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Liposomes loaded with histone deacetylase inhibitors for breast cancer therapy

Giorgia Urbinati[a,b], Véronique Marsaud[a,c], Vincent Plassat[a,c], Elias Fattal[a,b,c], Sylviane Lesieur[a,c], Jack-Michel Renoir[a,c,*]

[a] Centre National de la Recherche Scientifique, UMR 8612, Faculté de Pharmacie, 5 rue J.B. Clément, Châtenay-Malabry, F-92296, France
[b] Paris-SudUniversity, Orsay, F-91405, France
[c] IFR 141, Châtenay-Malabry, F-92296, France

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ABSTRACT

Histone deacetylase (HDAC) inhibitors (HDACi) of the class I trichostatin A (TSA), CG1521 (CG), and PXD101 (PXD) were incorporated at a high rate (~1 mM) in liposomes made of egg phosphatidylcholine/cholesterol/distearoylphosphoethanolamine-polyethylenglycol$_{2000}$ (64:30:6). Physicochemical parameters (size, zeta potential, loading, stability, release kinetics) of these HDACi-loaded pegylated liposomes were optimized and their cytotoxicity (MTT test) was measured in MCF-7, T47-D, MDA-MB-231 and SkBr3 breast cancer cell lines. In MCF-7 cells, TSA and PXD were efficient inducers of proteasome-mediated estradiol receptor α degradation and they both affected estradiol-induced transcription (TSA > PXD) contrary to CG. Moreover, TSA most efficiently altered breast cancer cell viability as compared to the free drug, CG-liposomes being the weakest, while unloaded liposomes had nearly no cytotoxicity. Pegylated liposomes loaded with TSA or PXD remained stable in size, charge and biological activity for one month when stored at 4 °C. All HDACi-loaded liposomes released slowly the encapsulated drug *in vitro*, CG-loaded liposomes showed the slowest release kinetic. These formulations could improve the efficacy of HDACi not only in breast cancers but also in other solid tumors because most of these drugs are poor water soluble and unstable *in vivo*, and their administration remains a challenge.

1. Introduction

Acetylation and deacetylation of histones have important roles in the modulation of chromatin topology and in regulating transcription in many tumor cell types. Transcriptionally active chromatin is generally associated with hyperacetylated histones, while silenced chromatin, in its condensed state, is linked to hypoacetylated histones (Nightingale et al., 2006; Wade et al., 1997). Histone deacetylase inhibitors (HDACi[1]) are a new class of antitumor agents which induces histone hyperacetylation and inhibits the proliferation of tumor cells by inducing cell cycle arrest, differentiation and/or apoptosis (Grunstein, 1997; Marks et al., 2000). Moreover, non-histone proteins, such as transcription factors like nuclear steroid hormone receptors, particularly the estrogen receptor alpha (ERα) (Vigushin et al., 2001) and the tumor suppressor p53 (Roy et al., 2005) are also targets for acetylation with varying functional effects. Several laboratories have shown that in response to HDAC inhibition, certain genes such as the *CDKN1A* gene (Huang et al., 2000), which encodes the p21$^{WAF1/CIP1}$ cyclin dependent kinase inhibitor are activated but others such as the *CCDN1* gene, which encodes cyclin D1, are repressed (Richon et al., 2000; Sandor et al., 2000). In addition, HDACi cause hyperacetylation of Hsp90, Raf, Akt, ErbB2 and Bcr-Abl leading to important antitumor effects (Kim et al., 2006). Thus, the transcriptional and non-transcriptional effects of HDACi render this class of molecules attractive for clinical developments in cancer therapy as drugs which target multiple pathways (Bolden et al., 2006; Liu et al., 2006; Smith and Workman, 2009).

Several HDACi are currently in early phase of clinical development as potential treatments for solid and hematological cancers, either as monotherapy or in association with other anticancer agents (Bolden et al., 2006). However, since most of them are insoluble and/or unstable *in vivo* (like trichostatin A – TSA (Elaut et al., 2002; Vanhaecke et al., 2004)) we decided to incorporate them in pegylated liposomes to allow their i.v. administration. Pegylated liposomes (Stealth® property) are second generation devices that, contrary to non-pegylated liposomes, are less rapidly captured by the reticulo-endothelium system (RES) presenting an enhanced pharmacokinetic profile. They take advantage from the enhanced permeability and retention effect (EPR) for increasing their tumor

Abbreviations: HDAC, histone deacetylase; HDACi, HDAC inhibitor; TSA, trichostatin A; ER, estrogen receptor; DSPE-PEG$_{2000}$, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt); ePC, egg phosphatidylcholine; Chol, cholesterol; MTT, 3-[4,5-dimethylthiazol-2-yl]diphenyltetrazolium bromide.

* Corresponding author at: Centre National de la Recherche Scientifique, UMR 8612, Faculté de Pharmacie, 5 rue J.B. Clément, Châtenay-Malabry, F-92296, France. Tel.: +33 1 46 83 58 31, fax: +33 1 46 83 58 32.
E-mail address: michel.renoir@u-psud.fr (J.-M. Renoir).

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accumulation when they are intravenously injected (Drummond et al., 1999). Such a type of nanocarrier is supposed to improve the pharmacokinetic and pharmacodistribution of the encapsulated drug (Moghimi and Szebeni, 2003).

In the present study, we compared the capacities of pegylated liposomes to load three class I and II HDAC inhibitors (CG1521, PXD101 and TSA), characterized their physicochemical and biological properties and evaluated their toxicity towards several ER-positive and ER-negative human breast cancer cell lines. These formulations revealed to be stable for one month, both in terms of their physicochemical characteristics (size and zeta potential) and of their biological activity *in vitro* (capacity to induce histone H4 acetylation). In addition, they revealed slow drug release kinetics which is fundamental to allow the nanosystem to accumulate in tumor tissues once intravenously injected. These preliminary results encourage further *in vivo* evaluation in appropriate xenograft models.

2. Material and methods

2.1. Chemicals

TSA was obtained from Alomone Labs (Jerusalem, Israel); CG1521 (CG) and PXD101 (belinostat) were obtained from Errant laboratories and Topo Target CuraGen Corp., respectively. They were solubilized in ethanol at 1–3 mM final concentration and stored at −20 °C. EPC and DSPE-PEG_{2000} were purchased from Avanti Polar Lipids (Alabaster, AL, USA); FITC-dextran, MTT tetrazolium salt and Chol were obtained from Sigma Aldrich (St. Quentin-Fallavier, France). Spectra/Por dialysis tubing was obtained from Spectrum Laboratories (Rancho Dominguez, CA, USA). All other chemicals were reagent grade, purchased from standard suppliers.

2.2. Liposome fabrication

Liposome suspensions (50 mM lipids) were prepared by a procedure previously described (Maillard et al., 2005). Lipid film hydration was performed at the following molar ratio: ePC/Chol/DSPE-PEG_{2000} (64:30:6). Incorporation of HDACi in liposomes was obtained by mixing the drugs (from 0.5 to 1.5 mM initial concentration) with chloroformic lipid solution. The lipid film was formed by removing the organic solvent under reduced pressure before being hydrated in Hepes buffer (Hepes 10 mM, NaCl 145 mM, pH 7.4). The resulting multilayer vesicles were then extruded (extruder Whitley, Lipex, Vancouver, Canada) sequentially through 0.2 and 0.1 μm polycarbonate membranes (Millipore, USA). Unincorporated HDACi were eliminated by ultracentrifugation (300,000 × *g* × 14 h at 4 °C; L8-70, Rotor 50.4 Ti, Beckman, USA). For FITC-dextran containing liposomes, the lipid film was hydrated with Hepes buffer containing FITC-dextran (10 mg/mL).

2.3. Physicochemical characterization of liposomes

Liposome size and zeta potential were determined with a Zetasizer 4 Malvern (Malvern Inst., UK) from three independent samples. The concentration of encapsulated HDACi in liposome was determined by UV-spectrophotometry at λ_{max} of 336 nm, 342 nm or 268 nm for CG1521, TSA and PXD101, respectively, after ethanol solubilization of purified drug-loaded liposomes. The drug loading efficiency (DLE) was calculated as follows: DLE1% = amount of drug in purified liposomes/amount of drug initially added × 100 or DLE2% = amount of drug in purified liposomes/amount of drug measured before purification of liposomes × 100.

2.4. Cell culture treatments and transcription measurement

MCF-7, T47-D A 1-2, SKBr-3 and MDA-MB-231 cell lines (ATCC, Molsheim, France) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Vervier, Belgium) supplemented with penicillin (5 IU/mL), streptomycin (50 IU/mL), 10% fetal calf serum (FCS). MELN cells are MCF-7 cells stably expressing the ERE-βglobin-luciferase construct (Balaguer et al., 1999). Prior to treatment, the medium was replaced with phenol red-free DMEM medium containing 10% stripped FCS (charcoal Norit A 1%, dextran T70 0.1%, 30 min at room temperature) for at least 48 h. For transcription measurements, cells were seeded on 3 cm diameter plates 32 h before treatments in phenol red-free DMEM supplemented with 10% dextran-coated charcoal stripped FCS. Cells were stimulated with 0.1 nM E_2 in the presence or not of various concentrations of either CG, or PXD (0.5–5 μM) or TSA (0.05 to 1 μM) for 16 h, then harvested and lysed in 250 μL of luciferase buffer (25 mM Tris, H_3PO_4 pH 7.8, 10 mM $MgCl_2$, 10% Triton X-100, 15% glycerol, 1 mM EDTA, 1 mM DTT) for 30 min at 4 °C. Luciferase buffer (50 μL), supplemented with 100 mM ATP and 87 μg luciferine/mL, was added to 50 μL of cell extract and luciferase activity was quantified in a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, DE). The protein concentration of each sample was determined by Biorad assay and luciferase activity was normalised with respect to protein concentration.

For measurement of HDACi activity, MCF-7 and MDA-MB-231 cells at 50–60% confluence in 3 cm diameter plates were exposed or not during 20 h to various concentrations of inhibitors (0.05–10 μM). Cells were then lysed in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100) with protease inhibitors (Complete Reagent, Roche Diagnostics, Indianapolis, IN, USA) for 20 min and then boiled for 10 min in Laemmli sample buffer prior Western blotting (see below).

2.5. In vitro drug release

In a first experiment, drug-encapsulated liposomes (750 μL) were placed into a dialysis tubing (MW cut-off = 10,000 Da), then dialysed against 250-fold excess of Hepes buffer (10 mM Hepes, 0.15 M NaCl, pH 7.4) supplemented with 30% FCS for 24 h at 37 °C. At various times, 50 μL aliquots were removed for drug quantification by spectrophotometry. Physicochemical characteristics of liposomes were further evaluated by quasi-elastic light scattering.

The drug release rates were also determined in MELN cells ($0.5–2 \times 10^6$ cells at 50–60% confluence). Liposomes were placed in inserts (NUNC Anapore, membrane 0.02 μm, Nalge Nunc Inc., USA) with or without 0.1 nM E_2 and the extent of increase of luciferase (LUC) activity was compared to that of equivalent concentrations of free HDACi. Only free HDACi and E_2 could pass through the insert membrane. At different times, inserts were removed from the dishes and cells were maintained at 37 °C for 24 h. LUC activity was quantified as described previously (Maillard et al., 2006).

2.6. In vitro liposome uptake

MCF-7 cells were plated onto a sterile cover-slip paced in 6-well plates and let to adhere for 24 h at 37 °C. Each test was carried out with cells at 50% confluence. FITC-dextran loaded liposomes were added (20 μL) to 3 mL DMEM in each test well for incubation (12 h at 37 °C or 4 °C). For control, cells were treated with free FITC-dextran and incubated similarly. After three washes with cold PBS (10 min), fresh PBS was added and the cover slips were placed on a microscope slide. The slides were then examined using a Nikon eclipse TE2000-S fluorescent microscope (Nikon, Champigny/Marne, France) at ×200 magnitude coupled to a Nikon