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In Silico and Experimental Studies of Concanavalin A: Insights into Its Antiproliferative Activity and Apoptotic Mechanism

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Abstract Concanavalin A (ConA), a mannose/glucose-binding legume lectin, has been reported to induce tumor cell death via a mitochondria-mediated autophagic pathway; however, the precise mechanism by which induces cell death remains to be discovered. In this study, we simulated the three-dimensional structure of ConA monomer, its dimer, and tetramer forms and reported its molecular dynamics simulations and phylogenetic analysis. Subsequently, we showed that ConA possessed remarkable antiproliferative effects on HepG2 cells. Further data showed that there was a link among its hemagglutinating, sugar-binding, and antiproliferative activities. In addition, we found that ConA induced apoptosis in HepG2 cells. Then, we demonstrated that the treatment of ConA caused mitochondrial transmembrane potential (MMP) collapse, cytochrome *c* release, and activation of caspase. In conclusion, we demonstrate that there is a positive correlation between carbohydrate-binding activity and antiproliferative activity of ConA. In addition, we confirm that ConA induces HepG2 cell death through a mitochondrial apoptotic pathway.

Keywords Concanavalin A (ConA) · Molecular structure · Phylogenetic analysis · Antiproliferative · Apoptosis · Mitochondrial pathway

Introduction

Lectins are carbohydrate-binding proteins that bind carbohydrates reversibly and possess the ability to agglutinate cells or precipitate polysaccharides and glycoconjugates [1]. These are widely distributed in animals, plants, and microorganisms and have attracted great interest because of their various biological activities such as cell agglutination, antitumor, immunomodulatory, antifungal, antiviraland antiinsect activities

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[2, 3]. Concanavalin A (ConA), the first plant lectin with a mannose/glucose-binding specificity, was found in the jack bean (*Canavalia ensiformis*) [4]. Monomeric units of ConA that have been identified have a molecular weight of approximately 27,000 Da [5]. Between pH2 and 5.5, ConA exists as a dimer of two covalently linked subunits, and at pH values above 5.5, it exists as a tetramer [6]. Additionally, ConA is the first plant lectin for which the three-dimensional structures have been established and is being widely studied for a variety of biological activities, especially the antiproliferative activity in ConA-treated cancer cells [7].

It is well known that cancer is associated with programmed cell death (PCD), which is an evolutionary conserved process that plays a key role in the homeostasis of cells [8]. Apoptosis, as type I PCD, is characterized by condensation of the cytoplasm and nucleus, DNA fragmentation, chromatin merging in the nuclear periphery, cell contraction, dynamic membrane blebbing, and cell phagocytosis. Moreover, apoptosis is also regarded as one of the most important mechanisms for tumor cell suicide [9]. Autophagy, as type 2 cell death, is independent of phagocytes and differs from apoptosis by the presence of autophagosomes, autolysosomes, and an intact nucleus in the cell [10]. In addition, autophagy is a significant molecular mechanism for tumor cell suicide induced by radiation or chemotherapy [11].

Previous studies reported that ConA induced several hepatoma cell deaths through a mitochondria-mediated autophagic pathway; the apoptosis and molecular mechanism of cell death have not been demonstrated in the aforementioned investigation [12]. Moreover, the relationship between the sugar-binding activity of ConA and its antiproliferative activity are not clear. Therefore, in our study, we reported in silico analyses of ConA and its antiproliferative activity on the sugar-binding sites, indicating that there is a close positive correlation between its carbohydrate-binding activity and antiproliferative activity. Subsequently, we demonstrated that ConA induced HepG2 cell death through a mitochondrial apoptotic pathway.

Materials and Methods

In Silico Methods

The structural data of ConA were acquired from the GenBank database (PDB code: 3cna). MODELLERv7 [13] was utilized to build the molecular modeling of ConA. Molecular dynamics simulations were performed with GROMACS software package (v3.3.1) [14]. Program CLUSTALW (version 1.81) was used to make a multiple alignment of ConA [15]. Phylogenetic analysis was exerted by the Molecular Evolutionary Genetics Analysis (MEGA) package [16].

Reagents

ConA was purchased from Sigma Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), NBSF, 2,3-butanedione, PMSF, TNBS, DEPC, mannose, glucose, D-fructose, and thyroglobulin were purchased from Sigma Chemical. Rabbit polyclonal antibodies against caspase-3, caspase-9, and β-actin, mouse polyclonal antibodies against cytochrome *c*, and horseradish peroxidase-

conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

The HepG2 cell lines were provided by Medical Sciences Center of West China in Sichuan University. The cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS and 0.03% L-glutamine (GIBCO) and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

Cell Growth Inhibition Assay

The HepG2 cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 5×10^4 cells/ml. After 24-h incubation, the cells were treated with or without various inhibitors at given concentrations 1 h prior to the administration of the ConA. The effects of ConA on cell viability/proliferation were determined using the MTT assay with a plate reader (Bio-Rad, Hercules, CA, USA) as described previously with some modifications [17].

Carbohydrate-Binding Activity Assay

To assess the effect of carbohydrates on ConA-induced HepG2 cell death, the MTT assay was determined as above except that the lectin was pre-incubated at 37 °C for 30 min with mannose, glucose, D-fructose, and thyroglobulin that inhibited or uninhibited the hemagglutinating activity of ConA at 24 h.

Microscopic Observations of Apoptotic Changes

The HepG2 cells were incubated with 20 μg/ml ConA for 24 h. The cellular ultrastructure was observed under transmission electron microscopy (Hitachi 7000, Japan).

Lactate Dehydrogenase Activity-Based Cytotoxicity Assay

Lactate dehydrogenase (LDH) activity was assessed using a standardized kinetic determination kit (Zhongsheng, LDH kit, Beijing, China) as the method previously described with some modifications [18].

Detection of Mitochondrial Membrane Potential

After incubation with ConA for the indicated time periods, the cells were stained with 1 μg/ml rhodamine 123 and incubated for 15 min at 37 °C. The fluorescence intensity of cells was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) [19].

Western Blot Analysis

The HepG2 cells were treated with 20 μg/ml ConA for 0, 6, 12, and 24 h. Both adherent and floating cells were collected, and then Western blot analysis was carried out by the method as described previously with some modifications [20].

Statistical Analysis

All the data were confirmed in at least three independent experiments. These data were expressed as mean ± SD. Statistical comparisons were made by Student's *t* test. $P<0.05$ was considered statistically significant.

Results

In Silico Analysis of ConA

The amino acid sequence of ConA shares the significant similarity with other lectins involving *Sophora alopecuroides* lectin, *Sophora flavescens* lectin, *Cladrastis kentukea* lectin, *Sophora japonicum* lectin, and *Cladrastis kentukes* lectin (Fig. 1). Also, the secondary structure prediction of ConA was shown that it was mainly composed of beta-strands (Fig. 1). To trace the evolutionary relationship of this lectin, the phylogenetic evolutionary tree was constructed as shown in Fig. 2. Due to the available structure data, we firstly built the three-dimensional structure of ConA monomer. As shown in Fig. 3a and b, ConA exhibited the canonical 12-stranded beta-sandwich structure. Furthermore, between pH2 and 5.5, ConA existed as a dimer of two covalently linked subunits whereas at pH

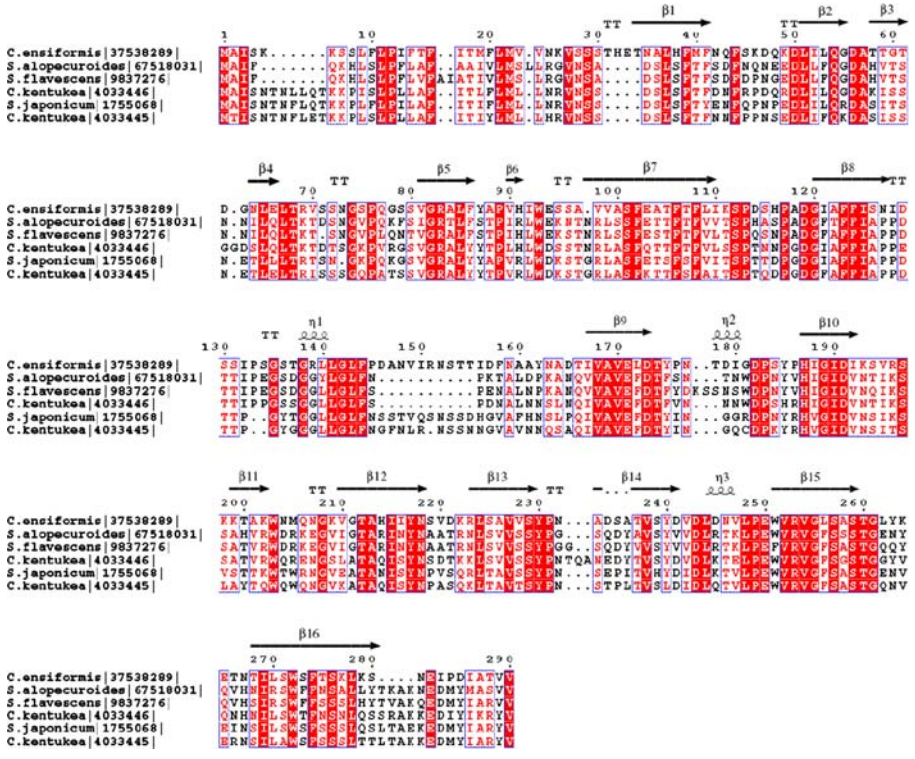

Fig. 1 Multiple alignment and secondary structure predication of ConA. The amino acid sequence of ConA shared the significant similarity with other relative lectins, and the secondary structure of ConA was mainly composed of beta-strands

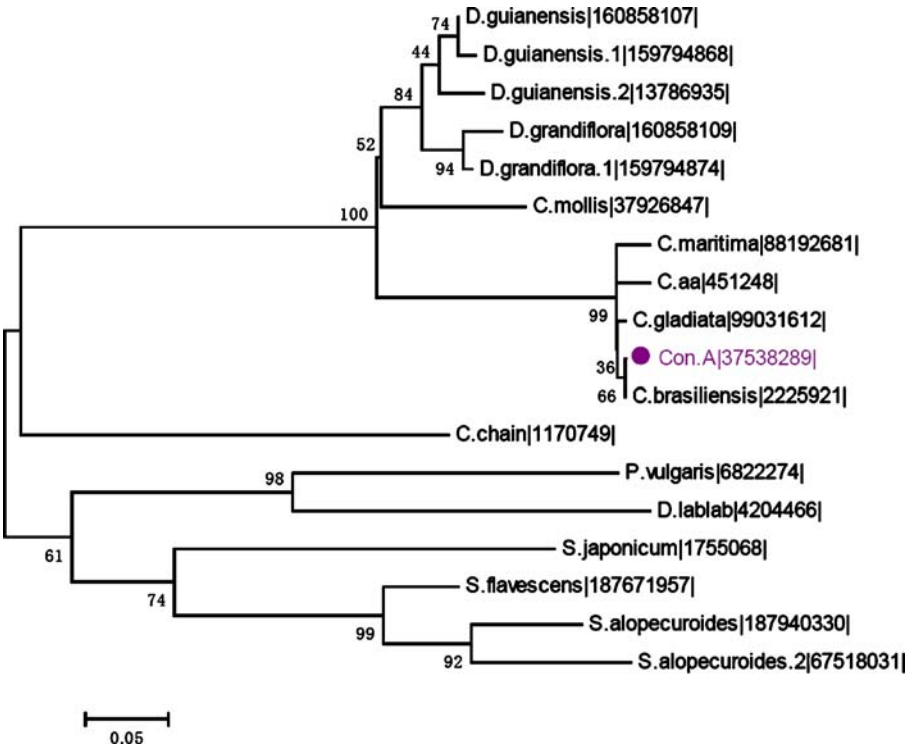

Fig. 2 Phylogenetic analysis of ConA. The phylogenetic evolutionary tree of ConA was constructed

values above 5.5, it existed as a tetramer. Thus, we also build the dimmer and tetramer of ConA by comparing modeling, respectively (shown in Fig. 3c and d). Finally, we simulated molecular dynamics of ConA, which subsequently showed that the ConA dimer and tetramer assumed the stable conformations, respectively, as shown in Figs. 4 and 5.

Antiproliferative Effect of ConA on Hepg2 Cells

The antiproliferative effect of ConA on HepG2 cells was evaluated by the MTT assay. As shown in Fig. 6a, the exposure to ConA caused inhibition of HepG2 cell growth in a dose-dependent manner.

Effects of Sugar-Binding Activity on Antiproliferative Activity

To assess the relationship between sugar-binding activity of ConA and its antiproliferative activity, we completely inhibited the sugar-binding activity after we pre-incubated with ConA in buffer containing mannose and glucose whereas we added D-fructose and thyroglobulin that uninhibited the sugar-binding activity at different concentrations, respectively, as shown in Fig. 6b. Under these conditions, the two types of mannose and glucose completely inhibited the sugar-binding activity of ConA and thus led to the disappearance of the hemagglutinating activity. Then, the cell inhibitory effect was also lost at the corresponding level with the hemagglutinating activity. It was suggested that there was a close link among the sugar-binding, the hemagglutinating, and the antiproliferative activities.

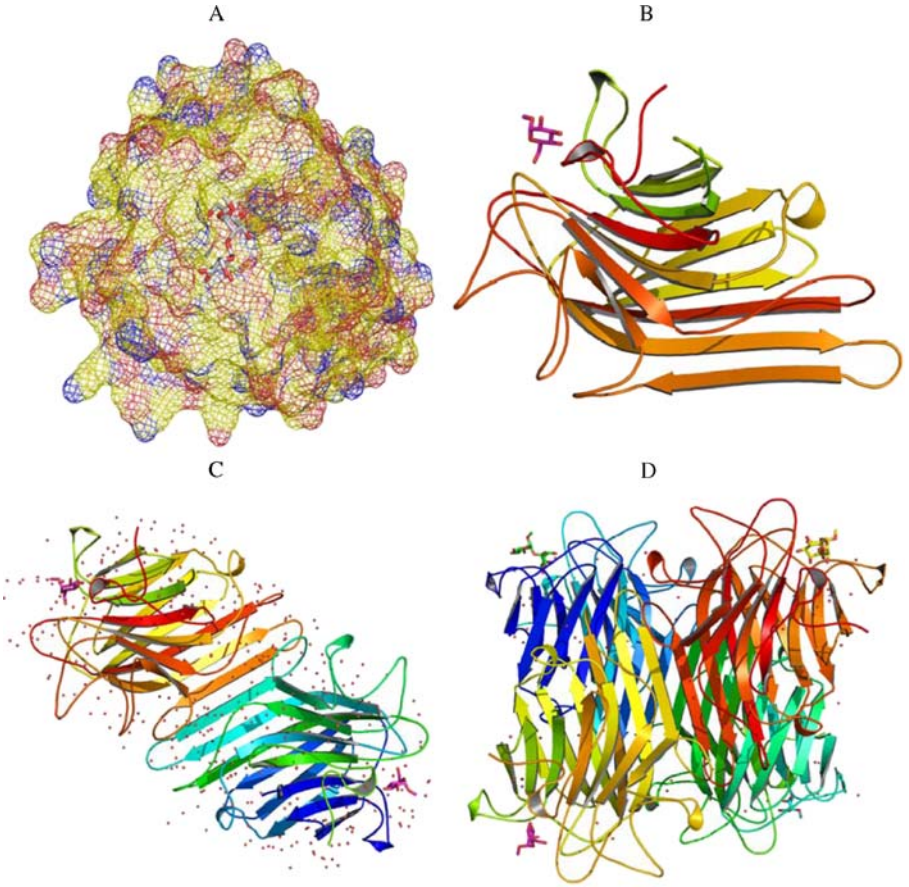

Fig. 3 Molecular modeling of ConA. The three-dimensional structure of monomer ConA (**a**, **b**). The dimer form of ConA (**c**). The tetramer form of ConA (**d**)

ConA Induced HepG2 Cell Apoptosis

To characterize the ConA-induced HepG2 cell growth inhibition, we observed the morphologic changes in the cells. When the cells were cultured with 20 μg/ml ConA for 24 h, marked morphologic alterations of apoptosis, including membrane blebbing and nuclear condensation, were observed under electron microscopy (Fig. 7a). Next, the ratio of LDH released from viable cells, floating dead cells, and the culture medium were compared. In Fig. 7b, the number of apoptotic cells was unregulated, with lower number of necrotic cells after progressively increasing concentration of ConA, suggesting that major cause of ConA-induced HepG2 cell death was not necrocytosis but apoptosis.

ConA Induced Cell Death via a Mitochondrial Apoptotic Pathway

The integrity of mitochondrial membranes was measured by rhodamine 123 staining. In Fig. 8a, ConA decreased the fluorescent intensity of rhodamine 123 staining indicative of MMP collapse, indicating that ConA induced mitochondrial dysfunction. Next, we found

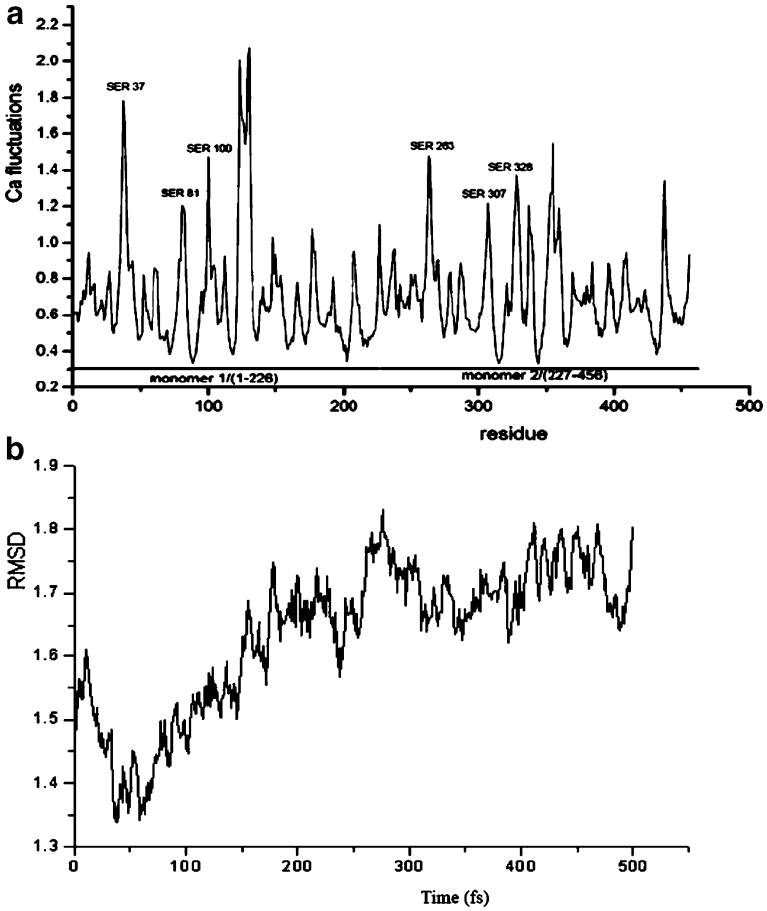

Fig. 4 **a**–**b** Molecular dynamics (MD) simulations evolution conducted of dimer and tetramer forms. Root mean square fluctuations (RMSF) for the C-alpha atoms of dimer

that the level of cytochrome *c* was increased in a time-dependent manner. Since the release of cytochrome *c* from mitochondria can activate caspase cascade, we investigated the involvement of caspase-9 and caspase-3 in ConA-induced apoptosis. In Fig. 8b, the levels of caspase-9 and caspase-3 were increased during the ConA treatment. It was concluded that ConA activated both initiator and executioner caspase in a time-dependent manner.

Discussion

Hitherto, ConA, one of the most widely studied plant lectins, has been drawing much attention in light of recent investigations concerning its antiproliferative activity and potential therapeutic applications in cancer therapy [12, 21]. In the current study, we carried out a series of in silico analysis of ConA for exploring the second structure, three-dimensional structure, molecular dynamics simulations, and phylogenetic tree of ConA.

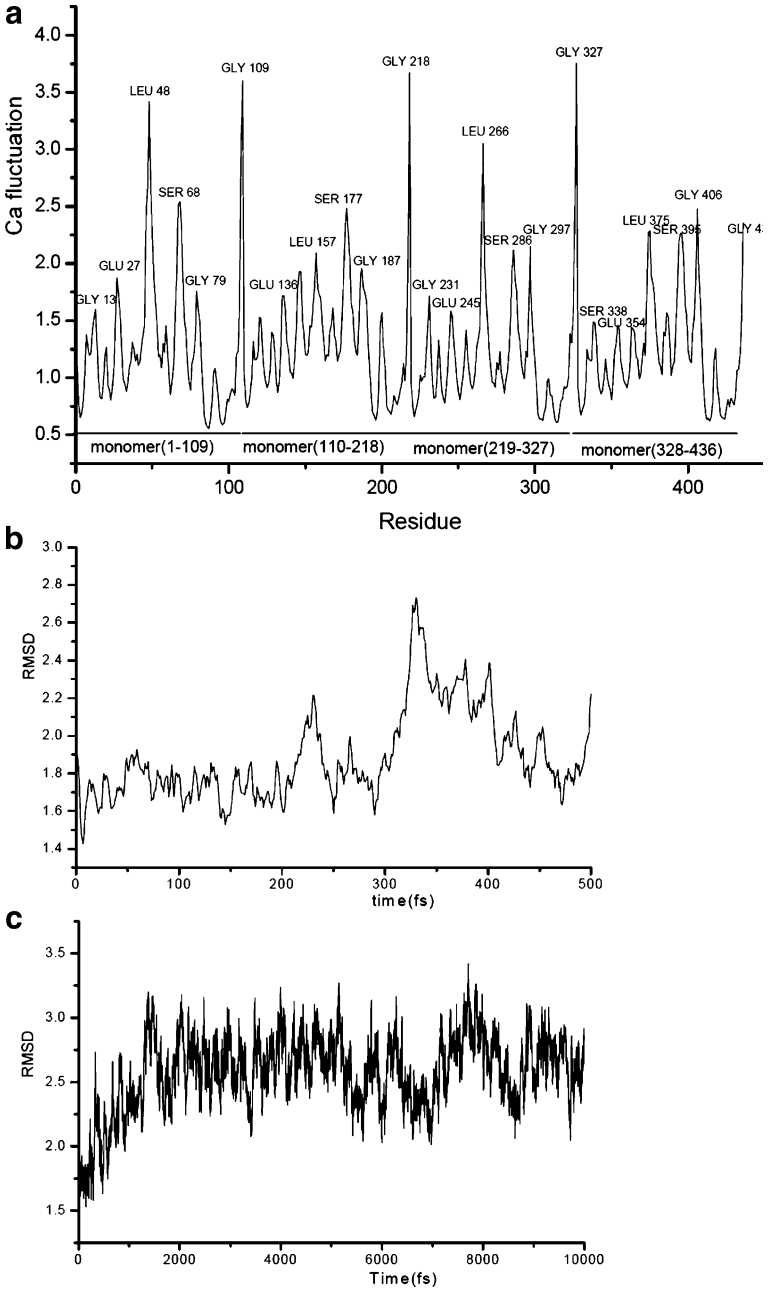

Fig. 5 **a**–**c** Molecular dynamics (MD) simulations evolution conducted of tetramer forms. Root mean square fluctuations (RMSF) for the C-alpha atoms of tetramer

Subsequently, we found that ConA was remarkable inhibitory or cytotoxic to HepG2 cells (IC_{50}=20 μg/ml, 24 h). And, we exerted carbohydrate-binding activity experiments to further obtain an insight into how its sugar-binding activity and antiproliferative activity were related with each other. Our data suggested that the hemagglutinating activity had

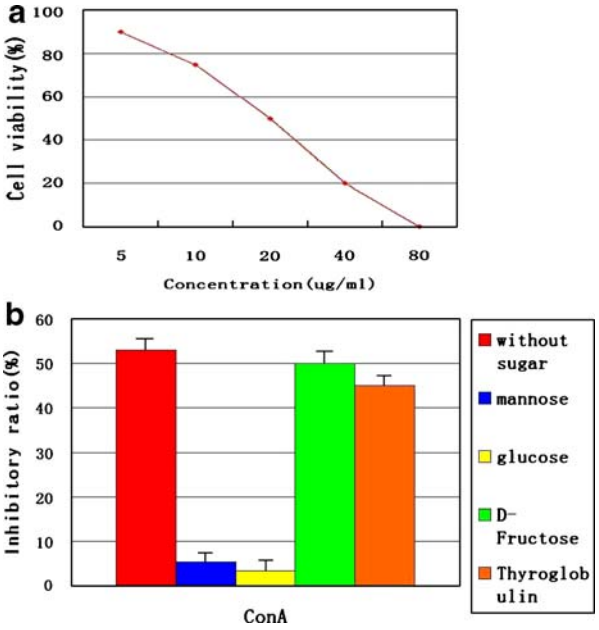

Fig. 6 Cell viability assay and sugar inhibition assays. The HepG2 cells were treated with various doses of ConA for 12, 24, 36, and 48 h. The inhibition ratio was measured by the MTT assay. $n=3$. Means ± SD (**a**). ConA was pre-incubated at 37°C for 30 min with different types of sugars; the cell inhibitory ratio was measured by the MTT assay ($x\pm$SD, $n=3$; **b**)

an important influence on the antiproliferative activity. Also, these results were in good agreement with the subsequent carbohydrate-binding activity assay, which in turn indicated that the sugar-binding activity of ConA overlapped the hemagglutinating active center. That is to say, the carbohydrate-binding activity of ConA may affect its antitumor activity. Due to the data of sugar-binding activity, we demonstrated that there was also an important correlation between the sugar-binding site and antitumor activity. Thus, we concluded that there was a link between its sugar-binding, hemagglutinating, and antiproliferative activities.

Previous studies have reported that mistletoe lectins, which belong to the type II ribosome-inactivating protein (RIP II) family and are composed of a catalytically active A-chain with rRNA N-glycosidase activity and a B-chain with carbohydrate-binding properties, exerted potent cytotoxic effects on several typical tumor cells [22]. Moreover, mistletoe lectins were reported to induce apoptosis through both caspase-8/FLICE independent of death receptor pathway and p53-independent pathway [23, 24]. And, they were also reported to induce apoptotic death in cancer cells involving a remarkable generation of intracellular hydrogen peroxide and activations of caspase-9 and caspase-3 cascade [25]. Compared to the above-mentioned investigations, ConA, a typical legume lectin, was shown to possess a distinctive three-dimensional structure with partially similar sugar-binding sites. Therefore, our studying results may be partially in agreement with the apoptotic mechanisms of mistletoe lectins. In this study, we found that ConA induced apoptosis in HepG2 cells. However, we also found the MMP collapse and cytochrome *c* release are associated with caspase-9, which act at multiple steps in the pathway to modulate the subsequent apoptosis and eventually activate caspase-3. These results indicate that the ConA-induced apoptotic mechanism is via a mitochondrial pathway as reported

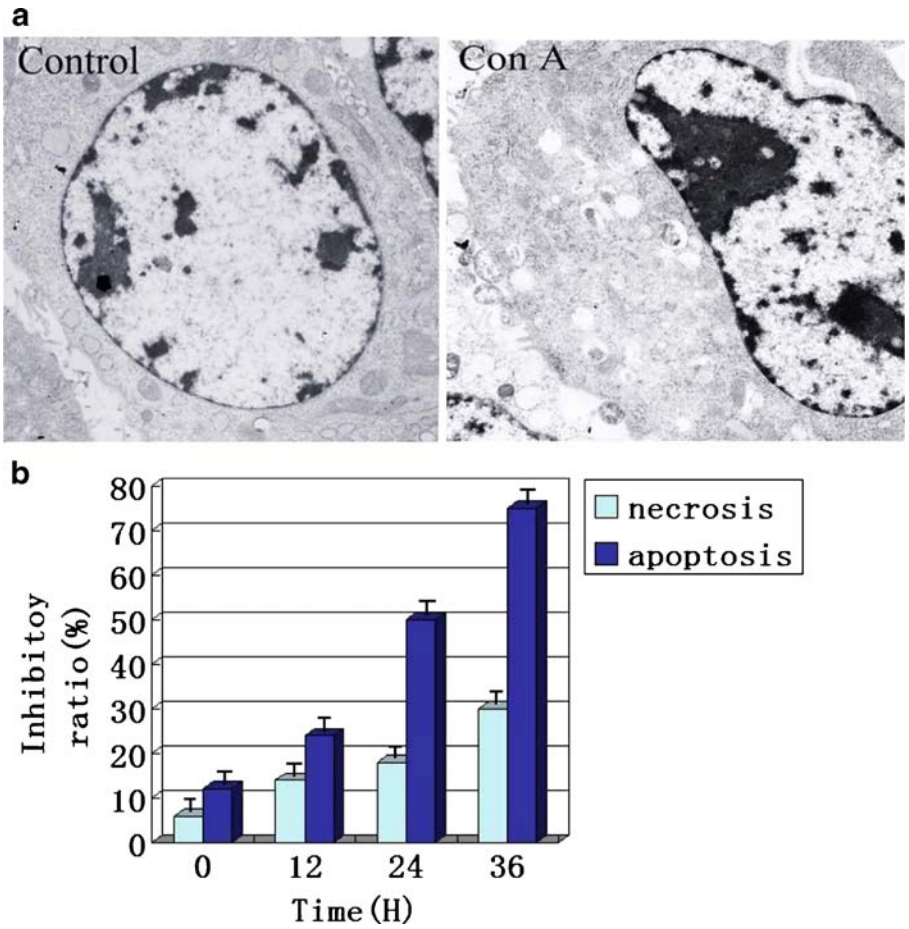

Fig. 7 ConA induced apoptosis in HepG2 cells. The cellular ultrastructural morphology was observed without ConA (*Control*) or with 20 μg/ml ConA for 24 h (*ConA*) under electron microscopy (**a**). The HepG2 cells were treated with 25 μg/ml ConA for 12, 24, or 36 h, and apoptotic and necrotic ratio was measured by LDH activity-based assay ($x \pm SD$, $n=3$; **b**)

studies of mistletoe lectins. Thus, it might be explained into the similarity of carbohydrate-binding active sites between mistletoe lectins and ConA.

A recent study has reported that a legume lectin named *S. flavescens* lectin (SFL) can induce tumor cell death through a caspase-dependent apoptotic pathway [26]. Another recent report has demonstrated that *Phaseolus coccineus* lectin, which is also a legume lectin with specificity toward sialic acid, possess marked cytotoxicity and induces apoptosis in murine fibrosarcoma L929 cells [27]. Interestingly, it was found that there was a significant correlation between sialic acid-specific activity of *P. coccineus* lectin and its antiproliferative activity, which is in good agreement with our present study of ConA. Similarly, we also demonstrated that ConA could induce tumor cell death through this apoptotic pathway, suggesting that these legume lectins might be speculated to possess some identical or similar biological activities and molecular mechanisms.

Autophagy is not only a survival response to either growth factor or nutrient deprivation but an important mechanism for tumor cell suicide [28, 29]. Previous studies have reported

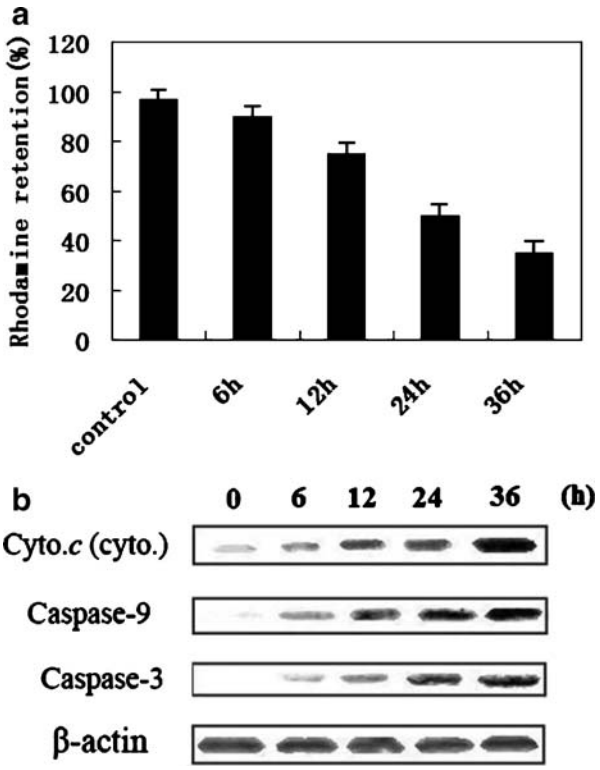

Fig. 8 ConA induced HepG2 cell death through a mitochondrial apoptotic pathway. After being treated with 20 μg/ml ConA for various time periods, the cells were loaded with 1 μg/ml rhodamine-123 at 37°C for 30 min and analyzed by flow cytometry ($x \pm SD$, $n=3$; **a**). Cell lysates were separated by 12% SDS-PAGE, and the levels of cytochrome *c*, caspase-9, and caspase-3 were detected by Western blot analysis. β-Actin was used as an equal loading control (**b**)

that ConA is cytotoxic or inhibitory to hepatoma cells, which can be mediated by a mitochondrial autophagic pathway [12, 30]. However, the typical apoptosis was not observed in ConA-treated cancer cells [30]. In contrast to the few prior studies, we reported for the first time that ConA not only possessed remarkable antiproliferative activity but also induced HepG2 cell apoptosis. Furthermore, we demonstrated that the treatment of ConA caused MMP collapse, cytochrome *c* release, and caspase activations, suggesting that ConA induces cell death via a mitochondrial apoptotic pathway. These distinctive data may be the results of the difference in the tumor cell types, experimental conditions, and different methods for plant lectin investigations.

In summary, we report herein that there is a close link between sugar-binding activity of ConA and its antiproliferative activity. Subsequently, we also demonstrate that ConA induces apoptosis in HepG2 cells. Finally, we show that the apoptotic mechanism is a mitochondrial pathway in ConA-treated HepG2 cells. These inspiring findings would provide more novel compelling evidence for ConA as a potential antitumor drug in treatment of human hepatoma disease.

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References

1. Sharon, N. (2007). *Journal of Biological Chemistry, 282*(5), 2753–2764.
2. Liu, B., Min, M. W., & Bao, J. K. (2009). *Autophagy, 5*(3), 432–433.
3. Liu, B., Zhang, B., Min, M.W., Bian, H.J., Chen, L.F., Liu, Q., Bao, J.K. (2009). *Biochimica et Biophysica Acta (BBA)—General Subjects.* doi:10.1016/j.bbagen.2009.04.020.
4. Jones, D. B., & Johns, C. O. (1916). *Journal of Biological Chemistry, 28*, 67–75.
5. Wang, J. L., Cunningham, B. A., & Edelman, G. M. (1971). *Proceedings of the National Academy of Sciences, 68*(6), 1130–1134.
6. Liu, B., Li, C. Y., Bian, H. J., Min, M. W., Chen, L. F., Bao, J. K. (2009). *Archives of Biochemistry and Biophysics, 482*, 1–6.
7. Ralph, B., Colton, E., Roger, M., & Ashok, G. (2006). *Biosensors and Bioelectronics, 22*(2), 275–284.
8. Baehrecke, E. H. (2002). *Nature Reviews Molecular Cell Biology, 3*(10), 779–787.
9. Cheng, Y., Qiu, F., Ye, Y. C., Guo, Z. M., Tashiro, S. I., Onodera, S., et al. (2009). *FEBS Journal, 276*(5), 1291–1306.
10. Wadsworth, V. K., & White, E. (2007). *Autophagy, 3*(6), 610–613.
11. Crighton, D., Wilkinson, S., & Ryan, K. M. (2007). *Autophagy, 3*(1), 172–174.
12. Chang, C. P., Yang, M. C., Liu, H. S., Lin, Y. S., & Lei, H. Y. (2007). *Hepatology, 45*(2), 286–296.
13. Sali, A., & Blundell, T. L. (1993). *Journal of Molecular Biology, 234*(3), 779–815.
14. Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A. E., & Berendsen, H. J. (2005). *Journal of Computational Chemistry, 26*(16), 1701–1718.
15. Thompson, J. D., Higgins, D. G., & Gibson, T. (1994). *Nucleic Acids Research, 22*(22), 4673–4680.
16. Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). *Molecular Biology and Evolution, 24*(8), 1596–1599.
17. Liu, B., Xu, X. C., Cheng, Y., Huang, J., Liu, Y. H., Liu, Z., et al. (2008). *BMB Reports, 41*(5), 369–375.
18. Cheng, Y., Qiu, F., Huang, J., Tashiro, S., Onodera, S., & Ikejima, T. (2008). *Archives of Biochemistry and Biophysics, 475*(2), 148–155.
19. Cheng, Y., Qiu, F., Tashiro, S., Onodera, S., & Ikejima, T. (2008). *Biochemical and Biophysical Research Communications, 376*(3), 483–488.
20. Liu, B., Cheng, Y., Zhang, B., Bian, H. J., & Bao, J. K. (2009). *Cancer Letters, 275*(1), 54–60.
21. Liu, B., Bian, H. J., & Bao, J. K. (2009). *Cancer Letters,* . doi:10.1016/j.canlet.2009.05.013.
22. Martin, L., Babette, M., Ju¨rgen, E., Holger, Z., & Hans, L. (1999). *Biochemical and Biophysical Research Communications, 264*(3), 944–948.
23. Hostanska, K., Vuong, V., Rocha, S., Soengas, M. S., Glanzmann, C., Saller, R., et al. (2003). *British Journal of Cancer, 88*, 1785–1793.
24. Heike, B., Engels, I. H., Wolfgang, V., Klaus, S. O., & Sebastian, W. (1999). *Cancer Research, 59*(9), 2083–2090.
25. Myung, S. K., Jienny, L., Kang, M. L., Sei, H. Y., Sujinna, C., Sang, Y. C., et al. (2003). *Life Sciences, 73*(10), 1231–1243.
26. Liu, Z., Liu, B., Zhang, Z. T., Zhou, T. T., Bian, H. J., Min, M. W., et al. (2008). *Phytomedicine, 15*(10), 867–875.
27. Chen, J., Liu, B., Ji, N., Zhou, J., Bian, H. J., Li, C. Y., et al. (2009). *Phytomedicine, 16*(4), 352–360.
28. Levine, B., & Klionsky, D. J. (2004). *Developmental Cell, 6*(4), 463–477.
29. Gozuacik, D., & Kimchi, A. (2004). *Oncogene., 23*(16), 2891–2906.
30. Lei, H. Y., & Chang, C. P. (2007). *Autophagy, 3*(4), 402–404.