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Full Length Research Paper

The antitumor effects of Araloside A extracted from the root bark of *Aralia elata* on human kidney cancer cell lines

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***Aralia elata* was used as therapeutic medicine in oriental courtiers a few hundred years ago. As one important constituent extracted from *Aralia elata*, Araloside A has been shown to treat gastric ulcers, hepatitis, and arrhythmias. It is not yet known, however, whether Araloside A may induce cell apoptosis in renal cell carcinoma. We therefore examined the antitumor effects of Araloside A on human kidney cancer cell lines GRC-1 and 786-O. We found that Araloside A 1, 3, 10, 30 and 100 μM caused a considerable reduction of cellular viability of GRC-1 1 and 786-O cells in a dose- and time-dependent manner. The number of Tunnel-positive cancer cells was also higher in cells treated with Araloside A than untreated cells. The real-time polymerase chain reaction (PCR) techniques showed that Araloside A was able to increase the expression of bax mRNA and inhibited the expression of bcl-2 mRNA. Conclusively, Araloside A has a remarkably antitumor effect on kidney tumor cells through regulating bax/bcl-2 ratio.**

Key words: Araloside A, antitumor, bax/bcl-2.

INTRODUCTION

Aralia elata has been used as folk medicine in Oriental courtiers such as Japan and Korea several hundred years ago (Chung et al., 2003; Hu and Wang, 2009; Satio et al., 1993). The bark and root cortex of *A. elata* were shown to be able to treat diabetes, hepatics, stomach pain, etc (Chung et al., 2005; Hu and Wang, 2009; Kim et al., 2009). The effective components of *A. elata* included saponins, alkaloids, glycosides, palmitic acid, linoleic acid, etc (Lee et al., 2005). Previous study has shown that ethanol extract of *A. elata* exhibited antioxidant activity by increasing activities of GST and SOD, and lowered decrease serum lipids in rats, which indicated its strong hypocholesterolemic and hypolipidemic actions (Chung et al., 2003). *In vivo* studies confirmed that the water extract of *A. elata* can suppress aldose reductase in STZ-induced diabetic rats (Chung et al., 2005). It was speculated demonstrated in previous studies that saponins and glycosides from *A. elata* contributed to the lowering of blood glucose levels by preventing sugar absorption (Yoshikawa et al., 1996). The water extract of mixture of *Phellodendron cortex* and *A. elata* have inhibitory effects on oxidative stress in kidney of diabetic rats (Lee et al., 2000). The methanolic extract of *A. elata* Seem exhibited strong antioxidant properties by inhibiting the production of prostaglandin E2, interleukin-1 and interleukin-6 and suppressing the expression of iNOS and COX-2 (Kim et al., 2009). Cardiac dysfunction in diabetic rats can be effectively prevented by total aralosides of *A. elata* Seem through increasing calcium channel currents and decreasing the expression of CTGF (Xi et al., 2009). The exercised-induced renal dysfunction also can be ameliorated by *A. elata* through inhibiting iNOS activity, and reducing production of inductivity NO (Li and Chang, 2009).

As a major araloside isolated from *A. elata* Seem, Araloside A also might have a series of biological actions in human and animals. It was reported that Araloside A

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significant decreased HCl-induced gastric lesions and aspirin-induced gastric ulcers (Lee et al., 2005). However, litter is known about whether Araloside A produces the antitumor effects on several human caner cell lines. To test this hypothesis, the present study was designed to observe the anticancer role and explore its molecular mechanism.

MATERIALS AND METHODS

Cell cultures

Human renal granular cell carcinoma cell line GRC-1 was provided by Department of urology, Beijing University, China. Human renal carcinoma cell line 786-O was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). These two kinds of cancer cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 16% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2% L-glutamine and 1% penicillin–streptomycin (Invitrogen). The cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in a plastic flask. Cultured medium was replaced twice every week.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide) assay

The cellular viability of human renal cancer cell lines GRC-1 and 786-O was determined by MTT assay. CRC-1 and 786-O cells were seeded in 96-well plates and allowed to attach overnight in 16% fetal bovine serum-containing RPMI 1640 medium. Human renal cancer cells were rendered quiescent by incubation in serum-free media for 24 h. Then renal cancer cells were exposed to three concentration of Araloside and were continuously cultured for 48 h. Afterward, media were removed and replaced with fresh media containing 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for an additional four hours. The supernatant was then removed, and reduced MTT product was solubilized by adding 100 μM DMSO (Sigma-Aldrich, USA). The absorbance was read on a microplate reader at 570 nm with background subtraction at 650 nm.

TUNEL assay

TdT-mediated dUTP nick-end labeling (TUNEL) assay was used to observe the effects of Araloside A on apoptosis of two human renal tumor cell lines by an *in situ* 'cell death detection kit'. Cancer cells were plated in dishes and then exposed to different concentrations of Araloside A for 24, 48 and 72 h. Afterwards, cultured cells were washed with PBS, and then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at 25°C. Permeabilization of cells was achieved by incubation with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, followed by incubation in freshly prepared TUNEL reaction mixture for 1 h at 37°C in the dark. TUNEL assay was then carried out according to the manufacturer's instructions (*in situ* cell death detection Fluorescein kit, Roche, Penzberg, Germany) (Catalog No. 11684795910). Data are expressed as percentage of the TUNEL-positive cells among the total number of cells counted.

Quantitative real-time PCR analysis

According to the guideline of the manufacturer, the total RNA from GRC-1 and 786-O cells was isolated by Trizol (Invitrogen) and purified by RNeasy Mini Kit and RNase-free Dnase Set (Qiagen, Valencia, CA). Total RNA from two kinds of cells was subjected to first-strand cDNA synthesis using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). The methods to determine the bax and bcl-2 mRNA level in cancer cells is just as described previously. The forward and reverse primers for Bax (Fw: 5'-AGAGGATGATTGCCGCCGT-3', Rev: 5'-CAACCACCCTGGTCTTGGATC-3', product size 243 bp) (Yogev-Falach et al., 2006). The forward and reverse primers for Bcl-2 (Fw: 5'-TCATGTGTGTGGAGAGCGTCA-3', Rev: 5'-ACAGTTCCACAAAGGCATCCC-3', product size 137 bp) (Yang et al., 2008). The forward and reverse primers for GAPDH (Fw: 5'-GAAGGTGAAGGTCGGAGTC-3', Rev 5'-GAAGATGGTGATGGGATTTC-3', product size 236 bp) (Jung-Hynes et al., 2009). The samples were cycled once for 95°C for 2 min then 40 cycles of 95, 58, 72°C for 15, 30, and 45 s respectively with a final extension for 5 min at 72°C. Relative mRNA for each gene was calculated by the comparative CT method (DDCT) using GAPDH as an endogenous control and untreated samples as the calibrator.

Statistical analyses

Statistical data are given as mean ± SD. The significance of differences among groups was determined using ANOVA. All statistical analysis was done by SPSS 13.0 software. Statistical significance was defined as $p<0.05$.

RESULTS

Effect of Araloside A on the cellular viability of GRC-1 and 786-O

As displayed in Figure 1A, exposure to Araloside A 1, 3, 10, 30 and 100 μM for 24 h significantly decreased the cellular viability of GRC-1 from 96.3±4.0 to 82.3±5.7, 75.7±6.8, 59.3±5.6, 51.6±6.0 and 43.3±6.2%, respectively (n=6 independent experiments, $p<0.05$). Araloside A 10 μM also strongly reduced the cellular viability of GRC-1 to 50.4±6.9 and 42.9±7.1 after 48 and 72 h incubation (Figure 1B). Likewise, Araloside A 1, 3, 10, 30 and 100 μM also decreased the cellular viability of 786-O from 97.1±4.3 to 90.5±5.7, 81.5±6.3, 71.5±6.2, 65.7±6.1 and 47.9±7.3% (n=6 independent experiments, $p < 0.05$) after 24 hours incubation, respectively (Figure 1C). The increasing time of Araloside A incubation promoted the decrease of cellular viability of 786-O (Figure 1D). These implied that Araloside A could induce apoptosis in GRC-1 and 786-O in a concentration and time-dependent manner.

Araloside A induced the apoptosis of GRC-1 and 786-O

Figure 2 demonstrated that Araloside A was able to induce the apoptosis in GRC-1 and 786-O. Incubation with Araloside A 1, 3, 10, 30 and 100 μM for 24 h obviously increased the percentage of TUNNEL-positive cells from 6.3±1.7 to 14.9±3.1, 23.4±3.0, 28.3±3.6, 40.7±5.1 and 48.6±6.3% in GRC-1 cancer cells, and from

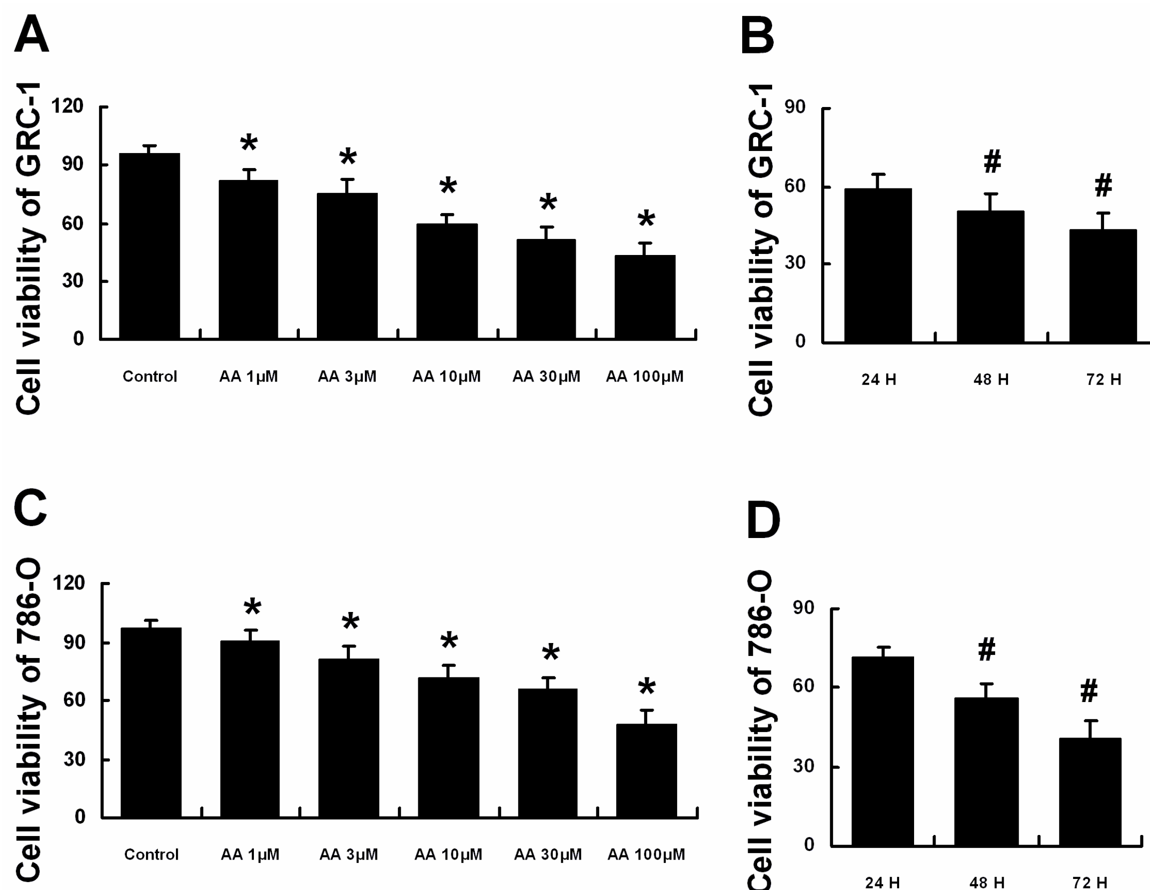

Figure 1. The effects of Araloside A (AA) on the cellular viability of GRC-1 and 786-O. Araloside A 1, 3, 10, 30 and 100 μM significantly reduced the cellular viability of GRC-1 (1A) and 786-O (1C) after 24 h incubation. The increase of incubation time from 24 hours to 48 and 72 hours obviously decreased the cellular viability of GRC-1 (1B) and 786-O (1D). n=6 independent experiments, *p < 0.05 vs Control. # p < 0.05 vs 24 h.

5.1±2.3 to 12.3±4.4, 19.9±3.6, 27.1±4.8, 38.3±6.3 as well as 53.7±7.9%, respectively in 786-O cell line (n=5 independent experiments, p<0.05) (Figures 2A and C). Exposure to Araloside A 10 μM for 48 and 72 h also can gradually increase the TUNNEL-positive cell number of GRC-1 to 45.3±5.9 and 60.1±7.2% (n=5 independent experiments, p<0.05) (Figures 2B and D).

Araloside A affected the bax/bcl-2 ratio in GRC-1 and 786-O cells

As shown in Figure 3A, both GRC-1 and 786-O cells exhibited a dramatic increase of bax mRNA expression after incubation with Araloside A 1, 3, 10, 30 and 100 μM for 24 h (n=5 independent experiments, p<0.05). The level of bcl-2 mRNA was remarkably decreased in both GRC-1 and 786-O cells by Araloside A after 24 h incubation (n=5 independent experiments, p<0.05) (Figure 3B). The bax/bcl-2 ratio was strongly augmented by Araloside A 1, 3, 10, 30 and 100 μM after 24 h treatment (Figure 3C).

DISCUSSION

Cancers presented the highest incidence occurring in the more developed countries. As one of cancers, the incidence of renal carcinoma was increased by approximately 2% in recently years. It is necessary to look for new therapeutic drugs for kidney cancers (Ljungberg et al., 2007). The stem and hook of *A. elata* was employed as medicine to treat a few diseases such as diabetes, gastric ulcers, hepatitis in Oriental nationals for several hundreds years ago (Chung et al., 2005; Satio et al., 1993). The methanolic (MeOH) extract of seeds of *A. elata* Seem exhibited strong antioxidant properties in different antioxidant tests, and also can produce obviously inhibitory effect on the production of prostaglandin E2, interleukin-1 and interleukin-6, and suppress the expression

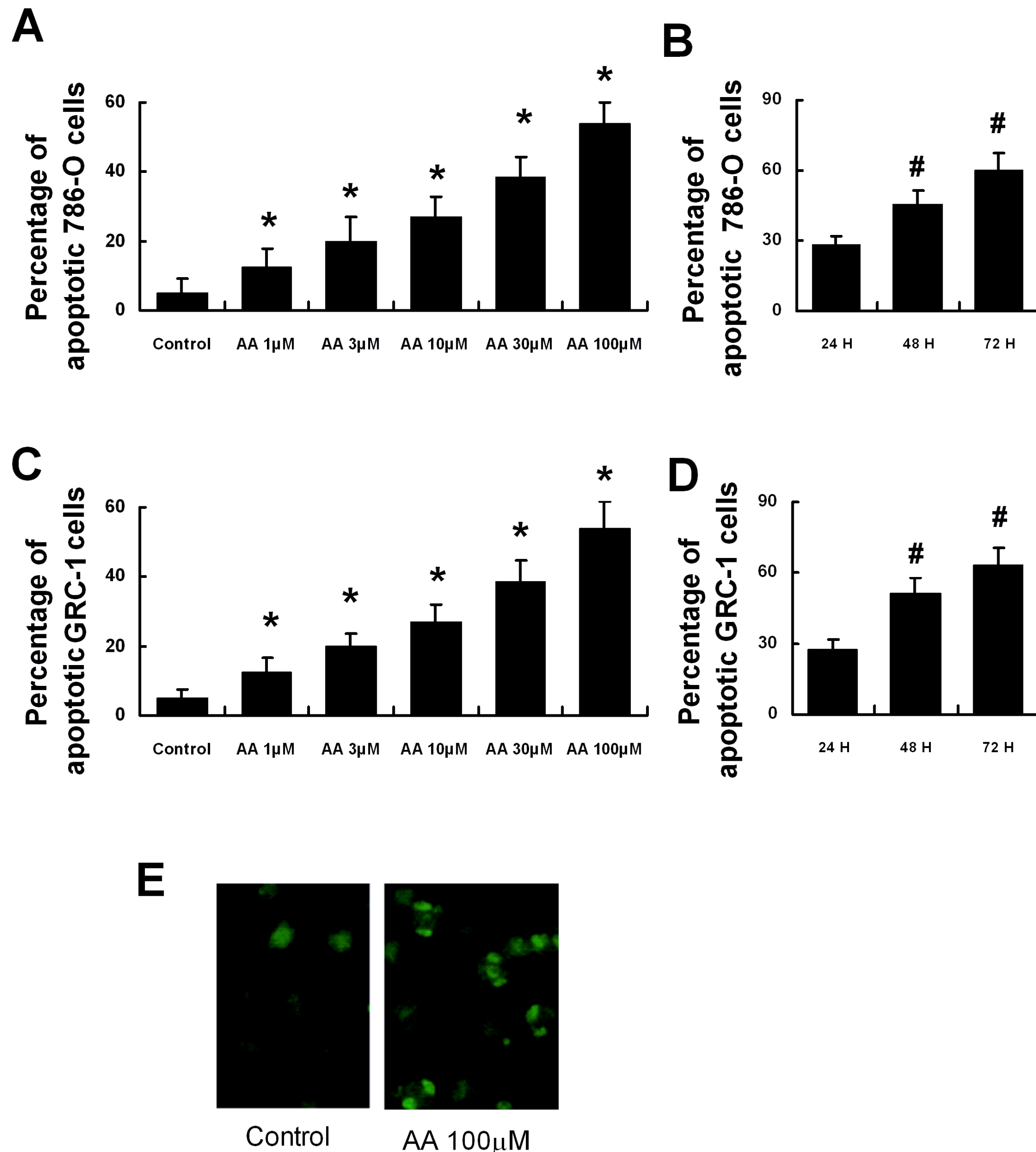

Figure 2. The effect of Araloside A (AA) on the apoptosis of GRC-1 and 786-O. Araloside A 1, 3, 10, 30 and 100 μM significantly induced the apoptosis of GRC-1 (2A) and 786-O (2C). Araloside A 10 μM also caused the apoptosis of GRC-1 and 786-O in a time-dependent manner (2B and 2D). The image of TUNNEL-positive cells in control and the presence of Araloside A 100 μM. n=5 independent experiments, *$p < 0.05$ vs Control. # $p < 0.05$ vs 24 h.

of iNOS and COX-2 mRNA transcription (Hu and Wang, 2009). Total aralosides of *A. elata* Seem also can prevent cardiac dysfunction and pathological damage in diabetic rats through increasing calcium channel currents and decreasing the expression of CTGF (Xi et al., 2009). Triterpenoid Compounds from the Buds of *A. elata* significantly inhibited fMLP-, PMA- and arachidonic acid-induced superoxide generation, and PMA-induced phosphorylation (Zhang et al., 2006). *A. elata* also could prevent kidney against exercised-induced impairment of renal function through lower iNOS activity, reduce production of inductivity NO, then relief oxide damage (Li and Chang, 2009).

Plenty of progress has been made in the understanding of biological actions of *A. elata* Seem (Sim et al., 2005). However, as a main compound from *A. elata,* little information is available about the pharmacological role of Araloside A. So, we observed the antitumor effects of

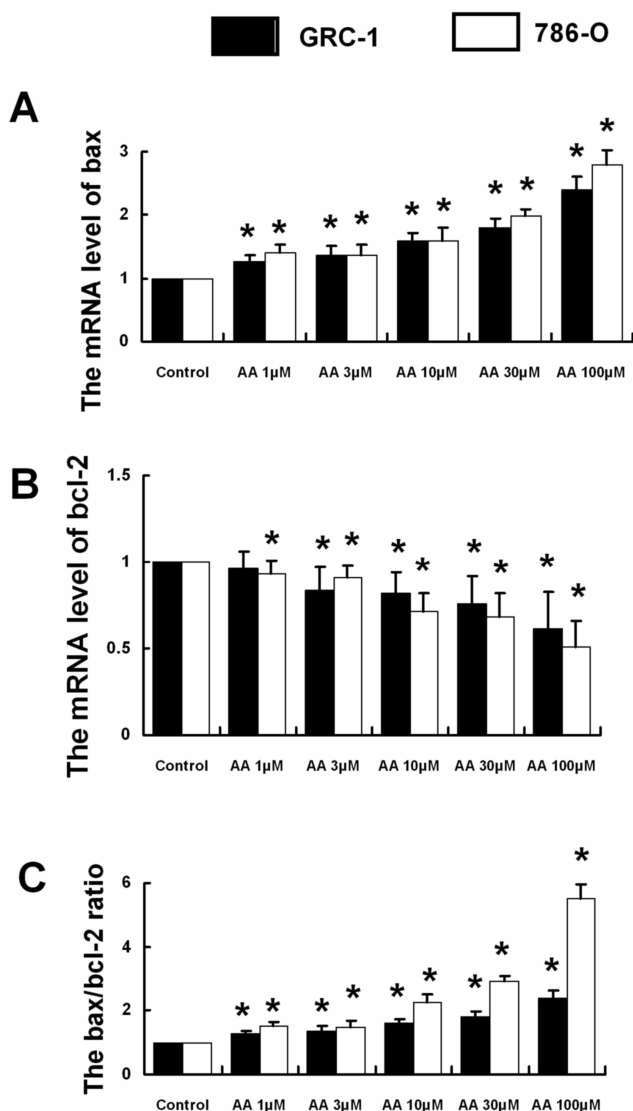

Figure 3. The effect of Araloside A (AA) on the bax/bcl-2 ratio of GRC-1 and 786-O. The level of bax mRNA in GRC-1 and 786-O in the absence and presence of Araloside A was shown in Figure 3A. The effects of Araloside A on the bcl-2 mRNA level were shown in Figure 3B. Figure 3C showed the bax/bcl-2 ratio in the presence of Araloside A in GRC-1 and 786-O. n=5 independent experiments, *p < 0.05 vs Control.

Araloside A on human carcinoma cell lines GRC-1 and 786-O. Our research found that Araloside A significantly inhibited the cellular viability of GRC-1 and 786-O, played an obvious inhibitory role in the proliferation of cancer cells. Additionally, Araloside A markedly increased the TUNNEL-positive cell number in two kinds of renal cancer cell lines. It may be suggested that Araloside A induced apoptosis of cancer cells.It is well known that bcl-2 and bax played a crucial regulatory role in the apoptosis of tumor cells (Johnston et al., 1997; Konturek et al., 2001). As one proapoptotic member, bax can induce tumor apoptosis. On the contrary, bcl-2 has an anti-apoptotic action. In this study, we found that Araloside A produced strongly inhibitory influences on the bcl-2 mRNA level in concentration-dependent manner in GRC-1 and 786-O cells, and at the same time considerably increase the expression of bax mRNA expression. The bax/bcl-2 mRNA ratio was significantly enhanced after exposed to Araloside A. It suggested that the dysregulation of bax/ bcl-2 ratio was the potential mechanism for the apoptosis of GRC-1 and 786-O induced by Araloside A. These findings may provide us one new clue for understanding therapeutic effects of Araloside A on renal cancers.

In summary, the major findings in the present study were that Araloside A has antitumor effect in renal cancer cell lines, and its underlying mechanism is through inhibiting bcl-2 mRNA and increasing bax mRNA.

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