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NANOMEDICINE

Characterization of PEGylated nanoliposomes co-remotely loaded with topotecan and vincristine: relating structure and pharmacokinetics to therapeutic efficacy

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ABSTRACT

Recently, developing drug delivery systems exhibiting controlled drug release at the tumor sites emerged as an attractive option for enhancing anticancer therapeutic efficacy. It seems, however, unlikely that single agent therapies will prove effective enough against the myriad cells present within the malignancy. Therefore, next generation nanotherapeutics must not only find their way to the solid tumor but also must effectively destroy the diverse populations of cells promoting tumor growth. Nanoliposomes offer an important advantage in the delivery of a combination of drugs acting synergistically in cancer treatment. They allow controlling the pharmacokinetics and biodistribution of the drugs by uniform time and spatial co-delivery of the agents. However, successful translation of such complex formulations into the clinic relies on understanding critical physicochemical characteristics. These include: liposomes' membrane phase and dynamics, size distribution, state of encapsulated drug, internal environment of liposome, state of grafted polyethylene glycol at the liposome surface, and *in-vivo* drug release rate. They determine the pharmacokinetics of the formulation and the bioavailability of the drugs. We encapsulated the combination of vincristine (VCR) and topotecan (TPT) in the same nanoliposome (LipoViTo). Our *in-vitro* and *in-vivo* characterization of LipoViTo provides an explanation for the good therapeutic efficacy that was previously reported by us. Moreover, we have described how to study these critical features for a two-drug in one nanoliposome formulation. This characterization is an important step for a rational clinical development and for how to ensure liposome product quality of LipoViTo and other liposomal formulations alike.

1. Introduction

In its simplest model, a solid tumor may be viewed as an organ containing multiple cell types that act in concert to promote tumor growth [1,2]. Thus, drugs that target a single type of cell for therapeutic intervention may only provide a marginal anti-tumor effect. Furthermore, in many cases solid tumor cells exhibit a continuum of phenotypes during disease progression and/or in response to therapy. It is therefore unlikely that single-agent therapies, regardless of the ability of the nanoparticle delivery system to sequester them in the solid tumor, will be effective against the myriad cells in malignancy. To overcome this limitation, the next generation nanotherapeutics must find their way to the solid tumor while effectively destroying the diverse populations of cells.

Drug combination when compared with a single drug is expected to result in a better therapeutic efficacy as it helps to overcome inherent or acquired resistance to drugs [3,4]. Most anticancer drugs will probably never reach their full potential unless they are given together with other drugs against a second or even a third target. However, controlled combination therapy is difficult to achieve as the individual agents in a conventional anticancer drug combination (as free drugs) will distribute and be eliminated independently of each other. Nanoliposomes offer an important advantage in the delivery of agents acting synergistically in cancer treatment. They allow synchronizing and controlling the pharmacokinetics and biodistribution of the drugs [3,4], and uniform time and spatial co-delivery of two agents [5].

We used the combination of vincristine (VCR) and topotecan (TPT), which we co-encapsulated in the same nanosterically stabilized liposome (nSSL), in a formulation named LipoViTo [5]. It was shown *in-vivo* that LipoViTo is more efficacious than: nSSL with one drug or two nSSL with a different drug in each, or free drugs [7]. Many anticancer liposomal formulations showed superior therapeutic efficacy in preclinical studies [6–8]. Yet, since the clinical approval of liposomal doxorubicin in 1995 [3], only one new anticancer liposomal drug was approved by the FDA (liposomal cytarabine) [9].

As was learned from our Doxil® experience [3], a crucial part and a major challenge in developing a nanoparticle drug-delivery system is understanding its critical physicochemical properties and their relevance to the disease to be treated. This crosstalk will have a major

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impact on the *in-vivo* performance of the formulation. To achieve this goal one has to establish the analytical tools that can adequately characterize *in-vitro* and *in-vivo* properties. The critical characteristics include size distribution, liposomes' membrane phase and dynamics, state of encapsulated drug, internal environment of liposome, state of grafted polyethylene glycol (PEG) at the liposome surface, *in-vitro* loading and leakage kinetics, *in-vivo* drug release rate and drug biodistribution. All these determine the supramolecular structure of the liposomal drug formulation and ensure consistent drug delivery to cancer tissues [9]. Hence, corresponding analytical techniques were applied to determine these characteristics of the LipoViTo formulation, in order to understand better the critical *in-vitro* and *in-vivo* characteristics of this formulation. Since LipoViTo contains two anticancer drugs that are co-remotely loaded in the liposome by an ammonium sulfate gradient, it is significantly more complex than most of the liposomal formulations that were developed so far.

In this paper, we further investigated the reasons for the efficient and stable co-loading of VCR and TPT into liposomes in which the drug loading is based on a transmembrane ammonium sulfate gradient as a drug loading driving force. In particular, we used solution X-ray scattering to study the structure of the liposomes with and without the grafted lipopolymer and of the 2 drugs encapsulated in the interior aqueous phase. Thus, our characterization is an important step for supporting further clinical development and to ensure liposome product quality of LipoViTo and other liposomal formulations containing more than one drug.

2. Materials and methods

2.1. Drugs

Vincristine (VCR) sulfate was obtained from Avachem Scientific (San Antonio, TX); topotecan (TPT), from Sinova (Bethesda, MD); radiolabeled vincristine sulfate [^{3}H] from ARC (St. Louis, MO).

2.2. Lipids

Phospholipon® 100 H (HSPC, T_m 55 °C) was obtained from Phospholipid (Hermesberg, Germany). It has an iodine value of 1.0, ~85% stearic acid (C18:0), ~15% palmitic acid (C16:0), and <1% other acyl chains. Cholesterol was obtained from Sigma (St. Louis, MO); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG^{2k}) from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol hexadecyl ether (CHE) radiolabeled with [^{14}C] from ARC (St. Louis, MO).

2.3. Preparation of liposomes

Nanoliposomes composed of Phospholipon 100H, cholesterol, and PEG-DSPE^{2k} (54:41:5 mole ratio) were prepared as previously described [5]. LipoViTo and other liposomes used in this study were stored in sterile dextrose 5% with 10 mM histidine buffer, pH 6, at 4 °C.

2.4. Physicochemical characterization of liposomes

The structure of the nSSL with the drug precipitate was measured by solution small angle X-ray scattering (SAXS) and analyzed using both *X+*program [10] and cryo-TEM images of the liposomes [5]. SAXS measurements were performed in our in-house, state-of-the-art setup descried elsewhere [11], or using the SOLEIL synchrotron, SWING beam-line or Elettra, 5.2 L SAXS beam-line, as described [10–13]. High-resolution solution SAXS was used to determine the electron density profile of the bilayers, their head-to-head thickness ($2\varepsilon_{head}$, see Fig. 2B), the PEG layer thickness, and the characteristic repeat distances of the forming phases. Those parameters were obtained by fully analyzing both the form factor and the structure factor of the radially integrated scattering curves, using models of a stack of infinite flat slabs, having a Gaussian electron density profile along the normal z direction [13], implemented in the analysis software *X+*, developed in our laboratory [10].

Membrane "fluidity" of the liposomes was determined by fluorescence anisotropy of the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) [14,15]. The DPH was added to the liposomes formulation, followed by 30 min incubation in the dark at 37 °C to achieve complete insertion of the DPH into the hydrophobic region of the liposome bilayer [14–16]. The degree of DPH anisotropy ($r = \frac{I_{II}-I_{\perp}}{I_{II}+2I_{\perp}}$) in the labeled liposomes in PBS was calculated from the fluorescence intensity at the parallel (I_{II}) and perpendicular ($I_{\perp}$) planes, using the Synergy 4 fluorescent plate reader (BioTek, USA), at excitation/emission wavelengths of 360/430 nm.

2.5. Drug remote loading

Topotecan (TPT) and vincristine (VCR), both anticancer drugs, were mixed with the preformed nSSL dispersion exhibiting a transmembrane ammonium salt gradient. For the loading kinetics studies, the remote loading was achieved by incubation of the above liposomes for 10 min - 8 h at 37–65 °C. For the other studies, the remote loading was achieved by incubation of the liposomes for 30 min at 55 °C. Next, the liposomes were cooled to 4 °C, followed by dialyzing against 5% dextrose to remove ammonia and residual unloaded drug. Alternatively, in some cases, unloaded drug and ammonia (released during the loading process) were removed by the cation exchange resin Dowex 50WX-4 [17,18].

2.6. In-vitro release of drugs from nanoliposomes

For studying the effect of biological fluids on drug release, drug loaded nSSLs were incubated up to 96 h at 37 °C in adult bovine serum (Biological Industries, Beit Haemek, Israel). Aliquots were taken from the incubated liposomes at selected time points, and the released drugs were efficiently removed from the drug loaded nSSL by cation exchange resin Dowex 50WX-4 [17,18]. ^{14}C CHE Liposomes and ^{3}H vincristine concentrations were determined by liquid scintillation counting, while TPT concentrations were determined by HPLC equipped with a fluorescence detector.

2.7. Chemical characterization of LipoViTo and LipoViTo chemical stability

Chemical characterization of LipoViTo and its stability were studied by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) [19], due to space restriction, the TLC results are described below while the HPLC results are described in the supplementary data. For the TLC, we used a thin layer of silica gel 60 on aluminum plates (Merck, Darmstadt, Germany). The plates were loaded with the samples, and were developed in a tank containing the mobile phase of chloroform/methanol/water in a ratio of 85:15:1.5 (v/v/v). TPT was visible as is by its fluorescence. VCR, Cholesterol and Phospholipon 100H were visualized by spraying the TLC plate with a solution of 10% copper sulfate in 10% phosphoric acid, and then heating the plate to 180 °C for 6 min. Under these conditions, the detection limits of 1-stearoyl-2-hydroxy-3-phosphocholine and stearic acid are 10 and 4 μg, respectively.

2.8. In-vivo drug release and biodistribution

NUDE-Hsd:Athymic mice (Harlan Laboratories, Jerusalem, Israel) with medulloblastoma tumor (~8 mm diameter) were used. ^{14}C-CHE labeled nSSL (62.3 mCi/mol of PL) and ^{3}H-VCR (23.9 mCi/g of VCR)