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In vitro evaluation of the genotoxicity of poly(anhydride) nanoparticles designed for oral drug delivery

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ABSTRACT

In the last years, the development of nanomaterials has significantly increased due to the immense variety of potential applications in technological sectors, such as medicine, pharmacy and food safety. Focusing on the nanodevices for oral drug delivery, poly(anhydride) nanoparticles have received extensive attention due to their unique properties, such as their capability to develop intense adhesive interactions within the gut mucosa, their modifiable surface and their biodegradable and easy-to-produce profile. However, current knowledge of the possible adverse health effects as well as, toxicological information, is still exceedingly limited.

Thus, we investigated the capacity of two poly(anhydride) nanoparticles, Gantrez® AN 119-NP (GN-NP) and Gantrez® AN 119 covered with mannosamine (GN-MA-NP), and their main bulk material (Gantrez® AN 119-Polymer), to induce DNA damage and thymidine kinase ($TK^{+/-}$) mutations in L5178Y $TK^{+/-}$ mouse lymphoma cells after 24 h of exposure.

The results showed that GN-NP, GN-MA-NP and their polymer did not induce DNA strand breaks or oxidative damage at concentrations ranging from 7.4 to 600 μg/mL. Besides, the mutagenic potential of these nanoparticles and their polymer revealed no significant or biologically relevant gene mutation induction at concentrations up to 600 μg/mL under our experimental settings.

Considering the non-genotoxic effects of GN-NP and GN-MA-NP, as well as their exceptional properties, these nanoparticles are promising nanocarriers for oral medical administrations.

1. Introduction

Small nanoparticles (NPs) are able to reach the nucleus and directly interact with the DNA causing genetic damage (Magdolenova et al., 2014). However, NPs do not need to be in direct contact with the DNA to induce genotoxic effects. NPs can negatively interact with cellular proteins, as well as with proteins involved in DNA replication, transcription or repair, cell division or mitotic spindle formation and generate high amounts of reactive oxygen species (ROS) inside the cells, which may cause indirect DNA damage (Magdolenova et al., 2014). Moreover, it has been shown that some NPs are deposited on the cellular surface, or inside the cell, and induce oxidative stress signaling cascades (Manke et al., 2013). Nowadays, it is known that oxidative stress is a crucial factor in NP toxicity (Ahmad et al., 2012; Kumar et al., 2011; Nel et al., 2006). Moreover, increased DNA damage has been

Abbreviations: CE, cloning efficiency; DAPI, 4,6-diamidino-2-phenylindole; ECACC, European collection of cell cultures; FPG, formamidopyridine DNA glycosilase; GEF, Global Evaluation Factor; GN, Gantrez® AN 119; GN-MA, Gantrez® AN 119 coated with mannosamine; HS, heat inactivated horse serum; MF, mutants frequency; ML, mouse lymphoma L5178Y $TK^{+/-}$clone 3.7.2C; MLA, mouse lymphoma assay; MMS, methyl methanesulfonate; MTT, Thiazolyl blue tetrazolium bromide; NPs, nanoparticles; OECD, Organization for Economic Cooperation and Development; PBS, phosphate buffered saline; PDI, polydispersity index; PLGA-PEO, poly-lactic-co-glycolic acid-poly-ethylene oxide copolymer; ROS, reactive oxygen species; RSG, relative suspension growth; TFT, 5-trifluorothymidine; TK, thymidine kinase; TSG, total suspension growth.

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associated with higher frequency of cancer (Hoeijmakers, 2009) and other health issues, including infertility and genetic disorders (Aitken and Krausz, 2001). Therefore, evaluation of the genotoxic potential of NPs should be exhaustive.

Poly(anhydride) NPs have been considered promising carriers for oral drug delivery (Agüeros et al., 2011; Calleja et al., 2015; Zhang et al., 2015). These NPs have received widespread attention due to their singular properties, such us their modifiable surface, which can enhance or reduce bioadhesion to specific target cells (Ensign et al., 2012). Furthermore, poly(anhydride) NPs are biocompatible, biodegradable, and capable of releasing drugs in a sustained way (Calleja et al., 2015). The copolymers between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) are an excellent example of this group of poly (anhydride) NPs (Arbós et al., 2002). Their surface can be modified with different ligands in order to modify their physico-chemical properties to improve *in vivo* distribution (Agüeros et al., 2009; Inchaurraga et al., 2015; Salman et al., 2006). For example, when Gantrez® AN 119 NPs are coated with mannosamine, their already strong bioadhesive interactions with the intestinal mucosa are enhanced (Salman et al., 2005, 2009). It has also been shown that NPs of Gantrez® AN 119 coated with mannosamine presented the highest ability to diffuse through a mucus layer, when compared to Gantrez® AN 119 NPs coated with other ligands (i.e. dextran, aminodextran, cyclodextrin or polyethylene glycol) (Iglesias et al., 2017). This property is especially advantageous in nanocarriers designed for oral drug delivery, since the residence time of the drug in the organism, as well as its availability, will be greater.

It has also been demonstrated that Gantrez® AN 119 based NPs, when orally administered, remain localized in the lumen of the gastrointestinal tract, indicating that these NPs are not absorbed or translocated (Agüeros et al., 2009; Arbós et al., 2002; Inchaurraga et al., 2015; Porfire et al., 2010). Furthermore, previous studies showed that Gantrez® AN 119 nanoforms are capable of establishing adhesive interactions with Caco-2 cells without being internalized (Ojer et al., 2013). However, Salman et al. (2006) observed that this nanoform in combination with mannosamine was uptaken by Peyer's patches, probably due to the presence of mannose receptor in this tissue.

Commercial bulk Gantrez® AN 119, as well as, bulk mannosamine have been recognized as safe for human health (Moreno et al., 2014). Nevertheless, the safety of Gantrez® AN 119 based-NPs and their different ligands have not been thoroughly studied, although some studies showed no effect on viability, cell metabolism, membrane integrity or DNA in Caco-2 cells after 24 h exposure at high concentration (Iglesias et al., 2017). In general, the toxicity of Gantrez® AN 119 nanoforms is considered low or even innocuous to the organism since these NPs are biodegradable and biocompatible (Landsiedel et al., 2012). However, their safety has not been thoroughly studied.

Nowadays, detection of chromosome or DNA damage represents an important tool for prioritizing compounds early in the drug development process since DNA alterations are clearly related to cancer development (Hoeijmakers, 2009). The comet assay is the most commonly used method in nanogenotoxicity studies (Azqueta and Dusinska, 2015). It is a simple method for measuring DNA damage, such as single strand breaks and double strand breaks, and alkali-labile sites (ALS) (purinic and apyrimidinic) (Azqueta and Collins, 2011). The assay has been modified to detect oxidized bases, by incorporating lesion specific enzymes (Dusinska and Collins, 1996). The use of these repair enzymes increases the sensitivity and specificity of the assay; recognizing specific base damages and creating additional DNA breaks which increases the amount of DNA that migrates from the nucleoids (Azqueta et al., 2013).

The use of mammalian genotoxicity tests as, the mouse lymphoma test (MLA) and the Ames test, were recommended by the OECD Working Party on Manufactured Nanomaterials in 2009 (OECD 476, 1997). The Ames test is not suitable for testing NPs due to the limited or no uptake through the bacterial wall (Azqueta and Dusinska, 2015). However, MLA could be a useful tool for genotoxicity assessment in NPs since it is performed on eukaryotic cells. MLA uses the endogenous thymidine kinase (TK) locus transcription to detect a wide spectrum of genetic damage, including both, point mutations and chromosomal alterations. This assay has been validated as a component of the genotoxic testing battery used for evaluating the mutagenicity potential of chemicals (ICH, 2011), and the Organisation for Economic Co-operation and Development (OECD) has recently updated the guideline for this assay (OECD 490, 2015). It has already been used for the assessment of mutagenicity of NMs in some studies (Gábelová et al., 2017).

Therefore, the aim of the present study was to explore the *in vitro* genotoxicity activity associated with the exposure of two poly (anhydride) NPs, Gantrez® AN 119 (GN-NP) and Gantrez® AN 119 covered with mannosamine (GN-MA-NP), after 24 h treatment using the alkaline comet assay and the MLA in L5178Y TK $^{+/-}$ cells. Furthermore, Gantrez® AN 119-polymer (GN-Polymer) was tested as an additional control to distinguish the possible genotoxic potential of the NPs from their bulk material form. Moreover, viability of the cells treated with NPs was evaluated using the proliferation assay.

2. Material and methods

2.1. Chemicals and reagents

NPs preparation: poly methyl vinyl ether-co-maleic anhydride or poly(anhydride) (Gantrez® AN 119; MW: 200000 g/mol) was provided by ISP (Spain). Mannosamine was purchased from Sigma (Spain). Acetone was obtained from VWR Prolabo (France). Deionized water (18.2 Ω resistivity) was obtained by a water purification system by Wasserlab (Spain). Nitrogen gas (ultra-pure, >99%) was produced using an Alltech Nitrogen generator by Ingeniería Analítica (Spain).

Comet assay and MLA: Fischer's medium, glutamine, sodium pyruvate, penicillin, streptomycin, phosphate buffer saline and heat-inactivated horse serum (HS) were purchased from Invitrogen (Spain). Hypoxantine, glycine, methotrexate, sodium carbonate anhydrous, tymidine and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltatrazolium bromide (MTT), methyl methanesulfonate (MMS), and 5-trifluorothymidine (TFT) were obtained from Sigma-Aldrich (Spain).

Comet assay: low-melting point agarose, standard agarose, Triton X-100, Tris, HEPES, EDTA and BSA were provided by Sigma. NaCl, NaOH and KCl were purchased from Panreac (Spain). Photosensitiser Ro 19-8022 kindly supplied by Hoffmann-La Roche (Switzerland). Formamidopypiridine DNA-glycosylase (FPG) was kindly provided by Professor Andrew Collins (Department of Nutrition, University of Oslo, Norway).

2.2. Preparation and characterization

2.2.1. Conventional poly(anhydride) NPs (GN-NP)

The setup of this formulation was carried out as previously reported with slight modifications (Irache et al., 2005; Ojer et al., 2012, 2013).

Briefly, 600 mg of the copolymer (Gantrez® AN 119) were dissolved in 60 mL acetone and desolvated by the addition of a hydroalcoholic mixture under magnetic stirring at room