Evaluation of synthetic/reconstituted high-density lipoproteins as delivery vehicles for paclitaxel

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Reconstituted (synthetic) high-density lipoprotein particles carrying paclitaxel (rHDL/PTX) were prepared with substantially higher PTX content than reported earlier. The rHDL/PTX complexes seemed to be primarily spherical nanoparticles when examined via electron microscopy, with a constant composition, molecular weight and exceptional stability even after ultracentrifugation and storage for up to 6 months. The rHDL/PTX nanoparticles had superior cytotoxicity against several cancer cell lines (MCF7, DU145, OV1063 and OVCAR-3), the half maximal inhibitory concentration (IC_{50}) having been found to be 5–20 times lower than that of the free drug. Studies with mice showed that the rHDL/PTX nanoparticles were substantially better tolerated than the corresponding dosages of either Taxol or Abraxane. ***Anti-Cancer Drugs*** **19:183–188**

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Introduction

A number of challenging factors, including poor water solubility, toxic side effects and drug resistance, have long interfered with the efficacy of cancer chemotherapeutic agents. Lipoproteins have been considered appropriate drug-delivery vehicles capable of overcoming these problems [1], owing to their structural features [2], biocompatibility and targeting capability via receptor-mediated mechanisms [3]. Our laboratory focused on the preparation and evaluation of reconstituted high-density lipoprotein (rHDL)/drug complexes, primarily for the purpose of enhancing the delivery of anticancer agents [4–6]. Our more recent efforts, reported here, resulted in substantially more enhanced paclitaxel (PTX)-carrying capabilities, physical/chemical properties and in-vivo tolerability than our previously reported formulation [4].

Materials and methods

Materials

Sodium cholate, egg yolk phosphatidyl choline (PC), free cholesterol, cholesteryl oleate, potassium bromide (KBr) and PTX were purchased from Sigma-Aldrich chemicals (St Louis, Minnesota, USA). [^{14}C]PTX was purchased from Sigma. Abraxane was a gift from Dr Ray Page, Department of Pharmacology, University of North Texas Health Science Center, Texas, USA (UNTHSC). For the initial phase of these studies, the apolipoprotein A-I (apo A-I) was provided by ZLB Bioplasma (Berne, Switzerland). For the latter portion of the studies, the apo A-I was isolated from human plasma as described by Brewer *et al.* [7].

Methods

Preparation of reconstituted (synthetic) high-density lipoprotein/paclitaxel complex with sodium cholate dialysis

The cholate dialysis procedure used was based on the procedures described earlier for discoidal rHDL particles [8,9]. After testing a variety of formulations of ingredients, a mixture of PC in $CHCl_3$ with free cholesterol, cholesteryl oleate and PTX was prepared with a molar ratio of apo A-I:cholesterol:cholesteryl oleate: PC = 1 : 5 : 1.3 : 115. This formulation was based on preliminary studies, after varying the levels of the respective components to optimize the incorporation of PTX into rHDL. The lipid mixture (PC, C and cholesteryl oleate) and 2 mg of PTX were dried under N_2 to a thin film and dispersed in 60 µl of dimethylsulfoxide [(DMSO) Sigma-Aldrich] and 1.4 ml of buffer (10 mmol/l Tris, 0.1 mol/l KCl and 1 mmol/l EDTA; pH 8.0; Sigma-Aldrich). About 140 µl of sodium cholate [100 mg/ml stock in 0.15 mol/l NaCl, 0.003 mol/l KCl and 0.15 mol/l KH_2PO_4, pH 7.4, designated as phosphate-buffered (0.008 mol/l Na_2HPO_4, 0.002 mol/l KH_2PO_4) saline (PBS; 0.14 mol/l NaCL; 0.01 mol/l KCl); pH 7.4] was added, to produce mixtures with a final PC to the cholate molar ratio of ~1 : 1.6. Apo A-I (12.7 mg/ml) in 0.4 ml of PBS was added to this mixture, and the final volume was adjusted to 2 ml with PBS. The lipid/protein/

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cholate mixture was then incubated for 12 h at 4°C, followed by dialysis against 2 l of PBS for 2 days, with three buffer changes. Using [^{3}H]cholate as a tracer, we determined that after 48 h of dialysis, < 2% of the cholate remained in the rHDL/drug preparations. The PTX-containing rHDL fractions were isolated by single-step density gradient ultracentrifugation [10,11], and were dialyzed overnight against PBS, pH 7.4. These rHDL preparations can be stored at 4°C for at least 60 days.

Preparative ultracentrifugation

KBr was added to 3 ml of rHDL/drug preparation, to achieve a density of 1.22 g/ml. The sample was placed in a centrifuge tube, and KBr solutions of decreasing density were layered on top of the sample as follows: 1 ml, 1.22 g/ml; 4 ml, 1.063 g/ml and 3 ml, 1.019 g/ml [10,11]. The layered sample was subsequently centrifuged for 24 h at 39 000 rpm in a Beckman Optima LE 80K (Beckman Coulter Inc., Fullerton California, USA) ultracentrifuge at 4°C in a swinging bucket rotor (SW 40). Fractions (1 ml) were collected from the top and analyzed for protein, phospholipids and cholesterol. Fractions were designated as follows and analyzed as described earlier [12]: those corresponding to very low-density lipoprotein [fraction 1–2, buoyant density (d) < 1.007 g/ml]; to intermediate-density lipoprotein (fraction 3, d = 1.007–1.02 g/ml); to low-density lipoprotein (fractions 4–6, d = 1.020–1.060 g/ml); to HDL_2 (fractions 7–8, d = 1.060–1.15 g/ml); to HDL_3 (fractions 9–10, d = 1.15–1.235 g/ml) and to lipoprotein-deficient serum (fractions 11–12, d > 1.235 g/ml).

Electron microscopy

After dialysis against a buffer containing 0.125 mol/l ammonium acetate, 2.6 mmol/l ammonium carbonate and 0.26 mmol/l EDTA (pH 7.4), the isolated rHDL samples were negatively stained with 2% sodium phosphotungstate (pH 7.2) and placed on Formvar/carbon-coated 200-mesh nickel grid support films (Ted Pella Inc., Reading, California, USA), as previously described by Forte *et al.* [13]. The particles were visualized (magnification of 50 000) using a Zeiss 910 (Carl Zeiss SMT Inc., Peabody, Massachusetts, USA) transmission electron microscope. The photographs obtained were enhanced, and the particle diameter was determined with Adobe Imageready CS2 (Adobe Systems Inc., San Jose, California, USA) software. For the estimation of the diameter of the rHDL/PTX nanoparticles, 50 images with distinct margins and symmetrical shapes were measured.

Cell culture

Established cancer cell lines were obtained from the American Type Culture Collection (Manassas Virginia, USA) and grown according to the procedures provided by the American Type Culture Collection, employing the respective recommended media, including the mixture of 1% penicillin and streptomycin and 10% fetal bovine serum (Gibco/Life Technologies, Invitrogen Corp, Carlsbad, California, USA). All the cells were grown in 75-cm^2 flasks and split, using 0.25% trypsin to release the cells from the medium, once 80–90% confluency was reached. Cells were counted and plated onto 96-well microtiter plates, 1 day before being used for assays.

Assessment of the cytotoxicity of the reconstituted (synthetic) high-density lipoprotein/paclitaxel complexes

The cytotoxic effect of the rHDL/PTX preparations on cancer cells was assessed by the MTT assay [14]. Cells were plated in 96-well plates (5000 cells/well) in their respective media. Next day, the monolayers were washed with PBS (pH 7.4) twice, and then incubated at 37°C for 24 h with rHDL/PTX complexes in serum-free media. The following day, 25 µl of MTT (1 mg/ml) was added to each well and incubated for 3 h at 37°C. Plates were centrifuged at 1200 r.p.m. for 5 min. The medium was removed, the precipitates were dissolved in 200 µl of DMSO and the samples were read at 540 nm in a microtiter plate reader.

Determination of reconstituted (synthetic) high-density lipoprotein components

Total cholesterol (Thermo DMA, Arlington, Texas, USA), free (unesterified) cholesterol and phospholipids (Wako Pure Chemical Industries Ltd, Osaka, Japan) were determined by the respective enzymatic reagent kits, using microtiter plate assays [15,16]. The concentration of cholesteryl esters was obtained as the difference between total and free cholesterol values. Protein determinations were carried out using Bradford reagent kits supplied by Bio-Rad Laboratories (Hercules, California, USA).

Molecular weight determination

The molecular weight of the rHDL/PTX complex was determined by fast protein liquid chromatography on a 10 × 300 mm Superose 6 (Amersham Biosciences, Piscataway, New Jersey, USA) column using apoferritin, alcohol dehydrogenase and serum albumin as standards. The molecular weight of the rHDL/Ptx complex was calculated on the basis of the principles described by Ackers [17].

Determination of the maximum tolerated dose

Female C57BL6 mice (6–8 weeks, 18–21 g, Harlan) were housed, no more than four/cage, in accordance with the Institutional Animal Care and Use Committee guidelines. The mice were given standard mouse chow and water *ad libitum*, and the bedding was changed once a week. The mice were held for 1–2 days before the injection schedule began. Subsequently, the mice were randomized and grouped the day before injection. The injection volume in all the dosage groups was 1.5 ml via the intraperitoneal

Table 1 Composition of rHDL/PTX particles (weight %) prepared by the described cholate dialysis method

	Protein	PC	FC	CE	PTX
Distribution (%)	33.5 + 1.1	58.8 + 1.1	1.3 + 0.3	0.34 + 0.15	9.9 + 0.7

The data represent the mean ± SEM of three determinations.
CE, cholesteryl oleate; FC, free (unesterified) cholesterol; PC, phosphatidyl choline; PTX, paclitaxel; rHDL, reconstituted high-density lipoproteins.

route, and injections were administered on days 1, 2 and 3. The dosage for Taxol injection was 30 mg/kg ($n = 6$), for Abraxane, 70 mg/kg ($n = 6$) and for rHDL/PTX, 100 mg/kg ($n = 6$). A control group was injected with the rHDL vehicle ($n = 4$). The weights and the health of the mice were monitored for 30 days. Weighings were performed once a day for the first 7 days and twice a week for the remaining monitoring period. These studies were approved by the Animal Use Committee of the UNTHSC.

Statistical methods

Statistical analysis was carried out to evaluate the data, when applicable. Accordingly, the data in Table 2 and Fig. 5 represent mean ± standard error of the mean (SEM).

Results

Formulation of reconstituted (synthetic) high-density lipoprotein/drug

A series of formulation studies were conducted to establish the optimal composition of the rHDL particles, to accommodate the maximum amount of PTX/particle. These studies were based on the protein (apoA-I) concentration of the rHDL complex, the largest component of these particles (Table 1). The preliminary data established the most favorable mixture of egg yolk phosphatidyl choline, cholesterol and cholesteryl oleate of the rHDL/PTX particles, which has been detailed under Methods (preparation of the rHDL/PTX complex).

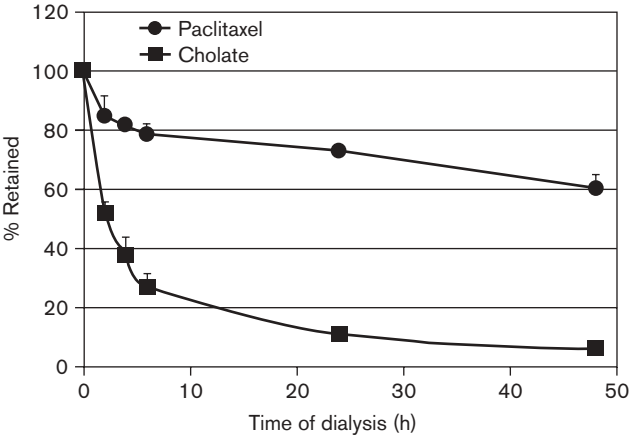
Figure 1 shows the pattern of incorporation of PTX into the rHDL particles.

The loading of PTX into the rHDL particles seemed to be maximal when about 2 mg of PTX was added to the reaction mix that contained the optimal amounts of the other ingredients. At this point, the incorporation efficiency of the drug was about 50%, with the maximal incorporation of 1 mg of PTX corresponding to a 2-mg initial load.

Characterization of the reconstituted (synthetic) high-density lipoprotein/paclitaxel complexes

The PTX incorporation represented about 10% of the total mass of the rHDL complex (Table 1). Based on the estimated molecular weight of rHDL (~170 000), approximately 25 PTX molecules were incorporated into the rHDL/PTX complexes/particle. The overall composition of these rHDL/PTX complexes showed some resemblance to that of native human HDL, although their PC content was substantially higher and their cholesterol content substantially lower than that of their circulating counterpart. During preliminary studies, the optimal PTX incorporation was observed with relatively low cholesterol-containing rHDL particles.

Fig. 1

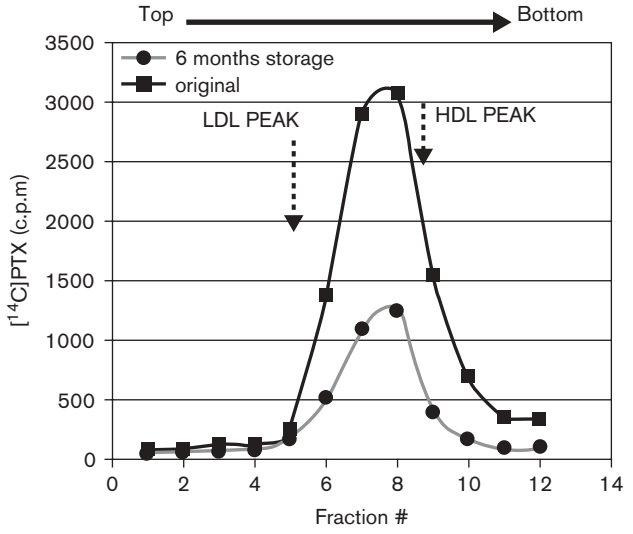


Retention of paclitaxel (PTX) vs. sodium cholate by the reconstituted high-density lipoprotein (rHDL)/PTX complex during the dialysis process.

The retention of PTX within the rHDL complex vs. the retention of sodium cholate is shown in Fig. 1. The data show an approximately 60% retention of the PTX vs. a less than 5% retention of the sodium cholate, on the basis of the starting concentrations before dialysis. The rHDL/PTX particles were further characterized by ultracentrifugation and electron microscopy. In Fig. 2, the ultracentrifuge pattern of the rHDL/PTX preparation shows that the synthetic HDL/drug complex had flotation properties very similar to those of native HDL, as indicated by the arrows for the peak distribution of native human HDL and low-density lipoprotein. These flotation properties were maintained after 6 months' storage at 4°C. The lower peak, representing the stored sample, is due to the application of correspondingly lower starting material for the ultracentrifuge run.

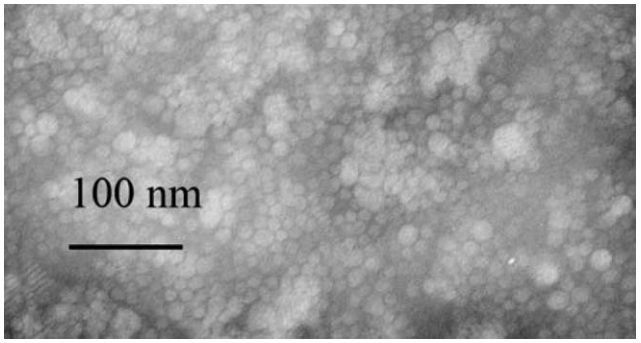
Figure 3 shows the electron microscopy of the rHDL/PTX particles on phosphotungstate negative staining. These data show that, although some of the rHDL/PTX

Fig. 2



Distribution of the radioactivity of [^{14}C]paclitaxel (PTX), incorporated into reconstituted high-density lipoprotein (rHDL), along with unlabeled PTX (see Methods) upon density gradient ultracentrifugation. The peak representing the rHDL/PTX preparation after 6 months' storage is smaller because approximately half of the original amount was used for the post-6-month centrifugation. LDL, low-density lipoprotein.

Fig. 3



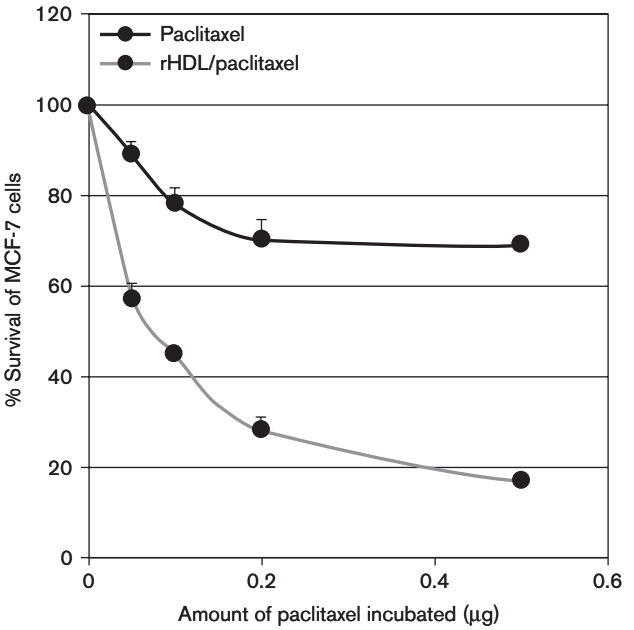
Electron micrograph of the negatively stained reconstituted high-density lipoprotein/paclitaxel nanoparticles.

represent discoidal particles, the majority of the structures are spherical, with a mean diameter of 11.4 ± 3.1 nm (range 7.4–20.7 nm).

Assessment of the cytotoxicity of the reconstituted (synthetic) high-density lipoprotein/paclitaxel complexes

The MTT assay, as described under Methods, was used in these studies. First the MCF7 breast cancer cell line was used to evaluate the cancer cell-killing potential of the rHDL/PTX nanoparticles (Fig. 4). This was followed by a more detailed assessment using several cancer cell lines (Table 2). The progressively cytotoxic effect of the

Fig. 4



Cytotoxicity of the reconstituted high-density lipoprotein (rHDL)/paclitaxel (PTX) nanoparticles vs. free PTX estimated by the MTT assay (see Methods).

Table 2 Comparison of IC_{50} values for selected cancer cell lines with rHDL/PTX and free PTX

Cell lines used	IC_{50} for rHDL/PTX (μmol/l)	IC_{50} for PTX (μmol/l)
OV1063	1.2	14.2
OVCAR 3	14.1	70.3
DU145	1.8	14.2
MCF7	0.6	14.2

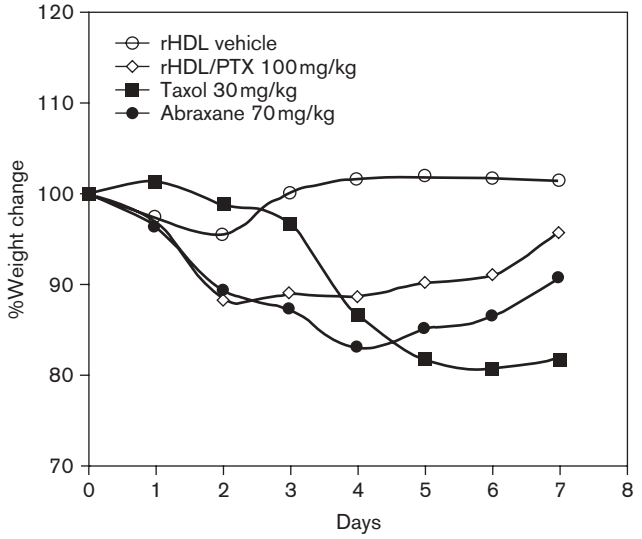
The cells were exposed to increasing concentrations of the respective drug preparations for 24 h before the performance of MTT cell viability assay.
PTX, paclitaxel; rHDL, reconstituted high-density lipoproteins.

rHDL/PTX nanoparticles with increasing drug concentrations is shown in Fig. 4. Clearly, the rHDL-delivered PTX was much more effective than the free PTX in this regard. A broader view of several cancer cell lines in Table 2 shows that the IC_{50} values for the rHDL-encapsulated PTX were 5–20-fold lower than those for the free PTX.

Animal studies

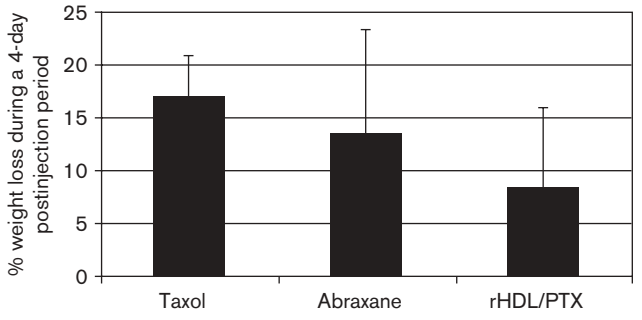
The purpose of these studies was to compare the respective tolerance levels of mice toward rHDL/PTX, Abraxane and Taxol. To design an effective protocol, we have chosen to use the concentrations of PTX at the established respective MTDs (30 mg/kg for Taxol and 70 mg/kg for Abraxane). The dosage for rHDL/PTX was chosen to exceed those of both the other formulations because we anticipated better tolerance of this formulation by the mice. The results of these experiments (in Fig. 5) show the time course of the mean weight change for the three groups of PTX-treated mice and the

Fig. 5



Weight loss incurred by female C57Bl/6 mice after three consecutive injections of paclitaxel (PTX) as Taxol, Abraxane and the reconstituted high-density lipoprotein (rHDL)/PTX formulation, respectively. Results are presented as the mean weight loss for each group treated with PTX (Taxol, Abraxane and the rHDL/PTX formulation, respectively) and the controls (rHDL only).

Fig. 6



Weight loss data (mean ± SD) combined for days 4–7 for each paclitaxel (PTX)-treated group [Taxol, Abraxane and reconstituted high-density lipoprotein (rHDL)/PTX].

controls. On the basis of these data, the rHDL/PTX formulation proved to be markedly superior to both Abraxane and Taxol, as the 15% weight loss that occurred had been reached or exceeded at the 30-mg/kg dose for Taxol and at the 70-mg/kg dose for Abraxane. In contrast, the rHDL/PTX formulation did not reach the 15% weight loss even at the 100-mg/kg dose level of PTX. These data (in Fig. 5) also show that the initial weight loss (during the first 3-day period) might have been partially due to the trauma associated with the injections. Therefore, the weight changes for each treated group were combined for days 4–7 and presented as mean ± SD (Fig. 6). The weight loss in the rHDL/PTX group was significantly less than in the Taxol group ($P < 0.0005$), whereas it was substantially, but not significantly, lower than in the Abraxane group ($P = 0.084$).

Discussion

Poor solubility, toxic side effects and limited antitumor potential have consistently hampered the development of new chemotherapeutic agents. This study involves a practical approach to employ rHDL as drug-delivery agents for cancer chemotherapy. Lipoprotein-based formulations have long been thought of as attractive candidates for delivering anticancer drugs to tumors [18,19], primarily because of their biodegradable components and the potential for receptor-mediated uptake of the transported drug by endocytosis [20] or by the selective uptake of core components [21]. The overexpression of lipoprotein receptors by malignant cells and tumors has been widely reported [22–24], including the selective delivery of the toxic anticancer agents to tumors [25], further enhancing the utility of lipoprotein-based delivery platforms in cancer chemotherapy. In addition, recent studies show that lipoproteins can be targeted to specific tumors by attaching homing ligands to their surface components [26]. For these and other reasons, we have focused our efforts on developing a synthetic HDL-type nanoparticle that is particularly suitable for the delivery of anticancer agents to tumors [4–6]. This report includes the description of a reconstituted rHDL/drug complex with superior drug-carrying capacity, compared with our earlier model [4]. In addition, we report, for the first time, the generation of spherical particles containing ingredients of human HDL via the cholate dialysis method (without the use of physical force). The spherical configuration of the rHDL/drug nanoparticles is an essential feature enabling enhanced drug loading and stability, an essential requirement for a practical formulation to be used for cancer chemotherapy.

Several attempts have been reported, over the last several decades, to use lipoprotein-type structures as drug-delivery vehicles [27–29]. No reports are, however, available of these formulations being employed in a clinical setting or even undergoing phase I trials. The main obstacle in developing successful lipoprotein-based formulations has likely been the availability of the respective apolipoprotein components (apo B-100 and apo A-I) needed for these lipoprotein/drug formulations in commercially suitable quantities. This situation has, however, changed recently, with the development of processes for producing recombinant apo A-I via bacterial [27] or plant vehicles (*http://www.sembiosys.com/Main.aspx?id=15*), improving the prospects for preparing synthetic HDL-type drug-delivery vehicles. Additional attempts have been reported to overcome this problem by using small peptides as surrogates for apolipoprotein B-100 [28–30].

The data presented in this study open the way for further indepth investigations, to explore the antitumor potential of rHDL and other lipoprotein-based formulations, and to take advantage of the receptor/tumor-targeting potential of these nanoparticles [26].

Acknowledgements

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