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

Qiao-Jun He, Bo Yang, Yi-Jia Lou, Rui-Ying Fang

Department of Pharmacology, College of Pharmaceutical Science, Zhejiang University, Hangzhou 310031, China

Abstract

Aim: To evaluate the antiproliferative activity of contracegestazol (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-dimethylthiazol-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 (IC50 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 G1 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
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-hydroxy-steroid 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 °C, in a humidified atmosphere of 5 % CO2/95 % 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 (IC50).

2.4 Cell cycle analysis

PC3 cells (5 × 10^6 cells/mL, 5 mL) were cultured in complete medium in 25 cm² 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 Epics Elite flow cytometer.

2.5 Western blotting analysis

Proteins were extracted in radioimmunoprecipitation assay buffer (50 mmol/L NaCl, 50 mmol/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 mm³. Tumor volume (mm³) was calculated as (W² × 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 IC50 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 G0/G1 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-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent assay method. Points represent mean ± SD.](image1)

![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.](image2)

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of animals</th>
<th>Body weight (g)</th>
<th>Tumor size (mm³)</th>
<th>Tumor weight (g)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Start</td>
<td>End</td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>18 ± 2</td>
<td>17 ± 3</td>
<td>70.8 ± 12.4</td>
<td>473.4 ± 104.3</td>
</tr>
<tr>
<td>CTX 30.00 mg/kg</td>
<td>8</td>
<td>19 ± 1</td>
<td>19 ± 2</td>
<td>78.3 ± 10.4</td>
<td>191.4 ± 34.2³</td>
</tr>
<tr>
<td>DL111-IT 1.25 mg/kg</td>
<td>8</td>
<td>18 ± 2</td>
<td>19 ± 2</td>
<td>71.4 ± 14.2</td>
<td>252.3 ± 46.9⁴</td>
</tr>
<tr>
<td>DL111-IT 5.00 mg/kg</td>
<td>8</td>
<td>19 ± 2</td>
<td>20 ± 2</td>
<td>70.5 ± 14.2</td>
<td>187.4 ± 46.1⁴</td>
</tr>
<tr>
<td>DL111-IT 20.00 mg/kg</td>
<td>8</td>
<td>18 ± 2</td>
<td>19 ± 2</td>
<td>77.4 ± 14.4</td>
<td>157.3 ± 49.5⁴</td>
</tr>
</tbody>
</table>
DL111-IT inhibited prostate cancer cell line PC3

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.

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 G0/G1 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 G1 phase in a dose-dependent manner. Thus, DL111-IT-mediated accumulation of hypophosphorylated pRb, through downregulating cyclin D1 and CDK4, induced G0/G1 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

This study received financial support from the National Natural Science Foundation of China (No. 30000209).

References