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·Original Article·

Contragestazol (DL111-IT) inhibits proliferation of human androgen-independent prostate cancer cell line PC3 *in vitro* and *in vivo*

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Abstract

Aim: To evaluate the antiproliferative activity of contragestazol (DL111-IT) on the human prostate cancer cell line PC3 *in vitro* and *in vivo* and to elucidate its potential molecular mechanisms. **Methods:** The cell killing ability of DL111-IT was measured by the 3-(4,5-dimethylthia-zol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent assay method and the tumor xenograft model. The cell cycle was analyzed by flow cytometry and protein expression, including retinoblastoma (pRb), cyclin-dependent kinase 4 (CDK4) and cyclin D1, was detected by Western blotting. **Results:** DL111-IT exhibited high efficiency on cell growth inhibition of the human androgen-independent prostate cancer cell line PC3. The drug concentration that yielded 50 % cell inhibition (IC_{50} value) was 9.9 mg/mL. In the PC3 tumor xenograft study, DL111-IT (1.25 mg/kg–20.0 mg/kg) given once a day for 10 days significantly inhibited tumor growth, with the inhibition rate ranging from 21 % to 50 %. Flow cytometric analysis indicated that DL111-IT could cause G_1 arrest in the PC3 cell line, but not apoptosis. DL111-IT enhanced pRb expression and down-regulated CDK4 and cyclin D1 expression, suggesting that cell cycle regulation might contribute to the anticancer property of DL111-IT. **Conclusion:** DL111-IT inhibits the proliferation of human androgen-independent prostate cancer cell line PC3 *in vitro* and *in vivo* by a cell cycle regulation pathway. *(Asian J Androl 2005 Dec; 7: 389–393)*

Keywords: DL111-IT; prostate cancer; pRb; cyclin-dependent kinase 4; cyclin D1; PC3; cell line

1 Introduction

The human prostate gland, a male sexual accessory tissue involved in seminal fluid production, has a remarkably high incidence of neoplastic disease. Prostate cancer remains the most common non-cutaneous malignancy in the developed world and is the second-highest cause of cancer death in males [1]. As local prostate cancer rarely causes symptoms, 38 %–51 % of patients present with locally extensive or metastatic disease at the time of diagnosis. Between 10 % and 50 % of clinically localized cases inevitably progress and the patients die from metastatic disease [2, 3]. Therefore, the development of novel anti-prostate cancer agents is an emergent issue.

Contragestazol (DL111-IT), 3-(2-ethylphenyl)-5-(3-methoxypheyl)-1H-1,2,4 triazole, was originally reported

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as a non-hormonal antifertility agent [4], and displayed a high activity of arresting early pregnancy in animals. Our previous study also demonstrated that DL111-IT inhibited progesterone synthesis by inactivating 3β-hydroxysteroid dehydrogenase, induced apoptosis in corpus luteum, and inhibited the growth of embryos [5–7]. As embryo and tumor cells share the same strong proliferation behavior, we hypothesized that DL111-IT could exert its cell killing ability on cancer cells. Based on the pilot study of DL111-IT effects on cancer, we confirmed that DL111-IT exhibited antiproliferation activity on various cancer cell lines both *in vitro* and *in vivo*. Here, we reported the effects of DL111-IT on human androgen-independent prostate cancer cell line PC3 *in vitro* and *in vivo*, and its potential mechanisms of the antiproliferation.

2 Materials and methods

2.1 Compounds

DL111-IT was synthesized by the Department of Medicinal Chemistry, College of Pharmaceutical Science at Zhejiang University, Hangzhou, China. Injectable oleum camelliae (IOC) was manufactured by Zhejiang Xianju Pharmaceutical Co., Xianju, China.

2.2 Cell lines

PC3 from bone was obtained from the Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37 ºC, in a humidified atmosphere of 5 % CO_2/95 % air and serially passaged in RPMI-1640 medium (Sigma Chemical Co., St. Louis, USA), supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.3 MTT assay for cell growth and viability

Cell growth and viability were tested using the 3-(4, 5-dimethylthia- zol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma) assay. PC3 cells were seeded (5000 cells/well) in 96-well microtiter plates (100 μL/well). After 24-hour incubation in RPMI-1640 medium, the cells were treated with various concentrations of DL111-IT (2.5 μg/mL–40.0 μg/mL) for 48 h. The medium was removed at the end of incubation and 0.5 mg/mL of MTT was added to the medium. After 4-hour incubation, dimethyl sulfoxide (200 μL) was added to each well, and the optical density was read at 570 nm. Cell sensitivity to a drug was expressed as the drug concentration that yielded 50 % cell inhibition (IC_{50}). Experimental conditions were tested in sextuplicate (six wells of the 96-well plate per experimental condition). All of the experiments were performed in triplicate.

2.4 Cell cycle analysis

PC3 cells (5×10^4 cells/mL, 5 mL) were cultured in complete medium in 25 cm^2 flasks, with or without DL111-IT (5 μg/mL–20 μg/mL) for 48 h. The cells were then harvested, washed in phosphate-buffered saline, centrifuged and re-suspended in 1 mL of 0.1 % sodium citrate containing 0.05 mg propidium iodide and 50 μg RNase for 30 min at room temperature in the dark. DNA content was measured with a Coulter Epicas Elite flow cytometer.

2.5 Western blotting analysis

Proteins were extracted in radioimmunoprecipitation assay buffer (50 mmol/L NaCl, 50 nmol/L Tris, 1 % Triton X-100, 1 % sodiumdeoxycholate, and 0.1 % sodium dodecylsulfate) and 50 μg of total protein was loaded per lane. Proteins were fractionated on 12 % Tris-glycine gels, transferred to nitrocellulose membrane (Pierce Biotechnology Inc., USA), and probed with primary antibodies (retinoblastoma [pRb], cyclin-dependent kinase 4 [CDK4] and cyclin D1) then horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Antibody-positive bands were visualized using ECL Western blot detection reagents (Pierce Biotechnology Inc., USA).

2.6 Antitumor activity in prostate cancer xenografted in athymic mice

Tumors were established by injection of PC3 cells (5×10^6 cells/animal) s.c. into the armpit of 4- to 5-week-old Balb/c female athymic nude mice (National Rodent Laboratory Animal Resource, Shanghai, China). Treatments were initiated when tumors reached a mean group size of 70 mm^3. Tumor volume (mm^3) was calculated as $(W^2 \times L)/2$, where W = width and L = length, as measured with calipers. DL111-IT was formulated in IOC and was given i.m. once a day for 10 consecutive days at 1.25 mg/kg, 5.0 mg/kg and 20.0 mg/kg DL111-IT, respectively. The positive control group was given i.m. once a day for 10 consecutive days at 30.0 mg/kg cyclophosphamide (CTX). Animal body weights and tumor volumes were recorded every two days until the mice were killed at day 13. Animal care was in accor-

dance with institutional guidelines.

2.7 Statistics

Significance (unpaired two-sided *t*-test) was determined by Microsoft Excel 2000 software.

3 Results

3.1 Cytotoxicity assay of DL111-IT on PC3 cell line

The dose response of human androgen-independent prostate cancer cell line PC3 to DL111-IT (0–40.0 μg/mL) was shown in Figure 1. The IC_{50} value of DL111-IT (95 % confidence interval) was 9.9 μg/mL (6.6 μg/mL–16.3 μg/mL).

3.2 Tumor growth of PC3 xenografts treated with DL111-IT

Tumor volumes were recorded every two days until the animals were killed at day 13. At day 4, tumor volumes were significantly inhibited ($P < 0.05$–0.01) in the 5.00 mg/kg and 20.00 mg/kg DL111-IT groups. DL111-IT (1.25 mg/kg) initiated a reduction in tumor growth rate ($P < 0.05$) at day 6. From day 1 to day 13, tumor volumes in the control group achieved a 6.7-fold increase, whereas tumor volumes in the DL111-IT treatment groups obtained 3.5-fold (1.25 mg/kg), 2.6-fold (5.00 mg/kg), and 2.0-fold (20.00 mg/kg) elevations, respectively (Figure 2). At day 13, DL111-IT showed a significant effect on tumor weight, but not on animal body weight (Table 1). The inhibition rates of tumor weight caused by DL111-IT (1.25 mg/kg–20.00 mg/kg) ranged from 21 % to 50 % (Table 1).

3.3 Induction of G_0/G_1 arrest by DL111-IT

The cell cycle profiles treated with DL111-IT and the proportions in each phase (%) are shown in Figure 3.

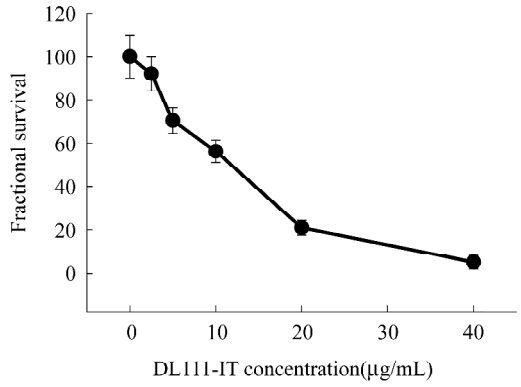

Figure 1. Dose-dependent activity of DL111-IT on PC3 cell line. Cells were treated with DL111-IT (2.5 μg/mL–40.0 μg/mL) for 48 h, and cytotoxicity was analyzed using the 3-(4,5-dimethylthia- zol, 2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent assay method. Points represent mean ± SD.

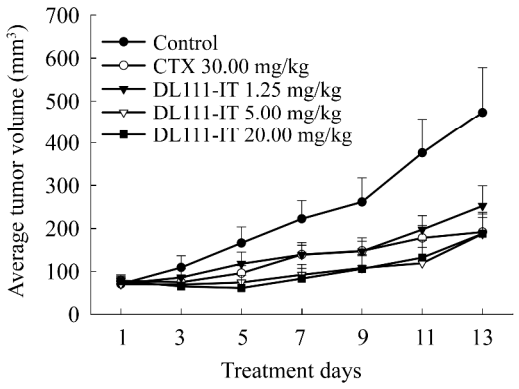

Figure 2. Tumor growth of PC3 xenografts treated with DL111-IT. DL111-IT was given i.m. once a day for 10 consecutive days at 1.25 mg/kg, 5.00 mg/kg, and 20.00 mg/kg, respectively. The positive control group was given i.m. cyclophosphamide (CTX) at 30.00 mg/kg once a day for 10 consecutive days.

Table 1. Effects of DL111-IT on athymic mice body weight, tumor volume and tumor weight at pre-dose and post-dose. $^{b}P < 0.05$; $^{c}P < 0.01$; $^{f}P < 0.01$ compared with control. CTX: cyclophosphamide.

Groups	No. of animals	Body weight (g)		Tumor size (mm^3)		Tumor weight	Inhibition rate
		Start	End	Start	End	(g)	(%)
Control	10	18 ± 2	17 ± 3	70.8 ± 12.4	473.4 ± 104.3	0.48 ± 0.14	/
CTX 30.00 mg/kg	8	19 ± 1	19 ± 2	78.3 ± 10.4	191.4 ± 34.2^{d}	0.26 ± 0.05^{c}	45.8
DL111-IT 1.25 mg/kg	8	18 ± 2	19 ± 2	71.4 ± 14.2	252.3 ± 46.9^{c}	0.38 ± 0.07	20.8
DL111-IT 5.00 mg/kg	8	19 ± 2	20 ± 2	70.5 ± 14.2	187.4 ± 46.1^{d}	0.28 ± 0.08^{b}	41.7
DL111-IT 20.00 mg/kg	8	18 ± 2	19 ± 2	77.4 ± 14.4	157.3 ± 49.5^{d}	0.24 ± 0.04^{c}	50.0

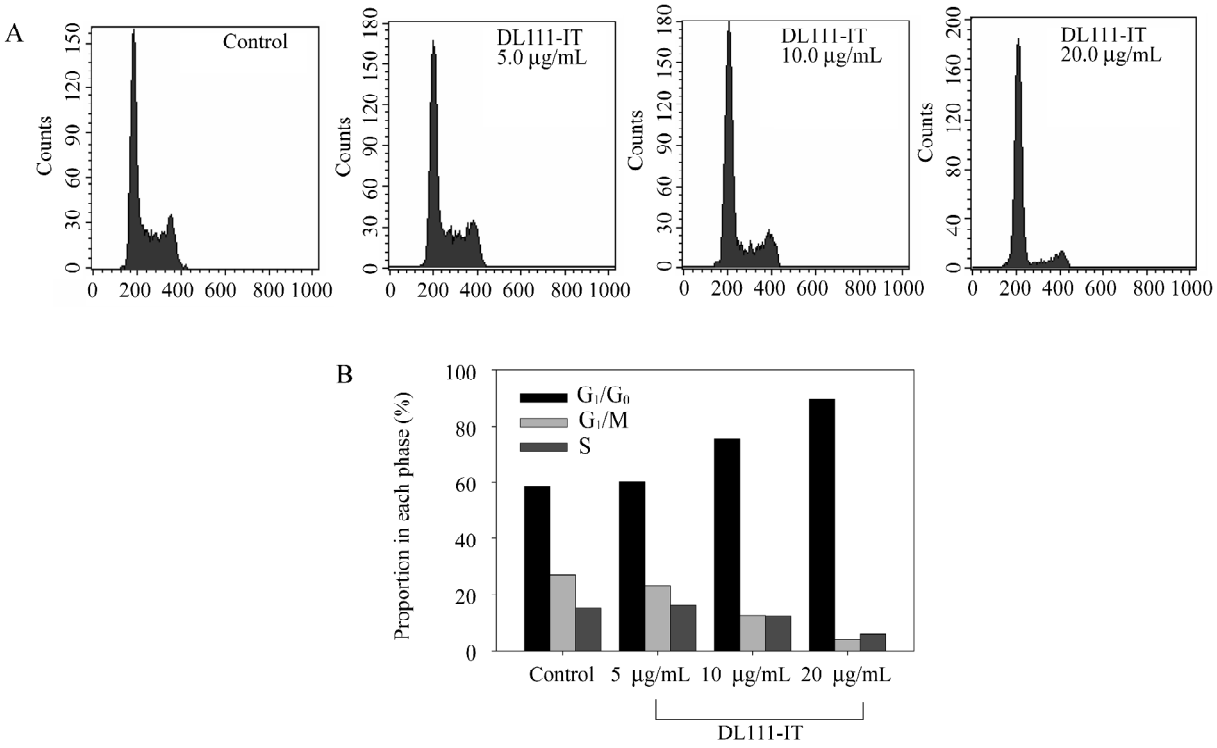

Figure 3. DL111-IT induced G_1 arrest in PC3 cells. (A): The profiles show the cell cycle after treatment with DL111-IT. (B): The proportions in each phase (%). DL111-IT caused cell cycle arrest in the G_0/G_1 phase.

DL111-IT caused G_0/G_1 arrest in a dose-dependent manner. The percentage of cells in the G_2/M and S phases declined as the concentrations of DL111-IT increased.

3.4 Expression of pRb, cyclin D1 and CDK4

The basal and DL111-IT-treated expression of pRb, cyclin D1 and CDK4 was measured by immunoblotting. As shown in Figure 4, DL111-IT (5.00 mg/mL–20.00 mg/mL) enhanced pRb protein levels in the PC3 cell line after 24-hour exposure. DL111-IT (10.00 mg/mL–20.00 mg/mL) obviously reduced cyclin D1 and CDK4 expression. The DL111-IT-mediated regulations of protein expression mentioned above were in dose-dependent patterns.

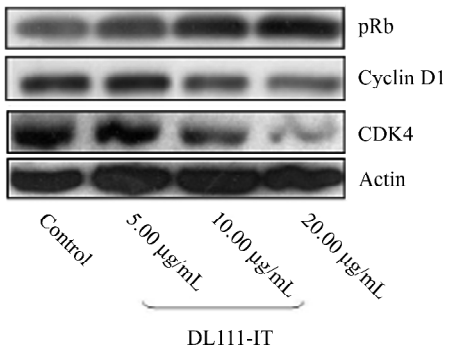

Figure 4. Protein expression of pRb, cyclin D1 and CDK4. PC3 cells were treated with DL111-IT (5.00 mg/mL–20.00 mg/mL) for 24 h. Expression of pRb, cyclin D1 and CDK4 was detected by Western blot analysis. Overexpression of pRb and reduction of cyclin D1 and CDK4 expression, mediated by DL111-IT in a dose–response pattern, were observed.

4 Discussion

DL111-IT was originally reported as a non-hormonal contraceptive. One of its key antifertility mechanisms is apoptosis in corpus luteum [7]. As triggering apoptosis is a critical property for a promising anticancer drug, we proposed DL111-IT had potential anticancer activity. In this study, we tested the sensitivity of PC3 to DL111-IT. The IC_{50} value was 9.86 mg/mL (6.60 mg/mL–16.30 mg/mL) *in vitro*, and DL111-IT (1.25 mg/kg–20.00 mg/kg)

-mediated tumor inhibition rates in the PC3 xenograft model was 21 %–50 %, without causing significant loss of animal body weight. Contrary to expectation, DL111-IT killed PC3 cells without causing apoptosis, but by inducing G_0/G_1 arrest.

Cyclin D1 mRNA levels have been shown to be augmented in six prostate cancer cell lines and in 25 %–30 % of prostate cancer samples examined. Accordingly, it is suggested that modification of cyclin D1 is implicated in the pathogenesis of prostate cancer [8, 9]. Cyclin D1 functions by activating CDK4 and CDK6, which in turn phosphorylate the product of the retinoblastoma tumor-suppressor gene (*pRb*), resulting in the loss of pRb grip on the E2F transcription factor. The latter is thus released and enabled to activate its own transcription, in parallel with the transactivation of important genes for S-phase entry [10, 11]. Overexpression of cyclin D1 enhances cell growth and is constitutively expressed in PC3 cells [12]. These previous data demonstrated that overexpression of cyclin D1 contributes to androgen-independent growth of prostate cancer cells. Our study has demonstrated that DL111-IT elevates pRb expression, downregulates cyclin D1 and CDK4 expression, and increases the percentage of G_1 phase in a dose-dependent manner. Thus, DL111-IT-mediated accumulation of hypophosphorylated pRb, through downregulating cyclin D1 and CDK4, induced G_0/G_1 arrest, inhibited PC3 cell proliferation, and ultimately exerted anticancer activity.

In conclusion, as a lead compound, DL111-IT exhibited a high level of anti-prostate cancer cell proliferation both *in vitro* and *in vivo*, mainly through the cell cycle regulation pathway, thereby implicating triazole series compounds as potentially promising agents in prostate cancer therapeutics.

Acknowledgment

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References

1 Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics. CA Cancer J Clin 1999; 49: 8–31.
2 Vercelli M, Quaglia A, Marani E, Parodi S. Prostate cancer incidence and mortality trends among elderly and adult Europeans. Crit Rev Oncol Hematol 2000; 35: 133–44.
3 Gao HW, Li YL, Wu S, Wang YS, Zhang HF, Pan YZ, *et al*. Mass screening of prostate cancer in a Chinese population: the relationship between pathological features of prostate cancer and serum prostate specific antigen. Asian J Androl 2005 Jun; 7:159-63.
4 Galliani G, Luzzani F, Colombo G, Conz A, Mistrello L, Baroae D, *et al.* On the mode of action of a new contragestational agent (DL111-IT). Contraception 1986; 33: 263–83.
5 Yang B, Fang RY. Synergistic effects on pregnancy-termination activity of DL111-IT in combination with mifepristone. Acta Pharmacol Sin 1996; 17: 361–5.
6 He QJ, Yang B, Wang WF, Wu HH, Fang RY. Synergistic effects of DL111-IT in combination with mifepristone and misoprostol on termination of early pregnancy in preclinical studies. Contraception 2003; 68: 289–95.
7 Yang B, Cao L, Fang RY, Gu ZP. Luteolytic effects of DL111-IT in pregnant rats. Eur J Pharmacol 1999; 380: 145–52.
8 Han EK, Lim JT, Arber N, Rubin MA, Xing WQ, Weinstein IB. Cyclin D1 expression in human prostate carcinoma cell lines and primary tumors. Prostate 1998; 35: 95–101.
9 Wang L, Liu D, Ahmed T, Chung FL, Conaway C, Chiao JW. Targeting cell cycle machinery as a molecular mechanism of sulforaphane in prostate cancer prevention. Int J Oncol 2004; 24: 187–92.
10 Cao MY, Lee Y, Feng NP, Al-Qawasmeh RA, Viau S, Gu XP, *et al*. NC381, a novel anticancer agent, arrests the cell cycle in G0-G1 and inhibits lung tumor cell growth *in vitro* and *in vivo*. J Pharmacol Exp Ther 2004; 308: 538–46.
11 Maddison LA, Sutherland DW, Darrios RJ, Greenberg NM. Conditional deletion of Rb causes early stage prostate cancer. Cancer Res 2004; 64: 6018–25.
12 Chen Y, Martinez LA, LaCava M, Coghlan L, Conti CJ. Increased cell growth and tumorigenicity in human prostate LNCaP cells by overexpression to cyclin D1. Oncogene 1998; 16: 1913–20.