Effect of Octadecadienoic Acid on Proliferation and Apoptosis of Glioma Cells and Its Mechanism

Mingren XIE, Xia YUAN, Shan QI, Lei YU, Farong YU*

School of Public Security, Gansu University of Political Science and Law, Lanzhou 730070, China

Abstract [**Objectives**] To explore the inhibitory effect of octadecadienoic acid (ODA) on proliferation and apoptosis of glioma cells and its mechanism. [**Methods**] Cultured human glioma cells (cell density 2×10^6 cells/L) were divided into three groups: solvent control group (DMSO, 30 μL/L), 5-FU group (10 mg/L) and octadecadienoic acid group (0.3, 0.6, 1.2 mg/L). The toxic effects of ODA on glioma cells were detected by trypan blue and thiazolium blue (MTT). The expression of P53, PI3K, P21, PKB/Akt and caspase-9 protein in glioma cells were detected by enzyme-linked immunosorbent assay (ELISA). [**Results**] The cell count under optical microscope showed that the inhibition rate of cell proliferation in low, medium and high dose ODA groups and 5-FU group was significantly higher than that in solvent control group ($P<0.01$), but there was no significant difference compared with 5-FU group ($P>0.05$). The results of MTT showed that compared with the solvent control group, the inhibition rate of cell proliferation in low, medium and high dose ODA groups and 5-FU group significantly increased ($P<0.01$); compared with 5-FU group, the inhibition rate of cell proliferation in high dose ODA group significantly increased ($P<0.01$). The results of flow cytometry showed that compared with the solvent control group, the number of cells in G_0/G_1 phase increased significantly ($P<0.05$, $P<0.01$), the number of cells in G_2/M phase decreased significantly ($P<0.01$) and the apoptosis rate increased significantly ($P<0.01$) in the low, medium and high dose ODA groups and 5-FU group; compared with 5-FU group, the number of cells in G_2/M phase decreased significantly ($P<0.01$) and the apoptosis rate increased significantly ($P<0.01$) in ODA group. ELISA testing results showed that the expression levels of P53, PI3K and PKB/Akt in low, medium and high dose ODA groups and 5-FU group were significantly lower than those in solvent control group ($P<0.01$), and only the expression level of protein in high dose ODA group was significantly lower than that in 5-FU group ($P<0.01$); the expression levels of P21 and caspase-9 in low, medium and high dose ODA groups and 5-FU group were significantly higher than those in solvent control group ($P<0.05$, $P<0.01$), but the expression level of protein in high dose ODA group was significantly higher than that in 5-FU group ($P<0.01$). [**Conclusions**] ODA can obviously inhibit the proliferation of glioma cells and induce apoptosis. The mechanism is related to up-regulation of P21, caspase-9, down-regulation of P53, PI3K, PKB/Akt, inhibition of cell division cycle and decrease of PI3K-Akt signal transduction pathway.

Key words Octadecadienoic acid, Glioma cells, Inhibition effect, Apoptosis

1 Introduction

The normal growth of higher animal and human tissue cells is regulated by different signal transduction systems, among which phosphatidylinositol 3 kinase/protein kinase B (PI3K-Akt) signal transduction pathway is closely related to cell division and proliferation[1]. ZOU *et al.*[2] found that cell growth or inhibition was regulated and controlled by PI3K-Akt signal transduction pathway, which was involved in many physiological and pathological changes. *In vitro* studies have shown that the use of PI3K-Akt signal transduction pathway inhibitors can reverse the drug resistance of tumor cells[3] and improve the efficacy of anti-tumor drugs[4-5]. PI3K-Akt signal transduction pathway plays an important role in tissue angiogenesis and cell growth, proliferation, metabolism, migration, differentiation and apoptosis[6]. When PI3K-Akt signal transduction pathway is activated, it promotes cell proliferation; when PI3K-Akt signal transduction pathway is inhibited, apoptosis is promoted[7]. The study of PI3K-Akt signal transduction pathway, its mechanism and PI3K protein expression has certain reference value for tumor diagnosis, treatment and prognosis judgment[8]. Zhong *et al.*[9] administered dihydroartemisinin (DHA) to SGC 7901 cells and inhibited the activation of PI3K-Akt signal transduction pathway, which blocked the cell at G_0/G_1 phase and inhibited the cell proliferation, suggesting that PI3K-Akt signal transduction pathway could be a potential target for tumor therapy. At present, the principle of clinical treatment of brain tumor (glioma) is to combine radiotherapy and chemotherapy on the basis of surgical treatment. Because radiotherapy and chemotherapy have great toxic and side effects, they bring unbearable pain to patients. Based on previous studies, octadecadienoic acid (ODA) was administered to human glioma cells to investigate the inhibitory effect of ODA on the proliferation of tumor cells and the expression of related proteins in PI3K-Akt signal pathway and its mechanism, in order to provide a reference for clinical application.

2 Materials and methods

2.1 Materials Human glioma cells were preserved and donated by Pharmacology and Toxicology Laboratory of Gansu Academy of Medical Sciences, and octadecadienoic acid was extracted and separated from the root tuber of *Euphorbiakansui* by the Institute of Natural Plant of Lanzhou University. The molecular formula is

Received: April 13, 2023 Accepted: July 18, 2023

Supported by Gansu Natural Science Foundation (21JR7RA571); Lanzhou Science and Technology Plan Project (2019-1-48); Major Project of Gansu University of Political Science and Law (GZF2021XZD06).

* Corresponding author. E-mail: SG3979019@163.com

$C_{18}H_{32}O_2$, the molecular weight is 280.46, the purity is 94.13%, and it is colorless and transparent liquid. In the experiment, dimethyl sulfoxide (DMSO) was used to help dissolve and RPMI1640 culture solution was added to prepare the required concentration (DMSO content was 30 μL/L); 5-fluorouracil (5-FU) was purchased from Shanghai Xudonghaipu Pharmaceutical Co., Ltd., trypan blue and thiazolium blue (MTT) were purchased from Shanghai Zhongtai Chemical Reagent Co., Ltd., DMSO was purchased from Tianjin Guangfu Fine Chemical Research Institute, enzyme-linked immunometric meter was purchased from Shanghai Leibo Analytical Instrument Co., Ltd. (MULTISKAN MK3), and P53, PI3K, P21, PKB/Akt and caspase-9 kits were purchased from Shanghai Biotechnology Development Co., Ltd.

2.2 Glioma cell grouping and administration Subcultured human glioma cells (cell density 2×10^6 cells/L) were inoculated into 96-well plates, in which RPMI 1640 culture medium was added to 2 wells, 100 μL per well as blank control, and 90 μL of cell suspension was added to the other wells, respectively. Low (ODA-L), medium (ODA-M) and high (ODA-H) dose octadecadienoic acid groups were treated with 0.3, 0.6 and 1.2 mg/L octadecadienoic acid, 10 μL per well; in 5-FU group, 10 μL of 5-FU (10 mg/L) was added to each well; 10 μL of DMSO was added to the solvent control group. There were 4 repeated wells in each group.

2.3 Detection of cell survival by trypan blue The cell plates were placed in a 5% CO_2 incubator at 37 ℃ and cultured in saturated humidity for 6, 12, 18, 24 and 48 h, with trypan blue 10 μL added to each well. The number of living cells was counted under optical microscope, and the average value was taken for drawing. The inhibition rate (%) of ODA on glioma cell survival was calculated according to the formula of inhibition rate = (1 − Number of living cells of the drug administration group/Number of living cells of the solvent control group) × 100%.

2.4 Detection of the inhibition rate of cell proliferation by MTT Cells were cultured for 48 h, 10 μL of MTT was added to each well and it was incubated for 4 h. 100 μL of dimethyl sulfoxide (DMSO) was added to each well, and was placed in a gas bath constant temperature oscillator at 37 ℃ for 10 min. Absorbency (A) was measured at 450 nm by enzyme-linked immunometric meter. According to the formula of inhibition rate = [1 − Absorbency (A) of the drug administration group/Absorbency (A) of the solvent control group] × 100%, the inhibition rate (IR, %) of ODA on glioma cell proliferation was calculated.

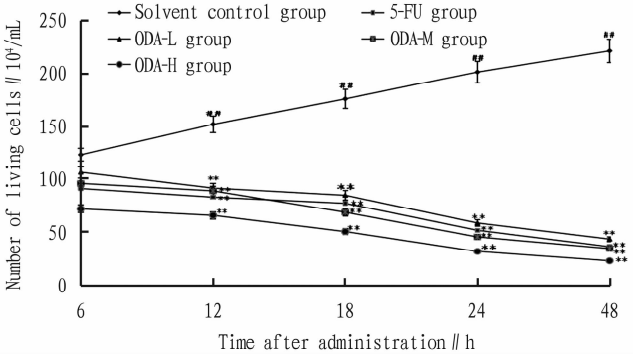
2.5 Detection of cell cycle and apoptosis rate by flow cytometry Cell fluid (cell density 2×10^5 cells/L) was added into cell culture flasks (containing 6 mL of RPMI 1640 culture medium), with 2 mL per flask. 0.8 mL of octadecadienoic acid (ODA) was added per flask in low, medium and high dose groups; in 5-FU group, 0.8 mL of 5-FU was added to each flask; in the solvent control group, 0.8 mL of DMSO was added to each flask. After 48 h culture, the cells were collected by centrifugation, and the cell number and apoptosis rate in each phase of division were measured by flow cytometry.

2.6 Detection of protein expression level by ELISA The cells were cultured for 48 h, and the protein expression levels of P53, PI3K, P21, PKB/Akt and caspase-9 were detected according to ELISA kit.

2.7 Statistical processing All data were expressed by mean ± standard deviation ($\bar{x}\pm s$), and the statistical processing was conducted by SPSS 20.0 software. One-way ANOVA analysis was used for comparison between groups.

3 Results and analysis

3.1 Toxic effect of ODA on glioma cells The results of cell counting by optical microscope showed that the number of living cells decreased significantly with the increase of drug concentration and the prolongation of culture time in the treatment group ($P<0.01$, Fig. 1). The inhibition rates of cell survival were 80.06%, 84.27%, 89.35% and 83.27% in the low, medium, high dose ODA groups and 5-FU group, respectively, which were significantly higher than those in the solvent control group (22.64%) ($P<0.01$), but there was no significant difference compared with the inhibition rate of 5-FU group (83.27%) ($P>0.05$).



Note: $^{**}P<0.01$ vs solvent control group; $^{\#\#}P<0.01$ vs 5-FU group.

Fig. 1 Toxicity of ODA on human glioma cells

3.2 Inhibitory effect of ODA on proliferation of glioma cells The results of MTT showed that the inhibition rate of cell proliferation in low, medium and high dose ODA groups and 5-FU group was 44.28%, 46.73%, 63.89% and 48.53%, respectively, which was significantly different from that in solvent control group (19.47%) ($P<0.01$); there was no significant difference in cell proliferation inhibition rate between 5-FU group and low and medium dose ODA groups, but the cell proliferation inhibition rate in high dose ODA group was significantly higher than that in solvent control group ($P<0.01$).

3.3 Effect of ODA on cell cycle and apoptosis of glioma cells The results of flow cytometry showed that compared with the solvent control group, the number of cells in G_0/G_1 phase increased significantly ($P<0.05$, $P<0.01$), the number of cells in G_2/M phase decreased significantly ($P<0.01$) and the apoptosis rate increased significantly ($P<0.01$) in the low, medium and high dose ODA groups and 5-FU group. There was no significant differ-

ence in the number of cells in G_0/G_1 phase, the number of cells in G_2/M phase and the rate of apoptosis between 5-FU group and low, medium and high dose ODA groups. In the high dose ODA group, the number of cells in G_2/M phase decreased significantly ($P<0.01$) and the rate of apoptosis increased significantly ($P<0.01$, Table 1).

Table 1 Effects of ODA on human glioma cell cycle and apoptosis ($\bar{x}\pm s$)

Group	Division cycle of glioma cells			Apoptosis rate//%
	G_0/G_1	S	G_2/M	
Solvent control	48.86 ±4.14#	21.62 ±5.06	29.51 ±3.17##	0.18 ±0.02##
5-FU	64.06 ±3.31*	23.28 ±2.52	12.66 ±3.02**	17.14 ±1.26**
ODA-L	54.11. ±2.17*	27.13 ±3.22	14.69 ±2.34**	15.41 ±2.14**
ODA-M	65.26 ±3.13*	21.41 ±3.03	13.33 ±3.22**	18.38 ±2.46**
ODA-H	67.62 ±3.16**	23.31 ±5.06	9.07 ±2.12**##	20.54 ±3.17**##

Note: $^{*}P<0.05$, $^{**}P<0.01$ vs solvent control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 5-FU group.

3.4 Effect of ODA on protein expression in glioma cells The results of ELISA showed that the protein expression level of P53, PI3K, PKB/Akt decreased significantly ($P<0.01$), while the protein expression level of P21, caspase-9 increased significantly ($P<0.05$, $P<0.01$) in the low, medium and high dose ODA groups and 5-FU group compared with the solvent control group.

There was no significant difference in the protein expression level of P53, PI3K, PKB/Akt, caspase-9 and P21 between 5-FU group and low and medium dose ODA groups, the protein expression level of P53, PI3K and PKB/Akt in high dose group significantly decreased ($P<0.01$), and the protein expression level of P21 and caspase-9 significantly increased ($P<0.01$, Table 2).

Table 2 Effect of ODA on protein expression level of glioma cells ($\bar{x}\pm s$)

Group	P53//pg/L	PI3K//pg/L	P21//ng/L	PKB/Akt//ng/L	Caspase-9//μg/L
Solvent control	2.893 ±0.013##	1.553 ±0.017##	2.623 ±0.009##	2.185 ±0.013##	121.275 ±0.781##
5-FU	1.828 ±0.009**	0.887 ±0.017**	3.355 ±0.013**	1.425 ±0.013**	154.125 ±0.741**
ODA-L	1.968 ±0.017**	1.093 ±0.013**	3.133 ±0.017*	1.513 ±0.015**	138.828 ±0.085*
ODA-M	1.775 ±0.013**	0.923 ±0.013**	3.473 ±0.017**	1.467 ±0.017**	163.423 ±0.463**
ODA-H	1.325 ±0.013**##	0.638 ±0.017**##	4.575 ±0.013**##	0.978 ±0.013**##	187.635 ±0.637**##

Note: $^{*}P<0.05$, $^{**}P<0.01$ vs solvent control group; $^{\#\#}P<0.01$ vs 5-FU group.

4 Discussion

Cell growth or inhibition is regulated by phosphatidylinositol 3 kinase/protein kinase B (PI3K-Akt) signal pathway[10]. When PI3K-Akt signal pathway is inhibited, apoptosis is promoted[11]. According to the report of Veloso[12], under normal physiological conditions, environmental factors in vivo and in vitro combine with cell membrane receptors to activate the expression of P53 gene, P53 protein and P21 gene directly regulate the expression of P21 protein, promoting the separation of P21 from GDP and the combination with GTP to form P21-GTP complex, which induces the opening of intracellular signal transduction system and the beginning of cell division and proliferation. The results of this study showed that ODA inhibited the activity of P53 gene and down-regulated the expression of P53 protein in glioma cells, while ODA up-regulated P21 in glioma cells. P21 has GTP enzyme activity, which can hydrolyze GTP to GDP. P21 is inactivated by binding with GDP, which closes the intracellular signal transduction system and inhibits cell proliferation, migration and invasion[13]. Wang Yu[14] gave different concentrations of shikonin to SMMC-772 cells and tumor-bearing mice, and the results showed that shikonin could significantly inhibit the proliferation of SMMC-7721 cells and the growth of tumor in tumor-bearing mice, inhibit the phosphorylation of AKT and PI3K proteins, inhibit the proliferation of SMMC-7721 cells and induce apoptosis by affecting PI3K/AKT signal pathway. The results of this study suggested that the expression level of PI3K, PKB/Akt and P53 in glioma cells was down-regulated after ODA administration, which decreased the activity of PI3K-Akt signal transduction pathway and promoted the apoptosis of glioma cells. Ding Lu[15] and Chen Yanan[16] cultured SGC-7901 cells with different concentrations of betulinic acid for 48 h respectively, and found that the cells shrank, the nucleus lysed and apoptotic bodies appeared, the rates of early and late apoptosis increased significantly, and the mitochondrial membrane potential decreased significantly. The trypan blue staining results showed that the number of living glioma cells decreased with the increase of ODA concentration and the prolongation of action time. The principle of MTT detection is that succinate dehydrogenase in mitochondria of living cells can reduce exogenous MTT to water-insoluble blue-purple crystals and deposit them in cells. Dimethyl sulfoxide (DMSO) was able to dissolve formazan in cells, and its absorbance (A) was measured by enzyme-linked immunometric meter at 450 nm wavelength. The mitochondrial structure of glioma cells was damaged by ODA, and exogenous MTT could not be reduced to water-insoluble formazan. The proliferation inhibition rate of glioma cells increased with the increase of ODA concentration. Liu Jiupeng[17] and Fan Hua[18] cultured MGC-803 cells for 48 h, respectively. In the final concentration of 10 – 30 μg/mL, the clone formation rate of MGC-803 cells decreased significantly, the growth inhibition rate increased significantly, the cell proliferation ability decreased significantly, the cells were blocked in G_0/G_1 phase, and the protein expressions of caspase-3, caspase-9 and

(To page 34)

cluded linear range, *T. asiatica* precision, stability, repeatability, and spiked recovery test. The results showed that the method is easy to operate, and has good repeatability, and the detection results are accurate and reliable, so it can be used for the determination of flavonoid content in *T. asiatica* leaves.

References

[1] Food and Drug Administration of Guangxi Zhuang Autonomous Region. Quality standards of Zhuang medicine, volume 2 (2011 edition)[S]. Nanning: Guangxi Science and Technology Press, 2011. (in Chinese).

[2] ZHU H, WEI SJ. Pharmacognosy of Zhuang medicine[M]. Nanning: Guangxi Nationalities Publishing House, 2006. (in Chinese).

[3] Health Department, Logistics Department, Guangzhou Army. Handbook of commonly used Chinese herbal medicines[M]. Beijing: People's Medical Publishing House, 1969. (in Chinese).

[4] ZHU H, TIAN H, CAI Y. Zhuang pharmacy[M]. Nanning: Guangxi Science and Technology Press, 2015. (in Chinese).

[5] ZHU H, FU P. Study on the Zhuang medicinal materials of *Tetracera asiatica*, Caryopteris incana, Sphenomeris chinensis, and Hedyotis auricularia[M]. Nanning: Guangxi Science and Technology Press, 2016. (in Chinese).

[6] DAI HF, MEI WL. Ethnic medicine records of Li nationality, volume 1 [M]. Beijing: Science and technology of China Press, 2008. (in Chinese).

[7] DA FF. Application of *Tetracera asiatica* and its extract in the preparation of drugs for the treatment and/or prevention of alcoholic liver injury[P]. Guangxi Zhuang Autonomous Region: CN110787196B, 2022-03-22. (in Chinese).

[8] ZHU H, DA FF, DAI ZH, *et al.* Application of *Tetracera asiatica* and its extract in the preparation of drugs for the treatment and/or prevention of chemical liver injury [P]. Guangxi Zhuang Autonomous Region: CN110787195A, 2020-02-14. (in Chinese).

[9] ZHU H, DA FF, FU P, *et al.* Application of *Tetracera asiatica* and its extracts in the preparation of drugs for the treatment and/or prevention of liver cancer[P]. Guangxi: CN107982292A, 2018-05-04. (in Chinese).

(From page 26)

Cyt-c were significantly up-regulated, inducing apoptosis. The results of flow cytometry showed that the number of G_0/G_1 phase cells in ODA group was significantly higher than that in solvent control group, while the number of G_2/M phase cells significantly decreased, and the apoptosis rate increased with the increase of drug concentration. ELISA detection results showed that ODA up-regulated the expression of caspase-9 in glioma cells, initiated the apoptosis signal pathway, led to apoptosis of glioma cells, and then exerted the anti-tumor effect of ODA[19].

In this paper, the inhibitory mechanism of ODA on proliferation of glioma cells was explored. ODA can damage cell membrane structure, increase cell membrane permeability, induce Cyt-c release from mitochondria, induce up-regulation of P21, caspase-9 protein expression, down-regulation of P13K, PKB/Akt, P53 protein expression, decrease the activity of PI3K-Akt signal transduction pathway, block cell division cycle, and lead to apoptosis and death of glioma cells. ODA can be used as a new drug resource for P53, P21, PI3K and PKB/AKT target therapy for nervous system tumors.

References

[1] LIU H, WANG J, TAO Y, *et al.* Curumol inhibits colorectal cancer proliferation by targeting miR-21 and modulated PTEN/PI3K/Akt pathways [J]. Life Sciences, 2019(221): 354 – 361.

[2] ZOU SJ, CHEN ZF, LI M. Expression of PI3K and AKT2 in gastric cancer and its elationship with clinicopathological features and survival of patients[J]. Journal of Practical Oncology, 2011, 26(4): 346 – 350.

[3] FU XQ, FENG JR, ZENG D, *et al.* PAK4 confers cisplatin resistance in gastric cancer cells *via* PI3K/Ak and MEK/Erk-dependent pathways[J]. Bioscience Reports, 2014, 34(2): 59 – 67.

[4] SHI J, YAO DM, LIU W, *et al.* Highly frequent PIK3CA amplification is associated with poor prognosis in gastric cancer[J]. BMC Cancer, 2012, 12(1): 50 – 61.

[5] GUO D, ZHANG B, LIU S, *et al.* Xanthohumol induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI3K/Akt/m TOR-kinase in human gastric cancer cells[J]. Biomed Pharmacoth, 2018(106): 1300 – 1306.

[6] ZHOU J, PEI Y, CHEN G, *et al.* SPC24 regulates breast cancer progression by PI3K/AKT signaling[J]. Gene, 2018(675): 272 – 277.

[7] CORTI F, NICHETTI F, RAIMONDI A, *et al.* Targeting the PI3K/AKT/mTOR pathway in biliary tract cancers: A review of current evidences and future perspectives[J]. Cancer Treatment Reviews, 2019 (72): 45 – 55.

[8] PARK S, KIM YS, KIM DY, *et al.* PI3K pathway in prostate cancer: All resistant roads lead to PI3K[J]. Biochimica Et Biophysica Acta-reviews On Cancer, 2018, 1870(2): 198 – 206.

[9] ZHONG X, WANG AJ, WANG HY. DHA-inhibited prolifera-tion through the PTEN/PI3K/Akt pathway in gastric cancer SGC 7901 cells[J]. Chinese Journal of Clinical Oncology, 2013, 40(4): 190 – 194.

[10] CAO WG, YANG WP, FAN R, *et al.* MiR-34a regulates cisplatin-induce gastric cancer cell death by modulating PI3K/AKT/survivin pathway [J]. Tumor Biology, 2014, 35(2): 1287 – 1295.

[11] ZHANG HY, CHEN JB, LU HZ. Research progress on the role of PI3K/Akt signaling pathway in tumor angiogenesis[J]. Shandong Medical Journal, 2012, 52(47): 98 – 100. (in Chinese).

[12] VELOSO M, FRITZ W, KLAUS K. P53 gene status and expression of p53, mdm2, and 21Wafl/Cipl proteins in colorectal cancer[J]. Virchous Arch, 2000, 437(3): 241 – 247.

[13] LI HX, LIU F, XIA JH. Effects of miR-670-5p on proliferation, migration and invasion of lung cancer cells[J]. Chinese Journal of Applied Physiology, 2021, 37(5): 500 – 505. (in Chinese).

[14] WANG Y, HE SY, ZHU RT, *et al.* The effects of shikonin on liver cancer cells SMMC-7721 apoptosis and its mechanism[J]. Chinese Journal of Applied Physiology, 2021, 37(4): 415 – 418. (in Chinese).

[15] DING L, SHAO SH, HE MQ, *et al.* Effects of Betulinic acid on apoptosis in human gastric cancer SGC-7901 cells[J]. Chinese Journal of Applied Physiology, 2021, 37(5): 495 – 499. (in Chinese).

[16] CHEN YN, SHAO SL, HE MQ, *et al.* Effects of Betulinic acid on proliferation in human gastric cancer SGC-7901 cells[J]. Chinese Journal of Applied Physiology, 2020, 36(6): 628 – 632. (in Chinese).

[17] LIU JP, SHAO SL, HE MQ, *et al.* Effect of betulinic acid on proliferation of human gastric cancer MGC-803 cell line[J]. Chinese Journal of Applied Physiology, 2021, 37(3): 257 – 261. (in Chinese).

[18] FAN H, SHAO SL, HE MQ, *et al.* Effects of betulinic acid on apoptosis of human gastric cancer MGC-803 cells[J]. Chinese Journal of Applied Physiology, 2021, 37(4): 419 – 422. (in Chinese).

[19] YU R, YU BX, CHEN JF, *et al.* Anti-tumor effects of at-ractylenolide I on bladder cancer cells[J]. Journal of Experimental Clinical Cancer Research, 2016, 35(40): 1 – 10.