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Biochimica et Biophysica Acta 1515 (2001) 167–176

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Targeting Stealth liposomes in a murine model of human small cell lung cancer

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Received 21 June 2001; received in revised form 16 August 2001; accepted 30 August 2001

Abstract

Tumor accumulation and therapeutic activity of Stealth liposomes loaded with doxorubicin (DXR) were examined in Balb/c nude mice xenografts inoculated subcutaneously with the human small cell lung cancer (SCLC) cell line, H69. Mice were treated with non-targeted liposomes (SL) or liposomes targeted with antagonist G coupled to the liposome surface (SLG). SLG showed 30–44-fold higher binding to H69 cells harvested from H69 xenografts than SL. At 48 and 72 h post injection, tumor accumulation of [^{125}I]tyraminylinulin-containing liposomes was shown to be dependent on liposome size but independent of the presence of the targeting ligand. Maximum tumor uptake of either SLG or SL ranged from 2 to 4% of injected dose/g of tissue. In therapeutic studies, mice received three weekly injections of 3 or 6 mg free DXR/kg or 3 or 10 mg liposomal DXR/kg at initial tumor volumes of either 7 or 33 mm^3. The therapeutic efficacy of DXR-containing SL or SLG was significantly improved over free DXR, but SLG did not improve anti-tumor efficacy relative to SL. Stealth liposomes containing DXR have potential as a therapy against human SCLC tumors.

Keywords: Pegylated liposome; Doxorubicin; Targeting; Antagonist G; Small cell lung cancer

1. Introduction

Lung cancer is still the leading cause of cancer-related deaths in the developed world [1]. Small cell lung cancer (SCLC) accounts for 25% of all lung neoplasms and is characterized by early and widespread metastasis with a 5 year survival rate of less than 5% [2]. The aggressiveness of this disease is related to its anchorage-independent proliferation [3], and to the autocrine and paracrine growth loops that govern SCLC [4,5]. SCLC cells secrete multiple neuropeptides that bind specific receptors on the surface of those same SCLC cells, triggering a cascade of intracellular signals that culminates in DNA synthesis and cell proliferation [5]. The understanding of these mechanisms has created new opportunities for

Abbreviations: SCLC, small cell lung cancer; SL, sterically stabilized liposomes; SLG, antagonist G-coupled sterically stabilized liposomes; mAb, monoclonal antibody; DXR, doxorubicin; HSPC, fully hydrogenated soy phosphatidylcholine; PEG-DSPE, methoxypoly(ethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine; CHOL, cholesterol; PDP-PEG-DSPE, *N*-(3′-(pyridyldithio)propionoyl)aminopoly(ethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine; MAL-PEG-DSPE, maleimide-derivatized poly(ethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); EMCS, ε-maleimidocaproic acid *N*-hydroxysuccinimide ester; MES, 2-(*N*-morpholino)ethanesulfonic acid; PL, phospholipid; [^{3}H]CHE, [1α,2α(n)-^{3}H]cholesteryl hexadecyl ether; TI, tyraminylinulin

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0005-2736/01/$ – see front matter
PII: S0005-2736(01)00411-4

therapeutic intervention, leading to the development of broad spectrum neuropeptide antagonists, notably the hexapeptide analogue of the neurotransmitter substance P, [D-Arg^{6},D-Trp^{7,9},N^{me}Phe^{8}]-substance P (6–11), antagonist G. Antagonist G competitively blocks the mitogenic action of neuropeptides like vasopressin, gastrin-releasing peptide and bradykinin at the receptor level [6,7], and has been shown to inhibit SCLC proliferation in vitro and in vivo [5,6,8]. As a result, antagonist G is entering phase II clinical trials for the treatment of SCLC [9]. Although broad spectrum neuropeptide antagonists might be useful antiproliferative agents against SCLC, at present chemotherapy, along with radiotherapy, is still the major treatment modality [10].

Stealth liposomes (SL), sterically stabilized with methoxypoly(ethylene glycol)distearoylphosphatidylethanolamine conjugates (PEG-DSPE), have long circulation half-lives following intravenous injection, resulting in increased tumor accumulation of associated drugs and improved therapeutic efficacy relative to standard therapy [11–13]. Although these features led to the clinical approval of doxorubicin-containing Stealth liposomes for the treatment of a number of solid tumors including Kaposi's sarcoma [14] and ovarian cancer [15], its efficacy against SCLC has not yet been evaluated. The therapeutic efficacy of Stealth liposomal drugs has been shown to be further improved in some model systems when ligands like monoclonal antibodies (mAb) that selectively bind to internalizing receptors on cancer cells are attached to the distal ends of PEG chains grafted to the liposomal surface [16,17]. This strategy has resulted in therapeutic improvements over non-targeted formulations when treatments were started at the early stages of tumor development when cells were easily accessible in the lung [18,19], or when cells were within the vasculature [20] or in the peritoneal cavity [20]. In the case of more advanced solid tumors, some mAb-targeted liposomal drug formulations have failed to show any therapeutic advantage over non-targeted liposomes [19,21], while other antibody-targeted formulations have had an advantage [22].

We have recently shown that antagonist G could be covalently coupled to the end of PEG-grafted Stealth liposomes (SLG). This resulted in improved cytotoxicity of doxorubicin (DXR)-containing SLG (DXR-SLG) against the SCLC H69 cell line relative to non-targeted liposomes [23,24]. In this work, we studied the biodistribution of sterically stabilized liposomes, with or without antagonist G, in Balb/c nude mice bearing subcutaneous xenografts of the SCLC cell line, H69. The therapeutic activity of DXR-containing SL, compared to SLG or free drug, was evaluated in the treatment of SCLC primary tumors.

2. Materials and methods

2.1. Materials

Antagonist G was synthesized by Alberta Peptide Institute (Edmonton, AB, Canada). Fully hydrogenated soy phosphatidylcholine (HSPC), methoxypoly(ethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine (PEG-DSPE) and *N*-(3′-(pyridyldithio)propionoyl)aminopoly(ethylene glycol) (M_r 2000) distearoylphosphatidylethanolamine (PDP-PEG-DSPE) were generous gifts of Alza (Mountain View, CA, USA). Maleimide-derivatized PEG_{2000}-DSPE (MAL-PEG-DSPE) was synthesized by Shearwater Polymers (Huntsville, AL, USA). Cholesterol (CHOL) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). ε-Maleimidocaproic acid *N*-hydroxysuccinimide ester (EMCS), 2-iminothiolane and Sigmacote were purchased from Sigma (St. Louis, MO, USA). Sephadex G-50 and Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden). RPMI 1640, penicillin-streptomycin and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). DXR was obtained from Faulding (Vaudreuil, PQ, Canada). [1α,2α(n)-^{3}H]Cholesterylhexadecyl ether, 1.48–2.22 TBq/mmol ([^{3}H]CHE) was purchased from Mandel Scientific (Guelph, ON, Canada). Na^{125}I and ACS scintillation fluid were purchased from Amersham (Oakville, ON, Canada). Iodobeads were obtained from Pierce (Rockford, IL, USA). Tyraminylinulin (TI) was synthesized and [^{125}I]tyraminylinulin ([^{125}I]TI) was prepared as previously described [25]. All other chemicals were of analytical grade purity.

2.2. Cell line

The human classical SCLC cell line NCI-H69

(ATCC HTB-119) was purchased from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were maintained within their exponential growth phase.

2.3. Preparation of liposomes

Liposomes were composed of HSPC:CHOL:PEG-DSPE plus either PDP-PEG-DSPE or MAL-PEG-DSPE at a 2:1:0.08:0.02 molar ratio (total PEG lipid was 5 mol% of phospholipid). For [^{125}I]TI-loaded liposomes, the aqueous space label was added during liposome hydration at 65°C [26]. DXR-containing liposomes were prepared by the ammonium sulfate gradient method using 250 mM ammonium sulfate (pH 5.5) in the interior of the liposomes and 100 mM sodium acetate, 70 mM NaCl buffer (pH 5.5) as the exterior buffer [27]. The amount of encapsulated DXR was determined from its absorbance at 492 nm following dissolution in methanol. Liposomes were sized by extrusion through Nucleopore filters and normally measured approx. 100 nm in diameter, as determined by dynamic light scattering.

Preparation of SLG from liposomes containing PDP-PEG-DSPE was carried as previously described [16]. Activation of antagonist G was performed by reacting it with EMCS at a 1:1 molar ratio in 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 140 mM NaCl buffer (pH 6.5) for 30 min at room temperature [28]. To prepare SLG liposomes containing MAL-PEG-DSPE, thiolated derivatives of antagonist G were first obtained by reacting the peptide with 2-iminothiolane at a 1:4 molar ratio in 25 mM HEPES and 140 mM NaCl (pH 8.0) for 1 h at room temperature [29]. Liposomes in HEPES buffer (pH 6.5) were then incubated overnight at room temperature with the activated peptide [30]. Activation and coupling of antagonist G took place in an inert N_2 atmosphere in silicon-coated glassware (Sigmacote). Free maleimide groups were quenched with an excess of 2-mercaptoethanol for 30 min at room temperature. Unbound antagonist G and 2-mercaptoethanol were removed by chromatography on a Sepharose CL-4B column, equilibrated in 25 mM HEPES and 140 mM NaCl (pH 7.4). The amount of antagonist G on the liposomes was determined by fluorimetry at $\lambda_{em} = 330$ nm, $\lambda_{ex} = 288$ nm.

Phospholipid (PL) concentration was determined from either the specific activity counts of a [^{3}H]CHE tracer or by the Bartlett colorimetric assay [31].

2.4. Cellular association

SLG were prepared by either the PDP-PEG-DSPE method or the MAL-PEG-DSPE method. SL or SLG were radiolabeled with [^{3}H]CHE, 28 kBq/μmol PL, and incubated for 1 h at 37°C with 1×10^6 H69 cells, either grown in culture or harvested from H69 xenografts, and the cellular association was determined by a previously described method [20].

2.5. Animals

Four- to 5-week-old female Balb/c nu/nu (nude) mice were purchased from Charles Rivers Canada (St. Constant, PQ, Canada). After arrival, they were given a minimum acclimation period of 1 week and received autoclaved food and water. The mice were housed in a virus antigen-free unit in the Health Sciences Laboratory Animal Services facility, University of Alberta. All animal protocols were approved by the Health Sciences Animal Policy and Welfare Committee, University of Alberta, and were in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

2.6. Xenografts

The SCLC H69 xenograft was derived by implantation of 10^7 exponentially growing H69 cells into the flanks of female nude mice. The xenograft was maintained as a subcutaneous tumor in the flanks of these animals. Tumors from donor animals were aseptically dissected and mechanically minced and implanted subcutaneously by trochar needle as 2–3 mm^3 pieces of viable tumor tissue into the flanks of recipient animals under methoxyflurane (Metofane, Janssen Pharmaceutica, Toronto, ON, Canada) anesthesia. Xenografts used in the experiments were pas-

saged 4–11 times in normal female nude mice. Histological analysis confirmed the pathology of the xenografts, which was checked every passage.

2.7. Orthotopic reconstitution of human SCLC in severe combined immunodeficient (SCID) mice

Based on previously published work [32,33], we made several attempts at an orthotopic reconstitution (intravenous model) of human SCLC after intravenous injection of 5×10^6 SCLC H69 cells (either grown in culture or harvested from subcutaneous xenografts grown in nude mice) in C.B. 17 SCID mice. Histological examination revealed that cells did not seed either in lungs or in any other main tissue, like liver. Although SCID mice do not produce functional B or T cells, the presence of natural killer cells might play a critical role in the eradication of circulating tumor cells.

2.8. Biodistribution in tumor-bearing mice

Xenografts were allowed to grow to a mean volume of approx. 43 mm^3 before starting biodistribution studies. Biodistribution of both targeted and non-targeted liposomes, encapsulating [^{125}I]TI, was determined as previously described [16]. Data were expressed as the percentage of injected dose/g of tissue.

2.9. Therapeutic studies

Animals were given ear tags to allow individual identification and tumor growth was assessed twice a week by caliper measurements for 52 days. Tumor volumes (V) were estimated as $V=\pi/6\times\text{length}\times\text{width}\times\text{height}$ [34]. The results were expressed as relative tumor volume, V_t/V_0 (where V_0 is the tumor volume at the start of the treatment and V_t is the tumor volume at any given time point) and was calculated for each individual tumor at every time point (mean relative tumor volume is a mean of relative tumor volumes, computed for each time point and for each individual mouse within a group) [8]. Treatment groups contained 5–6 mice and treatments (given intravenously via the tail vein) were started when tumors reached a mean volume of approx. 7 or 33 mm^3; the first day of treatment was designated as day 0. Treatment groups for each tumor burden consisted of three weekly doses of 3 mg DXR/kg of body weight of either free DXR, DXR-SL or DXR-SLG. In some experiments mice received 10 mg DXR/kg of DXR-SL or DXR-SLG and 6 mg/kg of free DXR. The reduction in the dose of free DXR was necessary to avoid toxicity. All experiments included an additional untreated control group of mice that received pH 7.4 HEPES buffer given under the same conditions.

2.10. Statistical analysis

Student's t-test was used to measure statistical significance between pairs of samples. Multiple comparisons were performed using analysis of variance (ANOVA). Data were considered significant at $P<0.05$.

3. Results

3.1. Biodistribution studies in tumor-bearing mice

The biodistribution of [^{125}I]TI-labeled SL or SLG was evaluated in Balb/c nude mice at 48 and 72 h post injection. SLG were prepared by either the PDP-PEG-DSPE or the MAL-PEG-DSPE coupling methods (SLG_{PDP} and SLG_{MAL}, respectively). Although a thiol ether bond was formed between the peptide and PEG in each method, in spite of the amount of peptide being equal (1 μg antagonist G/μmol PL), there were differences in the final mean diameters of the liposomes, with the SLG_{PDP} being larger than the SLG_{MAL} (170 vs. 124 nm, respectively). After 48 or 72 h, the spleen uptake of either SLG_{PDP} or SLG_{MAL} was approx. 5–10 times higher than that of SL (Fig. 1). No differences between uptake of SL vs. SLG occurred in other tissues, and with the exception of liver, uptake into other tissues was very low. After 48 h the blood levels of both SL and SLG_{MAL} (SLG_{PDP} was not determined at 48 h) were around 6.5–9% of injected dose/g of tissue and decreased to 1.3–1.5% of injected dose/g of tissue after 72 h (Fig. 1).

The tumor accumulation of both SL and SLG_{MAL} after 48 and 72 h was similar ($P>0.05$) and reached approx. 4% injected dose/g tissue by 72 h. The accumulation of SLG_{PDP} was approx. 12-fold less

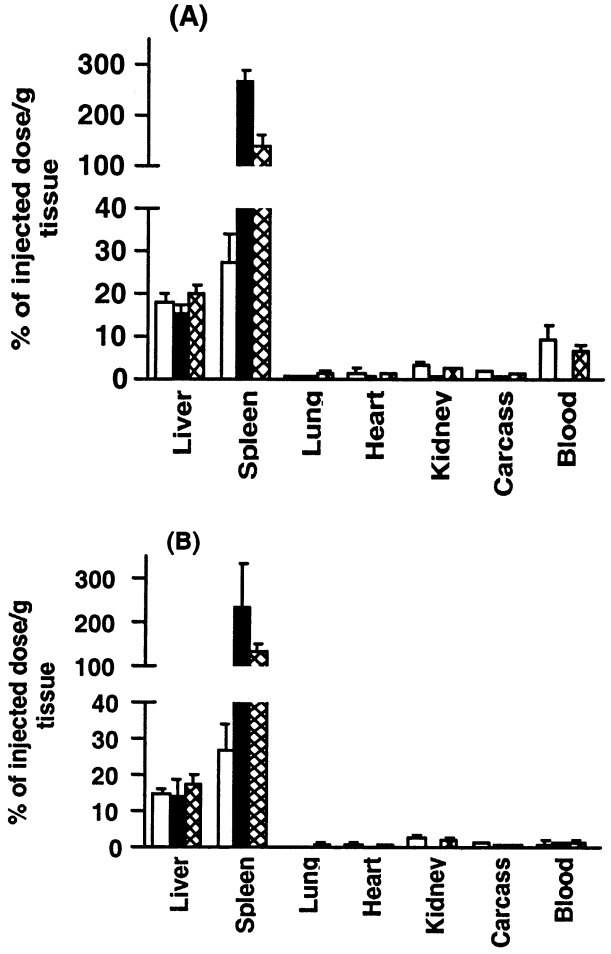

Fig. 1. Tissue distributions of liposomes in Balb/c nu/nu mice xenografted subcutaneously with H69 cells. [^{125}I]TI-labeled non-targeted (open bar) or antagonist G-targeted liposomes prepared by either the PDP-PEG-DSPE method (black bar) or the MAL-PEG-DSPE method (cross-hatched bar) were injected via the tail vein as a single bolus dose (0.5 μmol PL/mouse). After (A) 48 h or (B) 72 h, major organs and blood were collected and counted for ^{125}I label. Results were expressed as the percentage of injected dose/g tissue. Each point represents the average of three mice (± S.D.).

($P < 0.001$) at 72 h, which may be related to the larger size and higher splenic accumulation of these liposomes (Fig. 2).

3.2. Cellular association of liposomes

Since the tumor accumulation of SLG_{MAL} was significantly less than that of SLG_{PDP}, we looked for differences in the cellular association of these two samples. The term 'cellular association' is used to indicate a combination of binding to the cell surface plus internalization of the liposomes. For H69 cells grown in culture, the cellular association of SLG_{MAL} was the same as SLG_{PDP} (Fig. 3A). However, due to the poor tumor uptake of SLG_{PDP}, only SLG_{MAL} were used in further experiments.

Given that receptor expression on the surface of tumor cells is crucial for receptor targeting-based strategies, the cellular association of SLG_{MAL} with H69 cells harvested from 4th and 11th generation subcutaneous xenografts was tested. Cellular association of SLG_{MAL} with H69 cells harvested from a 11th generation tumor was slightly lower than that from a 4th generation tumor, but was still approx. 40-fold higher than the cellular association seen for SL, indicating that receptors for antagonist G were still being expressed at levels sufficient for targeting (Fig. 3B).

3.3. Therapeutic studies

Balb/c nu/nu mice bearing subcutaneous H69 cell xenografts were treated with free DXR, non-targeted liposomal DXR (DXR-SL) or antagonist G-targeted liposomal DXR (DXR-SLG). No DXR dose resulted in more than 20% weight loss and no treatment-related deaths were observed. In these mice, the maximum tolerated dose for free DXR was 6 mg/kg,

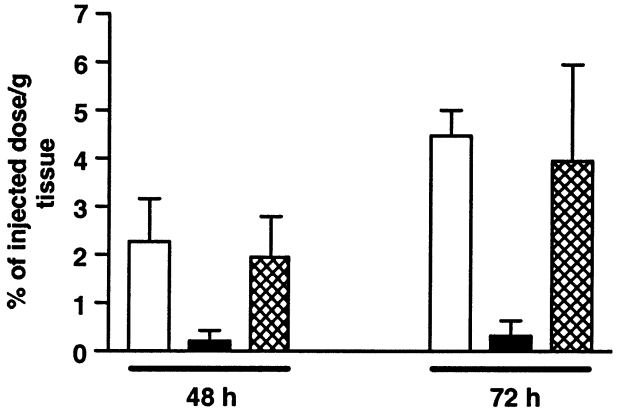

Fig. 2. Tumor accumulation of liposomes in Balb/c nu/nu mice xenografted subcutaneously with H69 cells. Conditions and symbols are as in Fig. 1. After 48 or 72 h, tumors were collected and counted for ^{125}I label. Results were expressed as the percentage of injected dose/g tissue. Each point represents the average of three mice (± S.D.).

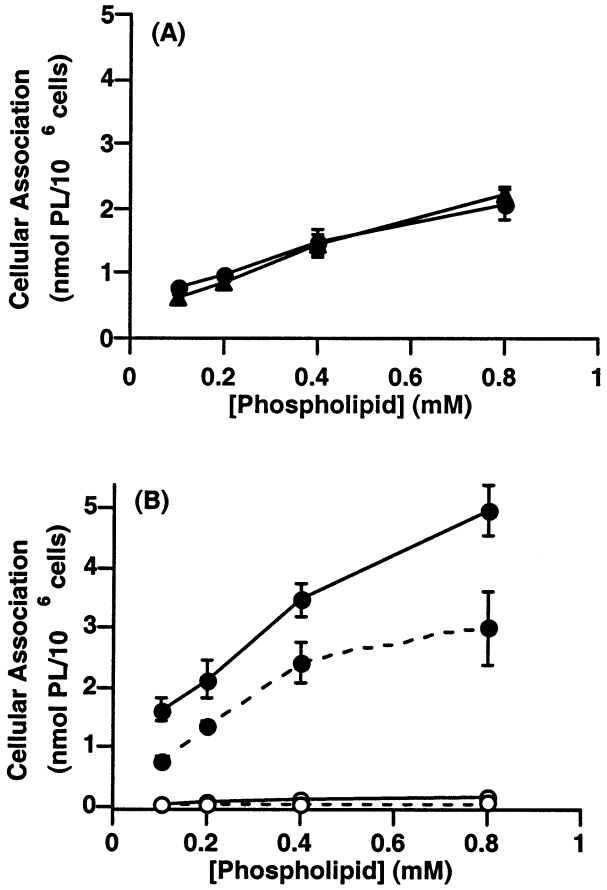

Fig. 3. Cellular association of [^{3}H]CHE-labeled liposomes with H69 cells. Liposomes composed of HSPC:CHOL:PEG-DSPE:PDP-PEG-DSPE or MAL-PEG-DSPE at a 2:1:0.08:0.02 molar ratio (0.1–0.8 mM PL/well), were incubated with 1×10^6 H69 cells. (A) SCLC H69 cells grown in culture were incubated at 37°C for 1 h with either SLG_{PDP} (▲) or SLG_{MAL} (●). (B) SCLC H69 cells harvested from H69 xenografts obtained after four passages or 11 passages were incubated with SL (——○—— and - - -○- - -, for tumors with four and 11 passages, respectively) or SLG_{MAL} (——●—— and - - -●- - -, for tumors with four and 11 passages, respectively). The amount of [^{3}H]CHE-liposomes associated with cells was determined from the initial specific activity of [^{3}H]CHE-liposomes. Data were expressed as nmol of PL/10^6 cells. Each point is the mean of three samples, ± S.D.

although doses of 10 mg DXR/kg of the liposomal drug were well tolerated. Mean relative tumor volumes for the various treatments were determined (Figs. 4 and 5).

Treatment of tumor-bearing mice with DXR-SL or DXR-SLG at low DXR doses (3 mg DXR/kg) when the tumor was at the beginning of its exponential growth phase (mean tumor volume of approx. 7 mm^3), caused a slight decrease in the tumor growth rate relative to non-treated tumors or free DXR, but no differences between the targeted and the non-targeted liposomes were observed (Fig. 4A). Treatment of mice with DXR-SL or DXR-SLG at 10 mg DXR/kg, at a tumor volume of 7 mm^3, almost completely inhibited tumor growth, although no differences were observed between the two liposomal treatments. Free DXR, at its maximum tolerated dose of 6 mg/kg, was significantly less effective than SL or SLG (Fig. 4B).

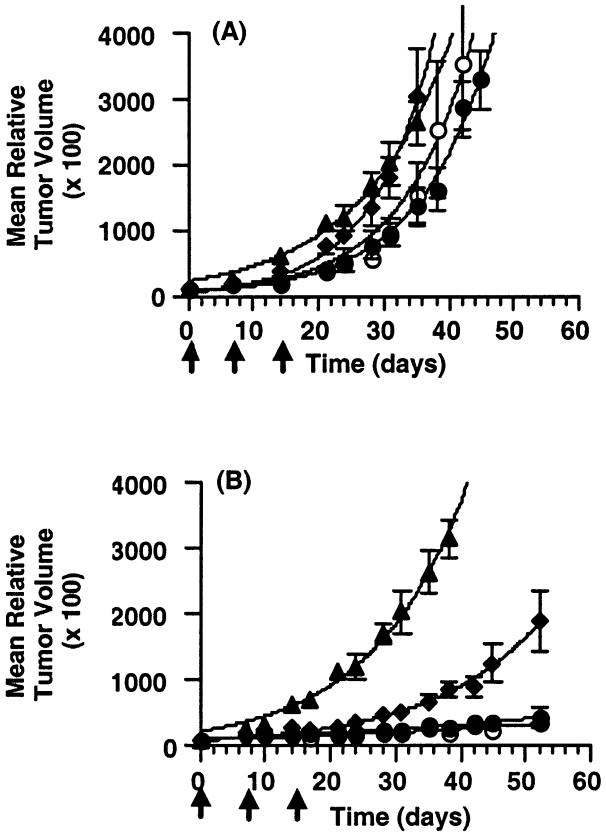

Fig. 4. Antitumor efficacy of various formulations of DXR against early SCLC H69 tumor xenografts grown in Balb/c nu/nu mice. Treatments started when the mean tumor volume reached approx. 7 mm^3 and the first day of treatment was designated as day 0. Mice were treated on days 0, 7 and 14 with: (A) 3 mg DXR/kg of body weight of either free DXR (♦), DXR-SL (○) or DXR-SLG (●); (B) 6 mg DXR/kg of body weight of free DXR (♦), and 10 mg DXR/kg of body weight of either DXR-SL (○) or DXR-SLG (●). Untreated controls received pH 7.4 HEPES buffer (▲). Arrows indicate the time points of treatment. The results were expressed as mean relative tumor volume. Each point represents the average of 5–6 mice ± S.E.M.

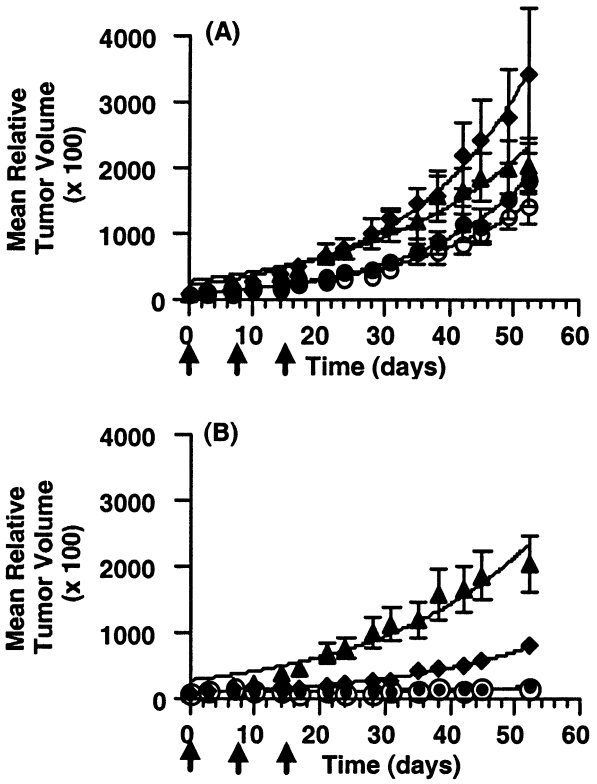

Fig. 5. Antitumor efficacy of various formulations of DXR against advanced SCLC H69 tumor xenografts grown in Balb/c nu/nu mice. Treatments started when the mean tumor volume reached approx. 33 mm^3 and the first day of treatment was designated as day 0. Mice were treated on days 0, 7 and 14 with: (A) 3 mg DXR/kg of body weight of either free DXR (♦), DXR-SL (○) or DXR-SLG (●); (B) 6 mg DXR/kg of body weight of free DXR (♦), and 10 mg DXR/kg of body weight of either DXR-SL (○) or DXR-SLG (●). Untreated controls received pH 7.4 HEPES buffer (▲). Arrows indicate the time points of treatment. The results were expressed as mean relative tumor volume. Each point represents the average of 5–6 mice ± S.E.M.

For treatments at 3 mg DXR/kg with higher tumor burdens (mean volume of approx. 33 mm^3, Fig. 5A), free DXR did not have any therapeutic activity. Treatment with either DXR-SLG or DXR-SL caused some inhibition of tumor growth, but no differences were observed between the two liposomal groups. Treatment with 10 mg/kg DXR-SLG or DXR-SL resulted in significant tumor growth inhibition (Fig. 5B). Treatment with free DXR at its maximum tolerated dose of 6 mg/kg was less effective than either liposomal formulation, but more effective than untreated control (Fig. 5B).

4. Discussion

The aggressiveness of SCLC, along with the low survival rate of patients with this type of tumor, demands the development of novel and more efficient therapeutic strategies. The unique pathophysiological features exhibited by most solid tumors, such as angiogenesis, leaky blood vessels and impaired lymphatic drainage provide an opportunity for the selective accumulation of liposomes. The long circulating half-lives of drugs like DXR entrapped in PEG-grafted liposomes, their in vivo stability in plasma and their improved vascular permeability are necessary for the high tumor accumulation [35] and improved therapeutic efficacy seen for these formulations [11].

Some evidence exists for further improvements in therapeutic effects when targeting ligands, like antibodies, were attached to the PEG-liposomes [20]. However, whole antibodies are probably not the best targeting molecules. Use of whole antibodies attached to the PEG terminus of Stealth liposomes enhanced mononuclear phagocyte system uptake of the liposomes via an Fc receptor-mediated mechanism [36] relative to either non-targeted liposomes or to liposomes targeted via Fab′ fragments that lack the Fc region of the antibody [37]. This resulted in a decrease of the blood residence time of the targeted liposomes, which would likely reduce tumor uptake [37,38]. Even if the targeted liposomes are distributed to solid tumors, their penetration into the tumor interior might be compromised by their binding to cell surface receptors at the tumor periphery [39]. Tumor penetration may be improved through the use of small ligands with reduced avidity for their target (relatively to whole antibodies), like antibody Fab′ fragments or single chain antibody fragments, or peptides [40].

Previous work in our laboratory has demonstrated improved cytotoxicity against the human SCLC H69 cell line of DXR-containing antagonist G-targeted liposomes, relative to non-targeted liposomes [23,24]. These results prompted us to evaluate the tumor accumulation and the therapeutic efficacy of these formulations against H69 xenografts in Balb/c nude mice. Small peptides have the advantage (over antibodies) of being non-immunogenic, chemically

defined and able to be manufactured in large quantities without biological contaminants.

Two different coupling methods were used to prepare the antagonist G-targeted liposomes in these experiments. Liposomes made by the PDP-PEG-DSPE method had a larger size, a greater splenic uptake, and a reduced tumor uptake relative to liposomes made by the MAL-PEG-DSPE method. The reduced tumor uptake of SLG_{PDP} is likely related to both their more rapid clearance into the spleen (resulting in fewer liposomes available to be distributed to tumors) and to their larger size. Previous studies have shown that the extravasation of PEG-grafted liposomes from the vascular compartment into the tumor interstitium was size-dependent, and also dependent on the type of tumor, and mainly limited by their ability to diffuse through the 100–1200 nm pores in the vessel wall [42,43].

Although there is a substantial increase in the cellular association of SLG with H69 cells harvested from H69 xenografts (Fig. 3B), no improvement in tumor accumulation was observed for SLG over SL (Fig. 2). This confirms observations by other authors that targeting of liposomes does not lead to increased accumulation of the liposomes in solid tumors [37,44,45]. It would appear that the accumulation of targeted liposomes in solid tumors is governed by the same processes that govern the tumor accumulation of non-targeted liposomes, i.e. passive diffusion and extravasation, and that peptide-targeted liposomes may also suffer from the 'binding site barrier', like antibody-targeted liposomes.

Recently, Fab′ fragments of a fully humanized version of the murine mAb 4D5, directed against the glycoprotein $p185^{HER2}$, were used to target PEG-grafted liposomal DXR towards HER2-overexpressing human breast cancer cells (BT-474 tumor xenografts) [41,44]. Even though the total uptake of the liposomes into tumor was similar for the targeted and the non-targeted liposomes in this model, tumor regression was observed [22]. The occurrence of increased tumor regression for the targeted liposomes relative to the non-targeted liposomes could be explained by differences in the intratumoral localization between non-targeted and targeted liposomes. Liposomes prepared with Fab′ fragments of mAb 4D5 were widely distributed in the intercellular spaces throughout HER2-positive tumor tissue as well as within the cytoplasm and in the perinuclear spaces of HER2-positive cancer cells. Non-targeted liposomes were mainly found in tumor-resident macrophages and in perivascular areas [44].

In the therapeutic experiments with the HER2 model, even though no increase in tumor accumulation was observed between targeted and non-targeted liposomes, similar to our observations in the SCLC model, the targeted liposomes resulted in an increased therapeutic effect relative to the non-targeted liposomes [22]. In our experimental model, while the liposomal formulations were superior to treatment with free drug, no differences in therapeutic effect were observed between DXR-SL and DXR-SLG. A similar result was obtained when DXR-containing liposomes were targeted with the N12A5 mAb towards the HER2 antigen in a gastric cancer model, possibly due to an absence of liposome internalization [45]. However, as previously demonstrated, SLG are internalized by H69 cells [23,24], so lack of internalization cannot explain the data.

One explanation might be poor vascularization of the H69 xenograft relative to the BT-474 xenografts, which were well vascularized [44]. Histological examination of the SCLC tumors revealed that they are poorly vascularized, thus confirming macroscopic observation. This seems to be a common feature of SCLC xenografts [48]. This would certainly limit the amount of liposomal drug that could accumulate in tumors (which was 2-fold lower for the SCLC tumors than for the BT-474 tumors), but one could argue that it would affect the accumulation of non-targeted and targeted liposomes to a similar degree. As shown in Fig. 2, there was no difference in total tumor uptake for the non-targeted versus the targeted (MAL-PEG-DSPE) liposomes, so the similar therapeutic activity for the two formulations is not surprising. Another explanation could be related to the presence of lower receptor densities on the H69 cells, relative to the BT-474 cells, or to down-regulation of the receptors on the H69 cell in vivo, relative to that seen in vitro. The cellular association experiments for SLG carried out with H69 cells harvested from H69 subcutaneous xenograft demonstrated that the in vivo propagation of the tumor over several generations did not compromise receptor expression for antagonist G.

Antagonist G is a chemical analogue of the neuro-

transmitter substance P with the structure 'H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2'. The biological action of this peptide depends on its ability to bind receptors (namely, vasopressin receptor) on the surface of H69 cells, via the amino acid sequence D-Trp-N^{me}Phe-D-Trp-Leu [6]. The in vivo metabolism of antagonist G has been fully characterized [46]. It has been shown that antagonist G is metabolized mainly by deamidation (Met-NH_2) followed by carboxypeptidase removal of methionine. Therefore, under these conditions the hydrophobic core of the peptide, which is important for its binding to tumor cells, is left intact [46]. Moreover, in vitro studies have shown that the metabolites retain peptide growth factor antagonist activity against vasopressin of the same order of magnitude as antagonist G [47]. These data strongly suggest that antagonist G is able to maintain its receptor binding properties in vivo and lead us to hypothesize that other factors must contribute to the similarities in the therapeutic activity of DXR-SL and DXR-SLG.

Our results emphasize that, even when ligands against internalizing epitopes are used for targeting, vascular density and/or degree of perfusion within a solid tumor can be major factors governing the therapeutic success of targeted liposomal drugs. Our previous successes in treating metastatic cells in blood, or cells that had only recently formed metastatic lesions in tissues [18–20], suggest that we should try the antagonist G-targeted liposomes to eliminate migrating cells in a metastatic or pseudometastatic model. Since SCLC has the highest metastatic potential of any of the solid tumors in humans [3], antagonist G-targeted liposomal DXR could be useful as an adjuvant therapy against SCLC metastatic cells migrating in blood; however, an appropriate model to test this hypothesis needs to be developed. Nevertheless, the results presented here point out that DXR-containing Stealth liposomes may have potential for the treatment of human SCLC tumors.

Acknowledgements

J.N.M. was the recipient of a Portuguese grant from Praxis XXI (Ref.: BD/5600/95). The technical assistance of Elaine Moase, M.Sc., and histopathological examination of the mice by Dr. N. Nation from the Health Sciences Laboratory Animal Services, University of Alberta, Edmonton, Canada, were greatly appreciated.

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