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Encapsulation of curcumin within poly(amidoamine) dendrimers for delivery to cancer cells

Lu Wang · Xiaoping Xu · Yi Zhang · Yuanqing Zhang · Ying Zhu · Jiye Shi · Yanhong Sun · Qing Huang

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Abstract Curcumin has anti-proliferative and pro-apoptotic properties against a variety of cancer cells in vitro. Unfortunately, the water-insolubility and instability leads to its low bioavailability in vivo tests. Here, we report a general approach to using poly(amidoamine) dendrimer with acetyl terminal groups to encapsulate curcumin (G_5-Ac/Cur) for drug delivery to cancer cells. The solubility, release kinetics, anticancer activity, and apoptotic-related protein expression (Bax and Bcl-2) were investigated in detail. Comparing with curcumin, the water-solubility value of G_5-Ac/Cur increased 200-fold, and the release of curcumin from the complexes was in a sustained manner. G_5-Ac/Cur showed higher anti-proliferative activity against A549 cell lines and had the better effect on the generation of intracellular reactive oxygen species, the mitochondrial membrane potential and cell apoptosis. Furthermore, the ratio of Bax/Bcl-2 was higher in samples treated with G_5-Ac/Cur. The results indicated that the G_5-Ac drug delivery system could improve the solubility and anti-cancer effect of curcumin.

Lu Wang and Xiaoping Xu contributed equally to this paper.

L. Wang · X. Xu · Y. Zhang · Y. Zhang · Y. Zhu · J. Shi · Y. Sun (✉) · Q. Huang
Laboratory of Physical Biology, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, 2019 BaoJia Road, Shanghai 201800, China
e-mail: sunyanhong@sinap.ac.cn

L. Wang · X. Xu · Y. Zhang · Y. Zhang
University of Chinese Academy of Sciences, Beijing 100049, China

J. Shi
UCB Pharma, 208 Bath Road, Slough SL1 3WE, UK

1 Introduction

Curcumin,1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione, is a natural and crystalline compound isolated from the plant *Curcuma longa*. This compound has gained wide public attention due to its growth inhibition and apoptosis induction in a variety of cancer cells lines [1–3]. On the other hand, curcumin has been proved to be pharmacologically safe even at very high doses in many clinical studies. Recently, it has emerged as one of the most potent chemo-preventive and chemotherapeutic agents [4, 5]. However, curcumin is generally found inactive in clinical trials primarily due to its water-insolubility and instability [6, 7]. Specifically, it degrades quickly in neutral and alkaline conditions with a half life ($t^1/_2$) less than 10 min in PBS at pH 7.2. Curcumin is proved to be hydrophobic under acidic conditions, resulting in extremely low bioavailability in both vascular and oral administration [8]. The aqueous insolubility and instability prevent curcumin from successful anticancer applications [9].

Nano drug delivery systems has become a promising approach to overcome these disadvantages of curcumin. As a drug delivery system, poly(amidoamine) (PAMAM) dendrimer can be easily modified and conjugated with a variety of interesting guest molecules such as targeting molecules and drugs [10–12] with desired water solubility and biocompatibility. In addition, the relatively hydrophobic interior of the dendrimers allows for physical encapsulation or complexation of hydrophobic cancer drugs to improve the water solubility and bioavailability of theses drugs. In this present study, we utilized acetyl-modified generation 5(G_5) PAMAM dendrimers to encapsulate curcumin. The encapsulation efficiency, release kinetics, the anti-proliferative and pro-apoptotic protein expression (Bax and Bcl-2) were investigated in detail.

Compared to other strategies of curcumin analogues syntheses [13–15], this approach is simpler and the solubility and the bioavailability of curcumin were improved significantly.

2 Materials and methods

2.1 Chemicals and reagents

Curcumin, dendrimer PAMAM generation 5 (G_5), triethylamine, acetic anhydride, were purchased from Aldrich Co., Ltd. The surface group of G_5 is –NH2, and the number average molecular weight of PAMAM were found to be 28824 g/mol. Annexin V-APC and 7-AAD were purchased from BD company. Fetal bovine serum (FBS), RPMI-1640, and penicillin–streptomycin were obtained from Gibco-BRL (Gaithersburg, MD, USA). Bcl-2 and Bax antibodies were purchased from Epitomcis. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and curcumin were obtained from Sigma-Aldrich (St.Louis, MO, USA).

2.2 Synthesis

2.2.1 Partial acetylation of generation 5 PAMAM dendrimer (G_5-Ac)

Partially acetylated PAMAM dendrimer generation 5 (G_5-Ac) was performed according to previously reported procedure [16]. Briefly, acetic anhydride (70 %ratio of primary amine numbers of G_5PAMAM dendrimer, 14.65 μL) was slowly added to the dendrimer G_5 solution (50 mg, 1.73 μmol dendrimer G_5 dissolved in 6 mL methanol) in the presence of triethylamine (1.25 equivalent of acetic anhydride, 27.00 μL). The mixture was stirred under N_2 atmosphere at room temperature. After 18 h, methanol was evaporated on a rotary evaporator. The residue was dissolved in water and dialyzed (using cellulose membrane with 3500 MWCO) against PBS buffer and double distilled water for 3 days. The obtained sample G_5-Ac was lyophilized and stored in a dry place before further modification and characterization. Yield: 94.7 %.

2.2.2 Encapsulation of curcumin within G_5-Ac

Two mL curcumin in methanol (1 mg/mL) was added into 2 mL G_5-Ac dendrimers solution (2 mg/mL), and then mixed. The mixture solution was vigorously stirred for 2 days to allow the evaporation of methanol solvent. The dendrimer/curcumin mixture solution was centrifuged (10000 rpm for 10 min) to remove the precipitates related to non-complexed free curcumin, which is insoluble in water. The precipitate was collected and dissolved in 1 mL methanol for UV–Vis spectroscopy analysis. The supernatant was stored at 4 °C, protected from light.

2.2.3 In vitro release of curcumin from G_5-Ac/Cur

Five mL solution of G_5-Ac/Cur complex in water or pure curcumin in ethanol was placed in a dialysis bag with MWCO of 10,000, hermetically tied, and suspended into 45 mL of aqueous medium, which was PBS buffer (pH 7.4) or acetate buffer (pH 5.0). The entire system was kept in a vapor-bathing constant temperature vibrator at 37 °C with gentle agitation. Then, 3.0 mL of the dissolution medium was taken out at intervals of 0.5, 1, 2.5, 3, 3.5, 4, 4.5, 5, 7, 10, 12, 24 h and replaced with the same volume of fresh dialysis medium. The curcumin content of samples was measured by HPLC.

2.3 Biology assays

2.3.1 Cell culture and cytotoxicity assay

Human lung adenocarcinoma A549 cells obtained from Shanghai Cell Bank (Shanghai, China) were grown in PRIM-1640 (Gibco, Invitrogen, UK)) with 10 % heat-inactivated FBS (Gibco, Invitrogen, UK) and antibiotics (100 g/mL of streptomycin and 100 g/mL of penicillin) at 37 °C in 5 % CO_2.

The effects of curcumin and G_5-Ac/Cur on A549 cells proliferation and cytotoxicity were measured using MTT assay. In brief, A549 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. The medium in each well was replaced with 100 μL of culture medium containing different concentration of treatments (5, 10, 20 μM) and cultured for another 24 h. The incubation medium was then replaced with 100 μL of fresh medium and 10 μL MTT reagent each well. After 4 h, 100 μL of SDS was added to each well and incubated overnight at 37 °C in the dark until all the crystals dissolved. The absorbance was measured at 570 nm using a microplate reader (Bio-rad model 550). Cell viability is expressed as a percentage of control.

2.3.2 Cellular uptake of curcumin and G_5-Ac/Cur

A549 cells were plated into 6-well plates at 2×10^5 cells per well in 2 mL of RPML-1640 medium containing 10 % FBS. After incubating for 24 h at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2, the medium was replaced with 2 mL of fresh medium containing 5 μM curcumin and 5 μM G_5-Ac/Cur respectively and cultured for 2 h. The medium was then removed and washed twice

with PBS and replaced with 2 mL fresh medium. ZEISS fluorescence microscope was used to observe the fluorescence at 530 nm excited at 425 nm and expressed as green.

2.3.3 Measurement of the change of mitochondrial membrane

The cells were treated with or without 5 μM curcumin and 5 μM G_5-Ac/Cur for 24 h in 6-well and then the medium was discarded. Twenty minutes before the termination of incubation, JC-1 dye (final concentration 10 μM) was added to the media and the samples were incubated in a CO_2 incubator for 20 min at 37 °C in the dark. Then, remove the supernatant and wash the cells with JC-1 staining buffer twice. Finally, after adding another 2 mL fresh medium, the staining solution was removed by centrifugation. Mitochondrial membrane potential was quantified using fluorescent microscope.

2.3.4 Measurement of intracellular reactive oxygen species (ROS) levels

Generation of intracellular ROS was detected by using oxidative-sensitive fluorescent probe 2, 7-dichlorodihydrofluoroscein diacetate ($DCFH_2$-DA). $DCFH_2$-DA is able to diffuse into cells readily, where it is hydrolysed enzymatically by intracellular esterases to form non-fluorescent $DCFH_2$. Following ROS generation, $DCFH_2$ is oxidized and transferred into highly fluorescent 2, 7-dichlorofluoroscein (DCF). Consequently, the intracellular ROS generation can be measured by $DCFH_2$-DA. Briefly, after treatment with curcumin or G_5-Ac/Cur, the cells were washed twice with PBS and then loaded with 10 μM $DCFH_2$-DA in serum free medium at 37 °C for 30 min. The cells were again washed twice with PBS and centrifuged at 1000 rpm to remove the extracellular $DCFH_2$-DA. The trapped fluorescent probe (DCF) inside the cells was used to detect intracellular ROS with an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a HITACHI-4500 spectrofluorometer.

2.3.5 Cell apoptosis measured by flow cytometry

Cell samples are divided into 24 well plates and the number of cells in each treatment group is not less than 100,000. The staining dyes are Annexin V-APC and 7-AAD. We set the cell samples as untreated, positive control (Annexin V-APC single dye, 7-AAD single dye and double dyed) and samples. The SSC, FSC and gate setting were selected according to untreated cells. The positive treated cells which were stained with single dye were used to adjust light compensation. The positive treated cells which were stained with double dyes were used to divide the state of the selected cells.

2.3.6 Western blot analysis

Cell lysate preparation and western blots were performed as described earlier using primary antibodies (Bcl-2, 1:1000; Bax, 1:1000 and GAPDH, 1:10000) and peroxidase-conjugated goat anti-rabbit secondary antibodies. The immunoreactive proteins were detected using an ECL Western Blotting Detection system (Millipore, USA). Bands obtained were densitometrically analyzed using Image J software (NIH, USA) and expressed as densitometric units in gray values.

2.3.7 Statistical analyses

The data are presented as the mean ± SD value. Statistical significance was calculated using the t test for paired samples. $P < 0.05$ was regarded as significant and $P < 0.01$ as highly significant.

3 Results

3.1 Acetylation of generation 5 PAMAM dendrimer (G_5-Ac)

The degree of acetylation was measured by ^{1}H NMR. ^{1}H NMR spectrum of the acetylated dendrimer showed the proton signal at d 2.44 ppm, which corresponded to the methylene protons of –NH–CH2– in dendrimer PAMAM G_5. The specific signal at d 1.99 ppm corresponded to the methyl protons of introduced acetyl groups. The integration ratio of these two kinds of proton signals in the acetylated dendrimer suggested that an average of 89 acetyl groups is present on the surface of each G_5 PAMAM dendrimer (Ac89-G_5).

3.2 Encapsulation of curcumin within G_5-Ac(G_5-Ac/Cur)

Curcumin shows very low water solubility (solubility of curcumin in water was determined to about 0.6 μg/mL [17]). Curcumin's full pharmacological potential is limited owing to its water solubility. Using G_5-Ac/Curcumin complexes as a formulation could overcome the water-insolubility and improve the bioavailability of this drug. The solubility of the complexes was 125 ± 5.8 μg/mL, which increased about 200-folds compared to curcumin. The formed G_5-Ac/Cur complexes were characterized with fluorescence spectroscopy (Fig. 1). Curcumin was excited with 425 nm and the emission wavelength was 530 nm.

After encapsulation with G_5-Ac, the G_5-Ac/Cur complexes have a peak at the same emission wavelength as curcumin. This indicates that curcumin has been successfully encapsulated into the G_5-Ac dendrimers. At pH 7.4, surface potential of G_5-Ac is 9.89 ± 3.12 mV and the values of G_5-Ac-Cur complex is 1.67 ± 0.79 mV. The changes of the surface potential also demonstrated that the curcumin was successfully encapsulated into the G_5-Ac.

3.3 In vitro release of curcumin from G_5-Ac/Cur

The release of curcumin from the complexes showed that the drug was released in a sustained manner (Fig. 2). In contrast, the pure curcumin drug dissolved in ethanol was quickly released to the outer phase of the dialysis bag in PBS buffer. About 70 % was released within just 5 h. The prolonged release profile of curcumin from the complexes under both different buffer conditions implied that PAMAM was extremely important for effective encapsulation and retention of curcumin. The release rate of curcumin from the complexes under pH 5.0 was higher than that under pH 7.4. This is helpful to release the curcumin from the complexes in the tumor region (pH is lower than 7.0).

3.4 In vitro cytotoxicity and cellular uptake of G_5-Ac/Cur to A549 cell lines

We studied the cytotoxicity of curcumin and G_5-Ac/Cur against A549 cell lines by using standard MTT assay. Comparable inhibition of cell proliferation was observed for free and encapsulated curcumin at different concentration (5, 10, 20 μM). Curcumin was used as a control by dissolving it in DMSO at a concentration of 10 mmol/L,

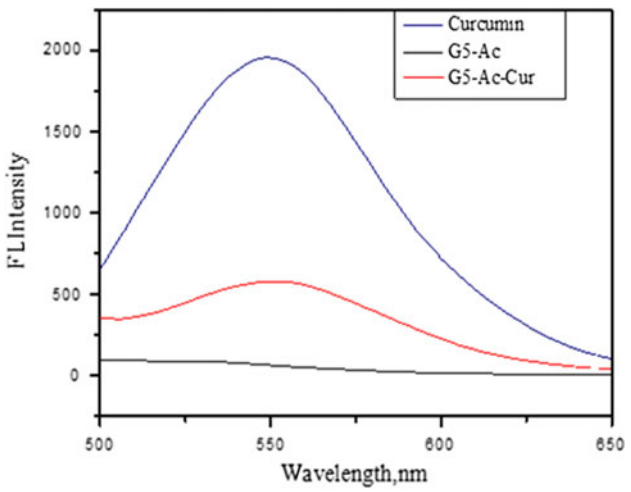

Fig. 1 Fluorescence emission spectrum of curcumin and G_5-Ac/Cur

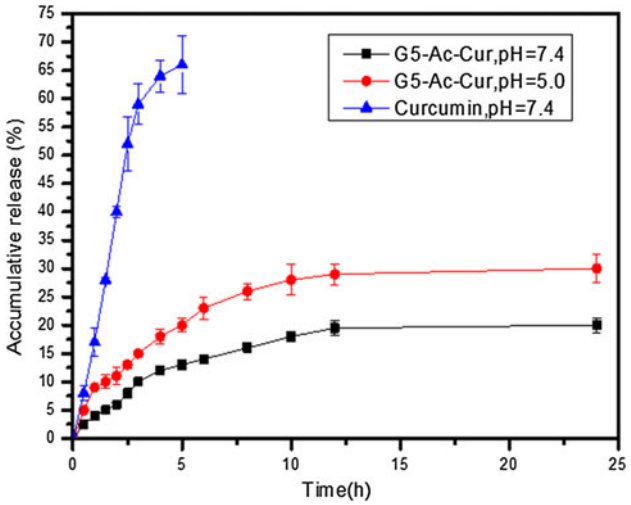

Fig. 2 The release of curcumin from G_5-Ac/Cur complex in PBS buffer (pH 7.4 and pH 5.0)

followed by dilution in medium to the needed concentration (final DMSO concentration is 0.1 %). The results are shown in Fig. 3. Evidently, G_5-Ac/Cur complexes had higher anti-proliferative activity than curcumin itself at each concentration. A significant difference was found between curcumin and G_5-Ac/Cur even at a concentration of 5 μM ($P < 0.01$). Thereby, 5 μM Curcumin and 5 μM G_5-Ac/Cur were selected for further analysis.

Cellular uptake of G_5-Ac/Cur was initially detected using fluorescence microscope. Curcumin had a green fluorescence and thus the fluorescence was used to monitor the intracellular curcumin and G_5-Ac/Cur complex. Result is shown in Fig. 4, the image of A549 cells treated with G_5-Ac/Cur (5 μM) for 2 h showed a stronger green fluorescence than that of curcumin (5 μM). Thereby, G_5 PAMAM increased curcumin's ability to enter the cancer cells, and this will strengthen the lethality of curcumin on cancer cells.

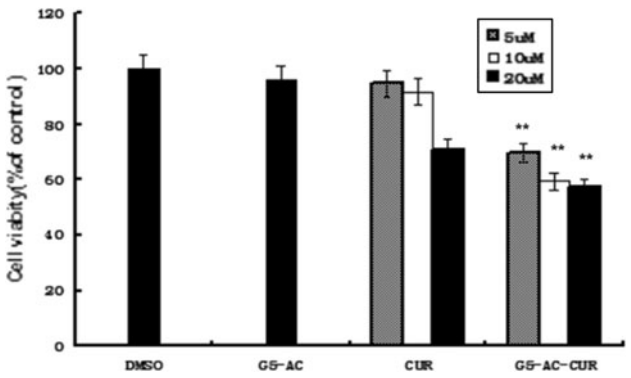

Fig. 3 The effect of curcumin and G_5-Ac/Cur on cell proliferation. The cells were incubated for 24 h. The data are expressed as mean ± SD. $^{**}P < 0.01$

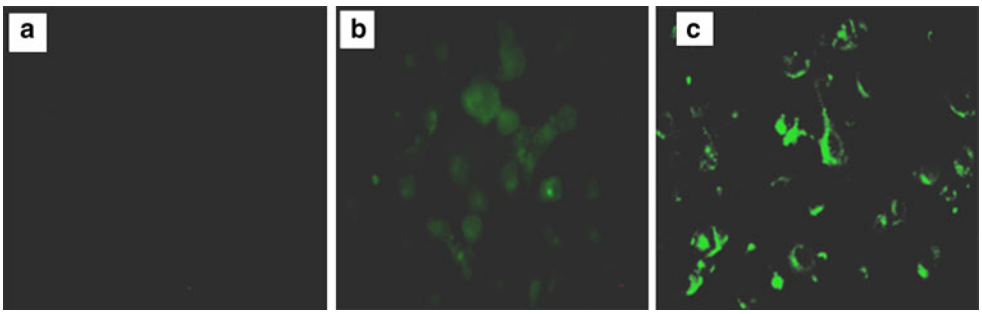

Fig. 4 Images of A549 cells as visualized under fluorescent microscope. **a** Untreated. **b** Curcumin. **c** G_5-Ac/Cur. Cells were incubated with 5 μM curcuminand G_5-Ac/Cur for 2 h and the fluorescence levels were determined by a fluorescence microscope

3.5 Effects of G_5-Ac/Cur on mitochondrial membrane potential and generation of ROS

Using JC-1 staining, changes in mitochondrial transmembrane potential were monitored by fluorescence microscopy. Figure 5 showed representative JC-1 fluorescence in both red and green. A red fluorescence was predominant in untreated cells, suggesting that JC-1 existed in the aggregated form in mitochondrial membranes at resting potential. By contrast, following the treatment with 5 μM curcumin and G_5-Ac/Cur respectively, the cells tended to reveal a green fluorescence, indicating the existence of free JC-1 at the depolarized mitochondrial membrane potential. Moreover, the green fluorescence induced by G_5-Ac/Cur was almost as strong as positive control treated with 400 uM H_20_2, meaning that G_5-Ac/Cur has much more capacity in regard of the depolarization of mitochondrial membranes than curcumin.

The production of intracellular ROS was measured by DCFDA stains using spectrofluorometer. These results (Fig. 6) indicated that curcumin could induce the generation of ROS, and the encapsulated curcumin complexes revealed stronger ability than free curcumin.

3.6 G_5-Ac/Cur induced apoptosis of A549 cell lines

The pro-apoptotic activity of curcumin and G_5-Ac/Cur in A549 cells lines was evaluated using Annexin V-APC/7-AAD assay by flow cytometry. H_2O_2 (400 μM) was used as a positive control. After 24 h treatment with curcumin and G_5-Ac/Cur at the concentration of 5 μM, the proportion of AnnexinV-APC positive cells was detected. Apoptosis represents the percentage of double-positive thymocytes positive for Annexin V staining, as is shown in Fig. 7. At the concentration of 5 μM, the percentage of apoptotic

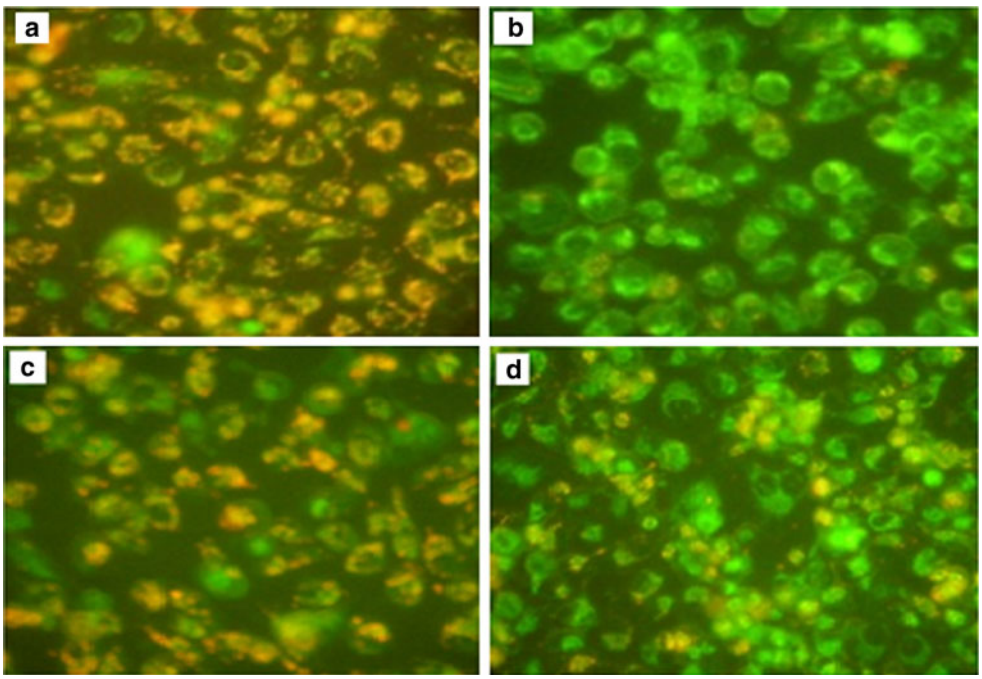

Fig. 5 The effect of curcumin and G_5-Ac/Cur on mitochondrial membrane potential (MMP). **a** Untreated. **b** Positive. **c** Curcumin. **d** G_5-Ac/Cur

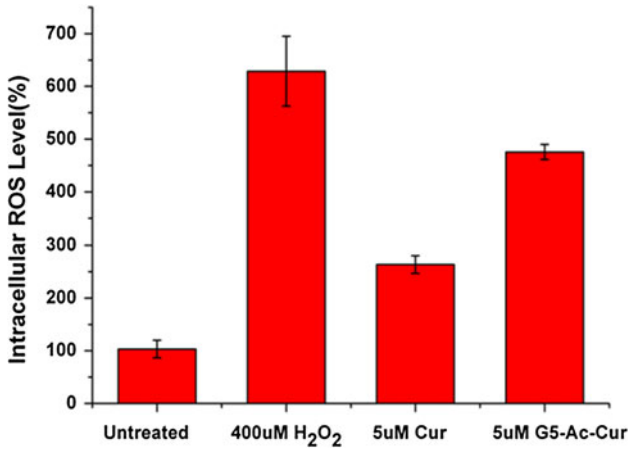

Fig. 6 The effect of curcumin and G_5-Ac/Cur on reactive oxygen species (ROS) generation. A549 cells were treated by different samples for 24 h. The data are expressed as mean ± SD

cells was 66.1 % for the treatment with G_5-Ac/Cur, whereas in the case of curcumin only it was 47.86 %.

3.7 Effects of G_5-Ac/Cur on the expression of Bcl-2 family members

To determine the apoptosis pathway induced by G_5-Ac/Cur in A549 cells, we examined the expression of those pro-apoptotic proteins (Bax) and anti-apoptotic proteins (Bcl-2) using western blot analysis. All the protein levels were measured by quantitative western blot analysis after normalizing with the expression of GAPDH. The results (Fig. 8) indicated that the levels of Bcl-2 decreased, while the expressions of Bax were increased, and the ratio of Bax/Bcl-2 in A549 cells treated with G_5-Ac/Cur was much higher than free curcumin.

4 Discussion

Curcumin-induced apoptosis has been demonstrated in A549 cells by accumulating evidence [18]. However, as a water-insoluble compound, curcumin is hard to be absorbed by organism [19, 20]. The present study demonstrates that PAMAM dendrimers could improve the solubility of insoluble drugs. We encapsulate the curcumin into PAMAM dendrimer with acetyl terminal groups. The solubility of G_5-Ac/Cur increased about 200 times. The sustained release effect of curcumin from dendrimers has been shown. Preliminary cell assay demonstrated the dendrimer could improve the inhibitory effect of curcumin on tumor cells. After making comparison between some typical concentration, G_5-Ac/Cur has higher anti-proliferative activity than free curcumin. The higher cytotoxicity maybe due to the water-soluble and sustained release of curcumin.

Previous studies have indicated that mitochondria play a crucial role in the process of apoptosis in various of

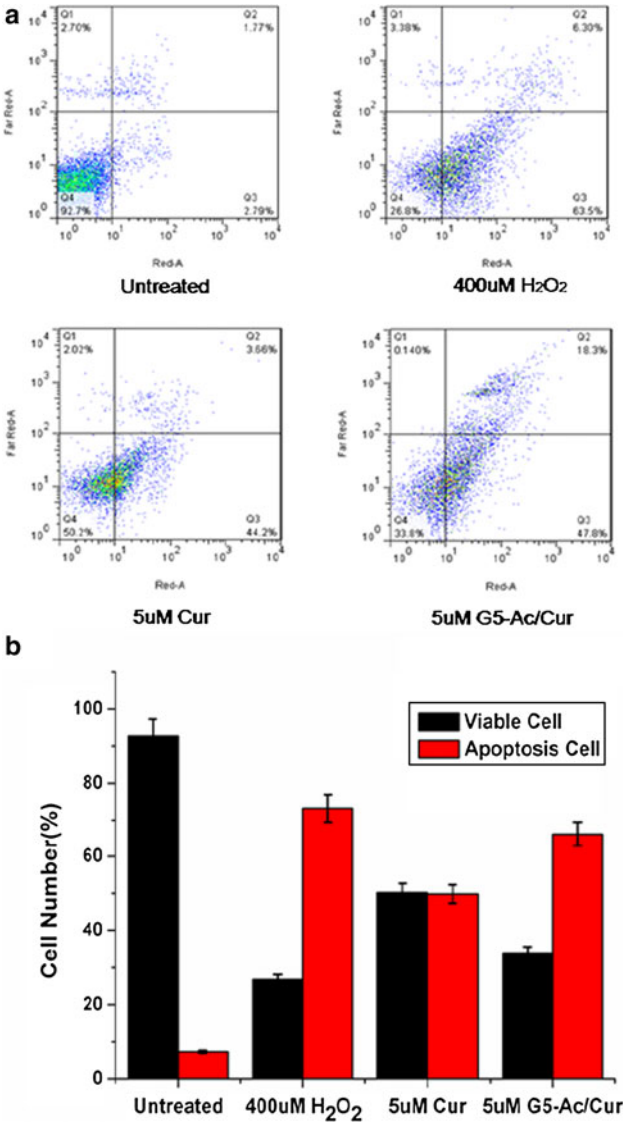

Fig. 7 Apoptosis cells detected by flow cytometry with Annexin V-APC/7-AAD. **a** The numbers shown in the lower and upper right quadrants are the percent of cells staining for apoptosis. **b** Graphic representation of results presented in **a**

mammalian cells [21]. Loss of mitochondrial membrane potential is considered as a signal at the early stage of apoptosis. Mitochondrial is a major place that generate intracellular ROS [22]. Earlier reports have suggested that curcumin could induce the loss of mitochondrial membrane potential in a dose-dependent manner and increase generation of ROS, which was described as an early event of curcumin-induced apoptosis [23, 24]. In this study, 5 μM G_5-Ac/Cur significantly reduced the mitochondrial membrane potential and increased the generation of intracellular of ROS, suggesting that the mitochondrial pathway play an important role in mediating apoptosis induced by G_5-Ac/Cur. Apoptosis is promoted by some of the Bcl-2 homologues (Bax, Bak, Bad, and $BclX_5$), whereas other members of the Bcl-2 family (Bcl-2, Bc1XL, and Bag-l) form

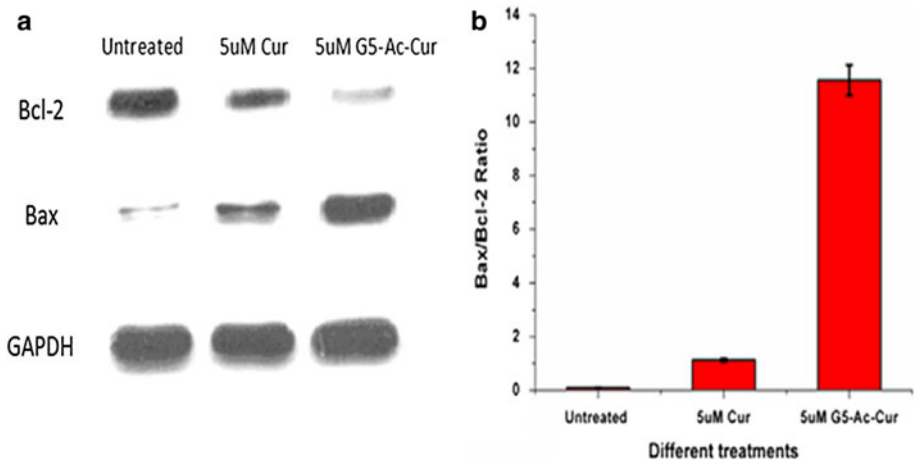

Fig. 8 The effect of curcumin and G_5-Ac/Cur on Bcl-2 and Bax protein expression. **a** Western blot was performed for Bcl-2 and Bax protein expression. Bax protein expression level in cells treated with G_5-Ac/Cur was higher than that of Cur in the same contration. While the Bcl-2 protein expression level was down-regulated. **b** The ratio of Bax/Bcl-2 in A549 cells treated with G_5-Ac/Cur was much higher than free curcumin

dimers with the apoptosis-promoters and inhibit their activity, hence suppressing apoptosis [25–27]. The ratio of Bax/Bcl-2 has been suggested to predispose cells to accelerated or suppressed apoptosis in response to external stimuli. Our data showed that the ratio of Bax/Bcl-2 increased significantly after treatment with G_5-Ac/Cur (Fig. 8), implying that G_5-Ac/Cur decreased mitochondrial membrane potential through a Bcl-2 family-mediated pathway.

5 Conclusion

In summary, PAMAM was used to encapsulate curcumin to improve its solubility and bioavailability. Results showed that the water-solubility value of G_5-Ac/Cur increased 200-fold compared to curcumin. G_5-Ac/Cur showed higher anti-proliferative activity against A549 cells lines, and had better effect on generation of intracellular ROS, reduction of mitochondrial membrane potential (MMP) and induction of cell apoptosis. Furthermore, the ratio of Bax/Bcl-2 was higher in samples treated with G_5-Ac/Cur which indicated that the G_5-Ac drug delivery system could improve the solubility and anti-cancer effect of curcumin. Compared with other strategy to synthesis curcumin analogues, this approach is simpler and the solubility and the bioavailability of curcumin were improved significantly.

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