Towards Dendrimers as Anticancer Drug Carriers: from *in Silico* to *in Vivo* Characterization

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Abstract
Dendrimers are highly branched macromolecules of low polydispersity that provide many exciting opportunities for design of novel drug-carriers, gene delivery systems and imaging agents. They hold promise in tissue targeting applications, controlled drug release and, moreover, their interesting nanoscopic architecture might allow easier passage across biological barriers by transcytosis. However, from the vast array of structures currently emerging from synthetic chemistry, it is essential to design molecules that have real potential for in vivo biological use. Under these perspectives, some of us have recently synthesized a new, consistent set of polyamidoamine dendrimers, which were subsequently tested for tumor cell specific uptake and cytotoxicity. In this paper, we then report the results of an extensive series of atomistic molecular dynamics simulations performed on all the new dendrimers, with the purpose of broadening the knowledge of potential structure/activity relationships for these highly promising compounds.

Introduction
Dendrimers are highly branched macromolecules of low polydispersity that provide many exciting opportunities for design of novel drug carriers, gene delivery systems, and imaging agents. They hold promise in tissue targeting applications, controlled drug release, and, moreover, their interesting nanoscopic architecture might allow easier passage across biological barriers by transcytosis. However, from the vast array of structures currently emerging from the skillness of synthetic chemists, it is essential to design molecules that have real potential for *in vivo* biological use.
The growing body of clinical data arising form the development of polymer therapeutics suggests that polymer-protein and polymer-drug conjugates constitute an important new class of anticancer drugs. This has aroused considerable interest in the design of a second generation polymer-based anticancer treatments, and also in the potential use of polymer therapeutics for management of other diseases. Although, in this respect, dendrimers offer particular advantages with respect to linear polymers, as their surface chemistry can be tailor-made, and their reduced structural density in the intramolecular core is amenable to host-guest entrapment with opportunities fro subsequent controlled release, the vast majority of dendrimers so far described were not intended for pharmaceutical use. Indeed, they were water-insoluble and, generally, quite toxic. Nonetheless, recently dendrimers have been prepared with surfaces modified by biological residues such as carbohydrates, multiple arrays of peptidyl epitopes for use as vaccines, as dendritic boxes that encapsulates guest molecules and in the form of dendrimer-protein and dendrimer-antibody conjugates. In recent times, we have become particularly interested in the study and development of dendrimers as carriers for anticancer agents. Indeed, there are still very few studies that have systematically investigated the basic molecular and biological properties of these novel macromolecules. For a dendrimeric carrier to be suitable for *in vivo* applications, it is essential that the carrier is not toxic and nonimmunogenic, and it should preferably be biodegradable. Moreover, it must display an inherent body distribution that will allow appropriate tissue targeting. Cisplatin and carboplatin are important anticancer agents (see Figure 1). They are widely used in the treatment of difference cancer types (e.g., lung and ovarian tumors), although low water solubility and small tumor cell selectivity still constitute serious drawbacks.

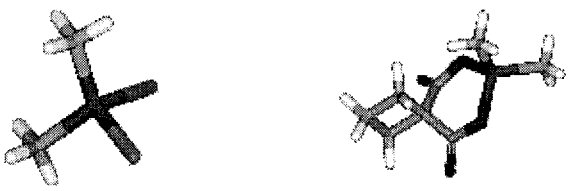

Figure 1. Structures of cis-diaminedichloroplatinum(II) (cisplatin, left) and cis-diamine(1,1-cyclobutane-dicarboxylate)platinum(II) (carboplatin, right).

As an example of the possible use of dendrimers as potential carrier and drug delivery systems for anticancer therapeutics, featuring tailor-made surface chemistry, some of us have recently synthesized a new series of polyamidoamine (yet not the well–known PAMAM) dendrimers (Fuchs et al., 2003). To this purpose, quaternized amines (set A), natural, proteinogenic aminoacids such as L-phenyilalanine (Phe), L-methionine (Met) and L-aspartic acid (Asp) (set B), diaminopropionic acid (Dap) (set C), and 5-dimethylamino-naphtalene-1-sulphonyl chloride (dansyl) motif (set D) were employed. Dendrimer sets A-C were chosen as starting systems for a systematic exploration of the influence exerted by surface derivatization on the toxicity of these molecules towards cell cultures. Moreover, some of the modified dendrimers of these sets feature chelating ligands for Pt(II), and could thus be of potential use for binding cisplatin-like complexes. The dansyl moiety was selected, to detect cellular uptake and intracellular distribution (by confocal fluorescence microscopy) of dendrimers D, for its high fluorescence level and polar nature.
All these application potentials, however, will not fully be realized before the understanding of the physical properties is considerably advanced and, consequently, the related structure-properties relationships have been uncovered. Therefore, in our attempt of exploring in greater detail the underlying mechanism of the potential biological functions of these newly synthesized molecules, we performed a comprehensive characterization of the dendrimeric sets A-C in a solvated environment, by computer-aided simulations.

Computational details

All molecular mechanics (MM) and dynamics (MD) simulations were performed by using the program packages Cerius[2] (v. 4.2), Discover and Materials Studio (v. 2.2) (all from Accelrys Inc., San Diego, USA), and in-house developed codes (stand-alone and add-on to the commercial software). The Amber all-atoms force field by Cornell et al. (1995), as imported by us in the above-mentioned suites, was used throughout the calculations. The generation of accurate dendrimer model structures was conducted as follows. For each dendrimer generation, the molecule was built and its geometry optimized via a combined steepest descent – conjugate gradient algorithm, using as a convergence criterion for the energy gradient the root-mean-square of the Cartesian elements of the gradient equal to 10^{-4} kcal/(mol Å). Long-range nonbonded interactions were treated by applying suitable cutoff distances, and to avoid the discontinuities caused by direct cutoffs, the cubic spline switching method was used. Van der Waals distances and energy parameters for nonbonded interactions between heteronuclear atoms were obtained by the 6^{th}-power combination rule proposed by Waldman and Hagler (1993).
Such a straightforward molecular mechanics scheme is likely to trap the simulated system in a metastable local high-energy minimum. To prevent the system from such entrapments, the relaxed structures were subjected to a combined molecular mechanics/molecular dynamics simulated annealing (MDSA) protocol (Fermeglia et al., 1999 and 2002; Pricl et al., 2001 and 2003). Accordingly, the relaxed structures were subjected to 5 repeated temperature cycles (from 298 K to 1000 K and back) using constant volume/constant temperature (NVT) MD conditions. At the end of each annealing cycle, the structures were again energy minimized to converge below 10^{-4} kcal/(mol Å), and only the structures corresponding to the minimum energy were used for further modeling.
The calculation of molecular surfaces was performed using the so-called Connolly dot surfaces algorithm (Connolly, 1983 and 1985). Accordingly, a probe sphere of given radius p_r, representing the solvent molecule, is placed tangent to the atoms of the molecule at thousands different position. For each position in which the probe does not experience van der Waals overlap with the atoms of the molecule, points lying on the inward-facing surface of the probe sphere become part of the molecule *solvent-accessible surface* (SAS). According to this procedure, the molecular surface generated consists of the van der Waals surface of the atoms which can be touched by a solvent-sized probe sphere (thus called *contact surface*), connected by a network of concave and saddle surfaces

(globally called *reentrant surface*), that smoothes over crevices and pits between the atoms of the molecule. The sum of the contact and the reentrant surface forms the so-called *molecular surface* (MS); this surface is the boundary of the *molecular volume* (MV) that the solvent probe is excluded from if it is not to undergo overlaps with the molecule atoms, which therefore is also called *solvent-excluded volume*. Finally, performing the same procedure by setting the probe sphere radius equal to zero, the algorithm yields the *van der Waals surface* (WS).

For the calculation of the molecular properties in the aqueous environment at T = 310K, each dendrimer molecule was solvated by a cubic box of TIP3P water molecules (Jorgensen et al., 1983), extending at least 10 Å in each direction from the solute. The periodic boundary conditions at constant pressure were applied and, in order to minimize the artifact of periodicity, a cutoff distance was set equal to half the box length. The resultant structures were relaxed via MM; in this case, the particle mesh Ewald technique was employed in handling nonbonded interactions. Further, to limit the effects due to a peculiar distribution of the water molecules in the cubic box, ten independent structures for each dendrimeric generation considered in this study were generated according to the procedure described above. The properties reported below are then to be considered as ensemble averaged from the appropriate set of ten structures.

Each molecular dynamics run was started by assigning initial velocity for atoms according to Boltzmann distribution at 2 x T. Temperature was controlled via weak coupling to a temperature bath (Berendsen et al., 1984) with coupling constant τ_T=0.01 ps. The Newton molecular equations of motion were solved by the Verlet leapfrog algorithm (Verlet, 1967), using an integration step of 1 fs and for a total simulation time of 3 ns.

Results and discussion

According to Fuchs et al. (2003), it is reasonable to assume that most of the present dendrimer amino groups behave as independent primary amine and, thus, are protonated at neutral pH. Therefore, all molecules have been modeled in their fully protonated state. Further, all synthesized dendrimers were fully soluble in water and in buffered cell culture media; accordingly, they were readily tested for cytotoxicity at different concentrations, using the human breast cancer cell line MCF-7. The results obtained by Fuchs et al. (2003) are summarized in Table 1.

Table 1. Summary of observed cytotoxicity for all dendrimers considered.

Set	G0	G1	G2
A	NT	T	HT
B			
Asp	NT	NT	-
Met	NT	MT	-
Phe	T	T	-
C			-
Dap	NT	NT	-

Legend: NT = non toxic; MT = moderately toxic; T = toxic; HT =highly toxic.

Although the polycationic nature of these compounds could be invoked to explain the concentration and generation dependence of their cytotoxicity (Roberts et al.,1996; Malik et al., 2000), this property alone cannot fully account for the observed influence on cellular growth and survival in every case. Undoubtedly, some structural factors should play a role.

Table 2 reports the MD averaged values of the radius of gyration R_g and of the aspect ratio of the largest to the smallest principal moment (I_z/I_x) for all dendrimers considered. Although undoubtedly more generations for each dendrimeric series are necessary to formulate some reliable structure-activity relationships (SARs), some speculations can be attempted. Indeed, from Table 2 two trends

can be envisaged: all dendrimers endowed with cytotoxicity are characterized by the highest values of the radius of gyration and by the lowest values of I_z/I_x (the relevant data are highlighted in bold in Table 1). In other words, a less compact and a more spherical shape seems to be a common feature of the cytotoxic behavior of these dendrimers. Interestingly Met-G1, which resulted in a slightly antiproliferative activity at the highest concentration considered (20 μM), features intermediated structural characteristics, as evidenced in italics in Table 2.

Table 2. Summary of observed cytotoxicity for all dendrimers considered.

Dendrimer-generation	R_g (Å)	I_z/I_x (-)
Set A		
A-G0	4.77	4.46
A-G1	**7.79**	**2.04**
A-G2	**10.68**	**1.03**
Set B		
Asp-G0	6.10	2.93
Asp-G1	9.43	2.46
Met-G0	6.38	2.88
Met-G1	*9.07*	*2.24*
Phe-G0	**7.07**	**1.43**
Phe-G1	**11.73**	**1.02**
Set C		
Dap-G0	6.69	2.99
Dap-G1	9.58	2.76

Figure 2 shows, as an example, the equilibrated MD structures of the first generation (G1) of two dendrimers belonging to sets A and C, respectively, from which both the enhanced compactness and the more spherical shape of the molecule of type A (cytotoxic) with respect to that of the Dap-based dendrimeric molecule (non cytotoxic) can be envisaged.

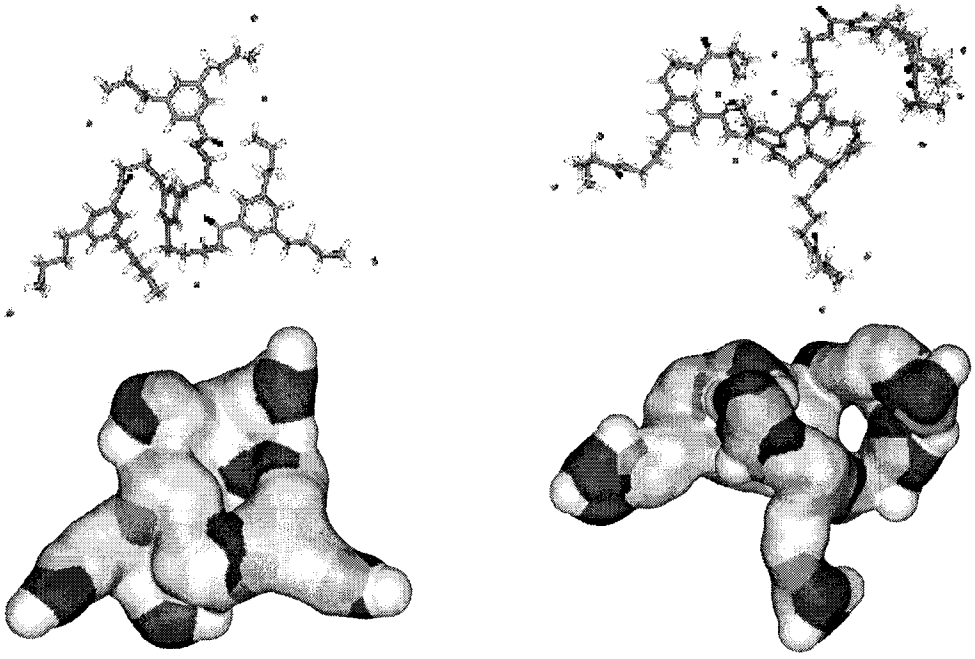

Figure 2. MD equilibrated structures of G1 of dendrimers belonging to sets A-C: (top left, molecular model dendrimer set A); (top right, molecular model Dap, set C); (bottom left, molecular surface dendrimer set A); (bottom right, molecular surface Dap, set C).

Another important problem in structural biology is the origin of specificity and recognition in molecular interactions. An essential step in this process is complementarity contact between approaching molecular surfaces. Surface representations of macromolecules such as dendrimers have provided a powerful approach for characterizing the structure, folding, interactions and properties of such molecules. A fundamental feature of surfaces that has not been characterized by these representations, however, is the texture (or roughness) of polymer surfaces, and its role in molecular interactions has not been defined. The degree of irregularity of a surface may be described by means of its fractal dimension D (Mandelbrot, 1983). As a surface becomes more irregular, the corresponding fractal dimension increases, starting from its lower limiting value $D = 2$, corresponding to an entirely smooth surface. The value of D can be obtained from the slope of the log(surface area) against log(probe size) used in the Connolly algorithm (see Computational details) to determine the molecular surface, as follows:

$$2 - D = \frac{d\log(SA)}{d\log(Rp)} \tag{1}$$

where SA and R_p are the molecular surface area and the probe radius, respectively. Table 3 lists the values of the calculated molecular surface area (SA), normalized by the number of atoms for each dendrimer generation, and the corresponding determined fractal dimension D for all dendrimers considered.

Table 3. Summary of observed cytotoxicity for all dendrimers considered.

Dendrimer-generation	SA/N (Å^2/atom)	D (-)
Set A		
A-G0	8.09	2.01
A-G1	**7.89**	**2.18**
A-G2	**7.76**	**2.21**
Set B		
Asp-G0	8.34	2.01
Asp-G1	8.14	2.05
Met-G0	8.08	2.09
Met-G1	*8.05*	*2.19*
Phe-G0	**8.01**	**2.25**
Phe-G1	**7.95**	**2.29**
Set C		
Dap-G0	8.35	2.00
Dap-G1	8.12	2.03

Another interesting series of considerations can be made upon observing the values reported in this Table. First, all cytotoxic dendrimers are characterized by the lowest values of the normalized molecular surface area (see values in bold in Table 3). Again, Met-G1 represents an intermediate behavior, congruent with the experimental evidence of the non toxicity of G0 and a moderate effect of G1. Even less subtle is the trend in the corresponding values of the fractal dimension D: the non toxic molecular species feature D values quite decidedly lower than their toxic counterparts. Interestingly, the dendrimeric families endowed with no cellular toxicity all present values of the surface fractal dimensions close to 2, i.e. are characterized by a regular, smooth surface. On the contrary, the toxic molecules have D values between 2.2 and 2.3, which fall in the range of the surface fractal dimensions exhibited by the vast majority of proteins (Zachmann, 1993). Finally, recent studies have reported that the average surface fractal dimension for cells is around 2.34 (DePetrillo et al., 2000). Already in 1967, Ryser hypothesized that the three-dimensional structure of compounds is important in their biological response on cell membranes (Ryser, 1967). Further,

branched molecules were found to be more efficient in neutralizing the cell surface charge than polymers with linear or globular structures, and rather rigid molecules were less toxic than linear or branched polymer (Fisher et al., 2003). Notwithstanding the necessity of more data to support our hypotheses, our data seems to be in agreement with these experimental findings, in that the cytotoxic dendrimers of series A (G1 and G2), the Phe-based dendrimers and G1 of Met-based compounds were all characterized by a more globular shape and a more branched surface pattern, as quantified by their fractal dimension *D*.

Conclusions

In this paper we reported the results obtained from a comprehensive characterization of newly synthesized dendrimeric molecules in a solvated environment, by computer-aided simulations. The evidences allowed us to formulate some SARs between the experimentally verified cytotoxicity/non-cytotoxicity of these compounds and some of their peculiar molecular features such as radius of gyration, shape and surface fractal dimensions. We are currently expanding our work to enlarge the data sets available and, hence, to obtain a definitive confirmation of the validity of the proposed SARs.

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