Preparation, Characterization and Anti-tumor Activity of Epirubicin Loaded Xyloglucan Nanoparticles for Nasal Delivery

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ABSTRACT

The main objective of this study was to develop a polymeric drug delivery system for epirubicin, intended to be administered nasally, capable of improving the therapeutic index of the drug and devoid of the adverse effects of drug. To achieve this goal epirubicin loaded polymeric nanoparticles were prepared by the high-pressure homogenization method. Our results demonstrate that the methodology of preparation allowed the formation of nanometric (<200 nm), homogeneous and negatively charged particles which are suitable for nasal administration. The in vitro anti-tumor activity of xyloglucan-based nanoparticles developed in this work was assessed by using KB cells and compared to the in vitro anti-tumoral activity of the drug alone. These results demonstrate that incorporation of epirubicin in nanoparticles strongly enhances the cytotoxic effect of the drug.

KEYWORDS: Nanoparticles; Xyloglucan; Epirubicin; Nasal; Anti-tumor activity.

Introduction

Since long, intranasal route has utilized for delivering drugs for systemic action as the drug directly reaches to blood by crossing the nasal mucosa (Graff and Pollack, 2005). In case of brain also, better targeting action can achieved due to direct movement of drug from the sub mucosal space of the nose into the CSF compartment of brain (Pardridge, 2007). Transnasal delivery is a non-invasive method of bypassing the BBB to deliver the drug substances and peptides to the CNS. The highly permeable nasal epithelium allows rapid drug absorption to the brain due to high total blood flow, porous endothelial membrane, large surface area and avoidance of first-pass metabolism. Trans nasal method can deliver a wide variety of therapeutic agents (small molecules and macromolecules) to the CNS (Costantino et al., 2007). Many agents active in the CNS are more effective when given nasally and provide the advantage of small dose, self-administration and avoidance of sterile techniques. It neither requires modification of the therapeutic agent nor coupling of drug to carrier. Transnasal delivery has some limitations including damage of nasal mucosa on frequent use of this route, rapid clearance from nasal cavity by mucociliary clearance system, interference due to nasal congestion, elimination of some quantity of drug absorbed systemically via normal clearance mechanism and possibility of partial degradation or irritation to the nasal mucosa (Ali et al., 2010).

From the last few decades, nanoparticles have attracted considerable interest in targeting drug molecules to brain. Nano delivery systems have great potential to facilitate the movement of drugs across barriers (e.g., BBB). Nanosystems employed for the development of nano drug delivery systems in the treatment of CNS disorders include polymeric nanoparticles (NP), nanospheres, nanosuspensions, nanoemulsions, nanogels, nano-micelles andnano-liposomes, carbon nanotubes, nanofibers and nanorobots, solid lipid nanoparticles (SLN), nano-structured lipid carriers (NLC) and lipid drug conjugates (LDC). In fact, nanotechnology has now emerged as an area of research for invention of newer approaches. The novel properties such as tiny size, tailored surface, better solubility, and multi-functionality of nanoparticles present the capability to interact with composite cellular functions in new ways (Alam et al., 2010).

Several decades of biomaterials research had led to a progressively sharp increased in the use of biodegradable polymeric NPs for drug delivery applications. The administration of biodegradable polymeric NPs offers controlled as well site-specific delivery. Various polymers employed in formulation of nano particulate delivery system due to their excellent biocompatibility, biodegradability, lower toxicity, greater stability and circulating time in the biological system. Among the new drug delivery systems, polymeric nanoparticles have been consider as promising were extensively characterized carriers for anticancer agents.
Xyloglucan is a natural polysaccharide isolated from seed kernel of *Tamarindus indica*. It possesses properties like high viscosity, broad pH tolerance and adhesivity. This leads to its application as stabilizer, thickener, gelling agent in food and binder in pharmaceutical industries (Glicksman, 1986). In addition to these, other important properties of xyloglucan have identified recently. They include non-carcinogenicity, muco-adhesivity, biocom-patibility, high drug holding capacity and high thermal stability.

Epirubicin is a newanthracycline derivative of doxorubicin, and a broad-spectrum antineo-plastic agent. It acts by intercalating DNA strands, resulting in complex formation, which inhibits DNA and RNA syntheses. Epirubicin is potent in inhibiting brain tumor cells in vitro. However, an optical in vivo imaging observation shows that, after intravenous administration, epirubicin could not be seen in the rat brain regions protected by the BBB, and just seen in the regions lacking the BBB.

In brain tumors, anticancer agents such as methotrexate (Wanget al., 2003), 5-fluorouracil (Sakane et al., 1997), and raltitrexed (Wang et al., 2005) have been delivered successfully to the brain using intranasal delivery. Shingaki et al., (1999) reported that intranasally delivered methotrexate reaches the CSF and reduces tumor weight in rodent glioma allografts. Intranasal drug targeting to the brain of the chemotherapeutic raltitrexed is significantly higher than that with intravenous administration (Wang et al., 2005). To achieve therapeutic efficacy, the drugs need of preferentially target brain tumor. Hence present study was carry out develop polymeric nanoparticles suitable for nasal administration and achieving targeting of drug to tumor site in brain.

Materials and Methods

Epirubicin was a kind gift from RPG Life Sciences Ltd. Mumbai India. Xyloglucan obtained from DSP-Gokyo Food and Chemical Co. Ltd. Fukusima JAPAN. All other chemicals were of analytical grade and used as received.

**Preparation of solid lipid nanoparticles**

Polymeric nanoparticles were prepared by using a High Pressure Homogenization and freeze drying technique. The procedure was as follows: 2.5 mg of Epirubicin HCl and 100 mg of Xyloglucan accurately weighed and dissolved in 100 ml of distilled water. The solution was stirred at 100 rpm for 20 minutes by using magnetic stirrer and mixture subsequently homogenized using high-pressure homogenizer (GEANEROSOAVI HPH) at pressures ranging from 300 bars to 1000 bars for 3–10 passes. After homogenization the particles were separated from the dispersing medium by centrifugation of the colloidal dispersion and nanoparticles were stabilized by freeze drying (Freeze dryer, Vir TisBenchtop, Italy), employing mannitol (0.5%) as a cryoprotectant.

**Particle size analysis and polydispersity index (PDI)**

Particle size (PS) distribution (mean diameter and polydispersity index) was determined by photon correlation spectroscopy (PCS) using a Malvern instrument (Nano ZS 90, Malvern Instruments, UK). Mean particle size and polydispersity index were calculated for each sample. Distilled water was used as dispersant and the system maintained at 25 °C.

**Zeta potential measurement**

The nanoparticles were dispersed in distilled water to get stock solution of 1% w/w. This dispersion was filled in zeta cell and zeta potential was determined using Zeta Sizer (Nano ZS 90, Malvern Instruments, UK) with the help of software.

**Scanning electron microscopy**

The morphology of Epirubicin HCl loaded polymeric nanoparticles observed using a scanning electron microscopy (SEM) (Jeol, JSM-6700F, Japan). A drop of the nanoparticle suspension placed on a graphite surface and dried. The sample was then coated with Pt by Ion Sputter (Jeol, JFC-1100, Japan). Coating was performed at 20 mA for 4 min, and observation was made at 15 kV (Barbara Luppi et al., 2010).

**Percentage of Entrapment efficiency**

For determination of drug entrapment efficiency, optimized formulation (about 2.5 mg of) is dissolved in distilled water. The drug concentration in the optically clear supernatant, obtained after centrifugation was determined by spectrophotometric measurements in water at 254 nm in an quartz cuvette by using a double-beam UV−vis (UV 1700, Shimadzu, Japan), against the standard solution of epirubicin hydrochloride (Hui-zhu Zhanget al., 2009). It is calculated by the formula,

\[
\% \, EE = \left( \frac{\text{total loaded drug weight}}{\text{initial drug weight}} \right) \times 100
\]

**In-vitro release studies**

Drug release studies by dialysis method were performed similarly to previously described protocol (Wolfram S, et al. 2011). Five milliliters of suspension was placed within a hydrated dialysis membrane (molecular weight cut-off size of 12.4 kDa, Sigma) and was dialyzed against 50 ml of PBS (pH 6.6) at 37 ± 0.5°C in a closed light-protected vessel upon gentle stirring (~100 rpm). The amount of released drug was determined by spectrophotometric measurements of the release medium at 254 nm (each aliquot was returned immediately after measurement to maintain constant volume of the release medium and to keep constant amount of drug in the system).

**Ex vivo permeation studies**

Fresh nasal tissues carefully removed from Nasal cavity of sheep obtained from the local slaughter house. Tissue sample placed in Franz type diffusion cell displaying a permeation area of 0.785 cm². Phosphate buffer solution (PBS) pH 6.6 (16 ml) at 37±0.5 °C was added to the accept or chamber. The temperature within the chambers maintained at 37 °C. After a pre-incubation
time of 20 minutes, formulation equivalent to 2.5 mg of Epirubicin HCl placed in the donor chamber. At predetermined time points, 2 ml samples withdrawn from the accept or compartment, replacing the sampled predetermined time points, 2 ml samples withdrawn. Epirubicin HCl placed in the donor chamber. At time of 20 minutes, formulation equivalent to 2.5 mg of Epirubicin HCl was diluted in RPMI 1640 culture medium to give final epirubicin concentrations of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml. The epirubicin HCl nanoparticles in RPMI had pH 6.6 after each sampling, for a period of 4 hours. The amount of permeated drug was determined using a UV-visible spectrophotometer at 254 nm (Barbara Luppi et al., 2011).

**Stability studies**

The stability studies were carried out in accordance with the ICH guidelines for new drug products at 5°C ± 3°C and at 25°C ± 2°C/60% ± 5% RH up to 6 months (M)(Q1A(R2), 2003). Optimized batch of formulation was subjected to stability studies. The NPs were filled in glass vials, closed with rubber closures and sealed with aluminum caps. The samples were withdrawn at predetermined periods and were examined visually for physical appearance. The contents of the vials evaluated for the PS zeta potential and drug content. For accelerated condition (i.e. 25°C ± 2°C/60% ± 5% RH) sampling was done at end of 1, 2, 3 and 6 months) and for 5°C ± 3°C sampling was done at end of 3 and 6 months. The drug content in the initial sample was considered as 100%. Data are expressed as mean ± SD, n = 3.

**Histological examination**

Histological studies carried out using isolated sheep nasal mucosa. Three sheep nas mucosa pieces (A, B and C) with uniform thickness were selected and mounted on Franz diffusion cells. A was treated with PBS pH 6.6 (negative control), B with isopropyl alcohol (Positive control), and C was treated with Epirubicin HCl loaded polymeric nanoparticles respectively. After treatment for 2 hours, all the samples washed with double distilled water, sectioned and stained with hematoxylin and eosin and photographed by optical microscope. Histological examination was carried out using isolated sheep nasal mucosa. Histological studies carried out using isolated sheep nasal mucosa. Three sheep nas mucosa pieces (A, B and C) with uniform thickness were selected and mounted on Franz diffusion cells. A was treated with PBS pH 6.6 (negative control), B with isopropyl alcohol (Positive control), and C was treated with Epirubicin HCl loaded polymeric nanoparticles respectively. After treatment for 2 hours, all the samples washed with double distilled water, sectioned and stained with hematoxylin and eosin and photographed by optical microscope (Jiang et al., 1995). The mucosa was dissected and subjected for histological examination to evaluate the toxicities of polymeric nanoparticles.

**Cytotoxicity assay**

KB cells (5×104 cells/ml) harvested from growing cells as a monolayer seeded onto 96-well plates at 10,000 cells/well and incubated for 24 hours. Epirubicin HCl polymeric nanoparticles and free Epirubicin were diluted in RPMI 1640 culture medium to give final epirubicin concentrations of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml. The epirubicin HCl nanoparticles in RPMI 1640 were diluted to 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, and 1.25 µg/ml, 2.5 µg/ml. At the end of the incubation period with the different concentration, cells were washed twice with phosphate-buffered saline solution (PBS, pH adjusted to 7.4) by centrifugation (10 min, 1200 rpm) and incubated with fresh RPMI 1640 and cultured in 5% CO2 at 37°C for 48 hours. Fresh growth media (100 µl) and 10 µl aliquots of MTT solution (5 mg/ml) used to replace the mixture in each well after 48 hours. The plates then returned to the incubator and maintained in 5% CO2, at 37°C for a further 4 hours. The growth medium and excess MTT in each well were then removed. DMSO (150 µl) was then added to each well to dissolve the internalized purple formazan crystals. The results expressed as a percentage of the absorbance of the non-treated cells (Hui-zhu Zhang et al., 2009).

**Results and Discussion**

**Particle size and size distribution analysis**

The high-pressure homogenization technique allowed the preparation of polymeric submicron particles. All NPs obtained were less than 500 nm in size. The particle size and size distribution analysis of polymeric nanoparticles are of great importance because they determine their key properties such surface area, packing density. Particle size of prepared lyophilized polymeric nanoparticles (batch A1–A5) was determined and results were shown in Table 1 along with polydispersity indices. A decrease in mean particle size from formulation A1 to A5 was simultaneous decrease in PDI. It is an due to the increase in pressures used in the high pressure homogenization (HPH) (Table 1). A varying in the polydispersity values shows a good size distribution. The particle size of polymeric nanoparticles is important factor as this determines the rate and extent of drug release as well as absorption. Formulation A5 has particle size 131.0 nm with low polydispersity index of 0.333. Hence, found to be suitable for nasal administration and brain targeting. Hence, considered as optimized as selected for further studies. Table 1 also shows that all the nanoparticles presented quite polydisperse populations with a mean diameter lower than 400 nm even after drug loading.

TABLE 1

<table>
<thead>
<tr>
<th>Formulation ingredients / Process Parameter</th>
<th>Formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle Size (d.nm)</strong></td>
<td>A1</td>
</tr>
<tr>
<td>Epirubicin HCl (mg)</td>
<td>2.5</td>
</tr>
<tr>
<td>Xyloglucan (mg)</td>
<td>100</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>100</td>
</tr>
<tr>
<td>Homogenization Pressure (bar)</td>
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<tr>
<td>No of Cycles</td>
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<tr>
<td>Particle Size (d.nm)</td>
<td>136.5</td>
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<tr>
<td>PDI</td>
<td>0.935</td>
</tr>
</tbody>
</table>

**Per cent entrapment efficiency**

The optimized composition (A5) shows 87.48% entrapment of drug. Such high entrapment efficiency of drug indicates the maximum amount of drug loaded in the formulation as result of homogenization process.

**Scanning electron microscopy (SEM)**

Figure 1 shows the morphology of the nasal inserts observed by scanning electron microscopy (SEM). As nanoparticles were obtained by freeze-drying, which consists of sublimation of the frozen water yielding to the formation of pores or channels in the polymer, all the samples were characterized by a sponge-like structure.
Zeta potential could greatly influence the stability of NPs in suspension through electrostatic repulsion between the particles. The xyloglucan microspheres prepared were negatively charged (zeta potential value -21.2 mV), indicating the presence of xyloglucan at the surface of all microspheres. Based on the zeta potential data from the previous studies for NPs, these absolute values of zeta potential indicate a high electrical charge on the surface of the NP that can cause strong repellent forces among particles to prevent aggregation of the NPs (Freitas and Müller, 1998).

**In vitro drug release studies**

The drug release study of batch A5 was carried out as it showed the suitable particle size. The dissolution profile showed biphasic behavior consisting of initial burst release followed by a slow release phase (Fig. 2). The initial burst release attributed to the presence of free drug in the external phase and drug adsorbed on the surface of particles while the slow release was due to drug encapsulated within polymeric matrix.

**Ex vivo permeation studies**

Ex-vivo nasal permeability study reveals drug diffusion at faster rate and the total percentage diffusion was much higher from the PNP system than pure drug. At the end of 4 hours, the percentage of drug diffusion was 69.96±1.99% from drug loaded polymeric nanoparticles and 40.47±3.60% from drug suspension.

**Stability studies**

The stability studies of the xyloglucan based polymeric NPs were performed in order to study the influence of varying environmental conditions on the parameters of the formulation influencing the stability. To ensure the NPs stability during storage, it is very important to maintain the critical parameters mean PS and size distribution up to the application. The stability studies carried out in accordance with the ICH guidelines for drug substances intended to be stored in a refrigerator. The results for the stability studies reveals that there was no significant change observed in PS, zeta potential and drug content NPs at 5 °C ± 3 °C for 6 M and 25 °C ± 2 °C/60% RH ± 5% RH for 3M.

**Histological examination**

It is necessary to examine histological changes in nasal mucosa caused by formulations, if it is to be considered for practical use. Histological studies show negative control mucosa (normal nasal mucosa) and positive control mucosa stained with hematoxylineosin and the effect of formulation on sheep nasal mucosa, 2 hours after applying the formulations (Fig. 3). No significant change in mucosal structure was observed when treated with drug loaded polymeric nanoparticle (A5) as compared to the both controls. The section of mucosa treated with formulation epirubicin HCl showed no changes in nasal epithelium. There was no sign of remarkable destructive effect of formulations on the treated nasal mucosa.

**Cyto toxicity assay**

KB cells grown in 96-well plates were exposed to serial dilutions of Epirubicin HCl loaded nanoparticles or free Epirubicin for 24 hr, and cell viability determined by the MTT assay following 48 hr further incubation. Epirubicin HCl concentrations leading to 50% cell killing (IC50) were determined from concentration-dependent cell viability curves. The in vitro anticancer cytotoxic activity of free Epirubicin HCl and Epirubicin HCl polymeric nanoparticles was shown in Fig. 4. Epirubicin HCl polymeric nanoparticles exhibited a higher cytotoxicity against KB cells than free Epirubicin HCl, stressing the key role of nanoparticles binding and internalization in enhancement of cytotoxic activity, the first reason related to the increased cellular uptake of Epirubicin. As a greater amount of Epirubicin could be delivered into cells in the form of nano-sized particles by endocytosis, the cells were more vulnerable to the cytotoxic effect of Epirubicin HCl. For free Epirubicin HCl, a multidrug resistant effect, out-fluxing Epirubicin HCl through the p-glycoprotein pump, might play an additional role in decreasing the intracellular concentration of epirubicin. In terms of the IC50 values, all the cancer cell lines retained different sensitivities to pure epirubicin and the nanoparticles formulation of epirubicin. With native epirubicin, KB cells showed the most sensitivity (IC50=0.7µM) while xyloglucan NPs showed somewhat similar but lower cytotoxicity (IC50=0.6µM).
Fig. 3. Microscopic images illustrate the histopathological condition of nasal mucosa after 2 hr exposure to PBS (A, negative control); isopropyl alcohol (B, positive control); drug-loaded polymeric nanoparticle (C).

Fig. 4. In vitro anti tumor activity.

**Conclusions**

The results obtained showed that the methodology selected in this work allowed the instantaneous and reproducible formation of nanometric (<200 nm), sphericals and homogeneous xyloglucan nanoparticles that exhibit high incorporation efficiencies of epirubicin. Moreover, it was shown that incorporation of epirubicin in the xyloglucan nanoparticles strongly enhances its anti-tumoral efficacy as compared to the free drug, this effect being more relevant for prolonged incubation times with cells. Based on these results, it can be concluded that the formulations developed in this work may be consider promising systems for in vivo epirubicin delivery. This polymeric nanoparticles system could be suitable for nasal administration to achieve targeting to brain tumor.

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**References**


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