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ORIGINAL ARTICLE

Polyamidoamine dendrimers as potential drug carriers for enhanced aqueous solubility and oral bioavailability of silybin

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Abstract

Purpose: In this study, the effect of polyamidoamine (PAMAM) dendrimers on the solubility of silybin was investigated. The in vitro drug release and the pharmacokinetics of silybin–dendrimer complex were also investigated. *Methods*: The solubilization of silybin by PAMAM dendrimers of generation G1.5, G2, G2.5, and G3 with different concentrations was determined and compared in different pH conditions. The in vitro release of silybin from the silybin–dendrimer complex was compared with pure silybin. Twelve rats randomized into two groups were separately orally administered silybin and silybin–PAMAM complex. *Results*: The water solubility of silybin was significantly improved by PAMAM dendrimers of generations G1.5, G2, G2.5, and G3 with different concentrations in different pH conditions ($P < 0.05$). The in vitro release of silybin from the silybin–dendrimer complex was significantly slower compared with pure silybin ($P < 0.05$). The pharmacokinetics parameters T_{max}, C_{max}, and $AUC_{0-\infty}$ of silybin and silybin–dendrimer complex were 10 minutes, 134.2 ng/mL, 654.6 (ng·h)/mL and 15 minutes, 182.4 ng/mL, 1298.7 (ng·h)/mL, respectively. The relative oral bioavailability of silybin–dendrimer complex calculated on the basis of $AUC_{0-\infty}$ was about 178% as compared with silybin. *Conclusion*: These results indicated that PAMAM dendrimers could increase the water solubility of silybin and improve its oral bioavailability.

Key words: Bioavailability, in vitro release, PAMAM dendrimers, pharmacokinetics, silybin, silybin–PAMAM complex, solubilization

Introduction

Dendrimers is a new class of artificial macromolecular compounds, which was first synthesized by Tomalia et al. in the mid-1980s[1]. Comparing with the traditional polymers, the layer-by-layer synthesis (expressed in 'generations') of dendrimers affords molecules with controllable size, structure, and peripheral groups, which lead to well-defined molecular structure, compact globular shapes, and extremely low polydispersity, as well as its ideal aqueous solubility[2–4]. Drug can be encapsulated in the macromolecule interior or linked to the surface functional end groups of dendrimers through electrostatic interactions or covalent bonds[5–7]; thus, the solubility and bioavailability of the drug can be greatly enhanced. Moreover, dendrimers possess good biocompatibility and biodegradability. All these characteristics enable the dendrimers to become a new generation of drug carrier and solubilizer for insoluble drug.

Polyamidoamine (PAMAM) is one of the most studied dendrimers with well-defined spherical structure and nanometer scale size (Figure 1). PAMAM possesses empty internal cavities and many surface functional end groups that are responsible for high solubility and reactivity. Furthermore, PAMAM has demonstrated its potential use as a drug delivery system. A variety of molecules, such as drugs and other therapeutic agents, can be embedded in the interior void space of PAMAM or adsorbed on the cationic surface to increase their cellular uptake[8,9]. PAMAM is capable of complexing with DNA and oligonucleotides and has been proven to enhance the cytosolic and nuclear uptake of nucleic acids[10,11]. Moreover, PAMAM has been reported as a solubility enhancer of drugs[12,13] and shown the potential in controlled drug delivery[14]. In addition, increasing evidence showed an important property of PAMAM: transepithelial and endothelial transport ability, this is of great interest to pharmaceutical researchers[15].

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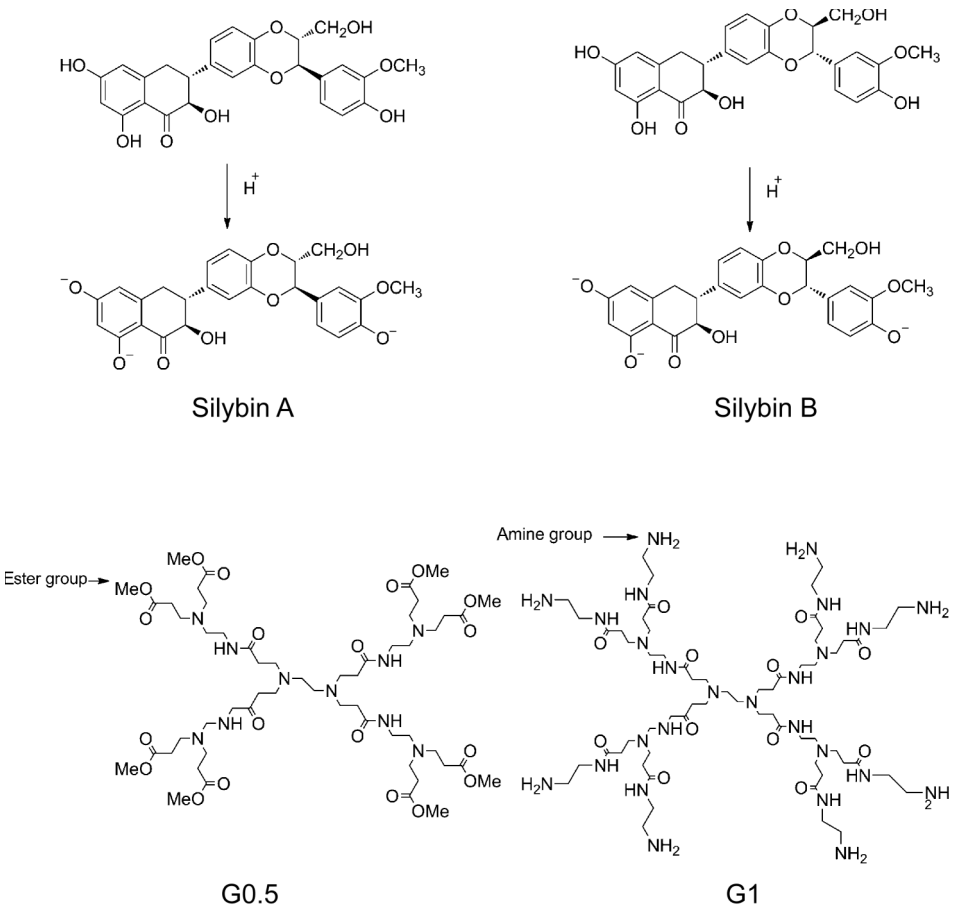

Figure 1. Molecular structures of silybin and PAMAM dendrimers with ester- (G0.5) and amine-terminated (G1) surface functional groups.

Silybin (Figure 1), derived from the milk thistle plant, *Silybum marianum*, has been used for centuries as a natural remedy in the treatment of hepatitis and cirrhosis, as well as in the protection of the liver from toxic substances[16,17]. However, the clinical use of silybin is limited by its extremely low solubility in both water and oil, and its poor absorption in the gastrointestinal tract, which result in the low bioavailability of silybin[18,19].

Over the past few years, various efforts have been made to improve the solubility and in vitro dissolution property of silybin. Because of the relatively low solubilization capability and bioavailability, the applications of solid dispersion, cyclodextrin complex, and phospholipid complex to deliver silybin are very limited. The aim of this work was to investigate the potential of PAMAM dendrimers, both amine-terminated full generation (G2 and G3) and ester-terminated half-generation (G1.5 and G2.5), as a solubility enhancer of silybin. The solubilization mechanism, the in vitro release behavior, and pharmacokinetics of silybin–PAMAM complex were investigated.

Materials and methods

Materials

PAMAM dendrimers (generation 1.5, 2.5 with $-COOCH_3$ end groups, generation 2, 3 with $-NH_2$ end groups, ethylenediamine core) were provided by East China University of Science and Technology (China). Silybin was kindly donated by Panjin Green Biological Development Co. Ltd. (Liaoning, China). 2-Naphthol was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Regenerated cellulose dialysis membrane with molecular weight cutoff (MWCO) of 3500 Da was obtained from Biosharp (St. Louis, MO, USA). Diethyl ether, acetic ether, methanol, acetonitrile, and other chemicals were all of analytical reagent grade.

Solubility studies

The water solubility of silybin was determined using the equilibrium solubility method. Excess drugs were added to 1 mL of each test solution (G1.5, G2, G2.5, and G3) to ensure the drug solution reaching saturation. The solution was mechanically shaken for 72 hours at 37°C and then centrifuged at 10,000 rpm is 1,118,000 g for 10 minutes. Aliquots of the supernatants were analyzed by a validated high-performance liquid chromatography (HPLC) method. The influence of pH on the solubility of silybin was evaluated by dropwise addition of either 1 M NaOH or 0.1 M HCl.

Incorporation of silybin in dendrimer

Silybin was dissolved in ethanol and PAMAM dendrimer was added. The sample was stirred overnight, and then

ethanol was removed by using rotary evaporator. The resultant traces were dried under vacuum to remove ethanol completely. Deionized water was added to the obtained traces. The mixture was stirred for another 12 hours to extract the silybin–PAMAM dendrimer complex and then filtered through 0.22 μm of cellulose acetate membrane filter. Finally, the filtrate was lyophilized to obtain silybin–PAMAM dendrimer complex.

In vitro release studies

In vitro release of silybin from drug–dendrimer complex was performed by dialysis method. Silybin–PAMAM complex was dissolved in deionized water at a concentration equivalent to 2 mg/mL silybin. Pure silybin was dissolved in little methanol, then diluted with more deionized water (2 mg/mL), and used as a control. Five milliliters of the samples was transferred immediately to the dialysis bags (MWCO = 3500). The bags were promptly placed in 500-mL glass beakers containing 400 mL of the dissolution medium maintained at 37°C. The outer phase was stirred continuously with a magnetic stirrer and samples (1 mL) were taken at specific time intervals followed by replenishment with 1 mL of fresh dissolution medium. The amount of drug in the samples withdrawn from the outer phase over a 12-hour period was determined by HPLC to characterize the release of silybin. The dissolution medium was simulated gastric fluid (pH 1.2), simulated intestinal fluid (pH 6.8), and deionized water, respectively.

^{1}H NMR studies

^{1}H NMR spectra of the silybin–G2 PAMAM complex with different molar ratios in 6d-dimethyl sulfoxide (DMSO) were obtained on 500.132 MHz at 298.2 ± 0.1 K (BRUKER AV-500 spectrometer, New South Wales, Germany).

Bioavailability studies

The animal experiments were conducted in full compliance with local, national, ethical, and regulatory principles for animal care. The pharmacokinetics of the silybin–G2 PAMAM complex was compared with a water suspension of silybin in rats in a randomized two-period crossover study after an oral dose equivalent to 12 mg/kg silybin. The washout period between administrations was 1 week. Twelve male rats weighing 220–250 g housed on standard laboratory diet at an ambient temperature and humidity in air-conditioned chambers were used for this study. Before the experiments the rats were fasted overnight. After administration, about 0.4 mL of blood was collected through the orbital sinus vein into heparinized tubes at the predefined times. The plasma obtained by centrifugation (10 minutes, 4000 rpm 1 788.8 g) was stored at –20°C until analysis.

Pharmacokinetic analysis was performed by the noncompartmental method, using the Kinetica4.4. C_{max} and T_{max} were observed as raw data. Area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal method. Area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/k$, where C_t and k were the last measurable concentration and the elimination constant, respectively.

HPLC analysis of silybin

The concentrations of silybin in the solubility studies, silybin–PAMAM dendrimer mixture, and in vitro release or rats' plasma were determined using a validated HPLC method. The HPLC system consisted of an isocratic pump (LC-10AT, Shimadzu, Kyoto City, Japan), with UV detector (SPD-10A, Shimadzu). The column used was a Diamonsil® C_{18} (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of acetonitrile: methanol: 0.03 M KH_2PO_4 (3:49:48, v/v/v), and pH was adjusted to 3.0 with phosphoric acid. The flow rate was 1.0 mL/min. Silybin was measured at 288 nm.

Sample extraction

For the pharmacokinetic study, 20 μL of internal standard solution (2-naphthol, 0.5 μg/mL), 50 μL of 10% acetic acid solution, 2 mL ether, and 0.3 mL acetic ether were added to 200 μL of plasma and vortexed for 1 minute. The mixture was then centrifuged at 4000 rpm is 1 788.8 g for 10 minute, then the supernatant was taken and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted with 100 μL of the mobile phase, and 60 μL of the final solution was injected in the HPLC system.

Statistics

Each experiment was repeated at least three times. Statistical data analysis was performed using Student's t-test with $P < 0.05$ as the minimal level of significance.

Results and discussions

Solubility study

The influence of dendrimer concentration on the solubility of silybin was measured at 37°C, and the results were shown in Figure 2. It was observed that the extremely low water solubility of silybin was significantly improved by PAMAM dendrimers ($P < 0.05$). The solubility of silybin in the dendrimer solutions increased in an approximately linear manner with an increase of dendrimer concentration. These results may be due to the increase in the number of surface amines and internal cavities, which could improve the interaction or encapsulation with silybin molecules. Large numbers of primary amines on PAMAM dendrimers surface could interact electrostatically with the phenolic hydroxyl groups of silybin molecules. Moreover, PAMAM dendrimers possess empty internal cavities and an open structure. The hydrophobic cavities in PAMAM dendrimers could keep hydrophobic silybin molecules inside, and the tertiary amines in these internal cavities

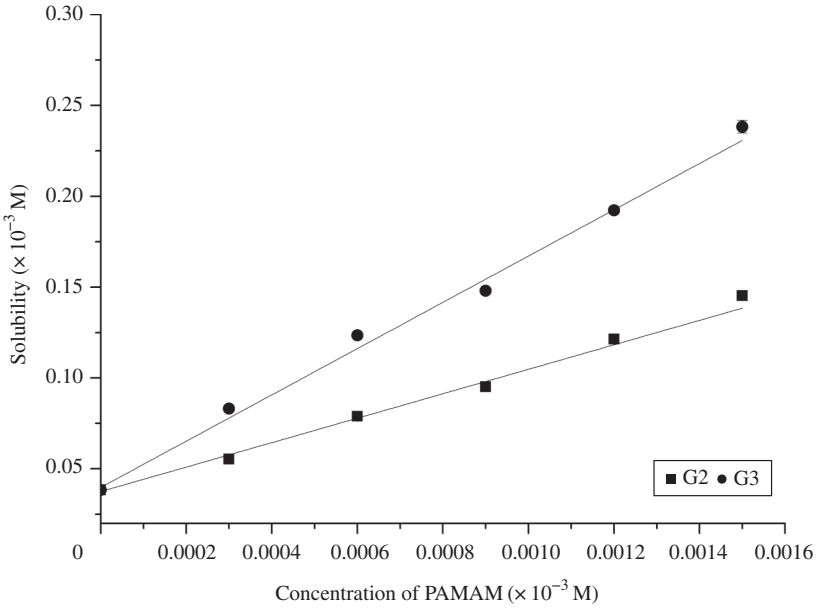

Figure 2. Solubility of silybin in the presence of increasing concentration of PAMAM dendrimers.

could interact with the atoms of the silybin molecules by hydrogen bond formation[20].

To ascertain the effect of pH of PAMAM dendrimers on the solubility of silybin, dendrimer solutions were produced at a series of pH values (pH 4.0, 7.0, 8.0, 9.0, and 10.0). The concentrations of G2 and G3 PAMAM dendrimers remain constant (the concentration of G2 and G3 is 4.6×10^{-7} M). Figure 1 shows the unionized and the ionized forms of silybin. The formation of the anion is responsible for the increase of solubility with increasing pH of the aqueous media. The solubility of silybin in PAMAM dendrimer solutions was highest at pH 10.0, with decreasing pattern at pH 9.0, 8.0, and 7.0, and the lowest at pH 4.0 (Figure 3). At acidic condition, there is no significant increase of solubility of silybin in dendrimer solution compared with that at higher pH conditions ($P > 0.05$). This may be because the silybin is unionized at pH value close to 4 and hence cannot interact electrostatically with the surface amine groups of dendrimer molecule.

Full-generation PAMAM dendrimers have primary amines on the surface and tertiary amines in their internal cavities, whereas half-generation dendrimers expose ester groups on their surface with internal tertiary amines (Figure 1). The reported p*K*a values of the primary amines (surface groups) are 7.0–9.0 and that of the

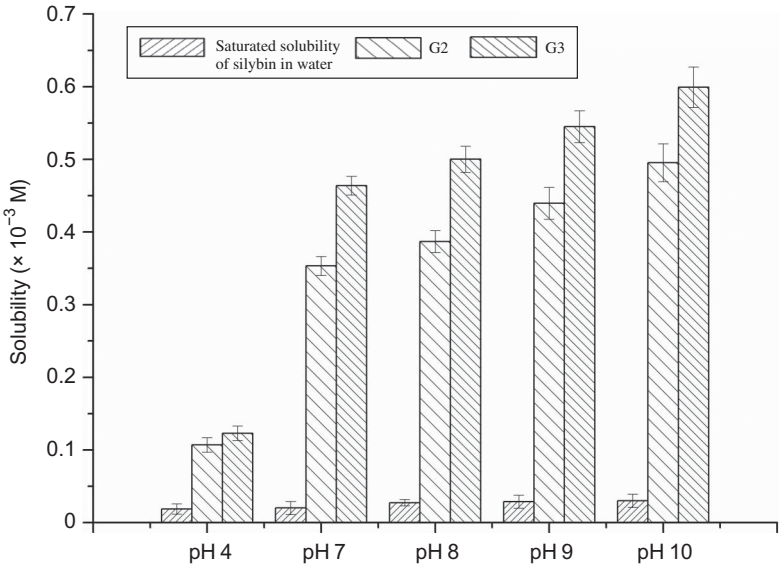

Figure 3. Solubility of silybin in the presence of different gevtnerations of PAMAM dendrimers (4.6×10^{-7} M) in different initial pH ($n = 3$).

interior tertiary amines are 3.0–6.0[21]. Therefore, positively charged moieties' value of the PAMAM could be altered by changing the pH of the solution, which in turn significantly affected the ability of the PAMAM dendrimer to interact with silybin ($P < 0.05$). Silybin, a weak acid with three ionizable phenolic hydroxyl groups, is not stable at the pH < 3 and pH > 8. The hydroxyl ionizable functional group might act as a counter ion for the dendrimer amine groups, thereby participating in the interaction between silybin and dendrimers. At pH 4.0, the solubility of silybin was not significantly increased compared with other pH ($P > 0.05$), because silybin was in the unionized form at this pH and could not interact electrostatically with the ionized dendrimer moieties. At pH 7.0–10.0 the silybin hydroxyl groups would be in their ionized form and therefore, an increase in the solubility of silybin was observed, because of the electrostatic interactions between the positively charged tertiary amines of the dendrimers and the negatively charged phenolic hydroxyl group anion of the silybin. In this pH range most of the surface amines groups and the interior tertiary amines of the full-generation PAMAMs will be positively charged. These results indicated that the electrostatic interaction between the external amines and the phenolic hydroxyl groups may be the major mechanism for the increased solubility of silybin in PAMAM dendrimers solution.

Incorporation efficacy

Drug could be either encapsulated or complexed to the dendrimer. The ability of different amounts of drug to be incorporated into dendrimer (PAMAM–G1.5–$COOCH_3$, PAMAM–G2–NH_3, PAMAM–G2.5–$COOCH_3$, and PAMAM–G3–NH_3) was studied to estimate the 'maximum' number of molecules that could be incorporated into a dendrimer molecule. The estimated number of silybin incorporated into dendrimers is summarized in Table 1. The initial molar ratios of silybin to PAMAM–G2–NH_2 were 10, 25, and 40. When molar ratios were greater than 20, only 20 molars of silybin could be incorporated. Similarly for PAMAM–G3–NH_2, when molar ratio was greater than 32, it can be seen that only 32 molecules of silybin were incorporated. In the case of half-generations, despite the increase in molar ratio, there was hardly any change in the amount of silybin in PAMAM–G1.5–$COOCH_3$ and PAMAM–G2.5–$COOCH_3$.

The number of silybin incorporated into PAMAM–G2–NH_2 and PAMAM–G3–NH_2 were 20 and 32, respectively. These values are consistent with the number of free amine groups present in PAMAM–G2–NH_2 (14) and PAMAM–G3–NH_2 (30). As discussed in previous studies, the solubility enhancement of silybin may be due to (a) electrostatic interaction between the surface amine groups of dendrimer molecule and the hydroxyl groups of silybin, (b) hydrophobic and open cavities in PAMAM dendrimers, and (c) hydrogen bond formations between tertiary amines in internal cavities of dendrimers and the atoms of guest molecules. Compared with the half-generations, full generations have extra electrostatical attachment between the surface amine groups and the opposite phenolic hydroxyl groups of silybin. Because of the serious steric hindrance on the surface of PAMAM dendrimers, the hydrophobic cavities in PAMAM could only occupy a few number of silybin molecules. This could well explain the differences between full generations and half-generations.

Table 1. The number of silybin molecules per PAMAM dendrimer molecule estimated incorporated ($n = 5$).

Generation	The number of silybin molecules
G1.5	4
G2	20
G2.5	6
G3	32

In vitro release studies

The strength and stability of the drug–dendrimer complex were investigated using in vitro release studies in simulated gastric fluid (pH 1.2), simulated intestinal fluid (pH 6.8), and deionized water, respectively. Because the external electrostatic interaction was found to be the major mechanism for drug complexation by PAMAM dendrimers, we can expect that the strength of electrostatic interaction determines the drug release behavior from dendrimers. The release of silybin from dendritic matrixes should be faster in lower pH conditions. As it can be seen from Figure 4, the lower the pH values the faster the release rate of silybin. This is due to the availability of positively charged proton to interact with the phenolic hydroxyl group of silybin molecules, which reduces the electrostatic interactions between the amine groups of dendrimers and the hydroxyl groups of the drug, thereby increasing the release rate of silybin from dendrimers. Alternatively, the positively charged ternary amine groups of dendrimers, which increase the polarity of the interior cavities of dendrimers, would contribute to the distinct release behavior of silybin in different pH conditions. The differences of drug release rate in different dissolution media can be correlated with a combination effect of the ionization state of the drug and the PAMAM dendrimers. These results strongly suggested that electrostatic interaction might play an important role in release of drugs from dendritic matrixes.

^{1}H NMR studies

NMR spectroscopy is one of the most powerful and precise techniques to investigate the structure of complexes and information on the formation of aggregates, ion pairing, encapsulation, and size variations at both molecular and atomic levels. Previous NMR studies have given useful information about the binding of different types of guests to dendrimers. Meijer et al. introduced methodology to get

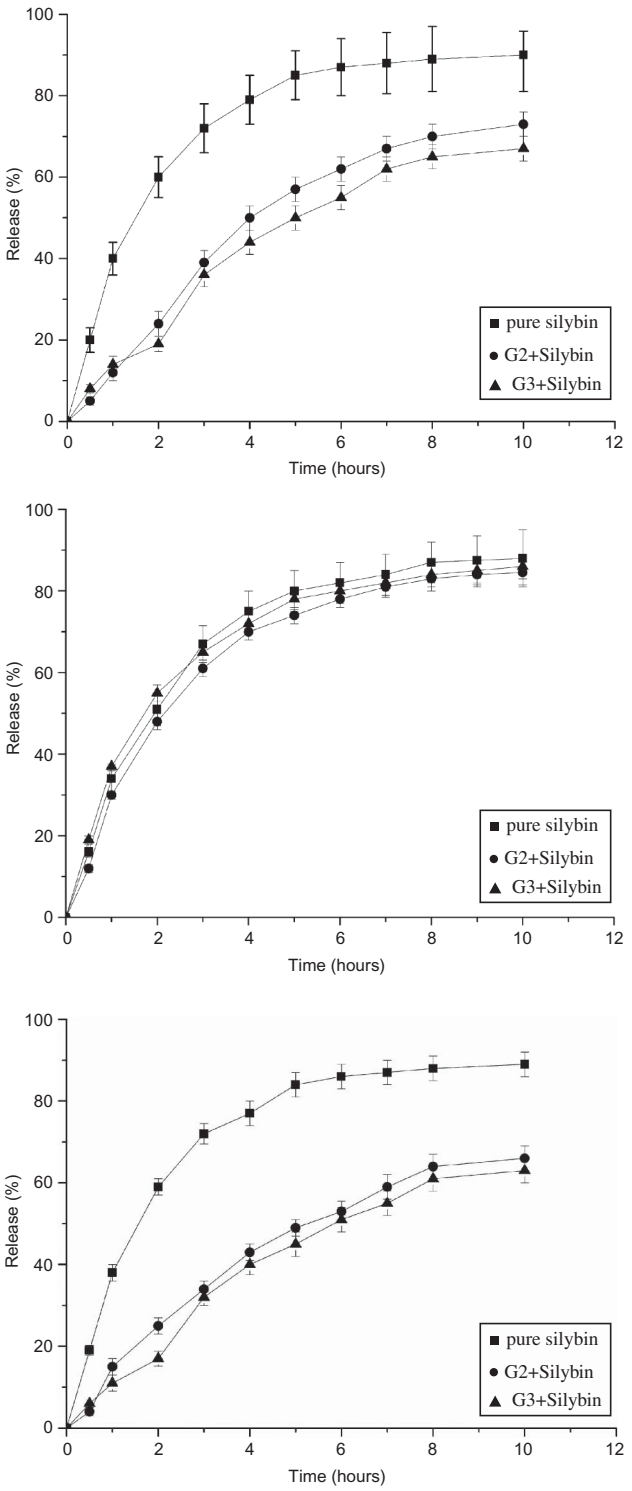

Figure 4. In vitro release of silybin from silybin–PAMAM dendrimer complex compared with the diffusion of a silybin solution: (a) simulated gastric fluid, pH 1.2; (b) simulated intestinal fluid, pH 6.8; (c) deionized water ($n = 3$).

insight into the binding of carboxylic and phosphonic acid-type guest molecules to dendrimers by ^{13}C NMR and ^{31}P NMR[22]. Astruc et al. investigated the interactions of cationic guests (acetylcholine, benzyltriethylammonium, and dopamine) with carboxylate-terminated anionic dendrimers by ^{1}H NMR[23]. The numbers of binding sites and the binding strength between dendrimers and guests were obtained in these studies by different quantitative analysis methods. Recently, Cheng's group investigated the host–guest chemistry of dendrimer-based drug formulations by 1D (^{1}H and ^{13}C) and 2D NMR (nuclear Overhauser enhancement spectroscopy) studies[24,25]. The following conclusions were made based on these studies: (1) higher generation dendrimers are more capable of encapsulating guests in their interior than lower ones, whereas lower generation dendrimers are much easier for electrostatic attachment of guests on the surface than higher ones; and (2) electrostatic interactions contributes much more to the solubility enhancement of poorly soluble drugs than hydrophobic interactions and hydrogen bond interactions.

The ^{1}H NMR spectrum of a PAMAM dendrimer has five kinds of 1 H peaks corresponding to the four methylene protons in the interior of the dendrimer (a, b, c, and d; δH 2.24 ppm for a, δH 2.43 ppm for b, δH 2.67 ppm for c, and δH 3. ppm for d, respectively; Figure 5a) and two methylene protons in the outermost layer of dendrimer (b′ and d′; Figure 5b). However, the peaks for protons d and d′ are overlapped for the G2 dendrimer in DMSO, and the chemical shift of proton b′ is similar with that of proton c. Therefore, only four broad 1 H peaks are observed in the ^{1}H NMR spectrum of a G2 dendrimer in DMSO.

^{1}H NMR spectra of the silybin–G2 PAMAM complexes with different molar ratios are presented in Figure 6. It was observed that significant changes in the chemical shifts of methylene protons of the G2 dendrimer were obviously changed with the addition of silybin. On account of the electrostatic interaction between the amine functional groups of the dendrimer and the phenolic hydroxyl groups of silybin, the outermost layer of the G2 dendrimer exhibits a downfield chemical shift. In comparison, the downfield shift of the interior methylene protons may be due to the following interaction mechanisms: the interior pockets of the PAMAM dendrimer can encapsulate silybin molecules by hydrophobic interactions or act as hydrogen bond donors/receptors.

Bioavailability study

The oral bioavailability of silybin from silybin–G2 PAMAM complex was assessed in rats and compared to that of silybin suspension. Figure 7 shows the mean silybin plasma concentration versus time plots of the two silybin formulations. The results indicated that silybin suspension was rapidly absorbed through the rat gastrointestinal tract with a C_{max} of 134.2 ng/mL at a T_{max} of 10 minutes. The administration of silybin–G2 PAMAM mixture achieved a C_{max} of 182.4 ng/mL at a T_{max} of 15 minutes, and the whole blood concentration of silybin declined more slowly than that following suspension of silybin.

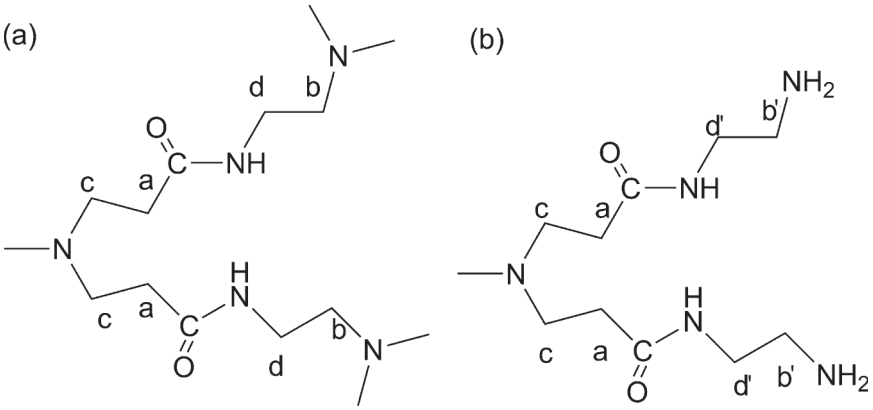

Figure 5. Chemical structure and atom labeling in the (a) G2 dendrimer interior repetitive unit; (b) G2 dendrimer outermost layer.

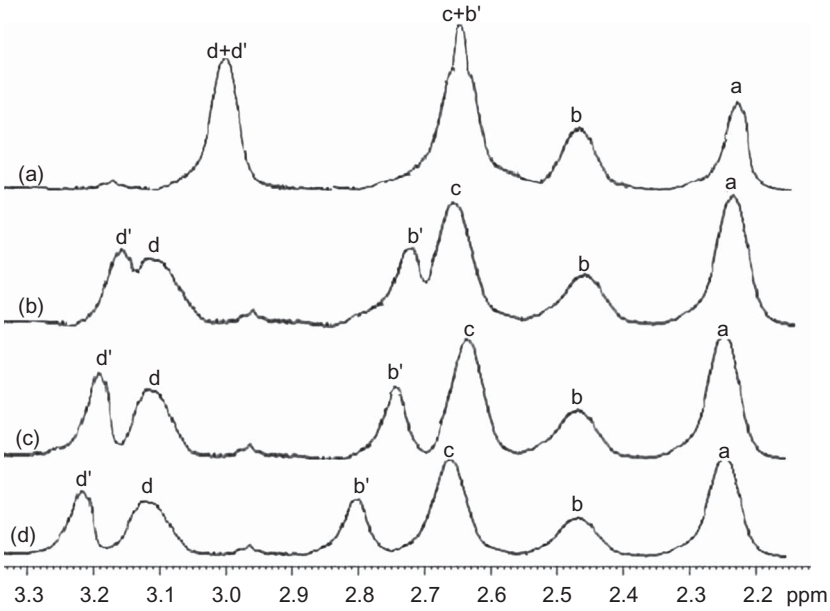

Figure 6. The ^{1}H NMR spectra of G2 and G2 dendrimer/silybin/6d-DMSO solutions at different molar ratios. The concentration of the G2 dendrimer was kept constant at 6.94×10^{-5} M. (a) the G2 dendrimer + 15 equiv of silybin; (b) the G2 dendrimer + 30 equiv of silybin; (c) the G2 dendrimer + 45 equiv of silybin; (d) the G2 dendrimer + 60 equiv of silybin.

The pharmacokinetic parameters obtained using the noncompartmental method is given in Table 2. A noncompartmental model can be employed to fit the experimental data of both silybin–G2 PAMAM complex and suspension of silybin with regression coefficients of 0.9747 and 0.9901, respectively. Calculated on the basis of the $AUC_{0-\infty}$ of each formulation, the oral bioavailability of silybin–G2 PAMAM mixture was about 178% as compared with that of silybin suspension.

Cytotoxicity of PAMAM dendrimers increases with the generation for cationic dendrimers[26], and the cytotoxicity of PAMAM G3 is reported to be low[27]. Weilun and colleagues approved that PAMAM at high concentration (>100 mM) caused a significant decrease in cell viability. This result indicated that the cytotoxicity of PAMAM is minimal at the concentration used in this study.

The results of pharmacokinetic parameters and oral bioavailability data demonstrated that drug–dendrimer complex could improve the oral absorption of silybin. Previous studies by Yulian and colleagues confirmed that PAMAM dendrimers at lower concentrations might be potential and safe absorption enhancers for improving absorption of poorly absorbable drugs from the small intestine[28]. However, the mechanisms whereby PAMAM dendrimers increase the small intestinal absorption of drugs are not fully understood. It has been suggested that PAMAM dendrimers decreased the transepithelial electrical resistance value by loosening the tight junction of Caco-2 cells[29]. The opening of tight

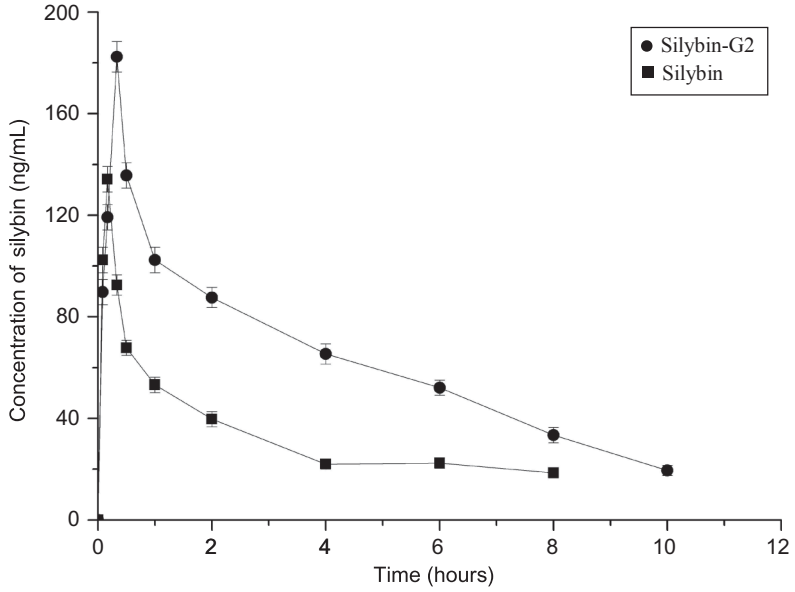

Figure 7. Rat plasma silybin concentrations versus time plot after a single oral dose of 12 mg/kg equivalent silybin–dendrimer complex or silybin ($n = 12$).

Table 2. Pharmacokinetic parameters after oral administration of silybin–dendrimer complex and suspensions of silybin calculated by a noncompartmental method ($n = 12$).

Formulation	T_{max} (minute)	C_{max} (ng/mL)	AUC_{0-t} [(ng h)/mL]	$AUC_{0-\infty}$ [(ng h)/mL)]	MRT [a] (hour)	Relative bioavailability (%)
Silybin–dendrimer complex	15 ± 0.8	182.4 ± 37.3	959.02 ± 136.4*	1298.7 ± 206.4*	3.76 ± 1.08*	178.2 ± 26.78*
Suspension of silybin	10 ± 0.7	134.2 ± 17.6	583.19 ± 98.5	654.6 ± 120.7	2.09 ± 0.76	100

*$P < 0.05$ versus suspension of silybin with the silybin–dendrimer complex; [a]mean residence time.

junction in the epithelium may increase the transport of drugs through a paracellular route. Furthermore, it has been reported that cationic PAMAM dendrimers are transported through a combination of the paracellular pathway and adsorptive endocytosis[30]. Low molecular-weight drugs may be encapsulated into the core of PAMAM dendrimers or interact with the surface group of PAMAM dendrimers. Therefore, the adsorptive endocytosis of the conjugation with PAMAM dendrimers may also contribute to the absorption enhancement effects of drug–PAMAM dendrimers complex.

It seems that multiple mechanisms, rather than a single mechanism – including paracellular transportation of drug–dendrimers complex across epithelium, enhanced contact with epithelium, and enhanced absorption through the adsorptive endocytosis process – may contribute to the enhanced oral bioavailability of silybin by PAMAM dendrimers.

Conclusion

In conclusion, the solubility of silybin was greatly enhanced in the presence of PAMAM dendrimer. The amine-terminated PAMAM–G2–NH_2 and PAMAM–G3–NH_2 may predominantly form a complex with the phenolic hydroxyl group from the silybin because of the electrostatic interaction, as shown by ^{1}H NMR. Complex of silybin with dendrimers led to sustained release of the drug in vitro and improved bioavailability in vivo. Although dendrimer drug delivery is in its infancy, it offers several attractive features, such as its easily controllable size, shape, branching length, and surface functionality that allows us to modify the dendrimers as per the requirements, and make this compound ideal carrier in many of the applications.

Declaration of interest

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