RESEARCH ARTICLE

Preliminary Evaluation of the *in vitro* Efficacy of 1, 2-di (Quinazolin-4-yl) Diselane against SiHa Cervical Cancer Cells

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Abstract

Cervical cancer is one the most common malignancies among females. In recent years, its incidence rate has shown a rising trend in some countries so that development of anticancer drugs for cervical cancer is an urgent priority. In our recent anticancer drug discovery screen, 1, 2-di (quinazolin-4-yl)diselane (LG003) was found to possess wide spectrum anticancer efficacy. In the present work, the *in vitro* anticancer activity of LG003 was evaluated in the SiHa cervical cancer cell line. Compared with commercial anticancer drugs 10-hydroxycamptothecin, epirubicin hydrochloride, taxol and oxaliplatin, LG003 showed better anticancer activity. Furthermore, inhibition effects were time- and dose-dependent. Morphological observation exhibited LG003 treatment results in apoptosis like shrinking and blebbing, and cell membrane damage. Lactate dehydrogenase release assay revealed that LG003 exerts such effects in SiHa cells through a physiology pathway rather than cytotoxicity, which suggests that title compound LG003 can be a potential candidate agent for cervical cancer.

Keywords: 1, 2-di (quinazolin-4-yl)diselane - cervical cancer - SiHa cells - anticancer activity

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Introduction

Cervical cancer is one the most common malignancy among females, and common age group of patients is 45-55 year (Srisuwan et al., 2014). There are several risk factors including Human papillomavirus (HPV), smoking, the interval between menarche and first sexual intercourse (Natphopsuk et al., 2012). it is concluded that high-risk HPV is a major risk factor of cervical cancer (Munoz, 2000; Natphopsuk et al., 2013; Zhang et al., 2013). In recent years, its incidence rate shows a rising trend, especially in developing countries, more than 85% of the global burden occurs in these regions (Ferlay et al., 2010; Simayi et al., 2013). In China, the incidence in females is 11.98/100, 000 in 2010, age-standardize incidence rate in china is 9.84/100,000 (Chen et al., 2014). In some countries, preventive and protective measures are compulsorily taken against HPV related diseases because these diseases have become a public health problem, especially cervical cancer which severely threatens female health (Yilmazel et al., 2014).

At present, early prevention, diagnosis and screening programs are effective measures against cervical cancer which are recommend by World Health Organization WHO. Although these measures are taken, there are still nearly 528.000 new diagnoses of cervical cancer and over 250.000 deaths annually (Wang et al., 2013; Wang and Wu, 2013; Yilmazel et al., 2014).

Main treatment regimens of cervical cancer are surgery, radiotherapy and chemotherapy. For bulky (stage IB2) or locally advanced cervical cancer, the effective treatment is concurrent chemoradiation (Mowa et al., 2009; Rai et al., 2014). Effective chemotherapy can improve the cure rate, decrease the risk of recurrence and metastasis, improve life quality of patients. There are some kinds of drugs used in cervical cancer chemotherapy, such as Oxaliplatin, cyclophosphamide, Fluorouracil, Bleomycin, Mitomycin, Adriamycin, Methotrexate and Cisplatin, and so on. Platinum compounds is standard chemotherapy for advanced and recurrent cervical cancer (Kesic, 2006), the response rates (RR) range from 20 to 30% (Cadron et al., 2007). Although these drugs display certain therapeutic efficacy in cervical cancer patients, the biggest problem with them is that their cytotoxicity is very high and efficacy relatively minute, specifically given the multidrug resistance and recurrence occuring which remains one of principal challenges facing cervical cancer (Hu et al., 2013). So we are looking forward to find new chemotherapy drugs that are more efficient, safe, low toxicity, and reduce the risk of recurrence and metastasis.

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In our recent anti-cancer drug discovery, 1, 2-di (quinazolin-4-yl)diselane (LG003) (Figure 1) was synthesized and exhibited significant *in vitro* antitumor effects in different tumor cell lines such as PC-3, A549, MDA-MB-435, MDA-MB-231, SiHa, Hela cells (Huang et al., 2014). This suggests compound LG003 could be a potential anti-tumor agent. In present study, we preliminarily investigated and evaluated the antitumor effects of compound LG003 on human cervical cancer cell line SiHa.

Materials and Methods

Cell line and reagents

Human cervical cancer cell line SiHa was supplied by professor Hongtao Wang (Department of Laboratory Medicine, Bengbu Medical College, Bengbu, China). LG003 was synthesized in School of Chemistry and Materials Science of Ludong University (Yantai, China). DMSO, MTT, Trypsine were all purchased from Sigma. RPMI 1640 was obtained from GIBICO. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd (Hangzhou, China). Lactate Dehydrogenase (LDH) test kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). 10-Hydroxycamptothecin was purchased from Huangshi Feiyun Pharmaceutical Co., Ltd. (Huangshi, China). Oxaliplatin was purchased from Jiang Su Heng Rui Medicine Co., Ltd. (Lianyungang, China). Epirubicin Hydrochlorideand was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. (Taizhou, China) and Taxol was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China).

Cell culture and drug treatments

SiHa cells were cultured in RPMI 1640 culture medium containing 10% FBS and grown at 37ºC in 5% CO_2 atmosphere. Tested agents were dissolved in DMSO as stock solution at first, the stock solution was directly diluted to required concentrations with culture medium before treatment. The solvent control group received an equivalent amount of DMSO and the final concentration of DMSO did not exceed 0.1% (v/v).

Assessment of cell inhibition rate

Cell inhibition rate was assessed through MTT assay (Huang et al., 2012). SiHa cells were exposed under either DMSO alone as negative control or different concentrations of tested agent for indicated treatment time. The inhibition rates of tested agents against SiHa cells were measured through an ELISA plate reader (BioTek-Synergy HT). The experiments were repeated three times independently. As a result, the inhibition rates of tested agents against SiHa cells were calculated as the following formula.

Morphological observation of SiHa cells (Huang et al., 2012)

SiHa cells were cultured in 96-well plates (2×10^3 or 3×10^3 cells per well in 0.1 mL medium) and incubated in 10% FBS/RPMI 1640 medium at 37ºC for 24 h.

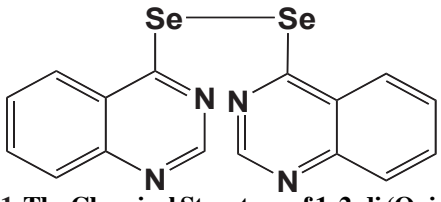

Figure 1. The Chemical Structure of 1, 2-di (Quinazolin-4-yl) Diselane

After cell attachment, the cells were treated with tested agents at indicated concentrations respectively. After required treatment time, the morphological observation was performed using inverted fluorescent microscope (Olympus).

Determination of lactate dehydrogenase (LDH) (Huang et al., 2014)

SiHa cells were cultured in 6-well plate (2 mL medium per well containing 8×10^4 cells), and maintained for 24h at 37ºC in 5% CO_2 atmosphere. The cells were treated with tested drugs at required concentrations for 24h, cytotoxicity of each drug was evaluated by measuring the release of LDH and enzyme activity. The positive control group was set through lysing cells with 2% (V/V) Triton X-100 in culture media at 37ºC, the negative control group was treated with 1/1000 (V/V) DMSO in culture media alone. The amounts of LDH in the supernatant were determined and calculated as kit instructions as cytotoxicity. All tests were performed in triplicate and three experiments were done independently with similar results.

Statistical analysis

All data were analyszed with SPSS 16.0. Results were shown in $\bar{x}\pm s$. The mean comparison among groups was performed using one-way analysis of variance (ANOVA). Statistical significance was defined as $p<0.05$. Each experiment had been done three times independently. Measurements from all the replicates were combined and analyzed.

Results

LG003 exhibits considerable inhibition effect on SiHa cells

To evaluate the inhibition efficacy of LG003 on SiHa cells, we measured the inhibition rates of LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin through MTT assay. Compared with DMSO control, LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride or Oxaliplatin treatment of 72h at indicated concentrations showed significant inhibition activities ($p<0.05$) against SiHa cells. Compared with Oxaliplatin (51.51±2.99) or 10-Hydroxycamptothecin (65.31±0.35) at 10 μM, LG003 (80.57±5.73) showed better inhibition activity ($p<0.01$); compared with Epirubicin Hydrochloride (89.77±9.37) or Taxol (89.08±9.2) at 10 μM, LG003 showed similar inhibition effects on SiHa cells ($p>0.05$) (Table 1, Figure 2). These results indicate that title compound LG003 may be a potential anticancer agent for human cervical cancer.

Morphological effects of LG003 and four commercial anticancer drugs on SiHa cells

In order to evaluate the effects of LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin on SiHa cells, we directly observed the morphological changes in SiHa cells treated with title agents. As can be seen in Figure 3, compared with DMSO control, after treatment of 24-48h at 1 μM, LG003 did not cause notable changes in cell number and morphology. LG003 treatment at same concentration for 72-96h mainly resulted in the decrease in cell population with moderate morphology changes. LG003 treatment at 10 μM resulted in significant inhibition and notable morphology changes. Prolonged treatments caused the decrease in cell population and apoptosis like shrinking and blebbing, and cell membrane damage, crumbling eventualy. Compared with LG003, 10-Hydroxycamptothecin or Oxaliplatin treatments at corresponding concentration for corresponding time caused similar effects; Taxol or Epirubicin Hydrochloride treatments at corresponding concentration for corresponding time mainly resulted in distinct damage effects on SiHa cells. As a whole, compared with other four commercial drugs, the damage effects of LG003 on SiHa cells are mild under the same dose and exposure time. These results suggest that LG003 possesses promising anticancer activity with mild cytotoxicity.

Table 1. Inhibition Rates of LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin Against SiHa Cells at 1 μM and 10 μM for 72h ($\bar{x}$±s)

Group	1 μM	10 μM
LG003	17.82±6.56	80.57±5.73
10-Hydroxycamptothecin	27.39±2.39*	65.31±0.35**
Taxol	67.54±1.9##	89.08±9.2
Epirubicin Hydrochloride	63.14±8.16△△	89.77±9.37
Oxaliplatin	18.05±4.28	51.51±2.99▲▲

Compared with DMSO control, compound LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride or Oxaliplatin showed significant inhibition (p<0.05); comparison between LG003 and 10-Hydroxycamptothecin, *p<0.05, **p<0.01; comparison between LG003 and Taxol, #p<0.05, ##p<0.01; comparison between LG003 and Epirubicin Hydrochloride, △p<0.05, △△p<0.01; comparison between LG003 and Oxaliplatin, ▲p<0.05, ▲▲p<0.01

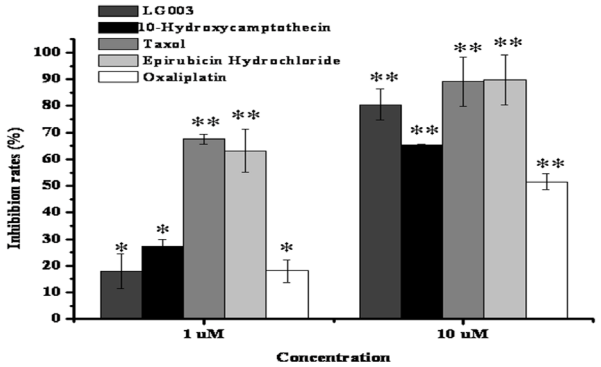

Figure 2. Inhibition Effects of LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin on SiHa cells at 1 μM and 10 μM for 72 h. SiHa cells were cultured in 10% FBS/RPMI 1640 medium in 96 well culture dishes (2000 cells per well) for 24h. After cell attachment, the cells were treated with DMSO (1/1000, V/V) alone as negative control or LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin for 72h. Final concentrations of tested agents are shown. Then cell viability was determined through MTT assay. All tests were performed in triplicate and three independent experiments were repeated. *p<0.05 *vs* DMSO control (0), **p<0.01 *vs* DMSO control (0)

LG003 exerts antitumor activity in a dose- and time-dependent manner in SiHa cells

In order to investigate the anticancer manner that LG003 executed *in vitro* inhibition activity in SiHa cells, the treatment time- and dose-effects of LG003 on SiHa cells were assessed through MTT assay as detailed above. As shown in Figure 4, compared with DMSO control (0 μM), LG003 treatment at 1 μM for 72h or 5 μM for 24h began to exhibit significant inhibition effects (p<0.05), and the inhibition activity increased in a dose- and time-dependent way (r_{24h}=0.91, r_{48h}=0.96, r_{72h}=0.93, r_{96h}=0.96, p<0.05) which was confirmed by synchronous morphological observation.

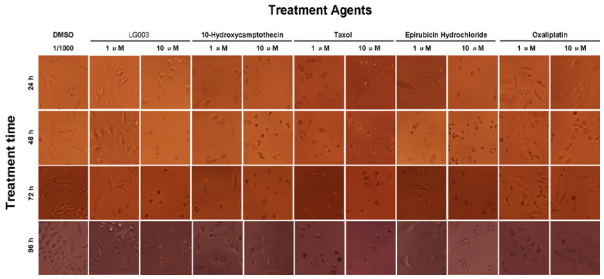

Figure 3. Comparison of Morphological Changes of SiHa Cells Treated by Compound LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin. SiHa cells were maintained in 96-well plates (2×10^3 cells per well) as detailed in "Materials and methods". The cells were treated with LG003, 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin at indicated concentrations respectively. After required treatment time, the morphological observation was performed using inverted fluorescent microscope (Olympus). Representative figures from three independent experiments are shown.

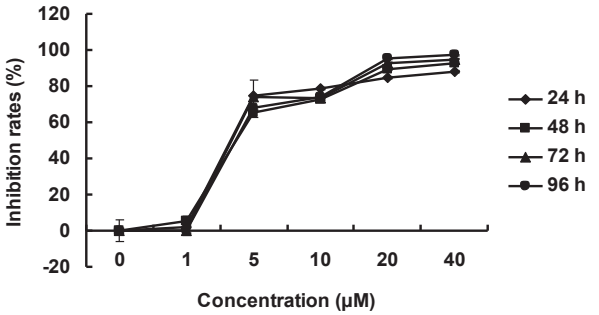

Figure 4. Dose- and Time-Dependent Manner of Anticancer Effects of LG003 in SiHa Cells. SiHa cells were cultured in 10% FBS/RPMI 1640 medium in 96 well culture dishes (3000 cells per well) for 24h. After cell attachment, the cells were treated with DMSO (1/1000, V/V) alone as negative control or LG003 for 24h, 48h, 72h, 96h. Final concentrations of LG003 are shown. Then cell viability was determined through MTT assay. All tests were performed in triplicate and three independent experiments were repeated

Treatment of LG003 results in the inhibition of cell proliferation and morphological changes in SiHa cells

It was found that, compared with DMSO control, LG003 treatment at 1 μM for indicated time did not show obvious effect on cell morphology and population size. However, LG003 applied at 5 μM dosage for 24h began to exhibit visible effects including antiproliferation and cell becoming round. LG003 applied more than 5 μM for 24h or at 5 μM for more than 24h resulted in significant inhibition of cell proliferation and notable cell shrinking and blebbing like apoptosis and crumbling eventualy. The effect of LG003 on SiHa cells increased with prolonged treatment and increasing concentration (Figure 5).

LG003 shows weak cytotoxicity in SiHa cells

In order to determine whether LG003 executed *in vitro* anticancer activity through cytotoxicity in SiHa cells, the level of lactate dehydrogenase induced by LG003 in supernatant was measured. The cytotoxicity of LG003 was assessed based on measuring the release of LDH and enzyme activity. As can be seen in Table 2, compared with negative control (spontaneous release well, 882.2±159.3), LG003 treatment at indicated concentrations did not cause significant defference in level of lactate dehydrogenase (p>0.05); compared with positive control (complete release well, 3649.3±198.0), LG003 treatment at indicated concentrations showed significantly lower level of LDH (p<0.05). In order to evaluate the cytotoxicity of LG003, we also tested the cytotoxicity of 10-Hydroxycamptothecin (as representative of Oxaliplatin and 10-Hydroxycamptothecin) and Taxol (as representative of Epirubicin Hydrochloride and Taxol).

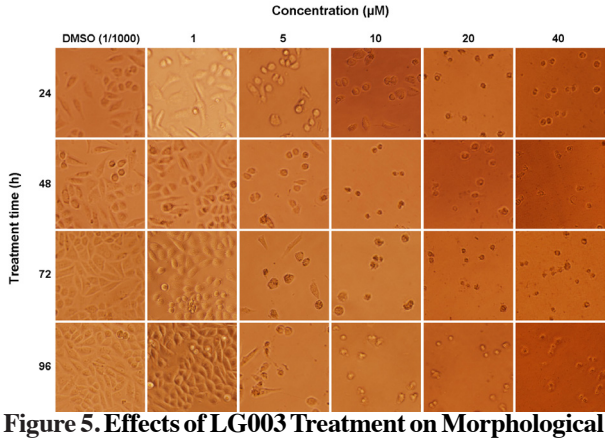

Figure 5. Effects of LG003 Treatment on Morphological Changes and Proliferation in SiHa Cells. SiHa cells were grown and treated as detailed above. The morphological changes were observed through inverted fluorescent microscope. Representative pictures from three independent experiments are shown

As shown in Table 2, compared with negative control, 10-Hydroxycamptothecin treatment at 10 μM or more showed significant cytotoxicity (p<0.05), Taxol at 5 μM or more showed significant cytotoxicity (p<0.05); compared with positive control, 10-Hydroxycamptothecin and Taxol at indicated concentrations showed significantly lower level of cytotoxicity (p<0.05); compared with LG003 treatment group at correspounding concentration, Taxol at 5 μM or more showed significant cytotoxicity (p<0.05), 10-Hydroxycamptothecin treatment at 10 μM or more showed significant cytotoxicity (p<0.05). These results suggest that LG003 has significantly low cytotoxicity and exerts antitumor activity in SiHa cells through antiproliferation or apoptosis inducing or other pathway rather than cytotoxicity.

It should be noted that, the cytotoxicity of LG003 treatment at 1-10 μM showed declining trend with the increase of the concentration (Table 2) which can be cell response to sustainable stress. At 20 μM the release of LDH induced by LG003 reached top level (1198.6±211.2) which can be cell adaptation. However, at 20-40 μM the release of LDH induced by LG003 showed a decreasing trend from 1198.6±211.2 to 942.8±105.7 which can be explained by compound-caused decrease in cell population. This phenomenon did not appear in 10-Hydroxycamptothecin or Taxol treated cells because the two agents possess so much cytotoxicity that these cells can not endure the stress.

Discussion

Today, the incidence of cervical cancer has been increasing which severely threatens women health (Baskaran et al., 2013). To develop effective therapy methdod for cervical cancer is urgent and essential (Wongwatcharanukul et al., 2014; McRae et al., 2014). Chemotherapy is a necessary component of the treatment (Sharma et al., 2012). Chemotherapy agents with considerable efficacy play a key role in treatment. There are some chemotherapy drugs used in cervical cancer, such as Oxaliplatin, cyclophosphamide, Fluorouracil, Bleomycin, Mitomycin, Adriamycin, Methotrexate, Cisplatin, and Epirubicin Hydrochloride, and so on. These agents are often of cytotoxicity. Although they display therapeutic effects, their side effects should not be ignored (Hu et al., 2013). In recent years, the development and achievements of human genome project and cancer science promote molecular diagnosis and targeted molecular cancer therapy (Chiranjeevi et al., 2014; Li et al., 2014). Small molecular-targeted anticancer drug discovery is given significant emphasis in anticancer research field. A number of biological molecules modulate angiogenesis in

Table 2. LDH Release of SiHa Cells Treated with Compound LG003, 10-Hydroxycamptothecin and Taxol at Different Concentrations for 24h (υ/L, $\bar{x}$±s)

Group	1 μM	5 μM	10 μM	20 μM	40 μM
LG003	1057.2±196.1*	997.2±76.7*▲	883.9±164.4*△▲	1198.6±211.2*△▲	942.8±105.7*△▲
10-Hydroxycamptothecin	899.2±159.9*	896.4±138.8*	1168.2±169.5*#	1358.8±104.3*#	1783.0±149.4*#
Taxol	1002.7±196.9*	1329.0±174.3*#	1246.0±128.3*#	1527.8±118.8*#	1999.9±143.5*#

Compared with positive control (complete release well, 3649.3±198.0), *p<0.05; compared with negative control (spontaneous release well, 882.2±159.3), #p<0.05; comparison between LG003 and 10-Hydroxycamptothecin, △p<0.05; comparison between LG003 and Taxol, ▲p<0.05

cervical cancer such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) (Yoysungnoen-Chintana et al., 2014). Quinazoline derivatives were reported possessing important biological activities of anticancer and antivirus, and mostly targeting EGFR (Huang et al., 2012). LG003, just a Quinazoline derivatives, was synthesized through introducing selenium into quinazoline lead structure and exhibited considerable *in vitro* antitumor activities in many cancer cell lines (Huang et al., 2014). In present work, we preliminarily investigated and evaluated its *in vitro* antitumor activity in SiHa cells. It was found that LG003 possessed remarkable efficacy which was better or similar than several commercial antitumor drugs. Synchronous observation indicated the damage caused by LG003 treatment was more moderate than 10-Hydroxycamptothecin, Taxol, Epirubicin Hydrochloride and Oxaliplatin at corresponding dosage and time. These results suggest that LG003 possesses promising anticancer activity with mild cytotoxicity. In order to investigate the treatment manner of LG003 in SiHa cells, we tested the effects of dosage and time on tested cells. It was found that LG003 executed *in vitro* anticancer activity in a dose- and time-dependent way. 5 μM dosage for 24h was its key effect point, at which LG003 exhibited visible antiproliferation and morphological effects. The effects of LG003 on SiHa cells increased with prolonged treatment and increasing concentration.

Furthermore, in order to determine whether LG003 executed *in vitro* anticancer activity through cytotoxicity in SiHa cells, the level of lactate dehydrogenase induced by LG003 in supernatant was measured. Normally LDH exists in almost all cytoplasm, it plays an important role in producing cell energe including cancer cells. (Seki et al., 2014). LDH can't be released into the extracellular through the cell membrance, but, this cytosolic enzyme can be released if the cell was injuried or dead. Therefore, the detection of LDH is used to evaluate the degree of cell damage (Berthier et al., 2002). Our present study showed, compared with negative control, LG003 treatment at indicated concentrations did not cause significant defference in level of lactate dehydrogenase ($p>0.05$) which suggests that LG003 has no or low cytotoxicity, comparison with positive control did show and confirm its faint cytotoxicity. Moreover, the cytotoxicity was significantly low compared with 10-Hydroxycamptothecin (at corresponding dose from 10-40 μM) and Taxol (at corresponding dose from 5-40 μM) ($p<0.05$).

It is interesting to note that the level of lactate dehydrogenase start to decrease at first and it reached to the minimum at the dose of 10 μM, and then increase to maximum at 20 μM. This may be due to the response to sustainable stress. Then these cells gradually endure and adapt the stress. But this adaptation is limited, when the stress well exceed cell capacity, cytotoxicity effect will continue to rise with increasing concentration. The cells under the exposure of 10-Hydroxycamptothecin or Taxol did undergo this way.

In summary, our current study demonstrates that LG003 has considerable antitumor activity in SiHa cells, and executes the inhibition effect through physiology pathway rather than cytotoxicity. This suggests that LG003 can be protential anticancer agents for human cervical cancer.

It should be noted that equal works have been done by our group in Non-small-cell lung cancer cell line A549 and human breast cancer cell lines MDA-MB-435. LG003 exhibited similar efficacy in these cells (unpublished data). Based on these knowlege, we conclude that LG003 possesses broad spectrum of anticancer activity. Our present study is still preliminary. LG003 should be extensively investigated in our fulture study to reveal its anticancer mechanism for the purpose of developing LG003 as a potential candidate anticancer drug.

Acknowledgements

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