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Research Paper

Genotoxic evaluation of poly(anhydride) nanoparticles in the gastrointestinal tract of mice

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ABSTRACT

Gantrez® AN 119-based NPs have been developed as oral drug carriers due to their strong bioadhesive interaction with components of the gastrointestinal mucosa and to their adaptable surface. The use of mannosamine to coat Gantrez® AN 119-based NPs results in a high mucus-permeable carrier, able to reach the gastrointestinal epithelium. Although their efficacy to transport a therapeutic agent has been demonstrated, their safety has not yet been thoroughly studied. They have proved to be non-cytotoxic, non-genotoxic and non-mutagenic in vitro; however, the in vivo toxicity profile has not yet been determined. In this study, the in vivo genotoxic potential of Gantrez® AN 119 NPs coated with mannosamine (GN-MA-NP) has been assessed using the in vivo comet assay in combination with the enzyme formamidopyrimidine DNA glycosylase in mice, following the OECD test guideline 489. To determine the relevant organs to analyse and the sampling times, an in vivo biodistribution study was also carried out. Results showed a statistically significant induction of DNA strand breaks and oxidized bases in the duodenum of animals exposed to 2000 mg/kg bw. However, this effect was not observed at lower doses (i.e. 500 and 1000 mg/kg which are closer to the potential therapeutic doses) or in other organs. In conclusion, GN-MA-NP are promising nanocarriers as oral drug delivery systems.

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1. Introduction

In the last two decades, the development of nanoparticles (NPs) has significantly increased due to the immense variety of potential applications in medicine, pharmacy and food safety (Salata, 2004). Among the various nanoparticles for oral drug delivery systems, polymeric NPs have received major attention due to their unique properties and their adaptable surface (Salman et al., 2006). Furthermore, they are biodegradable, biocompatible and easy to produce.

In many cases, their surface can be easily coated with different ligands in order to modify their physico-chemical properties, as well as their distribution in vivo (Agüeros et al., 2009; Inchaurraga et al., 2015; Salman et al., 2006). For instance, coating with the mannosamine ligand enhances the ability of Gantrez® AN 119 to develop stronger bioadhesive interactions with components of the intestinal mucosa (Salman et al., 2009). In line with this result, this coating showed the best mucus permeability compared with other ligands (i.e. dextran, aminodextran, cyclodextrin, and poly-ethylene glycol (PEG) of different molecular weights) in an in vitro transwell system approach (Iglesias et al., 2017a). This property is particularly interesting and advantageous for engineered products administered orally since it results in prolonged residence time of the formulation in close contact with the absorptive mucosal epithelium, thus increasing drug absorption and bioavailability. It is worth mentioning that after oral

Abbreviations: ALS, alkali-labile sites; DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ECVAM, european centre for the validation of alternative methods; EMA, european medicines agency; EMS, ethyl methanesulfonate; Fpg, formamidopyridine DNA glycosilase; GN-MA-NP, gantrez® 119 AN coated with mannosamine; GN-MA-NPAC5.5, gantrez® AN 119 coated with mannosamine labelled with Alexa Cy5.5; ICCVAM, interagency coordinating committee on the Validation of Alternative Methods; JACVAM, japanese center for the validation of alternative Methods; NPs, nanoparticles; OECD, organization for economic cooperation and development; PEG, poly-ethylene glycol; ROI, region of interest; SBs, strand breaks; SD, standard deviation.

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administration, Gantrez® AN 119-based NPs are not absorbed or distributed to other organs but remain in the gastrointestinal tract before being eliminated (Agüeros et al., 2009; Arbós et al., 2002; Inchaurraga et al., 2015; Porfire et al., 2010; Yoncheva et al., 2005). Nevertheless, Gantrez® AN 119 NPs coated with mannosamine are taken up by Peyer's patches, probably due to the presence of mannose receptor in this tissue (Salman et al., 2006).

Though there are several studies that demonstrate the efficacy of Gantrez® AN 119 coated with mannosamine to transport therapeutic agents (Salman et al., 2009), the safety of these NPs has not yet been thoroughly studied. Commercial Gantrez® AN 119, as well as the mannosamine ligand, have been recognized as safe to human health (Moreno et al., 2014). Their nanoform combination did not affect cell metabolism, membrane integrity or viability of Caco-2 cells after 24 h exposure at high concentrations (2 mg/mL) (Iglesias et al., 2017a).

Genotoxicity is one of the most relevant issues in toxicology due to its relation with the development of mutations and cancer. Furthermore, genotoxicity and oxidative damage have been described as the main mechanisms of toxicity of several NPs (Dusinska et al., 2015). Therefore, according to papers on the development of new nanomedicine products for human use published by the European Medicines Agency (EMA), as well as by several authors, the detection of DNA damage represents a crucially important endpoint for the safety assessment of NPs (Dusinska et al., 2015; EMA, 2006; Magdolenova et al., 2014). In addition, in order to induce genotoxic lesions, there is no need for a direct interaction of NPs with the DNA since they could also interact with proteins, membranes and other cellular components generating high amounts of reactive oxygen species (ROS), which can damage DNA (Magdolenova et al., 2014). Moreover, ROS can also be produced after an inflammatory reaction.

Gantrez® AN 119 NPs coated with mannosamine (GN-MA-NP) did not induce a significant level of DNA strand breaks or formamidopyridine DNA glycosylase (Fpg)-sensitive sites, measured by the comet assay, after 3 or 24 h of incubation in Caco-2 cells. Nevertheless, they induced a very slight increase in Fpg-sensitive sites at 24 h of treatment at very high concentrations (i.e. 1 and 2 mg/mL) (Iglesias et al., 2017a). Furthermore, GN-MA-NP did not show potential genotoxic or mutagenic activities in mouse lymphoma cells (Iglesias et al., 2017b).

The evaluation of the potential in vivo toxicity of polymeric NPs is also essential for acquiring more relevant information about human exposure to NPs. The in vivo mammalian alkaline comet assay is a simple method for measuring DNA damage in single cells from multiple animal (usually rodent) tissues that have been exposed to potentially genotoxic materials. This assay has been validated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Inter-agency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) resulting in the OECD test guideline 489 (Morita et al., 2015); OECD 489, 2014). The assay detects single and double strand DNA breaks at the level of single cells. The combination of the comet assay with the use of different enzymes from the DNA repair system allows the detection of different types of DNA damage. For instance, the comet assay can be modified for detecting oxidized purines, by incorporating Fpg (Dusinska and Collins, 1996). This bacterial DNA repair enzyme detects base damages and introduces additional DNA breaks, increasing the amount of migrated DNA, improving the sensitivity and specificity of the comet assay (Azqueta et al., 2013).

Thus, the present study was aimed at investigating the in vivo genotoxic potential of GN-MA-NP using the in vivo comet assay in combination with Fpg in mice. The comet assay protocol used in our study complies with the OECD test guideline 489. Time points and organs were selected after performing an in vivo biodistribution study using fluorescence-labelled GN-MA-NP.

2. Material and methods

2.1. Chemicals

For the preparation of the NPs, Gantrez® AN 119 and mannosamine were provided by ISP and Sigma, respectively. Acetone was obtained from VWR Prolabo and Alexa Cyanine5.5 carboxylic acid was provided by Interchim.

For the mincing solution, Mg^{++}, Ca^{++} and phenol red-free Hank's balanced salt solution were provided by Gibco, Na_2EDTA was from Sigma, and dimethyl sulfoxide (DMSO) was purchased from Panreac.

For the comet assay, low melting point agarose, standard agarose, Triton X-100, Tris, HEPES, EDTA, BSA and ethyl methanesulfonate (EMS) were provided by Sigma. NaCl, NaOH and KCl were purchased from Panreac. Fpg was a gift from Prof. Andrew Collins (University of Oslo).

For the anaesthesia of mice isofluorane was used (Forane).

2.2. Preparation of GN-MA-NP

NPs were prepared from the copolymer between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) and subsequently covered with mannosamine. The fabrication of this formulation was carried out as previously reported with slight modifications (Salman et al., 2006).

Briefly, 100 mg of the copolymer (Gantrez® AN 119) was dissolved and stirred in 5 mL acetone. In parallel, 5 mL water containing 10 mg mannosamine was added to the polymer solution under magnetic stirring and incubated for 30 min. NPs were obtained by the addition of 10 mL absolute ethanol under magnetic stirring. The organic solvents were evaporated under reduced pressure. Finally, the NPs were purified by tangential filtration (3000 rpm, 5 min, 4 °C) and spray-dried in a mini Spray dryer (Büchi B290). The resulting powder was then stored at room temperature until its use. The resulting NP was then called GN-MA-NP.

2.2.1. Preparation of fluorescently labelled NPs

For the biodistribution study, GN-MA-NP was fluorescently labelled with Alexa-Cy5.5 carboxylic acid (GN-MA-NPAC5.5). For this purpose, 2 mg Alexa-Cy5.5 carboxylic acid was dissolved per 50 mL of acetone containing the copolymer. The formulation was prepared as described above.

2.3. Characterization of NPs

Particle size, polidispersity index (PDI) and zeta potential of GN-MA-NP were determined by photon correlation spectroscopy and electrophoretic laser Doppler anemometry, respectively, using a ZetaPlus zeta potential analyzer with 90 Plus/BI-MAS Multi angle particle sizing option (Brookhaven Instruments Corporation). The diameter of these NPs was determined after their dispersion in ultrapurified water (1/10) and measured at room temperature using a scattering angle of 90°. The zeta potential of GN-MA-NP was determined by diluting the samples in a 0.1 mM KCl solution adjusted to pH 7.4. The particle size and PDI of GN-MA-NPAC5.5 were also measured.

2.4. Biodistribution study

Biodistribution studies were carried out in accordance with the institutional and national guidelines for the welfare of laboratory

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