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Research paper

Efficacy of multi-functional liposomes containing daunorubicin and emetine for treatment of acute myeloid leukaemia

Lene Myhren [a], Ida Mostrøm Nilssen [a,1], Valérie Nicolas [b], Stein Ove Døskeland [a], Gillian Barratt [c], Lars Herfindal [a,d,*]

[a] Department of Biomedicine, University of Bergen, Bergen, Norway
[b] Université Paris-Sud11, Plateforme d'Imagerie Cellulaire, IFR141-IPSIT, FR 92296, Chatenay-Malabry, France
[c] Insitut Galien Paris-Sud (UMR CNRS 8612), Université Paris-Sud 11, LabEx LERMIT, FR 92296, Chatenay-Malabry, France
[d] Translational Signalling Group, Haukeland University Hospital, Bergen, Norway

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ABSTRACT

Despite recent advances in chemotherapy against acute myeloid leukaemia (AML), the disease still has high mortality, particularly for patients who tolerate extensive chemotherapy poorly. Nano-formulations have potential to minimise the adverse effects of chemotherapy. We present here a liposomal formulation encapsulating both the anthracycline daunorubicin (DNR) and emetine (Eme) for enhanced cytotoxic effect against AML cells. Eme could be loaded into the PEGylated liposomes together with DNR by the acid precipitation principle, with a loading efficiency of Eme at about 50% of that of DNR. The liposome surface was modified with folate to enhance drug loading into cells, giving higher cytotoxic activity. Both intracellular drug loading and cytotoxic activity could be further increased by anti-folate treatment of AML cells with methotrexate (MTX). The combination of DNR and Eme also increased drug loading in MTX-treated cells compared to DNR alone. Liposomes with both DNR and Eme were particularly efficient against AMLs with deficient p53. In conclusion, we have produced a multi-functional liposomal anti-leukaemic drug formulation designed to overcome some of the problems in anthracycline chemotherapy: (1) Combination of DNR and Eme to diminish drug resistance. (2) Using PEGylated stealth liposomes to minimise adverse side-effects. (3) Molecules on the liposomal surface target proteins on AML-cells ensure selectivity, which was enhanced by priming the leukaemia cells with MTX.

1. Introduction

Acute myeloid leukaemia (AML) is a hematopoietic stem cell disorder that causes excessive proliferation and rapid accumulation of myeloid precursor cells in the bone marrow. If left untreated, death occurs within weeks or months after diagnosis. AML is a heterogeneous disease with regard to disease progression and therapy response. For the sub-type acute promyelocytic leukaemia (APL, WHO classification, 2008) differentiation therapy with all-trans retinoic acid, often in combination with cytostatics such as arsenic trioxide and daunorubicin (DNR) has proven successful [10]. However, for most AML patients the primary treatment regime still involves high doses of chemotherapeutics such as the cell cycle-specific inhibitor cytarabine (Ara-C) in combination with the cell cycle-unspecific inhibitor anthracycline DNR [28,39]. Complete remission is reached in about 60% of AML patients between 60 and 70 years old, with 2-year disease-free survival of about 30%, while for patients above 70 years, the outcome is even worse. [25,31]. For patients younger than 60 years, complete remission is reached in 79% of patients [31]. However, the relapse risk is in the range of 45–50% in older patients, and overall, AML is the leading cause of death due to leukaemia with a 5-year relative survival below 20% [4,19,25]. There is thus a need to develop treatments with improved therapeutic efficiency that is tolerable also for weak and elderly patients.

The search for novel AML therapy regimes made us undertake a study to investigate whether protein synthesis inhibitors such as

Abbreviations: AML, Acute myeloid leukaemia; DDS, Drug delivery system; DNR, Daunorubicin; DOX, Doxorubicin; Eme, Emetine; FA, Folate; FR2, Folic acid receptor 2 (β); HEPC, Hydrogenated egg phosphatidylcholine; MTX, Methotrexate; PE, Phosphatidylethanolamine; PEG, Polyethylene-glycol; PSMA, Prostate specific membrane antigen.

* Corresponding author. Department of Biomedicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway. Tel.: +47 55 58 63 81; fax: +47 55 58 63 60.
E-mail address: lars.herfindal@biomed.uib.no (L. Herfindal).
[1] Present address: Bergen Hospital Pharmacy, P.O. Box 1, 5021 Bergen, Norway.

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emetine could improve the efficacy of standard anthracycline therapy [16]. This was based on findings that AML cells show enhanced translation of mRNA coding for survival proteins upon DNR exposure [16]. As with all chemotherapy, the combination therapy DNR/protein synthesis inhibitor could include the risk of toxic side effects in normal tissue and organs. Thus, we needed to find a drug delivery system (DDS) that could spare normal tissue from excessive chemotherapy exposure, but still deliver the drugs to the AML cells.

Liposomal DDS are attractive tools to overcome problems associated with chemotherapy-induced toxicity by targeting the drug to the tumour cells, and diverting it away from normal tissue, thereby increasing the therapeutic window. Doxorubicin-loaded liposomes (Doxil®/Caelyx®) are used against solid tumours to reduce cardiotoxicity [35], and many other anti-cancer liposomal formulations are in clinical trials (see [1] for a recent review). Liposomes have the advantage that drugs can be loaded during or after production, allowing loading of several drugs with different chemical properties into the same nanocarrier. In AML, the short pulses of high doses daunorubicin pose a threat to the patients due to bone marrow depletion [3] and intestinal mucositis [7]. Interestingly, the presence of p53 appears to rescue normal tissue, rather than increasing the toxic effects [18]. Thus, by designing a therapy that selectively attacks p53-deficient cells, we can minimise toxic side-effects. As a result of the protection of exposure afforded by the liposomes, it is possible to increase the therapeutic window.

We wanted to create a formulation that allowed for the intracellular delivery of DNR and a protein synthesis inhibitor in one DDS. Emetine was chosen as the protein synthesis inhibitor since it has relatively equal toxic profile across the species [34] compared to cycloheximide, where the LD50 varies from 1 to 2 mg/kg in dog and rat to 130 mg/kg in mouse [22,37]. Also, Eme is already used against protozoal infections in man [26] and approved for use in humans. We also wanted to target leukaemia cells through liposome internalisation via the folate receptor 2 (FR2) [41], and aimed to increase drug delivery by further stimulating FR2-expression in AML cells.

In this study, we present a multifunctional liposomal DDS loaded with DNR and Eme for enhanced AML-cell toxicity, its surface being modified with PEG for "stealth" effect, and FA for targeting. Furthermore, we prove increased drug delivery to AML cells exposed to anti-folate therapy.

2. Materials and methods

2.1. Production of liposomes

Liposomes were prepared with hydrogenated egg phosphatidylcholine (HEPC, Lipoid EPC-3, T12508 from Lipoid KG, Ludwigshafen, Germany), cholesterol (Sigma, La Jolla, CA), and PEG-ylated distearoyl phosphatidylethanolamine (PEG-PE, PEG molecular weight 2000 Da, Avanti Polar Lipids, Alabaster, Alabama, US). All lipids were dissolved in chloroform in a molar ratio of 1.81:1:0.15, HEPC:Chol:PEG-PE, and a film was made by evaporation of the lipids. Liposomes carrying folate (FA) lipids were prepared by adding distearoyl-glycero-phosphoethanolamine-N-PEG5000-folate, PE-PEG-FA, Avanti Polar Lipids) at one tenth of the molar concentration of PEG-PE. Fluorescent liposomes were made by adding Rhodamine-labelled PE (Rh-PE, Molecular Probes) at concentration of one per cent of total PC.

The film was rehydrated in warm (60 °C) 250 mM $(NH_4)_2SO_4$ pH 6.5 at 0.5 mg lipids/ml. Between 10 and 15 glass beads (1 mm diameter) were added to facilitate the hydration by vortexing at maximum intensity until the film was totally hydrated. The sample was next stirred at 60 °C for 20 min before extrusion at 70 °C (LIPEX™ extruder; Northern Lipids, Burnaby, Canada) through membranes (Whatman, Kent, UK) with pore size of 0.8 μm (five times), 0.4 μm (five times) 0.2 μm (double membranes, ten times) and 0.1 μm (double membranes, ten times).

The liposomes were then passed through a column with G25-medium Sephadex (Amersham, Uppsala, Sweden) and eluted in PBS, pH 8.0. Empty liposomes were first run through the column to avoid unspecific binding of liposomes to the gel.

The liposomes were loaded with drugs based on the acid-precipitation concept [11,13]. In brief, DNR (Cerubidine, 20 mg DNR and 100 mg mannitol, Sanofi Aventis Lysaker, Norway) and emetine (Eme, Sigma–Aldrich, St. Louis, MO), were added to the liposome suspension at a ratio of 1:10 drugs:lipids (w/w). In some formulations, doxorubicin (DOX, Accord Healthcare, Gothenburg, Sweden) was used instead of DNR. DOX is the preferred anthracycline for solid tumours, whereas DNR and sometimes idarubicin (IDA) is used against AML. The liposomes and drugs were incubated for 1 h at 60 °C before gel filtration to remove non-encapsulated drug.

Size and zeta potential was measured by dynamic light scattering (DLS) using a Zetasizer (Malvern Instruments Ltd. Malvern, UK). For measurement of size, the liposomes were diluted in the suspension buffer (PBS or 250 mM $(NH_4)_2SO_4$) and measured three times. To measure zeta potential of loaded and unloaded liposomes, the suspension was diluted 1:10 in isotonic (5%) sucrose, since dilution with PBS gave very inconsistent results even between measurements of the same liposomal preparations. Under these conditions, we obtained similar size measurements to those recorded using PBS as suspension buffer.

2.2. Quantification of liposomal drug content by HPLC

Liposome suspensions or solutions of DNR or Eme for standard curves were evaporated under vacuum and the residue dissolved in 0.15 ml 3:1 0.1% aqueous TFA:MeOH. The solution was next injected into a reversed phase HPLC column (Kromasil 100-5 C18 250-4.6 mm, Akzo Nobel, Sweden) connected to a Merck-Hitachi LaChrome HPLC-system (VWR, WestChester, USA) with a L-7455 diode array detector. Mobile phase A was 3:1 0.1% aqueous TFA:-MeOH, and mobile phase B was acetonitrile. The flow rate was 1.0 ml/min and Eme was eluted during a 13 min isocratic mode with 100% A followed by a gradient to 100% B during 6 min, where DNR eluted. Standard curves from 0.005 to 0.12 mg/ml DNR or Eme were prepared, and drug content in liposomes quantified by integration of the peaks at 280 nm (Eme) and 480 nm (DNR).

2.3. Cell maintenance and experimental conditions

The NB4 [20], HL-60 (ATCC No.: CCL-240) and Molm-13 [27,33] leukaemia cell lines and the LNCaP (ATCC No.: CRL-1740) prostate cancer cells were cultured in RPMI 1640 medium enriched with 10% foetal bovine serum (FBS, Invitrogen, Carlsbad, CA). The MV4-11 cells were cultured in Iscove's medium added 8 mM L-glutamine and 10% FBS. All cell lines were cultured in media supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin (both from Cambrex, Belgium) in a humidified atmosphere (37 °C, 5% CO_2). All culture media were from Sigma (Sigma, La Jolla, CA). p53 knocked down Molm-13 cells (Molm-13 shp53) were generated by retroviral transfection for stable expression of shRNA against p53 [29]. The leukaemia cell lines represent different subclasses of AML (acute promyelocytic leukaemia (NB4 [20]), acute monocytic leukaemia with the poor prognostic factor FLT3 internal tandem duplication (Molm-13 [27]) and the commonly used HL-60 AML cell line, as well as cells with and without inducible p53 (Molm-13 and Molm-13 shp53).