Biomaterials 35 (2014) 7022–7031

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Dose ranging, expanded acute toxicity and safety pharmacology studies for intravenously administered functionalized graphene nanoparticle formulations

Shruti Kanakia [a], Jimmy D. Toussaint [a], Sayan Mullick Chowdhury [a], Tanuf Tembulkar [a], Stephen Lee [a], Ya-Ping Jiang [b], Richard Z. Lin [b], Kenneth R. Shroyer [c], William Moore [d], Balaji Sitharaman [a,*]

[a] Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY, USA
[b] Department of Physiology and Biophysics, Stony Brook University, Stony Brook, NY, USA
[c] Department of Pathology, Stony Brook University, Stony Brook, NY, USA
[d] Department of Radiology, Stony Brook University, Stony Brook, NY, USA

ARTICLE INFO

Article history:
Received 17 February 2014
Accepted 16 April 2014
Available online 20 May 2014

Keywords:
Graphene nanoplatelets
Dextran
Toxicity
Dose range
Biodistribution
Maximum tolerated dose

ABSTRACT

Graphene nanoparticle dispersions show immense potential as multifunctional agents for *in vivo* biomedical applications. Herein, we follow regulatory guidelines for pharmaceuticals that recommend safety pharmacology assessment at least 10 − 100 times higher than the projected therapeutic dose, and present comprehensive single dose response, expanded acute toxicology, toxicokinetics, and respiratory/cardiovascular safety pharmacology results for intravenously administered dextran-coated graphene oxide nanoplatelet (GNP-Dex) formulations to rats at doses between 1 and 500 mg/kg. Our results indicate that the maximum tolerable dose (MTD) of GNP-Dex is between 50 mg/kg $\leq$ MTD $<$ 125 mg/kg, blood half-life $<$ 30 min, and majority of nanoparticles excreted within 24 h through feces. Histopathology changes were noted at $\geq$250 mg/kg in the heart, liver, lung, spleen, and kidney; we found no changes in the brain and no GNP-Dex related effects in the cardiovascular parameters or hematological factors (blood, lipid, and metabolic panels) at doses $<$ 125 mg/kg. The results open avenues for pivotal preclinical single and repeat dose safety studies following good laboratory practices (GLP) as required by regulatory agencies for investigational new drug (IND) application.

1. Introduction

Carbon nanostructures such as fullerenes, metallofullerenes and carbon nanotubes have been widely investigated as multifunctional materials for applications in tissue engineering, molecular imaging, therapeutics, drug delivery, and biosensing [1–3]. Recently, graphene nanoparticles, (also known as graphene nanoplatelets (GNPs) or simply graphene oxide (GO), (herein referred as GNPs)) show promise *in vitro* and *in vivo* for drug/gene delivery and biological sensing/imaging applications due to their nanoscopic size, large specific surface area, and physicochemical properties [4–10]. For instance, GNPs could be loaded with aromatic drugs via Van der Waals (pi–pi stacking) interactions [5], or non-covalently complexed with cationic polymers such as polyethyleneimine via weak electrostatic interactions to facilitate plasmid DNA (pDNA) and small interfering RNA (siRNA) delivery [7]. GNPs could also be intercalated or covalently functionalized with important elements (e.g. manganese, iodine) in medicine to develop highly efficacious contrast agents for magnetic resonance imaging (MRI) [8,9], computed tomography (CT) [10], and their intrinsic electromagnetic properties could be harnessed towards the development of probes for fluorescence [4], photoacoustic and thermoacoustic imaging [11]. There is now a wide body of research documenting the toxicology and pharmacology of fullerenes, metallofullerenes and carbon nanotubes (CNTs) [1–3,12]. These studies on the various carbon nanostructures evaluate their safety for the above healthcare applications, or environmental/occupational health issues [12–14]. Reports to date show that the structure/shape (e.g. spherical, tubular), chemical composition (e.g. pristine,

* Corresponding author. Department of Biomedical Engineering, Bioengineering Building, Rm #115, Stony Brook University, Stony Brook, NY 11794-5281, USA. Tel.: +1 631 632 1810.
E-mail address: balaji.sitharaman@stonybrook.edu (B. Sitharaman).

http://dx.doi.org/10.1016/j.biomaterials.2014.04.066
0142-9612/

functionalized), synthesis method (e.g. chemical vapor deposition, oxidative exfoliation), and route of administration (e.g. intravenous, nasal) are key factors that influence toxicity and tissue response for carbon nanostructures [2,3,12,15,16]. For instance, multiwalled CNTs (MWCNTs) greater than 20 μm in length introduced directly into mesothelial lining along the body cavity of mice induced asbestos-like pathogenicity [13]. However, exposure of MWCNTs of lengths less than 20 μm did not induce a similar effect [13]. Multiple reports also recommend that pristine fullerenes or CNTs should be avoided for *in vivo* applications, and have emphasized the importance of chemical functionalization of these nanoparticles to impart water dispersibility, reduce aggregation and improve stability in physiologic fluid as well as facilitate adequate excretion rates to prevent accumulation in tissue [3,12,17,18]. Compared to CNTs and fullerenes, fewer studies have assessed the *in vitro* [19–21], and *in vivo* [22,23] biological effects of graphene nanoparticles.

Intravenous (IV) administration is a widely employed and preferred mode of systemically introducing pharmaceutical formulations for imaging, drug delivery or therapy. IV injections were employed in a subset of toxicological investigations of carbon nanostructures for such biomedical applications [1–3,23–25]. In general, the maximum dosages of a test formulation in toxicity and biodistribution studies depend on the concentration of the stock solution of the test formulation, and the maximum solution volume (typically 2 ml/kg for bolus and 4 ml/kg for slow IV injections) that can be injected without causing adverse side effects to the animals [26]. Hydrophobic carbon nanostructures have typically been covalently or non-covalently functionalized with moieties (functional groups, macromolecules) to improve water dispersibility and thus, allow higher doses. For small animal (rodents) toxicology studies that employed IV administration, the reported stock solution concentrations of water-dispersible carbon nanostructures are $\leq$ 10 mg/ml and maximum permissible doses (MPD) are in the units to low tens mg/kg range [2,3,23,27,28]. Additionally, for a given water-dispersed carbon nanostructure formulation, most investigations have focused on histopathology, and biodistribution, presenting little information on maximum tolerable doses (MTD), or assessment of other important issues such as respiratory and cardiovascular pharmacology safety. Consequently, the therapeutic indices of these formulations remain unknown. Furthermore, we found no published studies that explicitly followed the preclinical safety pharmacology guidelines of regulatory agencies such as the Food and Drug Administration (FDA), International Conference on Harmonization (ICH) and the European Medicines Agency (EMA) which recommend assessing toxicity at least 10 to 100 times higher than the projected therapeutic dose [29–032]. Such studies are necessary to receive regulatory approval for first-in-human trials for any carbon nanostructure-based IV formulations proposed for imaging, or therapeutic application.

Recently, we reported on the synthesis, physiochemical characterization and *in vitro* studies (see Table S1&S2 in the Supplementary Information section) of GNPs non-covalently functionalized with the FDA-approved natural polymer dextran (hereafter called GNP-Dex) [9]. GNP-Dex nanoparticles are disc-shaped with an average diameter of ~100 nm (Fig. S1), average thickness of ~3 nm and GNP:dextran weight ratio of 3:2 [9]. These nanoparticles are hydrophilic, and form stable colloidal dispersions in deionized water and biological fluids (buffers and blood) up to 100 mg/ml concentrations (Fig. S2). To the best of our knowledge, this is the highest level of dispersion achieved for any water-dispersible graphene nanoparticle. To date, *in vitro* and *in vivo* studies of other formulations of water-dispersible GNPs, synthesized via covalent (including covalent functionalization with dextran), and non-covalent functionalization strategies have reported maximum concentrations of up to 1 mg/ml [5,15,23,33,34]. Lastly, GNP-Dex dispersions are iso-osmolar (upon addition of mannitol) and iso-viscous to blood [9].

According to FDA and ICH guidance documents, prerequisites for first-in-human trials of the GNP-Dex for IV biomedical applications (e.g. drug delivery or imaging contrast agent) include *in vivo* pre-clinical safety pharmacology studies in a rodent and non-rodent model. Thus, as a part of the preclinical *in vivo* safety pharmacology evaluation, here, we report dose response, expanded acute toxicology, toxicokinetics, and respiratory/cardiovascular safety pharmacology assessment of GNP-Dex in rats.

2. Materials and methods

2.1. Animal care, dose ranges for expanded acute toxicity studies

All the experiments were performed according to the guidelines of Institutional Animal Care and Use Committee at Stony Brook University, NY. Acute toxicity (1-day, $n = 6$) and expanded acute toxicity (30-days, $n = 8$) studies were performed at the doses of 1, 25, 50, 125, 250, 500 mg/kg on Wistar male rats weighing 200–250 g. Mannitol (55 mg/ml), added to the GNP-Dex formulation to control the osmolality, was used as a control. The animals were anesthetized using isoflurane (1–2.5% mixture of O_2/air, via inhalation). Single doses of GNP-Dex were administered intravenously via the tail vein and the animals were monitored for any adverse effects for 1-day or 30-days. After injection, animals were transferred into metabolic cages to collect urine and feces samples.

2.2. Transthoracic echocardiography and blood pressure measurement

Transthoracic echocardiography with Doppler was performed with a high-resolution imaging System (Visual Sonics, Vevo 770, Toronto, Canada) using a 30-MHz linear array transducer (RMV 707B). GNP-Dex doses of 1, 25, 50, 125, 250 and 500 mg/kg and mannitol were injected into Wistar rats ($n = 4$ at each dose for 30-day expanded acute toxicity group) under anesthesia (1–2.5% mixture of O_2/air with isoflurane, via inhalation). Animals were placed on warming pads to maintain normothermia and their limbs were secured to electrocardiography sensors. Two-dimensional images were captured and recorded before injection as well as at 10 min and 2 h post injection in parasternal long- and short-axis projections with 2D guided M-mode recordings at the mid-ventricular level in both views. Left ventricular (LV) dimensions (mass, volume, wall thickness) were measured in the M-mode view and % fractional shortening was calculated. Additionally heart rate, respiration rate, blood velocity, and ejection fraction were recorded for both systolic and diastolic phase. An aortic arch or parasternal view (that is as parallel as possible with the sound beam) was used to obtain the pulsed-wave Doppler image to measure the blood flow velocity through the aorta. Visual Sonics software was used to calculate and analyze the data. Blood pressure was measured using a non-invasive occlusion type tail cuff system (Coda 6, Kent Scientific System, PA) 10 min and 2 h after administration of GNP-Dex.

2.3. Biodistribution and elimination

One day and 30-day animals for the biodistribution studies received IV injections at 1, 25, 50, 125, 250, 500 mg/kg doses. Urine and feces samples were collected at 8 and 24 h for the 1-day group, and daily up to a week for the 30-day group. Blood samples (~100 μl from tail vein) were collected at 0.5, 2, 4, 8 and 24 h after injection for the 1-day group, and every 48 h after injection up to one week post-injection for the 30-day group. At the end of the respective durations, animals were euthanized using 100% CO_2 and brain, heart, liver, lungs, spleen and kidneys were harvested. Mn^{2+} ions generated during the synthesis of GNPs from the oxidizing agent $KMnO_4$ get intercalated between graphene sheets. Hence, manganese served as a stable endogenous elemental tag to quantify GNP-Dex concentrations in blood, urine, feces and bio-distribution studies. The Mn^{2+} ions concentration in GNP-Dex, measured by inductively coupled plasma mass spectrometry (ICP-MS) was 0.064 ± 0.010% [9]. The Mn^{2+} concentrations in the blood, urine, feces and various tissues were determined using ICP-MS as described below. The % injected dose values in the blood, urine, feces or various tissues were calculated using the formula

$$\left[\left\{\left[Mn^{2+}(\text{experimental group})\right] - \left[Mn^{2+}(\text{sham control group})\right]\right\} \Big/ \left[Mn^{2+}(\text{injected doses})\right]\right] * 100.$$

2.4. ICP-MS

Blood, urine, feces and organs from different animals at the same dose from the same group (day 1 or day 30) were pooled together, and chemically digested using trace metal grade 70% nitric acid (HNO_3) (catalog # 02650, Sigma Aldrich, St Louis, MO), followed by trace metal grade 30% hydrogen peroxide (H_2O_2) (catalog # 95321, Sigma Aldrich, St Louis, MO) until only inorganic content was left. The digested