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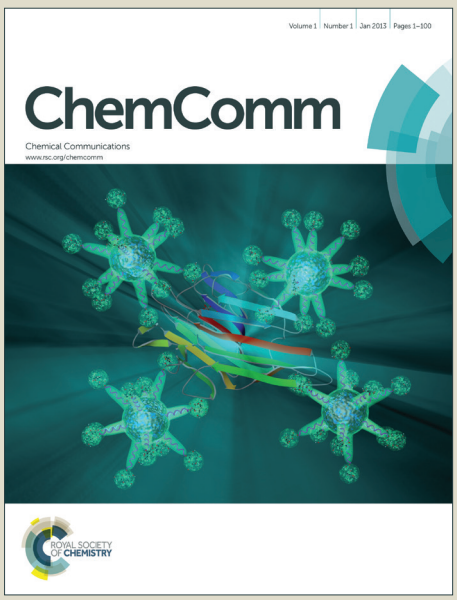

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COMMUNICATION

Novel Lysosome Targeted Molecular Transporter Built On Guanidinium-Poly-(propylene imine) Hybrid Dendron For Efficient Delivery of Doxorubicin Into Cancer Cells

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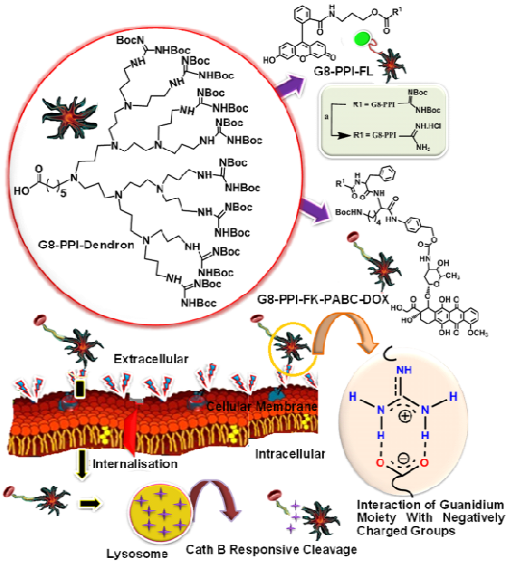
An efficient synthetic approach has been adopted to construct new dendron-based octa-guanidine appended molecular transporter with lysosomal targeted peptide-doxorubicin conjugate. Transporter alone (G8-PPI-FL) is found non-toxic, showed higher cellular uptake compared to Arg-8-mer, exhibited excellent selectivity towards lysosome in Cathepsin B expressing HeLa cells, while Dox-conjugate showed significant cytotoxicity to cancer cell without affecting the non cancerous cell.

Over the last decades target specific drug delivery of anticancer drugs have huge interest in pharma industries because some of them successfully delivered at the right target with required doses, which minimizes dose limiting toxicity[1]. Most of the widely used anticancer drugs are highly hydrophobic (poor bioavailability) in nature and lack of specificity to particular tumor site, causes severe toxicity to normal tissues and organs. It is therefore a challenging task of pharmaceutical and medicinal chemistry researcher to come up with a unique targeted drug delivery system (TDDS) which selectively ferried the cargo molecule to the diseased cells and tissues in order to achieve maximum therapeutic effect by possessing minimum pernicious concomitant effects to normal tissues. Encouraged by well-established cell-penetrating peptides (CPPs) like Tat (49-57) and arginine-octamer (Arg-8-mer)[2] that crossways biological barriers efficiently, research on molecular transporter significantly progressing towards TDDS development that directed to deliver intracellular organelles of the diseased cells and tissues[3]. Probable mechanism emphasized that guanidine group of arginine residue leads to the formation of bi-dentate hydrogen bond between cell surface phosphates, carboxylates and/or sulphates, which facilitates cellular entry[2]. Therefore, extensive work on guanidine appended synthetic molecular transporter[4,5] has been progressed over the years for the development of new delivery carriers with improved submissive targeting system that can actively reach to the cancer cells after extravasations[6]. Attempts have also been made for improving pharmacokinetic profile of poor bio available drugs[7-11].

Recently, focus on TDDS construction increases considering various cellular proteases as target sites. Among these proteases, cathepsin B (Cat B)[12], is a ubiquitous lysosomal cysteine protease plays active role in cancer invasion, which is up regulated in several malignant tumor cells, while extremely low in normal cells. Thus, Cat B considered as a vital candidate for targeted cancer therapy. Herein, we have introduced a new class of synthetic octa-guanidine molecular transporter built on poly-(propylene imine) dendron starting from aminocaproic acid as the focal point, G8-PPI (Scheme 1) for target specific delivery of Doxorubicin (Dox), an anthracycline-based DNA intercalator, is one of the most typical anticancer drugs commonly employed in the clinic.



Scheme 1. Thematic representation of the proposed mechanism of drug delivery by TDDS based on the G8- PPI Dendron scaffold and chemical structure of G8-PPI, G8-PPI-FL, G8-PPI-FK-PABC-DOX.

The straightforward synthetic construction initiated with PPI dendron growth by sequential Michael addition of acrylonitrile followed by reduction with Raney-Ni. Next, eight unit of free amine terminal is coupled with Boc-protected guanidine residue affording the G8-PPI skeleton. Finally, fluorescein (FL) is attached via amino propanol linker to the G8-PPI. Similarly the G8-PPI dendron further conjugated to Cat B specific small peptide substrate Phe(F)-Lys(K)[11] for synthetic ease as well as potential systemic stability, followed by 4-aminobenzyloxy carbonyl (PABC), a self-immolative spacer and DOX resulted the

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desired TDDS, G8-PPI-FK-PABC-DOX. In this synthetic construct the ratio of loading of drug to carrier is 1:1. Cellular internalization study has been compared with fluorescein label arginine-octamer (Arg-8-G-FL) as a standard cell-penetrating peptide obtained by solid phase peptide synthesise (SPPS) using manual coupling of Rink amide resin (ESI scheme 6). The final target transporter molecules G8-PPI-FL and G8-PPI-FK-PABC-Dox were transformed to HCl salt after deprotection of Boc group on the guanidine moieties by treating with ethyl acetate saturated with HCl gas (ESI scheme 4). The key intermediates and aimed products, G8-PPI-FL, G8-PPI-FK-PABC-DOX were characterized by HPLC, NMR spectroscopy and MALDI-TOF mass spectrometry (ESI Sec.No:2). The stability of the DOX conjugated carrier has been checked by HPLC in different time interval (1, 2, and 4 month) (ESI Fig. S1a (iv)), also *in vitro* stability in PBS buffer (7.4) containg 10% FBS (ESI Fig S7b). We have initially assessed the cellular uptake efficiency of the synthesized transporter G8-PPI-FL in HeLa cells by flow cytometery analysis and time dependent microscopy. In flow cytometer analysis suspension of 1-5×10^5 HeLa cells were treated with 2 μM G8-PPI-FL and visually differentiated with Arg-8-G-FL for various time periods (ESI Fig. S8a). After incubation at 37°C cells suspension was washed properly with PBS and re-suspended in colourless media and analyzed using BD LSRFORTESA instrument in FITC channels with emission filters at 530 nm. Flow cytometric analysis indicates that cellular uptake of G8-PPI-FL increases with time of incubation as we have observed green colour histogram shifted towards higher intensity (ESI Fig. S8a). Cellular uptake efficiency was further quantified and found to be significantly higher uptake of both G8-PPI-FL and G8-PPI-FK-PABC-DOX compared to Arg-8-G-FL (ESI Fig. S8b). Cellular uptake of G8-PPI-FL and Arg-8-G-FL in HeLa cells using fluorescent microscope for various time periods (such as 60, 120 and 240 min) reveals that green fluorescence intensity of G8-PPI-FL increases with time compared to Arg-8-G-FL in HeLa cells (ESI Fig. S10). Therefore, both flow cytometer analysis and microscopic images indicates that cellular uptake ability of G8-PPI-FL is significantly higher than that of Arg-8-G-FL. Furthermore, we have investigated the intracellular localization of transporter G8-PPI-FL and compared with Arg-8-G-FL in HeLa cells using intracellular staining dyes such as lysotracker and mitotracker. G8-PPI-FL was found to be highly co-localized with lysotracker and extent of co-localization is much higher than Arg-8-G-FL which reflects the lysosomal selectivity G8-PPI-FL carrier representing better delivery system of Dox (Fig 1, S11, S13, S14 and S15). Again, we have evaluated the co-localization potency of G8-PPI-FL and Arg-8-G-FL in mitochondria. From confocal data (Fig 1a, S13 and S14) of G8-PPI-FL found no significant co-localization with mitotracker. Therefore, above results clearly indicates that of G8-PPI-FL having significant selectivity towards lysosome of both normal and cancer cell. However, we did not find noticeable localization of G8-PPI-FL into the mitochondria.

Flow cytometry data reveals that uptake of Dox conjugate was maximum after 120 min incubation (Fig 2a-c). We have also quantified this result and observed significant release of Dox is occurred from conjugate compared to free Dox (Fig 2, ESI Fig. S9). Microscopic images revealed red colour fluorescence reaches to maximum intensity after 120 min incubation with G8-PPI-FK-PABC-DOX conjugate compared to free Dox (Fig 2d-f). However, the significant uptake of Dox-conjugate shown compare to free Dox which starts from 30 mins (Fig 2d). Moreover, merged pink colour microscopic images indicate that Dox releases at lysosome and reaches up to nucleus where it binds with DNA (Fig 2d-f merged). Therefore, above results clearly demonstrate that G8-PPI-FK-PABC-DOX conjugate serves as an efficient drug-conjugate for the delivery of Dox in cervical cancer cell line.

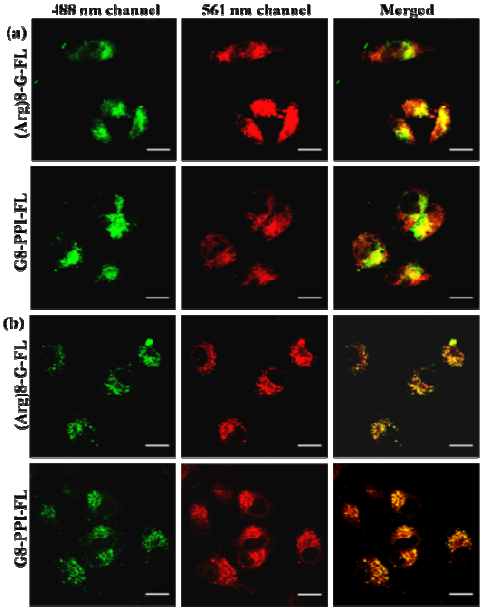

Fig. 1 Co-localization studies by confocal microscopy: Confocal images reveals co-localisation G8-PPI-FL and (Arg)8-G-FL (green) with mitotracker (red) (a) and lysotracker (red) (b) in HeLa cells. Scale bar corresponds to 20μm.

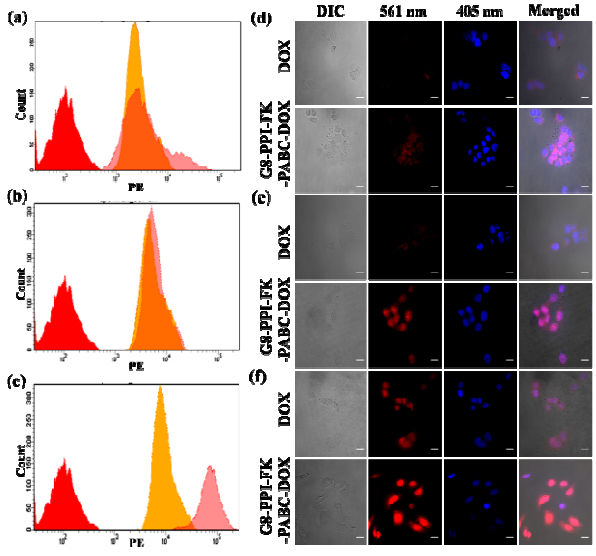

Fig. 2 Flow cytometric analysis of intracellular DOX release kinetics of G8-PPI-FK-PABC-DOX (shown in pink colour) and bare Dox (shown in yellow colour) at 30 (a), 60 (b) and 120 (c) mins. Microscopic analysis of intracellular Dox release kinetics of G8-PPI-FK-PABC-DOX and bare

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Dox at 30 (d), 60 (e) and 120 (f) mins. Scale bar corresponds to 20μm.

We have further examined the drug release ability of Dox conjugated carrier by incubating G8-PPI-FK-PABC-DOX in NaOAc /EDTA buffer with Cat B enzyme in the ratio of 9:1. Captivating the benefit of the intrinsic fluorescent property of Dox, its cumulative release from G8-PPI-FK-PABC-DOX was judged by fluorescence measurement at 590 nm (ESI Fig. S7a). Here we observed above 70% of Dox release occurred in the presence of enzyme after 28 h as we know that Cat B activity generally occurred in acidic pH which cleaves the specific peptide substrate, subsequently releasing Dox[12]. Furthermore, stability of the Cat B peptide substrate in TDDS was evaluated at different pH conditions, which confirmed no significant drug release even at physiological pH (ESI Fig. S7a(ii)). Next, we have investigated *in vitro* cytotoxicity profiles of G8-PPI-FK-PABC-Dox conjugate using MTT assay. In this study G8-PPI-FL and free Dox has been treated as control. HeLa and WI-38 cells were incubated with various concentrations (0.5-4 μM) of G8-PPI-FK-PABC-Dox, G8-PPI-FL and free Dox for 24 hours. Interestingly, we found that novel G8-PPI-FK-PABC-DOX conjugate showed higher cytotoxicity with IC_{50} value 0.40±0.01 μM compared to that free Dox with IC_{50} value 1.15±0.10 μM (Fig 3a) and non-cytotoxic against non-cancerous cell WI-38 (Fig 3h). We did not observe any cytotoxicity after treatment with transporter alone G8-PPI-FL. Therefore, above data clearly indicates that enhance cytotoxicity arises from release of Dox

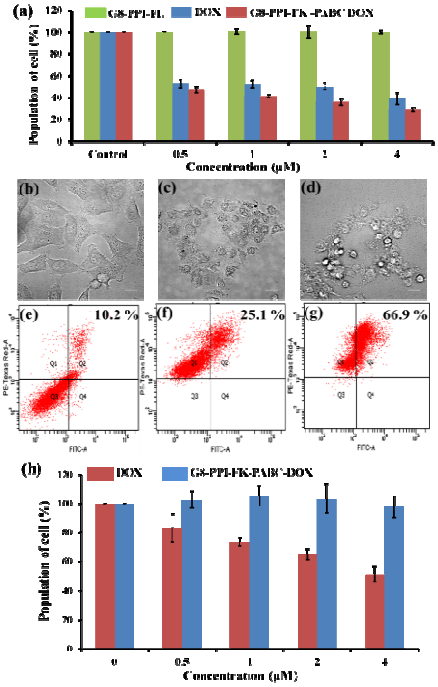

Fig. 3 Survival of HeLa cell line was assessed by MTT assay after treatment with G8-PPI-FL (transporter), doxorubicin (Dox) and G8-PPI-FK-PABC-DOX (a). Cellular morphology of HeLa cells incubated in absence of each drug (b), after treatment with doxorubicin (c) and after treatment with G8-PPI-FK-PABC-DOX (d). Scale bar corresponds to 20μm. FACS analysis of HeLa cells after treatment with annexinV and propidium iodide. Control Cells (e), cells treated with 2μM doxorubicin (f), cells treated with 2μM G8-PPI-FK-PABC-DOX (g). MTT assay indicates that G8-PPI-FK-PABC-DOX conjugate non-cytotoxic against non-cancerous cell (WI-38) while free DOX shows significant cytotoxicity (h).

from the conjugate only in cancer cell. Moreover, we have checked the cellular morphology after treatment with 4 μM of free doxorubicin and G8-PPI-FK-PABC-DOX conjugate for 4 h using Nikon Ti-U microscope with a 40X objective in bright field. The morphology of cells were compared with non-treated cells and we have found that extensive damage of cellular morphology in case of G8-PPI-FK-PABC-DOX treated cells compared to free Dox treated HeLa cells (Fig 3). Finally, we have investigated type of cell death using flow cytometer by annexin V and Propidium iodide (PI) method. Flow cytometry data clearly indicates higher population of apoptotic death of HeLa cells (66.99%) after treatment with G8-PPI-FK-PABC-DOX conjugate, while lesser population of apoptotic death of HeLa cells (25.1%) after treatment with free Dox (Fig 3). This data absolutely supports that Dox conjugate G8-PPI-FK-PABC-DOX is more potent compared to free Dox against HeLa cell.

In conclusion, we have designed and synthesized a poly-propylene imine) dendron based molecular transporter which showed significantly higher cellular uptake than Arg-8-mer and have excellent selectivity towards lysosome. Further, we have constructed TDDS and conjugated Dox through lysosomal targeting Cat B (over expressed in cancer cell) specific peptide sequence Phe-Lys with a self-immolative linker PABC (G8-PPI-FK-PABC-DOX), which showed targeted killing of cancer cell (HeLa) without affecting normal cell (WI-38). G8-PPI-FK-PABC-DOX shows equal uptake efficiency like G8-PPI-FL and enhanced cytotoxicity (IC_{50} value 0.40±0.01 μM) compared to free Dox (IC_{50} value 1.15±0.10 μM) and apoptosis in HeLa cell due to noticeable Dox release as shown by kinetic measurement (>70%).

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Notes and references

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Graphical Abstract

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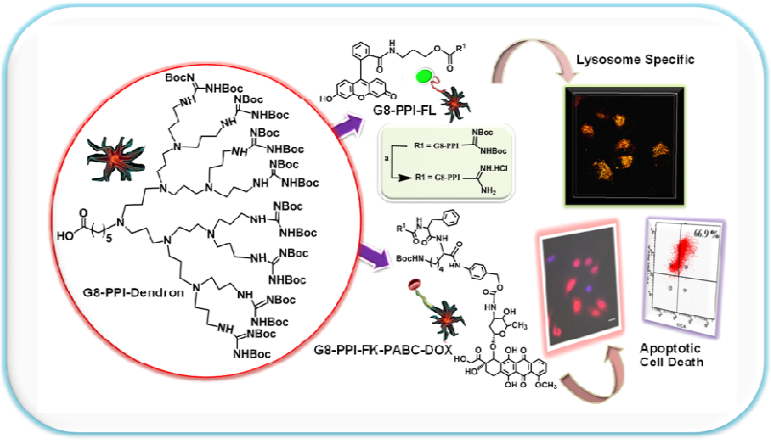

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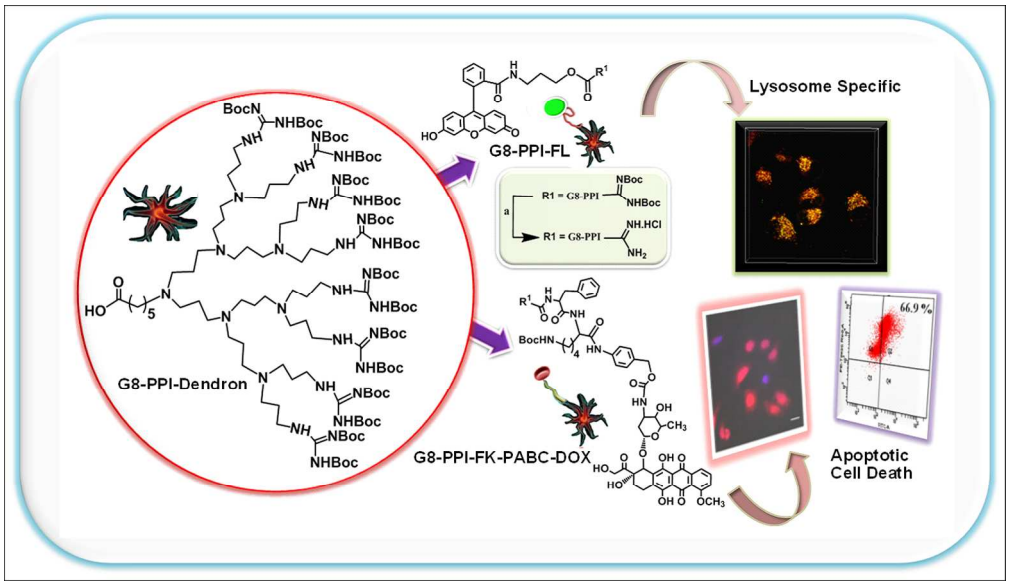

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