DNA LNCs, PEG was used (see the review of Huynh et al. (2010) describing the advantages and drawbacks of this polymer). The coating of the DNA-LNC surface with longer PEG chains ($PEG_{2000}$) improved nanocapsule *in vivo* circulation time on a mouse model of subcutaneously injected glioma cells (Morille et al., 2009a)

In this study, non-viral LNCs were used to deliver pHSV-tk in melanoma tumour cells after IV injection into mice. Considering the melanoma mice model, some *luc+* human melanoma cells were engrafted on nude mice. Thus, the localisation and the growth of the tumours could be followed by luminescence. Once the tumours were established, the biodistribution and localisation of intravenously injected DNA LNCs in this orthotopic melanoma mouse model were traced via *in vivo* fluorescence imaging. Bioluminescence and fluorescence images were then compared in order to localise DNA LNCs versus PEG DNA LNCs, and a treatment with ganciclovir (GCV) was carried out in order to evaluate the efficiency of the gene suicide approach.

## 2. Materials and methods

### 2.1. Preparation of nanocarriers

#### 2.1.1. Liposomes and lipoplexes

Solutions of DOTAP (1.2-DiOleoyl-3-TrimethylAmmonium-Propane) and DOPE (1.2-DiOleyl-sn-glycero-3-PhosphoEthanolamine) in chloroform (20 mg/ml) (Avanti Polar Lipids, Inc., Alabaster, USA) were first dried by an evaporation process under vacuum and the formed lipid film was then hydrated with deionised water over night at 4 °C. The next day, liposomes were sonicated for 20 min and lipoplexes were prepared. For their preparation, DOTAP/DOPE (1/1, M/M) liposomes were mixed with 660 μg of HSV-tk encoding plasmid (pORF-TK-ΔCpG; InvivoGen; 4.35 kb) at a charge ratio of 5 (+/−) in 150 mM NaCl.

#### 2.1.2. DNA-loaded lipid nanocapsules (DNA LNCs)

LNCs were composed of lipophilic Labrafac® WL 1349 (Gatefossé S.A., Saint-Priest, France), a mixture of caprylic and capric acid triglycerides, oleic Plurol® (polyglyceryl-6 dioleate) which was kindly provided by Gatefossé S.A. (Saint-Priest, France), NaCl (Prolabo, Fontenay-sous-Bois, France), water (obtained from a Milli-Q-plus® system, Millipore, Paris, France) and Solutol® HS-15 (30% of free polyethylene glycol 660 and 70% of polyethylene glycol 660 hydroxystearate (HS-PEG)) (BASF, Ludwigshafen, Germany). The formulation process is based on phase-inversions of a microemulsion described by Heurtault et al. (2002). Briefly, all the components, in a well defined ratio (3.9% of oleic Plurol® (w/w), 5.9% of Solutol® (w/w), 9.9% of Labrafac® (w/w), 78.9% of water (w/w) and 1.4% of NaCl), were mixed together under magnetic stirring and temperature cycles around the phase-inversion-temperature (PIT) were performed. In the last step, cold water was added (in a ratio 1:1.96) to dilute the emulsion and to form the LNCs. To formulate fluorescent DNA LNCs, lipoplexes were added instead of the formulation water (Vonarbourg et al., 2009), and a mixture of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, em. = 644 nm; exc. = 665 nm) (Invitrogen, Cergy-Pontoise, France) and Labrafac® replaced Labrafac® alone. To obtain the Labrafac®–DiD mixture, a solution of DiD in acetone at 0.6% (w/w) was prepared, incorporated in Labrafac® in a ratio of 1:10 (w/w) and acetone was evaporated before use (Garcion et al., 2006).

#### 2.1.3. Preparation of coated nanocapsules by post-insertion

Coated nanocapsules were prepared as previously described (Morille et al., 2009a). Briefly, fluorescent DNA LNCs were purified after their formulation, using PD10 Sephadex columns (Amersham Biosciences Europe, Orsay, France). To compensate for the dilution of our formulation and the desalting effect caused by this purification step, an ultrafiltration step was performed with MilliporeAmicon® Ultra-15 centrifugal filter devices (Millipore, St. Quentin-Yvelines, France) and the salt- and LNC-concentration were readjusted afterwards to obtain a physiologic concentration of NaCl (150 mM) and the initial concentration of LNCs (152 g/l). These purified fluorescent DNA LNCs were then mixed with 1,2-DiStearoyl-sn-glycero-3-PhosphoEthanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-$mPEG_{2000}$) (Mean Molecular Weight (MMW) = 2805 g/mol) (Avanti Polar Lipids, Inc., Alabaster, USA) to obtain a final polymer concentration of 10 mM. The mixture was incubated for 4 h at 30 °C and vortexed every 15 min.