The effect of PAMAM dendrimer concentration, generation size and surface functional group on the aqueous solubility of candesartan cilexetil

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ABSTRACT
This article investigates the aqueous solubility of the poorly soluble drug candesartan cilexetil (CC) in the presence of poly(amo)odamine) (PAMAM) dendrimers. The effect of variables such as concentration, generation size (G2–G4), and surface groups (NH2, COOH and TRIS) of PAMAMs on the aqueous solubility of CC was studied. A two-factor factorial (3 × 3) ANOVA design was used to study the effect of generation size and surface functional group of the PAMAMs. The results showed that the aqueous solubility of CC in the presence of carboxyl and TRIS-terminated PAMAMs was higher than those of amine-terminated PAMAMs, and the effect of surface functional group of the PAMAMs on the aqueous solubility of CC was dependent on the generation size (p < 0.05). The sequence of the observed solubility fold enhancement due to PAMAMs was G4.COOH (8378) > G3.COOH (3456) > G4.TRIS (2362) > G2.COOH (1013) > G3.TRIS (749) > G2.TRIS (293) > G4.NH2 (91) > G3.NH2 (50) > G2.NH2 (37). The CC-PAMAM dendrimer inclusion complexes were characterized by UV–Vis, attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and differential thermal analysis (DTA) techniques. Regarding the results of these techniques, improvement in the solubility of CC is expected primarily through the intermolecular hydrogen bonding between the drug and internal tertiary and surface functional groups of the studied PAMAMs.

INTRODUCTION
Candesartan cilexetil (CC), a prodrug of candesartan, (2-Ethoxy-3-[2-(1H-tetrazol-5-yl)-4-yl methyl] 3-benzoimidazole-4-carboxylic acid 1-cyclohexyloxy carbonyl oxy ethyl ester) (Figure 1) is an angiotensin II receptor blocker (ARB) and often used orally in patients with hypertension, kidney disease and heart failure (1–3). The major problem related to the formulation and effectiveness of the CC is its poor aqueous solubility (5 × 10⁻⁵ g/L), which might lead to low or irregular bioavailability in the living cells (4).

To date, several techniques have been used to enhance the solubility of CC such as solubilization by nanosuspensions (5), solid dispersions (6,7), self-emulsifying drug-delivery systems (emulsions) (8,9), surfactants (10), and polymeric materials (11). Among these techniques, complexation with cyclodextrins has gained much attention (12–14). However, the aqueous solubility of CC at the above-mentioned literature could not be improved significantly. For this reason, an alternative approach is necessary, and there still remains a need for finding an ideal candidate to enhance the solubility of CC.

Dendrimers are emerging as outstanding carriers with their globular, hyperbranched, monodispersed structures and supramolecular properties (15–17). Dendritic architecture has been found to be an appropriate carrier for various drugs including anticancer, anti-inflammatory, antimicrobial, antibacterial, etc with the capacity to enhance solubility, and therefore, bioavailability of poor soluble drugs (18–20). This could be attributed to the high level of control over dendritic architecture (size, branching density and terminal groups) for enhanced drug solubilization.

Poly (amidoamine) dendrimers (PAMAMs) are one of the best-known dendrimers in drug-delivery due to their charged or polar species in the physiological conditions of the body environment (21,22). A typical PAMAM dendrimer possesses three main components; namely core, layers of repeating units (generations) and terminal groups (end groups on the surface). End groups might have cationic, anionic or neutral charges, and are mainly responsible for the high solubility, reactivity and toxicity of the entire molecules (23). Numerous studies have demonstrated that cationic PAMAMs can have generation, concentration and surface-charge dependent toxicity (24–26). In particular, the cell toxicity of PAMAMs is attributed to the number and cationic charge of end groups (17,20,27,28). In spite of facilitating intracellular drug-delivery, the positive charge of PAMAMs can restrict their use in biological systems because of the interaction of them with the negatively charged biological membrane to lead holes and cell lysis (28). Nevertheless, recent studies have indicated that cationic PAMAMs can be considered as advantageous depending on their route of administration such as oral (29), nasal (30), and injection (31) or treated type of tissue (32). Also, it has been proposed that small cationic PAMAMs in solutions can act as safe absorption enhancers for small to average size compounds (29,33).

Toxicities of PAMAMs can be minimized by modifying their cationic surface groups into neutral or anionic groups so that dendrimers can become more biocompatible (27,34,35). Previous studies have shown that neutral and anionic PAMAMs do not interact with the biological environment and are more suitable for clinical applications (27). Overall, surface engineered PAMAMs can...
be good candidates to improve the bioavailability of drugs with poor solubility and may provide protection against potential enzymatic or hydrolytic breakdown within the body (18).

Architecture of PAMAMs is hydrophobic internally even though hydrophilic in nature externally. The internal cavities (core and repeat units: amine and amide linkages) of the PAMAMs can be host for small hydrophobic guest molecules (e.g. drugs, biological actives or hydrophobes) while outer peripheral groups are mainly responsible for the behaviors in solutions (36,37). These micelle-like properties of PAMAMs make them promising carriers for poor soluble drugs to make non-covalent inclusion complexes (38,39). On the other hand, if some drug molecules are trapped by the densely packed structure of the dendrimer, an encapsulation interaction occurs. Also, drugs can interact electrostatically and hydrophobically with the internal tertiary nitrogens and surface functional groups of the PAMAMs (19,37). Surface modification of PAMAMs with hydrophilic functional groups allows them to be used widely in pharmaceutical and drug delivery studies (36,37).

Over the past decades, amine-terminated PAMAMs (PAMAM-NH2) have been used in a broad range of applications for enhanced drug solubilization (18–20). Yiyun and Tongwen (40) used different generations (G3–G4) of PAMAM-NH2 dendrimers to encapsulate and increase the aqueous solubility of non-steroidal anti-inflammatory drugs such as ibuprofen (74-fold), diflunisal (26-fold) and naproxen (1570-fold). Chauhan et al. (41) reported higher encapsulation of indomethacin by factors of 29, 26, and 10-fold for amine (cationic), hydroxyl (neutral), and carboxyl (anionic)-terminated PAMAMs, respectively. In another study, Devarakonda et al. (42) reported significant increases in the solubility of nicosamide (an anthelmintic drug used in the treatment of disease) in the presence of different generations G0 (372-fold), G1 (1354-fold), G2 (1945-fold) and G3 (6176-fold) PAMAMs compared to hydroxypropyl-β-cyclodextrin (~10-fold enhancement).

Prospectively, Beezer et al. (43) modified the surface functional groups of ester-terminated half generation (G1.5 and G2.5) PAMAMs with hydroxyl groups by reacting them with tris(hydroxymethyl) aminomethane (TRIS) molecule. They tested resulting TRIS-terminated PAMAMs (PAMAM-TRIS) as the solubility enhancer of small acidic hydrophobic molecules, and proposed them as the significant drug-delivery systems for the future studies due to their ideal cell interactions. However, up to now, a little work has been conducted to investigate PAMAM-TRIS dendrimers as the solubility imposter of the poorly water-soluble molecules or drugs, and more work is needed (44).

Although the carboxyl-terminated PAMAMs (PAMAM-COOH) are attributed as less or negligible toxic compared to toxic PAMAM-NH2 dendrimers (45–47), PAMAM-TRIS dendrimers are expected to have greater potential for the prospective drug carrier systems as they exhibited ideal cell interactions (23,43). On the other hand, PAMAM-NH2 dendrimers are still commercially available and studies on them still continues in the various fields of science including pharmaceutics (17,48).

The purpose of this study is to investigate the aqueous solubility of CC in the presence of PAMAMs. The effect of anionic PAMAM-COOH and neutral PAMAM-TRIS dendrimers on the aqueous solubility of CC was examined as an alternative to cationic PAMAM-NH2 dendrimers. In addition to surface functional group (NH₂, COOH and TRIS), the effect of concentration and generation (G2–G4) of PAMAMs on the aqueous solubility of CC was also investigated.

**Materials and methods**

**Materials and apparatus**

Methyl acrylate, EDA, methanol, n-butanol, TRIS were purchased from Merck & Co. (Kenilworth, NJ). NaOH, 37% HCl, NaH₂PO₄, NaCl and KHP were supplied from Merck. CC was kindly supplied from DEVA Holding (Istanbul, Turkey) as a gift. All solutions were prepared using 18.2 MΩ Millipore Milli-Q deionized water (Merck Millipore Corporation, Billerica, MA). NaOH solutions were used as titrant after standardized with primary grade KHP. Standardized HCl against KHP was used as excess acid in order to adjust initial pH of dendrimer solutions. pH 4.0, 7.0 and 11.0 buffer solutions for the calibration were supplied from Merck. Dendrimer solutions were stored at 4°C. Unless otherwise stated all chemicals were in analytical grade and used without further purification.

A CEM Focused Microwave™ synthesis system, model, Discover (CEM Corporation, Matthews, NC) with a continuous microwave power output from 0–300 W (±30 W) programmable in 1 W increment, infrared temperature control system programmable from 25–250 °C, and 5–125 mL vessel capacity was used as microwave reactor. Spectroscopic titrations were carried out automatically by using TitroLine® 7000 (SI Analytics GmbH, Hattenbergstrabe, Germany) autotitrator equipped with thermostated titration vessel under nitrogen media and PG TG 70 UV–Vis spectrophotometer equipped with UVWinS Software version 5.0.5 (Tresser Instruments, Roßdorf, Germany), together. Differential thermal analysis (DTA) thermograms were recorded on a TG-TGA instrument (TA instruments, New castle, DE) calibrated with indium.

**Synthesis of PAMAMs**

Microwave-assisted synthesis (MAS) and characterization of PAMAM-NH2 (Gn.NH₂) and PAMAM-TRIS (Gn.TRIS) dendrimers from half generation ester-terminated PAMAMs (PAMAM-OCH₃) were performed according to our previous studies (49), and (50), respectively. On the other hand, PAMAM-COOH dendrimers (Gn.COOH) were prepared by slight modification of the literature procedure (51), and our previous study (52). General experimental and procedures for the preparation of synthesized PAMAMs are given in the supplementary file. Purity of PAMAMs was characterized by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), 1H-NMR, 13C-NMR, and supported by spectroscopic titrations (see supplementary file). Synthesized PAMAMs were used in the solubilization studies of CC.

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**Figure 1.** Molecular structure and some physicochemical properties of CC: $\lambda_{max} = \text{260 nm, } \log P = 6.1, \text{Mw}= 610.67 \text{g/mol}$. (5).
Preparation and characterization of drug-PAMAM dendrimer inclusion complexes

CC-dendrimer inclusion complexes were prepared according to a method described by Devarakonda et al. (53) and summarized briefly hereafter. CC was dissolved in methanol and then the PAMAM dendrimer was added. CC-PAMAM dendrimer complexes were prepared by stirring 1:1 (w/w, dendrimer/drug) initial ratio of CC to PAMAM dendrimer mixture at 200 rpm and 37 ± 0.1 °C for 24 h in methanol. Then, methanol was removed by a rotary evaporator. The precipitates were dried under vacuum to remove the methanol completely, followed by the addition of deionized water and stirred for another 2 h to extract out drug–dendrimer complex. This solution then filtered through the 0.45 μm cellulose acetate filter and lyophilized to remove the water completely. Resulting drug-PAMAM dendrimer inclusion complexes were subjected to ATR-FTIR (4000–600 cm⁻¹), UV–Vis (230–400 nm) and DTA.

UV characterization

The CC-PAMAM dendrimer inclusion complexes were diluted properly with methanol to the final concentration of 22 μg/mL. The UV-spectra of these complexes were recorded between the wavelength ranges of 230–400 nm. The maximum absorption wavelengths of CC in methanol are at 254 and 308 nm. Any suppression or shift in these strong absorption bands was assigned to the inclusion complex formation (53–55).

ATR-FTIR spectral studies

The drug-PAMAM dendrimer inclusion complexes, CC (alone) and PAMAMs (alone) were subjected to ATR-FTIR. The ATR-FTIR spectra were recorded after 20 scans at 4 cm⁻¹ in a wavenumber range of 4000–600 cm⁻¹.

DTA thermograms were recorded on a TG-TGA instrument calibrated with indium. Precisely weighed 5 mg samples of drug-PAMAM dendrimer inclusion complexes were packed in an aluminum pan and heated from 25 to 300 °C at a scanning rate of 10 °C/min. Inert atmosphere was maintained by purging nitrogen gas at a flow rate of 40 mL/min.

Solubility measurements

Solubility studies were carried out as per the method described by Higuchi and Connors (56) and summarized hereafter. An excess amount of CC was added to 5 mL amber colored vials containing increasing concentrations of PAMAMs (0.125–2.00 mM) and then the vials were sealed. The resulting suspensions were shaken with an orbital shaker at 200 rpm while maintained at 37 ± 0.1 °C in an incubator for 24 h. The obtained mixtures were centrifuged at 2500 rpm for 5 min, and insoluble excess CC was removed by filtering through 0.45 μm cellulose acetate filters. The concentration of CC was determined spectrophotometrically by using UV–Vis spectrophotometer in the wavelength ranges of 230–400 nm. UV-spectrum was taken at 254 nm for three repeated measurements. The absorbances were correlated with the calibration curve and the amounts of drugs were determined.

Statistical analysis

To study the effect of generation size and surface functional group of the PAMAMs on the aqueous solubility of CC, a two-factor factorial (3 × 3) ANOVA design (57) with n = 3 replicates was used where the aqueous solubility of CC was measured for three levels of factor A (generation size of the dendrimers: G2, G3 and G4) and three levels of factor B (surface functional group of the dendrimers: NH2, TRIS and COOH). Using this design, the effect of two factors on the aqueous solubility of CC was evaluated at a probability level of p = 0.05 (95% confidence interval).

In the presence of a significant interaction among the factors (independent variables), a one-way ANOVA (simple main effects test) was used in order to explore where these interactions exist. Finally, the differences between the two sample means were observed by pair-wise comparisons by using Benferroni post hoc test. Residual analysis was performed for the assumptions of the two-way ANOVA. Outliers were assessed by inspection of a box-plot, normality was assessed using Shapiro–Wilk’s normality test. Homogeneity of variances was assessed by Levene’s test. There were no outliers. Residuals were normally distributed (p > 0.05) and there was homogeneity of variances (p > 0.05). All the statistical analyses were performed by using a commercial software package (SPSS 20.0) (SPSS Inc., Chicago, IL) and data were presented as mean ± confidence interval.

Results and discussion

Structural characterization of PAMAMs

The PAMAMs were characterized structurally via 1H NMR, 13C NMR, ATR-FTIR, EA and their purity was also determined by spectroscopic titrations (see supplementary file). The results were in good agreement with the literature (49,50). Since the dendrimers are hygroscopic, the prepared PAMAMs were stored in methanolic solution (10% w/w) and stored at ±4 °C. Some selected properties and characterization data of PAMAMs were presented in Table 1. The abbreviations G2–G4 (second to fourth generation) were suggested for respective NH2, TRIS and COOH-terminated PAMAMs with molecular weights ranging from 1430 to 8864 g/mol.

As shown in Table 1, molecular weight and the number of tertiary amine groups of dendrimers increase exponentially with each generation. This also implies that the surface density of PAMAMs increases as the generation number increases. All the PAMAMs (G2–G4) evaluated in this study have tertiary amine groups at each branching point. The end groups vary as NH2, TRIS and COOH groups.

Possible mechanism of interaction between the PAMAMs and CC

Possible mechanism of interaction between the PAMAMs and CC could be through hydrophilic and hydrophobic interactions (59).

Table 1. Selected characteristics of EDA cored PAMAMs (58) and maximum number of CC molecules interacting with the dendrimer molecules determined experimentally.
All the PAMAMs evaluated in this study have tertiary amines in their internal cavities, which could act as hydrogen bond donors and acceptors (Table 1). Likewise, the periphery groups of the studied PAMAMs might act as hydrogen bond donors and acceptor.

CC can be encapsulated in the interior of PAMAMs or bound to the surface functional groups. Drug loaded PAMAM dendrimer solutions (2 mM) were used to get a better insight into drug-PAMAM dendrimer interactions. The maximum number of CC molecules which can associate with the PAMAMs are presented in Table 1. Investigation of the Table 1 reveals that the number of CC molecules interacting with the PAMAMs increased with an increase in the dendrimer generation size for all the NH2, TRIS and COOH-terminated PAMAMs. However, no obvious correlation or relation could be observed between the number of tertiary or terminal groups present in a single dendrimer molecule and the number of CC molecules.

The highest number of CC molecules was observed for G4.COOH which has 30 inner tertiary amines and 32 surface COOH groups. In total, 33.5 drug molecules were associated per G4.COOH molecule. In contrast, 9.4 and 0.4 drug molecules were interacted with G4.TRIS, which has 30 tertiary amines and 96 surface OH groups, and G4.NH2, which has 30 tertiary amines and 32 surface NH2 groups, respectively. This announced that there was a strong interaction between CC molecules, and COOH-terminated PAMAMs. When the number of CC molecules associated with each dendrimer molecule taken into consideration, it could be concluded that inclusion complexation was most probably a hydrophilic interaction between the interior dendrimer nitrogen groups or surface functional groups and drug specific binding sites with hydrogen bonding.

In addition to hydrogen bonding, hydrophobicity of dendrimers’ environment could also be a factor for the interaction. Former studies have shown that microenvironment in the dendritic microcavities is considerably less polar than the microenvironment of the bulk aqueous phase (60). Therefore, highly hydrophobic CC molecules could be solubilized in the hydrophobic or low polar cites of dendrimers by encapsulation. To overall, regarding the drug-PAMAM dendrimer hydrophilic and hydrophobic interactions, PAMAM-COOH dendrimers could be desirable for the solubility enhancer of small hydrophobic API, CC.

**Characterization of the PAMAM dendrimer inclusion complexes**

**UV characterization**

UV-spectra of CC, PAMAM dendrimer and CC-PAMAM dendrimer inclusion complexes were recorded in methanol, since suppressions and shifting in UV absorption have been reported as an evidence for the other drug-PAMAM dendrimer inclusion complexes (53–55). Figure 2 shows the UV-spectra of CC (1), PAMAMs (5–7) and CC-PAMAM dendrimer inclusion complexes (2–4) in methanol. The maximum absorption peaks of CC are at λmax = 254 and 308 nm. Investigation of the Figure 2 reveals that UV-spectra of CC-PAMAM dendrimer inclusion complexes do not display a new type of complexation band. The only difference in UV-spectra of CC alone and CC-PAMAM dendrimer inclusion complexes is the suppression of peaks present at 254 and 308 nm. These results indicate that inclusion complexation between the CC and the PAMAMs could be by non-covalent bonding, electrostatic interactions or encapsulation of drug molecules to the internal cavity of dendrimers.

**ATR-FTIR characterization**

The infrared spectral properties concerning CC-G3 PAMAM dendrimer inclusion complexes are presented in Figure 3. In Figure 3, the ATR-FTIR spectrum of pure drug CC displayed characteristic signals at 2941 (C–H stretching), 1751 (ester C=O stretching vibration), 1612 (N–H bending), 1348 (aromatic C–N stretching), 1315 and 1240 cm⁻¹ for C–O stretching of aromatic esters (61), 1074 (C–O ether stretching) and 744 cm⁻¹ aromatic C–H bending.

![Figure 2. UV-spectra of methanolic solutions (1) CC, 22 µg/mL; (2) CC-G4.COOH; (3) CC-G4.TRIS; (4) CC-G4.NH2; (5) G4.COOH (50 µg/mL); (6) G4.NH2 (50 µg/mL); (7) G4.TRIS (50 µg/mL).](image-url)
Important vibrations detected in the ATR-FTIR (600–1800 cm\(^{-1}\)) spectra of G3.NH\(_2\) were three characteristic bands at 3273 (stretching vibration of primary amine groups), 1635 and 1547 cm\(^{-1}\) (amide I and II), and at 1028 cm\(^{-1}\) (C–N stretching of primary amine groups). The ATR-FTIR spectrum of pure G3.TRIS showed about at 3271, 1643, 1560, 1392 and at 1026 cm\(^{-1}\) corresponding to the vibrations of O–H stretching, C=O stretching, N–H bending, C–O stretching and C–O stretch groups, respectively. The ATR-FTIR spectrum of G3.COOH displayed a band at 3271 cm\(^{-1}\) corresponding to the vibrational O–H stretching of the carboxylic terminal groups. Also, it showed significant three bands 1635 (C=O stretching), 1556 (N–H bending) and at 1396 cm\(^{-1}\) (C–O stretching).

After complexation of PAMAMs with CC, spectral shifts were observed for the dendrimer O–H stretching (0–2 °), C=O stretching (0–12), N–H bending (0–6), C–O stretching (0–8) and C–N stretching (0–10 cm\(^{-1}\)) because of the hydrophilic interactions with dendrimer polar groups (62,63). Similar patterns were observed for all CC complexes of any generations of PAMAMs. The observed spectral changes can be attributed to hydrophilic interaction of drug N–H group with dendrimer C=O, C–O and C–N/N–H groups. In other words, shifts at the position of bands could be explained by intermolecular hydrogen bonding between drug and dendrimer groups. Moreover, fully disappearance of the ester C=O stretching vibration band of CC (1751 and 1715 cm\(^{-1}\)) from the spectra of all CC-PAMAM dendrimer complexes allowed us to postulate that all the ester carbonyl groups (ester C=O) of CC were involved in hydrogen bonding with the specific binding sites of PAMAMs (drug polymer complexes (3,5,7); Figure 3(b)).

Additional proof for the drug-PAMAM dendrimer inclusion complexation through hydrophilic interactions comes from the major spectral shifting of the -OH bond vibrations from 3271 to 3273 cm\(^{-1}\) for both G3.COOH and G3.TRIS dendrimers. On the contrary, no shift at the position of NH stretching vibration (3273 cm\(^{-1}\)) of G3.NH\(_2\) was observed while complexing with CC (Figure 3(a)).

Figure 3. Expanded ATR-FTIR spectra of (1) CC; (2) G3.COOH; (3) CC-G3.COOH complex; (4) G3.TRIS; (5) CC-G3.TRIS complex; (6) G3.NH\(_2\); (7) CC-G3.NH\(_2\) complex.
This could be due to the absence of electrostatic or ionic interactions between the surface amine groups of PAMAM-NH₂ dendrimers with CC.

On the other hand, hydrophobic interactions were also investigated by examining the dendrimer antisymmetric and symmetric CH₂ stretching vibrations in the region 3000–2800 cm⁻¹. The antisymmetric and symmetric CH₂ bands of the G3.COOH, G3.TRIS and G3.NH₂ located at 2966 and 2840 cm⁻¹; at 2941 and 2825 cm⁻¹; at 2931 and 2827 cm⁻¹ were observed at 2968 and 2848 cm⁻¹; at 2946 and 2841 cm⁻¹; at 2935 and 2849 cm⁻¹ for CC-G3.COOH, CC-G3.TRIS and CC-G3.NH₂ complexes, respectively. The larger shifting (∆ν = 22 cm⁻¹) was observed for G3.NH₂ dendrimer. The observed spectral shifting of dendrimer CH₂ vibrations is pointing out the major hydrophobic interactions for CC-G3.NH₂ dendrimer.

The results of ATR-FTIR studies approved the inclusion complexation between PAMAMs and CC. Spectral changes in ATR-FTIR spectra occurred as a result of a serious of hydrophilic (intermolecular hydrogen bonding: dendrimer-dendrimer and CC-dendrimer) and hydrophobic interactions (encapsulation).

**Differential thermal analysis (DTA)**

DTA can be used for the recognition of inclusion complexes (64). When the guest molecules were embedded in the dendrimer cavity, their melting, boiling or sublimation points generally shifted to a different temperature (52,65). Figure 4 shows the change in the thermal behavior of CC, G3 PAMAM dendrimer, and CC-G3 PAMAM dendrimer inclusion complexes. As shown in Figure 4, the DTA curve of the pure drug exhibited a sharp endothermic peak at 170.6 °C corresponding to its melting point and indicating its crystalline nature (66) whereas the thermograms of neat G3.COOH, G3.TRIS and G3.NH₂ PAMAMs displayed broad characteristic endothermic peaks at 133.1, 138.4 and 104.8 °C, respectively. In the presence of G3 PAMAMs, the complete disappearance of the melting peak of CC (170.6 °C), and formation of new endothermic peaks referring to drug-PAMAM dendrimer inclusion complexes of CC-G3.COOH (114.4 °C), CC-G3.TRIS (123.5 °C), and CC-G3.NH₂ (123.9 °C) could be attributed to transformation of CC from its crystal form to amorphous form, and successive encapsulation of CC with the PAMAMs (65).

**Effect of PAMAM dendrimer concentration on the aqueous solubility of CC**

The effect of PAMAM dendrimer concentration and generation size on the aqueous solubility of CC was carried out by using different generation PAMAMs having NH₂, TRIS and COOH surface functional groups (Table 1), and the results were shown in Figure 5. It was observed a proportional increase in the solubility of CC with increasing dendrimer concentration (0–2 mM) and generation size (G2–G4). CC is practically water insoluble (0.0048 mg/mL). The increased solubility of CC could be expected to non-covalent interactions between the CC and PAMAMs involving a variety of driving forces such as hydrophobic bonding, hydrogen bonding and electrostatic interactions (56). In particular, encapsulation of CC molecules by PAMAMs could be another driving force for the solubility enhancement of CC. Investigation of the structures of PAMAMs with molecular simulations showed that lower generations (G < 4) displayed open structure while later or higher generations (G > 4) were characterized by densely packed closed structures (67). Due to these unique and specific properties, PAMAMs can entrap small CC molecules in their hydrophobic cavities. Also, the increased solubility of CC in the presence of PAMAMs could be presumably due to increase in the number of surface functional groups and internal cavities, which are rich in terms of tertiary amine groups and available to interact with CC molecules (68). On the other hand, theoretical studies since 1990 have shown that a dendritic structure made up from flexible bonds should exhibit maximum density at the center of the molecule with a decrease in density toward the periphery (69,70) so that lower generation PAMAMs (G < 4) could be successfully take role to encapsulate the drug molecules inside their internal cavities.

**Interaction effect between generation size and surface functional group of the PAMAMs**

An interaction effect occurs when the effect of one independent variable on a dependent variable is different at different levels of the other independent variable. In our case, independent variables are generation size and surface functional group while...
dependent variable is the solubility of CC. G2, G3 and G4 are the levels for generation size whereas NH2, TRIS and COOH are those for surface functional group of PAMAMs. Our results showed that the solubility of CC increases as the generation size increases in each generation of dendrimers having different surface functional groups (Figure 5). However, investigation of the Figure 5 revealed that a further evidence emerged that PAMAM-NH2, PAMAM-TRIS and PAMAM-COOH dendrimers display different solubilization effect on CC. Regarding the solubility of CC, the effect of generation size on the aqueous solubility of CC might not be the same for NH2, TRIS and COOH-terminated PAMAMs. For this reason, we decided to test this assumption by carrying out another study, which also takes into account the type of surface functional groups of the PAMAMs. In this point, our aim was to learn whether the effect of generation size on the aqueous solubility of CC might be different for NH2, TRIS and COOH-terminated PAMAMs. In other words, does the effect of generation size on the solubility of CC depend on the variety of surface functional groups? These questions could directly be answered by checking whether there is a statistically significant interaction effect (between the two independent variables: generation size and surface functional group) by carrying out a two-way ANOVA. Thus, we interested in determining whether the effect of generation size on the aqueous solubility of CC was different for PAMAM-NH2, PAMAM-TRIS and PAMAM-COOH dendrimers.

Two-way ANOVA results revealed that there was a significant interaction between generation size and the level of surface functional group on the solubility of CC ($p < 0.05$). The significance of the interaction term indicates that the effect of surface functional group of the PAMAMs on the aqueous solubility of CC depends on the generation size. That is, the simple main effects: generation size and surface functional group are different as we have statistically significant interaction effect. Thus, we have to carry out simple main effect tests in order to explore where these interactions exist.

**Simple main effects: generation size and surface functional group**

**Effect of generation size on the aqueous solubility of CC**

Effect of generation size of PAMAMs on the aqueous solubility of CC was investigated for three levels of surface functional groups (NH2, TRIS and COOH). The results were presented in Figure 6. We can obtain a basic perception of whether we have an interaction between the two independent variables from visual inspection of the profile plots (Figure 6). For instance, inspection of Figure 6 shows that the solubility profile lines are not parallel and have different patterns. Thus, we might expect that the interaction effect is statistically significant. On closer examination, it would appear that all generations have an increasing effect on the solubility of CC with NH2, TRIS to COOH surface functionality.

A two-way ANOVA simple main effect test for generation size was conducted and observed that there was a statistically significant difference in the solubility of CC between generations of all the surface functional groups ($p < 0.05$). Interestingly, it was not observed a statistically significant effect of generation size on the solubility of CC for amine-terminated PAMAMs ($p > 0.05$) (Figure 6). On the other hand, there were statistically significant differences in the mean solubility of CC between TRIS and COOH-terminated PAMAMs for generation G2–G4 ($p < 0.05$). The highest attained CC solubility for G4.COOH PAMAM dendrimer was 29.39 mg/mL (95% CI, 28.763–30.016) higher than the second close G4.TRIS PAMAM dendrimer ($p < 0.05$).
Effect of surface functional group on the aqueous solubility of CC

The effect of surface functional group of PAMAMs (NH2, TRIS and COOH-terminated) on the aqueous solubility of CC was investigated for three different generations (G2–G4). The solubility profile of CC as a function of increasing concentration of G4.NH2, G4.TRIS and G4.COOH dendrimer in aqueous vehicle is presented in Figure 7. The solubility of CC increased linearly with the increasing concentration of PAMAMs. The solubilization effect of PAMAMs on CC was in the order of G4.COOH > G4.TRIS > G4.NH2. Similar trends were observed for all generations. Also, the phase solubility profile of CC showed a similar trend to Higuchi’s A type curve for all types of end groups. However, it could not be guessed that to what extent the solubility of CC will be in linear correlation with the amount of added PAMAM concentration. Thus, it could be more pleasurable to use required amount and type of dendrimer as an excipient for the desired solubility of CC. For example, it could be observable from Figure 7 that 0.5 mM added amount of G4.COOH leads almost the same solubility enhancement of CC with 2 mM of G4.TRIS (see dashed reference line in Figure 7). A former study indicated that $A_N$ type of solubility profiles of drugs could be observable at higher concentrations due to aggregation/self-association of the drug–dendrimer complexes with charge masking (71). This would be inevitable if one continues to add dendrimer until obtaining a higher solubility enhancement. In other words, there is no end while deciding the amount of added dendrimer if one wants to aggregate the results while asserting he or she increased the solubility of an active ingredient pharmaceutical (API) up to ten, a hundred or a thousand fold. Despite all the attractiveness of the solubility enhancement by inclusion complexation, excess improvement in the solubility by using an excipient might lead to drawbacks such as toxicity of complexing agent and precipitation upon dilution with aqueous media having a pH at which the compound is less soluble (72). For this reason, it should be more important to report whether the effect of dendrimer concentration, generation and surface functional group on the aqueous solubility of any kind of drugs is statistically significant or any kind of statistically significant differences exists among these factors.

An analysis of simple main effects for surface functional group was performed with statistical significance receiving a Bonferroni adjustment ($p < 0.05$). Considering G4 dendrimer, where the maximum difference occurs in the solubility of CC, mean “solubility of CC” between G2, G3 and G4 dendrimers having either NH2, TRIS or COOH surface functional groups, respectively ($p < 0.05$). That is, the effect of surface-group functionality of the PAMAMs on the aqueous solubility of CC is dependent on the generation size.

As it can be seen from Figure 8, the gap between the solubility profile lines of COOH, TRIS and NH2-terminated PAMAMs expands as the generation increases. This is also the indicator of an interaction between the generation size and surface functional group. This could be attributable to an exponential increase in the number of specific binding sites of PAMAMs with altering generations and surface groups (Table 1).

Solubility studies

The effect of concentration, generation size and surface-group functionality on the aqueous solubility of CC was studied using PAMAMs G2-G4 with NH2, TRIS and COOH end groups. Figure 9 shows the comparison of the solubility enhancement of PAMAMs. As can be seen from Figure 9, the solubility of CC increased with the increasing generation number of all dendrimers. The sequence of the observed solubility fold enhancement in the presence of PAMAMs was G4.COOH (8378) > G3.COOH (3456) > G4.TRIS (2362) > G2.COOH (1013) > G3.TRIS (749) > G2.TRIS (293) > G4.NH2 (91) > G3.NH2 (50) > G2.NH2 (37). In a short former study (11), only the concentration effect of NH2-terminated G4 PAMAM dendrimer on the aqueous solubility of CC was investigated. In that study,
the solubility enhancement of CC was announced as 373 fold in the presence of 10 mg/mL G4 PAMAM dendrimer with no temperature record. It is interesting to note that solubility is a relative concept and can change depending on the amount of used excipient, temperature and equilibrium time where the maximum solubility of drug reached. In this sense, we have emphasized with statistical studies that it is more important to note whether a significant increase could be observable or not with a change in the used concentration and generation of PAMAM excipient. The results of our studies showed that surface functionality of PAMAMs serves as a statistically significant important factor for the solubility enhancement of CC (p < 0.05) while generation size has exceptionality only for NH$_2$-terminated PAMAMs that no considerable statistically significant solubility enhancement was observed with increasing generation number (p > 0.05). Apart from this, it was noteworthy that a considerable solubility enhancement of CC was obtained in the range of 8378–37 fold.

**Conclusion**

In this study, different generations G2-G4 PAMAMs with NH$_2$, TRIS and COOH surface functional groups were used for the first time in the solubility enhancement of poorly soluble and low bioavailable drug, CC. ATR-FTIR, UV-Vis, DTA characterization and solubility studies of the drug-PAMAM dendrimer inclusion complexes suggested that CC was successfully encapsulated in the PAMAM dendrimer cavity.

Effect of concentration, generation size (G2–G4) and surface groups (NH$_2$, TRIS and COOH) on the aqueous solubility of CC was examined. It was observed that the solubility of CC enhances within an increase in the concentration of the PAMAMs and effect of surface functional group on the aqueous solubility of CC was dependent on the generation size of PAMAMs. The increase in the solubility of CC was observed primarily due to intermolecular hydrogen bonding between drug and internal tertiary and surface functional groups of dendrimer. Resonance on the surface COOH groups of PAMAM-COOH dendrimers could gain them strong hydrogen bonding donor and acceptor abilities. This could be attributable to their highest solubility enhancement abilities.

To overall, an ideal interaction between the drug-delivery vehicle and cell is desired (43). PAMAM-NH$_2$ dendrimers could enhance the solubility of pure soluble drug CC to some extent. However, they might not be desirable for the future clinical applications of CC due to their positive surface charges. In this point, anionic PAMAM-COOH and neutral PAMAM-TRIS dendrimers could be alternatives to cationic PAMAM-NH$_2$ dendrimers. In particular, PAMAM-COOH and PAMAM-TRIS dendrimers investigated in this work could be good candidates as the solubility enhancer of poor soluble drug CC and this study might be useful for the future studies dealing with the formulation development and clinical applications of CC.

**Disclosure statement**

Authors declare that the major contribution in all parts of this study was provided by Ali Serol Ertürk’s correspondence, and there is no conflict of interest.

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