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EC PHARMACOLOGY AND TOXICOLOGY
Research Article

Synergistic Toxicity of PAMAM Dendrimers and Minocycline to Rainbow Trout Hepatocytes

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Received: March 22, 2017; **Published:** April 18, 2017

Abstract

Dendrimers are used as polycationic carriers for enhanced drug or gene delivery for the treatment of various illnesses. The potential release of these compounds in the environment has raised concerns about their ecotoxicity in non-target organisms such as fish. The purpose of this study was to determine the toxicity of G2 and G5 poly(amidoamine) dendrimers (PAMAM dendrimers) to rainbow trout hepatocytes in the presence of a representative antibiotic (minocycline) in treated wastewaters. Hepatocytes were exposed to increasing concentrations of G2 and G5 dendrimers both alone and in the presence of minocycline for 48 h at 15°C. Cell viability was determined by the microplate carboxyfluorescein retention assay. The data revealed that G5 PAMAM dendrimer was toxic at a threshold concentration of 7 µg/mL, while G2 PAMAM dendrimer was not toxic at concentrations of up to 20 µg/mL. Minocycline alone was not toxic to trout hepatocytes at 100 µg/mL, but when used in combination with G2 PAMAM dendrimer at a concentration of 20 µg/mL, it became toxic at a threshold concentration of 70 µg/mL. Minocycline was 2 times more potent in the presence of G5 PAMAM dendrimer relative to G2 PAMAM dendrimer, at a threshold concentration of 35 µg/mL. We conclude that dendrimers could enhance the toxicity of a representative environmental antibiotic to rainbow trout hepatocytes.

Keywords: *Hepatocytes; Oncorhynchus Mykiss; Cell Viability; PAMAM Dendrimers; Minocycline*

Introduction

Commercial applications of nanotechnology are increasing and pervading many sectors of our economy. These products range from electronic devices, paints/dyes, cosmetics and personal care products to biomedical applications such as imaging and drug/gene delivery strategies [1]. Any product that has at least one dimension between 1 and 100 nanometers (nm) is considered a nanomaterial (NM). Compounds produced at the nano scale offer new and useful emerging properties with tremendous potential for commercial applications. For example, the use of nanoparticles for drug delivery can permit the enhanced delivery of a given drug and can target drug release to specific sites in the body [2]. The toxicity of nanomaterials arises from the cumulative effects of four basic properties: 1) the leaching of low-molecular-weight molecules or ions from the nanomaterial, 2) the geometry (size and shape) of the nanomaterials including their aggregates, 3) the surface properties (reactivity), and 4) the vector effect [3,4]. The last property has been extensively researched in connection with the development of drug, gene and peptide delivery systems in therapeutics [2]. The cytotoxicity of Adriamycin to the Chinese hamster transformed cell line DC3F increased when it was associated with cyanoacrylate nanoparticles [5]. In addition, an Adriamycin-resistant strain of the DC3F cell line became more sensitive to nanoparticle-carried Adriamycin, which provides evidence of vector effects. In another study, carp accumulated nearly 3 times more cadmium in the presence of titanium dioxide nanoparticles [6].

From an environmental risk assessment perspective, this phenomenon is particularly worrisome since the toxicity of xenobiotics is likely to increase without any changes occurring in their concentrations in the aquatic environment.

The development and use of poly(amidoamine) (PAMAM) dendrimers for targeted and enhanced xenobiotic (drug) and gene delivery have been extensively examined [7,8]. The interest in these dendritic-branched nanoparticles stems from their structural properties such as uniformity, size, shape, monodispersity and functionalized surfaces [9]. Dendrimers are composed of an initiator amine core (-NH2) with attached amidoamine units that are radially distributed around the core (Figure 1). Each successive branching that forms a surface layer is termed a generation (G). Full-generation dendrimers (G1, G2, G3) have cationic amine-terminated groups, while half-generation dendrimers (G2.5, G3.5) have anionic carboxylic moieties at physiological pH. Finally, each successive generation has twice the number of terminal groups and increased diameter size [10]. Cationic dendrimers have been shown to exhibit cytotoxicity and hemolyzing properties which are dependent on size and surface charge (zeta potential) [11,12]. The generation number and number of primary amines were related to the production of ROS, cytotoxicity which includes both apoptosis and necrosis [13]. ROS production was co-located in mitochondria and could initiate apoptosis in a concentration and size dependent manner. Moreover, it appears that dendrimers produce small "nanoholes" or "nanopores" in membranes, which can perturb membrane potential and permeability [14]. Thus, the toxicity of dendrimers is associated with their surface properties (3rd basic property) in addition to the vectorization effect.

Studies on the ecotoxicity of PAMAM dendrimers are relatively scarce at present. PAMAM dendrimers were shown to increase production of reactive oxygen species in mouse macrophage cells [15]. Moreover, the increased production of ROS followed the number of cationic primary amino group at the surface. King-Heiden., *et al*. [16] showed that G4 PAMAM dendrimer was toxic to zebrafish embryos and decreased growth and larval development at sublethal concentrations. The vector properties of PAMAM dendrimers were also observed in algae exposed to the herbicide glyphosate, with increased toxicity of the glyphosate formulation being observed in the presence of G4 PAMAM dendrimer [17]. Algal toxicity was observed in response to glyphosate concentrations (7 to 15 µM) and to G4 PAMAM dendrimers (10 to 20 nM), which were not toxic on their own. Given that PAMAM dendrimers would be released in urban effluents (e.g., hospital wastewaters) which contain many pollutants, including antibiotics, it is relevant to assess the toxicity of a representative antibiotic to hepatocytes during exposure to dendrimers. Tetracyclines (minocycline) are commonly found in hospital and municipal wastewaters [18]. Minocycline concentrations may be as high as 530 µg/L in hospital effluents and may range from 95 to 920 µg/L in wastewater treatment plant effluents. This could lead to accumulation in non-target organisms if their exposure to such compounds exceeds their capacity to eliminate them. The hypothesis of this study consists in determining if the presence of PAMAM dendrimers could enhance the toxicity of an environmentally relevant antibiotic-minocycline in fish liver cells.

The purpose of this study was to investigate the cytotoxicity of G2 and G5 PAMAM dendrimers alone and in the presence of minocycline. G2 and G5 PAMAM dendrimers were used to maximize size dependence effects involved with these drug vectors. The nature of the interaction of G2 and G4 PAMAM dendrimers with minocycline is discussed in light of the observed responses.

Materials and Methods

Preparation and exposure of rainbow trout hepatocytes

Second and fifth generation PAMAM dendrimers were purchased from Sigma Chemical Company (Ontario, Canada). They were diluted in High Quality water at 200 mg/mL to perform dynamic light scattering (DLS) analysis in order to measure particle size distribution and Zeta potential and hepatocyte exposure. The analysis was done using a DLS) instrument with a gel electromobility option (Wyatt-Instrument Mobius, 532-nm laser). Zeta potential was determined from gel mobility data as described in Domingos., *et al*. [19]. Primary cultures of rainbow trout (*Oncorhynchus mykiss*) were prepared using an adaptation of the double perfusion method with citrate and serum bovine albumin for tissue dissociation and liberation of hepatocyte aggregates from the liver [20]. Briefly, the livers from 4 young-of-the year (8-

to 10-cm fork length) were used to prepare primary cultures of hepatocytes. The livers were first perfused with phosphate-buffered saline (PBS: 120 mM NaCl, 5 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM glucose) containing 10 mM citrate, pH 7.4, at 4°C until the livers were purged from red blood cells. The livers were then minced and placed in 10 mL of PBS containing citrate and 0.5% serum bovine albumin. The suspension was stirred slowly with a magnetic stirring bar (at 20 - 40 rpm) for 30 min at room temperature. After this period, the suspension was passed through a cell extraction sieve (30 - 50 µm diameter mesh, Sigma Chemical Company) and washed in PBS containing 0.1% serum bovine albumin, followed by centrifugation (200 × g for 5 min)/resuspension until a clear supernatant was obtained (usually after 3 - 4 times). A sample of the cell suspension was set aside for cell density and viability assessments. The cells were stained in 0.004% trypan blue in PBS and counted, and cell viability was determined using a hematocytometer under a microscope at 200X enlargement (viable cells stay clear). Hepatocytes appeared mostly as aggregates of 4 - 8 cells, and individual cells tended to group together, which is normal and healthy behaviour for hepatocytes. Hepatocytes were plated at n = 6 replicates per treatment in 48-well microplates at a density of 0.5 × 10^6 viable cells/mL in Liebovitz (L-15) cell culture media containing 10 mM HEPES-NaOH, pH 7.4, 50 units penicillin, 50 µg/mL streptomycin and 0.1 µg/mL amphotericin B. The cells were exposed to increasing concentrations of G2 and G5 PAMAM dendrimers (2.5, 5, 10 and 20 µg/mL) and to minocycline (12.5, 25, 50 and 100 µg/mL) alone under a sterile fume hood. These concentrations correspond to 0.075, 0.15, 3 and 6 µM for G2 dendrimer and 0.0875, 0.175, 0.35, and 0.7 µM for G5 dendrimer respectively. The molar concentrations of minocycline were 27.5, 55, 110, 220 µM. The cells were also exposed to both minocycline and individual dendrimers at each corresponding concentration: 2.5 µg/mL G2 or G5 dendrimer and 12.5 µg/mL minocycline, 5 µg/mL G2 or G5 dendrimer and 25 µg/mL minocycline, etc. (see Figure 2 legends). These concentrations were chosen based on an exploratory range following a semi-log distribution. The cells were exposed for 48 h at 15°C in a saturated humidity atmosphere. At the end of the exposure period, the microplates were centrifuged at 250 × g for 3 to 5 min at 4oC and the exposure media removed by aspiration. Cells were suspended in PBS as described above, but without albumin and citrate for cell density and viability assessments. Relative cell density was determined by measuring the absorbance at 600 nm.

Cell viability assessment

Hepatocyte viability was determined by the fluorescein dye retention assay as described elsewhere [20]. A portion (20 µL) of the cell suspension was mixed with 180 µL of 10 µM carboxyfluorescein diacetate in PBS containing 1 mM glucose in dark-coloured microplates with clear bottoms for 20 min at 20°C. The microplate was centrifuged at 250 × g for 5 min and the supernatant removed. The cells were then resuspended in 200 uL of PBS, their absorbance was measured at 600 nm for cell density, and fluorescence was measured at 485 nm excitation and 520 nm emission using a microplate reader (Chameleon II, Bioscience, USA). A positive control (100% mortality) was prepared by adding cells to separate wells in 25% DMSO during the 20-min incubation time to completely permeabilize the cells. The data were corrected for cell density (600 nm), normalized to controls and expressed as a fold change (reduction) in fluorescence.

Data analysis

The hepatocytes were exposed to n = 6 replicates of each concentration of the tested compounds. The data were checked for normality and homogeneity of variance using the Shapiro-Wilk Brown-Forsythe tests, respectively. The data were expressed as mean with the standard error and subjected to an analysis of variance and critical differences between treatments (including controls) were determined using Fisher's Least Square Difference test. The data were expressed in threshold concentration which is defined by the geometric mean of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC): threshold = $(NOEC \times LOEC)^{1/2}$. Interactions between PAMAM dendrimers and minocycline were determined by multiple regression analysis on cell viability data (i.e., to seek out trends between hepatocytes exposed to minocycline+dendriment and minocycline or dendrimer only). Multiple regression analysis and the above tests were performed using the Statistic software package (version 8.). Significance was set at $p < 0.05$.

Results and Discussion

The prepared test samples consisted of G2 and G5 PAMAM dendrimers organized around a diamine core (Figure 1). The G2 and G5 dendrimers have a theoretical diameter of 2.9 and 5.4 nm, respectively (Table 1). Although the size of these dendrimers did not change much, the number of functional amine groups ($-NH_4^+$) at the surface readily increased from 16 to 128 for G2 and G5 dendrimers, respectively. This was accompanied by an increase in molecular weight in such a manner that an equivalent 20 µg/mL solution consisted of 6 and 0.7 µM (one order of magnitude) of G2 and G5 dendrimers, respectively. Compared to the same amount of minocycline, the dendrimer concentrations were one to two orders of magnitude lower, i.e., minocycline was in excess compared to G2 and G5 dendrimers. Given the pKa values for the 2 amine groups of minocycline (5 and 9.5), it is expected that the molecule will be cationic at physiological pH and that binding on the surface of the dendrimers is unlikely to occur through electrostatic interaction but may occur through hydrogen bonding at other sites. This is consistent with what is known about these types of drug vectors, which have numerous surface area chemistries permitting many possible interactions of drugs with the nanoparticle. Dendrimers also induce pore formation, thereby permitting higher diffusion of drugs or xenobiotics into cells [21]. These properties complicate the classical risk assessment paradigm because nanoparticles could change the bioavailability of contaminants in the environment.

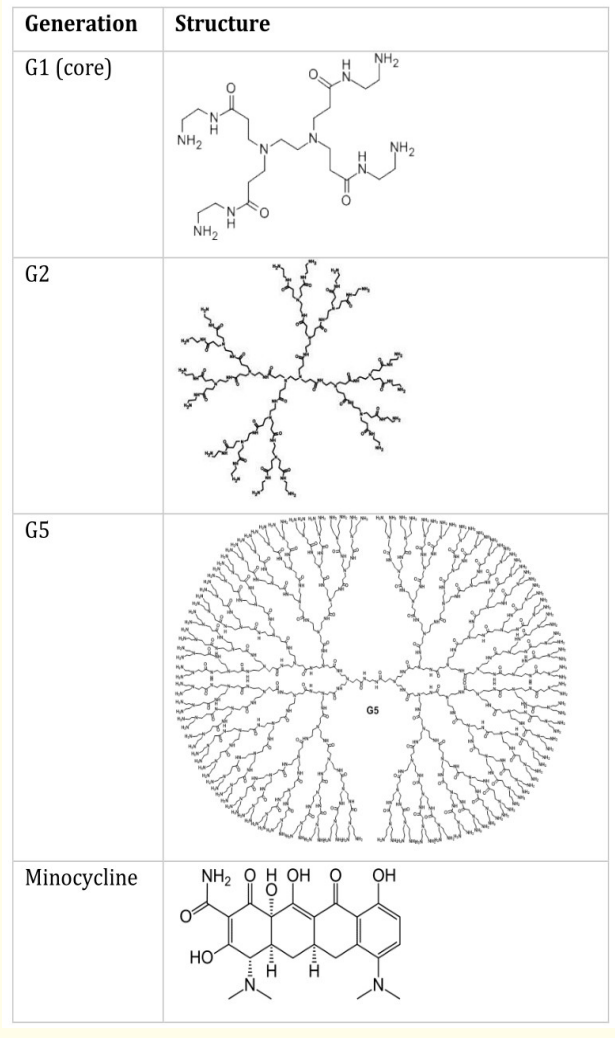

Figure 1: *Molecular structure of PAMAM dendrimers and minocycline.*

Compound	MW g/mol	Formula	# surface groups	Diameter (nm)	Zeta Potential (mvolt)
Minocycline	494	$C_{23}H_{27}N_3O_7 \cdot HCl$	--		--
G2 PAMAM	3256	$[NH_2(CH_2)_2NH_2]$:(G = 2); $PAMAM(NH_2)_{16}$	16	2.9	16.6 (HQ water)
G5 PAMAM	28826	$[NH_2(CH_2)_2NH_2]$:(G = 5); $PAMAM(NH_2)_{128}$	128	5.4	35 (HQ water)

Table 1: *Physico-chemical characteristics of minocycline, G2 and G5 dendrimers.*

Rainbow trout hepatocytes were exposed to increasing concentrations of PAMAM G2 dendrimer and minocycline both alone and in combination (Figure 2). Exposure to G2 alone did not produce any significant changes in cell viability. Minocycline did not produce significant decreases in cell viability either (ANOVA $p = 0.2$). When PAMAM G2 dendrimer and minocycline were combined, a significant reduction ($p = 0.02$) in cell viability was observed for concentrations between 20 and 100 µg/mL, with a toxicity threshold of 45 µg/mL. Multiple regression analysis of changes in cell viability induced by the PAMAM G2 dendrimer + minocycline group showed significant ($R = 0.67$; $p = 0.02$) correlations between PAMAM G2 dendrimer and minocycline. The analysis also showed that the partial correlation of PAMAM G2 dendrimer was significant ($p < 0.05$) but not with minocycline ($p > 0.05$), which suggests that the PAMAM G2 dendrimer had more bearing to lost of cell viability. For the PAMAM G5 dendrimer, cell viability decreased 0.75-fold relative to the controls at 10 µg/mL; when it was combined with a non-cytotoxic concentration of minocycline (50 µg/mL), cell viability levels decreased 0.65-fold relative to the controls (Figure 2). At the highest tested concentrations of PAMAM G5 dendrimers and minocycline (20 and 100 µg/mL, respectively), cell viability decreased 0.4-fold relative to the controls (Figure 3). The PAMAM G5 dendrimer alone accounted for the first decrease in cell viability, that is, a 0.68-fold decrease relative to the controls. Multiple regression analysis revealed that changes in cell viability associated with the combined exposure (PAMAM G5 dendrimer+minocycline) were significantly related ($R = 0.89$; $p < 0.01$) to minocycline and PAMAM G5 dendrimer treatments. Partial correlation analysis revealed that the PAMAM G5 dendrimer group ($\beta = 0.67$) was more correlated than the minocycline group ($\beta = 0.3$), suggesting that the PAMAM G5 effects also drove toxicity in the combined exposure group. The toxicity of the dendrimers was in the same range as in a previous study using *Daphnia magna* and two fish cell lines [22]. The reported toxicity ranged from 7.4 mg/mL to 230 mg/L; the Daphnia magna test was more sensitive than the trout cell lines. The toxicity of the dendrimers was also dependent of size (and zeta potential in this case), i.e., the G5 dendrimer was more toxic than the G2 PAMAM dendrimer (Figure 3). Minocycline was not toxic on its own, but its toxicity readily increased in the presence of dendrimers. These results reveal the possible interaction of nanoparticles with the toxicity of common contaminants such as antibiotics in municipal effluents and surface waters in urban areas. Although the nature of the interaction could not be determined in the present study, multiple regression analysis revealed that the dendrimers were the major drivers of toxicity, which suggests that dendrimer interactions were responsible for the combined minocycline-dendrimer cytotoxicity. The toxicity of dendrimers involves the production of reactive oxygen species, genotoxicity and apoptosis [23]. The production of reactive oxygen species is dependent on dendrimer size as with the formation of DNA strand breaks which preceded cell necrosis.

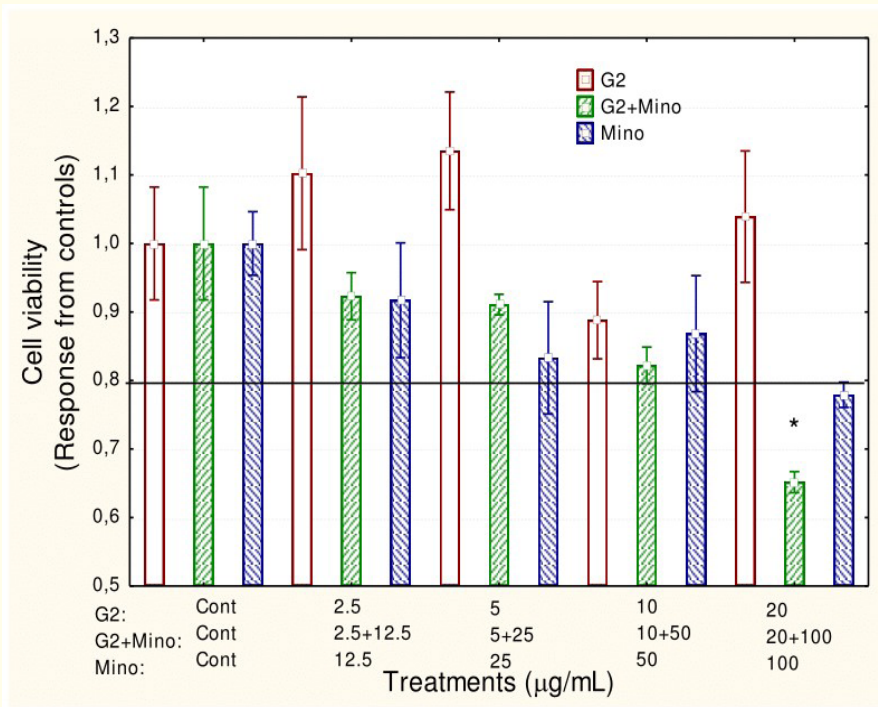

Figure 2: *Change in cell viability in trout hepatocytes exposed to G2 dendrimer and minocycline. Rainbow trout hepatocytes were exposed to increasing concentrations of G2 PAMAM dendrimer, minocycline and the combination of two. The data represent the mean with standard error. The line denotes a 20% drop in cell viability. The * symbol indicates significance at $p < 0.05$.*

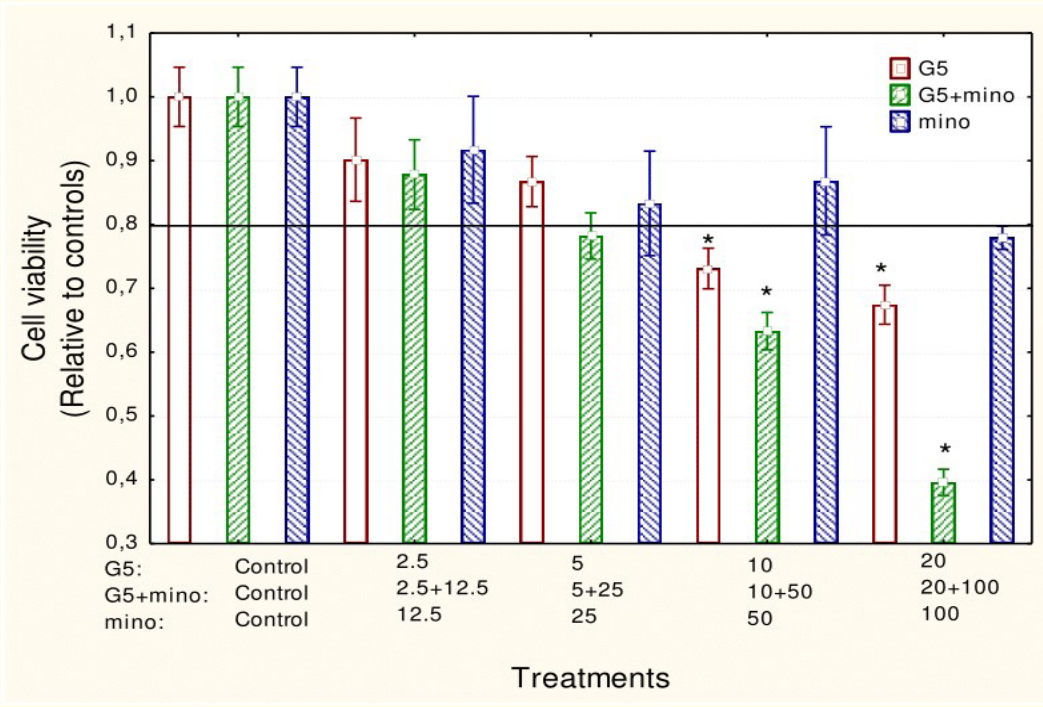

Figure 3: *Change in cell viability in trout hepatocytes exposed to G5 dendrimer and minocycline. Rainbow trout hepatocytes were exposed to increasing concentrations of G5 PAMAM dendrimer, minocycline and the combination of two. The data represent the mean with standard error. The line denotes a 20% drop in cell viability. The * symbol indicates significance at $p < 0.05$.*

The interactions between drugs (or contaminants) and PAMAM dendrimers represent a complex subject. Xenobiotic-dendrimer interactions are mainly driven by electrostatic bonds, hydrogen bonds, hydrophobic and van der Waals forces [24]. Minocycline has low hydrophobicity, and the expected interactions appear to involve hydrogen bonding since minocycline is cationic at physiological pH. Dendrimers also appear to have the capacity to bind proteins and DNA, given that the addition of serum albumin and calf thymus DNA was found to reduce the hemolyzing potential of 3rd to 6th generation PAMAM dendrimers towards red blood cells [25]. The number of positively charged groups at the surface of dendrimers was found to be responsible for hemolytic properties towards erythrocytes [26]. The capping of these cationic charges and the addition of anionic moieties at the surface of dendrimers were found to be effective in reducing the haemolytic and cytotoxic properties of the dendrimers. Based on tumor necrosis factor-α inhibition by dexamethasone, it was found that a PAMAM dendrimer-dexamethasone association was more efficient at delivering this drug *in vitro* than liposome encapsulated dexamethasone [27]. This suggests that hydrophilic traits are better than lipophilic ones for ensuring the efficient delivery of polar compounds (in the range of 0.02 > log kow > 4; minocycline has log kow =0.04) within cells. In another study, the cytotoxic concentration that decrease viability by 50% (CC50) for doxorubicin (log kow = 1.27) decreased four-fold when this drug was combined with G4 PAMAM dendrimers in MCF-7 cells [28]. This finding provides further evidence that hydrophilic interactions (hydrogen bonding and electrostatic interactions) are responsible for enhanced cytotoxicity. Another study showed that G2 PAMAM dendrimers were retained to a greater extent and penetrated more cells as compared to dendrimers whose surface was modified by acetylation or carboxylation (more hydro-

phobic and less positively charged form) [29]. From an (aquatic) environmental perspective, PAMAM dendrimers would likely interact with polar xenobiotics and increase bioavailability. However, they would also interact with natural organic matter (usually bearing negative charges), proteins, peptides and DNA naturally present in the environment. More research is required to gain a better understanding these processes and determine whether the toxicity of PAMAM dendrimers could be reduced, if not eliminated, by these natural ligands in the presence of environmental contaminants such as pesticides, surfactants and other polar compounds.

Acknowledgements

The work was supported by the Chemical Management Plan of Environment and Climate Change Canada. The technical assistance of Joana Kowazyk is duly recognized.

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