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Freeze-drying of squalenoylated nucleoside analogue nanoparticles

L. Bildstein[a], H. Hillaireau[a], D. Desmaële[b], S. Lepêtre-Mouelhi[b], C. Dubernet[a], P. Couvreur[a,*]

[a] Université Paris-Sud XI, Faculté de Pharmacie, UMR CNRS 8612, IFR 141, 92296 Châtenay-Malabry Cedex, France
[b] Université Paris-Sud XI, Faculté de Pharmacie, UMR CNRS 8076 BIOCIS, 92296 Châtenay-Malabry Cedex, France

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ABSTRACT

Nucleoside analogues are potent anticancer or antiviral agents that however display some limitations (rapid metabolism, induction of resistance). In order to overcome these drawbacks, we recently proposed new prodrugs, in which nucleoside analogues were covalently coupled to squalene (SQ). The resulting amphiphilic compounds spontaneously formed nanoparticles (NPs) and displayed a promising efficacy both *in vitro* and *in vivo*. Since long-term stability is essential for further clinical development we needed to develop a laboratory-scale freeze-drying protocol in order to improve the colloidal stability of those NPs. Squalenoylated gemcitabine (SQdFdC) has been successfully freeze-dried with trehalose (10%, w/w) as a cryoprotectant. Concentrations of SQdFdC up to 4 mg/mL after freeze-drying and rehydration have been obtained, which is necessary for *in vivo* studies. Stability measurements by dynamic light scattering showed that trehalose had a stabilizing effect on SQdFdC NPs, and that freeze-dried SQdFdC NPs could be stored up to four months at room temperature before rehydration, without loss of stability. *In vitro* cytotoxicity studies on three murine cell lines showed that SQdFdC NPs retained their cytotoxic activity after freeze-drying. We showed that this freeze-drying protocol could also be applied to squalenoylated didanosine (SQddI) and zalcitabine (SQddC). Overall, these results allow for the use of freeze-dried NPs in upcoming preclinical trials of the different squalenoylated compounds developed in our laboratory.

1. Introduction

Nucleoside analogues (NAs) are small molecules that impair nucleic acid synthesis and therefore display significant anticancer or antiviral activities, despite several limitations. They, indeed, experience a short plasmatic half-life, due to their rapid metabolization into inactive species. NAs are often too hydrophilic to diffuse through cell membranes and have to be taken up by membrane nucleoside transporters to enter the cells (Cass, 1995). Furthermore they need to be phosphorylated before exerting their cytotoxic or antiviral activity (Bergman et al., 2002). Impaired transport (Mackey et al., 1998) and/or altered kinase activity (Ruiz van Haperen et al., 1994; Dumontet et al., 1999) can induce resistance to the NAs, which is a major concern from a clinical standpoint.

In order to overcome these shortcomings, we previously coupled covalently squalene (SQ) to NAs like gemcitabine (difluorodeoxycycitidine, dFdC), didanosine (2′,3′-dideoxyinosine, ddI) and zalcitabine (2′,3′-dideoxycytidine, ddC) (Couvreur et al., 2006). Independently of the nature of the NA or the location of the squalene derivative, the resulting amphiphilic prodrugs were capable of forming nanoparticles (NPs) in water without need of surface active agents. Electron microscopy studies and X-ray diffraction investigations on squalenoylated gemcitabine (SQdFdC) and zalcitabine (SQddC) NPs have revealed original supramolecular inner organizations (Couvreur et al., 2008; Bekkara-Aounallah et al., submitted for publication). Under this form, SQdFdC displayed a significantly enhanced plasmatic half-life over dFdC, due to the protection of its metabolization-sensitive amine moiety by the SQ. SQdFdC also showed very promising preclinical results on various leukaemia-bearing murine models, with a considerable improvement over the parent drug, in terms of control of the disease and survival, without modification of the toxicological profile (Reddy et al., 2008a). This was explained by the prolonged release and decreased elimination of the drugs, as well as by their preferential accumulation in lymphoid organs which are the major sites of metastasis in case of leukaemia (Reddy et al., 2008b).

These promising results pave the way towards initiating further preclinical investigations on SQdFdC as this nanomedicine could be a potential candidate for clinical trials. However, SQdFdC nanoparticles are stable only for a few days in suspension, which is a significant drawback in a development perspective. Freeze-drying the suspension is the most common answer to the colloidal stability concerns frequently arising upon the development of nanoparticulate systems. This industrial process consists in sublimating the water from a frozen NP suspension. It may greatly enhance the physical and chemical stabilities of the formulation, provided that

* Corresponding author.
E-mail address: patrick.couvreur@u-psud.fr (P. Couvreur).

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some necessary precautions are taken in order to minimize the various stresses associated with this process (Abdelwahed et al., 2006).

The aim of the present study was to set up a laboratory-scale freeze-drying process of SQdFdC NPs in order to improve their physico-chemical stability. The impact of various factors such as the nature and concentration of the cryoprotectant and the concentration of SQdFdC has been evaluated. Further characterizations of the SQdFdC freeze-dried NPs in terms of stability after rehydration, shelf-life in solid form over several months and *in vitro* cytotoxic activity have been undertaken. Moreover, we have investigated whether this freeze-drying protocol could also be applied to other squalenoylated nucleoside analogues.

2. Materials and methods

2.1. Drugs and chemicals

Gemcitabine hydrochloride (dFdC), zalcitabine (ddC) and didanosine (ddI) were purchased from Sequoia Research Products Ltd. (UK). Glucose and trehalose were obtained from Sigma–Aldrich Chemical Co. (France), dextran 70 from Prolabo (France), absolute ethanol from Carlo Erba (Italy). Deionised and filtered MilliQ® water (Millipore, France) was used.

4-N-trisnorsqualenoyl-gemcitabine (SQdFdC), 4-(N)-trisnorsqualenoyl-dideoxycitidine (SQddC) and 5′-trisnorsqualenoyl-didanosine (SQddI) were synthesized and characterized as reported earlier (Couvreur et al., 2006) by covalent coupling of 1,1′,2-trisnorsqualenoic acid with respectively dFdC, ddC and ddI. The squalene derivative was coupled onto the amino group of the nucleoside heterocycle in case of dFdC and ddC, and onto the 5′ hydroxyl group of ddI's deoxyribose.

2.2. Preparation of the nanoparticles

Squalenoylated NA nanoparticles were prepared by nanoprecipitation. Briefly, SQdFdC, SQddC or SQddI was dissolved in 1 mL absolute ethanol and added dropwise in 2 mL of a cryoprotectant-containing aqueous phase under vigorous stirring. NP formation occurred immediately. Ethanol was then evaporated using a Rotavapor® and the nanoparticulate suspension was stored at 4 °C until further use.

2.3. Freeze-drying parameters

Weighed flat-bottom screw-cap glass vials filled with 500 μL of nanoparticulate suspension were used in all experiments. Two freezing processes were compared: flash freezing in liquid nitrogen for 5 min, followed by 2 h at −25 °C in a frost-free freezer, and slower on-shelf-freezing at −25 °C for 2 h. Vials containing the frozen NP dispersions were uncapped and transferred into the −20 °C pre-equilibrated chamber of an Alpha I-5 freeze-drier (Martin Christ, Germany), which was depressurized to 5 Pa using a Pascal 2010 pump (Adixen/Alcatel). Temperature was kept at −20 °C during 60 h of primary drying, and then stepwise increased to 25 °C over the last 24 h to ensure secondary desiccation. After reestablishment of ambient pressure, vials were capped, weighed and stored at room temperature or at 4 °C until further use.

2.4. NP characterization and stability measurements

Mean particle size and polydispersity index were determined by quasi-elastic light scattering (QELS) using a Malvern zetasizer nano ZS (Malvern Instrument, UK). The measurements were performed in triplicate, at 25 °C, after dilution of the NP to around 0.1 mg/mL with MilliQ® water.

The characteristics of the nanoparticulate suspensions were determined by QELS within one hour after nanoprecipitation or rehydration. Their stability was monitored over one week at 4 °C: aliquots were taken 1, 3 and 7 days after nanoprecipitation or rehydration, and immediately diluted and characterized by QELS.

Graphical representations of QELS measurements are reported in all figures as mean ± standard deviation of three separate measurements of the same diluted nanoparticulate suspension.

2.5. In vitro cytotoxicity assay

Sensitive and adriamycin-resistant P388 murine leukaemia cell lines (P388/s and P388/adr) were kindly provided by the "Institut de Recherche sur le cancer" (IRSC, France). The J774.A1 murine macrophage–monocyte cell line was obtained from ECACC (catalogue number 91051511). All cell lines were grown under humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (Gibco, France), supplemented with 10% foetal calf serum (Gibco, France), 50 U/mL penicillin and 50 μg/mL streptomycin. Culture medium for both P388 strains additionally received 20 nM 2-mercaptoethanol (Sigma, France).

The *in vitro* cytotoxic activity of control and freeze-dried SQdFdC NPs was evaluated on all three cell lines using the 3-[4,5-dimethylthiazol-2-yl]-3,5diphenyl tetrazolium bromide (MTT) test. Cells were seeded in 96-well plates and pre-incubated for 24 h to ensure exponential growth. Culture medium containing control SQdFdC NPs, freeze-dried SQdFdC NPs, or dFdC was added upon the cells, in triplicate. Concentration range was 1–1000 nmol/L (SQdFdC NPs) or 0.1–100 nmol/L (dFdC). After 48 h incubation, 20 μL of a 5 mg/mL MTT solution in phosphate buffer saline was added to each well. 2 h incubation later, the plates were centrifuged for 5 min at 250 × *g* and the culture medium was gently sucked out and replaced by 200 μL dimethylsulfoxyde in order to dissolve the formazan crystals. Absorbance at 570 nm, which is proportional to the number of living cells, was measured with a microplate reader (Metertech Σ 960, Fisher Bioblock, France). The percentage of surviving cells in each well was calculated as the absorbance ratio between treated and untreated control wells. The IC50s, drug concentrations which lead to 50% survival, were determined by non-linear least squares fitting. The sigmoid-shaped survival data (normalized as a percentage of an untreated control) were modelized by a three-parameter Hill function, *H*, whose values are given by the following equation:

$$H([\text{drug}]) = \frac{A}{1 + ([\text{drug}]/B)^C}$$

where [drug] is the drug concentration and *A*, *B* and *C* are the parameters of the Hill function. The IC50 directly appears in this form of the equation as the parameter *B*. The Microsoft Excel® built-in "solver" algorithm was used to fit the model to experimental data, which yields the optimized values of *A*, *B* and *C*.

3. Results and discussion

3.1. Optimization of SQdFdC freeze-drying conditions

Numerous reports state that optimization of cryoprotectant and process parameters is a prerequisite for successfully freeze-drying nanoparticles. Mono-, di- or poly-saccharides are commonly used cryoprotectants which generate a glassy matrix upon water sublimation, therefore preventing NP aggregation during storage, provided that the nature and concentration of the cryoprotectant have been optimized (Abdelwahed et al., 2006). They can also interact with the surface of the NPs, which protects it from structural alterations (Crowe et al., 1996). Process characteristics like freezing

Table 1
Size increase in SQdFdC NPs after rehydration of the freeze-dried suspensions, as a function of SQdFdC and trehalose concentrations, averaged over several experiments ($n \geq 3$).

	Trehalose concentration (w/w)				
	5%	7.5%	10%	15%	20%
SQdFdC concentration (mg/mL)					
1	Incomplete redispersion	ND	+10%	ND	ND
2		+112%	+24%	ND	
4		ND	Incomplete redispersion		

Grey boxes: incomplete redispersion of the solid, strongly aggregated NPs (ND: experiment not done).

rate can have a tremendous impact on the properties of rehydrated NPs (van Winden et al., 1997).

In our experiments glucose, trehalose and dextran 70 were chosen as cryoprotectants. In order to avoid excessive NP dilution, the cryoprotectants were dissolved in the nanoprecipitation aqueous medium before adding the ethanolic SQdFdC solution. Glucose up to 10% (w/w) or dextran 70 up to 1% (w/w) led to a collapse of the freeze-dried cake and massive NP aggregation upon rehydration. On the contrary, trehalose at 10% concentration efficiently protected SQdFdC NPs from aggregation up to 2 mg/mL concentration (Table 1), while at 7.5% concentration it ensured NP redispersion but led to rapid aggregation. Various authors have underlined the interactions between trehalose and the surface of liposomes (see among others Liu et al., 1997; Sum et al., 2003; Crowe et al., 2001 or Lenne et al., 2006). Since SQdFdC NPs are of lipidic nature as well, we hypothesize that trehalose interacts more with the polar part of SQdFdC surface molecules than glucose and dextran, which explains its superior cryoprotective ability.

The impact of the freezing rate was investigated on SQdFdC nanoparticles prepared at 1 or 2 mg/mL in 10% trehalose solution. Fast liquid nitrogen freezing followed by 2 h at −25 °C was compared with slower on-shelf-freezing at −25 °C for 2 h, on four independent experiments. Although both freezing rates led to similar increases in size and polydispersity over the course of the process (14.5% size increase, and 54.5% polydispersity index increase), the slower one resulted in more reproducible sizes of the NPs upon rehydration (data not shown). Since liquid nitrogen freezing negatively influenced the freeze-drying behaviour of the NPs, on-shelf-freezing at −25 °C has been retained as the operating condition for all the experiments described hereafter.

The preclinical *in vivo* evaluations are the long-term focus of the present work. Due to the limitation concerning the volume that can be injected in mice for preclinical studies, only SQdFdC concentrations above 4 mg/mL may be used. Since the direct freeze-drying of SQdFdC dispersions above 2 mg/mL had proven impossible, whatever the trehalose concentration (Table 1), lyophilized cakes were rehydrated with smaller amounts of water as compared to the initial suspension volume in order to increase the final nanoparticle concentration. With this method, the target concentration of 4 mg/mL could be reached by rehydrating a freeze-dried 2 mg/mL SQdFdC (with 10% trehalose) at half of its initial volume, without hampering NP redispersion (Table 2). In our experience, the resulting 20% (w/w) final trehalose concentration was found to be well tolerated by the animals despite hypertonicity. If higher SQdFdC concentrations after rehydration would be necessary, e.g. for maximum tolerable dose studies, further optimization will be required in order to get stable NPs at higher concentrations using acceptable trehalose concentration.

3.2. Characterization of freeze-dried SQdFdC nanoparticles

3.2.1. Stability of rehydrated NPs

Stability of the rehydrated NPs over the course of a few days is essential for successful *in vivo* preclinical studies. Thus, the effect of freeze-drying (using 10% trehalose) on the size and polydispersity of the NPs has been investigated. The consequences of the rehydration of SQdFdC NPs with smaller volumes of water were evaluated as well.

The influence of the trehalose on the stability of non-freeze-dried SQdFdC NPs has been evaluated by performing the nanoprecipitation step directly in a trehalose solution (10%) instead of in pure water. This has led to an average 11.3% size increase as compared with the mean diameter of NPs prepared in pure water, as determined in 4 independent experiments. Interestingly, trehalose significantly improved the stability of the NP suspension (Fig. 1) over one week, which vastly offsets its initial adverse effect. As an internal control, a concentrated trehalose solution has been added to NPs freshly prepared in pure water, to reach a final 10% trehalose concentration. Initial size increase and long-term stabilization of the NPs similar to those of NPs prepared directly in a trehalose solution were observed (data not shown). It was concluded that trehalose influenced the colloidal balance of SQdFdC NPs and increased their overall stability, independently of its presence before or after the nanoprecipitation step.

After freeze-drying and subsequent rehydration, NP size and polydispersity index increased slightly. Nevertheless, one day after rehydration freeze-dried SQdFdC NPs were actually found more stable than non-freeze-dried control NPs dispersed in 10% trehalose solution. When 2 mg/mL NP suspension was rehydrated in half of its initial volume, to reach a 4 mg/mL final concentration,

Table 2
Evolution of SQdFdC NPs during the freeze-drying and rehydration steps, averaged over several experiments ($n \geq 3$), ±standard deviations.

Concentrations		Mean diameter (nm) (Polydispersity index)		
[SQdFdC] mg/mL	[Trehalose] % (w/w)	Before freeze-drying	After freeze-drying and rehydration	Deviation, in percentage
1	10%	112 ± 6 (0.121 ± 0.049)	123 ± 8 (0.160 ± 0.042)	+10.2%
2	10%	128 ± 12 (0.110 ± 0.048)	158 ± 14 (0.149 ± 0.027)	+24.0%
2 *before*/4[a] *after*	10% *before*/20%[a] *after*	120 ± 18 (0.111 ± 0.048)	138 ± 25 (0.120 ± 0.022)	+15.5%

Z-avg: means particle size (nm); Pdi: polydispersity index.
[a] Rehydration of a lyophilized 2 mg/mL NP suspension with a reduced water volume to achieve a final concentration of 4 mg/mL of SQdFdC.

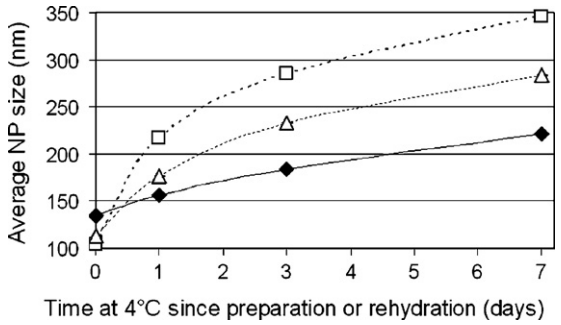

Fig. 1. Stability of SQdFdC NPs at 4 °C over a week after nanoprecipitation or rehydration. All dispersions at 1 mg/mL SQdFdC. NP either nanoprecipitated in water (□), in 10% trehalose (△) or in 10% trehalose and subsequently freeze-dried and rehydrated (♦).

it displayed exactly the same size evolution as the controls (i.e. non-freeze-dried NPs and freeze-dried NPs resuspended at a final concentration of only 2 mg/mL) (Fig. 2), despite a superior size immediately after rehydration. These results confirm that trehalose contributes to the stabilization of the colloidal suspension, and that its effect was reinforced by the freeze-drying process. This was especially true when trehalose concentration was of 10% for SQdFdC 1 mg/mL and of 20% for SQdFdC 4 mg/mL, leading to only a slight increase in the particles size that remained below 250 nm after a week.

It is crucial that the diameter of the rehydrated NPs remained below ca. 250 nm in order to maintain the *in vivo* biodistribution and anticancer efficacy of SQdFdC NPs (Reddy et al., 2008b). Our results show that 4 mg/mL rehydrated SQdFdC NPs offered an administration window of several days after rehydration, which was the target in a preclinical perspective. Increasing the concentrations of trehalose clearly stabilized SQdFdC nanoparticulate suspensions, to the point of counterbalancing the mild destabilizing effect of the freeze-drying process, as observed on the 2 mg/mL SQdFdC NPs.

3.2.2. Shelf-life over several months

Long-term storage of the nanoparticles must be ensured before larger-scale preclinical trials are undertaken, because they will necessitate a scaling up of SQdFdC NPs preparation.

The stability of 4 mg/mL suspension of SQdFdC NPs (rehydrated from 2 mg/mL freeze-dried NPs suspension) was monitored after storage times up to 4 months, at room temperature or at 4 °C. No macroscopic change of the freeze-dried cake was observed within the 4 months. *In vivo*-compatible rehydrated NPs (i.e. sizes below 200 nm) were obtained whatever the temperature and duration of

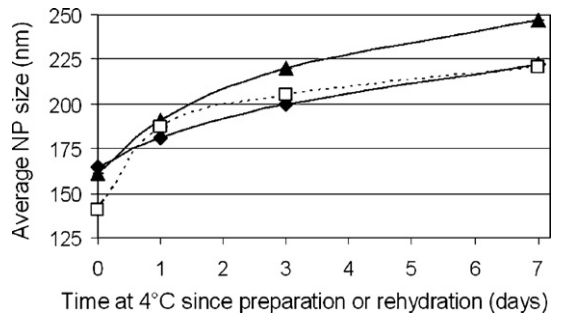

Fig. 2. Stability of SQdFdC NPs at 4 °C over a week after nanoprecipitation or rehydration. Samples coming from the same batch of 2 mg/mL SQdFdC NPs nanoprecipitated in 10% trehalose: control NPs (□); freeze-dried and rehydrated NPs (▲); freeze-dried NPs rehydrated with a smaller amount of water to increase SQdFdC concentration to 4 mg/mL (♦).

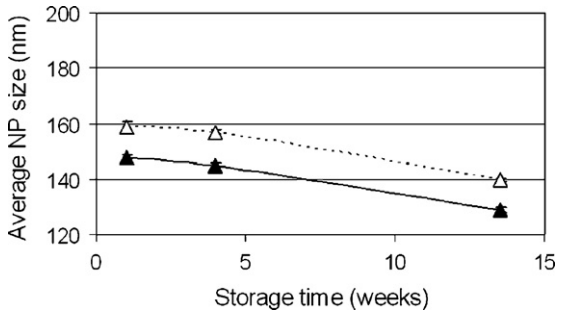

Fig. 3. Average size of SQdFdC NPs freeze-dried and rehydrated to reach a concentration of 4 mg/mL. Storage conditions: at 4 °C (△) or at room temperature (▲).

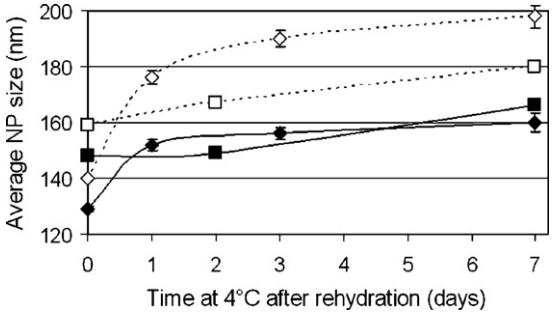

Fig. 4. Influence of the storage in freeze-dried form on the stability of SQdFdC NPs at 4 °C over a week after rehydration. 1-week- (□■) or 4-months- (◊♦) storage, at room temperature (—■♦—) or at 4 °C (----□◊----).

the storage (Fig. 3). Although slightly smaller NPs were obtained after storage at room temperature, additional experiments are required to highlight why room temperature leads to a somewhat better size control.

Interestingly, it was observed that the NPs displayed a slow but steady decrease in rehydrated mean size with increasing storage time. This shrinkage of the NPs could come from a progressive diffusion out of the NPs of the water molecules which were found to be trapped within the inverted hexagonal phase formed by the stacking of the SQdFdC molecules (Couvreur et al., 2008). In order to confirm this hypothesis, the evolution of the size of the NPs stored either 1 week or 4 months in solid form was monitored after rehydration. As seen in Fig. 4, despite inferior sizes after rehydration, the 4 months-stored NPs experience a faster size increase than their 1 week-stored counterparts, and after 24 h they display a similar stability profile when stored at room temperature. This observation suggests that the particles would not be altered after 4 months but only desiccated, and that they would recover their normal size a few hours after rehydration.

3.2.3. In vitro cytotoxicity

The *in vitro* cytotoxicity profile of the freeze-dried SQdFdC nanoparticles was evaluated on three murine cell lines. Cytotoxicity measurements of freeze-dried and control NPs arising from the

Table 3
Cytotoxicity of dFdC, control, and freeze-dried SQdFdC NP after 48 h exposure, on three murine cell lines.

Cell line	IC50 values (nmol/L)			IC50 ratio: control/freeze-dried
	dFdC	Control NPs	Freeze-dried	
P388/s	2.3	35.9	30.0	1.20
P388/adr	1.5	26.3	28.3	0.93
J774.A1	5.1	79.7	83.2	0.96

dFdC and SQdFdC IC50 values in nmol/L.

Table 4
Size of SQddC and SQddI NPs, before and after freeze-drying with different trehalose (w/w) concentrations, averaged over two experiments, ±standard deviation.

Molecule	[Trehalose] % (w/w)	Mean diameter (nm) (polydispersity index)		
		Before freeze-drying	After freeze-drying and rehydration	Deviation, in percentage
SQddC	5%	119 ± 2 (0.094 ± 0.010)	772 ± 152 (0.326 ± 0.137)	+584%
	10%	118 ± 0 (0.057 ± 0.009)	151 ± 5 (0.087 ± 0.011)	+28.0%
SQddI	5%	176 ± 1 (0.266 ± 0.021)	187 ± 3 (0.243 ± 0.007)	+6.3%
	10%	176 ± 1 (0.266 ± 0.021)	191 ± 1 (0.240 ± 0.022)	+8.7%

Z-avg: mean particle size (nm); Pdi: polydispersity index.

same initial batch were performed simultaneously, and compared to the cytotoxicity of dFdC.

As shown in Table 3, freeze-drying and rehydration did not alter the cytotoxic activity of the NPs, which legitimates the use of freeze-dried SQdFdC NPs for *in vitro* and *in vivo* preclinical studies.

This observation also suggests that no chemical degradation of SQdFdC has occurred over the course of the process, since the hydrolysis by-product dFdC displays a very different cytotoxicity profile. The eventuality of a chemical degradation of SQdFdC in the freeze-dried NPs could be definitively overruled by an analytical technique such as the liquid chromatography–tandem mass spectroscopy technique developed for the simultaneous determination of dFdC and SQdFdC (Khoury et al., 2007).

3.3. Freeze-drying of SQddC and SQddI nanoparticles

SQddC and SQddI are two squalenoylated nucleoside analogues structurally close to SQdFdC that form stable nanoparticles under similar conditions. In order to broaden the scope of the present investigation, we tested whether the freeze-drying process coined for SQdFdC could be applied to other squalenoylated NAs.

The size of the particles before and after freeze-drying reported in Table 4 clearly points out the differences between the two drugs. While successful freeze-drying of the SQddC NPs could only be achieved using the same experimental conditions as for SQdFdC (10% trehalose and around 25% size increase), 5% trehalose was enough to ensure successful freeze-drying of SQddI with only 6.3% size increase after rehydration.

Chemical differences exist between the 3 nucleosides analogues that may contribute to their different behaviours during the freeze-drying process. Whereas ddC and dFdC share the same pyrimidine heterocycle, ddI is a purine nucleoside analogue. Moreover, the squalene derivative is linked to ddI through an ester bond on the ribose 5′ end, while it is located on the heterocycle of the two other squalenoylated NAs (Couvreur et al., 2006). This could lead to differences between the cytidine and inosine analogues in terms of hydrophilicity of the prodrug and surface properties of the NPs. The latter could allow for enhanced stabilizing interactions with trehalose in the glassy state in the case of SQddI, which would in turn explain the efficient cryoprotection of SQddI NPs even at 5% trehalose concentration. Further studies of the interaction between trehalose and different squalenoylated compounds by differential scanning calorimetry could be a worthwhile tool in order to understand or predict their freeze-drying behaviour.

4. Conclusion

This study has shown that squalenoylated nucleoside analogue nanoparticles could be successfully freeze-dried, and that the squalenoyl gemcitabine nanoparticles could be stored in solid form for several months while retaining their *in vitro* cytotoxic activity as well as their physico-chemical properties. Once scaled up, this freeze-drying protocol will improve the reliability and facilitate the upcoming preclinical studies of this promising nanomedicine.

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