Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 281 (2004) 129–132
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

In vitro and in vivo evaluation of a melamine dendrimer as a vehicle for drug delivery☆

Michael F. Neerman[a], Wen Zhang[a], Alan R. Parrish[b,1], Eric E. Simanek[a,*]

[a] *Department of Chemistry, Texas A&M University, College Station, TX 77843, USA*
[b] *Department of Medical Pharmacology and Toxicology, Texas A&M University Health Science Center, College Station, TX 77843, USA*

Received 9 October 2003; received in revised form 23 April 2004; accepted 23 April 2004

Abstract

Cell-based and acute and subchronic in vivo toxicity profiles of a dendrimer based on melamine reveal that this class of molecules warrants additional study as vehicles for drug delivery. In cell culture, a substantial decrease in viability was observed at 0.1 mg/mL. For the acute studies, mice were administered 2.5, 10, 40 and 160 mg/kg of dendrimer via i.p. injection. At 160 mg/kg, 100% mortality was seen 6–12 h after injection. For the other cohorts, blood chemistry work revealed no renal damage was taking place at 48 h. Liver enzyme activity nearly doubled for the mice treated at 40 mg/kg suggesting hepatotoxicity. For the subchronic studies, three i.p. injections of 2.5–40 mg/kg of dendrimers were administered at 3-week intervals. No mortality was observed. Forty-eight hours following the last administration, blood chemistry revealed no renal damage, but liver damage was indicated by elevated serum enzyme activity at the highest dose. Histopathological data further confirms that doses up to 10 mg/kg show no hepatic damage at subchronic doses. However, subchronic doses at 40 mg/kg lead to extensive liver necrosis.

Keywords: Dendrimer; Drug delivery; Biocompatibility; In vivo; Toxicity

1. The note

The monodisperse, multivalent and globular nature of dendrimers has led many groups to consider their use as vehicles for drug delivery (Cloninger, 2002; Esfand and Tomalia, 2001; Patri et al., 2002; Aulenta et al., 2003). This use requires these architectures to display low toxicity and be biocompatible, non-immunogenic, and biodegradable or subject to ready clearance. These studies address the acute and subchronic toxicity of molecule **1**, a third generation, cationic dendrimer based on melamine (Fig. 1). We are pursuing this class of dendrimers because it offers exceptional potential for the control over surface groups: that is, any number of different and specific patterns of surface functionality can be obtained as a result of the differential reactivity of the triazine building block. The synthesis of **1** has been published (Zhang et al., 2003). In summary, the convergent route commences with the surface groups and relies on iterative reactions of cyanuric chloride and piperazine. At each stage of the synthesis, the intermediates are iso-

☆ Supplementary data associated with this article can be found at doi: 10.1016/j.ijpharm.2004.04.023.

* Corresponding author. Tel.: +1-979-845-4242; fax: +1-979-845-9452.
E-mail addresses: parrish@medicine.tamu.edu (A.R. Parrish), simanek@tamu.edu (E.E. Simanek).
[1] Co-corresponding author. Tel.: +1-979-458-1538; fax: +1-979-845-0699.

0378-5173/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2004.04.023

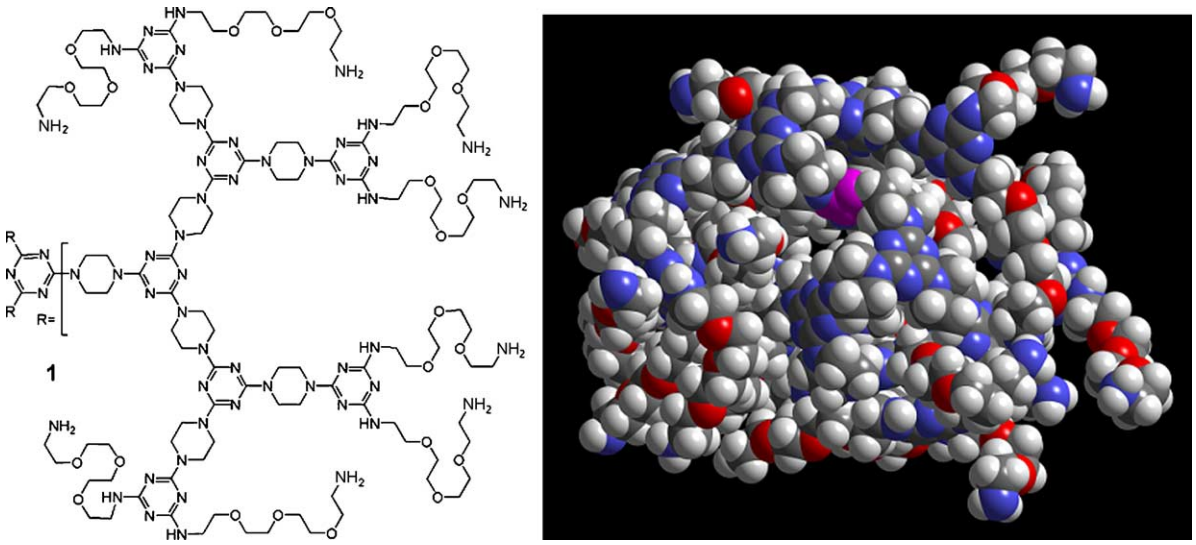

Fig. 1. Atomic and low energy structures of 1, a third generation melamine dendrimer (MW = 8067 Da) with 24 amine surface groups present on the periphery. The core is maroon.

lated and completely characterized by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy. Trimerization with trispiperazinyltriazine occurs in the penultimate step and is followed by deprotection of the groups on the periphery. Following isolation, molecule 1 appears to be a single chemical entity: no impurities are detected by mass spectrometry or ^{1}H or ^{13}C NMR spectroscopy. The aromatic triazines confer a hydrophobic environment while the amines on the periphery provide water solubility.

These studies address the toxicity of **1** in cell culture. The in vivo acute (48 h) and subchronic (6 weeks) studies to assess the short-term and long-term effects of our dendrimer. The goal of these studies is to determine whether melamine-based vehicles would be precluded due to the potential toxicity of this heterocyclic building block. If the toxicity of this molecule is sufficiently low, we can justify further studies of this system given the synthetic versatility available in the construction of these molecules. We find that **1** displays in vivo toxicity comparable with other polycations, notably PAMAM dendrimers, that are attracting attention as drug delivery vehicles.

1.1. In vitro evaluation

The toxicity of **1** to cultures of Clone 9 cells was determined and compared with dextran. Dendrimer **1** shows an onset of toxicity at 0.1 mg/mL (Fig. 2). Other studies have found that most cationic polymers, including dendrimers (DAB, DAE, PAMAM) are cytotoxic to cells (B16F10: IC_{50} = 0.1 mg/mL by the MTT assay) and hemolytic (hemolysis at >1 mg/mL) at similar concentrations (Malik et al., 2000), although the in vivo acute and subchronic toxicities were not established.

1.2. Acute study

The acute studies show that 160 mg/kg is a lethal dose: 100% mortality was observed 6–12 h after i.p. injection. The effects of 1 on renal function were evaluated by changes in blood urea nitrogen

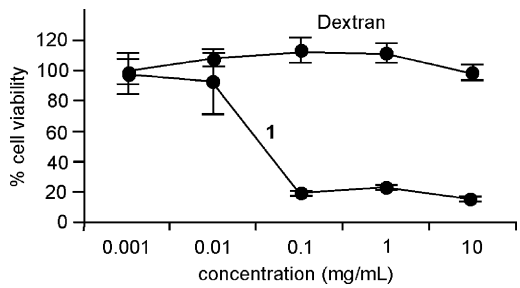

Fig. 2. Viability of Clone 9 cells with increasing concentrations of 1 or dextran.

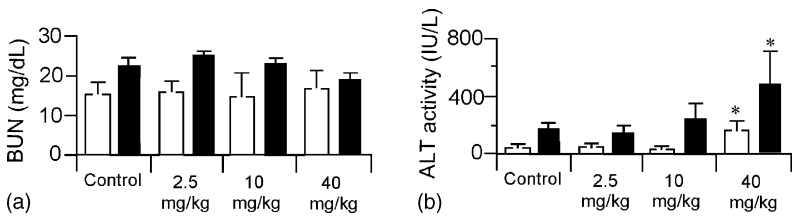

Fig. 3. BUN (panel a) and ALT (panel b) data from the acute (white) and subchronic (black) studies. Results were done in groups of $n = 5$ and expressed as the mean ± S.D. *Statistically different when compared to the control group ($P < 0.05$).

(BUN) levels. None of the treatment groups showed any significant changes ($P > 0.05$) in BUN levels when compared to the control group (Fig. 3, panel a) treated with phosphate buffered saline (500 μl, 0.1 M, pH 7.4). The effects of **1** on hepatic function were evaluated by changes in alanine transaminase (ALT) activity in serum. Doses up to 10 mg/kg led to no significant difference in ALT activity compared to the control group 48 h after injection (Fig. 3, panel b; $P > 0.05$). However, a statistically significant increase in ALT was observed after 48 h in those mice receiving 40 mg/kg ($P < 0.05$).

1.3. Subchronic study

BUN levels following three doses every 3 weeks over a 6-week period revealed no changes in renal function (Fig. 3, panel a) when compared to the controls ($P > 0.05$). Histopathological assessment also confirmed that **1** was not nephrotoxic at multiple doses up to 40 mg/kg. Serum ALT levels varied with dose. There was no significant increase in ALT activity up to doses of 10 mg/kg (Fig. 3, panel b; $P > 0.05$). However, there was a significant increase in ALT activity at 40 mg/kg ($P < 0.05$). Histopathological assessment revealed that 2.5 and 10 mg/kg did not induce necrosis (Fig. 4). However, extensive necrosis was seen at 40 mg/kg, which coincides with the significant increase in ALT activity found in the serum. We are unable to comment at this time on the mechanism of liver damage.

These studies add to the paucity of data available on the in vivo behavior of dendrimers. The results show that **1** has low toxicity to mice in single i.p. doses up to 40 mg/kg and at subchronic i.p. doses up to 10 mg/kg. This toxicity is consistent with other dendrimers reported in the literature that are currently being considered as drug delivery vehicles. Preliminary investigations of PAMAM found toxicity commenced at 40 mg/kg i.p. (Roberts et al., 1996). Biodistribution studies with ^{125}I-labelled PAMAM dendrimers in vivo indicated that both cationic and anionic dendrimers primarily partition to the liver from the blood, although the anionic form has longer circulation times (Malik et al., 2000). This accumulation was independent of delivery pathway: i.v. and i.p. delivery resulted in similar distributions. Accumulation of charged dendrimers in the liver may emerge as a general feature of these architectures. Biodistribution studies with cationic PAMAM dendrimers have also

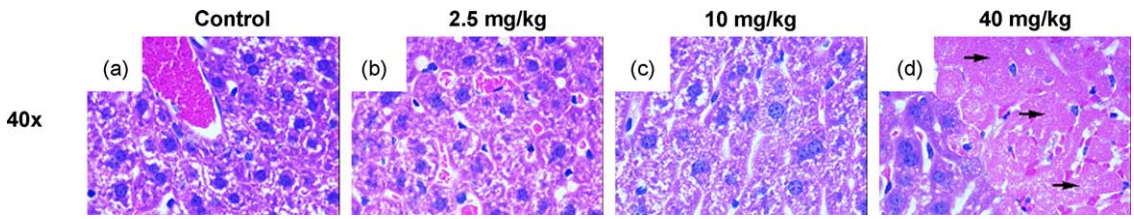

Fig. 4. Microscopic observation of mouse liver at 40× (panels a–d) with increasing dose. Liver structure appears normal up to 10 mg/kg following multiple doses over a 6-week period. Multiple doses of 40 mg/kg led to extensive hepatic necrosis, as assessed by loss of nuclei shown with arrows.

suggested a generation-specific accumulation in either the kidneys or pancreas (Roberts et al., 1996). Neutral dendrimers may behave differently. Two neutral polyesters were found to be rapidly cleared when administered by i.v. injection (Ihre et al., 2002; Padilla De Jesus et al., 2002). Liver accumulation of a third neutral polyester was observed, although this could be attributed to either loss of the label from the dendrimer or receptor-mediated targeting. The lack of toxicity of these polyesters is notable: the study was conducted at 1.3 g/kg i.v.

In summary, these studies suggest that the toxicity of this melamine-based dendrimer (and, potentially, related architectures) is comparable to the cationic PAMAM dendrimers. The choice of surface groups on the dendrimer, whether cationic or anionic or neutral, consistently emerges as the determining factor for in vivo and in vitro behavior of the dendrimer. This trend is encouraging as it suggests that general synthetic solutions may be available including acylation (Quintana et al., 2002; Jevprasesphant et al., 2003; Bhadra et al., 2003). Reducing the number (Roberts et al., 1996; Malik et al., 2000; El-Sayed et al., 2002) or density of cationic groups (Fischer et al., 2003) also reduces toxicity. Anionic and neutral dendrimers do not show a marked dose or generation-dependent toxicity in vivo or in vitro.

Additional study of these structures will continue because this class of dendrimers offers two potential advantages over most other classes including PAMAM. First, the aromatic interior of these molecules makes them significantly more hydrophobic likely favoring noncovalent sequestration of hydrophobic guests. Second, and more importantly, the synthetic versatility of this system through the tailoring or the surface groups in a non-statistical fashion with multiple different groups suggests that more "composition space" can be examined with these molecules. The results of these studies will be reported in due course.

Acknowledgements

The National Institutes of Health (GM 64650) and the Center for Microencapsulation and Drug Delivery at Texas A&M University supported these investigations.

References

Aulenta, F., Hayes, W., Rannard, S., 2003. Dendrimers: a new class of nanoscopic containers and delivery devices. Eur. Polym. J. 39, 1741–1771.

Bhadra, D., Bhadra, S., Jain, S., Jain, N.K., 2003. A PEGylated dendritic nanoparticulate carrier of fluorouracil. Int. J. Pharm. 257, 111–124.

Cloninger, M.J., 2002. Biological applications of dendrimers. Curr. Opin. Chem. Biol. 6, 742–748.

El-Sayed, M., Ginski, M., Rhodes, C., Ghandehari, H., 2002. Transepithelial transport of poly(amidoamine) dendrimers across Caco-2 cell monolayers. J. Controlled Release 81, 355–365.

Esfand, R., Tomalia, D.A., 2001. Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. Drug Discov. Today 6, 427–436.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Ihre, H.R., Padilla De Jesus, O.L., Francis Jr., C., Fréchet, J.M.J., 2002. Polyester dendritic systems for drug delivery applications: design, synthesis, and characterization. Bioconjugate Chem. 13, 443–452.

Jevprasesphant, R., Penny, J., Jalal, R., Attwood, D., McKeown, N.B., D'Emanuele, A., 2003. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. Int. J. Pharm. 252, 263–266.

Malik, N., Wiwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weener, J.W., Meijer, E.W., Paulus, W., Duncan, R., 2000. Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of ^{125}I-labelled polyamidoamine dendrimers in vivo. J. Controlled Release 65, 133–148.

Padilla De Jesus, O.L., Ihre, H.R., Gagne, L., Frechet, J.M.J., Szoka Jr., F.C., 2002. Polyester dendritic systems for drug delivery applications: in vitro and in vivo evaluation. Bioconjugate Chem. 13, 453–461.

Patri, A.K., Majoros, I.J., Baker Jr., J.R., 2002. Dendritic polymer macromolecular carriers for drug delivery. Curr. Opin. Chem. Biol. 6, 466–471.

Quintana, A., Raczka, E., Piehler, L., Lee, I., Myc, A., Majoros, I., Patri, A.K., Thomas, T., Mule, J., Baker Jr., J.R., 2002. Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor. Pharm. Res. 19, 1310–1316.

Roberts, J.C., Bhalgat, M.K., Zera, R.T., 1996. Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst^{TM} dendrimers. J. Biomed. Mater. Res. 30, 53–65.

Zhang, W., Jiang, J., Qin, C., Thomson, L.M., Parrish, A.R., Safe, S.H., Simanek, E.E., 2003. Triazine dendrimers for drug delivery: evaluation of solubilization properties, activity in cell culture, and in vivo toxicity of a candidate vehicle. Supramol. Chem. 15, 607–615.