BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
12 (2016) 2043–2060

Original Article

nanomedjournal.com

The ligand (s) anchored lipobrid nanoconstruct mediated delivery of methotrexate: an effective approach in breast cancer therapeutics

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Received 31 December 2015; accepted 1 May 2016

Abstract

The present study was designed to engineer surface-anchored and methotrexate loaded lipobrid nano-constructs for targeting breast cancer. Ligands (fucose, galactose and mannose) anchored lipobrid nano-constructs were used to compare and assess delivery efficiency in breast cancer cell lines as well as in DMBA induced breast cancer animal model. The developed and characterized formulations were used to comparatively assess cellular uptake, cell-viability, apoptosis, lysosomal membrane permeability, bioavailability, bio-distribution, changes in tumor volume and animal survival. Our results show greater cellular uptake, cytotoxicity at low IC50, apoptosis with altered lysosomal membrane permeability and greater rate of degradation of lysosomal membrane. We saw better bioavailability and tumor targeting efficiency with minimum secondary organ drug distribution. The significant reduction was seen in tumor burden with ligand anchored lipobrids in comparison to plain and MTX-lipobrid formulations. In conclusion, fucose anchored MTX-lipobrid formulation showed promising results, and warrants to explore the development of therapeutic interventions for breast cancer. Published by Elsevier Inc.

Key words: Lipobrids; Methotrexate; Breast cancer targeting; Lectin receptors; Carbohydrate ligands

The chemotherapeutic drugs used in cancer treatment have faced several limitations including undesirable bio-distribution, rapid drug clearance and non-specific targeting that leaves adverse effects on healthy cells.[1–3] Nanoparticles (NPs) have emerged as a promising alternative to deliver anti-cancer drugs through enhanced permeation and retention (EPR) effect.[4–7] Furthermore, NPs with a size range of 10-500 nm are seen advantageous over conventional drug delivery.[8–11] The efforts were made to develop nano-drug delivery vehicles such as polymeric NPs,[12–15] nanostructured lipid carriers (NLCs),[16,17] solid lipid nanoparticles (SLNs),[18] and liposomes[19,20] for controlled and targeted drug delivery. The lipid and polymer based nano-carriers have shown the effective delivery potential of chemotherapeutic drugs. The lipid polymer hybrid NPs have been designed to explore all attributes in one delivery system[21–23] to achieve controlled and targeted delivery. Recently, self-assembled polymer–lipid hybrid NPs were shown to overcome the limitations observed with conventional drug delivery; rapid blood clearance of drug, controlled drug

Abbreviations: MTX, methotrexate; F, fucose; G, gaalactose; M, mannose; NPs, nanoparticles; EPR, enhanced permeation and retention; PL S100, Phospholipon S100; PCL, polycaprolactone; C-6, coumarin-6; DMF, dimethyl formamide; DCM, dichloromethane; PDI, Polydispersity Index; HR-TEM, high resolution transmission electron microscope; CLSM, confocal laser scanning microscopy; EBr, ethidium bromide; AO, acridine orange; LMP, lysosomal membrane permeability; AUC, area under the curve; C_{max}, maximum plasma concentration; $t_{1/2}$, half-life; MRT, mean residence time; T_{max}, time to reach maximum plasma concentration.

Author disclosures and funding: All authors declare that they do not have any conflict of interest statement/financial support. The research fellowship was offered to the first author & Varun Kushwah by CSIR-HRDG, New Delhi, India with letter reference no: 09/135 (0667)/2012-EMR-I.

We acknowledge BASF, Mumbai, India and AIIMS, New Delhi, India for providing poloxamers as a gift sample and HR-TEM analysis of NPs respectively.

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http://dx.doi.org/10.1016/j.nano.2016.05.008
1549-9634/Published by Elsevier Inc.

release, and tumor-specific delivery. These attributes of polymer–lipid hybrid NPs were enough to draw considerable attention for drug and gene delivery.[24,25]

Although NPs have shown improved targeted drug delivery with minimal systemic adverse effects,[6] surface of NPs are conjugated with targeting molecules which may be recognized by the receptors present on cancer cells[26] to achieve better drug targeting efficiency. NPs are effective in ligand conjugation for receptor mediated targeting of drug delivery, and offer novel and much better cancer therapeutic approaches.[5,6,11,27] The active targeting of NPs increase the probability of drug availability at the target site which eventually reduces chances of exposure of healthy cells. It will minimize the adverse effects[28] because of drug therapy.

The exploitation of overly-expressed membrane receptors (lectin receptors/LRs), as drug targets, during tumor condition has been a promising approach for NPs.[29–32] The presence of lectin (carbohydrate) moieties on the surface of therapeutic NPs can efficiently enhance specificity and binding affinity, and can eventually lead to significantly higher cellular uptake through receptor-mediated endocytosis.[29,31–35] The lectin receptor-mediated targeting employs the interaction of endogenous ligands with different sugar moieties such as galactose (G), mannose (M), fucose (F), fructose and lactose.[33–36] The sugar moieties anchored to different drug nanocarriers resulted into glycosylated carriers having carbohydrate as stratum ligands which are known for quick internalization through lectin receptor-mediated endocytosis.[29–32]

Recently, we have reported a novel single step method for the synthesis of self-assembled MTX loaded lipid polymer hybrid NPs.[21] MTX plays an important role in treating brain, breast and ovarian cancer, and also several leukemia cases.[37] However, clinical applications of MTX are limited due to its pH dependent solubility, dose-related cytotoxicity, non-specific rapid diffusion throughout the body, short half-life in blood-stream, and development of resistance by target cells.[38,39] Therefore, we took advantage of targeting potential of mannose (M), galactose (G) and fucose (F) to deliver anti-cancer agents as these ligands may be conjugated to free $-NH_2$ functional groups NP's surface.[34,35,40]

Present study was designed to investigate the formulation, and comparison of cancer-targeting potential of F, M and G-anchored lipid polymer hybrid (lipobrid). To our knowledge, we are the first group to report the targeted and controlled delivery of MTX through F/G/M conjugated lipobrid to develop therapeutic approaches against breast cancer. This study encompasses *in vitro* and *in vivo* comparison of targeting potential of the said ligands. Collectively, carbohydrate based ligand anchored targeted lipobrids were formulated and their delivery potential was assessed with both *in vitro* and *in vivo* models to better meet the needs of breast cancer treatment.

Methods

Materials

MTX was obtained as a gift sample from IPCA, Pvt. Ltd., Mumbai, India. The phospoholipid Phospholipon S100 (PL S100) was received as a gift from Lipoid GmbH, Ludwigshafen, Germany. Polycaprolactone (PCL; MW ~ 14,000, Mn ~ 10,000), 7,12-dimethylbenz[*a*] anthracene (DMBA), stearyl amine (SA), L-fucose, D-mannose, and D-galactose were purchased from Sigma-Aldrich, Chemical Co. (St. Louis, MO, USA). Lutrol® F-87 was obtained as gift sample from BASF, Mumbai, India. Tween 80 and DMF were procured from Thermo Fisher Scientific Pvt. Ltd, Mumbai, India and Central Drug House (P) (CDH) Ltd., New Delhi, India, respectively. All other chemicals, reagents and solvents were of analytical grade. Methotrexate marketed injection (MTX-MF) (25 mg/ml) was purchased from local drug house, Chandigarh, India. The protocols for animal experiments were approved by Institutional Animal Ethics Committee (IAEC), Punjab University, Chandigarh, India (Letter Ref. No. PU/IAEC/S/14/95).

Preparation of lipobrids

MTX loaded lipobrids (MTX-lipobrid) were prepared by a single step nano-precipitation method as reported earlier.[21] Briefly, MTX, polycaprolactone (PCL) was dissolved in 3 ml DMF and heated at 60-70 °C. Phospholipid S100 (20% PCL) and stearyl amine (10% mol ratio of PL) was dissolved in 2 ml DCM: DMF (1:1) and heated at 60-70 °C. Both heated solutions were mixed and stirred continuously for 10 min. This solution was added drop by drop into surfactant solution of Lutrol® F-87 (0.5% w/v) at a constant flow rate of 1 ml/min. The solution was stirred on magnetic stirrer (Remi, Mumbai, India) at 800 rpm for 2-3 h. The organic phase was then removed by dialysis (MWCO 10 kDa, Himedia, Mumbai) against the double distilled water for 12 h. The coumarin-6 (100 μg/50 mg polymer) was added in organic phase (DMF) and coumarin-6 co-encapsulated MTX-lipobrid formulations with and without ligands were prepared using the above-mentioned protocol.

Preparation and characterization of ligand anchored lipobrids

Ligand conjugated lipobrids were prepared by post insertion method as earlier reported,[34] by incubating the suspension of lipobrid with equal molar ratio (equivalent to stearyl amine used in lipobrid) of activated F, G and M solution in saline individually. This method is associated with activation of ligands (F/M/G) by ring opening at acidic pH (4.0) followed by the reaction of its aldehyde group of activated ligands with free amino groups present at the outward of prepared lipobrid (Supporting Information Section 1.1).

MTX-lipobrids were characterized with respect to their size, PDI, zeta potential, surface morphology and drug entrapment efficiency. Size, zeta potential and PDI of NPs were determined by Zetasizer (PCS, Nano ZS90 zetasizer, Malvern Instruments Corp, UK). The *in vitro* release and stability studies of lipobrids were carried out (Supporting Information Section 1.2). Surface morphology of lipobrids was assessed by high resolution transmission electron microscope (HR-TEM) analysis. A drop of diluted lipobrid suspension was placed on a membrane coated colonial grid surface and immediately stained with a drop of 1% phosphotungstic acid. The excess fluid was removed after 1 min, and grid surface was air dried, and was examined under HR-TEM, TecnaiG^2 20 TEM (Fei Company, Netherland). Direct lysis method of lipobrids (sonication for short duration) was utilized to calculate % age drug entrapment efficiency. The mixture was filtered and diluted suitably with mobile phase, and MTX content was estimated by RP-HPLC.[41] The percentage