European Journal of Pharmaceutics and Biopharmaceutics xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Charge affects the oral toxicity of poly(amidoamine) dendrimers

Giridhar Thiagarajan [a,c], Khaled Greish [b,c,1], Hamidreza Ghandehari [a,b,c,*]

[a] Department of Bioengineering, University of Utah, Salt Lake City, UT, United States
[b] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT, United States
[c] Utah Center for Nanomedicine, University of Utah, Salt Lake City, UT, United States

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Oral drug carriers
Acute toxicity
In vivo
Surface charge
PAMAM dendrimers
Drug delivery

ABSTRACT

Poly(amidoamine) (PAMAM) dendrimers have been evaluated for the influence of surface functionality and size on the epithelial barrier of the gut with the goal of identifying safe carriers that can be used for oral drug delivery. Limited studies are conducted to date, however, to assess the toxicity of PAMAM dendrimers *in vivo* when administered by the oral route. The goal of this research was to conduct an oral acute toxicity study of PAMAM dendrimers as a function of size and charge in immune competent CD-1 mice. Maximum tolerated doses (MTD) of PAMAM dendrimers as a function of size and surface functionality were established and clinical signs of toxicity monitored. Results demonstrate that positively charged dendrimers caused more toxicity, whereas their anionic counterparts were tolerated at ten times higher doses. Severe signs of toxicity observed for large (G7) cationic amine- or hydroxyl-terminated dendrimers include hemobilia and spleenomegaly. The MTD for these dendrimers ranged from 30 mg/kg to 200 mg/kg. Anionic G6.5 or smaller molecular weight carboxyl-, amine-, or hydroxyl-terminated dendrimers (G3.5-COOH, G4-NH_2, G4-OH) on the other hand were tolerated at doses of up to 500 mg/kg (300 mg/kg in some cases) with minimal or no signs of toxicity. Establishing the MTD of orally delivered PAMAM dendrimers and the influence of surface functionality and size on toxicity aids in the rational design of PAMAM-drug conjugates for oral drug delivery applications.

1. Introduction

Dendrimers are branched polymeric architectures that have been used extensively for drug delivery applications [1–3]. One such branched polymer, that is, poly(amidoamine) dendrimers, has been extensively investigated for oral drug delivery [4–9]. These studies, largely conducted in *in vitro* models of intestinal epithelial barrier, such as the everted sac and Caco-2 cell monolayers, have clearly shown the influence of size, surface functionality, and charge on toxicity and transepithelial transport. A general trend has been observed that cationic PAMAM dendrimers are more toxic than their anionic counterparts, larger dendrimers are more toxic compared to smaller dendrimers of similar surface functionality, and that masking cationic residues with non-charged groups improves tolerability of PAMAM dendrimers and their uptake by the epithelial cells [10,11]. While the *in vitro* studies of PAMAM toxicity and transepithelial transport provide guidelines for the design of these carriers for oral drug delivery applications, much needs to be done to establish a size and charge window where oral administration of these constructs in an *in vivo* setting is safe. The present study is an initial attempt in this direction and aims to understand the influence of size and surface charge on maximum tolerated dose and toxicity of PAMAM dendrimers in mice.

2. Materials and methods

2.1. Preparation and characterization of PAMAM dendrimers

PAMAM dendrimers (Table 1) were fractionated and characterized as previously described [12]. Briefly, PAMAM dendrimers (G3.5-COOH, G4-NH_2, G4-OH, G6.5-COOH, G7-NH_2, and G7-OH) with ethylene diamine core were purchased from Sigma (St. Louis, MO). Dendrimer samples were further fractionated by a preparative Sephadex Hiload 75 size exclusion column (GE Healthcare Biosciences, Piscataway, NJ) as necessary to remove small molecular weight impurities. All dendrimers were characterized at physiologically relevant pH by dynamic light scattering (DLS) on a DAWN HELEOS II (Wyatt Technologies, Santa Barbara, CA) at a concentration of 5 mg/ml and their zeta potential recorded on a Malvern Zetasizer (Malvern Instruments Inc., Westborough, MA) at a concentration of 10 mg/ml in triplicate. Zeta potential was measured in double distilled water (DDW) with pH adjusted to 7.4 using

* Corresponding author. Utah Center for Nanomedicine, Nano Institute of Utah, University of Utah, Salt Lake City, UT 84112, United States. Tel.: +1 801 587 1566; fax: +1 801 581 6321.
E-mail address: hamid.ghandehari@pharm.utah.edu (H. Ghandehari).
[1] Present address: Department of Pharmacology & Toxicology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand.

0939-6411/$ - see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.ejpb.2013.01.019

Table 1
Physicochemical characterization of PAMAM dendrimers.

Dendrimer	#Of surface groups[a]	Size (diameter) in nm	Zeta potential in mV (conductivity in mS/cm)[d]
G3.5-COOH	64	3.2 ± 0	–[b]
G4-NH_2	64	3.4 ± 0.22	–[b]
G4-OH	64	2.6 ± 0	–[b]
G6.5-COOH	512	8.5 ± 0.61	−42.0 ± 1.2 (1.794)[c]
G7-NH_2	512	8.1 ± 0.42	64.8 ± 3.2 (0.264)[c]
G7-OH	512	6.4 ± 0	27.7 ± 1.1 (0.269)[c]

[a] Provided by manufacturer.
[b] Dendrimers were below detection limit.
[c] Zeta potential was measured in double distilled water at pH 7.4 (not buffered).
[d] Mean conductivity values.

Table 2
Acute toxicity doses administered orally to CD-1 mice.

Treatment group	No. of mice	>10% Body weight loss	Other signs of toxicity
G7-NH_2			
50 mg/kg	5	1	Hemobilia
30 mg/kg	5	0	No
G7-OH			
100 mg/kg	5	0	No
300 mg/kg	5	1	Splenomegaly
200 mg/kg	5	0	No
G6.5-COOH			
100 mg/kg	5	0	No
300 mg/kg	5	0	No
500 mg/kg	5	0	No
G4-NH_2			
50 mg/kg	5	0	No
100 mg/kg	5	0	No
300 mg/kg	5	0	No
G4-OH			
100 mg/kg	5	0	No
300 mg/kg	5	0	Elevated BUN level in 1 mice
500 mg/kg	5	0	No
G3.5-COOH			
100 mg/kg	5	0	No
300 mg/kg	5	0	No

BUN – blood urea nitrogen.

HCl and NaOH (not buffered). In addition, PAMAM dendrimers were characterized for absence of small molecular weight impurities by high performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA) on a C18 (4.6 × 250 mm, 5 μm) column (Waters, Milford, MA) in an acetonitrile: water mixture (27:73) with 0.14% trifluoro acetic acid and size exclusion chromatography (SEC) on an analytical Superose 6 10/300 GL column (GE Healthcare Biosciences, Piscataway, NJ). Elution buffer was PBS: acetonitrile (80:20) with 0.1% sodium azide.

2.2. Oral acute toxicity studies

All oral acute toxicity studies were carried out in 4–6 weeks old female CD-1 mice weighing about 25 g purchased from Charles River Laboratories (Boston, MA) and used strictly according to the rules and guidelines of the University of Utah Institutional Animal Care and Use Committee. Animals were fed normal diet during the course of all studies. The dose escalation study started at 100 mg/kg (except for cationic dendrimers which started at 50 mg/kg). Detailed list of dose administered under each treatment group is provided in Table 2. Each dose of PAMAM dendrimer or saline control was prepared in a total volume of 0.2 ml/mice with physiological saline. Samples were filtered through 0.2 μm filters and administered by oral gavage using appropriately sized curved feeding needles. To exclude the presence of endotoxin in nanoparticle samples, an endpoint LAL assay (Lonza, Basel, Switzerland) was performed according to the manufacturer's instructions. Immediately after the single dose administration, animals were observed for 30 min for post-injection reaction. Body weight was recorded, and systemic clinical observations for signs of toxicity such as unusual locomotion, bleeding in any orifice, ruffling of fur/skin, lacrimation/ redness of the eye, vasodialation, vasoconstriction, and coldness of body [12] were carried out twice daily for a period of 10 days. Unless animals showed signs of toxicity (greater than 10% animal weight loss consistently for more than 2 days or other clinical signs of toxicity [12]), the acute toxicity study progressed to completion (10 day period). Ten days after administration, mice were individually euthanized using 70% CO_2 in oxygen, with euthanasia confirmed by lack of breathing for 30 s. Blood was taken via inferior vena cava (IVC) stick and drawn into a heparinized syringe through a 23G needle and deposited into a blood tube. Blood samples were examined for clotting and/or hemolysis upon collection. Organs (heart, lungs, liver, spleen, kidney, and GI) were removed, weighed and % weight of organ to total body weight calculated to determine organ atrophy/hypertrophy in response to dendrimer administration. Complete blood counts (CBC) were performed within 2 h of blood collection using a CBC-DIFF (Heska, Loveland, CO) blood count analyzer. Following CBC, samples were centrifuged at 10,000 rpm for 2.5 min. The collected serum samples were used to measure blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) using a DRI-CHEM (Heska, Loveland, CO) veterinary blood chemistry analyzer to examine kidney and liver toxicity. Maximum tolerated dose (MTD) was considered as the maximum dosage of a particular dendrimer that resulted in less than 10% animal weight loss over a period of 10 days or did not manifest any clinical signs of toxicity. At a given dose of a particular dendrimer when overt toxicity was observed, the animal was euthanized by CO_2 asphyxiation, and the dosage of that particular dendrimer was reduced to midway between the current lethal dose and earlier determined maximum dose that was tolerated.

3. Results and discussion

3.1. Physiochemical characterization of PAMAM dendrimers

PAMAM dendrimers were chosen in two different size ranges (~3 nm and 8 nm), and each of these groups in turn had three different surface groups (hydroxyl, carboxyl, and amine) making it a total of six dendritic nanoconstructs (G3.5-COOH, G4-NH_2, G4-OH, G6.5-COOH, G7-NH_2, and G7-OH) that were studied. Dendrimers were characterized for hydrodynamic size and zeta potential, results of which are presented in Table 1. The absence of small molecular weight impurities was confirmed by size exclusion chromatography and high performance liquid chromatography which have been reported elsewhere [12].

In order to understand results from *in vivo* toxicity studies, it was necessary to employ probes that were well characterized for their physiochemical properties. Interesting to note in the physicochemical characteristics was the fact that in both size ranges evaluated the hydroxyl-terminated dendrimers were smaller than their carboxyl or amine-terminated counterparts (diameter measured by DLS) as shown in Table 1. This was probably due to the fact that the amine- or carboxyl-terminated surface groups on the respective dendrimers repel each other at the terminal ends

Q1 Please cite this article in press as: G. Thiagarajan et al., Charge affects the oral toxicity of poly(amidoamine) dendrimers, Eur. J. Pharm. Biopharm. (2013), http://dx.doi.org/10.1016/j.ejpb.2013.01.019