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Targeted delivery via avidin fusion protein: Intracellular fate of biotinylated doxorubicin derivative and cellular uptake kinetics and biodistribution of biotinylated liposomes

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ABSTRACT

In this study, avidin–biotin technology was combined with a multifunctional drug carrier modality i.e. liposomes to achieve an active and versatile targeting approach. The anti-cancer drug doxorubicin (DOX) was modified with direct biotinylation (B-DOX) (Allart et al., 2003), or encapsulated in biotinylated sterically stabilized pH-sensitive liposomes (BL-DOX), and targeted to the lentiviral vector transduced cells expressing an avidin fusion protein on the cell membrane (Lehtolainen et al., 2003; Lesch et al., 2009). The direct biotinylation of doxorubicin improved cell internalization in rat glioma (BT4C) cells expressing avidin fusion protein receptor but cell toxicity was reduced by 78-fold due to impaired nuclear localization. In contrast, liposomal formulations restored the biological activity of the DOX in several cell lines. However, mainly due to uptake via non-specific pathways the active targeting of BL-DOX was negligible in both *in vitro* and *in vivo* studies. Active targeting with multifunctional drug carrier systems is challenging and further studies will be needed to optimize the properties of targeted drug carrier and receptor expression systems.

1. Introduction

The poor efficiency and severe adverse effects of cancer treatment are mainly due to systemic drug distribution and the multidrug resistance mechanisms appearing in cancer cells. These undesirable properties could be largely avoided by targeting chemotherapeutics to the cancer tissue. Targeting is intended to increase the drug concentration in the tumor tissue while reducing the concentration elsewhere in the body i.e. improving the efficacy of the treatment and minimizing detrimental side effects.

Nanocarriers, such as polymers, nanoshells and liposomes, alter the pharmacokinetics and biodistribution of anticancer drugs. The nanocarriers used for targeting purposes are generally coated with hydrophilic polyethylene glycol (PEG) chains, which hinder the opsonization process and subsequently reduce the plasma clearance (Klibanov et al., 1990). Due to their prolonged circulation time and the enhanced permeability and retention (EPR) effect, nanocarriers are efficiently accumulated in the tumor tissue (Maeda, 2001). The specificity against the tumor tissue can be further enhanced by active targeting approaches that utilize antigens and receptors, which are over-expressed on the target cell membrane (Peer et al., 2007). With these approaches, the active targeting moiety, such as a ligand or a monoclonal antibody, can be coupled directly to a drug molecule or in most cases to the distal end of the PEG chain on the surface of the nanoparticle. The ligand need to possess the appropriate conformation and high affinity towards the corresponding receptor in order to evoke receptor-mediated endocytosis. On the other hand, the expression of the target receptor should be cell specific, high (density 10^4–10^5 copies per cell) (Lopes de Menezes et al., 1998; Park et al., 2002), stable and homogenous in the target tissue to mediate the active targeting. Some naturally over-expressed receptors such as epidermal growth factor (EGF) receptor, folate receptors (FRs) and transferrin (Tf) receptors, are commonly used in targeted cancer therapies (Daniels et al., 2006; Hynes and Lane, 2005; Low and Kularatne, 2009; Nicholson et al., 2001). However, in many cases, these same target receptors are also expressed, albeit at lower levels, in fast-

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growing healthy cells such as fibroblasts, epithelial and endothelial cells (Ekblom et al., 1983; Peer et al., 2007). Moreover, the expression level of the endogenous receptors may vary between individuals. Actively targeted nanocarriers have been studied extensively and, at the moment, there are multiple clinical trials in progress. However, despite huge efforts in the field only a few actively targeted formulations have been approved for clinical use. Therefore, there is still the need to develop new strategies for use in targeted drug therapies.

Avidin, isolated originally from chicken eggs, has an extremely high affinity for the coenzyme biotin (affinity constant $7 \times 10^{14}\,M^{-1}$) (Green and Toms, 1973; Green, 1990). This feature has made the avidin–biotin technology popular for wide-range of biotechnological applications such as imaging and drug targeting. In multi-step targeting approaches, avidin is coupled to antibodies or used as a linker between a biotinylated antibody and a biotinylated therapeutic agent (radionuclide, drug, etc.) (Lesch et al., 2010). In addition, biotin can be covalently linked to almost any kind of molecule via its relatively inert side chain without affecting the avidin binding properties (Richards, 1990; Wilchek and Bayer, 1988). Previously, we have described an avidin fusion protein expression system utilizing avidin–biotin technology (Lehtolainen et al., 2002, 2003). The approach is based on using a virus-mediated transgene vector that leads to the expression of the avidin fusion protein receptor on the surface of the target cell. This protein can selectively bind biotinylated compounds and subsequently trigger endocytosis. The advantage of the avidin fusion protein receptor is its ability to be expressed in any type of tumor tissue (Lehtolainen et al., 2003), which enables the usage of one single ligand instead of requiring different ligands for each tumor type. Previously, we have shown that the avidin fusion protein can be expressed in the target tissue (Lehtolainen et al., 2002, 2003; Lesch et al., 2009), and the functionality of the receptor to bind the biotinylated compounds has been demonstrated under both *in vitro* and *in vivo* conditions (Lehtolainen et al., 2002, 2003). In addition, preliminary results of the potential usage of the avidin fusion protein for therapeutic purposes revealed that biotinylated nanoparticles containing paclitaxel were more cytotoxic towards transduced rat glioma (BT4C) cells than non-targeted nanoparticles or paclitaxel alone. However, a similar trend was observed in the control cells, and the effect seemed to be concentration dependent (Lesch et al., 2009). Thus, avidin fusion protein technology can be considered to be as a promising tool for the active targeting of biotinylated therapeutic compounds even for other applications in addition to cancer.

In this study, the avidin–biotin technology was applied in a two-step targeting approach. First the avidin fusion protein receptor was expressed on the cell membrane and second, transfected cells were treated with either the biotinylated doxorubicin (B-DOX) (Allart et al., 2003) or sterically stabilized pH-sensitive liposomes encapsulated with doxorubicin (BL-DOX). The cytotoxicity of B-DOX was analyzed in BT4C, and the intracellular distribution was evaluated by fluorescence microscopy. Then, the cytotoxicity of BL-DOX was evaluated in several cancer cell lines *in vitro*, which were characterized in terms of efflux protein expression at the RNA level. The cellular uptake kinetics of BL-DOX was also measured quantitatively by high performance liquid chromatography mass spectrometry (HPLC-MS/MS) in BT4C cells. Furthermore, the *in vivo* biodistribution of the BL-DOX in nude mice bearing subcutaneous glioma tumor stably expressing the avidin fusion protein was determined by HPLC-MS/MS.

2. Materials and methods

2.1. Cell lines and culture conditions

Human glioblastoma–astrocytoma (U-87 MG; American Type Culture Collection, Manassas, VA, USA), human glioblastoma (U-118 MG; American Type Culture Collection) and rat glioma (BT4C) (Tyynela et al., 2002) cell lines were maintained in Dulbeccós Modified Eaglés Medium (DMEM; Sigma–Aldrich®, St. Louis, MO, USA) and human renal clear carcinoma (Caki-2; American Type Culture Collection) cell line in McCoy's 5A+GlutaMAX™-I medium (GIBCO®, invitrogen™, Grand Island, NY, USA). The growth media were supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS; GIBCO®, Carlsbad, CA, USA) and 0.1 U/ml penicillin G/0.1 μg/ml streptomycin (Euroclone®, Siziano, Italy). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured twice a week.

2.2. Preparation of the drug-conjugate (B-DOX) and liposomes (BL-DOX)

The biotinylated doxorubicin derivative (B-DOX; MW 869) was prepared as described earlier (Allart et al., 2003). The liposomes were composed of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; Avanti® Polar Lipids, Inc., Alabaster, AL, USA), cholesteryl hemisuccinate (CHEMS; Sigma–Aldrich®), 1,2-diastearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}; Avanti®) and 1,2-diastearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG_{2000} Biotin; Avanti®) and they were prepared with the ammonium sulfate gradient method (Haran et al., 1993) at molar ratios of 6:4:0.3 and 6:4:0.2:0.1 of DOPE/CHEMS/DSPE-PEG_{2000} (L-DOX) and DOPE/CHEMS/DSPE-PEG_{2000}/DSPE-PEG_{2000} Biotin (BL-DOX), respectively. In brief, after evaporation, the lipid film was rehydrated in 250 mM ammonium sulfate (pH 8.5). The liposomes formed were extruded 23 times through two stacked polycarbonate membranes with a pore size of 100 nm (Whatman®, Kent, UK). Then, the liposomes were eluted through a Sephadex® G50 (Pharmacia Biotech AB, Uppsala, Sweden) column which was equilibrated with 10% sucrose in 25 mM Trizma buffer (pH 8.5) in order to exchange the outer buffer of the liposomes. Doxorubicin hydrochloride (DOX; Sigma–Aldrich®) was loaded into the liposomes in a mass ratio of DOX/DOPE 0.2:1 through an ammonium sulfate gradient. Non-capsulated DOX was removed by elution through a Sephadex® G50 (Pharmacia Biotech AB) column equilibrated with 150 mM NaCl in 20 mM HEPES buffer (pH 7.4).

2.3. Characterization of the liposomes

2.3.1. Size distribution

The Gaussian size distribution of the liposomes (app. 80 ± 30 nm) was evaluated by number weighting by employing Nicomp™ 380 ZLS Zeta particle sizer (NICOMP Particle Sizing Systems, Inc., Santa Barbara, CA, USA).

2.3.2. Charge

The charge of the liposomes (L-DOX −2.32 ± 0.27 mV and BL-DOX-0.03 ± 1.00 mV) in 20 mM Hepes 150 mM NaCl buffer (pH 7.4) was measured by Nicomp™ 380 ZLS Zeta particle sizer (NICOMP Particle Sizing Systems, Inc.) (electric field strength 10 V/cm and scattering angle 14.7°).

2.3.3. Phospholipid ratio

The phospholipid ratio of the liposomes was measured as recommended by Fiske and Subbarow (1925).

2.3.4. Liposomal DOX concentration

The liposomal DOX concentrations were determined by measuring the fluorescence intensity of the drug (λ_{ex} 470 nm; λ_{em} 585 nm) against a standard curve of free DOX by using a plate reader Victor^2 1420–012 Multilabel Counter (Perkin–Elmer, Inc., Waltham, MA, USA). The encapsulation efficiency was always more than 90%.