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Research Paper

Plasma membrane targeting by short chain sphingolipids inserted in liposomes improves anti-tumor activity of mitoxantrone in an orthotopic breast carcinoma xenograft model

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ABSTRACT

Mitoxantrone (MTO) is clinically used for treatment of various types of cancers providing an alternative for similarly active, but more toxic chemotherapeutic drugs such as anthracyclines. To further decrease its toxicity MTO was encapsulated into liposomes. Although liposomal drugs can accumulate in target tumor tissue, they still face the plasma membrane barrier for effective intracellular delivery. Aiming to improve MTO tumor cell availability, we used short chain lipids to target and modulate the tumor cell membrane, promoting MTO plasma membrane traversal. MTO was encapsulated in liposomes containing the short chain sphingolipid (SCS), C_8-Glucosylceramide (C_8-GluCer) or C_8-Galactosylceramide (C_8-GalCer) in their bilayer. These new SCS-liposomes containing MTO (SCS-MTOL) were tested *in vivo* for tolerability, pharmacokinetics, biodistribution, tumor drug delivery by intravital microscopy and efficacy, and compared to standard MTO liposomes (MTOL) and free MTO.

Liposomal encapsulation decreased MTO toxicity and allowed administration of higher drug doses. SCS-MTOL displayed increased clearance and lower skin accumulation compared to standard MTOL. Intratumoral liposomal drug delivery was heterogeneous and rather limited in hypoxic tumor areas, yet SCS-MTOL improved intracellular drug uptake in comparison with MTOL. The increased MTO availability correlated well with the improved antitumor activity of SCS-MTOL in a MDAMB-231 breast carcinoma model. Multiple dosing of liposomal MTO strongly delayed tumor growth compared to free MTO and prolonged mouse survival, whereas among the liposomal MTO treatments, C8-GluCer-MTOL was most effective. Targeting plasma membranes with SCS improved MTO tumor availability and thereby therapeutic activity and represents a promising approach to improve MTO-based chemotherapy.

1. Introduction

Breast cancer is the second most commonly diagnosed cancer among women after non-melanoma skin cancer, and is the second leading cause of cancer death after lung cancer [1,2]. Chemotherapy is frequently part of the treatment and is applied prior to surgery (neoadjuvant) to decrease tumor size or after surgery (adjuvant) to eliminate possibly remaining or metastasized tumor cells. Chemotherapy is also applied in advanced and recurrent disease. Anthracyclines are often used in breast cancer chemotherapy, next to taxanes, cyclophosphamide, methotrexate, fluorouracil, carboplatin and more recently introduced targeted drugs [3]. Mitoxantrone (MTO) has gained importance in the treatment of metastatic breast cancer as compared to anthracyclines [4,5] due to its similar therapeutic activity and lower drug toxicity at equal doses [6,7].

Nuclear DNA is the major target for MTO. Binding of MTO to DNA causes DNA condensation and inhibition of replication and RNA transcription. MTO is also a potent inhibitor of topoisomerase II, an enzyme involved in control of DNA topology through breaking and rejoining double-stranded DNA [8,9].

MTO, like other amphiphilic drugs crosses the cell membrane by a relatively slow process involving a flip flop [10]. Next, MTO preferentially associates with the inner leaflet of the membrane

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among the phospholipid head groups, rather than inside the lipid bilayer [10]. However, the complex and heterogeneous composition of the plasma membrane and the presence of caveolae and lipid rafts, make the plasma membrane a mosaic-like patchwork [11] with a differentiated lipid distribution that may reduce or prevent drug passage [12]. Poor accumulation of cytotoxic drugs in tumor cells is a major limitation in cancer therapy and membrane lipid and proteins play an important role in drug resistance. At the cellular level resistance can be caused by drug pumps such as the transmembrane P-glycoprotein that competes actively with passive drug transport across the membrane, limiting intracellular drug uptake and efficacy [13]. In addition, more and more evidence suggests that tumor cell membrane lipid composition and its biophysical state contribute to multidrug resistance [12]. To overcome these barriers, we questioned whether the membrane barrier function could be modulated to specifically enhance drug membrane traversal and increase its intracellular accumulation and thus its therapeutic efficacy. Here we aim to apply our previously described concept of targeting the plasma membrane lipid composition using short chain sphingolipids (SCS) [14] as novel drug delivery strategy to improve MTO chemotherapy for breast cancer. To achieve this we made use of a nanovesicle platform with pegylated-bilayer-inserted SCS and MTO encapsulated in its aqueous core [14]. Liposomes composed of DSPE-PEG2000 have good solubility properties in aqueous media preventing liposomal clearance by Mononuclear Phagocytic System (MPS) in spleen and liver by inhibition of nanocarrier recognition. Thereby, liposomes remain longer in circulation and by virtue of the enhanced permeability and retention effect (EPR) in solid tumors, liposomes are able to accumulate at the tumor site for drug delivery (passive targeting) [15–17]. Whereas liposomal encapsulation prolongs systemic circulation and decreases drug toxicity, it is less beneficial for effective delivery of bioavailable drug into tumor cells limiting therapeutic efficacy [18]. However, inserting SCS, such as C_8-Glucosylceramide (C_8-GluCer) or C_8-Galactosylceramide (C_8-GalCer) in the liposomal bilayer improves drug co-delivery to tumor cells through membrane permeabilization [19–22]. This approach combines reduced toxicity through liposomal encapsulation with the advantage of enhanced intracellular drug delivery by SCS-mediated tumor cell membrane traversal.

Here, we present efficacy studies in an orthotopic breast cancer model using SCS-liposomal based MTO chemotherapeutic treatment in comparison with free MTO and optimized standard MTOL. Pharmacokinetics and biodistribution of the MTO formulations were studied. Intratumoral drug delivery was imaged by intravital microscopy.

2. Material and methods

2.1. Lipids and chemical reagents

Hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylethanolamine (DSPE)-PEG_{2000} were from Lipoid (Ludwigshafen, Germany). Short-chain sphingolipids, D-glucosyl-β-1,1′ N-octanoyl-D-erythro-sphingosine (C_8-GluCer), D-galactosyl-β-1,1′ N-octanoyl-D-erythro-sphingosine (C_8-GalCer) and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rho-PE) were from Avanti Polar Lipids (Alabaster, AL, USA).

Polycarbonate filters were from Northern Lipids (Vancouver, BC, Canada) and PD-10 Sephadex columns were from GE Healthcare (Diegem, Belgium). Cholesterol, and HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid) were from Sigma Aldrich (Zwijndrecht, The Netherlands). Hoechst was from Molecular Probes (Leiden, The Netherlands). Phosphate-buffered-saline was from Boom and FACS flow fluid from BD Biosciences. Mitoxantrone-dihydrochloride, 2 mg ml^{-1} (OnKotrone) was from Baxter. Hormonal pellets, 1.5 mg β estradiol 17-Acetate was from Innovative Research of America, Sarasota, Florida, USA. Matrigel membrane Matrix was from BD Biosciences. Hypoxia specific marker HP3-1000 Kit Pimonidazole Hydrochloride, Hypoxyprobe-1 Omni Kit PAb2627AP and Rabbit antisera were from HPI (Burlington, MA, USA). Goat anti Rabbit AF488 and DAPI were purchased from Invitrogen, Carlsbad, CA, USA. Fluoromount-G was from Southern Biotech, AL, USA.

2.2. Liposome preparation

Liposomes were composed of HSPC/Cholesterol/DSPE-PEG_{2000} in a molar ratio of 1.85:1:0.15. To the mixture of lipids 0.1 mol of SCS was added per mole of total amount of lipid (including cholesterol). Standard liposomes and SCS-enriched liposomes containing MTO were prepared by lipid film hydration and extrusion method using a thermobarrel extruder (Northern Lipids, Vancouver, Canada) at 65 °C [23]. Lipids were dissolved in chloroform methanol (9:1 v/v), and a lipid film was created under reduced pressure on a rotary evaporator and subsequently dried under a stream of nitrogen. Lipid film was hydrated by addition of 250 mM of $(NH_4)_2SO_4$, pH 5.5 and liposomes were sized by sequential extrusion through 100-, 80-, and 50 nm polycarbonate filters (Northern Lipids, Vancouver, Canada). Non-encapsulated $(NH_4)_2SO_4$ was removed by gel filtration chromatography using PD-10 Sephadex column, eluted with 135 mM NaCl, 10 mM Hepes buffer, pH 7.4 and drug loading was performed for 1 h at 65 °C, at a drug to phospholipid ratio (D:PL) of 0.07 (w/w) [14]. Size and polydispersity index (PDI) were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) at 25 °C. Lipid concentration was measured by phosphate assay [24].

After separation of free non-encapsulated MTO from liposome-encapsulated MTO, the amount of entrapped drug was measured by fluorimetry ($\lambda_{excitation}$ 607 nm; $\lambda_{emission}$ 684 nm) and measured after entire liposome solubilization with 1% (v/v) Triton in water in relation to a MTO calibration curve.

Fluorescent labeled liposomes were prepared using fluorescent lipid Rhodamine at 0.1 mol% of total amount of lipid.

2.3. Mitoxantrone quantification in plasma and tissue by HPLC-UV

A high performance liquid chromatography (HPLC) assay was developed and validated for the quantification of MTO in mouse plasma and tissue samples of liver, spleen, kidney, heart, lungs and tumor to determine *in vivo* MTO pharmacokinetics and biodistribution after 4 and 24 h [25]. The HPLC separations were performed on a stainless steel analytical GraceSmart RP18 column, 150 mm × 2.1 mm packed with internal diameter 5 µm particle size C-18 material, preceded by a guard column holding an AJ0-A286 C18 cartridge (Phenomenex, Torrance, CA, USA). The isocratic mobile phase was 27:73 (v/v) acetonitrile:ammonium formate (160 mM) with hexanesulfonic acid (35 mM), adjusted to pH 2.7 with formic acid and running at a flow rate of 0.2 ml/min. UV absorption at 655 was monitored using a SF757 UV/VIS detector (Kratos, NJ, USA). Peak detection and integration were done with a Chromeleon data system version 6.8 (Dionex, Sunnyvale, CA, USA).

Standard of MTO, 1000 ng/ml was prepared by dilution of MTO stock solution 2 mg ml^{-1} in water/acetonitrile (80:20), aliquoted and stored at −20 °C.

To determine MTO plasma levels, fresh calibration standards of 30, 100, 300, 1000, 3000 and 10,000 ng/ml were prepared in human plasma for each analytical run in duplicate. Quality control