Supporting Information

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

Jyothi B Nair¹, #, Saswat Mohapatra², #, Surajit Ghosh*, ², Ž, Kaustabh K Maiti*, ¹, §

a) CSIR-National Institute for Interdisciplinary Science & Technology (NIIST), Industrial Estate, Pappanamcode, Thiruvananthapuram- 695019, Kerala, India; Chemical Sciences & Technology Division (CSTD), Organic Chemistry Section
b) Chemistry Division, CSIR-Indian Institute of Chemical Biology, Jadavpur, Kolkata-700032, India.

S.No Contents Page No:
1 Synthesis 2
1.1 Materials and methods 2
2 Experiments and spectral Data’s 3
2.1 Synthesis of G8-OA-PPI 4
2.2 Synthesis of G8-PPI 6
2.3 Synthesis of G8-PPI –FK-PABC-DOX 7
2.4 Synthesis of G8-PPI –FL 9
2.5 Synthesis of (Arg)-8-G-FL 11
2.6 HPLC , Zetapotential 12
2.7 NMR data 13
3 a) In Vitro drug release kinetics, b) In vitro stability 16,18
4 In vitro cellular assay. 19
4.1 Flow cytometric detection of cellular uptake and kinetics 19
4.2 Detection of cellular uptake and kinetics using fluorescent microscope 21
4.3 Confocal analysis of cellular localization of G8-PPI-FL and (Arg)-8-G-FL 23
4.4 Cell viability assay 27
4.5 Analysis of cellular morphology 28
4.6 Detection of cellular death by flow cytometer 28
5 References 29
1. Synthesis.
   1.1 Materials and methods

   Unless otherwise indicated, all non-hydrolytic reactions were carried out in oven-dried glassware under an inert atmosphere of dry argon or nitrogen. Protected amino acids were purchased from Nova bio chem. Doxorubicin Hydrochloride was obtained from Cal biochem. Cathepsin B was acquired from Enzo life science. All the other chemicals and solvents were purchased from sigma Aldrich, Merk, and Specrochem, used without further purification. Analytical TLC was performed on a Merck 60 F254 silica gel plate (0.25mm thickness), and visualization was done with UV light (254nm and 365nm), or by spraying with a 5% solution of phosphomolybdic acid or ninhydrine solution followed by charring with a heat gun. Column chromatography was performed on Merck 60 silica gel (60-120 or 100-200 mesh). HPLC was performed by Shimadzu HPLC system consisting of SCL-10Avp system controller, two LC-8A solvent delivery units, SPD-M20A UV-vis photo diode array (PDA) detector, equipped with Multi PDA- LC solution (software) on a 250 mm x 4.6 mm i.d, 5µm, YMC-Pack R&D ODS analytical column (9YMC Co., Ltd. Japan). NMR spectra were recorded on a Bruker AMX 300 (1HNMR at 300MHz; 13C-NMR at 75 MHz) and Bruker AMX 500 (1H-NMR at 500MHz; 13C-NMR at 125MHz) spectrometers. Tetra methyl silane was used as reference for 1H NMR, and the chemical shift were reported in ppm and the coupling constant in Hz. High resolution mass spectra were determined on a HR-EMI analysis of Thermo Scientific Exactive system, and MALDI-TOF mass spectra on a Shimadzu Biotech, AXIMA-CFR PLUS system. Liophilization was performed with lyophilizer Tenesis Wizard 2.

   For biological studies 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) was purchased from Himedia. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dulbecco’s modified eagle’s medium (DMEM) trypsin-EDTA, dimethylsulfoxide (DMSO) for cell culture solution were purchased from Sigma Aldrich. Penicillin-Streptomycin, neutravidin, alexa fluor 568-carboxylic acid succinimidyl ester and fetal bovine serum (FBS), Mito Tracker red CMX Ros were purchased from Invitrogen. Annexin V and PI apoptosis detection kit were purchased from Santa Cruz Biotechnology. Bisbenzimide H 33258 (hoechst) was purchased from Calbiochem. Lyso Tracker Deep Red was purchased from Life technologies.

   **Cell and Cell Culture:** Human cervical cancer cell line HeLa (adenocarcinoma) and Human lung fibroblast normal cell line WI-38 has been obtained from National Centre for Cell Science (NCCS) Pune, India. Cells are cultured on 25 cm² tissue culture flask in Dulbecco’s modified eagle’s medium (DMEM) containing 10% fetal bovine serum, kanamycin sulfate (110 mg/L), penicillin (50 units/mL), streptomycin (50 µg/mL) at 37°C in the sterile condition of 5%
CO₂ incubator with humidified atmosphere. Cells are exponentially cultured as monolayer up to 70-80% confluence. After optimal growth cells were detached using trypsin-EDTA (1X). All the cell culture work has been performed under sterile condition.

2 Experiments and spectral Data’s

Scheme 1: Schematic representation of G8-PPI-FL, G8-PPI-FK-PABC-DOX and (Arg)8-G-FL. Synthetic procedures: ESI scheme 2, 3, 4, 5 & 6. h) HCl(g), EtOAc, RT, 4 h. 17) Control compound prepared by solid phase peptide synthesis.
2.1 Synthesis of G8-OA-PPI

2.1.1 Synthesis of compound 1

6-aminohexanoic acid (5g, 0.038mol), glacial acetic acid (43.7mL, 0.764mol) and acrylonitrile (188.58mL, 2.867mol) were added and kept in oil bath (105°C), Refluxed for 30h under N\textsubscript{2} atmosphere, the excess acrylonitrile was concentrated under reduced pressure and co-evaporated repeatedly with toluene to remove residual acetic acid. Then the residue was dissolved in ethyl acetate washed several times with water, dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated. The crude product was purified by silica gel column chromatography to obtain compound 1 as brownish yellow sticky solid, Yield 84%. \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 1.260 (m, 2H), 1.383 (m, 2H), 1.670 (m, 2H), 2.357 (t, J = 5Hz, 2H), 2.545 (t, J = 6Hz, 4H), 2.859 ppm (t, J = 4Hz 6H). HRMS m/z calculated 237.1477, C\textsubscript{12}H\textsubscript{19}N\textsubscript{3}O\textsubscript{2}, found 238.2316 [M+H\textsuperscript{+}].

2.1.2 Synthesis of compound 2

To a solution of 1 (9.6g) in anhydrous ethanol (125ml) were added 1N NaOH solution 15ml and approximately 4g Raney-Ni catalyst. The mixture was hydrogenated in a Parr apparatus at 50 psi for 24 h at room temperature. The catalyst was filtered through
celite column and eluted with ethanol. After the evaporation of ethanol under rotary evaporator to yield yellow sticky solid with 82 % yield.  

**1H NMR (CD$_3$OD):**  $\delta$ 1.242 (m, 2H), 1.425 (m, 2H), 1.654 (m, 4H), 2.390 (m, 2H), 2.522 (m, 2H), 2.666 (t, $J = 7.2$Hz, 4H), 3.330 (m, 4H), 3.460 (t, $J = 7.5$Hz, 2H). HRMS m/z calculated 245.2103, C$_{12}$H$_{27}$N$_3$O$_2$ found 246.3412 [M+H]$^+$.  

### 2.1.3 Synthesis of compound 3

**1H NMR (CDCl$_3$):**  $\delta$ 1.384 (m, 2H), 1.397 (m, 2H), 1.646 (m, 4H), 2.319 (t, 6.5Hz, 10H), 2.531 (t, $J = 7$Hz .8H), 2.640 (t, $J = 5.5$Hz, 4H), 2.714 (t, $J = 6.5$Hz, 4H), 3.212 ppm (t, $J = 7.5$Hz, 6H); Yield=78%, HRMS m/z calculated, C$_{24}$H$_{39}$N$_7$O$_2$, 457.3165, found 458.5634 [M+H]$^+$ as sticky solid.  

### 2.1.4 Synthesis of compound 4

**1H NMR (CD$_3$OD):**  $\delta$ 1.12 (m, 2H), 1.337 (m, 2H), 1.526 (m, 2H), 1.673 (m, 6H), 2.190 (t, $J = 7.5$Hz, 2H,), 2.489 (t, $J = 7.5$Hz, 20H), 2.676 (t, $J = 6.5$Hz, 10H), 5.115 (br. s, 8H); Yield= 84%, HRMS m/z calculated: C$_{24}$H$_{39}$N$_7$O$_2$, 473.4417, found 474.8978 [M+H]$^+$as yellow solid.  

### 2.1.5 Synthesis of compound 5

**1H NMR (CDCl$_3$):**  $\delta$ 1.228 (m, 2H), 1.434 (m, 2H) , 1.649 (m, 2H) ,1.540 (m, 12H) 1.873 (m, 12H) , 2.326 (t, $J = 6$Hz, 2H), 2.525 (t, $J = 6.5$Hz, 10H), 2.538 (t, $J = 7.5$Hz, 8H), 2.813 (t, $J = 6.5$Hz, 8H), 3.414 (m, 4H), 3.552 (m, 4H) , 3.655 (m, 4H) , 3.751ppm (m, 4H) . Yield=78%, HRMS m/z calculated: C$_{48}$H$_{79}$N$_{15}$O$_2$, 898.6541 found 899.7802 [M+H]$^+$as yellow sticky solid.
2.1.6 Synthesis of compound 6 (G8-OA-PPI)

Yellow sticky solid, \( ^1H \) NMR (CD\(_3\)OD): \( \delta \) 1.075-1.641 (m, 28H), 2.478 (m, 40H), 2.585 (m, 16H), 2.722,-2.819 ppm (m, 20H); Yield 74%; HRMS m/z calculated C\(_{48}\)H\(_{111}\)N\(_{15}\)O\(_2\), 929.9045, found 953.0321 [M+Na].

2.2 Synthesis of G8-PPI

![Chemical Structure](image)


2.2.1: Synthesis of compound 7

To a solution of 6 (176 mg, 0.19 x10\(^{-3}\) mol) in 10ml of water ,1N HCl solution was added drop wise to a neutral pH .To this solution dioxane were added (40 mL) and then N,N-di-Boc-N-trifluorometanesulfonylguanidine (600 mg, 1.5 x10\(^{-3}\) mol) and triethylamine (0.13 ml, 0.95 x10\(^{-3}\) mol). After stirring for 72 hrs at RT, the reaction mixture was evaporated and residue was diluted with Ethyl acetate, water and brine. The organic layer was dried over Na\(_2\)SO\(_4\) and concentrated. Yield 67%; \( ^1H \) NMR (CDCl\(_3\)): \( \delta \) 1.187 (m, 8H), 1.370 (m, 4H), 1.431 (s, 144H),1.523 (m, 10H), 2.489 (m, 20H), 2.537 (m, 16H), 3.088 (m, 16H), 3.349 (m,
2.3 Synthesis of G8-PPI –FK-PABC-DOX

2.3.1 Synthesis of compound 8

Fmoc-Lys (Boc) OH (1g, 0.0078 mol) and N- Ethoxy carbonyl 1- 2- ethoxy - 1, 2 dihydroquinoline, EEDQ (1.9g, 0.0078 mol) was dissolved in anhydrous dichloromethane (100 mL). After stirring for few minutes at room temperature 4- amino bezyl alcohol (PAB-OH) (0.96g, 0.0078 mol) was added and stirring was continued for 24 hrs. Completion of the reaction was monitored by TLC and DCM was evaporated under rotary evaporator and residue was purified on silica with CHCl3: MeOH) 0.5% to afford 8 as white powder. HRMS m/z calculated C_{33}H_{39}N_{3}O_{6}, 573.6832 found 578.7643 [M+H]^+.

8 (1.2g) was treated with a solution of 20% piperidine in DMF ( 5mL ) and reaction was stirred for 10- 15 minutes at room temperature . The product was precipitated with di-ethyl ether (150 mL) and washed with the same and dried over vacuum pump to afford 9 yield 83% as yellow powder. HRMS m/z calculated C_{18}H_{29}N_{3}O_{4}, 351.2234, found 352.2342 [M+H]^+.

In the next step Fmoc-Phe-OH (1.14g, 2.96 mol) and N- Hydroxysuccinimide (NHS) (0.34g, 2.96 mol) were dissolved in anhydrous DCM (50 mL) stirred for 0 °C for 5 minutes EDC was added and stirred for 16 hrs and precipitate was filtered off. 9 (0.7g, 2.96 mol) and triethyl amine TEA (0.22 mL, 2.96 mol) was added to the filtrate stirred for 4 h. The volatiles were removed under reduced pressure and purified by silica. To a solution of the above (1.6g, 2.28 mol) and 4-Nitrophenylchloroformate (1.8g, 9.12 mol) in anhydrous DCM 30 mL, N, N' di isopropyl ethyl amine (0.9 mL, 6.84 mol) was added at 0 °C and reaction was stirred for 48 h, the volatiles were removed under reduced pressure and Purified by silica afforded 10. HRMS m/z calculated C_{49}H_{51}N_{3}O_{11} 885.36, found 886.45 [M+H]^+. 1H NMR (CDCl3): δ 1.25 (m, 2H), 1.43 (s, 9H), 1.518, (m, 2H), 1.90 (m, 2H), 3.125 (d, 2H), 3.266 (t, J=4.75 Hz,
1H), 4.709 (d, J=7.32 Hz, 2H), 4.692 (t, J=6.56 Hz, 1H), 5.075 (t, J=4.78 Hz, 1H), 5.103 (s, 2H), 7.16-7.71 (m, 23H), 8.03 (br.s, 4H) ppm.

To a solution of 10 (5mg, 5.6 x 10⁻⁶ mol) in anhydrous DMF (2 mL) Doxorubicin Hydrochloride (3.3 mg, 5.6 x 10⁻⁶ mol) and DIPEA (7µL) were added and the reaction was stirred for 48 h to yield Fmoc- Phe- Lys- PABC – DOX, 11 as red sticky solid. MALDI-TOF MS m/z calculated C₇₀H₅₇N₅O₁₉ 1290.3723 found 1313.8945 [M+Na]⁺. 11 was treated with 20% piperidine in DMF and stirred for 30 minutes at room temperature and precipitated with diethyl ether and dried under vacuum. A solution of 11 in dry DCM, EDC (2 mg, 1.123 x 10⁻⁵ mol) were added under N₂ atmosphere and was stirred for 10 minutes and already prepared G8-PPI (16 mg, 6.72 x 10⁻⁶ mol) was added and stirred for 24 hrs the product was concentrated in rotary
evaporator and purified by silica gel column chromatography to afforded 12. Yield=70%, MALDI-TOF MS m/z calculated C_{191}H_{318}N_{36}O_{50} 3918.78, found 3919.56 [M+H]^+. Finally all the Boc deprotections were carried out using saturated HCl (g)- Ethyl acetate solution at room temperature. After 4 h stirring the solution was concentrated and residue was washed with ethyl acetate and dried over vacuum pump. The residue was purified by supelclean LC -18 reverse phase silica gel, Sigma Aldrich. The purified product was dissolved in de-ionized water filtered through PTGE syringe filter (0.450 µm) and checks purity by HPLC and lyophilized to give final product 13 as brown sticky solid. Yield= 68%, MALDI-TOF MS m/z calculated C_{106}H_{182}N_{36}O_{16}, 2216.8102, found 2239.4512. [M+Na]^+. ^1H NMR (CD$_3$OD): δ. 1.285 (m, 12H), 1.413 (m, 2H), 1.569 (m, 17H), 1.661 (m, 13H), 2.441-2.878 (m, 64H), 3.27-3.36 (m, 10H), 4.35-4.50 (m, 15H), 5.029 (s, 2H), 7.038-7.825 (m, 12H), 8.034 (br, s, 8H).

2.4 Synthesis of G8-PPI –FL

Fluorecein (221mg, 1.5x10$^{-4}$ mol) in DMF were activated with EDC (230 mg, 3x10$^{-4}$ mol), HOBT (162 mg, 3x10$^{-4}$ mol) for 2 mins then amino propanol (50 mg, 1.5x10$^{-4}$) was added, stirred under nitrogen atmosphere for 36 hrs. Completion of the reaction was
monitored by TLC and purified by silica gel column chromatography to yield 14, yield=85%. 14 (26 mg, 6.8x10⁻⁵ mol) were coupled with G8-PPI(11mg,5.2x10⁻⁵mol) in presence of EDC (19 mg, 10.4x10⁻⁵ mol), DMAP (1mg, 10mol%) to yield 15, ¹H NMR (CDCl₃): δ 1.25 (m, 4H), 1.439 (s, 144H), 1.344 (2H, m), 1.72 (m, 2H), 1.657 (2H, m), 1.539 (m, 12H), 2.41 (t, J=7.85 Hz, 40H), 2.227 (t, 2H), 3.041 (t, J=5.66 Hz, 2H), 3.611 (t, J=5.67Hz, 2H), 2.953 (t, J=4.55 Hz, 16H), 6.532-7.711 (m, 10H), 8.22 (s, 8H). MALDI-TOF MS m/z calculated C₁₅₈H₂₇₀N₃₂O₃₈, 3225.25 found 3326.34 [M+H]⁺. Finally all the Boc protected groups were removed using saturated HCl (g) Ethyl acetate solution at room temperature. After 4 h stirring the solution was concentrated and residue was washed with ethyl acetate and dried over vacuum pump. The residue was purified by supelclean LC -18 reverse phase silica gel. The purified product was dissolved in de ionized water filtered through PTGE syringe filter (0.450 μ) and checks purity by HPLC and lyophilized to give final product 16 as yellow solid. ¹H NMR (CD₂OD): δ 1.286 (m, 4H), 1.3224 (m, 4H), 1.563 (m, 6H), 1.4535 (m, 6H), 1.574 (m, 10H), 1.721 (m, 8H), 1.821 (m, 8H), 2.176 (m, 2H), 2.656 (m, 26H), 2.75 3(m, 8H), 2.922 (m, 12H), 3.045 (m, 2H), 3.161-3.826 (m, 6H), 4.11 (m, 4H), 6.404-7.934 (m, 10H), 8.157 (s, 8H). MALDI –TOF –MS m/z calculated C₇₈H₁₄₂N₃₂O₆, 1624.1729 found 1625.9403 [M+H]⁺, Yield 65%.
2.5 Synthesis of (Arg) 8-Gly-FL

**SI Scheme 6: Solid phase peptide synthesis of Arg-8-mer**

Synthesis of amide terminal (Arg) 8-G-FL started with deprotecting Fmoc group of Rink amide resin using 20% piperidine in DMF for 20 minutes. Then the resin was washed with DMF (3 x 3 mL), Fmoc-Arg(pbf)OH (276 mg, 4.2 x 10^{-4} mol.) dissolved in DMF along with activating agent HBTU (161 mg, 4.2 x 10^{-4} mol) and 2-3 drops of DIPEA charged to the resin bed. The reaction was continued for 8 h with shaking, progress of the reaction was monitored by the Kaiser test. After completion of the coupling, the resin was washed with DMF (3 x 3 mL), and the Fmoc protection group was removed by treatment with piperidine in DMF (20%, 3 x 2 mL, 3 x 15 min). The reaction cycle was continued in a similar manner again seven times with Fmoc Arg(pbf)OH and Fmoc-Gly-OH (126mg, 4.2 x 10^{-4} mol). Finally fluorecein free acid (20mg, 8.52 x 10^{-5} mol) activated with DIC (10 mg, 8.52 x 10^{-5} mol) and HoBt (11 mg, 8.52 x 10^{-5} mol) was added to the resin and shaken for overnight. Peptide was released from the resin bed by treatment with the most common cleavage cocktails that is, 95% trifluoro acetic acid, 2.5% triisopropyl silane, 2.5% water. The resin washing was combined and concentrated under reduced pressure, and the residue was co-evaporated with toluene and precipitated with cold ether (3 mL) and filtered the residue
peptide that afforded as shiny yellow solid. MALDI –TOF –MS m/z calculated C\textsubscript{70}H\textsubscript{112}N\textsubscript{34}O\textsubscript{13}, 1645.8583 found 1646.9023 [M+H]+.

2.6 HPLC, Zeta potential measurement

![HPLC chromatogram](image)

**Fig. S1a:** HPLC chromatogram of G8-PPI-FL (i), G8-PPI-FK-PABC-DOX (ii) and (Arg)\textsubscript{8}-G-FL (iii), at different period of time stability of G8-PPI-FK-PABC-DOX(iv)

The HPLC analysis was conducted using Shimadzu RP- HPLC ODS column with mobile phase consisting of in acetonitrile (A) and in water (B). The gradient was linearly increased from 0% to 80% B for 35 minutes at the flow rate of 1 mL/min at ambient temperature. UV-VIS detection was monitored simultaneously at 254 nm and 480 nm wavelengths.
Zeta-potential of G8-PPI-FL, G8-PPI-FK-PABC-DOX was measured using a Malvern Zeta Sizer Nano-ZS ZEN 3600. 1mM of DDS3 was dissolved in milli Q water for evaluation.

**Fig. S1b:** Zeta potential of G8-PPI-FL, G8-PPI-FK-PABC-DOX

2.7 NMR data
Fig. S2: $^1$H NMR spectra of compound 5

Fig. S3: $^1$H NMR spectra of compound 6

Fig. S4: $^1$H NMR spectra of compound 7
Fig. S5: H$^1$ NMR spectra of compound 13

Fig. S6: H$^1$ NMR spectra of compound 16
3.  **a) In Vitro drug release kinetics**

Detailed studies were carried out by checking the Dox release from Dox-conjugated carrier. At first we incubated 0.05 mM G8-PPI-FK-PABC-DOX in NaOAc /EDTA buffer (with 50 mM NaOAc and 1 mM EDTA, pH=5.05) with cathepsin B enzyme (62.5 ng/µL) 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 as judged by fluorescence measurement at 590 nm. 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. The result indicated that the above 70% of Dox release occurred in the presence of enzyme after 28 hrs. 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. A blank was carried out without cathepsin B enzyme, using 100 µL of G8-PPI-FK-PABC-DOX buffer solution. All measurements were carried out at different pH conditions (5.05, 7, 7.4 and 9) at specific time intervals from 0 to 30 hrs. Fluorescence was measured at different time intervals using BioTec Synergy 4 spectrophotometer at 590 nm, which has been reflected as % drug release (plotted in Y- axis) as shown in Fig. S2.
Fig. S7a: i) Line graph showing the release of doxorubicin from G8-PPI-FK-PABC-DOX in the presence of cathepsin B enzyme at pH 5.05. WE denotes with enzyme and WOE denotes without enzyme. ii) Drug release at different pH conditions (5.05, 7, 7.4 and 9).
b) In Vitro stability

To evaluate the stability of G8-PPI-FK-PABC–DOX in blood circulation, we have dispersed the TDDS in PBS (pH=7.4) containing fetal bovine serum (FBS) and incubated at 37°C. and checked HPLC at different intervals of time. The result indicates that there is no significant drug degradation providing possibility of enhancing drug accumulation in tumour tissue.

Fig. S7b: The stability of G8-PPI-FK-PABC-DOX in PBS (pH=7.4) containing 10% FBS at 37°C for 24 hrs
4. *In vitro* cellular assay

4.1 Flow cytometric detection of cellular uptake and kinetics

To analyze the transporter G8-PPI-FL, cellular uptake kinetics was studied using flow cytometer. 1-5×10^5 density of cells were detached using trypsin and resuspended in 200 µL cell culture serum free media containing G8-PPI-FL and (Arg) 8-G-FL at the concentration 2 µM for different time periods up to 4 h mentioned in figure legend. After incubation at 37 °C cells suspension was diluted with 2 mL of PBS and centrifuged at 800×g. The cell pellet was washed twice with PBS by centrifugation and resuspended in 500 µL of serum free media. Fluorescence analysis was performed with BD LSRFORTESA using FITC channels and emission filters at 530 nm. In order to characterize the drug (DOX) delivery efficiency of G8-PPI-FK-PABC-DOX, cellular uptake kinetics of DOX was analyzed with respect to free drug DOX. We have followed above mentioned treatment protocol for G8-PPI-FK-PABC-DOX and DOX at the concentration 4 µM. After incubation for different time up to 120 min (mentioned in figure legend) cell suspension was washed twice with PBS. Fluorescence analysis was performed with BD LSRFORTESA using PE channels and emission filters at 561 nm.
Fig. S8a: Flow cytometric analysis of cellular uptake kinetics of G8-PPI-FL (transporter) (shown in green colour histogram) and (Arg)8-G-FL (shown in violet colour histogram) in 60 (i), 120 (ii), 240 (iii) mins. Red graph represents untreated control of HeLa cells. (iv) Bar diagram of FACS data reveals kinetics of cellular uptake of G8-PPI-FL and (Arg)8-G-FL.

Fig. S8b: Quantitative plot represents cellular uptake kinetics of HeLa cells after treatment with (Arg)8-G-FL, G8-PPI-FL and G8-PPI-FK-PABC-DOX for 30, 60 and 120 min of incubation. Data represents G8-PPI-FL and G8-PPI-FK-PABC-DOX has similar uptake efficiency.
**Fig. S9:** Bar diagram of FACS data reveals kinetics of DOX release with time in case of G8-PPI-FK -PABC-DOX

### 4.2 Detection of cellular uptake and kinetics using fluorescent microscope

For qualitative analysis and localization of fluorescence tagged G8-PPI-FL and (Arg)8-G-FL in HeLa cells, exponentially growing cells were seeded and harvested onto a cover glass bottom dish at a density of about 3000-5000 cells per dish. Then cells were either incubated with G8-PPI-FL or (Arg)8-G-FL for different time period up to 4h (mentioned in figure legends) in serum free media. Cell DNA was stained with Hoechst 33258 (1 µg/mL). Cells were washed by PBS and live cell imaging was carried out after different time using a Nikon Ti-U microscope on 37°C stage with a 40X objective in 405 and 488 nm channel for FITC and Hoechst 33258 respectively and DIC mode for bright field.
Fig. S10: Microscopic images reveal cellular uptake kinetics of FITC labeled (Arg)8-G-F1 and G8-PPI-FL (transporter) after 60 (a), 120 (b), 240 (c) mins incubation with HeLa cells. Scale bar corresponds to 20 µm.

For analysis of DOX release from G8-PPI-FK-PABC-DOX conjugate in HeLa cells, similar protocol of kinetics study has been used as described above. Here, treatment of G8-PPI-FK-PABC-DOX was carried out in serum free medium to 3000-5000 cells, already harvested in cover glass bottom dish. Bare DOX also treated in parallel experiment in similar way as G8-PPI-FK-PABC-DOX. After the incubation for different time period up to 120 min cells were washed. Nucleus was stained with Hoechst 33258. To visualize the cellular release, auto florescence of DOX was analyzed using Nikon Ti-U microscope after different time with a 40X objective in 561 nm channel. Nuclear stain was analyzed by 405 nm channel.
4.3 Confocal analysis of cellular localization of G8-PPI-FL and (Arg)8-G-FL

To understand the mechanism of release of drug using G8-PPI-FL transporter containing cathepsin substrate, sub-cellular localization of this transporter need to be analyzed. HeLa cells and WI-38 at a density of 3000 were seeded on cover glass bottom dish for 24 h followed by incubation with either in absence (negative control) or presence of fluorescence labeled G8-PPI-FL and (Arg)8-G-FL for 2h in serum free medium. After a single wash with serum free media live cells were treated with either mitotracker or lysotracker at the concentration of 100 µM or Hoechst for 1 h. Cell imaging was carried out using And or spinning disc confocal microscope using 60X objective (Olympus) and an Andor iXon3 897 EMCCD camera in 488 and 561 nm wavelength lasers light.

**Fig. S11: 3D Confocal merged image of Co-localization of Arg-8-mer (a) and G8-PPI-FL (b) (green) with lysotracker (red) in HeLa cells**
**Fig. S12:** Confocal images indicate no signal of co-localisation in absence of both (Arg)8-G-FL (a) and G8-PPI-FL (b) with lysosome (red) in HeLa cells. Scale bar corresponds to 20 µm.
**Fig. S13** Colocalization analysis with JACoP, image randomization of green and red pair (green and red channel) generates colocalisation map shown as white overlay on merge of the green and red channel. Brown Arrow represents non-colocalised (exclusion) signal. (Arg)8-G-F shows more colocalization with mitochondria (a) in comparison to G8-PPI-FL. G8-PPI-FL shows more colocalization with lysosome (b) in comparison to (Arg)8-G-F. Scale bar corresponds to 20 µm.
**Fig. S14** Curves (a-d) represents Van Steensel’s cross correlation functions (CCFs) for (Arg)8-G-FL colocalization with mitochondria (a) and lysosome (b); G8-PPI-FL colocalization with mitochondria (c) and lysosome (d).

**Table 1:** Pearson’s coefficient, CCF and P-value of colocalization analysis represents higher colocalization of G8-PPI-FL with lysosome than the mitochondria in comparison to (Arg)8-G-FL.
**Fig. S15**: Confocal images indicates co-localisation (Arg)8-G-FL (green) and G8-PPI-FL (green) with lysotracker (red) in non cancer WI-38 normal cell. Without treatment control represents as negative control. Scale bar corresponds to 20 µm.

### 4.4 Cell Viability assay:

To analyze percentage of cell death MTT assay was performed after drugs treatments. 10000 cells were seeded onto 96 well tissue culture plates in sterile conditions. After harvesting for overnight cells were incubated for 24 h in the absence or presence of 4 different concentrations (4 µM, 2 µM, 1 µM and 0.5 µM) of G8-PPI-FL (transporter), Doxorubicin (DOX) and G8-PPI-FK-PABC-DOX. Treated cells were incubated for 4h in presence of MTT solution prepared from 5 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide in PBS. 1:1 (v/v) DMSO/MeOH was used as MTT solvent and
cell population was analyzed by observing absorbance at 550 nm using microplate ELISA reader. Data analysis was performed using following formula:

\[
\% \text{ of Viable cells} = \left( \frac{(A_{550}^{TC} - A_{550}^{B})}{(A_{550}^{UC} - A_{550}^{B})} \right) \times 100
\]

(TC-Treated cells, B-Background, UC-untreated cells)

4.5 Analysis of cellular morphology:

To study cellular morphology 3000 cells were seeded overnight onto a cover glass bottom dish. Then cells were incubated for 4 h in absence and presence of 4 µM of DOX (Doxorubicin) and G8-PPI-FK-PABC-DOX. After washing with fresh media cells were incubated in colorless complete media for microscopic imaging. Live cells imaging was carried out using a Nikon Ti-U microscope with a 40X objective in bright field mode at 37°C.

4.6 Detection of cellular death by flow cytometer

Cell death was analyzed by flow cytometer using annexin V and PI method. 1-5×10⁵ density of HeLa cells were harvested on a 6 well plate for 24-48 h. Cells were first incubated with 2 µM of DOX and G8-PPI-FK-PABC-DOX for 24 h under sterile condition. After treatment, media containing drug was washed twice with PBS. Then cells were detached using trypsin-EDTA (1X) and washed once with 10% FBS containing DMEM. Cells pellet was resuspended in a 1X assay buffer (Santa Cruz Biotechnology). 5-6 ×10⁶ cells in 100 µL assay buffer were incubated for 15-20 min with 2.5 µL of Propidium iodide (PI) (stock conc. of 50 µg/mL) and annexin V (stock conc. of 200 µg/mL). Before analysis 400 µL of assay buffer has been added to make total volume of 500 µL of cell suspension. Flow cytometer analysis was carried out with respect to FITC fluorescence from annexin V-FITC and PI auto fluorescence intensity using FITC and PI channels of BD LSRSORTESA flow cytometer with emission filters at 530 and 610 nm respectively. To differentiate fluorescence and
background noise both annexin V-FITC and PI positive and negative cells were analyzed. The untreated both positive controls were used to characterize the normal cells population.

**Data analysis:** All the images were analyzed using Image J software.

5. References


