Original Article

Investigation of the Inhibitory Effect of Platycodin D in Human Transitional Cell Carcinoma Cell Line 5637

(platycodin D / transitional cell carcinoma / proliferation / anti-tumour / migration)

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Abstract. Platycodin D is an active component isolated from Chinese herb *Platycodonis radix* with various pharmacological activities, such as antitussive, expectorant, anti-inflammatory, and analgesic effects. Interestingly, platycodin D also exerts anticancer effects against several types of cancer. However, few studies on the anti-tumour effects of platycodin against urinary bladder cancer have been reported. In this study, we explored the anti-tumour effect of platycodin D against human bladder cancer and its mechanisms *in vitro* and *in vivo*. We found that platycodin D had significant anti-proliferative effects on four types of cancer cells, especially the 5637 bladder cancer cell line, and exerted these effects by preventing cell cycle progression from G_0/G_1 to S phase, down-regulating Ki-67 and cyclin D1 protein expression and up-regulating P21 protein expression. Furthermore, platycodin D inhibited 5637 cell migration by decreasing twist-related protein 1 (Twist1) and matrix metallopeptidase 2 (MMP2) expression and exerted significant tumour-suppressive effects in tumour-bearing nude mice. Platycodin D also increased caspase-9, caspase-8, caspase-3, and p53 expression and decreased Bcl-2 expression in tumour tissues. Taken together, our results provide a theoretical basis for application of platycodin D in treating urinary bladder cancer.

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Abbreviations: CSP – cisplatin, DMSO – dimethyl sulphoxide, IC50 – half-maximal inhibitory concentration, iNOS – inducible nitric oxide synthase, IR – inhibition ratio, MAPK – mitogen-activated protein kinase, MIBC – muscle-invasive bladder cancer, MMP2 – matrix metallopeptidase 2, NMIBC – non-muscle-invasive bladder cancer, PBS – phosphate-buffered saline, PD – platycodin D, Twist1 – twist-related protein 1.

Introduction

Urinary bladder cancer can be caused by smoking, benzidine exposure, or excess meat and alcohol intake, with a genetic background, and is one of the most common diseases of the urinary system (Siegel et al., 2019). Various therapeutic strategies, such as surgical management and bladder-preserving adjuvant therapies, have been applied based on the diverse manifestations of bladder cancer, including non-muscle-invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC), and metastatic bladder cancer (Park et al., 2014a; Babjuk et al., 2017). Although these treatments are partially effective in tumour elimination and improving patient survival, some undesirable outcomes still pose practical clinical challenges. The probability of NMIBC recurrence, which can then progress to MIBC, is associated with high mortality (Sylvester et al., 2006).

Radical cystectomy with pelvic lymph node removal is a standard operation for MIBC; however, it is often unsatisfactory because the quality of life is compromised (Gore et al., 2010). A bladder-preservation therapy, combining chemo- and radiotherapy, is therefore often preferred (Bodgi et al., 2019), although it is linked with radiation-related intestinal complications (Langsenlehner et al., 2010) and compromised immune responses (Sio et al., 2014). Development of molecular targeted drugs and immunotherapy acting against urinary bladder cancer is promising (Thompson et al., 2015; Smolensky et al., 2016), but could be strengthened by identification of new effective drugs.

Some herbs may become active compounds for urinary bladder cancer treatment. *Platycodon grandiflorum* (*P. grandiflorum*) is a perennial dicotyledonous herb that grows in the Changbai Mountain area of China. One of the most active pharmacological ingredients in *P. grandiflorum* is platycodin D, which has been shown to have anti-inflammatory, expectorant and cough-relieving effects, as well as blood lipid-lowering effects (Zhao and Kim, 2004). Recently, the anti-tumour and immunomodulating effects of platycodin D were reported. Platycodin D displayed toxic effects against breast cancer (Tang et al., 2014), liver cancer (Li et al., 2015b),

prostate cancer (Zhou et al., 2015), and lung cancer (Li et al., 2016) cells by activating multiple cellular mechanisms (Khan et al., 2016).

The growth of lung tumours was inhibited by platycodin D due to increased inducible nitric oxide synthase (iNOS) and TNF-α immunoreactivity in the tumours or inhibition of Bcl-2 expression and telomerase activity (Park et al., 2005, 2014b). Li et al. (2015b) confirmed that platycodin D-induced hepatocellular cell proliferation was inhibited, and apoptosis was further amplified by co-treatment with autophagy inhibitors and platycodin D. In addition, most anti-tumour drugs are synthetic chemicals that damage both tumour cells and normal cells. However, platycodin D may minimize possible damage to tissues, as it was shown to ameliorate cisplatin (CSP)-induced renal injury by decreased intraluminal cast formation and diminished epithelial desquamation (Kim et al., 2012). Platycodin D was also shown to exert a protective effect against liver injury in alloxan-induced diabetic mice via regulation of the Treg/Th17 cell balance (Chen et al., 2015).

Cell apoptosis can be triggered by intrinsic and extrinsic apoptotic pathways, which involve different sets of proteins. In the intrinsic apoptotic pathway, the Bcl/Bax family of proteins can promote or inhibit apoptosis by destroying or maintaining the integrity of the mitochondrial membrane. Additionally, p53 can act on Bcl-2 and inhibit its expression. Furthermore, caspase-9 activation cleaves and activates procaspase, producing effector caspase-3. There are also many initiating factors for the extrinsic apoptotic pathway. TNF and Fas are initiating factors, and caspase-8 activation further mobilizes apoptosis executors caspase-3, 6, and 7 (Pfeffer and Singh, 2018; Xu et al., 2019).

In the current study, the anticancer effects of platycodin D against human bladder cancer and its mechanisms were explored both *in vitro* and *in vivo*. Platycodin D inhibited cell proliferation and migration by activating dual apoptotic pathways. Our data provide unprecedented evidence for the application of platycodin D in the treatment of bladder cancer.

Material and Methods

Cell culture

A human cervical cancer cell line (HeLa), human liver cancer cell line (HepG-1), and human bladder carcinoma transitional cell lines (T24 and 5637) were purchased from Cusabio (Beijing, China). The HeLa and T24 cells were cultured in DMEM (Gibco; Beijing, China), and the HepG-1 and 5637 cells were cultured in RPMI 1640 medium (HyClone; Beijing, China) supplemented with 10% foetal bovine serum (Thermo Scientific; Rockford, IL) at 37 °C and cultured under 5% CO_2. Platycodin D was purchased from Shifeng Biological Technology (Shanghai, China) and dissolved in saline.

MTT assay

Cells were seeded into 96-well plates at a density of 10,000 cells per well. After 4 h of incubation, platycodin D was added at the indicated concentration (5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, or 80 μg/ml) for further incubation for 20 h, with four replicate wells for each group. Saline was added to the wells as a negative control group. Then, 100 μg/well MTT was added, followed by incubation for another 4 h. Finally, the solution was removed, and 150 μl of dimethyl sulphoxide (DMSO) was added. The plates were shaken for 10 min, and the absorbance values at 492 nm were read using an RT-2100C microplate reader (Rayto, Shenzhen, Guangdong, China). The inhibition ratio (IR) was calculated using the following formula:

$$\text{inhibition ratio (\%)} = (1 - A_{\text{Experiment}} / A_{\text{Control}}) \times 100\%$$

To measure the growth curve of 5637 transitional carcinoma cells, 20 μg/ml platycodin D was added for further incubation (12, 24, 48, or 72 h). Then, the absorbance values were measured.

Flow cytometry

In this experiment, 5×10^6 5637 cells were seeded into a culture flask. After treatment with platycodin D or saline for 24 or 48 h, the cells were collected and washed three times with cold phosphate-buffered saline (PBS). The cells were resuspended in 1 ml of cold PBS and stained with a Cycle Test™ Plus DNA Kit (Bogoo; Shanghai, China). The cell cycle distribution was analysed with Mod Fit 3.0 software using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ).

Wound-healing assay

5637 cells were cultured in 6-well plates at a density of 1×10^6 cells/well. Once the confluence rate reached approximately 80 %, a wound was carefully made in the cell monolayer using a pipette tip. The cells were washed three times with PBS for 3 min and treated with platycodin D. Images of the wound were taken and recorded from the same well at 0, 24 and 48 h after the different treatments.

Immunocytochemical staining

After treatment with platycodin D or saline for 24 h, fixed cells or tissues were washed with PBS, permeabilized with 1% Triton X-100 for 20 min, and then incubated with 10% goat serum for 20 min. The monolayer cells were incubated with antibodies against cyclin D1 and Ki-67 (Santa Cruz; Wuhan, Hubei, China). Tissues were incubated with antibodies against caspase-8, caspase-9, caspase-3, p53 and Bcl-2 (1:200, Bogoo) overnight at 4 ℃. The cells were washed three times in PBS for 5 min and then incubated with secondary antibodies. Slides were incubated with streptavidin-horseradish peroxidase at 37 °C for 30 min and stained with diaminobenzidine (DAB) at room temperature without light for

10 min. Then, they were dehydrated using a graded ethanol series, mounted with neutral gum, and observed by optical microscopy. The positivity rate was calculated with the following formula:

positivity rate (%) =
= (positive cell number/total cell number) × 100%

Western blot analysis

Treated cells were harvested and lysed with RIPA buffer (Biomed; Beijing, China) on ice for 30 min. Proteins were extracted by centrifugation, and protein concentrations were measured using the DC™ Protein Assay (Bio-Rad; CA). Then, 40 μg of protein was loaded into each well of 10% SDS-PAGE gel, and the separated proteins were transferred onto PVDF membranes (ZSGB-BIO, Beijing, China). The membranes were blocked in 5% milk for 1 h and then incubated with primary antibodies against P21, cyclin D1, MMP2, Twist1 (1 : 250, Santa Cruz Biotechnology; Dallas, TX), or β-actin (1 : 250, Cell Signaling Technology; Danvers, MA) for 2 h. The PVDF membranes were covered with secondary antibodies (1 : 500) for 1 h, and target protein bands were visualized with ECL developing solution (Solarbio, Beijing, China).

Animal experiments

All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85.23, revised 1985). Four-week-old male BALB/c nude mice weighing approximately 18–20 g were purchased from the Laboratory Animal Center of Peking Union Medical College (Beijing, China). The mice were maintained under aseptic conditions (temperature: 18–22 °C, humidity: 50–60 %). Then, 5637 cells (1×10^7 cells suspended in 100 μl of PBS) were subcutaneously injected into the left thigh of each mouse. The tumours were allowed to grow for 2 weeks, and the model mice were used for subsequent experiments. The mice (N = 5 per group) were orally administered 1 ml of saline or platycodin D at a dose of 300 mg/kg daily for 2 weeks. The anti-tumour rate was calculated based on tumour weight with the following formula:

anti-tumour rate (%) =
= ($weight_{Control}$ – $weight_{Experiment}$) / $weight_{Control}$ × 100%

Statistical analyses

All data are expressed as the mean ± standard deviation and were compared for statistical significance by the *t*-test (2 groups) or ANOVA (> 2 groups) using SPSS 13.0 software. Sidak's multiple comparison test was used following ANOVA. Differences for which P values < 0.05 were considered significant. Each experiment was performed three times.

Results

Platycodin D had an inhibitory effect on the proliferation of cancer cells

Several cancer cell lines were used to detect the anti-proliferative effects of platycodin D, as shown in Fig. 1A. HeLa, HepG-1, T24 and 5637 cells were treated with different concentrations of platycodin D, and all inhibition rates increased upon platycodin D treatment in a dose-dependent manner (P < 0.0001). We found that the inhibitory effect was more obvious in 5637 cells, in which the half-maximal inhibitory concentration (IC50) of platycodin D was 18.53 μg/ml. Platycodin D at 20 μg/ml was chosen as the experimental drug concentration for the next experiment to reduce errors in drug administration. As shown in Fig. 1B, the proliferation rate of 5637 cells treated with platycodin D for 72 h was almost

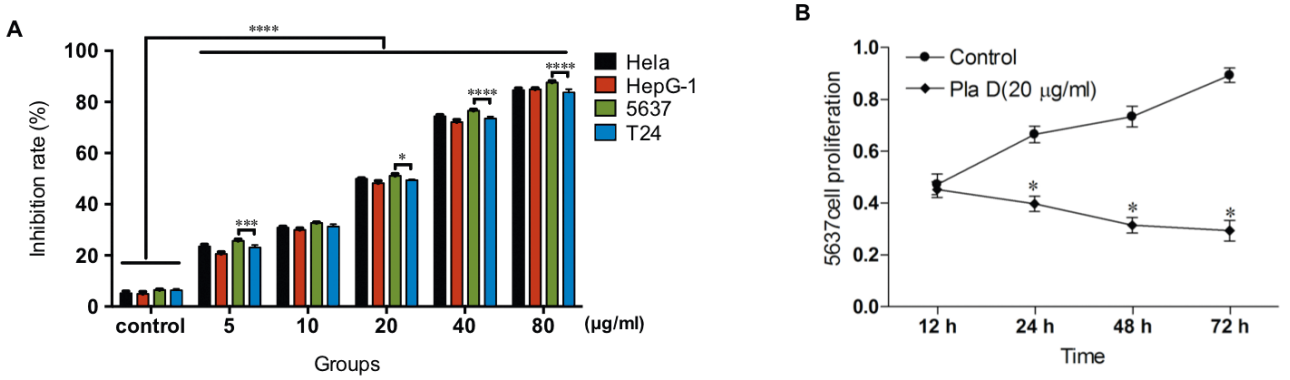


Fig. 1. Platycodin D inhibited growth of 5637 cancer cells. Cells were treated with saline or 20 μg/ml platycodin D (Pla D). (**A**) Platycodin D inhibited HeLa, HepG-1, 5637, and T24 cell proliferation. (**B**) Platycodin D inhibited 5637 cell proliferation in a time-dependent manner. Inhibition rates were measured by MTT assay. Data from three independent experiments are presented as the mean ± SD, and asterisks (*) indicate a significant difference between groups (*P < 0.05; ***P < 0.001; ****P < 0.0001).

40 % ($P < 0.05$). The proliferation ability of the 5637 cells gradually decreased in a time-dependent manner after platycodin D treatment.

Platycodin D inhibited 5637 cell proliferation by altering the cell cycle

5637 cell cycle profiles were detected after platycodin D treatment using flow cytometry. As shown in Fig. 2A, the proportion of cells at G_0/G_1 phase after platycodin D treatment for 48 h was approximately 65.85 ± 0.92 %, which was significantly higher than that of the control group ($P < 0.01$). Correspondingly, the proportion of cells in the platycodin D treatment group at S phase was significantly lower than that of the control group at 48 h ($P < 0.01$). Meanwhile, platycodin D treatment led to a decline in the proliferation index at 24 and 48 h (Fig. 2B). Thus, platycodin D inhibited 5637 cell proliferation by preventing cell cycle progression from G_0/G_1 to S phase.

Ki-67 and cyclin D1 play a pivotal role in cell proliferation (Scholzen and Gerdes, 2000; Seiler et al., 2013). We examined the expression of Ki-67 and cyclin D1 in 5637 cells using immunocytochemical staining (Fig. 2C). After 24 h of treatment, the proportions of 5637 cells treated with platycodin D that were positive for Ki-67 and cyclin D were significantly lower than those among the control group (Fig. 2D; $P < 0.05$). These results suggest that platycodin D inhibited 5637 cell proliferation by suppressing the protein expression of Ki-67 and cyclin D1.

Furthermore, we used western blot analysis to detect the expression of cyclin D1 and p21. As shown in Fig. 2E, the expression of cyclin D1 in the control group was higher than that in the experimental group at 24 and 48 h ($P < 0.05$), which was consistent with the results of immunocytochemical analysis. However, the expression of p21 increased after platycodin D treatment, but there was no significant difference between its expression after 24 and 48 h. Our data further suggest that platycodin D inhibited 5637 cell proliferation by up-regulating the expression of p21.

Platycodin D inhibited 5637 cell migration by suppressing MMP2 and Twist1 expression

To investigate the inhibitory effect of platycodin D on cell migration, a wound-healing assay was performed to measure the migration of 5637 bladder cancer cells with or without platycodin D treatment. As shown in Fig. 3A and B, 5637 cells in the control group showed substantial migration, and the distance between 5637 cells at the wound edges was small after 48 h, while the cells in the control group treated with platycodin D migrated more slowly, suggesting the anti-migration effect of platycodin D.

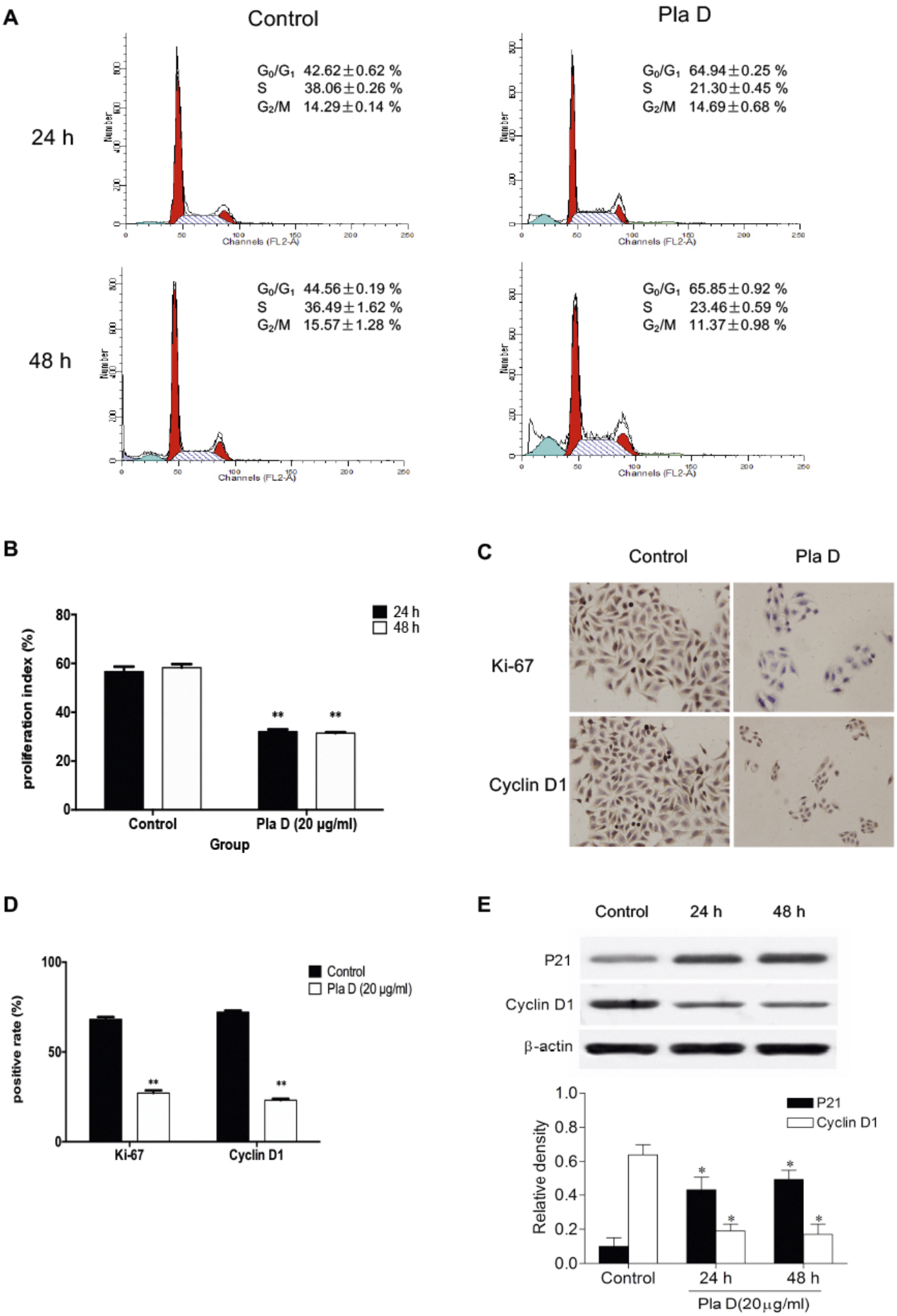
Additionally, we confirmed that the expression levels of essential proteins MMP2 and Twist1 correlated with tumour aggressiveness. As shown in Fig. 3C, western blot analysis revealed a marked decrease in the expression of MMP2 and Twist1 in 5637 cells after platycodin D treatment at 24 and 48 h, but these two proteins were highly expressed in the control group. The results above indicate that platycodin D suppressed 5637 cell motility by down-regulating MMP2 and Twist1 expression.

Platycodin D suppressed tumour growth by activating proteins involved in apoptosis

To examine the tumour-suppressive effect of platycodin D, 5637 cell tumour-bearing mice were orally given saline or platycodin D after tumour formation, and changes in tumour weight were measured. As shown in Table 1, the tumour weight was dramatically reduced by platycodin D treatment compared to that in the control group, and the platycodin D anti-tumour rate was approximately 57.63 %. Additionally, the administration of platycodin D significantly suppressed tumour growth compared with that in the control group (Fig. 4A).

In addition, to better understand the anti-tumour mechanism of platycodin D, we detected the expression levels of several proteins associated with cancer development (apoptotic initiators caspase-8/9, executor caspase-3, and anti-apoptotic proteins Bcl-2 and p53) using immunocytochemical staining (Table 2). As shown in Fig. 4B, cells in the platycodin D-treated group showed a stronger brown colour than those in the control group, indicating increased caspase-8/9, caspase-3 and p53 levels. As shown in Fig. 4C, the expression levels of these proteins were significantly higher than those in the control group ($P < 0.01$). However, the expression of anti-apoptotic protein Bcl-2, which was highly expressed in the control group, declined after treatment with platycodin D ($P < 0.01$). Taken together, these data indicated that platycodin D efficiently exerted an anti-tumour

Fig. 2. Anti-proliferative effect of platycodin D against 5637 cancer cells. 5637 cells were treated with saline or 20 μg/ml platycodin D. (**A**) Platycodin D interfered with the cell cycle profile. The distributions of cells at each phase of the cell cycle were determined by flow cytometric analysis. (**B**) Platycodin D decreased the proliferation index of 5637 cells, which was calculated as follows: proliferation index = (S + G2 / M) / (G0 / G1 + S + G2 / M). (**C**) Platycodin D down-regulated expression of Ki-67 and cyclin D1. The expression levels of Ki-67 and Cyclin D1 were determined at 48 h after treatment by immunocytochemical staining. Representative images are shown; original magnification, × 200. (D) Platycodin D reduced the expression of Ki-67 and cyclin D1. (E) The expression of p21 and cyclin D1 in 5637 cells was determined by western blot analysis. β-Actin acted as a loading control. Asterisks (*) indicate a significant difference between ▶▶ groups (*$P < 0.05$; **$P < 0.01$).


A
Control
Pla D
24 h
48 h
G0/G1 42.62±0.62 %
S 38.06±0.26 %
G2/M 14.29±0.14 %
G0/G1 64.94±0.25 %
S 21.30±0.45 %
G2/M 14.69±0.68 %
G0/G1 44.56±0.19 %
S 36.49±1.62 %
G2/M 15.57±1.28 %
G0/G1 65.85±0.92 %
S 23.46±0.59 %
G2/M 11.37±0.98 %
Number
Channels (FL2-A)
B
proliferation index (%)
24 h
48 h
Control
Pla D (20 μg/ml)
Group
C
Control
Pla D
Ki-67
Cyclin D1
D
positive rate (%)
Control
Pla D (20 μg/ml)
Ki-67
Cyclin D1
E
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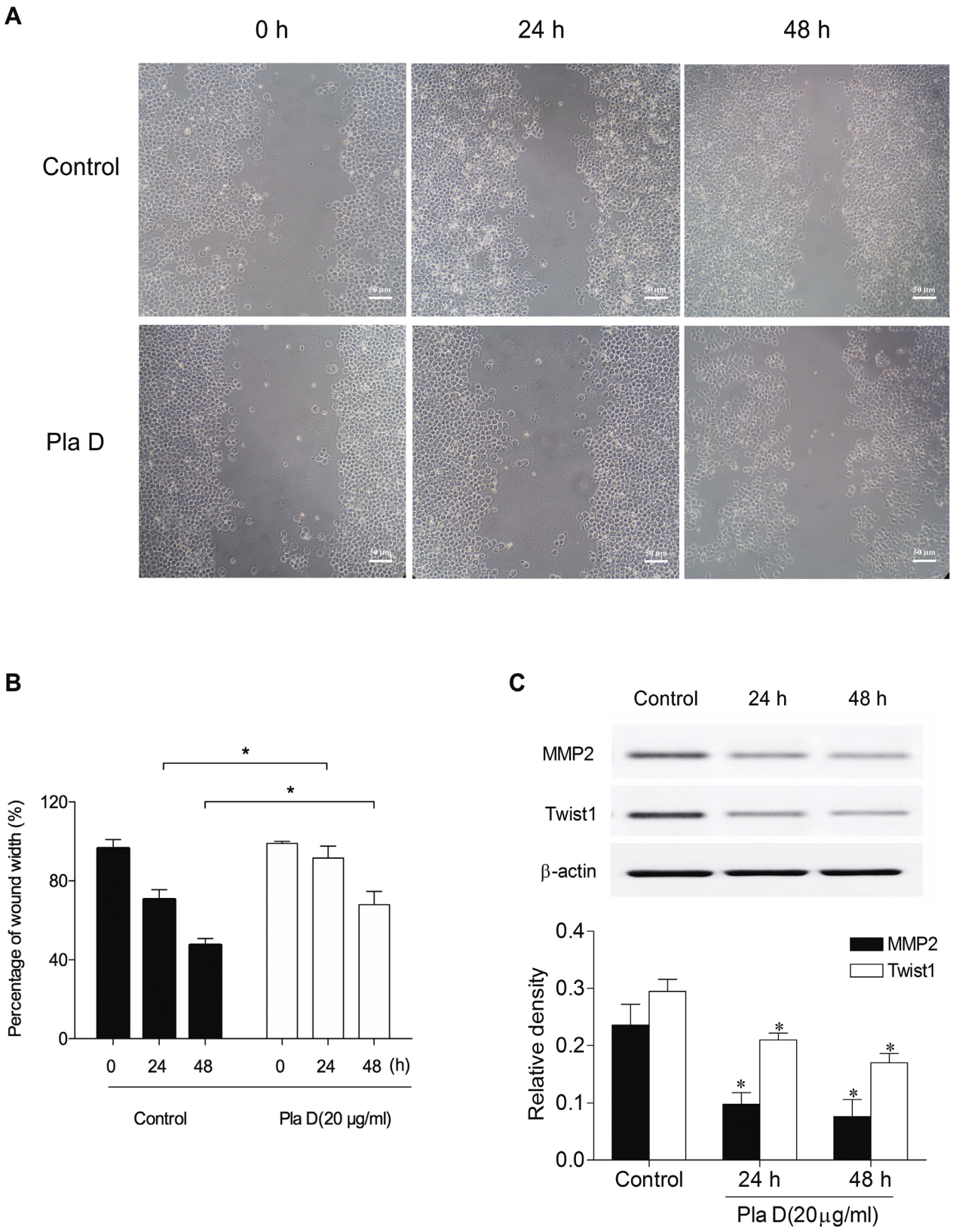

Fig. 3. Platycodin D inhibited 5637 cancer cell migration. 5637 cells were treated with saline or 20 μg/ml platycodin D for 0, 24 or 48 h. (**A**) 5637 cell migration was evaluated using a wound-healing assay. Cell migration was inhibited by platycodin D; original magnification, × 100. (**B**) The percentage of the remaining wound width was decreased in the platycodin D group compared to the saline group. Data are presented as the mean ± SD (*: $P < 0.05$). (**C**) Platycodin D suppressed expression of MMP2 and Twist1. Protein expression levels were determined by western blot analysis. β-Actin was applied as the loading control. Data are presented as the mean ± SD, and asterisks (*) indicate a significant difference between groups (*$P < 0.05$).

Table 1. Anti-tumour rates of the saline and platycodin D groups

Group	Weight (g)	Anti-tumour rate (%)
Control	4.34 ± 0.77	–
Pla D	1.84 ± 0.47*	57.63 %

Compared to the control group, *P < 0.05

Table 2. Molecules in the extrinsic and intrinsic apoptotic pathways

Classic apoptosis pathway	Molecules
intrinsic	p53 Bcl-2 caspase-9
extrinsic	caspase-8

effect by up-regulating multiple proteins involved in the apoptotic pathway.

Discussion

Bladder cancer is one of the most common diseases of the urogenital system. The main types of this cancer are NMIBC and MIBC. Over 90 % of bladder cancers can be characterized as transitional cell carcinoma. Appropriate treatments and prognosis are evaluated depending on how deeply the cancer has invaded the bladder wall and the histological findings (Koroltchouk et al., 1987). Modern medical technologies for treating bladder cancer, such as complete endoscopic resection with or without intravesical therapy, radical cystectomy with urinary diversion, chemo- or radiotherapy and bladder preservation, help palliate patients' symptoms. However, a new pharmacological therapy is still needed due to the various toxic effects and side effects of existing therapies and high frequency of recurrence and mortality (Metts et al., 2000; Prado et al., 2014).

Previous studies have shown that platycodin D may have an anti-proliferative effect against various cancers, including prostate cancer cells, leukaemia cells, and lung cancer cells, and promote cell apoptosis by activating multiple pathways (Kim et al., 2008; Zhao et al., 2015; Zhou et al., 2015). However, studies concerning the toxic effect of platycodin D on bladder cancer have not been reported. Thus, our study mainly explored the anticancer effects of platycodin D on bladder cancer, with a focus on three features: the anti-proliferative, anti-migration and tumour-suppressive effects of platycodin D. We found that platycodin D exerted an effective toxic effect on bladder cancer.

According to our data, platycodin D suppressed proliferation of cervical and liver cancer cells, which is consistent with the previously reported results of an MTT assay (Li et al., 2015b; Zeng et al., 2016). It also inhibited growth of the NMIBC cell line 5637 and MIBC cell line T24. However, its anti-proliferative effect on 5637 cells was greater, and the migration of the 5637 cells was gradually decreased by platycodin D, which indicates that platycodin D might be an effective drug for bladder cancer therapy. In addition, platycodin D disturbed the 5637 cell cycle profile, as evidenced by flow cytometry results, which showed that the number of 5637 cells at the G_2/M and S phases obviously declined after platycodin D treatment.

The Ki-67 protein is a proliferation marker that regulates cell cycle progression in human tumour cells. It can be detected at late G_1 phase and peaks at M phase of the cell cycle (Gerdes et al., 1984; Scholzen and Gerdes, 2000). Cyclin D1 is over-expressed in malignant cancer and plays a critical role in the regulation of cell cycle progression by binding the Cdk4 protein to shorten the G_1 phase (Fong et al., 2000; Wang et al., 2014). Cancer-suppressor gene *p21* is an important mediator that halts cell cycle progression by repressing the activity of the cyclin D1/Cdk4 complex when p21 is in the cell nucleus, thereby blocking DNA synthesis (Chen et al., 1995; Zhao et al., 2014). In this study, we confirmed that platycodin D could decrease cyclin D1 expression in 5637 cells, which prolonged G_0/G_1 phase. The accumulation of 5637 cells in G_0/G_1 phase may be associated with a decrease in the expression of indicator Ki-67 due to platycodin D. Platycodin D significantly increased p21 expression, which offers further evidence that p21 is a regulator involved in cell cycle control, and platycodin D arrested 5637 cells at G_1 phase by regulating expression of multiple related proteins.

In addition, numerous studies have shown that disturbances in the regulation of caspase activation play a key role in the acceleration of cancer cell death both *in vitro* and *in vivo.* Whichever pro-apoptotic pathway is activated by induction factors, effector caspases (caspases-3, 6 and 7) exert an apoptotic effect. Additionally, caspases-2, 8, 9 and 10 are initiator caspases. Both caspase-8 and caspase-9 can activate the effector caspase-3, causing disintegration of the cellular structure (Green and Kroemer, 1998; Koff et al., 2015; Shalini et al., 2015). Moreover, the *p53* gene regulates apoptosis by interacting with Bcl-2 family members, which play an important role in intrinsic mitochondrial pathways. Additionally, the *Bax* gene expression is deregulated by p53 via direct transcriptional activation of the *Bax* promoter with concomitant down-regulation of Bcl-2 (Song et al., 2014).

Our previous study confirmed that platycodin D promoted 5637 cell apoptosis *in vitro.* Apoptotic bodies, lipid droplets and damaged mitochondria were observed by transmission electron microscopy. Platycodin D increased protein expression of the related apoptotic proteins caspases-8, 9, and 3 and mRNA expression of p53 and Bax. In contrast, platycodin D decreased protein expression of survivin and livin and mRNA expression of Bcl-2. Thus, we concluded that platycodin D promot-

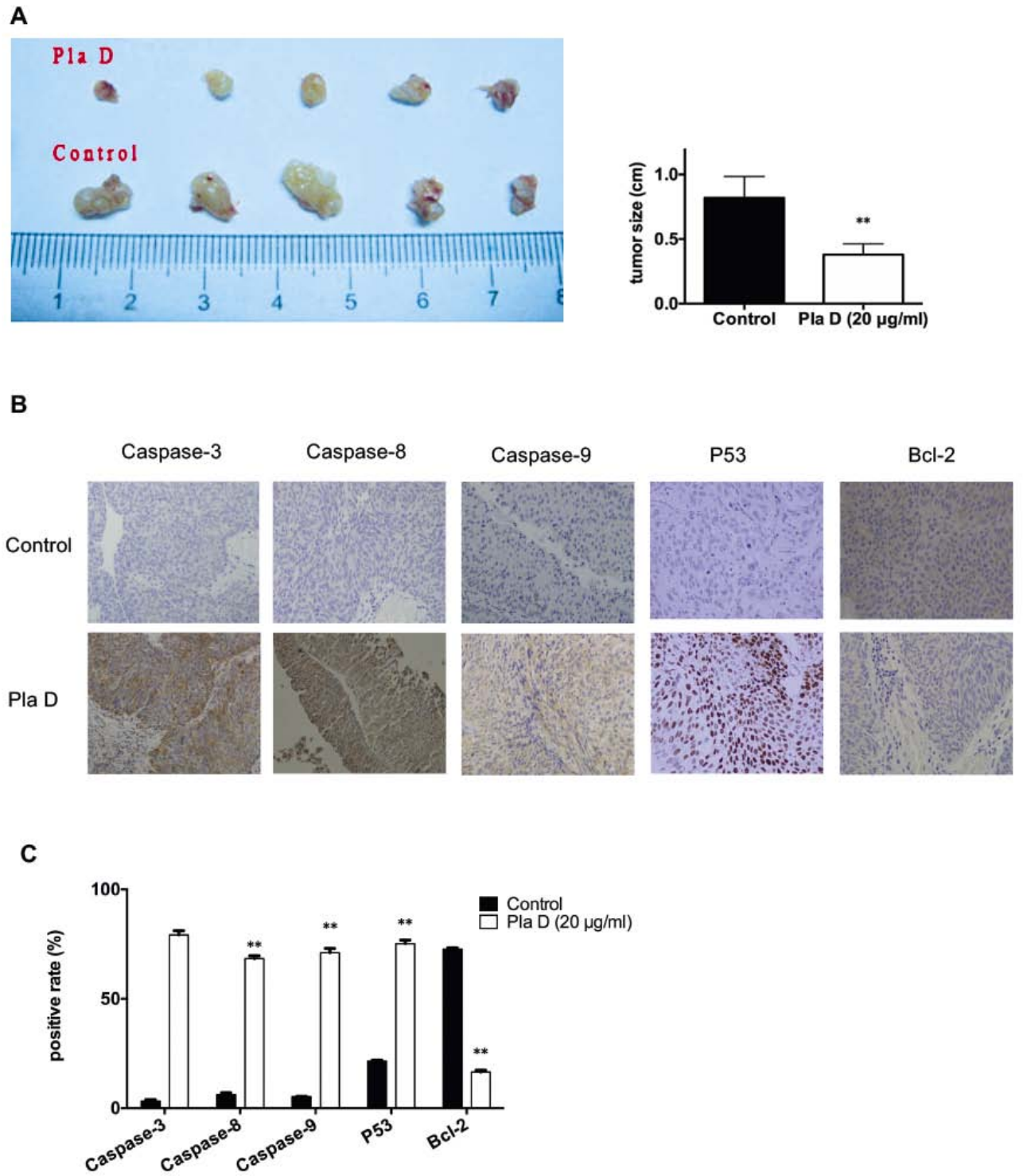

Fig. 4. Platycodin D suppressed tumour growth by regulating expression of apoptosis-related proteins. Saline or platycodin D was orally administered to 5637 tumour-bearing mice for 2 weeks. (**A**) Tumour sizes are shown. (**B**) Platycodin D up-regulated expression of caspases-3/8/9 and p53 and down-regulated expression of Bcl-2. The expression levels of proteins in tumour tissues were determined by immunocytochemical staining. Representative images are shown; original magnification, × 200. (**C**) Platycodin D altered apoptosis-related protein expression. Asterisks (*) indicate a significant difference between two groups (**P < 0.01).

ed 5637 cell apoptosis via mitochondrial and death receptor pathways. Additionally, platycodin D can inhibit other proliferation factors in addition to the caspase family (Li et al., 2018). In this study, we focused on its anti-tumour effect *in vivo.* We found that platycodin D effectively suppressed bladder tumour growth, with a decrease in tumour weight observed in mice orally treated with platycodin D. This was consistent with our *in vitro* finding that platycodin D promoted expression of caspases-8 and 9, which in turn triggered activation of caspase-3 in tumour tissues. Down-regulated Bcl-2 expression and up-regulated p53 expression were also observed in the bladder tumours after platycodin D treatment. Thus, combined with previous data, our data suggest that platycodin D may exert its anti-tumour effect against bladder cancer through extrinsic receptor-mediated and intrinsic mitochondrial pathways.

The treatment options for metastatic bladder cancer are limited because of its complicated multistep mechanism. However, our data demonstrated that platycodin D effectively inhibited migration of bladder cancer 5637 cells. Twist1 is thought to be a master regulator of metastasis because high Twist1 expression is correlated with invasive carcinoma (Yang et al., 2004). The invasive ability of tumours decreased when the expression of Twist1 was inhibited (Mahmoud et al., 2016). MMP2, also called gelatinase A, leads to metastasis by degrading the extracellular matrix, including the basement membrane (Corcoran et al., 1996). Combined with the results of the wound-healing assay, our findings suggest that platycodin D reduced the migration of 5637 cells by down-regulating Twist1 and MMP2 expression.

Platycodin D fights cancer by targeting multiple signalling pathways that involve proliferation and survival inhibition, apoptosis induction, cell cycle arrest, angiogenesis, and metastasis inhibition (Khan et al., 2016). The ERK pathway was found to be activated and regulate autophagy in platycodin D-treated HepG2 and BEL-7402 cells (Li et al., 2015a,b). In MCF-7 breast cancer cells, reactive oxygen species (ROS) generation and mitogen-activated protein kinases (MAPKs), which play important roles in apoptosis, were activated by treatment with platycodin D. Furthermore, platycodin D induced activation of ASK-1, which further triggered p38 and JNK activation by phosphorylation and promoted transcription of apoptosis-related genes *FasL*, *Bim*, and c-*Jun* (Yu and Kim, 2010). Platycodin D also decreased the tumour microvessel density of HCT-15 xenografts in mice by blocking the VEGFR2-mediated signalling pathway (Luan et al., 2014). Our recent experiments have verified the anticancer activity of platycodin D against bladder cancer through activation of the intrinsic and extrinsic apoptotic pathways, which has not been investigated before. However, more studies on specific potential mechanisms involving the expression of related protein kinases and growth factors are still needed for an in-depth exploration in the future.

In conclusion, we show that platycodin D inhibited 5637 NMIBC cell proliferation and migration and tumour growth by activating multiple signals and pathways. These results may provide the basis for further research on platycodin D in clinical bladder cancer therapy.

Competing interests

The authors have no conflicts of interest to declare.

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