LG308, a Novel Synthetic Compound with Antimicrotubule Activity in Prostate Cancer Cells, Exerts Effective Antitumor Activity

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ABSTRACT

Microtubule plays many different essential roles in the process of tumorigenesis in many eukaryotes, and targeting mitotic progression by disturbing microtubule dynamics is used as a common strategy for cancer treatment. Microtubule-targeted drugs, including paclitaxel and Vinca alkaloids, were previously considered to work primarily by increasing or decreasing the cellular microtubule mass. The tubulin/microtubule system, which is an integral component of the cytoskeleton, is a therapeutic target for prostate cancer. In this study, we found a novel synthetic compound, 8-fluoro-9-carboline (LG308), which disrupted the microtubule organization via inhibiting the polymerization of microtubule in PC-3M and LNCaP prostate cancer cell lines. Further study proved that LG308 induced mitotic phase arrest and inhibited G2/M progression significantly in LNCaP and PC-3M cell lines in a dose-dependent manner, and these were associated with the upregulation of cyclin B1 and mitotic marker MPM-2 and the dephosphorylation of cdc2. Besides, the cell proliferation and colony formation of PC-3M and LNCaP cells were effectively inhibited by LG308. Furthermore, LG308 induced apoptosis and cell death in PC-3M and LNCaP cell lines in vitro. In vivo, LG308 dramatically suppressed the growth and metastasis of prostate cancer in both xenograft and orthotopic models. All these data indicate that LG308 is a promising anticancer candidate with antimitotic activity for the treatment of prostate cancer.

Introduction

In all eukaryotic cells, microtubule, as one of the main components of the cell cytoskeleton, plays many different essential roles, including the maintenance of cell shape, intracellular transport, cell motility, meiosis, and mitosis (Amos, 2000; Jordan and Wilson, 2004). Previous studies have shown microtubule dynamics to be crucial for cell function (Wilson and Jordan, 1995). In addition, microtubule is extremely important in the process of mitosis in orchestrating the separation and segregation of chromosomes, which sheds lights that microtubule is an important target for the research and development of anticancer drugs (Risinger et al., 2009; Stumpff et al., 2014). All microtubule-targeted drugs can disrupt the dynamics of microtubule and induce arrest of mitosis of tumor cells (McIntosh and Hering, 1991). Antimitotic drugs can be classified into two groups: one that inhibits microtubule polymerization, such as colchicine and vinorelbine; and the other, which stimulates microtubule depolymerization, such as paclitaxel and docetaxel (Desbene and Giorgi-Renault, 2002).

Prostate cancer (PCa) is a kind of common malignant tumor in the male genitourinary system. Among men, PCa is the most frequently diagnosed new cases of cancer and the second leading cause of cancer death in the United States (Siegel et al., 2015). With the development of society and the improvement in living standards, as well as the changed dietary structure and the aging of the population and hormone misuse, it can be doubtlessly estimated that the worldwide incidence of PCa will increase rapidly, attracting even more attention (Ren et al., 2013). At present, surgical treatment, radiation, hormone treatment, and chemical therapy are four most common therapeutic methods for PCa patients. Hormone treatment, which is also termed androgen-deprivation therapy, is the critical therapeutic option for advanced PCa patients of the preceding four therapies (Jani and Hellman, 2003; Ponholzer et al., 2011). Unfortunately, hormonal therapy has a palliative benefit in the early stage of...
PCa treatment, but most patients eventually progress to androgen-independent or hormone-refractory PCa, which means androgen-deprivation therapy is no longer effective, and the cancer continues to develop (Miyamoto et al., 2004). Therefore, chemotherapy of the advanced hormone-refractory PCa has drawn much more attention over the last few decades. Some microtubule-targeted agents have demonstrated overall survival benefit clinically (Suzman and Antonarakis, 2014). Nowadays, some chemodrugs that target microtubule and tubulin, including docetaxel, cabazitaxel, epothilones, and vinorelbine, have been used clinically as chemotherapy for androgen-independent PCa (Sewak et al., 2010; Kearns et al., 2013; Morganti et al., 2013). Nevertheless, drug resistance often appears after prolonged treatment. Therefore, researching and developing some novel antimicrotubule candidates are potential strategy for hormone-refractory PCa (Aneja et al., 2010).

In this study, a novel compound with tetrahydro-β-carboline scaffold, named sulforhodamine B (SRB; LG308) (Fig. 1A; mol. wt: 308.3495), was synthesized, screened, and identified as an anticancer agent in our laboratory. Our results indicated that LG308 induced G2/M cell-cycle arrest by inhibiting microtubule polymerization and disrupting the microtubule assembly. Further study suggested that LG308 inhibited the proliferation of PCa cells and induced cell apoptosis and cell death. More important is that LG308 suppressed xenograft and orthotopic PC-3M tumor growth and metastasis.

Materials and Methods

Synthesis of LG308. To a solution of 2-fluorophenylhydrazine hydrochloride (0.81 g, 5.00 mM) in 30 ml of EtOH/H2O (v/v = 5/1) 4-chloro-1,1-dimethoxybutane (0.84 g, 5.50 mM) was added and heated to reflux for 2 hours. Then the solvent was removed under vacuum, and the residue was chromatographed through silica gel eluting with CH2Cl2/MEOH (30:1) to give the 7-fluorotryptamine (0.37 g) as an oil. Subsequently, aq. HCHO (1.56 ml) was added to the solution of 7-fluorotryptamine (0.37 g, 2.08 mM) in CH2C(OH) (15 ml) in a dropwise manner. The mixture was stirred at room temperature for 12 hours at the atmosphere of N2; then the solvent was removed under vacuum, and the residue was chromatographed through silica gel eluting with CH2Cl2/MEOH (40:1) to afford the 8-fluoro-1,3,4,9-tetrahydro-β-carbonile (0.36 g) as a white powder. Finally, to a solution of phenylacetic acid (283 mg, 2.08 mM) in anhydrous N,N-Dimethylformamide (3 ml) were added 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (518 mg, 2.70 mM) and N-Hydroxybenzotriazole (309 mg, 2.29 mM) at 0°C, and then 8-fluoro-1,3,4,9-tetrahydro-β-carbonile (0.36 g, 1.89 mM) was added to the mixture after stirring for 15 minutes. The mixture was stirred for another 3 hours, diluted with H2O, and extracted with EtoAc. The combined organic phase was dried over anhydrous Na2SO4, concentrated, and chromatographed over silica gel to give 0.54 g of LG308. The synthesis route of LG308 is described in Supplemental Fig. 1A.

Cell Lines, Animals, and Reagents. Human PCa cell lines PC-3M, DU145, 22RV1, and PC3 were purchased from American Type Cell Culture (Manassas, VA). Human normal prostate epithelium immortalized cell line PNT1A (a human normal prostate epithelium immortalized cell line) and human PCa cell line LNCap was gifts from Professor Hanyi Zhuang of Shanghai Jiao Tong University. PC-3M cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Cleveland, OH). LNCap, PNT1A, PC3, DU145, and 22RV1 cells were maintained in RPMI-1640 medium (Gibco). Both media were supplemented with 10% fetal bovine serum (Wist, St. Bruno, QC, Canada). In addition, PC-3M cells were transfected with pGL4 vector (Promega, Madison, WI) and selected in G418 (C6H4NO4-O10.2H2SO4) for stable PC-3M-luc cell line. All cells were incubated at 37°C with 5% humidified CO2. Mice were obtained from National Rodent Laboratory Animal Resources, Shanghai Branch of China. All animal experimental protocols were approved by the Animal Investigation Committee of the Institute of Biomedical Sciences, East China Normal University. The antibodies used in this study were anti-cdc2, p-cdc2, cdc2e 25c, cyclin B1, caspase 3, poly ADP ribose polymerase (PARP), β-actin (Cell Signaling Technology, Danvers, MA), α-tubulin (Epitomics, Burlingame, CA), and CD31 (Abcam, Cambridge, UK) and anti-phospho-Ser/Thr-Pro MPM-2 (Millipore, Billerica, MA). The compound LG308 was synthesized by our laboratory with purity of more than 98%.

Cell Proliferation Assay (Sulfonhodamine B Assay). Cell proliferation inhibition assay was determined by sulforhodamine B (SRB) assay. Briefly, 4000 cells/well were seeded in 96-well plates. After 24 hours, cells were exposed to five different doses (0, 1, 5, 10, 20 μM) of LG308 for 72 hours. Cells were fixed with 10% trichloroacetic acid for 1 hour at 4°C, washed five times with flowing water, and then air-dried. Then stained with 50 μl 0.4% (w/v) SRB for 20 minutes at room temperature, washed five times with 1% acetic acid, and then air-dried. Then 100 μl 10 mM Tris was added per well, and absorbance was measured at 515 nm.

Colony Formation Assay. Cells were split with trypsin and seeded 2000 (PC-3M, PC3, DU145) or 4000 (LNCap, 22RV1) per well in six-well dishes. Cells were allowed to attach overnight and then exposed to different doses of LG308 for 1 week. After fixation with 4% paraformaldehyde for 20 minutes at room temperature, cells were stained with 0.2% crystal violet. The number of cell colonies was calculated and analyzed as the ratio of the number of treated samples to untreated samples. Triplicate wells were set up for each dose.

Cell-Cycle Distribution Analysis. Cells were initially seeded in 6-cm dishes. After incubation for 24 hours, cells were treated with four different doses (0, 5, 10, and 20 μM) of LG308 for 24 hours. After being washed with PBS and digested with trypsin; adherent and floating cells were collected, washed once with PBS, and then fixed in cold 70% ethanol overnight in 4°C. After ethanol fixation, cells were washed in PBS once and suspended in PBS with 200 μg/ml ribonuclease and 50 μg/ml propidium iodide in the dark for 30 minutes. Then cells were analyzed by flow cytometry (FACS Calibur; BD Biosciences, Franklin Lakes, NJ).

Immunofluorescence Staining. Immunofluorescence staining was performed according to a previous report (Zhang et al., 2011). Briefly, cells were seeded on gelatin-coated glass coverslips; 24 hours later, cells were treated with different doses of LG308 for 24 hours. Then cells were incubated with 4% paraformaldehyde for 20 minutes, washed with PBS, treated with 0.5% Triton-X 100, washed with PBS. After blocking in 0.5% bovine serum albumin, cells were incubated with primary antibody overnight at 4°C before further incubation with secondary antibody at 37°C for 1 hour in the dark. Then 4’,6-diamidino-2-phenylindole (DAPI) was added for 5 minutes in the dark. Images were recorded by confocal microscopy (Leica, Oskar-Barnack-Straße 11, Germany).

Mitotic Index Assay. Mitotic index assay was performed according to previous report (Chang et al., 2011) with modification. Cells were seeded on gelatin-covered glass coverslips; 24 hours after, cells were treated with different doses of LG308 for 24 hours. Then cells were incubated with 4% paraformaldehyde for 20 minutes at room temperature, cells were permeabilized with 0.2% Triton-X 100 in PBS for 5 minutes. The nuclei were stained with DAPI. After being washed with PBS, cells were visualized and photographed with Leica microscope. Five random fields were counted and analyzed. Mitotic index was calculated by dividing the total number of examined cells by the number of cells in mitosis.

Western Blotting. Cells were exposed to five different doses (0, 1, 5, 10, 20 μM) of LG308 for the indicated times and lysed in RIPA buffer [50 mM Tris- HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 30 mM Na3P04, 50 mM NaF, 1 mM Na2VO4] containing protease/phosphatase inhibitors (Roche, Indianapolis, IN).
LG308 (C_{19}H_{17}FN_2O)  
MW: 308.3495

Fig. 1. (A) Chemical structure, molecular formula and 308.3495 mol. wt. of LG308. (B) LG308 more potently inhibited the proliferation of several PCa cells than normal prostate epithelium cells. Cells were incubated with different doses of LG308 for 72 hours in 96-well plates, and cell viability was tested by SRB assay (n = 3); **P < 0.01; ***P < 0.001. (C) LG308 inhibited colony formation of PCa cells PC-3M and LNCaP. After treatment by different doses of LG308 in six-well plates for a week, cells were fixed and stained with crystal violet, and the numbers of cell colonies were counted. n = 3; ***P < 0.001. (D) LG308 induced cell-cycle arrest of LNCaP and PC-3M cell lines. Cells were analyzed by flow cytometry after treatment of LG308 with different doses for 24 hours. (E) Effect of LG308 on the expression of G2/M transition related proteins. After incubation with different doses of LG308 for 24 hours, cells were lysed and cdc2, p-cdc2, cdc25c, cyclin B1, and β-actin were measured by Western blotting analysis with their specific antibodies.
Lysates were mixed with sample loading buffer and heated at 100°C for 10 minutes. After separated by SDS-PAGE, extracted protein was transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in PBS and 0.1% Tween-20 and then incubated with specific primary antibodies over night at 4°C. Then membranes were exposed to secondary antibodies for 2 hours at room temperature. Immunoreactive proteins were visualized using the Odyssey Fluorescence Scanner (LI-COR Bioscience, Inc., Lincoln, NE).

Apoptosis Analysis. Apoptosis analysis was carried out by flow cytometry (FACS Calibur; BD Biosciences). Cells were treated with four different doses (0, 5, 10, 20 μM) of LG308 for 48 hours or with 10 μM LG308 for different times. Cells were washed with PBS, harvested by 0.25% trypsin, and washed with PBS again, followed by resuspension in binding buffer, after which 5μl of Annexin V fluorescein isothiocyanate and 5 μl of propidium iodide were added, and the mixture was kept in the dark for 15 minutes at room temperature, and then 400 μl of binding buffer was added and analyzed immediately with flow cytometry (FACS Calibur; BD Biosciences).

Viability Assay. Viability assay was performed using the live/ dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR). This kit contains calcine-AM to stain the living cells (green) and ethidium homodimer-1 to stain the dead cells (red). Briefly, cells were exposed to five different doses (0, 1, 5, 10, 20 μM) of LG308 for 48 hours, and then 2 μM calcine AM and 4 μM ethidium homodimer-1 working solution was added. After incubation at room temperature for 30 minutes, the green living and the red dead cells were visualized by fluorescence microscopy and photographed. Cells from three random areas per sample were counted for statistical analysis.

In Vivo Microtubule Polymerization Assay. In vivo microtubule polymerization assay was performed according to previous reports (Anjei et al. 2010; Chang et al., 2011; Shi et al., 2013; Zhao et al., 2012; Kuo et al., 2004) with modification. PC-3M and LNCaP cells were seeded on six-well plates. After 24 hours, cells were exposed to four different doses (0, 5, 10, 20 μM) of LG308, 50 nM colchicine, and 50 nM paclitaxel for 24 hours, respectively. Cells then were washed with PBS three times before adding lysis buffer containing 20 mM Tris-HCL (pH 6.8), 1 mM MgCl2, 2 mM EGTA, and protease inhibitor and phosphatase inhibitor and 0.5% NP40. Supernatants were collected after centrifugation at 14,000 rpm for 10 minutes. Supernatants and pellets were dissolved in SDS-PAGE sampling loading buffer at 95°C for 10 minutes. Supernatants containing soluble tubulin and the pellets (the polymerized tubulin lysates) were subjected to 10% SDS-PAGE before Western blotting.

Xenograft Model of Human Prostate Cancer PC-3M Tumor. PC-3M cells (2×107) were implanted s.c. on the right side of the dorsal area of 4-week-old male nude mice. Ten days later, mice were randomly divided into two groups (n = 8). Using the appropriate dose according to the preliminary test, LG308 (25 mg/kg) was injected i.p. each day. Control group was treated with dimethyl sulfoxide (DMSO). Twenty days later, mice were sacrificed, tumors were removed, and images were taken. The growth rate of the tumor xenograft was evaluated by determining the tumor volume using digital caliper every day. Tumor growth rate was measured as the following equation: volume = length × width2 × 0.52. Mice were continually observed until they were sacrificed (Dong et al., 2010).

PC-3M Orthotropic Tumor Growth and Metastasis. Male nude mice were anesthetized and placed under a sterile cover in a supine position. An incision was made 3 mm above the pubic symphysis, and the bladder and seminal vesicles were carefully lifted to expose the dorsal prostate. The PC-3M-luc cells suspension (1 × 105 cells in 0.05 ml of PBS) was injected into the dorsal prostate (Zhang et al., 2010; Pettaway et al., 1996; Tuomela et al., 2008). After inoculation, the abdominal muscle layer and skin were closed and sewed up, respectively. One week later, the mice were injected with 0.25% trypsin, and washed with PBS again, followed by resuspension in binding buffer, after which 5μl of Annexin V fluorescein isothiocyanate and 5 μl of propidium iodide were added, and the mixture was kept in the dark for 15 minutes at room temperature, and then 400 μl of binding buffer was added and analyzed immediately with flow cytometry (FACS Calibur; BD Biosciences).

H&E and Immunohistochemistry. H&E staining and immunohistochemistry were performed as reported previously (Zhang et al., 2012). Tumors, liver, and kidney were removed from sacrificed mice, fixed with 10% formaldehyde, and then embedded in paraffin. Paraffin-embedded tissues were sectioned (i.e., 5-μm sections). Tumor tissues were stained with MPM-2 (Millipore) and CD31 (Abcam). H&E staining of liver and kidney was performed according to standard protocol. Images were taken with Leica microscope. The results were analyzed using Image-Pro Plus 6.0 software.

Determination of LG308 in Nude Mouse Plasma. Blood samples were collected from mice at 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 8 hours after i.p. administration. The concentration of LG308 in plasma was determined by an Agilent 1290 LC system coupled with 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Chromatography separation was performed on a Phenomenex (Torrance, CA) Kinex XB-C18 column (3.0 × 100 mm, 2.6 μm). The mobile phase consisted of water (A) and acetonitrile (B) using gradient elution at a flow rate of 0.3 ml/min, and the injection volume was 4 μl. Detection of the ions was performed in the multiple reaction monitoring mode, monitoring the transition of mass-to-charge ratio (m/z) 307.2 precursor to the m/z 159.9 product ion for LG308 and m/z 401.3 precursor to m/z 254 product ion for IS (an analog of compound). The fragmentor and collision energy were optimized for LG308 and IS, respectively. A liquid-liquid extraction method by ethyl acetate was used to isolate LG308 from plasma.

Statistical Analysis. Dose- and time-dependent effects were analyzed using one-way analysis of variance. The analysis of tumor volume and body weight varies with time in animal models; the Wald test was used. Other parameters were defined with an unpaired student’s t test; P < 0.05 was considered significant. P values for all experiments analyzed were indicated.

Results

LG308 Inhibits Proliferation and Colony Formation of PCa Cells. First, the antiproliferative effect of LG308 against normal prostate or PCa cells was determined by SRB assay, as indicated in PC-3M, LNCaP, PC3, DU145, and 22Rv1 cell lines (PCa cell line) and PNT1A cell line. In these assays, LG308 inhibited the proliferation of PC-3M, LNCaP, PC3, DU145, and 22Rv1 cells, whereas it just showed a slight effect on the PNT1A cell (Fig. 1B). For example, after treatment of LG308, the half-maximal inhibitory concentration (IC50) were about 5 μM against LNCaP and PC-3M PCa cell lines, compared with about 90% viability of normal prostatic cell PNT1A at the same concentration. When treated with 20 μM of LG308, the viability of LNCaP and PC-3M cells was dramatically decreased to 30%, whereas the increase for PNT1A cells was only about 20%, which indicated that LG308 showed a more potent cytotoxic effect on PCa cells than on normal prostate epithelium cells. In addition, colony formation assay was performed for illustrating the ability of cell proliferation more specifically. In accordance with the SRB assay, LG308 inhibited colony formation in a dose-dependent manner, and the most significant difference in colony formation between LG308-treated cells and control cells was at 5 μM LG308 (Fig. 1C and Supplemental Fig. 1B). All these results demonstrated that LG308 inhibited the proliferation of PCa cells and possessed great selectivity between prostate normal cells and PCa cells.

LG308 Induces Cell-Cycle Arrest of LNCaP and PC-3M Cell Lines. To verify the causal relation of the inhibitory cell proliferation and cell-cycle arrest of LNCaP and PC-3M cells after treatment with LG308, cell-cycle distribution was...
analyzed by flow cytometry. LG308 increased the cell number at G2/M phase after 24-hour treatment with increased concentration, accompanied by a decreased cell number at the S phase and G1 phase in LNCaP and PC-3M cell lines (Fig. 1D); a similar cell-cycle arrest effect was also detected in PNT1A cells (Supplemental Fig. 1C). Furthermore, proteins responsible for G2/M transition were measured by Western blotting. As expected, the rational results were consistent with the cell-cycle assay. LG308 caused dose-dependent decreases in phosphorylated cdc2 and total cdc25c and an accumulation of cyclin B1 in PC-3M and LNCaP after 24-hour treatment (Fig. 1E). Meanwhile, the level of total cdc2 was not affected by the treatment of LG308 (Fig. 1E). Conclusively, these results indicated that LG308-induced cell cycles were arrested in G2/M phase before cell death occurred.

LG308 Arrests Cell-Cycle in Mitotic Phase. Next, the mitosis index assay was performed referred to the published report by staining the cells with DAPI to confirm whether cells were arrested at G2 or M phase (Chang et al., 2011). As shown in Fig. 2A, with an increasing dose of LG308, more cells were accumulated at M phase as indicated by condensed nuclei in PC-3M, and LNCaP showed the similar M-phase cell accumulation in response to LG308 treatment (Supplemental Fig. 2A). Furthermore, we conducted the Western blotting assay to detect the MPM-2, a marker for mitotic cells. As Fig. 2B shows, MPM-2 was upregulated, accompanied by an increase in LG308 concentration. Upregulation of MPM-2 was also observed in LNCaP cells (Supplemental Fig. 2B).

LG308 Disrupts Microtubule Organization of PC-3M and LNCaP Cells. Microtubules are composed of α-tubulin and β-tubulin heterodimers and play critical roles in regulating cell cycle and cell proliferation (Jordan and Wilson, 2004). Conventionally, α-tubulin and β-tubulin heterodimers exist in two forms: soluble monomer and polymerized tubulin heterodimers. Our results showed that inhibitory effects of LG308 on PCa cell growth were related to cell mitosis arrest, which might be regulated by microtubular dynamics. The in vivo microtubule polymerization assay was performed on PC-3M and LNCaP cells to investigate the effect of LG308 on microtubule polymerization. After extraction of soluble and polymerized tubulin, we discovered that polymerized tubulin decreased in a dose-dependent manner in response to LG308 concentration at 72 hours in PC-3M cells (Supplemental Fig. 2C). Meanwhile, a cell viability assay was also carried out on PC-3M and LNCaP cells using the live/dead viability/cytotoxicity kit (Molecular Probes) to investigate whether LG308 resulted in the death of PCa cells. As revealed in Fig. 3C, the percentage of dead cells increased with the increase in LG308 concentration. These data indicate that LG308 also provoked apoptosis and cell death in addition to its effect on cell cycle.

LG308 Inhibits the Growth of PC-3M Tumor Xenograft. The preceding data suggest that LG308 inhibits PCa cells in vitro; thus, we tested the effects of LG308 (25 mg/kg daily) on PC-3M tumor xenograft to investigate the effect of LG308 on PCs in vivo. As shown in Fig. 4A, LG308 inhibited the growth of PC-3M tumor compared with the control group. The average tumor size of control group was 2510.62 ± 663.10 mm³, whereas the volumes of 10 and 25 mg/kg daily treated group was 178.16 ± 52.86 mg, At the same time, the treatment of LG308 at the given concentration had little effect on the body weights of the LG308-treated mice (Fig. 4A), and the H&E staining results of liver and kidney confirmed that LG308 presented no obvious influence on the anatomic morphologies of mice administrated LG308 (Fig. 4C) compared with the control group. These data suggest that LG308 had low toxicity to mice at the curative dose. At the same time, less cell proliferation, as indicated by probes anti-Ki-67 and accumulation of M-phase cells, as indicated by probes anti-MPM2 (Fig. 4B), was detected in LG308-treated tumors, but no significant change in angiogenesis was indicated by anti-CD31 probes (Supplemental Fig. 4A). Thus, LG308 exerted potent antitumor efficacy toward PC-3M tumor xenograft in vivo.

LG308 Suppresses PC-3M Orthotopic Tumor Growth and Metastasis In Vivo. Next, we evaluated the impact of LG308 on PC-3M orthotopic tumor growth and a metastasis model. A luciferase-expressing PC-3M cell line (PC-3M-luc) was constructed before cells were injected into the dorsal prostate. As Fig. 5A shows, on the day the nude mice were sacrificed, the average normalized photon flux of the group treated with 10 mg/kg per day of LG308 and the group treated with 25 mg/kg per day of LG308 were 35.27% ± 18.45% and 11.04% ± 9.95% that of the control group, respectively. The average tumor volume of the control group was 1230.71 ± 663.10 mm³, whereas the volumes of 10 and 25 mg/kg daily of the LG308-treated group were 388.18 ± 46.98 mm³ and
209.95 ± 144.47 mm³, respectively (Fig. 5D). The tumors of the control group spread to other organs, like the intestine, liver, kidney, and spleen; however, the LG308-treated group did not develop obvious metastasis (Fig. 5B). At the same time, treatment of LG308 with the given concentration had little influence on the body weights of the LG308-treated mice.
compared with the control group (Fig. 5C). In conclusion, these results indicate that LG308 not only inhibits the growth of PC-3M tumor xenograft, but it also hinders PC-3M orthotopic tumor growth and metastasis in vivo.

Concentration of LG308 in Nude Mouse Plasma. As shown in Supplemental Fig. 4B, the concentrations of LG308 in nude mouse plasma at 10 minutes were 6885.01 ng/ml and 3580.55 ng/ml after i.p. administration of LG308 with doses of 25 mg/kg and 10 mg/kg, which far exceed its effective concentration in vitro, and it could still reach 362.19 ng/ml and 38.25 ng/ml after 8 hours, respectively. These data could support its pharmacologic effects in vivo.

Discussion

In the present study, we described a small molecular compound, LG308, which inhibits prostatic tumor growth and metastasis. LG308 inhibited the proliferation of androgen-dependent (LNCaP, 22RV1) or -independent (PC-3M, DU145, PC3) PCa cells in vitro and suppressed PC-3M tumor growth and metastasis via blocking microtubule polymerization.

In the past few years, great strides have been made for identifying effective and new therapies for PCa (Shore et al., 2012), but most PCa cases ultimately relapsed and progressed to intractable castration-resistant PCa (CRPC) (Xu and Zhang, 2014). Traditional chemotherapy was applied clinically for the treatment of CRPC. Docetaxel and cabazitaxel are well established antimicrotubule chemotherapy agents with significant survival benefits in CRPC treatment (McKeage, 2012). The successful use of docetaxel and cabazitaxel in PCa chemotherapy suggests that microtubule-targeting agents may be a feasible strategy for treatment; however, although they have been extensively used, these agents continue to...
Fig. 4. (A) PC-3M tumor-bearing mice were treated i.p. with LG308 (25 mg/kg daily) or DMSO (served as control) for 20 days. Tumor volume and mice body weight were measured 3 times a week during LG308 administration, and the weight of the removed tumor was also measured. n = 8; ns, not significant; *P < 0.05; ***P < 0.001. (B) Expression of Ki-67 decreased, whereas MPM-2 increased in the LG308 treatment group. For Ki-67 and MPM-2, IHC staining, sections cut from the paraffin blocks of PC-3M xenograft were carried out using Ki-67 or MPM-2 antibody, and the positive cells (brown) of each group were counted. n = 3; ***P < 0.001. (C) Liver and kidney H&E staining of control and LG308 treatment group.
have many problems, including drug resistance and concomitant side effects (Kavallaris, 2010). So, it is necessary to develop new agents that overcome these hurdles. In our study, we showed that LG308 inhibited the polymerization of tubulin in a concentration-dependent manner. Polymerized tubulin decreased significantly when responding to LG308 exposure in the microtubule polymerization assay in PC-3M and LNCaP cells. We also observed disorganized microtubule distribution in interphase and disrupted spindle in mitotic phase of PC-3M and LNCaP cells. Moreover, the scattered staining increased with the concentration increase of LG308. In accordance with the fact that microtubule-targeting agents

Fig. 5. (A, B) PC-3M-luc tumors were imaged by IVIS, and tumor volume was represented by the normalized photon flux; separated tumors, metastasis, and organs were also imaged by IVIS. n = 8; **P < 0.01. (C) Mice body weight was measured every 2 days during LG308 treatment. ns, no significant. (D) Orthotopic tumors were removed and images were taken, and the volume of the tumors was measured. n = 8; **P < 0.01.
arrest the cell cycle at the G2/M phase, our results showed that LG308 induced a G2/M blockade, as indicated by flow cytometry analysis (Fig. 1D). We further investigated the in-depth molecular mechanism of cell-cycle arrest induced by LG308. Cyclin B and cdc2 kinase regulated the start of M phase (King and Cidlowski, 1995). Previous research has shown that the activation of cdc2 kinase is dependent on accumulation of cyclin B and dephosphorylation of cdc2 (Chiang et al., 2013). We observed an obvious dose-dependent decrease in phosphorylated cdc2 protein and total cdc25c protein in PC-3M and LNCaP and an accumulation of cyclin B1 protein in PC-3M. Meanwhile, the level of total cdc2 was not affected by the treatment of LG308. Previous studies have shown that MPM2 was a protein related to mitosis (Scatena et al., 1998), and the MPM-2 upregulation accompanied by the increase of LG308 concentration was detected in our study. Cell-cycle arrest at the G2/M phase might be an upstream event leading to apoptosis (Jordan and Wilson, 2004). Significant cell apoptosis and cell death induced by LG308 were detected by flow cytometry (Jordan and Wilson, 2004). Mitotic phase might be an upstream event leading to apoptosis (Jordan and Wilson, 2004). Meanwhile, the level of total cdc2 was not affected by the treatment of LG308. Previous studies have shown that MPM2 was a protein related to mitosis (Scatena et al., 1998), and the MPM-2 upregulation accompanied by the increase of LG308 concentration was detected in our study. Cell-cycle arrest at the G2/M phase might be an upstream event leading to apoptosis (Jordan and Wilson, 2004). Significant cell apoptosis and cell death induced by LG308 were detected by flow cytometry analysis and cell live/dead assay in PCa cells (Fig. 3, A–C). In conclusion, LG308 is a promising anticancer candidate with antimicrotubule activity for the treatment of prostate cancer according to our research.

Besides the primary advantage of LG308 that it possesses remarkable anticancer effectiveness in vitro and in vivo, another advantage of LG308 is its acceptable toxicity. In fact, drugs possessing antimicrotubule activity have been used clinically as anticancer drugs for several years (Zhang et al., 2015), but many obstacles to effective treatment with currently approved agents remain, including significant side effects (Risinger et al., 2009; Novio et al., 2014). According to our research, LG308 has acceptable toxicity. Although LG308 induced cell-cycle arrest in PNT1A, a human normal prostate epithelium immortalized cell line, as well as PCa cell lines, and it shows only slight proliferation inhibition in PNT1A cells with the effective concentration in PCa cell lines. LG308 at the effective concentration had little effect on the body weight of the LG308-treated mice in vivo compared with the control group in s.c. xenograft tumor model and orthotopic growth and metastasis model (Figs. 4A and 5C). No significant difference in H&E staining results of liver and kidney between the LG308-treated group and the control group (Fig. 4C).

Our study had a few limitations. Although LG308 had an inhibitory effect on microtubule polymerization, we still did not know the specifics. It was not clear whether LG308 exerted its inhibitive effect on microtubule polymerization by direct combination with tubulin or through some other means. Meanwhile, apoptosis and cell death induced by antimitic agents were known to be related to alterations in cellular signaling pathways, but the specific mechanisms of LG308 in PCa were not clear enough. Additionally, we also tested some symbol parameters of the pharmacokinetics and pharmacodynamics of LG308 in vivo; more studies are needed to determine more of these specific characteristics for its preclinical study in the future.

In conclusion, our data demonstrated that LG308, a novel small-molecule compound was designed and synthesized, and it was efficacious in suppressing the growth and metastasis of PCa both in vitro and in vivo via perturbing the microtubule polymerization primarily. As a result, LG308 can be considered a new potential drug candidate in for PCa, and it was worth further research and investigation.

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Authorship Contributions


References

LG308 Suppresses PCa by Blocking Microtubule Polymerization


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