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# A Lysosome - Targeted Drug Delivery System based on Sorbitol Backbone towards Efficient Cancer Therapy

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*A straightforward synthetic approach has been 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 major objective is to deliver the potential anti-cancer drug, doxorubicin to the target sites efficiently, thereby minimizing dose-limiting toxicity. Three TDDS vectors have been 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. Cytotoxicity of DDS1 has been assessed and was found to be non toxic. Cellular internalization and co localization studies of all the 3 systems have been 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, since 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 the growing need of 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 demonstrated widely 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 of solid tumors and 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 (HMPA) 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 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 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 best 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, as membrane-bound and soluble forms.[16,17] Among these proteases, cathepsin B (Cat B), a lysosomal cysteine protease, is highly up regulated in malignant tumors and premalignant lesions at the mRNA and protein levels.[18] Overexpression of Cat B has been associated with oesophageal adenocarcinoma, breast cancer and other tumors.[19-21] Since 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.[22] Targeting Cat B enzyme in Cat B - enriched tumor cells, enhances efficacy of the anti-cancer drug, whilst minimizing toxicity to normal tissues. With this consideration in mind, we developed a synthetic strategy of a TDDS using Cat B peptide sequence GLPG, in conjugation with sorbitol core linked to multiple guanidine groups targeting to 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.[23, 24] 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 transport of disparate cargos (molecular drugs, proteins, nucleic acids). Additionally, sorbitol occurs naturally in plants especially in apples, pears, cherries and largely devoid of any toxicity. It is also postulated that positively charged guanidine groups shows association with cell -surface negatively charged phospholipids and other negatively charged residues by electrostatic interaction via hydrogen bond formation, facilitating cellular entry through the lipid bilayer. [25] Our key interest is to deliver doxorubicin, a potential anti-cancer 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 has been illustrated in Fig. 1.

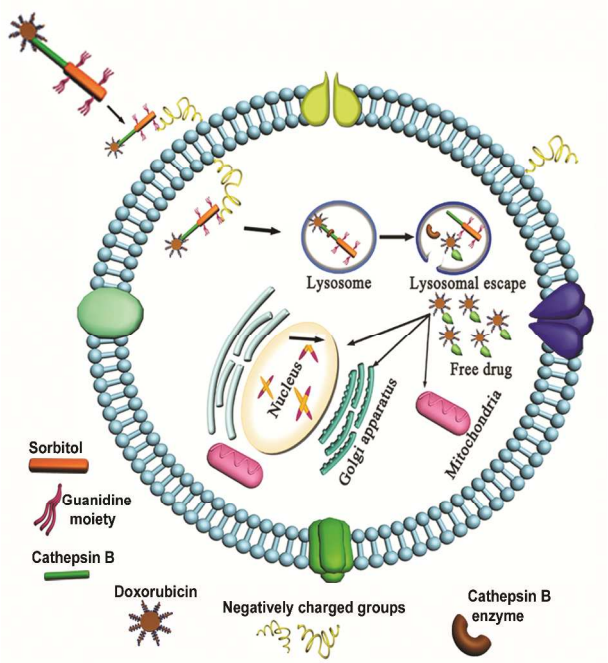
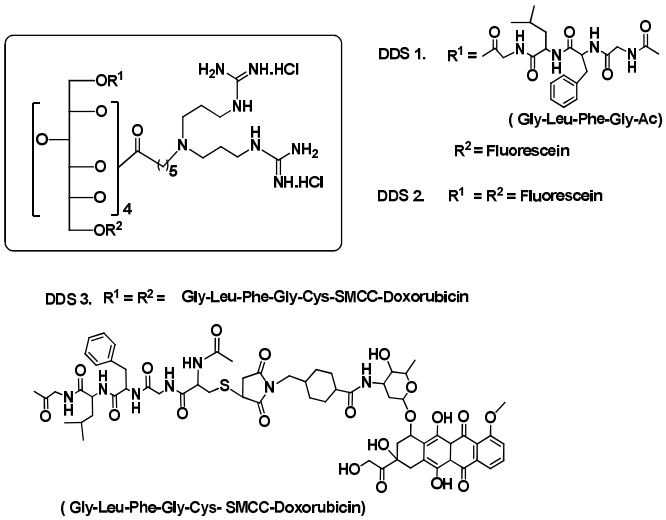



**Fig. 1** Schematic representation of the proposed mechanism of drug delivery by the TDDS based on sorbitol scaffold. The TDDS is internalized through the lipid bilayer by electrostatic interaction between the guanidine moieties and negatively charged groups such as phospholipids / sulphates on the cell surface. The TDDS then enters into lysosomes, where doxorubicin is released by lysosomal cysteine protease, cathepsin B.

## 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 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) cylohexane-1-carboxylate (SMCC).




**Scheme 1.** Synthetic construct of sorbitol based octa-guanidine carriers DDS1, DDS2 and DDS3

At one end of SMCC, Dox is coupled by amide bond, while at the other end, cysteine residue of GLPGC is linked to the maleimide group of SMCC. In this synthetic construct, Dox has been covalently conjugated to the carrier and the ratio of loading of drug to carrier is 2: 1. Cat B peptide sequence has been synthesized by solid phase synthesis using manual coupling of HMPB-MBHA resin (supporting information (SI), sec. 1.1). All the three DDS constructs have been 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 have been described in SI sec. 1.2 to 1.5).

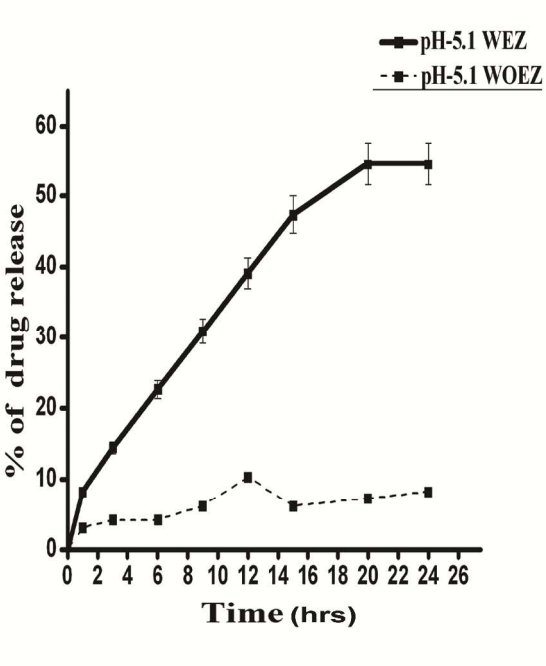



**Fig. 2** Line graph showing the release of doxorubicin from DDS3 in the presence of cathepsin B enzyme at pH 5.1. The analysis was based on the percentage increase in the intrinsic fluorescence of doxorubicin caused due to its release. WEZ denotes *with enzyme* and WOEZ denotes *without enzyme*. Data represented as mean ± standard deviation (SD), n=3.

To investigate the drug release of Dox conjugated carrier, DDS3, we incubated DDS3 (60µg/100 µL, in 50mM NaOAc and 1mM EDTA, pH=5.1) with cathepsin B enzyme (62ng/1µL) 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 SI. sec. 1.7). Dox release generally occurred 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 Figure 2, above 50 % of Dox release occurred in the presence of enzyme at 20 h. Moreover, stability of the Cat B peptide substrate[27] in DDS3 has been evaluated at different pH conditions, which confirmed no significant drug release even at physiological pH (SI; Fig. S2).

*In vitro* cell- based assays have been carried out in HeLa (human cervical cancer cell line) cells expressing cathepsin B [28] that examined the uptake and targeting efficiency of the carriers. DDS1 was first tested for its toxicity in HeLa cells by MTT assay (details of protocol described in SI.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 concentrations (data not shown). We next investigated the cellular internalization of DDS1 and DDS2 by flow cytometry (details in SI 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. 3). DDS1 uptake is evident by a shift in the fluorescence peak towards the right with regard to untreated control. A further shift in the peak with regard to DDS1 uptake reveals DDS2 cellular internalization (Fig. 3).

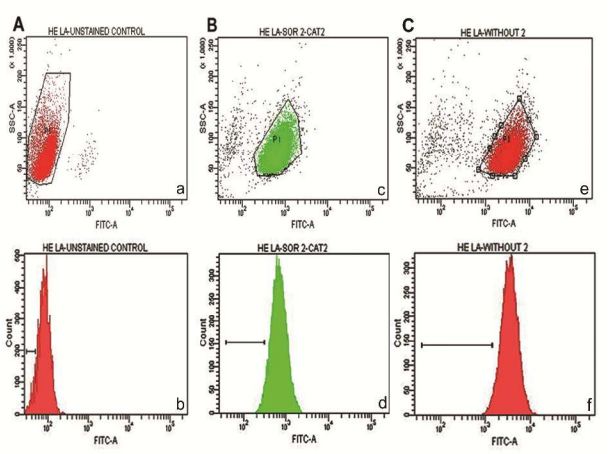



**Fig. 3** Mean fluorescence (530nm emission) levels measured by flow cytometry demonstrating cellular uptake of DDS1 and DDS2 compared to untreated control cells. Dot plots and corresponding histograms: A (a & b) denote untreated control (shown in red), B (c & d) denote DDS1 (shown in green), C (e & f) denote DDS2 (shown in red).

Further support for cellular uptake came from fluorescent imaging (details in SI 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 the 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 specific localization of DDS1 in intracellular organelles by selective permeabilization of the plasma membrane using digitonin (details in SI sec.2.0). Prior to digitonin treatment, we could observe uniform green cytoplasmic fluorescence corresponding to the cytosolic probe, calcein, in all the cells (Fig. 4A). But calcein fluorescence was completely lost within 10min of digitonin treatment demonstrating 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 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). But a red punctiform fluorescence was retained for DDS3 showing its localization in intracellular organelles similar to DDS1 (Fig. S5). These organelles were presumed to be the lysosomes, which were further confirmed by co localization studies using confocal microscopy (details in SI sec. 2.1).

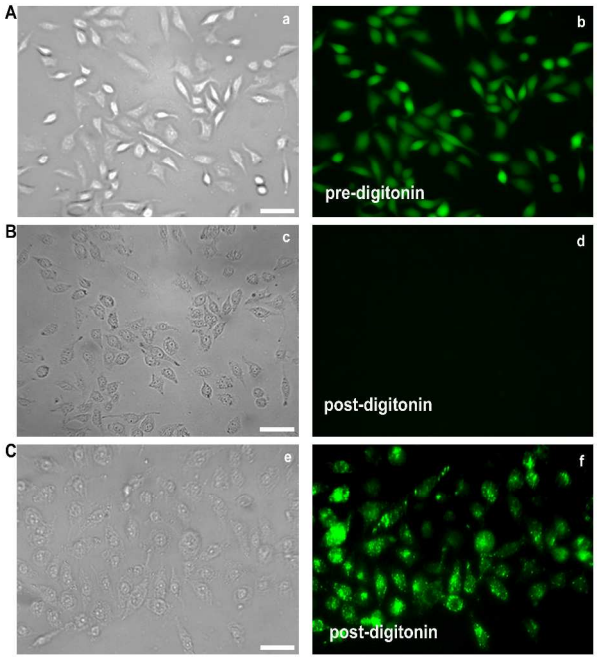



**Fig. 4** Specific localization of DDS1 in intracellular organelles: transmitted light and corresponding fluorescence images. A-b denotes calcein (515 nm emission) incorporated cells prior to permeabilization by digitonin. B-d denotes loss of calcein fluorescence within 10min of digitonin treatment in cells loaded only with calcein, demonstrating permeabilization of plasma membrane. C-f denotes punctiform green fluorescence of DDS1 (530nm emission) retained even after 2 hrs of digitonin treatment in cells loaded with both DDS1 and calcein. A-a, B-c and C-e show the transmitted light images of A-b, B-d and C-f respectively. Scale bar:25µm

DDS1 was found to localize significantly in the lysosomes as evident from the merged/ overlayed 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 organellar localization (data not shown). Overall, our results provide information that DDS1 and DDS3 confined to the lysosomes. Furthermore, the intrinsic fluorescent property of Dox has been exploited here to visualize the sub cellular localization of DDS3. [29]

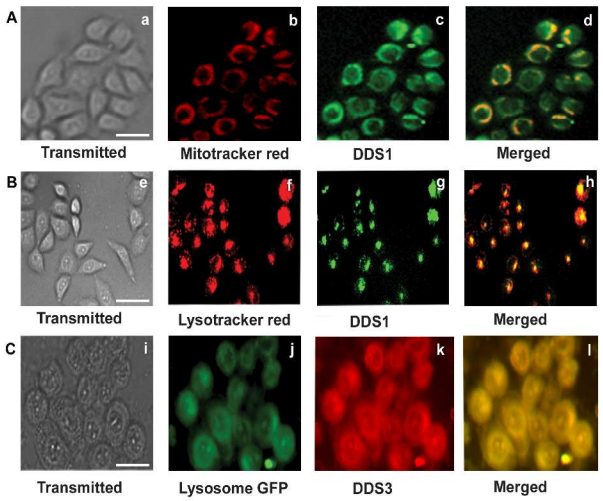



**Fig. 5** Co localization studies by confocal microscopy: A. A little, but insignificant co localization of DDS1 (30µM) observed within the mitochondria, indicated by the merged image [A-d] of mitotracker red (100nM) [A-b] & DDS1 [A-c]. B. Significant co localization of DDS1 within the lysosomes, indicated by the merged image [B-h] of lysotracker red (50nM) [B-f] & DDS1 [B-g]. C. Significant colocalization of DDS3 (30µM) within the lysosomes, indicated by the merged image [C-l] of lysosome GFP [C-j] & DDS3 [C-k]. A-a, B-e and C-i denote the corresponding transmitted light images. Scale bar: 25µm.

Flow cytometric analysis of DDS3 indicated its cellular uptake by the mean fluorescence levels in the PE-A histograms (Fig. 6). The therapeutic efficiency of DDS3 has also been disclosed by the concentration - dependent increase in cell death that it provoked. At 30µM, DDS3 induced 62.5± 6.3 % cell death. The percentage of cells showing the intrinsic red fluorescence of DDS3 was found to decrease with increasing concentrations (5µM: 58%, 10µM: 49.9%, 20µM: 38.5%, 30µM: 23.4%) suggesting increase in cell death (Fig. 6).

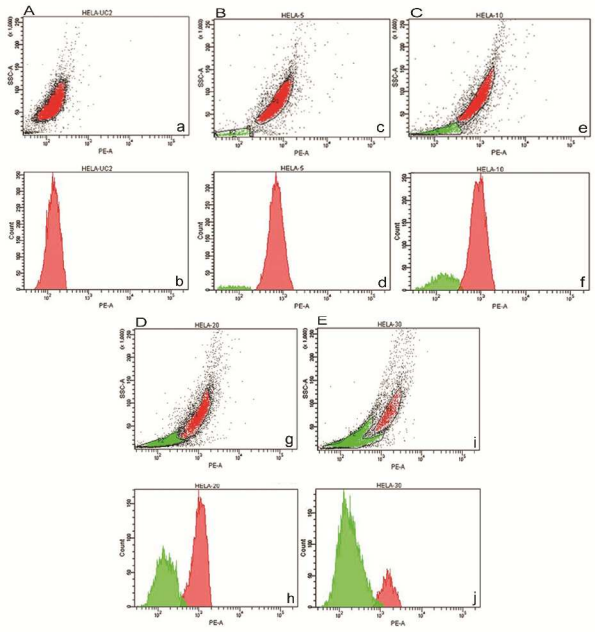


**Fig. 6** Flow cytometric data showing cellular uptake as well as cytotoxicity induced by different concentrations of DDS3. Cellular uptake has been demonstrated by the mean fluorescence levels in the PE-A histograms (red peaks; 570nm emission). Dot plots and corresponding histograms of A(a&b)-untreated control cells, B(c&d) - 5μM, C (e&f) -10μM, D (g&h) -20μM and E (i & j)-30μM. 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 has been gated and is shown as population P2 (green).

We next investigated the beneficial effect of DDS3 in comparison to free Dox by MTT assay. The results summarized in Fig. 7 demonstrate that 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).

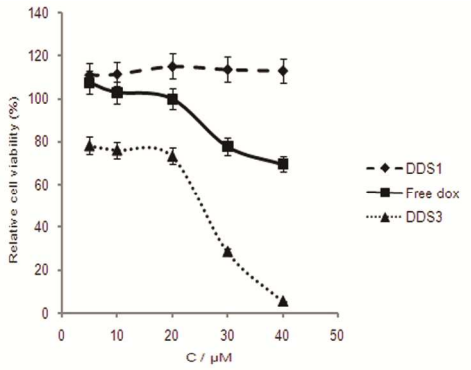



**Fig. 7** MTT assay showing relative viability of HeLa cells on incubation with DDS1, free DOX and DDS3 individually. The line graphs show that DDS1, the free carrier, does not induce any cytotoxicity, whereas the cytotoxicity stimulated by DDS3, the conjugated DOX, is higher than that of free DOX. *C* stands for concentration. Data expressed as mean±SD, n=6.

In summary, as a proof-of-concept, we have demonstrated a lysosome - targeted drug delivery system that has been constructed utilizing a sorbitol backbone with an octa-guanidine unit responsible for efficient cellular uptake. For lysosomal targeting, we have introduced Cat B tetrapeptide sequence into the sorbitol carrier. The release of Dox from the drug conjugate, DDS3, in the presence of cathepsin B enzyme, has been monitored by kinetics measurement based on fluorescence. Cellular internalization and targeting efficiency have been examined in HeLa cells that express cathepsin B. The targeting efficiency of DDS1 to intracellular organelles has been made obvious by selective permeabilization of the plasma membrane, whereas the specific lysosomal targeting efficacy has been unveiled by co localization 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 free Dox. However, DDS1 did not show any noticeable toxicity even at high concentrations.

## Conclusions

Hence the synthetic targeted carrier conjugated with Dox has been suggested to have the following applied advantages: 1) efficient targeted delivery of the anti-cancer drug as a result of its intracellular release, probably by enzymatic cleavage of Cat B peptide 2) enhanced cytotoxicity via Dox- attached carrier in the tumor tissues and reduced undesirable side effects in normal cells and tissues; this may reduce dose-limiting toxicity as in chemotherapy. Supportive to this, a previous study demonstrated that a cathepsin-B cleavable doxorubicin prodrug (Ac-Phe-Lys-PABC-DOX) had increased anti-metastatic effects and reduced side effects, especially cardiotoxicity in a hepatocellular carcinoma model system.[30] Nonetheless, we believe that *in vitro* studies are not just adequate and the results obtained in this study provide a firm foundation for future investigations of pharmacokinetic profile using *in vivo* / xenograft model.

## Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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