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ORIGINAL ARTICLE

Cytotoxicity and inhibition of lipid peroxidation activity of resveratrol/cyclodextrin inclusion complexes

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Abstract Resveratrol (Res) is a plant-based polyphenol compound and is known to inhibit the growth of a variety of cancer cells and protect lipoproteins against oxidative damage. However the poor solubility and labile property may constitute a serious problem for its bioavailability. The problem could be overcome by the formation of inclusion complexes with cyclodextrins (CDs). The aim of this work is to include Res by β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-CD) to form the Res/β-CD and Res/HP-CD inclusion complexes and evaluate their cytotoxicity on cancer cells and inhibition of lipid peroxidation activity. The complexes are characterized by powder X-ray diffraction, fourier transform infrared spectroscopy and scanning electron microscopy. The cytotoxicity of the two complexes has been evaluated by methylthiazoletetrazolium reduction assay on two cancer cell lines (cervical carcinoma cells HeLa and hepatocellular liver cancer cells Hep3B) and one normal cell line (umbililical vein endothelial cell HUVEC). The results showed that the two complexes exhibit high cytotoxicity on two cancer cells, especially for Hep3B, and show no significant effect on normal cells. The Res/HP-CD complex shows higher cytotoxicity on the two cancer cells than that of the Res/β-CD complex. The inhibition of lipid peroxidation induced by Fe^{2+}/ascorbate of the two inclusion complexes has been determined by thiobarbituric acid assay. The inhibition rate shows a linear increase with the increase of CDs concentration, and the Res/HP-CD complex shows stronger inhibition activity than that of the Res/β-CD complex. The results of this work indicate a potential for using the Res/CD complexes to inhibit human cancer growth and lipoproteins peroxidation.

Keywords Resveratrol · Cyclodextrin · Inclusion complex · Characterization · Cytotoxicity · Lipid peroxidation

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Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene, Res) belongs to a group of naturally polyphenol compounds found in more than 70 plant species [1], a number of which are dietary components, such as grapes, mulberries, and peanuts [2]. It is produced by plants in response to exogenous stress factors, such as injury, fungal infections or UV irradiations. Recently, Res has attracted great interest due to the "French paradox". In France despite fat-rich diets, mortality from coronary heart disease is lower than in other countries due to the moderate consumption of red wine [3]. Epidemiological studies suggested that Res was one of the active ingredients of red wine responsible for decreased coronary heart disease mortality [4]. Res has been found to display many pharmacologic effects, such as modulation of lipid metabolism, protection of low-density lipoproteins against oxidative and free radical damage [5, 6], and inhibition of platelet activation and aggregation [7]. The most interesting biological activity of this compound is its strong anti-carcinogenesis effect [8]. It can block the carcinogenesis stages of initiation, promotion, progression and has potent chemopreventive

effect on multiple carcinogenesis models such as skin cancer, breast cancer, lung cancer, prostate cancer [9]. Although the molecular basis for the biological activity of Res is not well understood, it was known that Res exhibits cyclooxygenase-2- and p53-dependent apoptosis-inducing effects [10] and antioxidant effects [6]. However the utilization of beneficial effects of highly lipophilic Res is limited by its poor solubility in water [11] and its rapid metabolism and elimination [12]. In addition, it is susceptible to oxidative degradation [13], and the half-life of *trans*-Res is only 30–45 min [14]. It is also an extremely photosensitive compound [15], where 80–90% of *trans*-Res in solution is converted to *cis*-Res if exposed to light for 1 h [16]. Taking into account its poor solubility, short biological half-life, labile property and rapid metabolism and elimination, it is highly desirable to stabilize Res while preserving its biological activity and to enhance its bioavailability. It is case where formation of inclusion complexes with cyclodextrins (CDs) may prove useful.

Cyclodextrins are cyclic oligosaccharides and composed of glucopyranose units assuming a truncated cone structure with a hydrophobic cavity. The hydrophobic cavity forms inclusion complexes with a wide range of guest molecules [17–20]. Inclusion complex formation can modify some physico-chemical properties, such as solubility, stability and bioavailability, of poorly water-soluble drugs [18, 21].

Up to now, inclusion of Res by CDs has been reported in few publications. Bertacche et al. [22] first prepared and characterized the inclusion complexes of Res with native (α-, *β*-, *γ*-) and modified CDs (2-hydroxypropyl-*β*-CD (HP-CD), dimethyl-*β*-CD (DM-CD)). The apparent formation constants of the Res complexes by native (α-, *β*-, *γ*-) and modified CDs (HP-CD, DM-CD, methyl-*β*-CD (M-CD), ethyl-*β*-CD (E-CD), maltosyl-*β*-CD, carboxymethyl-*β*-CD and acetyl-*β*-CD) have been studied by different methods, such as nuclear magnetic resonance (NMR), enzymatic, solubility and fluorimetric assays [22–25]. Thermodynamic parameters and driving forces of the inclusion processes of Res by α-, *β*-, *γ*-CD and HP-, M- and E-CD have been determined [25–27]. From the point of research on inclusion of Res by CDs, most of the above studies have focused on the demonstration of their structures and apparent formation constants, but very little dealt with their bioavailability. Lucas-Abellán et al. [28] determined the oxygen radical absorbance capacity of Res complexed by HP-CD and concluded that the antioxidant activity of Res is prolonged on complexation. Berta et al. [29] reported that the Res/2-hydroxypropyl-*β*-cyclodextrin (HP-CD) cream and mouthwash can significantly improved anti-proliferative activity of hamster oral squamous cell carcinoma cell line HCPC I and related animal model. In our previous work, the scavenging capacity of Res in free form towards stable radical DPPH shows little difference when compared with its complexed by *β*-cyclodextrin (*β*-CD) and HP-CD [30]. However, the cytotoxicity on representative cancer cells, such as human cervical carcinoma cell line HeLa, human hepatocellular liver cancer cell line Hep3B and the inhibition activity of lipid peroxidation of the Res/CD complexes have not been reported.

The present work reports on the preparation of inclusion complexes of Res with *β*-CD and HP-CD and characterization of the complexes using powder X-ray diffraction (XRD), fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM). The cytotoxicity of the inclusion complexes was determined by methylthiazoletetrazolium (MTT) reduction assay on two cancer cell lines (human cervical carcinoma cell line HeLa and human hepatocellular liver cancer cell line Hep3B) and one normal cell line (human umbililical vein endothelial cell line HUVEC). The inhibition of lipid peroxidation induced by Fe^{2+}/ascorbate of the two inclusion complexes has been determined by thiobarbituric acid (TBA) assay.

Experimental

Materials

Resveratrol, *β*-CD, HP-CD (1.0 molar substitution degree) and 3-(4,5-dimethylthiazolyl)-2, 5-diphenyl- tetrazolium bromide (MTT) were obtained from Sigma Aldrich. TBA, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), lecithin (from egg yolk) and *L*-ascorbic acid were from Sinopharm Chemical Reagent Co. Ltd. All other reagents were of analytical reagent grade. Deionized water from a Milli Q system apparatus (Millipore, USA) was used throughout the experiments.

Preparation of the inclusion complexes

The inclusion complexes between Res and CDs were prepared by wet technologies [22]. Each CDs (0.1 mmol) was finely suspended in water (5 mL) at room temperature with vigorous stirring and an equimolar amount of Res (0.1 mmol) was directly added to the suspension. To minimize photochemical degradation the flasks were covered with aluminium foil. After stirring for 120 h, the suspensions were filtered through 0.45 μm cellulose acetate membrane filters to remove undissolved solid. The water was removed by evaporation in vacuum. Free CDs and Res (0.1 mmol) were also suspended in water with vigorous stirring for 120 h, and then evaporated the water in vacuum. The solid complexes, CDs and Res were characterized by XRD, FT-IR and SEM.

Characterization of the inclusion complexes

X-ray diffraction patterns of Res, CDs and inclusion compounds were recorded on a Bruker AXS D8 Advanced

X-ray diffractometer (Cu Kα radiation, $\lambda = 1.5406$ Å). The scanning rate is 1°/min in the 2θ range from 5 to 35°.

FT-IR spectra of Res, CDs and inclusion compounds were collected using a Thermo Nicolet 520 FT-IR spectrophotometer in a spectral region between 4,000 and 450 cm^{-1}. Samples were mixed in a mortar with KBr (1:100) and pressed to small tablets and placed in the infrared beam.

The SEM images of Res, CDs and inclusion compounds were taken on LEO 1530 SEM operating at 20 kV.

Cytotoxicity assay

Human cervical carcinoma cell line HeLa, human hepatocellular liver cancer cell line Hep3B and human umbililical vein endothelial cell line HUVEC were obtained from China Center for Type Culture Collection, Wuhan University. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere at 37 °C in 5% CO_2.

The morphology of cells was observed on LECIA DMIL inverted microscopy. Cytotoxicity was measured using MTT assay. The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitochondrial and cell viability [31]. Briefly, after being harvested from culture flasks, cells were diluted to 1×10^5 cells/mL and seeded in each well containing 100 μL of the growth medium in a 96-well plate. After 24 h incubation, the medium was removed by suction and cells were treated with 100 μL mixed medium for 48 h, the mixed medium containing 95 or 90 μL growth medium and 5 or 10 μL Res (dissolved in 50% dimethyl sulphoxide, DMSO), β-CD or HP-CD (4 or 8 mmol/L, dissolved in water), Res/β-CD or Res/HP-CD complex (Res dissolved in 4 or 8 mmol/L β-CD or HP-CD). Then 20 μL of 5 mg/mL MTT in phosphate buffered saline (PBS) was added to each well and the plate was incubated for further 4 h. The solution in each well containing unbound MTT were removed by suction and 100 μL DMSO was added to each well. After incubation at 37 °C for 10 min, the absorbance at 490 nm of the clear solutions was detected on a Multiskan MK3 Microplate reader. All measurements were performed a minimum of three independent times, in five replicates. The percentage viability was calculated using the following equation.

$$\text{Cell viability, } \% = \left(\frac{A_s}{A_0}\right) \times 100 \qquad (1)$$

Where A_0 and A_s correspond to the absorbances at 490 nm of the control cells and the cells treated with Res, CDs and inclusion compounds, respectively.

Inhibition of lipid peroxidation activity

The inhibition activities of the Res/β-CD and Res/HP-CD complexes against lipid peroxidation induced by Fe^{2+}/ascorbate were estimated in liposomes by the measurement of malondialdehyde (MDA) formation, using TBA assay as described by Rakić et al. [32]. Liposomes were prepared from the egg lecithin, which diluted to 0.03 g lecithin/mL with water in an ultrasonic bath for 30 min. The reaction mixtures with a final volume of 4 mL contained 20 μL $FeSO_4$ (75 mmol/L), 50 μL of liposomes, 10 μL of Res/β-CD or Res/HP-CD complex, 20 μL of *L*-ascorbic acid (0.1 mol/L) and phosphate buffer (pH = 7.4). After incubation at 37 °C for 1 h, 0.2 mL of EDTA (0.1 mol/L) and 1.5 mL of TBA reagent (3 g TBA, 120 g TCA and 10.4 mL $HClO_4$ in 800 mL of water) were added to each mixture and heated at 100 °C for 15 min. After cooling to room temperature, the mixtures were centrifuged for 10 min (3,000 rpm) and the absorbance of supernatant was measured at 532 nm against a blank containing all the reagents except for the inclusion complex. The percentage of inhibition activity of lipid peroxidation of Res/β-CD or Res/HP-CD inclusion complex was calculated according to the following equation:

$$\text{Inhibition activity, } \% = \left(\frac{A_0 - A_s}{A_0}\right) \times 100 \qquad (2)$$

where A_0 and A_s correspond to the absorbances at 532 nm of the control (containing all reagents except for the tested complexes) and the tested complexes, respectively.

Results and discussion

X-ray diffraction

The powder XRD patterns of Res, CDs and their inclusion complexes are illustrated in Fig. 1. Res shows a diffractogram consistent with its crystalline nature, while Res/β-CD displays a different pattern in the 5–30° (2θ) area where the peaks assigned to Res almost disappear, demonstrating that the new species, in this case a Res/β-CD complex, is formed between Res and β-CD. A similar behavior is also found in the case of Res/HP-CD system.

Fourier transform infrared spectroscopy

The FT-IR spectra of Res, β-CD, HP-CD, Res/β-CD and Res/HP-CD are shown in Fig. 2. The spectrum of Res showed characteristic intense bands at 1592, 1385 and 974 cm^{-1}, which corresponded to C–C olefinic stretching, C–O stretching and typical *trans* olefinic band, respectively. However, the FT-IR spectra of inclusion complexes

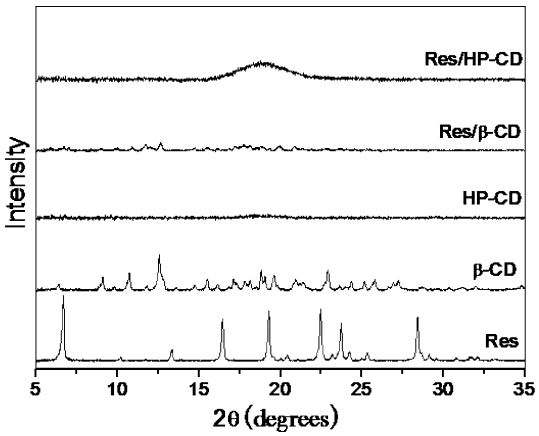

Fig. 1 XRD patterns of Res, β-CD, HP-CD and the inclusion complexes of Res/β-CD and Res/HP-CD

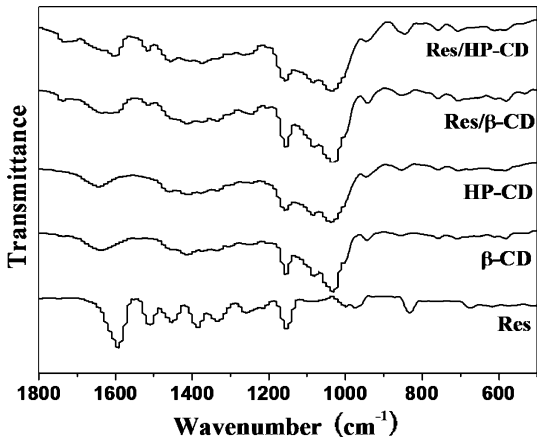

Fig. 2 FT-IR spectra of Res, β-CD, HP-CD and the inclusion complexes of Res/β-CD and Res/HP-CD

showed obvious changes in the spectral features of the guest molecule. It was found that the band intensity at 1592 cm^{-1} decreased while the bands at 1385 and 974 cm^{-1} disappeared, thus indicating the formation of inclusion complexes.

Scanning electron microscopy

The SEM images of Res, β-CD, HP-CD, Res/β-CD and Res/HP-CD were shown in Fig. 3. From the figure we see that free Res appeared as small agglomerate crystals, β-CD had rod-like structures and HP-CD appeared as broken sphere-like structures. After the inclusion process, the complexes showed a morphological modification and appeared amorphous structure.

Cellular morphology

Figure 4 shows the photographs of HeLa cells after treatment with free Res in water, CD solutions, and complexed Res solutions for 48 h, respectively. It can be seen that 4 mmol/L β-CD or 4 mmol/L HP-CD alone do not exert any significant effect on cellular morphology. A similar result is observed after incubation with free Res in water. After incubating on Res/4 mmol/L β-CD (Res in 4 mmol/L β-CD) or Res/4 mmol/L HP-CD (Res in 4 mmol/L HP-CD) solutions, the cells exhibit dramatic morphological alterations. The structure of the HeLa cells is damaged and the cell membrane is disrepaired. The damage exerted by the Res/HP-CD complex on HeLa cells is stronger than that of the Res/β-CD complex.

The results of free Res in water, CD solutions and the complexes of Res mixed with 4 mmol/L β-CD or 4 mmol/L HP-CD on the cellular morphology of Hep3B is similar to that of HeLa cell (result not shown). The cellular morphology of normal cell HUVEC did not change after incubation with free Res or Res mixed with 4 mmol/L β-CD or 4 mmol/L HP-CD (results not shown).

Cell cytotoxicity

The effects of Res and its complexes by β-CD or HP-CD on the viability of two human cancer cell lines (HeLa, Hep3B) and one human normal cell line (HUVEC) determined by MTT assay are shown in Fig. 5. It can be seen from Fig. 5a that the cytotoxicity of Res/β-CD and Res/HP-CD complexes on HeLa cells are stronger than those of the free components in water: β-CD, HP-CD and Res. Neither β-CD nor HP-CD has any significant effect on the viability of HeLa cells. Free Res in water shows little inhibition on the viability of HeLa cells, where the inhibitory rate is only 5% with 10 μL dosage (10% of the total medium volume). The complexes of Res with β-CD or HP-CD show significant inhibition on the viability of HeLa cells, the inhibitory rate is 40.4% with 10 μL Res/8 mmol/L β-CD complex and 39.0% with 10 μL Res/8 mmol/L HP-CD complex.

The cytotoxicities of Res/β-CD and Res/HP-CD complex on Hep3B cells are shown in Fig. 5b. It is clearly seen that the effects of β-CD, HP-CD and free Res in water on the viability of Hep3B cells are almost the same as those on HeLa cells. It is important to mention that the cytotoxicities of the two complexes on Hep3B cells are stronger than those exerted on HeLa cells. The inhibitory rate is 46.2% with 5 μL Res/8 mmol/L β-CD solution and 43.3% with 5 μL Res/8 mmol/L HP-CD solution. The cytotoxicities of the two complexes on human non-cancerous cell line HUVEC are shown in Fig. 5C. None of the samples: Res/β-CD or Res/HP-CD complex, free Res, β-CD and HP-CD, show any significant cytotoxicities on HUVEC cells.

From these results, the Res/β-CD and Res/HP-CD inclusion complexes significantly decrease the viabilities of

tested human cancer cells (Hep3B, HeLa) but do not have significant effect on human normal cells HUVEC. The effect of the two complexes on Hep3B is stronger than that on HeLa. The Res/HP-CD complex shows a stronger cytotoxicity on the two cancer cells than that of Res/β-CD complex. Free β-CD and HP-CD species exhibit no effect on the viability of cancer cells (Hep3B, HeLa) or normal cells HUVEC, which is in good agreement with the results of Leroy-Lechat et al. [33].

From the phase-solubility diagrams of the Res/β-CD and Res/HP-CD system in water [30], the concentration of free Res in water is about 0.077 mmol/L, when complexed in 4 mM CDs, the concentration of Res arrives to 0.658 mmol/L in β-CD and 1.872 mmol/L in HP-CD solutions, and it is about 8.55 and 24.31 times of free Res in water. When the concentration of CDs arrives to 8 mmol/L, the concentration of Res is 0.968 mmol/L in β-CD and 3.888 mmol/L in HP-CD, is about 12.57 and 50.49 times of free Res. Res alone in water has no significant effect on HeLa cellular morphology and shows little inhibition on the viability of HeLa and Hep3B cells is probably due to its very low concentration in water. The different damage effect on the cancer cellular morphology and different cytotoxicities exerted by the Res/β-CD and Res/HP-CD complexes is also likely due to their different inclusion ability.

Inhibition of lipid peroxidation activity

The activities of inhibition of lipid peroxidation by the two complexes were measured by the TBA method and the results are shown in Fig. 6. The percentages of inhibition of lipid peroxidation of the two inclusion complexes increase with increasing concentration of CDs. The

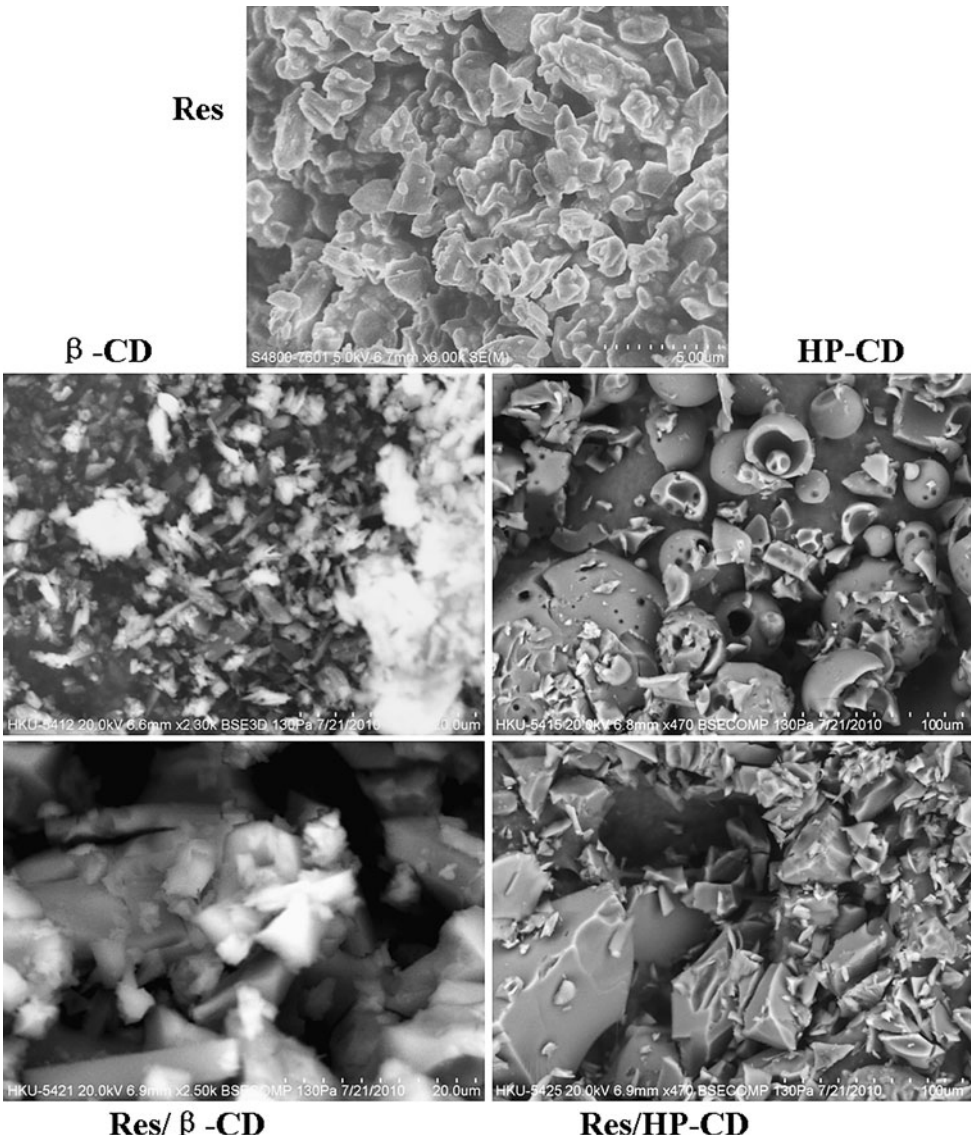

Fig. 3 SEM photomicrographs of Res, β-CD, HP-CD and the inclusion complexes of Res/β-CD and Res/HP-CD

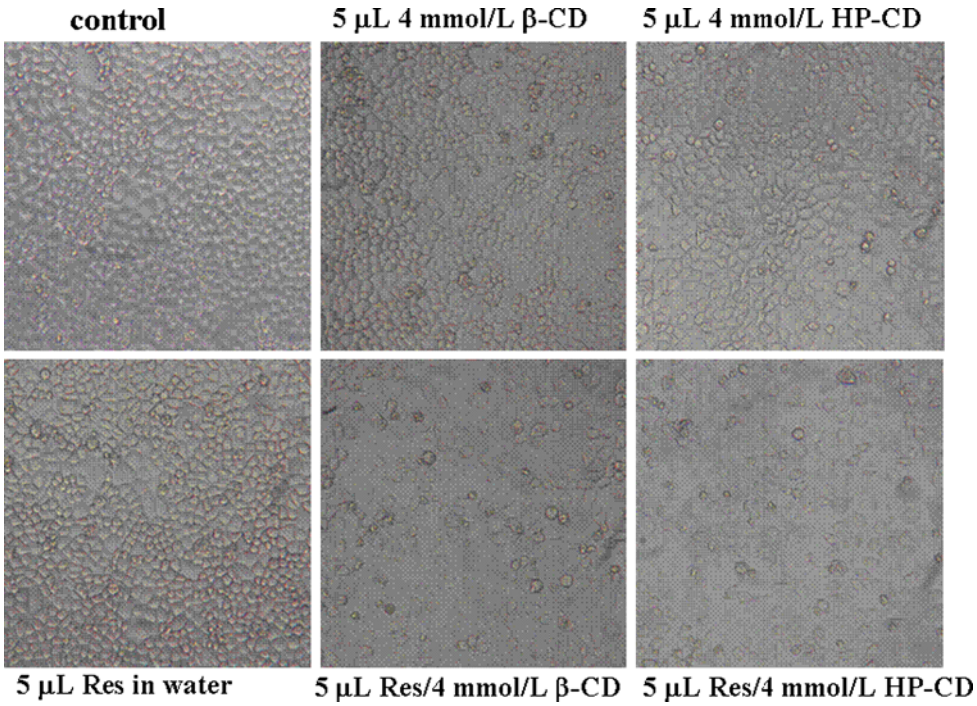

Fig. 4 Morphology of HeLa cells incubated with the Res/*β*-CD and Res/HP-CD inclusion complexes

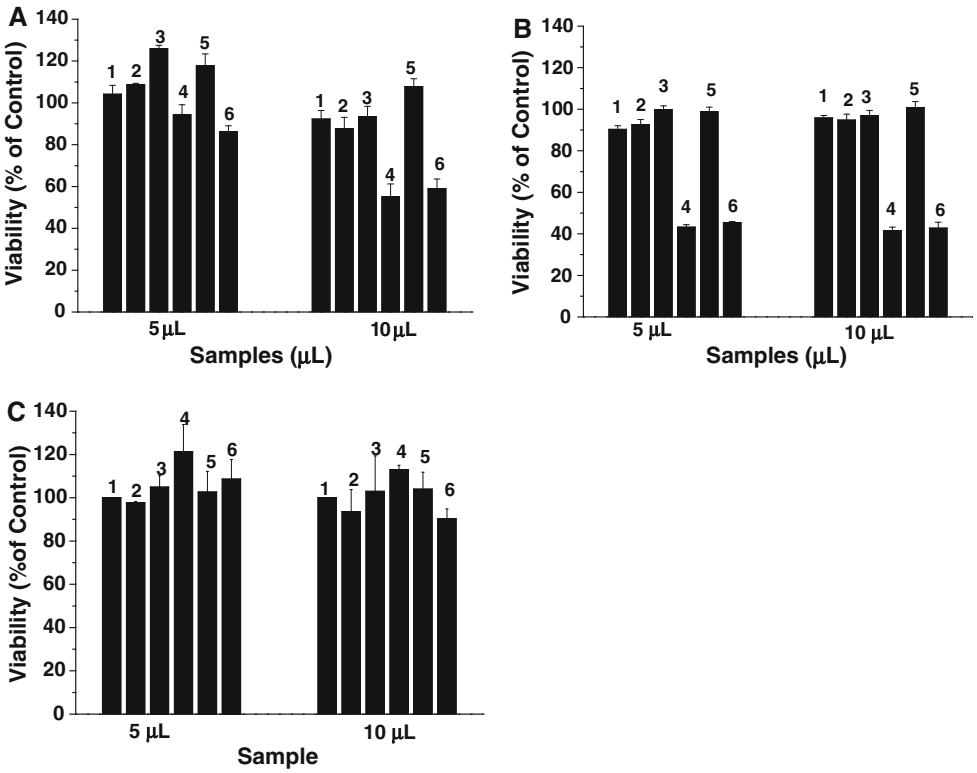

Fig. 5 Cell viabilities of human cancer cells (HeLa and Hep3B) and human normal cells (HUVEC) after treatment with free Res in water, free CDs and the corresponding inclusion complexes of Res/*β*-CD and Res/HP-CD. *1* Control; *2* 5 or 10 μL Res; *3* 5 or 10 μL 8 mmol/L *β*-CD; *4* 5 or 10 μL Res/8 mmol/L *β*-CD; *5* 5 or 10 μL 8 mmol/L HP-CD; *6* 5 or 10 μL Res/8 mmol/L HP-CD. **A** Hela cells **B** Hep3B cells **C** HUVEC cells

inhibition activity of the Res/HP-CD complex is larger than that of the Res/*β*-CD complex. The lipid peroxidation inhibition percentages of Res/*β*-CD and Res/HP-CD are 29.22 and 34.92%, respectively, at the CD concentration of 6 mmol/L. As the CD concentration is increased up to 8 mmol/L, the inhibition percentages rise to 50.07 and 59.98%, respectively. No inhibition effect was observed for free CDs (data not shown).

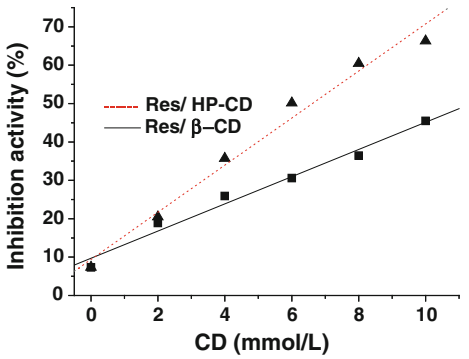

Fig. 6 Inhibition activity of lipid peroxidation of the Res/β-CD and Res/HP-CD complexes at 25 °C

Conclusion

The limited water solubility of Res can be overcome by the formation of inclusion complexes with CDs. The Res/β-CD and Res/HP-CD inclusion complexes show significant cytotoxicity on human cancer cells HeLa and Hep3B. At the same CDs concentration, the cytotoxicity of the Res/HP-CD complex is higher than that of the Res/β-CD complex. The cytotoxicities of the two complexes on Hep3B cells are larger than those exerted on HeLa cells. There are no significant cytotoxicities of the two complexes on human normal cells HUVEC. The inhibition of lipid peroxidation by the Res/β-CD and the Res/HP-CD inclusion complex both increase with the increase of CDs concentration. The inhibition activity of the Res/HP-CD complex is stronger than that of the Res/β-CD complex. The results of this work indicate a potential for using the Res/CD complexes to inhibit human cancer growth and lipoproteins peroxidation.

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