

Evaluation of pullulan-functionalized doxorubicin nanoparticles for asialoglycoprotein receptor-mediated uptake in Hep G2 cell line

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Received: 21 October 2010 / Accepted: 1 January 2011 / Published online: 27 January 2011
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Abstract The present study discusses evaluation of pullulan-functionalized doxorubicin nanoparticles for asialoglycoprotein receptor-mediated uptake in the Hep G2 cell line. Doxorubicin hydrochloride (DOX) nanoparticles using polymers of different hydrophobic character, polyethylene sebacate (hydrophobic) and poly (lactic-co-glycolic acid) (intermediate hydrophobicity) with high entrapment efficiency and particle size were prepared by modified nanoprecipitation, using Gantrez AN 119 as complexing agent. Nanoparticles of Gantrez AN 119 were also prepared to represent a hydrophilic polymer. Cell uptake of DOX nanoparticles was found to be comparable to DOX solution irrespective of DOX concentration, nanoparticles size, and pullulan concentration. Furthermore, uptake of nanoparticles functionalized with or without pullulan prepared with polymers of different hydrophobic character revealed comparable uptake. Comparable uptake of DOX solution and DOX nanoparticles functionalized with or without pullulan suggest extracellular release of DOX as the mechanism of uptake from the nanoparticles. In vivo evaluation in hepatic cancer model is therefore essential to

confirm the role of pullulan as asialoglycoprotein receptors ligand.

Keywords Doxorubicin · Asialoglycoprotein receptor · Pullulan · Hep G2 cells · Polyethylene sebacate

1 Introduction

Doxorubicin hydrochloride (DOX) is a drug of choice in the treatment of hepatic cancer. Insufficient concentration at the tumor site due to P-glycoprotein efflux coupled with cardiotoxicity, nephrotoxicity, myelosuppression, and topoisomerase II resistance are serious limitations of current DOX therapy (Patil et al. 2008). Efficient treatment of hepatic cancer may be facilitated through hepatocyte-specific delivery of DOX. Asialoglycoprotein receptors (ASGPR) are predominantly present in large numbers on the sinusoidal cell membrane of hepatocytes and internalize sugars such as galactose or lactose and glycoproteins with terminal galactose or N-acetylgalactosamine by endocytosis (Wu et al. 2002). Targeting to ASGPR using nanocarriers represents an attractive strategy for hepatocyte-specific delivery of DOX for the treatment of hepatic cancer.

Various ligands for ASGPR including its natural ligand asialo-feutin (Arangoa et al. 2003) or galactosylated or lactosylated polymers (Kawakami et al. 1998; 2000), glucoside (Maitani et al. 2001), glycolipids (Slidregt et al. 1999) when functionalized on to nanocarriers resulted in enhanced targeting to hepatocytes. Pullulan (PUL), a water-soluble polysaccharide polymer comprising three α -1, 4-linked glucose molecules that are repeatedly polymerized at α -1, 6-linkages on terminal glucose, was reported to be accumulated in the liver at significantly higher amounts than other water-soluble

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polymers (Yamaoka et al. 1993; 1995). Chemical conjugation of interferon (Suginoshita et al. 2002) and DNA (Hosseinkhani et al. 2002) with pullulan enabled enhanced targeting to the liver. The binding of iodine-labeled pullulan to isolated parenchymal cells was inhibited by known ligands of ASGPR suggesting ASGPR-mediated uptake. Further, the internalization of pullulan via ASGPR-mediated endocytosis has been confirmed by fluorescence microscopy (Kaneo et al. 2001).

Recently we have reported the design of pullulan-functionalized polyethylene sebacate (PES)-DOX nanoparticles for improved hepatic targeting. Comparative biodistribution study in rats using ^{99m}Tc -labeled formulations revealed high blood concentration and long circulating nature of nanoparticles, and hence the possibility of improved targeting to hepatocytes (Guhagarkar et al. 2010).

The aim of the present study is to evaluate *in vitro*, the ASGPR-mediated uptake of pullulan-functionalized DOX nanoparticles in Hep G2 cell lines, with the specific objective of understanding the role of nanoparticle size and composition on cell uptake. The polymers selected for design of DOX nanoparticles included hydrophobic polymer PES, polymer of intermediate hydrophobicity poly (lactic-co-glycolic acid) (PLGA) and hydrophilic polymer Gantrez AN 119 (copolymer of methyl vinyl ether and maleic anhydride).

2 Materials and methods

2.1 Materials

Doxorubicin hydrochloride was obtained from Hovid Sdn Bhd (Malaysia) as gift sample. Gantrez AN 119 ISP (Molecular weight 200000) was obtained as a gift sample from Anshul Agencies (Mumbai, India). PES was synthesized in our laboratory (molecular weight; 9,625). PLGA 50:50 (PDLG 5010; inherent viscosity midpoint of 1 dl/g) was purchased from PURASORB[®]. Tween 80, magnesium acetate, tetrahydrofuran (THF), acetone, and sodium lauryl sulfate were purchased from S.D. Fine Chemicals (Mumbai, India). Acetonitrile HPLC grade was provided as gift sample by Azeocryst Organics Pvt Ltd (Mumbai, India). Pullulan (molecular weight, 200 kD) from Hayashibara, Japan was supplied as gift sample by Gangwal Chemicals Pvt. Ltd. (Mumbai, India). Eagle's Minimum Essential Medium (EMEM), fetal bovine serum, EDTA, and trypsin was purchased from HIMEDIA[®]. Gentamycin injection IP (40 mg/ml) was obtained from local suppliers. Distilled water was used throughout the experiments. All other chemicals and reagents were either spectroscopic or analytical grade.

2.2 Synthesis of PES

PES was synthesized in our laboratory by a two-step reaction involving initial esterification between ethylene glycol and sebacic acid to form a diester, dihydroxy ethylene sebacate oligomer. This was followed by condensation reaction between two ester dimmers to form PES. PES was characterized by gel permeation chromatography, FTIR, $^1\text{H-NMR}$, differential scanning calorimetry, and X-ray diffraction. Toxicity studies including genotoxicity and mutagenicity have confirmed safety of PES for biomedical and pharmaceutical applications (More et al. 2009).

2.3 Preparation of nanoparticles

PES-DOX nanoparticle (NP) was prepared by modified nanoprecipitation. PES (20 mg) and Gantrez AN 119 (Gantrez; 5 mg) were dissolved in 10 ml of THF and acetone (1:1). DOX (10 mg) and tween 80 (10% *v/v*) were dissolved in water (25 ml). The organic phase was added drop-wise to the aqueous phase under magnetic stirring followed by addition of aqueous solution of magnesium acetate tetrahydrate (1 ml of 0.5% *w/v*) as a stabilizing agent for Gantrez. The resulting dispersion was stirred for a period of 4 h to ensure complete evaporation of the organic solvents. This was followed by addition of pullulan (nanoparticles: pullulan ratio 1:1/1:2) to prepared pullulan-functionalized nanoparticles. PLGA-DOX NP was prepared as above by simple replacement of PES with PLGA. Gantrez-DOX NP was prepared as above without addition of PLGA or PES in the organic phase.

PES-DOX NP dispersion was centrifuged (Z36HK Hermle, Germany) at 20,000 rpm for 30 min. The supernatant was analyzed for entrapment efficiency and the pellet obtained was dispersed in distilled water/distilled water containing pullulan (nanoparticles: pullulan ratio 1:1/1:2) by probe sonication (DP120, Dakshin, Mumbai, India) for a period of 10 min with a 15 s on/off cycle to obtain nanoparticles of larger size.

2.4 Evaluation of nanoparticles

2.4.1 Entrapment efficiency and drug loading

The supernatant obtained after centrifugation was suitably diluted and analyzed for free DOX by UV spectrophotometry (Shimadzu, Japan) at 478 nm. The percentage entrapment efficiency was calculated as follows:

% Entrapment efficiency

$$= \left[\frac{(\text{DOX})_{\text{total}} - (\text{DOX})_{\text{supernatant}}}{(\text{DOX})_{\text{total}}} \right] \times 100$$

Drug loading was calculated using the following formula

$$\% \text{ Drug loading} = \frac{[(\text{amount of DOX entrapped in nanoparticles}) / (\text{amount of DOX added} + \text{amount of drug and polymers added})] \times 100}$$

2.4.2 Particle size

Particle size was determined by photon correlation spectroscopy using the N4 plus submicron particle size analyzer (Beckman Coulter, USA) at 25°C. All measurements were taken by scattering light at 90°. The nanoparticulate dispersion was diluted with water (filtered through 0.22 µm membrane filter) to obtain final counts per second (intensity), 5×10^4 to 1×10^6 and the particle size was recorded.

2.4.3 Zeta potential

The Zeta potential of nanoparticles was determined using Zeta sizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The nanoparticulate dispersion was diluted and the zeta potential determined.

2.5 Cell uptake studies

Human hepatoma cells, HepG2, were used as the model cell line for evaluation of uptake of DOX nanoparticles. Cells were obtained from the National Institute of Cell Sciences, Pune, India. The cells were grown on EMEM containing 1 mM sodium pyruvate supplemented with 10% fetal bovine serum, and 40 mg/ml gentamycin, at 37°C. Cells were trypsinized on reaching 90% confluence.

Hep G2 cells were seeded in 24-well plates at densities of $3\text{--}4 \times 10^5$ cells/well and allowed to adhere by incubating for a period of 24 h at 37°C. The medium was discarded and replaced with DOX solution and DOX nanoparticles. The plate was then incubated at 37°C for a period of 1 h. At the end of 1 h, supernatant was collected and the cells lysed in the wells using 0.5% w/v sodium lauryl sulfate solution (200 µl). Trypsin-EDTA solution (100 µl) was added to facilitate cell detachment. The DOX content in the cell lysate was determined by high-performance liquid chromatography.

The liquid chromatographic system Jasco LC900, consisting of a Jasco PU-980 Intelligent HPLC pump (Jasco, Japan) coupled with a Jasco UV-975 Intelligent UV/VIS detector and a Rheodyne injector (model 7725) fitted with a 20-µl sample loop, was employed for the study. Data integration was done using Borwin Chromatography software version 1.21. Chromatography was performed on a Spherisorb® 250 × 4.6 mm HPLC cartridge prepacked with Spherisorb® 5 µm ODS2 (Waters, USA). The mobile phase

comprised of acetonitrile (40%) and water containing 0.1% w/v triethylamine with pH adjusted to 3 with ortho-phosphoric acid (60%). The mobile phase was degassed by sonication prior to use. Chromatography was performed at room temperature under isocratic conditions at a flow rate of 1.0 ml/min with UV detection at a λ_{max} of 254 nm.

Cell uptake of PES-DOX NP and PES-DOX-PUL was evaluated at two different concentrations of DOX viz. 10 and 50 µg/ml and nanoparticles/pullulan ratio 1:1 and 1:2 while nanoparticle size was maintained at ~200 nm. Comparative evaluation of nanoparticles of the three polymers PES, PLGA, and Gantrez functionalized with pullulan (nanoparticles: pullulan 1:1) was also carried out at DOX concentration of 10 µg/ml while nanoparticle size was maintained at ~125 nm. DOX nanoparticles without pullulan and DOX solution served as controls.

2.6 Statistical analysis

All data in tables and figures are expressed as mean ± standard deviation and mean ± standard error, respectively. Statistical analysis was performed using the one-way ANOVA with Dunnet test and Student's *t* tests. $p < 0.05$ was the criterion for statistical significance.

3 Results

The entrapment efficiency, drug loading, particle size, and zeta potential of nanoparticles are reported in Table 1. PES-DOX NP prepared by modified nanoprecipitation technique using Gantrez as a complexing agent revealed high entrapment efficiency and high drug loading. Entrapment efficiency and drug loading decreased with increased hydrophobicity of polymers and was in the order Gantrez-DOX-NP > PLGA-DOX NP > PES-DOX NP. Functionalization with pullulan did not influence the particle size and zeta potential of nanoparticles.

Cellular uptake of DOX from PES NP (~200 nm) is depicted in Fig. 1. Cell uptake of PES-DOX NP and PES-DOX-PUL was comparable to DOX solution ($p > 0.05$). Further, increase in pullulan concentration did not enhance the cellular uptake of nanoparticles. Although uptake of PES-DOX-PUL at 50 µg/ml was significantly higher ($p < 0.05$, *t* test) than PES-DOX-PUL at 10 µg/ml it was comparable to DOX solution (50 µg/ml). Decreasing particle size of PES NP to ~125 nm revealed no change ($p > 0.05$) in uptake. Comparative evaluation of nanoparticles of the three polymers (~125 nm) is depicted in Fig. 2. Surprisingly, the uptake with all the three polymers was comparable ($p > 0.05$) despite differences in their hydrophobic character. Moreover, functionalization with pullulan did not enhance cell uptake even at the higher concentration of pullulan.

Table 1 Entrapment efficiency, particle size, and zeta potential of nanoparticles (mean \pm SD, $n=3$)

Nanoparticles	Entrapment efficiency (%)	Drug loading (%)	Particle size (nm)	Zeta potential (mV)
PES-DOX NP	75.92 \pm 2.4	21.6 \pm 0.62	210.3 \pm 2.3	-22.1 \pm 2.4
PES-DOX PUL			215.5 \pm 11.8	-21.9 \pm 0.5
PES-DOX NP	75.92 \pm 2.4	21.6 \pm 0.62	125.5 \pm 3	-19.9 \pm 1.65
PES-DOX PUL			128.4 \pm 2.8	-20.33 \pm 0.80
PLGA-DOX NP	81.09 \pm 0.14	23.16 \pm 0.06	129.7 \pm 0.9	-21.7 \pm 0.87
PLGA-DOX PUL			124.4 \pm 1.07	-23.4 \pm 1.64
Gantrez-DOX NP	87.90 \pm 0.24	58.60 \pm 0.18	133.6 \pm 1.7	-20.6 \pm 1.68
Gantrez-DOX PUL			130.5 \pm 3.3	-21.2 \pm 2.42

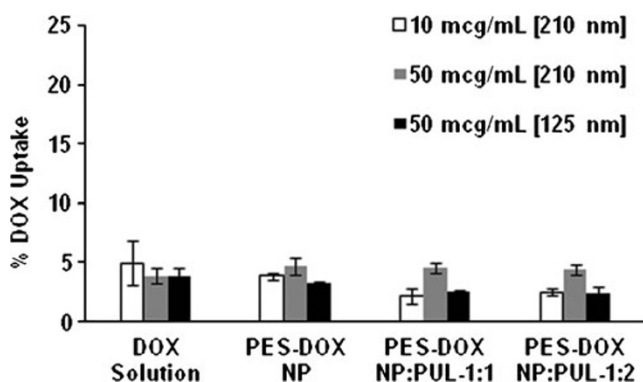
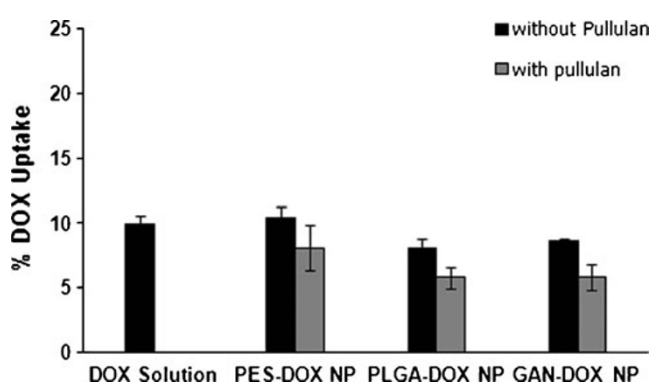
4 Discussion

Development of multidrug resistance and toxicity associated with higher doses, poses significant challenges in the treatment of hepatic cancer using DOX. However design of nanoparticulate carriers of DOX can address these issues by overcoming P-glycoprotein-mediated efflux thereby increasing intracellular drug concentration and drug cytotoxicity (Barraud et al. 2005). Previous studies have reported increased antitumor efficacy of DOX-PIHCA nanoparticles when evaluated in hepatic metastases model in mice. DOX-PIHCA nanoparticles were found to accumulate in the Kupffer cells of the liver which served as drug reservoir inducing release of DOX to the neighboring metastatic cells (Chiannilkulchai et al. 1990). However, this can result in toxicity to the Kupffer cells and other macrophages when conventional nanocarriers are used (Brigger et al. 2002). A significant dose of DOX in the hepatocytes, the site of action for the drug can be achieved by targeting to the ASGPR, abundantly present on hepatocytes. Moreover, ASGPR are reported to be overexpressed in hepatic cancer (Trouet and Jolles, 1984). DOX coupled to lactosaminated human serum albumin has been extensively studied as hepatotropic carrier to improve the chemotherapeutic index of DOX in the treatment of hepatic cancer (Stefano et al.

2006; Fiume et al. 2008). Poly[N-(2-hydroxypropyl)methacrylamide] copolymer bearing DOX and galactosamine, known as PK2 was found to enhance hepatic targeting when evaluated in primary hepatocellular tumors (Julyan et al. 1999; Seymour et al. 2002).

In the present study, pullulan-functionalized nanoparticles of DOX with high entrapment efficiency and high drug loading were designed for targeting to ASGPR. The high entrapment efficiency is attributed to ionic complexation between Gantrez and DOX and the modified nanoprecipitation method followed for the preparation of nanoparticles (Guhagarkar et al. 2010). The same was observed with PLGA-DOX NP. Negative zeta potential values can be attributed to the free carboxyl groups of Gantrez.

ASGPR is reported to be highly expressed on the surface of several human hepatoma cell lines such as Hep G2. Though most of the studies indicate enhanced uptake of small size nanoparticles, there appear to be conflicting reports regarding particle size and uptake by hepatocytes. While an upper particle size limit of 80 nm has been proposed by some researchers for high hepatocellular uptake (Rensen et al. 2001), efficient uptake of liposomes of size 227 nm by hepatocytes have been demonstrated by others (Maitani et al. 2001). Recently, Huang et al. (2008)

**Fig. 1** Effect of particle size and DOX concentration on uptake of PES-DOX NP in Hep G2 cell line (mean \pm SE, $n=3$)**Fig. 2** Effect of polymer type on uptake of DOX nanoparticles in Hep G2 cell line (mean \pm SE, $n=3$)

reported greater association of galactosylated superparamagnetic iron oxide nanoparticles of 278-nm size to Hep G2 cells. Zauner et al. (2001) observed internalization of only few particles of polystyrene microsphere of 93 and 220 nm in Hepa 1-6 and Hep G2 cell line. In the present study, no significant difference ($p > 0.05$) in the uptake was observed irrespective of size. Further functionalization with pullulan revealed no specific enhancement on the cell uptake.

Influence of surface hydrophobicity on cellular uptake is well documented. Cell attachment experiments revealed that interactions between Hep G2 cells and polymeric films increased with increase in hydrophobicity (Hu et al. 2007). On the hand decreased cellular uptake due to presence of residual poly(vinyl) alcohol on nanoparticle surface has also been reported (Sahoo et al. 2002). Pullulan-functionalized nanoparticles of three polymers of different hydrophobic character, revealed no significant difference in uptake by Hep G2 cells. Anchoring of pullulan on to the nanoparticles surface was however confirmed by lower hemolytic potential and long circulating nature of pullulan-coated DOX nanoparticles (Guhagarkar et al. 2010). Our data corroborates with an earlier report on DOX liposomes prepared using sterylglucosamine as ASGPR ligand which did not show enhanced cell uptake in Hep G2 cell line, nonetheless higher accumulation of DOX liposomes in liver was observed in vivo in mouse and rat liver cancer model (Shimizu et al. 1998; Maitani et al. 2001).

Affinity of ligand to ASGPR is strongly influenced by ligand type and density and spacing of the ligand. For instance, binding of N-acetylgalactosamine sugars to ASGPR is more effective than galactose. Clustering of glycosides was reported to enhance the affinity for ASGPR and found to be in order tetraantennary > triantennary >> biantennary >> monoantennary galactosides (Connolly et al. 1982). Higher ASGPR recognition of liposomes containing glycolipids with tris-galactoside was reported by Sliedregt et al. (1999). Moreover, appropriate spacing of sugar residues (20 >> 10 >> 4 Å) is essential for optimum receptor recognition (Biessen et al. 1995; Lee et al. 1983).

Degree of galactosylation of ligand also affected the cellular uptake. Managit et al. (2005) found that uptake of galactosylated lipid emulsion was dependent on concentration of galactosylated cholesterol derivatives used in the preparation of emulsion. They found that at least 4 mol% of galactosylated cholesterol derivatives was required for recognition by receptor and 6 mol% resulted in extensive ASGPR-mediated uptake in Hep G2 cell lines. Similar observations were reported by Ogawara et al. (1998) on hepatic uptake of galactosylated BSA derivatives in isolated perfused rat liver, and by Han et al. (1999) on uptake of methotrexate-carrying BSA conjugated with a greater number of galactose moieties. Hashida et al. (1997)

evaluated hepatic targeting of galactosylated macromolecules including galactosylated proteins, polysaccharides (dextran and amylase), and poly-amino acids. While good correlation was observed between hepatic uptake clearance and galactose density for galactosylated protein, such correlation was not observed with galactosylated polysaccharides and poly-amino acid. The linear structures of polysaccharides and poly-amino acids could decrease the apparent densities of galactose residues suggesting that polysaccharides and poly-amino acids require more number of galactose residues to be recognized by the ASGPR.

Chemically, pullulan is a linear polysaccharide and consists of three α -1, 4-linked glucose molecules that are repeatedly polymerized at α -1, 6-linkages on terminal glucose. Suboptimal configuration of the glucose units could have precluded extensive interaction of the glucose units with the ASGPR. As reported with dextran and amylase higher concentration of pullulan may be required to enhance the cellular uptake by Hep G2 cells via ASGPR due to its linear configuration.

Recently, Xu et al. (2010) evaluated the mechanism for cellular uptake of PLGA nanoparticles and reported that nanoparticles are not taken up by cells and deliver the drug to the cells by extracellular release and/or direct drug transfer to the contacting cells thus resulting in lower cellular concentration with nanoparticles. Comparable uptake of DOX solution and DOX nanoparticles with and without pullulan, suggests extracellular release of DOX as the mechanism of uptake from the nanoparticles in our study. It appears therefore that in vitro evaluation in Hep G2 cell lines may not be the best method to evaluate ASGPR-mediated uptake of polysaccharide-based ligands (Hashida et al. 1997)

Poor cellular uptake can also be related to negative surface charge of nanoparticles. In general, positively charged and neutral nanoparticles exhibit enhanced cellular uptake compared to negatively charged particles (Lorenz et al. 2006; Hu et al. 2007). Negative zeta potential of the DOX nanoparticles could have resulted in repulsion between particles and Hep G2 cells resulting in poor cellular uptake.

5 Conclusion

The in vitro cell uptake studies in Hep G2 cell line could not elucidate the role of pullulan as ASGPR ligand, however in vivo studies in hepatic cancer model are essential to confirm the role of pullulan as ASGPR targeting agent.

Acknowledgements Swati A. Guhagarkar is thankful to Department of Biotechnology (DBT), Government of India for Ph. D Research Fellowship.

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