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Design, synthesis and evaluation of *N*-acetyl glucosamine (NAG)–PEG–doxorubicin targeted conjugates for anticancer delivery

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ABSTRACT

Efficacy of anticancer drug is limited by the severe adverse effects induced by drug; therefore the crux is in designing delivery systems targeted only to cancer cells. Toward this objectives, we propose, synthesis of poly(ethylene glycol) (PEG)–doxorubicin (DOX) prodrug conjugates consisting *N*-acetyl glucosamine (NAG) as a targeting moiety. Multicomponent system proposed here is characterized by ^{1}H NMR, UV spectroscopy, and HPLC. The multicomponent system is evaluated for *in vitro* cellular kinetics and anticancer activity using MCF-7 and MDA-MB-231 cells. Molecular modeling study demonstrated sterically stabilized conformations of polymeric conjugates. Interestingly, PEG–DOX conjugate with NAG ligand showed significantly higher cytotoxicity compared to drug conjugate with DOX. In addition, the polymer drug conjugate with NAG and DOX showed enhanced internalization and retention effect in cancer cells, compared to free DOX. Thus, with enhanced internalization and targeting ability of PEG conjugate of NAG–DOX has implication in targeted anticancer therapy.

1. Introduction

Chemotherapeutic drugs like paclitaxel, doxorubicin (DOX), camptothecin, *etc.* are known to be highly potent; however, they are also known to induce severe systemic toxicity, intolerance, and resistance (Galletti et al., 2007). While the translation of such drugs into their polymeric prodrug conjugate offers reduction in systemic toxicity, improve therapeutic index, and enhanced targeting by a mechanism called enhance permeation and retention (EPR) effect (Matsumura and Maeda, 1986; Duncan, 2007). After following cellular uptake by the endocytic route they are potential to bypass mechanisms of drug resistance, leading to enhance tumor targeting (>10–100-fold) compared to free drug (Duncan, 2006). Quite often the polymeric prodrug approach delivers anticancer drug with increased concentration of a drug at a tumor site due to overall passive targeting mechanism (Maeda et al., 1992). It is now very eminent fact that the tumors relatively possess altered physiology and molecular expression at cellular level compared to the normal tissues.

Many studies have been focused in delivering anticancer drugs at the targeted sites using targeting peptides (*e.g.* LHRH (Dharap et al., 2005)), carbohydrates (*e.g.* fucose (Moriwaki and Miyosh, 2010), galactosamine (Pimm et al., 1993), sialic acid (Jayant et al., 2007), *etc.*), monoclonal antibodies (mAbs) (Singh et al., 1989) or ligands (*e.g.* folate) (Haizheng and Lin, 2008). The tumors are dense, with hampered heterogeneous vasculature and altered efflux mechanism thereby leading into lowering of drug disposition at the targeted site (Murray and Carmichael, 1995). Toward this direction, the criticalness in anticancer prodrug therapy is aimed with multifold of objectives: (a) enhance the aqueous solubility of the anticancer drugs, (b) design of suitable drug delivery system (DDS) to attain EPR effect (*e.g.* PEG, dendrimers, liposome, nanoparticle, *etc.*), and (c) utility of suitable targeting moiety with tumor cells internalization capacity. In addition, various synthetic strategies have been explored to improve the pharmacokinetic and pharmacodynamic profile for anticancer prodrug conjugates (Greco and Vicent, 2009). Recently, we reported multifunctional polymers in medicine to achieve cellular targetability and delivery (Khandare et al., 2012). Various architectures of polymers, assemblies, and prodrug conjugates having targeting components

Abbreviations: DOX, doxorubicin; NAG, *N*-acetyl glucosamine; PEG, polyethylene glycol; DMF, *N*,*N*-dimethyl formamide; DCM, dichloromethane; EDC.HCl, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride; DMAP, *N*,*N*-diisopropyl-ethylamine,4-(methylamino) pyridine; EPR, enhance retention and permeation; LHRH, luteinizing hormone–releasing hormone; mAbs, monoclonal antibodies; TFA, trifluoroacetic acid; MAL, maleimide; GTS, glutathione; DIEA, *N*,*N*-diisopropyethyl amine; MS, material studio; PBS, physiological saline buffer solution; Fmoc, *N*-(9-fluorenylmethoxycarbonyl); HPMA, poly-2-hydroxypropylmethacrylamide; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; DAPI, 4′,6-diamidino-2-phenylindole.

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are being reported to enhance the anticancer activity using folate, RGD peptide, VEGF, LHRH, *etc.* However, newer ligands are being explored and bioconjugation methodologies using established polymers (including, PEG and other newer dendritic polymers) are developed. Therefore in this work, NAG is being evaluated as a targeting moiety, considering NAG as a ligand for the NAG receptors (NAGRs) over expressed on cancer cells (Dhanikula et al., 2008; Vannucci et al., 2003). In addition, NAG being a carbohydrate is excessively aqueous soluble, thereby may increase the aqueous solubility to the resulting PEG conjugate and it is also likely to have greater cellular internalization capacity. Thus, NAG could be used as a targeting ligand for targeting anticancer drugs to tumors that over express NAG receptors.

Tumor cells multiply rapidly and may over-express certain receptors for enhanced uptake of nutrients, including folic acid, vitamins, and sugars (Jayant et al., 2007; Haizheng and Lin, 2008). While active targeting can be achieved by conjugating a tumor-specific ligand or a polymer to the chemotherapeutic drug *via* a cleavable linker (Khandare and Minko, 2006). Furthermore, specific tumor uptake can occur through receptor-mediated endocytosis, where upon binding of the ligand-modified polymer with the cell-surface receptor leads to internalization of the entire polymer–receptor complex and vesicular transport through the endosomes (Garnett, 2001). In addition, the targeting moieties on to the delivery system are known to enhance the specificity toward tumor with increase in penetrating property of DDS (Minko et al., 2004).

Overall, targeted prodrug conjugates can offer increase in efficacy and decrease in systemic toxicity in cancer therapy (Chun and Sidney, 2008). Therefore, the main objective of this work is to establish (a) design and synthesis polymeric prodrug conjugate containing PEG as a carrier, DOX as a anticancer drug, NAG as a targeting moiety and conjugation using a glutathione as a spacer, (b) to characterize the PEG conjugate, (c) evaluate cellular entry and dynamics for comparative internalization and localization, and (d) to evaluate and compare *in vitro* efficacy using human MCF-7 and MDA-MB-231 cell line.

In the past, we have reported targeted prodrug conjugates consisting anticancer drugs; camptothecin, DOX, and paclitaxel using poly (ethylene glycol) linear polymer and hyperbranched dendrimers (Jayant et al., 2007; Saad et al., 2008). Furthermore, we reported varied targeting and internalization ligands (*e.g.* LHRH, and sialic acid) for increased cellular internalization ability and multicomponent DDS (Dharap et al., 2005; Jayant et al., 2007).

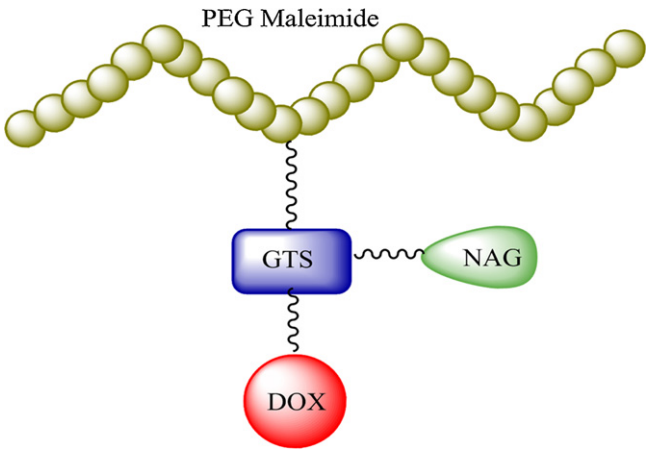

Fig. 1. Schematic depiction of targeted polymer prodrug conjugate. Glutathione (GTS), *N*-acetyl D-glucosamine (NAG), doxorubicin (DOX), and PEG-maleimide (30 kDa) (PEG).

Here we report design, synthesis, and *in vitro* evaluation of targeted PEG–DOX prodrug conjugates having NAG as a targeting moiety for enhanced cellular delivery ability and anticancer activity. We present PEGylated prodrug conjugate consisting PEG-maleimide 30,000 Da (MAL) as a carrier, DOX as an anticancer drug, and NAG as a targeting moiety and penetration enhancer (Fig. 1). In addition, we evaluated comparative cellular internalization dynamics of free DOX and PEG-drug conjugates using confocal microscopy. Drug release rate from the conjugates was analyzed by HPLC using esterase enzyme. Finally, *in vitro* cytotoxicity of the conjugates was evaluated and compared using human MCF-7 and MDA-MB-231 breast cancer cells.

2. Materials and methods

2.1. Materials

Doxorubicin was provided as a gift sample by RPG Life sciences, India. Methoxy PEG–maleimide (30 kDa) (PEG–MAL) was procured from Sunbright, UK. *N*-Acetyl D-glucosamine (NAG), glutathione (GTS), succinic anhydride, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimideHCl (EDC.HCl), *N,N*-diisopropyl-ethylamine, 4-(methylamino) pyridine (DMAP), and Sephadex G10 were purchased from Sigma–Aldrich, India. 9-Fluronyl methylcarbonyl formic acid (FmoC) was purchased from Merck, Germany. Dialysis membrane of molecular weight cut off 10–12 kDa was purchased from Hi-media, India. MCF-7 and MDA-MB-231 cells were procured from ATCC. 4′,6-Diamidino-2-phenylindole (DAPI) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were purchased from Invitrogen. All other chemicals and solvents were of analytical grade and used without purification.

2.2. Synthesis of PEG–DOX–NAG

Synthesis of PEG–DOX–NAG conjugate was achieved by two steps. First, synthesis of succinate derivative of DOX and further conjugation of synthesized DOX-succinate with PEG–GTS–NAG.

2.2.1. Synthesis of N-Fmoc-DOX-14-0-Succinate

Synthesis of *N*-Fmoc-DOX-14-*0*-succinate (compound **5**) conjugate is schematically represented in Scheme 1. DOX (compound **1**) was reacted with succinic anhydride (compound **4**) by two-step method as previously reported (Chena et al., 2003). DOX (92 μM) and sodium bicarbonate (280 μM) were dissolved in distilled water (5 ml). The resulting clear solution having pH 8–9 was stirred continuously on magnetic stirrer at 5–10 °C. 9-Fmoc chloride (compound **2**) (110 μM) in ethyl acetate (3 ml) was added drop wise into the above solution. The reaction mixture was maintained in the pH range of 7–8 with an addition of saturated solution of sodium bicarbonate. After 5 h, the product was extracted in ethyl acetate and further recrystallized in 0.1% trifluroacetic acid (TFA). The crystals were further washed with cold ether to remove excess of Fmoc. After drying in a desiccator, *N*-Fmoc-DOX was recovered. This intermediate was reacted with succinic anhydride (100 μM) in 3 ml of anhydrous DMF in the presence of triethylamine (150 μM). The mixture was stirred at room temperature. After 12 h, the reaction was stopped, and the solvent was evaporated under vacuum. The residue was dissolved in ethyl acetate (15 ml) and washed with water (25 ml). After drying over anhydrous sodium sulfate, the solvent was removed under vacuum. The red colored crude product was purified by a silica gel chromatographic column using ethyl acetate (95):hexane (5) as an eluent. The both reactions were carried out in dark.