



# Anticancer effect of the traditional Chinese medicine herb Maytenus compound via the EGFR/PI3K/AKT/GSK3 $\beta$ pathway

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**Background:** Cancer is a leading cause of death worldwide; folk anticancer medicinal plants have applied for cancer treatment. The Maytenus compound tablet as traditional Chinese compound medicine has been approved for alleviating hyperplasia of mammary glands, whether it can inhibit cancer still unknown. The study was to evaluate the anticancer activity of the Maytenus compound tablet.

**Methods:** MTS assay evaluated the anti-proliferation effect of the Maytenus compound on H226, A2058, 786O and HeLa cancer cells and two normal epithelial cell lines, namely, 16HBE and Hecate. Nude mouse xenograft tumor model using H226 and HeLa cells examined the drug's anticancer effect *in vivo*. Western blot assay studied the possible mechanism.

**Results:** The Maytenus compound indicated obvious ability to against proliferation in four strains of cancer cells, particularly against H226 cells by an IC<sub>50</sub> of 85.47±10.06  $\mu\text{g}/\text{mL}$  and against HeLa cells by an IC<sub>50</sub> of 128.74±17.46  $\mu\text{g}/\text{mL}$ . However, it had a low cytotoxicity in human normal epithelial cell lines 16HBE with an IC<sub>50</sub> of 4,555.86±25.21  $\mu\text{g}/\text{mL}$  and Hecate with an IC<sub>50</sub> of 833.56±181.88  $\mu\text{g}/\text{mL}$ . The Maytenus compound at the 2.45 g/kg oral dosages inhibited the proliferation of H226 cells and HeLa cells in nude mouse with inhibitory rates of 36.06% and 26.45%, respectively, and no organ toxicity. The Maytenus compound could significantly downregulate the expression of pEGFR, pPI3K, pAKT, pGSK3 $\beta$ ,  $\beta$ -catenin, and c-MYC and upregulate the protein expression of GSK3 $\beta$ .

**Conclusions:** The Maytenus compound has significant anticancer activities against human cancer H226 and HeLa cells both *in vitro* and *in vivo*, highlighting it may be an anticancer medicine.

**Keywords:** Maytenus compound; traditional Chinese medicine; anticancer; mechanisms

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## Introduction

Cancer mortality rate ranks the first around world and has become the major public health problem (1). Although there are many ways to treat cancer, it is still a difficult global problem to overcome. Nowadays, traditional anticancer medicinal plants have drawn growing interest as novel drugs because more and more studies have found that they have potentiality been as medicines for cancer prevention and therapy (2).

The Maytenus compound tablet is a folk traditional medicine used by Lahu minority people in Yunnan Province of China. It consists of six herb medicines, including Maytenus austroyunnanensis (S. J. Pei *et al.*, Y. H. Li) and Maytenus hookeri Loes (herein afterward referred to as Maytenus), Sargrassum, Oldenlandia Diffusa Willd, Balanophora Harlandii Hook.f, Tripterygium hypoglaucum Hutch and Paris polyphylla. These plants are predominantly distributed in Yunnan province of China. Maytenus is used in Chinese traditional medicine for therapy of digestive inflammatory disorders such as ulcers (3,4), and it has antiparasitic activities (5). In recent years, the leaf extract of Maytenus in Brazilian has been reported to possess anticancer activity (6). Previously, maytansine, the chemical constituent of Maytenus, played important roles in anticancer effect by affecting micro-tubules (7,8). T-DM1 (Trastuzumab emtansine) including DM1, one of the maytansine derivative, has strong effect against HER2-positive breast cancer as a novel drug (9). Trichothecin (TCN), isolated from an endophytic fungus of Maytenus, can induce G0/G1 cell cycle arrest and activate pro-apoptotic proteins, promoting cancer cell apoptosis (10).

The Maytenus compound tablet has been approved for alleviating hyperplasia of mammary glands [approval number: Dian drug (Z)05J02424] in China, and it is a traditional Chinese compound medicine, there are no reports about its anti-cancer function. Therefore, the goal of this study was to evaluate the anticancer activity of the Maytenus compound tablet in both *in vitro* and *in vivo*, further investigate the underlying mechanism.

## Methods

### Preparation of Maytenus compound

Per gram Maytenus compound tablet [(Z05J02424), Puer Traditional Ethnomedicine Institute, China] included Maytenus 235 mg, Sargrassum 176.5 mg, Oldenlandia Diffusa Willd 176.5 mg, Balanophora Harlandii Hook.f.,

176.5 mg, Tripterygium Hypoglaucum Hutch 176.5 mg, and Paris Polyphylla 59 mg. The tablets were grinded into powder and dissolved in Dimethyl Sulfoxide (DMSO; D2650, Sigma, USA) for further tests. And the control is 0 µg/mL of Mytenus, which medium add with Dymethyl sulfoxide.

### Cell lines

In this study, we used several strains of human cancer cell lines, involving lung cancer cell H226, melanoma cell A2058, kidney cancer cell 786O, cervical cancer cell HeLa, normal epithelial cell 16HBE and Hecate. All cancer cell lines were obtained from the ATCC Bioresource Center. H226, 786O and 16HBE cells were cultured with RPMI 1640 (Gibco, USA) including 10% FBS (Gibco, USA). A2058 cells were cultured with DMEM F12 (Gibco, USA) including 10% FBS. HeLa and Hecate cells were cultured with DMEM high glucose (Gibco, USA) including 10% FBS.

### Determination of IC50

Briefly, 3,000 cells and 150 µL medium were seeded each well in 96-well culture plates, each group had six duplicate wells. After cells were seeded for 24 h, they were treated with the Maytenus compound in designated doses (4,800, 2,400, 1,200, 600, 300, 150, 75, 37.5, 18.75, 9.375, 4.6875 and 0 µg/mL) for 72 h. Then, per well was added 30 µL of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; G111A, Promega, USA], and full wavelength scanner (Varioskan Flash, Thermo Fisher, USA) was used to measured absorbance of each sample at 490 nm after they were incubated in an cell incubator for 2 h. IC50 is the concentration that is required to inhibit 50% of cell growth; Bliss method was used calculate IC50 from survival curves (11).

### Cell proliferation

MTS assay was also used to quantify cell proliferation. In brief, 2,000 cells and 150 µL medium were seeded each well in 96-well culture plates, and each group had six duplicate wells, incubated in complete medium with different concentrations of the Maytenus compound. Over a 4-day period, every 24 h, each well was added 30 µL of MTS, and full wavelength scanner was used to measured absorbance of each sample at 490 nm after they were incubated in a cell incubator for 2 h.

### Clonogenic assays

One thousand and five hundred cells and 1.5 mL medium were seeded each well in 6-well culture plates each group had three duplicate wells. After cells were seeded for 24 h, they were treated with the different concentrations of Maytenus compound for 72 h. Then, cells were incubated continuously without the drug effect for approximately 10 days. After PBS washed cells three times, methanol fixed cells for 20 min and hematoxylin stained cells for 5 min, then cells were washed clean with ddH<sub>2</sub>O, aired, and naked clones were counted.

### In vivo antitumor tests

Five-week-old female athymic BALB/c nude mice were housed in pathogen-free conditions according to the Guide for the Care and Use of Laboratory Animals. We generated the xenograft model by injection of  $2 \times 10^6$  H226 cells or  $1 \times 10^6$  HeLa cells into the right armpit of nude mice. The mice were divided into control (ddH<sub>2</sub>O) and treatment group (2.45 g/kg dose of the Maytenus compound), randomly. And the 2.45 g/kg dose is determined according to the results of the toxicological experiment by Puer Traditional Ethnomedicine Institute (Puer, China). The ddH<sub>2</sub>O and the Maytenus compound were both intragastrically administered once a day, for a total of 30 (H226) or 22 days (HeLa). During this period, mice body weight and the long diameter (a) and short diameter (b) of tumors were measured and recorded every two days. Tumor volumes (cm<sup>3</sup>) were calculated according to the formula: volume =  $(a \times b^2)/2$ . The tumors, liver, spleen and kidney were collected and weighed after the mice were sacrificed by cervical dislocation. All procedures were supported by the Animal Care and Research Committee of Kunming Medical University.

All the isolated tumor tissues were cut into 4µm sections by fixing in the formalin and embedding in paraffin, sections were baked at 70 °C for 1 h, immersed in xylene for 20 min, and rehydrated in alcohol, followed by staining with hematoxylin-eosin.

### Western blotting

Cells treated with the Maytenus compound for 72 h were collected and lysed completely in cold RIPA buffer (89900, Pierce, USA), BCA protein assay kit (23227, Thermo Fisher, USA) was measured protein concentration.

For each sample protein, 50 µg were separated on SDS-polyacrylamide gels and transferred onto PVDF membranes (ISEQ00010, Millipore, USA) by electroblotting. Membranes were blocked with 8% non-fat milk in 0.1% TBST for 2 h at ambient temperature, then added primary antibody incubated for 15 h at 4 °C. Primary antibodies: EGFR (CST, #4405), pEGFR (CST, #4407), PI3K (ab189403), pPI3K (ab182651), AKT (ab8932), pAKT (ab179463), GSK3β (CST, #9832), pGSK3β<sup>Ser9</sup>(CST, #5558), β-catenin (CST, #8480) and c-MYC (ab56), GAPDH (CST, #2125). Secondary antibodies labeled with horseradish peroxidase (HRP) (1:8,000, CST, #7076) were incubated membranes for 1 h at room temperature. After above, the visualized blots were detected by ECL (RPN2135, GE healthcare, UK).

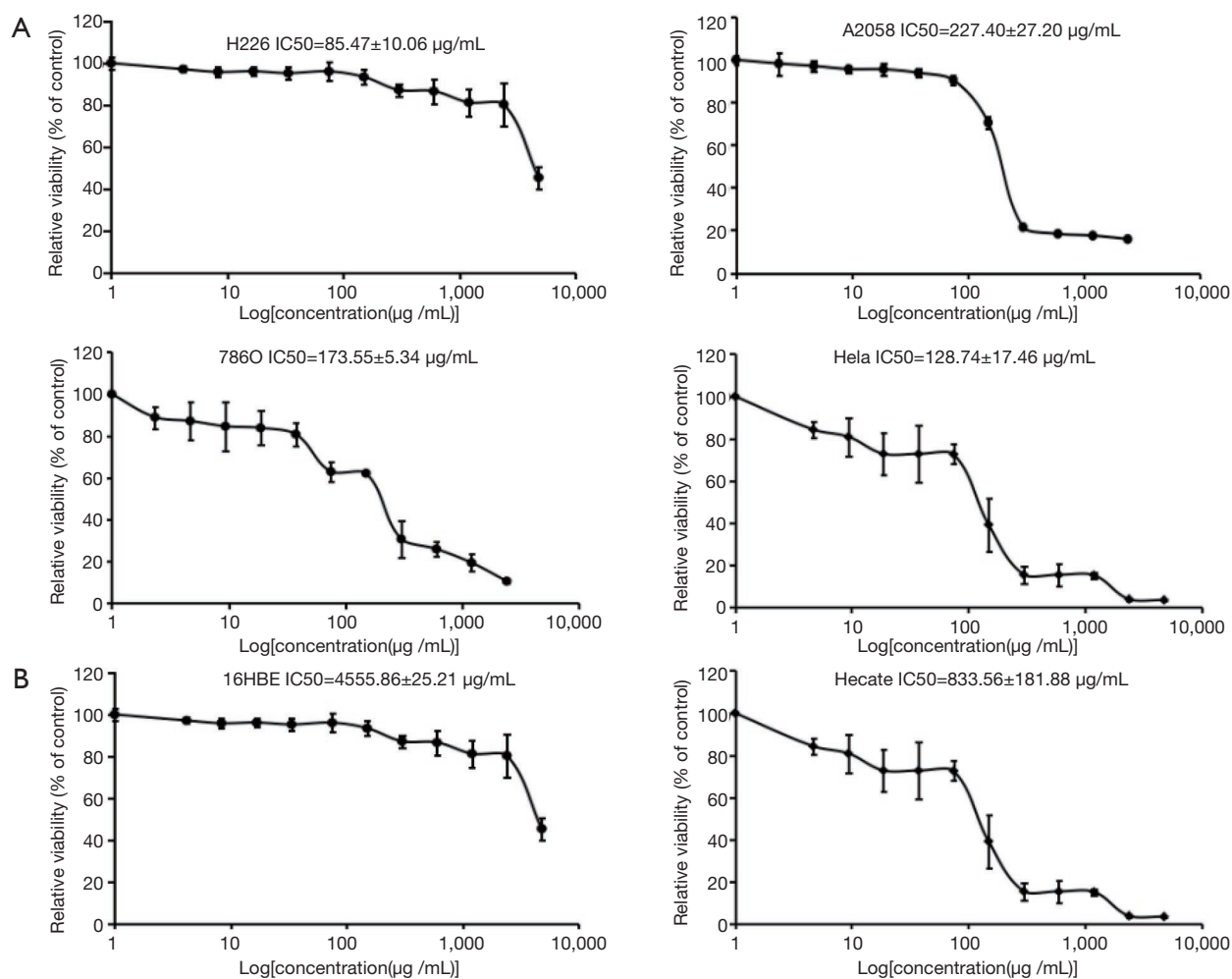
### Statistical analysis

Data are expressed as the mean ± SD (standard deviation) and evaluated by independent *t*-test with SPSS version 18.0 software and Prism 5 (GraphPad Software, USA). The differences were considered statistically significant at *P*<0.05.

## Results

### In vitro antitumor activity

Proliferation inhibitory effect at 72 h against four different human cancer cell lines (H226, A2058, 786O, HeLa) and human normal epithelial cell lines (16HBE, Hecate) were detected by MTS assay. As shown in *Figure 1A*, the Maytenus compound inhibited the growth of the four cancer cell lines in a dose-dependent manner. In addition, the Maytenus compound revealed a stronger proliferation inhibitory effect of H226 cells with an IC<sub>50</sub> of  $85.47 \pm 10.06$  µg/mL and of HeLa with an IC<sub>50</sub> of  $128.74 \pm 17.46$  µg/mL than of 786O cells with an IC<sub>50</sub> of  $173.55 \pm 5.34$  µg/mL and of A2058 cells with an IC<sub>50</sub> of  $227.40 \pm 27.20$  µg/mL, suggesting that the Maytenus compound could exhibit a favorable anti-lung cancer and anti-cervical cancer effect. However, the Maytenus compound had low cytotoxicity in the human normal epithelial cell lines 16HBE with an IC<sub>50</sub> of  $4,555.86 \pm 25.21$  µg/mL and Hecate with an IC<sub>50</sub> of  $833.56 \pm 181.88$  µg/mL (*Figure 1B*). According to the IC<sub>50</sub> values of the Maytenus compound in the four cancer cell lines at 72 h, we chose the H226 and HeLa cells lines to



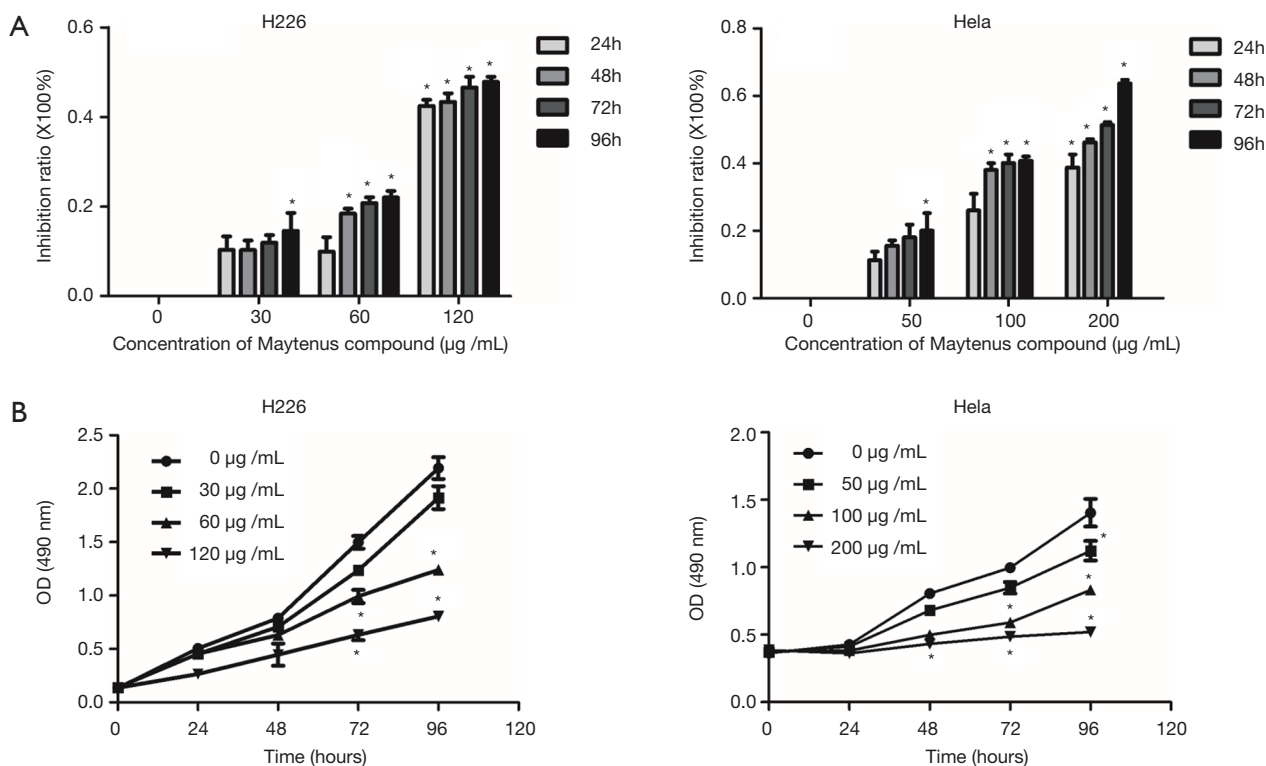
**Figure 1** Effect of Maytenus compound on human cancer cells and normal cells. (A) IC<sub>50</sub> of the Maytenus compound against H226, A2058, 786O and HeLa human cancer cells at 72 h (the mean ± SD, n=5); (B) IC<sub>50</sub> of the Maytenus compound against 16HBE and Hecate human normal epithelial cells at 72 h (the mean ± SD, n=5).

continue the study of antitumor activity of the Maytenus compound. Then, we selected 0, 30, 60 and 120 µg/mL Maytenus compound concentration to treat H226 cells and selected 0, 50, 100 and 200 µg/mL concentration to treat HeLa cells.

#### *The Maytenus compound had anti-activity effects on H226 and HeLa cells*

After H226 and HeLa cells were treated as mentioned above, MTS assay was detected the cell viabilities. As shown in *Figure 2A*, the Maytenus compound inhibited the cell growth significantly, the cell viabilities of H226 and HeLa cells were decreased with the increased in

Maytenus compound concentration and were decreased with the increased in Maytenus compound treatment time. In addition, as shown in *Figure 2B*, there was no statistically difference in H226 cells treated with 30 µg/mL Maytenus compound at 24, 48, 72 or 96 h. Additionally, there was no statistically difference in HeLa cells treated with 50 µg/mL Maytenus compound at 24, 48, 72 and 96 h. Additionally, there were no statistically differences in H226 cells treated with 60 and 120 µg/mL Maytenus compound at 48 h. However, the cell viabilities were significantly inhibited in H226 cells treated with 60 and 120 µg/mL Maytenus compound at 72 and 96 h and in HeLa cells treated with 100 and 200 µg/mL Maytenus compound at 48, 72 and 96 h.



**Figure 2** The Maytenus compound possessed anti-activity effects on cancer cells. (A) The Maytenus compound inhibited H226 and HeLa cells proliferation in a mode which is time and dose-dependent. The inhibition rate was calculated according to formula:  $1 - \text{Experimental group absorbance} / \text{Control group absorbance} \times 100\%$ ; (B) cell growth viability curves indicate that the Maytenus compound reduced the cells viability significantly. \*,  $P < 0.05$ .

### Clonogenic assays

As shown in *Figure 3*, after H226 and HeLa cells were treated with the Maytenus compound for 72 h, the cells' clone formation capacity was reduced significantly compared with that of the control cells. Additionally, the clone numbers of the H226 and HeLa cells decreased with the increase in the Maytenus compound concentration. It indicates that the Maytenus compound had anti-clonogenic cancer cell ability.

### In vivo antitumor activity

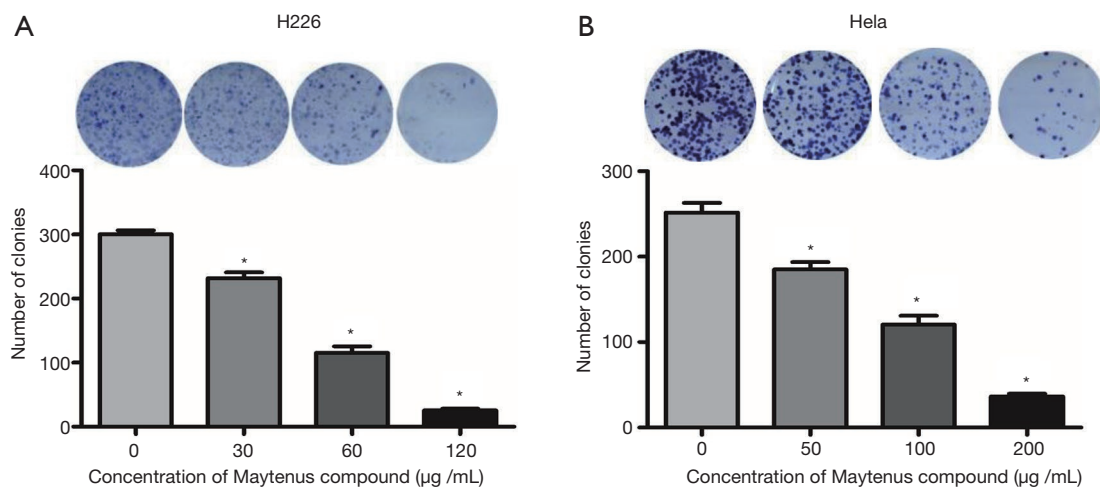
Nude mice were intragastrically administered the Maytenus compound at doses of 2.45 g/kg every day. As shown in *Figure 4*, the Maytenus compound inhibited H226 cells and HeLa cells tumor growth in the mouse models. Compared to control group, the inhibition effects in the experiment group were significant ( $P < 0.05$ ), the inhibition rates for tumor growth were 36.06% and 26.45% for the H226

mouse models and HeLa mouse models, respectively (*Table 1*). The tumor weight results also indicated the antitumor activity of the Maytenus compound *in vivo*. Furthermore, the Maytenus compound had no significant effect on the mouse bodyweight, liver index, spleen index and kidney index. These results clearly showed that the Maytenus compound had a significant anticancer effect *in vivo*.

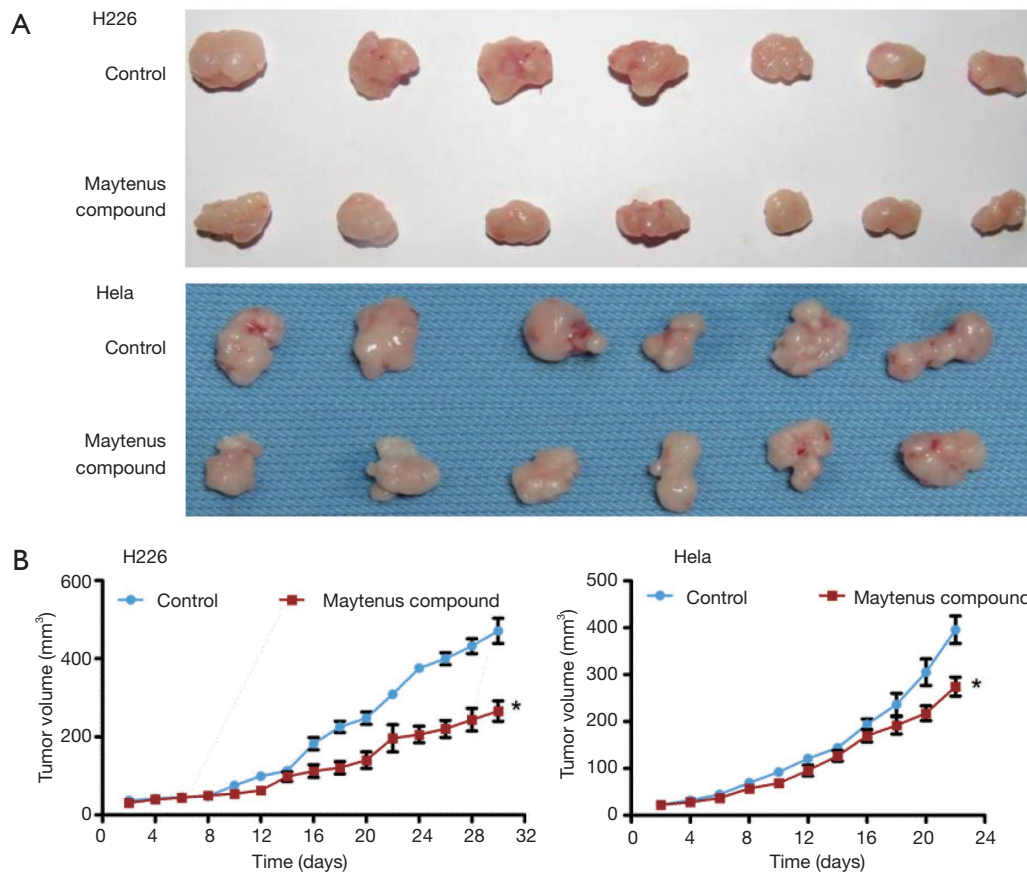
### HE staining analysis

As shown in *Figure 5*, in the control groups, the tumor cells volume was large and round, nucleoli large and distinct, the cell structure was clear and intact, suggesting good growth. However, in the Maytenus compound groups, the tumor cells showed degenerative changes, karyopyknosis and fibrosis; in the solid tumor from the HeLa Maytenus compound groups, necrosed area could be found, where the structure showed loss of nuclei and fragmentation of





**Figure 3** The Maytenus compound had anti-clonogenic effects on cancer cells, hematoxylin staining. (A) The Maytenus compound inhibited clone formation in H226 cells with a dose-dependent mode; (B) the Maytenus compound inhibited clone formation in HeLa cells with a dose-dependent mode. \*,  $P < 0.05$ .

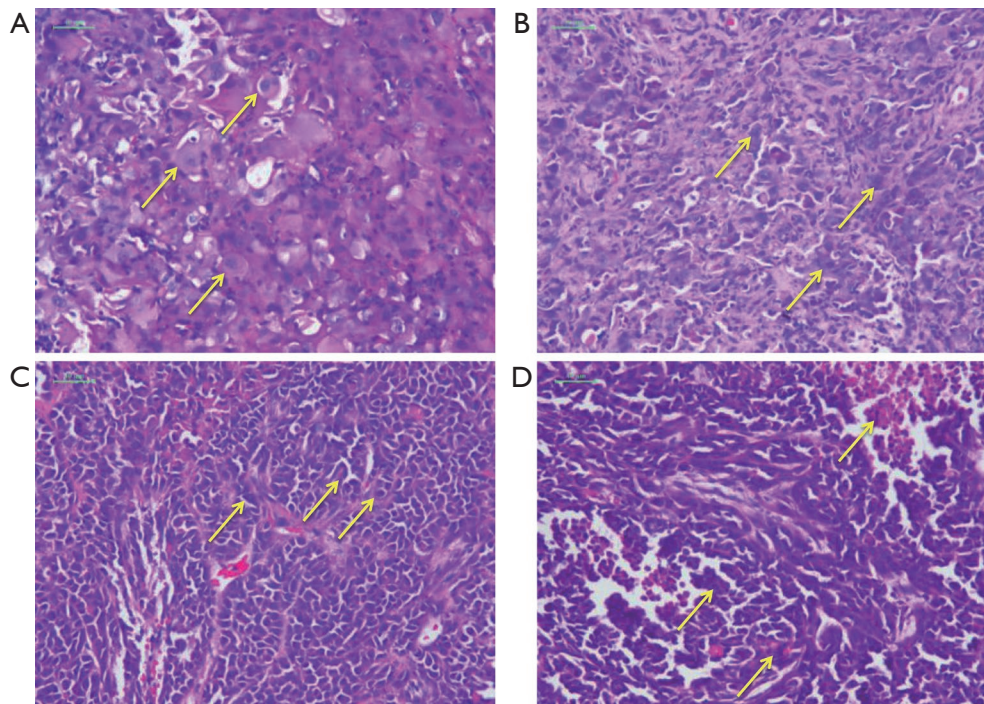


**Figure 4** Antitumor activity of the Maytenus compound *in vivo*. After inoculation with cancer cells for 1 week, mice were intragastrically administered the Maytenus compound at dose of 2.45 g/kg/day. (A) Tumor size was recorded; (B) tumor volume was recorded. Data are presented as the means  $\pm$  SD,  $n = 7$ . \*,  $P < 0.05$ .

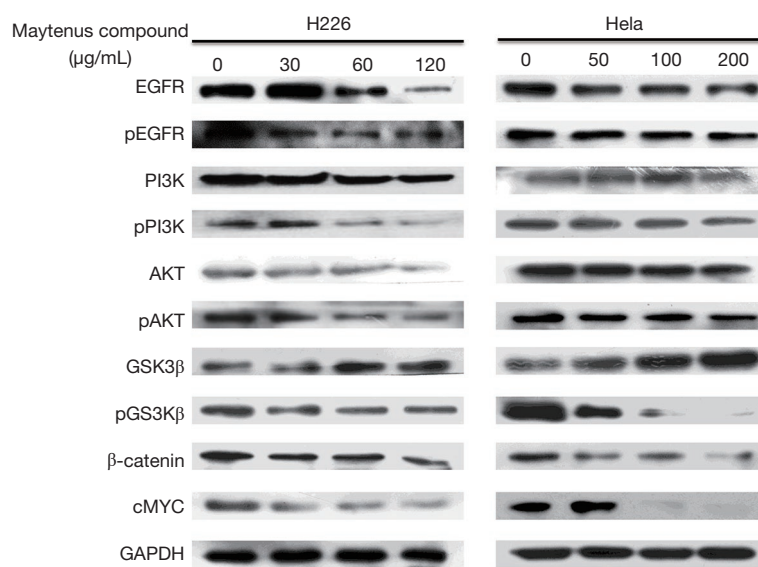
**Table 1** The antitumor effect of the Maytenus compound *in vivo*

Groups	H226		HeLa	
	Control	Maytenus compound	Control	Maytenus compound
Body weight (g) beginning	13.49±0.54	13.93±0.56	14.85±1.11	14.79±1.17
Body weight (g) ending	14.97±0.81	14.01±1.10	15.72±1.85	14.07±1.38
Tumor volume (cm <sup>3</sup> )	4.95±0.95	2.81±0.76	3.96±0.72	2.81±0.84
Tumor weight (mg)	0.23±0.07	0.15±0.04	0.54±0.12	0.40±0.09
Inhibitory rate (%)	–	36.06*	–	26.45*
Liver index	6.12±0.43	6.09±0.19	6.11±0.32	6.54±0.53
Spleen index	0.59±0.05	0.61±0.14	0.70±0.15	0.70±0.06
Kidney index	1.52±0.06	1.58±0.05	1.53±0.11	1.65±0.28

The Organ Index was calculated according to formula: Organ weight/Body weight × 100%. \*, P<0.05.



**Figure 5** HE staining of tumor tissue slices from mice (×200). (A) H226 control. Arrow indicating morphologically intact tumor cells with large cell volume, large and distinct nucleoli; (B) H226 Maytenus compound. Arrow indicating degenerative changes tumor cells with small cell volume, concentrated nucleus and plasma; (C) HeLa control. Arrow indicating morphologically intact tumor cells with large cell volume, large and distinct nucleoli; (D) HeLa Maytenus compound. Arrow indicating degenerative changes and necrotic tumor cells with small cell volume, concentrated nucleus and plasma.



**Figure 6** Western blot. Changes in expression levels of EGFR, pEGFR, PI3K, pPI3K, AKT, pAKT, GSK3β, pGSK3β, β-catenin and c-MYC proteins in H226 and HeLa cells after 72 h treatment with the Maytenus compound.

cells. The results indicated the Maytenus compound had anticancer ability *in vivo*.

### Western blot analysis

To further understand the antitumor activity of Maytenus compound on H226 and HeLa cells, western blot was used to analyze proteins change in cells treated with the Maytenus compound for 72 h. As shown in *Figure 6*, the Maytenus compound reduced the level of EGFR, pEGFR, pPI3K, pAKT, pGSK3β, β-catenin and c-MYC protein and increased protein expression of GSK3β in a dose-dependent manner. These results indicated that the Maytenus compound could inhibit proliferation of H226 and HeLa cells by down-regulating the phosphorylation levels of EGFR, PI3K, AKT and GSK3β.

### Discussion

Numerous valuable “treasures” from Chinese traditional medicine were discovered because of the development of science and technology, especially national herbal medicine (12,13). Some Chinese traditional medicine substances have been proven to possess anticancer properties without side effects, such as dihydroartemisinin (14). In addition, in China, Chinese herbal medicine products as antitumor agents have thousand-years of history. In our study, the

antitumor activity of the Maytenus compound against four cancer cell lines, including H226, A2058, 786O and HeLa, were confirmed in a dose-dependent manner, and these effects on H226 and HeLa were also confirmed in a time-dependent manner. Additionally, the Maytenus compound showed low toxicity in human normal epithelial cells, and the results are consistent with those of Nkosinathi, who reported that the methanolic root extracts of Maytenus procumbens exhibited moderate cytotoxic effects on the cancer and weak cytotoxicity in the normal cells (15). In addition, the Maytenus compound had the ability to control H226 and HeLa cell proliferation in nude mice *in vivo* without cytotoxicity. The result indicated that the Maytenus compound could be a potential natural and novel anticancer drug, especially for lung cancer and cervical cancer.

Lung cancer has high morbidity and mortality worldwide (1). Cervical cancer is the fourth in cancer related mortality among all cancer types and second in cancer related mortality among females (16,17), highlighting the importance of studying the antitumor mechanism of the Maytenus compound. Some reports have shown that some extracts of Maytenus have an antitumor mechanism, such as pristimerin, a natural triterpenoid isolated from *Celastrus* and *Maytenus* spp, which has been found to inhibit tumor cells *in vitro* and *in vivo*, and affect the proliferation, survival, angiogenesis and metastasis through the NF-κB, MAPK/ERK and AKT/mTOR pathways (18). Additionally,



the methanol extract of *Maytenus royleanus* leaves and its fractions possess anti-proliferative activity against prostate cancer by exhibiting cleavage of Caspase-3 and PARP (19). As the derivative of maytansine, T-DM1 combined with pertuzumab significantly inhibited the phosphorylation of HER3 or EGFR, and its downstream factor AKT inhibited gastric cancer proliferation (20).

The EGFR is a tyrosine kinase receptor that belongs to the Erb family, which is overexpressed in many tumor types, including lung cancer and cervical cancer (21,22). EGFR plays an important role in the physiological processes of the development and maintenance of epithelial tissues, and upregulated EGFR expression can activate cell proliferation and differentiation signaling pathways that lead to the progression of cancer, increase the metastasis and the suppression of apoptosis and angiogenesis (23-25). Drugs that affect the activity of EGFR have been used in clinical practice for cancer therapy (26,27). The major molecular mechanism of EGFR-induced tumor progression is that EGFR kinase phosphorylation activates the PI3K/AKT signaling pathway (28). PI3K phosphorylates the 3-hydroxyl of phosphatidylinositol, regulating proliferation, survival and metastasis signaling pathways, which are important for cancer (29-31). AKT (AK mouse plus transforming or thymoma), as a downstream component of the PI3K pathway, is a fundamental protein mediating signals from the receptor tyrosine kinases family, it is a frequent oncogene (32,33). Consistent with these data, western blot was used to evaluate the expression of the EGFR, pEGFR, PI3K, pPI3K, AKT, and pAKT proteins in the cells with the *Maytenus* compound treatment, and we obtained evidence that the *Maytenus* compound reduced the levels of the EGFR, pEGFR, PI3K, pPI3K, AKT, and pAKT proteins. These data collectively indicate that the *Maytenus* compound triggers the EGFR/PI3K/AKT signaling pathways.

The underlying downstream mechanism of the *Maytenus* compound in inhibiting the proliferation of cancer cells through the EGFR/PI3K/AKT signaling pathways still need to elucidate furtherly, many studies have reported that PI3K/AKT is the upstream regulatory pathway of the GSK3 $\beta$ / $\beta$ -catenin protein combination related with cell proliferation (34-36). Activated AKT translocates to the cell membrane, phosphorylating serine/threonine kinase glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) (37,38). Under normal physiological conditions, GSK3 $\beta$  is highly expressed, and its main role is to destroy the complex containing APC and Axin, promote the decomposition of  $\beta$ -catenin and weaken the stability of  $\beta$ -catenin; however, GSK3 $\beta$  is less expressed in cancer cells, which promotes the

stability of  $\beta$ -catenin (39,40). Then,  $\beta$ -catenin can enter the nucleus and activate its target genes, including c-MYC (41). Additionally, we found that the *Maytenus* compound significantly inhibited the expression of pGSK3 $\beta$ ,  $\beta$ -catenin and c-MYC and promoted the expression of GSK3 $\beta$ .

In conclusion, the *Maytenus* compound is a Chinese six-herb medicine, which possesses favorable inhibitory activity against the growth of human H226, A2058, 786O and HeLa cancer cells *in vitro*, especially against H226 and HeLa. The *Maytenus* compound inhibits the growth of H226 and HeLa xenografts *in vivo*.

In addition, we found that the *Maytenus* compound played a role in anticancer activity via the EGFR/PI3K/AKT/GSK3 $\beta$  pathway, and many of the traditional Chinese medicines have been shown to have anticancer activity through multiple targets (42,43); therefore, the *Maytenus* compound might have other pathways. Next, we further studied its anticancer mechanism. Maytansine has a high inhibition effect on cancer, but using it alone has hepatotoxicity, and it has been assessed in several oncology clinical trials (44). However, in this study, the *Maytenus* compound had a low cytotoxicity in human normal epithelium cells and no toxicity in mouse organs. Therefore, the *Maytenus* compound could be a potential treatment agent for lung cancer and cervical cancer. However, before it can proceed further in preclinical tests, considerations such as safety evaluations, pharmacokinetics and a suitable oral administration dosage should be investigated.

## Conclusions

Taken together, our results indicate that the *Maytenus* compound has significant anticancer activities against human cancer H226 and HeLa cells both *in vitro* and *in vivo*, highlighting it could be a novel potential anticancer drug.

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## Footnote

**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org>).

[org/10.21037/tcr.2019.09.30](https://doi.org/10.21037/tcr.2019.09.30)). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The animal experiments were performed in accordance with the institutional ethical requirements of the Kunming Medical University and approved by the Ethics Committee of Kunming Medical University (No. KMMU2019060). Individual informed consent was waived.

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