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Original Research

Antitumor mechanism of MAP30 in bladder cancer T24 cells, and its potential toxic effects in mice

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Abstract: To investigate the antitumor mechanism of MAP30 in human bladder cell line (T24) and its potential toxic effects in mice. In this study, the biological behavior of MAP30's influence on bladder cell was investigated to reveal the antitumor mechanism and role of MAP30 in bladder cancer. MAP30 gene sequence optimized by gene synthesis codon was inserted into the prokaryotic expression vector pET-28a to produce a large amount of target protein in *Escherichia coli*. The protein product was obtained after purification. Membrane hydration method was used to prepare MAP30 liposome in order to enhance its membrane permeability. The effects of MAP30 on the viability, apoptosis and migration of T24 cell were assessed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), flow cytometric and TUNEL assays, respectively. Mice were transfected with bladder cancer cells for 48 h. The expressions of apoptotic and non-apoptotic proteins were determined using Western blotting. Changes in tumor volume and occurrence of metastasis were assessed using luciferase assay. After 7 days, liver and kidney were excised for histological examination. The levels of reactive oxygen species (ROS), malondialdehyde (MDA), and reduced glutathione (GSH), and activities of catalase and glutathione peroxidase (GPx) were determined in serum or homogenate using enzyme-linked immunosorbent assay (ELISA). The yield of MAP30 after purification was significantly increased. The results of MTT assay showed that MAP30 significantly and concentration-dependently inhibited the proliferation and migration of T24 cells ($p < 0.05$). The prepared liposomes had uniform hydrated particle size of 132.6 nm, with encapsulation efficiency of 78 %. The inhibitory effect of MAP30 liposome on T24 cells was significantly higher than that of MAP30, and MAP30 significantly increased the number of apoptotic cells ($p < 0.05$). Western blotting showed that MAP30 significantly promoted the expression of caspase 3 ($p < 0.05$), but did not significantly affect the expressions of bcl-2 and bax ($p > 0.05$). It also significantly down-regulated the expressions of NF-$_k$B, JNK and MMP2 ($p < 0.05$). Tumor formation was significantly inhibited, and tumor volume reduced in bladder cancer-bearing mice after treatment with MAP30 ($p < 0.05$). Histological examination showed that MAP30 induced mild histological changes in the liver and kidney of mice, and significantly increased the level of MDA at day 1 ($p < 0.05$). It also significantly and time-dependently increased ROS, but reduced GSH levels and activities of catalase and GPx ($p < 0.05$). However, MAP30 had no significant effect on DNA ($p > 0.05$). The apoptotic effect of MAP30 in T24 cells is mediated via activation of caspase-3 signaling pathway. The protein produces mild histological changes in the liver and kidney of mice, but has no significant effect on DNA.

Key words: Bladder cancer; Bitter gourd; MAP30; Apoptosis; Toxicity.

Introduction

Bitter gourd (*Momordica charantia*) belongs to the *Cucurbitaceae* family, and is widely distributed in Asia, Africa and parts of South America (1, 2). Due to its rich taste and high nutritional value, its fruits are used as vegetable in Asia. Different parts of the plant are used to treat metabolic disorders and viral infections (3). In the last few decades, a number of nutrient/medicinal compounds in bitter gourd have been purified and characterized, including cucurbitane-type triterpenoids, linolenic acid, potato and pumpkin-type protease inhibitors, ribonucleases (RNase MC1 and MC2), type 1 ribosome-inactivating proteins (RIPs) (napin-like RIP, momorcharins and MAP30), and type 2 RIP (*M. charantia* lectin/MCL) (3-5). Some of these compounds possess antidiabetic, anti-HIV and antitumor potential.

Ribosome-inactivating protein (RIP) is an RNA glycosylase that cleaves adenine-ribose glycosidic bonds in conserved loop of 28S ribosomal RNA, which is required for binding elongation factors (6, 7). The representative RIP *Momordica* anti-HIV protein of 30 kDa (MAP30) was first purified by Lee-Huang in 1990, and was demonstrated to possess anti-HIV activity (8). It has also been reported to be effective against herpes simplex virus (HSV) (9-11). It inhibits the proliferation of HIV-associated lymphoma cells infected with Kaposi's sarcoma-associated virus via regulation of viral and cellular genes necessary for viral and cell proliferation and apoptosis (12). Similarly, recombinant MAP30 pro-

duced using vectors such as *E. coli* and pumpkin leaves are effective against HIV-1, HSV and HHV8 (13, 14). It has DNA glycosylation and RNA N-glycosidase (ribosome inactivation) effects. Its anti-HIV and anticancer properties are attributable to its DNA glycosylation and/or DNA depurination/depyrimidination potential (11, 15). Studies have shown that MAP30 inhibits proliferation of glioblastoma (U87GM), breast cancer (BT20), epidemic cancer (A431), melanoma Malme-3 M, myeloma U266, nerve maternal tumor (SK-N-SH), prostate cancer (DU145) and liver cancer (Hep 3B) cells (14). However, only a few in-depth investigations have attempted to investigate the mechanisms involved in these effects. One of such studies revealed that the antitumor effect of MAP30 against estrogen independent human breast cancer cells (MDA-MB-231) was exerted via the down-regulation of HER2 expression (15). In another study, it was shown that the antiproliferative and apoptotic effects of recombinant MAP30 on human colorectal cancer cells (LoVo) occurred via the upregulation of bax and downregulation of bcl-2 (16).

Liposomes (fat emulsions) are used to encapsulate/deliver MAP30 because MAP30 cannot readily permeate cell membrane due to its hydrophilic nature (17). The liposome-coated protein is usually prepared using membrane hydration method. The aim of this study was to investigate the mechanism involved in the antitumor effect of MAP30 in T24 cells, and its potential toxic effect in mice.

Materials and Methods

Prokaryotic expression and purification of MAP30

The MAP30 gene sequence optimized using whole gene synthesis codon was introduced into restriction enzyme cut sites BamHIand XhoIat both ends, and then inserted into the prokaryotic expression vector pET-28a. The MAP30 which C-terminal contained His-tag was fused and expressed in BL21(DE3) *E. coli.* The expression was carried out at 16 °C, and the protein product was purified using Ni magnetic beads (#16-266, Sigma-Aldrich). The target protein was eluted with 300 mM imidazole (I5513, Sigma-Aldrich), and was thereafter subjected to dialysis and ultrafiltration.

Liposomal assembly of MAP30

Liposome-encapsulated MAP30 was prepared using, membrane hydration method as follows: 10 mg dipalmitoylphosphatidylcholine (DPPC, P0763, Sigma-Aldrich), 1 mg cholesterol, and 1 mg distearyl phosphatidylacetamide-methoxy polyethylene glycol 2000 (DSPE-mPEG2000, 147867-65-0, corden) were dissolved in chloroform, and thoroughly mixed and evaporated to form a uniform film. The residual chloroform was completely evaporated under vacuum overnight, and 1 mL of phosphate-buffered saline (PBS, 2 mg/mL) containing protein was then added. The liposome membrane was ultrasonically detached within 20 min in ice bath, and then hydrated on a shaker for 5 min to form a turbid liquid. The resultant turbid liquid was transferred to an Eppendorf (EP) tube, and ultrasonically irradiated with a probe at 15 % power (135 W) for 30 min to obtain a transparent uniform blue colored liposomal suspension. Ultrafiltration with 10 kDa ultrafiltration tube at 12, 000 g at 4 °C was performed once every 5 min to obtain filtrate and liposome suspension which was then washed with PBS. The protein quality was spectrophotometrically assessed at 280 nm. The particle size of the prepared liposome was measured using transmission electron microscopy. The efficiency of encapsulation was calculated as shown in Equation 1:

$$Encapsulation\ efficiency = 1 - (protein\ content\ in\ the\ filtrate - initial\ liquid\ protein\ content)\ \% \quad \ldots\ldots (1)$$

Cell culture

Human bladder carcinoma cells (T24) were purchased from the American Type Culture Collection (ATCC). T24 were digested with 0.25 % trypsin and seeded in 6-well plates at a density of 2.0×10^5 cells/well. After attaining 70 - 80 % confluency, the cells were washed twice with PBS. The cells were then cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) with or without liposome-encapsulated MAP30 at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. The group without liposome-encapsulated MAP30 served as control.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay) was performed using TUNEL assay kits (ab66110, Abcam) in line with the kit protocols.

MTT assay

MTT assay was performed using MTT assay kits (CGD1, Sigma-Aldrich) in line with the kit protocols. Cells in logarithmic growth phase were seeded in 96-well plates at a density of 1×10^5 cell/well, and cultured in Dulbecco's modified Eagle's medium (DMEM) for 24 h. Then, MAP30 (14.3 and 28.6 μM) was added to the cells and incubated for 72 h. At the end of the third day, 20 μL of MTT solution (5 mg/mL) was added to the wells, followed by incubation at 37 °C for 2 h. The medium was finally replaced with 150 μL of 0.1 % dimethyl sulfoxide (DMSO) to completely dissolve the formazan crystals formed. The absorbance of the samples was read in a microplate reader at 490 nm. The assay was performed in triplicate. Cell viability was calculated as shown in Equation 2:

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ the\ experimental\ group \times 100}{Absorbance\ of\ the\ control\ group} \quad \ldots\ldots (2)$$

Cell proliferation/cytotoxicity assay

Cell proliferation/cytotoxicity assay was performed using cell counting kit 8 (CCK-8, CK 04, Dojindo).

Western blotting

The following antibodies used in this study were purchased from Abcam: Bax (ab32503), Caspase3 (ab13847), GAPDH (ab8245), Bcl-2 (ab32124), Actin (ab179467) and NF-$_k$B (ab16502). The following antibodies used in this study were purchased from CST: JNK (9252), MMP2(4022) and Caspase8 (8592). Human liver cancer cells (Hep G2) were incubated with graded concentrations of MAP30 (14.3 and 28.6 μM) at different time

points (24, 36 and 48 h). The cells were washed with PBS and lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor. The resultant lysate was centrifuged at 12, 000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using bicinchoninic acid (BCA) protein kit. A portion of total cell protein (30 µg) from each sample was separated on 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. The blots were incubated overnight at 4 °C with primary antibodies for bax, caspase 3, bcl-2, NF-$_k$B, JNK, matrix metalloproteinase 2 (MMP2), and β-actin, each at a dilution of 1 to 1000. Thereafter, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Bio-rad gel imaging system Respective protein expression levels were normalized to that of β-actin which was used as a standard.

Transfection of mice with bladder cancer cells

Male mice weighing 40 to 50 g (mean weight = 45.0 ± 4.11 g) and age 2 - 3 weeks (Cancer Institute of the Chinese Academy of Medical Science) were transfected with bladder cancer cells (3 x 10^6 cells) for 48 h using lipofectamine 2000 transfection kit.

Determination of tumor volume in bladder cancer-bearing mice

Changes in tumor volume and occurrence of metastasis were assessed using luciferase assay.

Determination of toxic effect of MAP30

The possible toxic effects of MAP30 was also assessed. The mice were intraperitoneally injected with 2 mg/kg body weight (bwt) MAP30 (1 µg/µL), while the control group received an equivalent dose of PBS. Blood samples were collected at intervals of 10 min on day 1, day 3 and day 7. The liver and kidney were excised for histological examination. Portions of the tissues were used to prepare 20 % tissue homogenate. The levels of ROS (HR006, G.fan), MDA (CEA597Ge-1, USCNK), and GSH (CEA294Ge-1, USCNK), and activities of catalase and GPx were determined using their respective assay ELISA kits.

Single cell gel electrophoresis assay (comet assay)

The level of DNA damage was assessed using comet assay (ab238544, Abcam). Agarose (15 mg) was dissolved in 1 mL of PBS and heated on a water bath at 97 °C until it completely melted, and then cooled to 37 °C. The cell suspensions resulting from the trypsinization of liver and kidney were centrifuged at 1000 rpm for 5 min. The resultant packed cells were resuspended in 1 mL PBS, and then equal volume of agarose was added to reduce the gel concentration to 7.5 mg/mL, and spread on 24-well plate cover. Subsequently, 60 µL aliquot was seeded in the wells. The plates were incubated at 40°C for 40 min, and thereafter cooled for 30 - 60 min. The plate cover was rinsed twice with PBS, and the lysate was blotted with absorbent paper, and thereafter subjected to constant pressure electrophoresis for 20 min. At the end of the electrophoresis, the plates were blotted dry, and SYBR green (50 µL) was added to each well. Then, the plates were incubated for 5 min in the dark and photographed.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (20.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

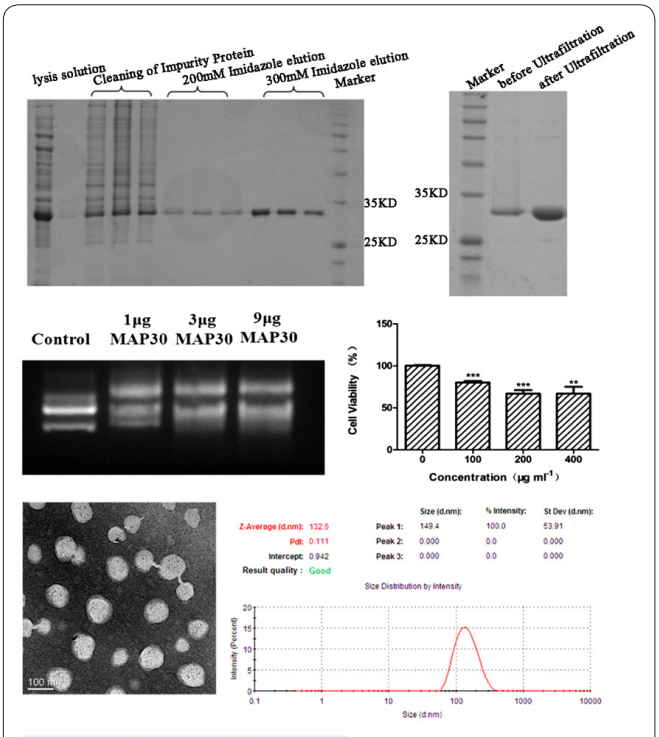
Results

Results of purification and construction of MAP30 liposome

The yield of MAP30 after purification was significantly increased. The purified protein exhibited DNA cleavage activity. The results of MTT assay showed that MAP30 significantly and concentration-dependently inhibited the proliferation of T24 cells ($p < 0.05$). The prepared liposomes had uniform hydrated particle size of 132.6 nm, and the encapsulation efficiency was 78 %. These results are shown in Figure 1.

Effects of MAP30 and MAP30 liposome on apoptosis of T24 cell

Both MAP30 and MAP30 liposome significantly



	crude solution	filtrate
A280	0.987	0.0737
volume (ml)	1	2.95

entrapment rate = (1-0.737 x 2.95/0.987) x 100%
= 78%

Figure 1. Purification and construction of MAP30 liposome . *p < 0.05; **p < 0.01; ***p < 0.001, when compared with control group.

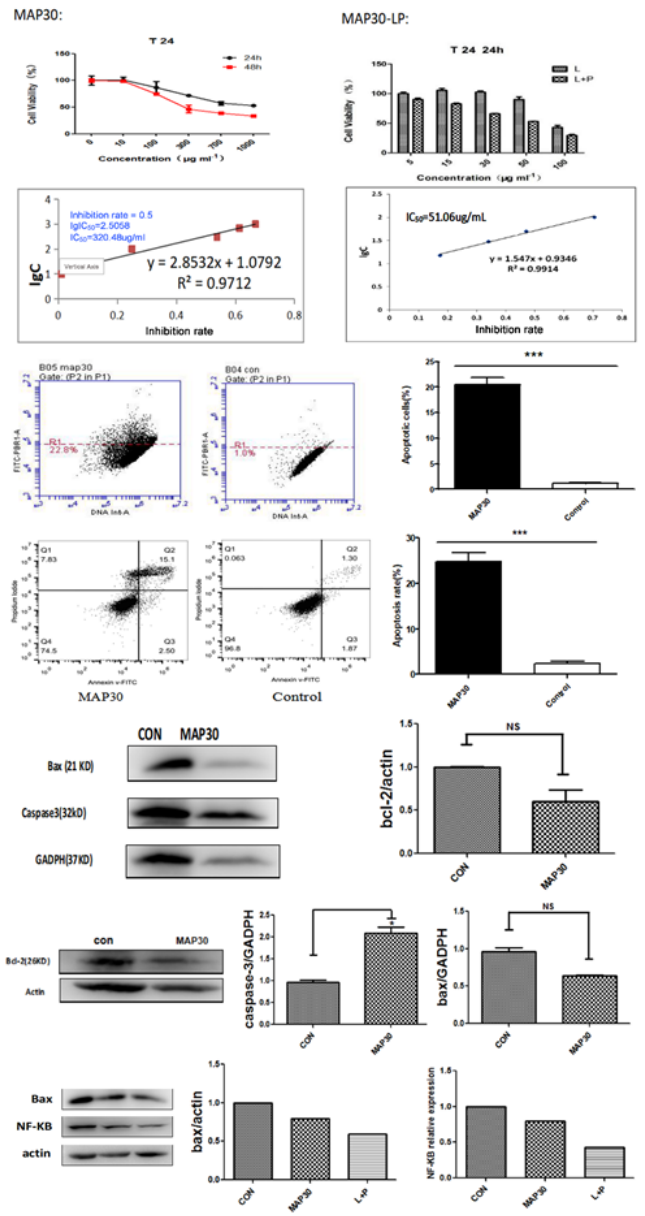


Figure 2. Effects of MAP30 on T24 cell apoptosis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

and concentration-dependently inhibited the proliferation of T24 cell ($p < 0.05$). The half maximal inhibitory concentration (IC_{50}) of MAP30 liposome (51.06 μg/mL) was significantly lower than that of MAP30 (320.48 μg/mL) ($p < 0.05$). The inhibitory effect of MAP30 liposome on T24 cells was significantly higher than that of MAP30, and MAP30 significantly increased the number of apoptotic cells ($p < 0.05$). The results of Western blotting showed that MAP30 significantly promoted the expression of caspase 3 ($p < 0.05$), but did not significantly affect the expressions of bcl-2 and bax ($p > 0.05$).

Effect of MAP30 on T24 cell proliferation and migration

As shown in Figure 3, treatment of T24 cells with MAP30 significantly down-regulated the expressions of NF-$_k$B, JNK and MMP2, and significantly reduced their migratory ability ($p < 0.05$).

Effect of MAP30 on tumor volume in bladder cancer-bearing mice

As shown in Figure 4, MAP30 significantly inhibited tumor formation and reduced tumor volume in bladder

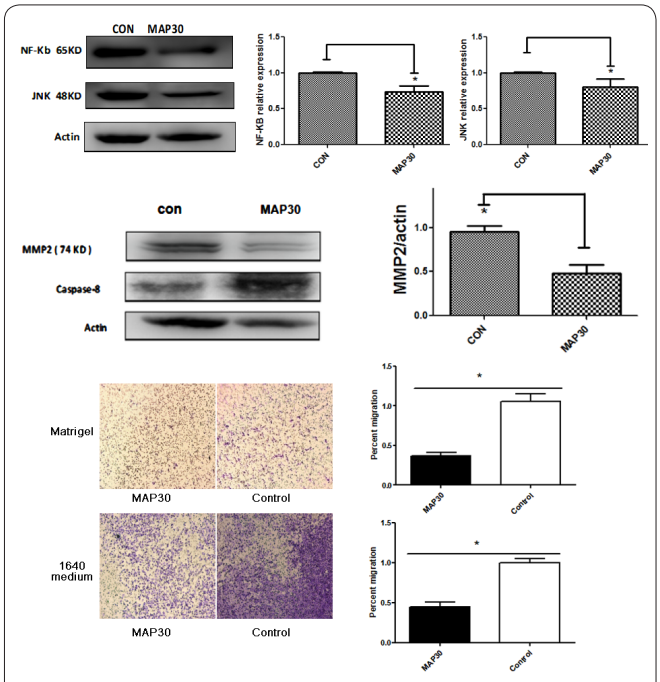


Figure 3. Effect of MAP30 on T24 cell proliferation and migration. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

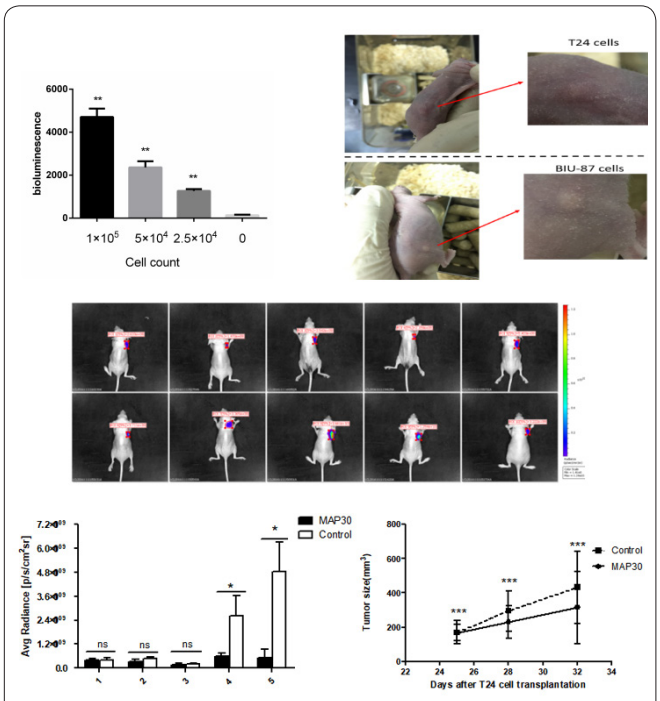


Figure 4. Effect of MAP30 on tumor volume. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

cancer-bearing mice ($p < 0.05$).

Toxicological effects of MAP30

As shown in Figure 5, MAP30 induced mild histological changes in the liver and kidney of mice, and significantly increased the level of MDA at day 7 ($p < 0.05$). It also significantly and time-dependently increased the level of ROS, but reduced the activities of catalase and GPx, and level of GSH ($p < 0.05$). However, MAP30 had no significant deleterious effect on DNA ($p > 0.05$).

Discussion

Bladder cancer is one of the most common tumors

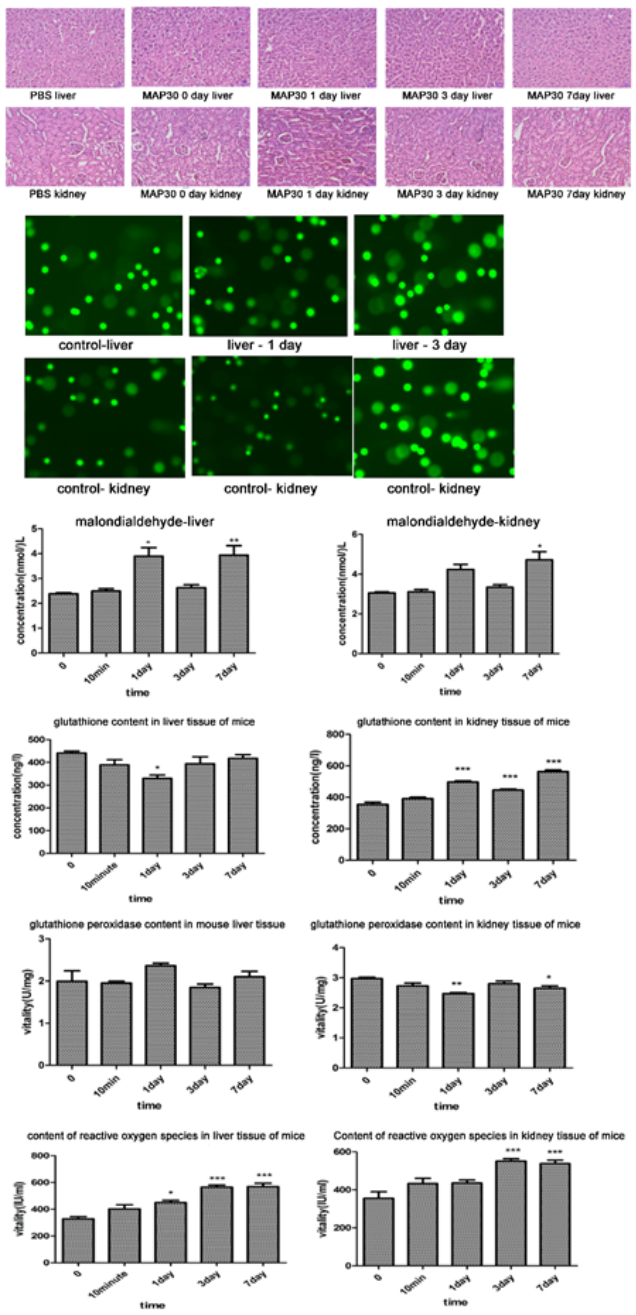


Figure 5. Toxicological effect of MAP30 protein. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with control group.

in the world (18-20). It is treated using surgical interventions and chemotherapy. Despite intensive treatment with combined multiagent chemotherapy and surgery, patients generally show poor prognosis and incurable relapse of the disease. Metastasis remains a huge challenge in patients with bladder cancer after chemotherapy and surgical intervention (31). Therefore, effective identification and development of novel molecular approaches to the diagnosis, treatment and prognosis of patients with bladder cance remain urgent clinical requirements.

Bitter gourd (Momordica charantia) is a common plant which widely distributed in Asia, Africa and parts of South America (1, 2). It has high medicinal value and are used to treat metabolic disorders and viral infections (3). Recent years, a number of nutrient/medicinal compounds in bitter gourd have been purified and characterized, including cucurbitane-type triterpenoids, linolenic acid, potato and pumpkin-type protease inhibitors, ribonucleases (RNase MC1 and MC2), type 1 ribosome-inactivating proteins (RIPs) (napin-like RIP, momorcharins and MAP30), and type 2 RIP (M. charantia lectin/MCL) (3-5).

Ribosome-inactivating proteins (RIPs) , an RNA glycosylase that cleaves adenine-ribose glycosidic bonds in conserved loop of 28S ribosomal RNA (6, 7), have attracted huge attention within the last few decades due to their ability to target a host of protein synthesis mechanism (21-25). Some RIPs have little or no detectable side effects since they recognize the unique dominant membrane characteristics of tumor cells (26-28). For instance, MAP30 was first purified by Lee-Huang in 1990, and was demonstrated to possess anti-HIV activity and be effective against herpes simplex virus (HSV) (8-11). Due to its DNA glycosylation and/or DNA depurination/depyrimidination potential (11, 15), MAP30 are effective against HIV-1. Artificially recombinant MAP30 has the same effect (13, 14), this also provides the possibility for the large-scale application of MAP30 in clinical practice. In recent years, the role of MAP30 in the occurrence and development of tumors has also attracted wide attention.Studies have shown that MAP30 has an inhibitory effect on a variety of tumors, but the specific molecular mechanism is still unclear (14).

MAP30 is not toxic to human sperm cells at doses that inhibit HIV-1 and HSV (11, 27). The MAP30 is effective against HIV and tumors. In this study, the protective effect of MAP30 against bladder cancer was investigated. It has been reported that MAP30 inhibits the growth of tumor cells including bladder cancer cells (30). It also inhibits the ability of cancer cells to form colonies in soft agar. It has been reported that extracts of bitter gourd fruits induce G2/M cell cycle arrest in MCF-7 breast cancer cells (29), and inhibit the expressions of G1/S-specific cyclins (D1, D2 and D3) in adrenocortical carcinoma cells (30). Under the same condition, cell cycle arrest at S phase was observed in the prostate cancer cells PC3 and LNCaP (31-33). The results of this study showed that MAP30 significantly and concentration-dependently inhibited the proliferation and migration of T24 cells. These results suggest that the cytotoxic effect of MAP30 may occur via cell cycle arrest at S phase.

Apoptosis has been shown to play a role in chemotherapy-induced tumor cell death (34). In this study, MAP30 significantly increased the number of T24 cells that became apoptotic, an indication that exogenously regulated caspase-3 may be involved in MAP30-induced apoptosis in T24 cell.

It has been speculated that apoptotic cell death is affected by a complex network of two subsets of pro-apoptotic proteins: multidomain pro-apoptotic proteins and BH3-only pro-apoptotic proteins, as well as the anti-apoptotic proteins of the bcl-2 protein family (20). The pro-apoptotic proteins bak and bid regulate mitochondrial membrane potential, inhibit cell cycle progression and cell survival, and promote apoptosis (35-37). The pro-survival protein bcl-2 provides a unique survival signal for cells and contributes significantly to tumor progression (38). In this study, the results of Western blotting showed that MAP30 significantly promoted the

expression of caspase 3, but did not significantly affect the expressions of bcl-2 and bax. These results suggest that MAP30- induced T24 cell apoptosis may not involve bcl-2.

The activation of NF-$_k$B and JNK promotes cancer cell proliferation and plays a role in the antitumor effect of certain chemotherapeutic agents (39). In this study, treatment of T24 cells with MAP30 significantly downregulated the expressions of NF-$_k$B, JNK, and MMP2. This may contribute to MAP30-induced cytotoxicity and caspase-3 mediated apoptosis. It is likely that MAP30 may inhibit cell migration by inhibiting MMP2.

Chemotherapeutic agents are known to exert their actions by targeting signaling proteins/membrane receptors or kinases. The membrane protein/receptor targeted by MAP30 in tumor cells remains unknown. However, studies on trichosanthin (TCS) may provide some clue. Trichosanthin (TCS) is a type I RIP isolated from the roots of *Trichosanthes kirilowii,* and it has 59 % sequence homology with MAP30 (7). Trichosanthin enters tumor cells via interaction with two receptor proteins: α2-macroglobulin receptor and low-density lipoprotein receptor-associated protein (LRP) (7). An understanding of the pathway through which MAP30 enters tumor cells may provide an important clue to the precise molecular mechanism involved in its anticancer effect.

The apoptotic effect of MAP30 in T24 cells is mediated via activation of caspase-3 signaling pathway. The protein produces mild histological changes in the liver and kidney of mice, but has no significant effect deleterious effect on DNA.

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Conflict of Interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Conghui Han and Lin Hao; Zhenduo Shi, Kun Pang, Wen Yang, Jiahe Zhou, Bingzheng Dong, Zhiguo Zhang, Yan Zhao, Lin Hao, Conghui Han collected and analysed the data; Zhenduo Shi, Kun Pang and Wen Yang wrote the text and all authors have read and approved the text prior to publication.

Zhenduo Shi, Kun Pang and Wen Yang contributed equally to this work and should be considered as co-first authors.

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