A lysosome-targeted drug delivery system based on sorbitol backbone towards efficient cancer therapy†

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A straightforward synthetic approach was adopted for the construction of a lysosome-targeted drug delivery system (TDDS) using sorbitol scaffold (Sor) linked to octa-guanidine and tetrapeptide GLPG, a peptide substrate of lysosomal cysteine protease, cathepsin B. The main objective was to efficiently deliver the potential anticancer drug, doxorubicin to the target sites, thereby minimizing dose-limiting toxicity. Three TDDS vectors were synthesized viz., DDS1: Sor-GLPG-Fl, DDS2: Sor-Fl (control) and DDS3: Sor-GLPGC-SMCC-Dox. Dox release from DDS3 in the presence of cathepsin B was studied by kinetics measurement based on the fluorescent property of Dox. The cytotoxicity of DDS1 was assessed and found to be non-toxic. Cellular internalization and colocalization studies of all the 3 systems were carried out by flow cytometry and confocal microscopy utilizing cathepsin B-expressing HeLa cells. DDS1 and DDS3 revealed significant localization within the lysosomes, in contrast to DDS2 (control). The doxorubicin-conjugated carrier, DDS3, demonstrated significant cytotoxic effect when compared to free Dox by MTT assay and also by flow cytometric analysis. The targeted approach with DDS3 is expected to be promising, because it is indicated to be advantageous over free Dox, which possesses dose-limiting toxicity, posing risk of injury to normal tissues.

Introduction

Targeted drug delivery system (TDDS) development is one of the challenging areas in pharmaceutical research that requires a multidisciplinary approach for the delivery of therapeutics to the site of action, without affecting healthy tissue or organ. Delivery systems constructed by utilizing target specific groups mainly small molecules like peptide substrates, heterocyclics, oligonucleotides and monoclonal antibodies have been widely demonstrated by many research groups.1-6 Focusing on cancer therapy, monoclonal antibodies against tumor-specific antigens have occasionally been successful in targeting tumors, but their irreducible bulk hinders the penetration into solid tumors and the excretion of unbound reagent. Moreover, elaborate reengineering is required to minimize immunogenicity.7,8 In recent years, drug delivery systems based on mesoporous silica nanoparticles (MSNPs) and polymeric carriers e.g. N-(2-hydroxypropyl)methacrylamide (HPMA) have been well studied.9-12 These carriers are known for passive targeting that takes the benefit of EPR effect (enhanced permeation and retention effect) of the tumor tissue. Such systems are simply distributed by blood circulation and are hardly selective. Hence, a majority of administered nanoparticles are known to accumulate in other organs, in particular, the liver, spleen and lungs. Keeping these in mind, researchers have attempted to construct drug delivery systems by considering various cellular proteases as target sites.13,14

It has been reported that lysosomal delivery is one of the potential targets for cancer treatment.7,15 Proteases of the cathepsin family are among the most studied lysosomal hydrolases. Although cathepsins are predominantly expressed and optimally active in acidic endosomal/lysosomal compartments, they are also found to be extracellularly active at physiological pH, in membrane-bound and soluble forms.16,17 Among these proteases, cathepsin B (Cat B), a lysosomal cysteine protease, is highly upregulated in malignant tumors and premalignant lesions at the mRNA and protein levels.18 An overexpression of Cat B has been associated with oesophageal adenocarcinoma, breast cancer and other tumors.19-21 Because Cat B expression is closely related to the invasive behavior of tumors, it could be a promising target for novel drug delivery systems designed against invading tumor cells.

Cat B cleaves various Cat B-specific peptide substrates viz., Leu, Arg-Arg, Ala-Leu, Phe-Arg, Phe-Lys, Ala-Phe-Lys, Gly-Leu-Phe-Gly, Gly-Phe-Leu-Gly and Ala-Leu-Ala-Leu, out of which
tetrapeptide, Gly-Leu-Phe-Gly (GLPG), has been proven to be the most effective with respect to both plasma stability and rapid hydrolysis in the presence of Cat B. Targeting Cat B enzyme in Cat B-enriched tumor cells enhances the efficacy of the anticancer drug, whilst minimizing toxicity to normal tissues. Considering this factor, we developed a synthetic strategy of a TDDS using Cat B peptide sequence GLPG, in conjugation with a sorbitol core linked to multiple guanidine groups targeting the lysosomes of tumor cells and tissues. Transporters constructed on a sorbitol scaffold linked to guanidine residues by a methylene spacer, mimicking the Arg-8-mer or Tat (residues 49–57), showed significant translocation across the cell membrane, mitochondria and blood–brain barrier efficiently. The major advantage of a carbohydrate scaffold-like sorbitol as the delivery carrier is that it possesses the highest density of functionality among organic compounds in terms of multiple hydroxyl groups. These groups are intended for divergent synthetic strategies facilitating the transport of disparate cargos (molecular drugs, proteins, nucleic acids). Additionally, sorbitol naturally occurs in plants especially in apples, pears, cherries and are largely devoid of any toxicity. It is also postulated that positively-charged guanidine groups shows association with negatively-charged phospholipids and other negatively charged residues on cell surface by electrostatic interaction via hydrogen bond formation, facilitating cellular entry through the lipid bilayer.

Our key interest is to deliver doxorubicin, a potential anticancer drug, utilizing this synthetic delivery system. The clinical applications of this drug have long been limited due to its severe dose-limiting toxicity. Taking advantage of the cleavable Cat B peptide sequence, a higher Dox concentration will be attained in tumor tissue when compared to normal tissue. The proposed mechanism of drug delivery is illustrated in Fig. 1.

Results and discussion

The TDDS synthesized on sorbitol scaffolds are represented as: Sor-GLPG-Fl (DDS1), Sor-Fl (DDS2) and Sor-GLPGC-SMCC-Dox (DDS3) (Scheme 1). DDS1 is the targeted delivery carrier where the two terminal primary hydroxyl groups of sorbitol have been utilized for the conjugation of (1) Cat B-specific tetrapeptide i.e. N-acyl protected tetrapeptide, Ac-Gly-Leu-Phe-Gly-OH denoted as GLPG and (2) a fluorophore i.e. fluorescein (Fl). DDS2 has been used as the control where both the primary hydroxyl groups of sorbitol have been attached to Fl molecules by ester bond. In DDS3, both the primary hydroxyl groups of sorbitol are conjugated with GLPGC, which are further linked to Dox via succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC).

At one end of SMCC, Dox is coupled by an amide bond, while at the other end, the cysteine residue of GLPGC is linked to the maleimide group of SMCC. In this synthetic construct, Dox is covalently conjugated to the carrier, and the ratio of loading of drug to carrier is 2 : 1. Cat B peptide sequence was synthesized by solid-phase synthesis using manual coupling of HMPB-MBHA resin (ESI, Sec. 1.1†). All the three DDS constructs were purified by reversed-phase (C18) column chromatography after Boc-group deportation from guanidine moiety. The key intermediates and target products, DDS1, 2 and 3 were characterized by HPLC, NMR spectroscopy and MALDI-TOF mass spectrometry (details of synthetic steps are described in ESI, Sec. 1.2 to 1.5†).

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To investigate the drug release of Dox-conjugated carrier, DDS3, we incubated DDS3 (60 µg/100 µL, in 50 mM NaOAc and 1 mM EDTA, pH = 5.1) with cathepsin B enzyme (62 ng/1 µL) at a ratio of 9:1, respectively. Taking advantage of the intrinsic fluorescent property of Dox, its release from DDS3 was assessed by fluorescence measurement at 590 nm (details of protocol given in ESI, Sec. 1.7†). Dox release generally occurs in the presence of lysosomal cysteine protease, Cat B in acidic pH. The protease cleaves the specific peptide substrate, subsequently releasing Dox.26 As shown in Fig. 2, above 50% of Dox release occurred in the presence of enzyme at 20 h. Moreover, the stability of the Cat B peptide substrate27 in DDS3 was evaluated at different pH conditions, which confirmed no significant drug release even at a physiological pH (ESI; Fig. S2†).

In vitro cell-based assays that examined the uptake and targeting efficiency of the carriers have been carried out in HeLa (human cervical cancer cell line) cells expressing cathepsin B.28 DDS1 was first tested for its toxicity in HeLa cells by MTT assay (details in ESI, Sec. 1.8†). Fig. S3† shows the relative cell viability on incubation with different concentrations of DDS1 for 24 h. DDS2 also showed high cell viability even at higher concentrations (data not shown). We next investigated the cellular internalization of DDS1 and DDS2 by flow cytometry (details in ESI, Sec. 2.2†). Both DDS1 and DDS2 were internalized by the cells demonstrated by the mean cell fluorescence levels in the FITC-A histograms (Fig. S4†). DDS1 uptake is evident by a shift in the fluorescence peak towards the right with regard to the untreated control. A further shift in the peak with regard to DDS1 uptake reveals DDS2 cellular internalization (Fig. 3).

Further support for cellular uptake came from fluorescent imaging (details in ESI, Sec. 1.9†). DDS1 was found to localize in definite regions of the cytosol, as observed from its fluorescence pattern, whereas DDS2 was found to accumulate in the entire region of the cell as fluorescence was found to be diffused, rather than localized (Fig. S4†). As the fluorescence of DDS1 was found to be localized in definite regions of the cell, we examined the specific localization of DDS1 in intracellular organelles by the selective permeabilization of the plasma membrane using digitonin (details in ESI, Sec. 2.0†). Prior to digitonin treatment, we could observe a uniform green cytoplasmic fluorescence corresponding to the cytosolic probe, calcein, in all the cells (Fig. 4A). But calcein fluorescence was completely lost within 10 min of digitonin treatment, demonstrating the selective permeabilization of the plasma membrane (Fig. 4B). On the contrary, a punctiform pattern of green fluorescence was observed in the cells even after 2 hours of digitonin treatment. This punctiform fluorescence that remained intact indicates an unambiguous localization of DDS1 in intracellular organelles (Fig. 4C). This punctiform fluorescence was absent for DDS2, demonstrating that DDS2 was localized only in the cytosol (Fig. S5†). However, a red punctiform fluorescence was retained for DDS3 showing its localization in intracellular organelles, which is similar to DDS1 (Fig. S5†). These organelles were presumed to be the lysosomes, which were further confirmed by colocalization studies using confocal microscopy (details in ESI, Sec. 2.1†).

DDS1 was found to significantly localize in the lysosomes as evident from the merged/overlaid image of lysotracker red and DDS1 (Fig. 5B). DDS3 was also found to localize within the lysosomes as evident from the merged image of lysosome GFP and DDS3 (Fig. 5C). Neither DDS1 nor DDS3 was found to...
concentrate in the nucleus (Fig. S6†). DDS2 did not show any specific organelle localization (data not shown). Overall, our results provide information that DDS1 and DDS3 are confined to the lysosomes. Furthermore, the intrinsic fluorescent property of Dox has been exploited here to visualize the subcellular localization of DDS3.29

Flow cytometric analysis of DDS3 indicated its cellular uptake by the mean fluorescence levels in the PE-A histograms (red peaks; 570 nm emission). Dot plots and corresponding histograms of A (a & b), untreated control cells; B (c & d), 5 µM; C (e & f), 10 µM; D (g & h); 20 µM; and E (i & j), 30 µM. A concentration-dependent increase in dead cell population has been shown by the green peaks in the histograms. The dead cell population in the dot plots was gated and is shown as population P2 (green).

 DDS3 stimulated significant cytotoxicity when compared to free Dox, which establishes the improved efficiency of targeted Dox-conjugated carrier over free Dox. This result is consistent with a previous study using chitosan/DOX/TAT where the conjugate was more effective than free Dox in killing CT-26 cells.29 In contrast, the free carrier DDS1 did not reveal any cytotoxicity under the same conditions (Fig. 7).
In summary, as a proof-of-concept, we demonstrated a lysosome-targeted drug delivery system which was constructed utilizing a sorbitol backbone with an octa-guanidine unit responsible for efficient cellular uptake. For lysosomal targeting, we introduced a Cat B tetrapeptide sequence into the sorbitol carrier. The release of Dox from the drug conjugate, DDS3, in the presence of cathepsin B enzyme was monitored by kinetics measurement based on fluorescence. Cellular internalization and targeting efficiency were examined in HeLa cells that express cathepsin B. The targeting efficiency of DDS1 to intracellular organelles was made obvious by selective permeabilization of the plasma membrane, whereas the specific lysosomal targeting efficacy was unveiled by colorization with lysotracker dye. Similarly, DDS3, the Dox-carrier conjugate, showed significant lysosomal localization. Cytotoxicity was evaluated for DDS1, DDS3 and free Dox. Interestingly, we observed enhanced cytotoxicity for DDS3 when compared to the free Dox. However, DDS1 did not show any noticeable toxicity even at high concentrations.

Conclusions

Hence, the synthetic targeted carrier conjugated with Dox is suggested to have the following built-in advantages: (1) efficient targeted delivery of the anticancer drug as a result of its intracellular release, probably by an enzymatic cleavage of the Cat B peptide and (2) enhanced cytotoxicity via Dox-attached carrier in the tumor tissues and reduction in undesirable side effects in normal cells and tissues; this may reduce dose-limiting toxicity in chemotherapy. In accordance, a previous study demonstrated that a cathepsin-B cleavable doxorubicin prodrug (Ac-Phe-Lys-PABC-DOX) has increased anti-metastatic effects and reduced side effects, especially cardiotoxicity in a hepatocellular carcinoma model system. Nonetheless, we believe that in vitro studies are not adequate, and the results obtained in this study provide a firm foundation for future investigations of pharmacokinetic profile using in vivo xenograft model.

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Notes and references