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ORIGINAL ARTICLE

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Antiproliferative activity of contragestazol (DL111-IT) in murine and human tumor models in vitro and in vivo

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Abstract *Purposes*: To evaluate the antiproliferative activity of contragestazol (DL111-IT) in vitro and in vivo and to elucidate potential molecular mechanisms. *Methods*: Cell killing ability of DL111-IT was measured by MTT/Trypan blue exclusion method and murine and human tumor models; cell cycle was analyzed by flow cytometry; pRb, CDK4 and Cyclin D1 expressions were detected by western blotting. *Results*: DL111-IT exhibited high efficiency on cell growth inhibition of 12 cancer cell lines, the IC_{50} values were 4.1–19.7 μg/ml. In Sarcoma-180 (S180) and Hepatoma-22 (H22) tumor bearing mice models, the inhibition rates were 55.9 and 55.6%, respectively, at the doses of DL111-IT 12.5–50.0 mg/kg for 9 days consecutive administration. Human ovarian carcinoma (HO-8910) xenograft study showed that, nine administrations (within 15 days) of DL111-IT (12.5–50.0 mg/kg) significantly inhibited tumor growth with the inhibition rates ranging from 17.0 to 64.3%. DL111-IT induced G1 arrest and overexpression of pRb, CDK4 and Cyclin D1 were observed in HO-8910 cell line, suggesting that cell cycle regulation might contribute to the anticancer property of DL111-IT. *Conclusions*: DL111-IT could inhibit the proliferation of cancer cells both in vitro and in vivo via a cell cycle regulation pathway.

Keywords DL111-IT · Anticancer activity · Cell cycle · Ovarian cancer

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Introduction

Contragestazol (DL111-IT), 3-(2-ethylphenyl)-5-(3-methoxypheyl)-1H-1,2,4 triazole (Fig. 1), was originally reported as a non-hormonal antifertility agent [1], and displayed a high activity on arresting early pregnancy in animals [2–4]. Our previous study also demonstrated that DL111-IT inhibited growth of embryos through inducing apoptosis in corpus luteum and declining uterine polyamine levels [5–7]. Since embryo and tumor cells shared the same strong proliferation behavior, and the polyamines acted a crucial role in tumor development [8], we proposed that DL111-IT could exert cell killing ability on cancer cells. The current study reported the antiproliferative activity of DL111-IT both in vitro and in vivo, and the potential mechanism of this action.

Materials and methods

Compounds

DL111-IT (purity: 99.4%) was synthesized by department of Medicinal chemistry, college of Pharmaceutical science of Zhejiang University. Injectable oleum camelliae (IOC) was manufactured by Zhejiang Xian-ju Pharmaceutical Co.

Cell lines

Murine leukemia (P388), Hepatoma (H22), and sarcoma (S180) cell lines, and human ovarian carcinoma (HO-8910), mouth epidermal carcinoma (KB), colorectal tumor (HCT116) cell line, erythromyeloid tumor (K562), nasopharyngeal carcinoma (CNE), Bladder Cancer (RD), non-small cell lung cancer (A549), cervix cancer (Hela), promyelocytic leukemia (HL60), androgen-independent prostate cancer (PC3), and hepatocellular

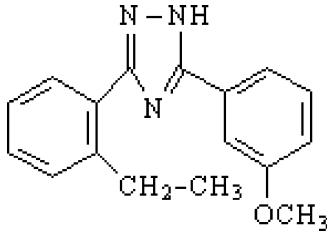

Fig. 1 The structure of DL111-IT

carcinoma (Bel7402) cell lines were obtained from the Cell bank, Chinese Academy of Science (Shanghai, China). Cells were maintained at 37°C, in a humidified atmosphere of 5% CO_2/95% air and serially passaged in RPMI 1640 medium (Sigma chemical Co.), supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Trypan blue exclusion and MTT assay for cell growth and viability

For the evaluation of cell growth inhibition in response to DL111-IT treatment, 2×10^5 cells/5 ml were incubated with DL111-IT 1.5 and 6.0 μg/ml for 24–144 h. Cell viability was assayed by trypan blue exclusion using a hemocytometer at 0, 24, 48, 72, 96, 120, and 144 h; triplicate samples were used for each treatment. Cell viability was estimated by the observation of morphology and by exclusion of 0.4% trypan blue solution.

Cell viability was tested by using the 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyltetra-zolium bromide (MTT) reagent (Sigma chemical Co.) assay. Cancer cells were seeded (5,000 cells/well) in 96-well microtiter plates (100 μl/well). After 24 h incubation in RPMI medium, cells were treated with various concentrations of DL111-IT (2.5–40.0 μg/ml) for 48 h. The medium was removed at the end of incubation, 0.5 mg/ml of MTT was added to the medium. After 4 h incubation, dimethyl sulfoxide (DMSO, 200 μl) was added to each well, and optical density (OD) was read at 570 nm. Cell sensitivity to drug was expressed as the drug concentrations that yielded 50% cell inhibition (IC_{50}). Experimental conditions were tested in sextuplicate (6 wells of the 96-well plate per experimental condition). All of the experiments were performed in triplicate.

Cell cycle analysis

To analyze cell cycle, HO-8910 cells (5×10^4 cells/ml, 5 ml) were cultured in complete medium in 25 cm^2 flasks, with or without DL111-IT (5.0–20.0 μg/ml) for 48 h. Cells were then harvested, washed in PBS, centrifuged, and resuspended in 1 ml of 0.1% sodium citrate containing propidium iodide (PI) 0.05 mg and 50 μg RNase for 30 min at room temperature in the dark. DNA content was measured with Coulter Epicas Elite flow cytometer.

Western blotting analysis

Proteins were extracted in radioimmunoprecipitation assay buffer (50 mM NaCl, 50 nM Tris, 1% Triton X-100, 1% sodiumdeoxycholate, and 0.1% SDS) 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 and then HRP-labeled secondary antibodies (Santa Cruz Biotechnoloogy Inc, USA). Antibody-positive bands were visualized using ECL western blotting detection reagents (Pierce Biotechnology Inc.).

Antitumor activity in ovarian cancer xenografted in athymic mice

Tumors were established by injection of HO-8910 cells (5×10^6 cells per animal, subcutaneously into the armpit of the athymic nude mice) in 4- to 5-week-old Balb/c female athymic nude mice (National rodent laboratory animal resource, Shanghai branch, China). Treatments were initiated when tumors reached a mean group size of 110 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 (12.5, 25.0, and 50.0 mg/kg, far below LD_{50}: 4,338 mg/kg in mice) was formulated in IOC and was administrated i.m. four times every week for 15 days. Positive control group was treated i.m. with 50.0 mg/kg cyclophosphamide (CTX) according to the same schedule as DL111-IT's for 15 days. Mice weight and tumor volume were recorded every 3 days until animals were sacrificed at day 16. Animal care was in accordance with institutional guidelines.

Antitumor activity in S180 and H22 tumor bearing mice [9, 10]

Tumor bearing mice models were established by s.c. injections of S180 or H22 cells (5×10^6 cells per animal, subcutaneously into the armpit) in male ICR mice (National rodent laboratory animal resource, Shanghai branch, China). Treatments were initiated 24 h after the injections of tumor cells. DL111-IT (12.5, 25.0, and 50.0 mg/kg) was formulated in IOC and was administrated i.m. once a day for nine consecutive days. CTX (50.0 mg/kg) was administrated i.m. once a day for nine consecutive days as positive control. Tested mice were sacrificed and tumor weights were recorded at day 10. Animal care was in accordance with institutional guidelines.

Statistics

Significance (unpaired two-sided Student's *t* test) was determined by Microsoft Excel 2000 software.

Results

Cytotoxicity assay of DL111-IT on human and murine cancer cell lines

All tested cancer cell lines (11 human cancer cell lines: HO-8910, KB, HCT116, K562, CNE, RD, A549, Hela, HL60, PC3, and Bel7402; 1 murine cancer cell line: P388) exhibited dose-dependent sensitivity to 48 h exposure with DL111-IT (0–40.0 μg/ml). The IC_{50} values ranged from 4.1 to 17.4 μg/ml and were listed in Table 1 with 95% confidence limit.

Cell proliferation assay of DL111-IT on human HO-8910 ovarian carcinoma cell line

Human HO-8910 ovarian carcinoma cells were treated with a single dose of DL111-IT (1.5 or 6.0 μg/ml) for 24–144 h. DL111-IT 1.5 μg/ml exhibited somewhat cytotoxicity, while DL111-IT 6.0 μg/ml exerted higher cell killing ability in a time-dependent manner; at day 6, the inhibition rates reached 20.5% ($P < 0.05$) and 41.0% ($P < 0.01$), respectively (Fig. 2).

Antitumor activity in S180 and H22 tumor bearing mice

S180 and H22 tumor bearing mice were treated with DL111-IT (12.5, 25.0, and 50.0 mg/kg, i.m.) once a day for nine consecutive days. Tested mice were sacrificed and tumor weights were recorded at day 10. Compared to the control group, tumor weights of S180 in 25.0 and 50.0 mg/kg DL111-IT groups reduced significantly ($P < 0.01$), and the inhibition rates were 50.0 and 55.9%, respectively. In H22-bearing mice model, DL111-IT exhibited similar anticancer behavior, and the inhibition rates were 52.0% (25.0 mg/kg, $P < 0.01$) and 56.6% (50.0 mg/kg, $P < 0.01$) (Table 2).

Tumor growth of HO-8910 xenografts treated with DL111-IT

At the initiation of treatment (day 1), tumor size was approximately 110 mm^3. DL111-IT and CTX were administrated i.m. four times every week for 15 days. Mice weight and tumor volume were recorded every 3 days until animals were sacrificed at day 16. At day 7, tumor volumes were significantly inhibited ($P < 0.01$–0.001) in 25.0 and 50.0 mg/kg DL111-IT treatment groups. DL111-IT (12.5 mg/kg) failed to reduce tumor volume ($P > 0.05$). From day 1 to day 15, tumor volumes in the control group achieved a 9.5-fold increase, while tumor volumes in DL111-IT treatment groups obtained 7.7-fold (12.5 mg/kg), 5.3-fold (25.0 mg/kg), and 3.9-fold (50.0 mg/kg) elevation, respectively (Fig. 3). At day 16, DL111-IT showed significant effect on tumor weight, but not on athymic mice body weight. The inhibition rates caused by DL111-IT (12.5–50.0 mg/kg) ranged from 17.1 to 64.8% (Table 3).

Table 1 IC_{50} values after 48 h exposure of DL111-IT

Cell line	IC_{50} (μg/ml)	95% confidence limit
P388	4.1	1.3–13.5
HO-8910	15.1	12.5–18.4
KB	10.7	7.4–15.6
HCT116	6.2	1.8–21.6
K562	9.9	5.3–18.4
CNE	17.3	10.5–28.5
RD	12.6	8.0–20.0
A549	17.4	13.0–23.4
Hela	9.9	4.8–19.6
HL60	14.1	7.4–26.7
PC3	9.9	6.6–16.3
Bel7402	5.6	1.4–15.7

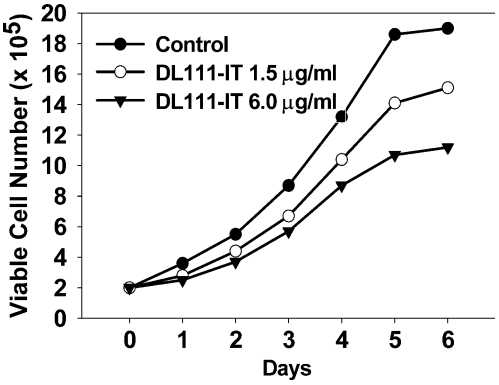

Fig. 2 The effect of DL111-IT on proliferation of human HO-8910 ovarian carcinoma cells. Human HO-8910 ovarian carcinoma cells were treated with DL111-IT (1.5 or 6.0 μg/ml) for 24–144 h. DL111-IT exerted cell killing ability in a time-dependent manner

Induction of G0/G1 arrest by DL111-IT

5×10^5 cells were treated with DL111-IT (5.0–20.0 μg/ml) for 48 h, and the DNA content was analyzed by flow cytometry. The cell cycle profiles treated with DL111-IT and the proportions in each phase (%) were shown in Fig. 4. DL111-IT caused G0/G1 arrest with a dose-dependent trend, the percentages of G0/G1 phase were 43.8% (5.0 μg/ml), 66.2% (10.0 μg/ml), and 76.2% (20.0 μg/ml). The proportion (%) of S phase declined with the concentrations of DL111-IT raised.

Expression of pRb, hyperphosphorylated pRb, Cyclin D1 and CDK4

The basal and DL111-IT-treated expression of pRb, hyperphosphorylated pRb Cyclin D1 and CDK4 was measured by immunoblotting. As shown in Fig. 5, DL111-IT (5.0–20.0 μg/ml) enhanced pRb protein level in HO-8910 cell line after 24 h exposure, and decreased hyperphosphorylated pRb. DL111-IT (10.0–20.0 μg/ml) obviously reduced Cyclin D1 and CDK4 expression.

Table 2 Effects of DL111-IT on S180 and H22 tumor bearing mice

Groups	No. of animals	S180		H22	
		Tumor weight (g)	Inhibition rate (%)	Tumor weight (g)	Inhibition rate (%)
Control	12	1.52 ± 0.57	–	2.00 ± 0.49	–
CTX 50.0 mg/kg	12	0.83 ± 0.25*	45.4	1.06 ± 0.50*	47.0
DL111-IT 50.0 mg/kg	12	0.67 ± 0.35*	55.9	0.87 ± 0.33*	56.6
DL111-IT 25.0 mg/kg	12	0.76 ± 0.40*	50.0	0.96 ± 0.46*	52.0
DL111-IT 12.5 mg/kg	12	1.01 ± 0.54	33.6	1.51 ± 0.51	24.5

S180 and H22 tumor bearing mice were treated with DL111-IT (12.5, 25.0, and 50.0 mg/kg, i.m.) once a day for nine consecutive days, respectively. Tumors were dissected and weighed, and inhibition rates were calculated at day 10 * $P < 0.01$ versus control

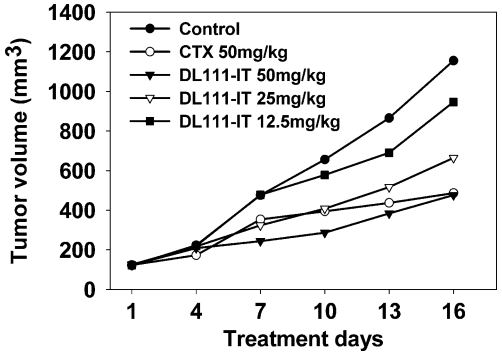

Fig. 3 Tumor growth of HO-8910 xenografts treated with DL111-IT. At the initiation of treatment, tumor size was approximately 110 mm^3. DL111-IT was formulated in IOC and was administrated i.m. four times every week for 15 days with 12.5, 25.0, and 50.0 mg/kg DL111-IT, respectively. 50.0 mg/kg cyclophosphamide (CTX) was treated i.m. according to the same schedule as DL111-IT's for 15 days as positive control. Mice weight and tumor volume were recorded every 3 days until animals were sacrificed at day 16. DL111-IT significantly restrained HO-8910 cell growth in athymic mice

DL111-IT-mediated regulations in protein expression mentioned above were in dose-dependent patterns.

Discussion

DL111-IT was originally reported as a non-hormonal contraceptive, and one of the key antifertility mechanisms was DL111-IT-mediated apoptosis in corpus luteum [5]. In this study, we set out to determine whether DL111-IT had antiproliferation activity in tumor cells and the mechanisms. Twelve cancer cell lines were employed to do the cytotoxicity assay, and the IC_{50} values of DL111-IT ranged from 4.1 to 19.7 μg/ml in vitro, indicating that DL111-IT exhibited anticancer property. In our previous pharmacokinetic study in rats, 24 h after the injection of 50 mg/kg DL111-IT, the highest tissue concentration was detected in ovary, achieving 34 ± 13 μg/g fresh tissue, about 2.5-fold higher than this in the other tissues (Data not shown). Therefore, we proposed that DL111-IT could exert cell killing ability in ovarian tumor in vivo, though, among the 12 tested cancer cell lines, human ovarian cancer cell HO-8910 was not the most sensitive cell line to DL111-IT. In the HO-8910 xenograft study, 25.0 and 50.0 mg/kg DL111-IT significantly (both $P < 0.001$) suppressed the tumor growth, and the tumor inhibition rates achieved 44.8 and 64.8%, respectively. In the S180- and H22-bearing mice study, 10 days treatment of 25.0 or 50.0 mg/kg DL111-IT also significantly (both $P < 0.001$) inhibited tumor growth, all of the tumor inhibition rates reaching > 50%, higher than those in CTX treatment group. In all experiments in vivo, DL111-IT did not induce a loss in tested mice body weight. Whole animal data suggested that, DL111-IT had potential antiproliferation property with less toxicity, and had a promising clinical trial value.

Previous antifertility study reported that DL111-IT could induce apoptsis in corpus luteum, so we checked the DNA content after the HO-8910 cells treated with

Table 3 Effects of DL111-IT on athymic mice body weight, tumor volume and tumor weight

Groups	No. of animals	Body weight (g)		Tumor size (mm^3)		Tumor weight (g)	Inhibition rate (%)
		Start	End	Start	End		
Control	15	20 ± 1	19 ± 1	102 ± 18	1,045 ± 316	1.05 ± 0.32	–
CTX 50.0 mg/kg	8	20 ± 1	17 ± 1	111 ± 12	456 ± 165	0.47 ± 0.21*	55.2
DL111-IT 50.0 mg/kg	8	19 ± 1	19 ± 1	118 ± 42	467 ± 153	0.37 ± 0.15*	64.8
DL111-IT 25.0 mg/kg	8	19 ± 1	19 ± 1	105 ± 15	659 ± 82	0.58 ± 0.18*	44.8
DL111-IT 12.5 mg/kg	8	20 ± 1	19 ± 2	123 ± 16	997 ± 268	0.87 ± 0.25	17.1

At the initiation of treatment, tumor size was approximately 110 mm^3. DL111-IT and CTX were administrated i.m. four times every week for 15 days. Animals were sacrificed at day 16, tumors were dissected and weighed, and inhibition rates were calculated * $P < 0.001$ versus control

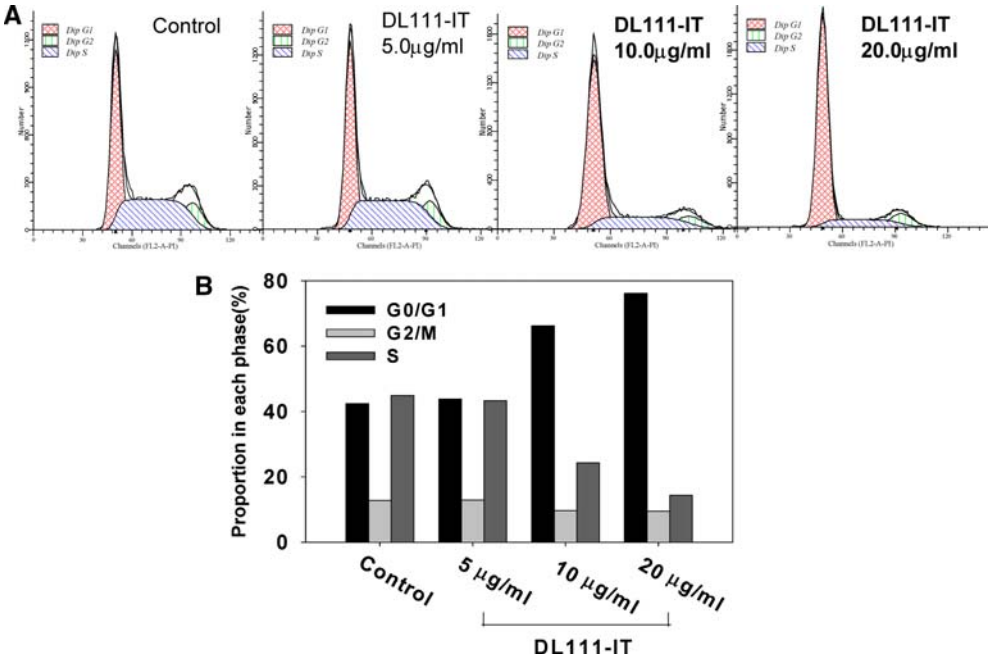

Fig. 4 DL111-IT induced G1 arrest in HO-8910 cells. HO-8910 cells were treated with DL111-IT (5.0–20.0 μg/ml) for 48 h, and the DNA content of 10,000 events was analyzed by flow cytometry. The profiles show the cell cycle after treated with DL111-IT in (**A**); the proportions in each phase (%) were shown in (**B**). DL111-IT caused a cell cycle arrest in G0/G1 phase

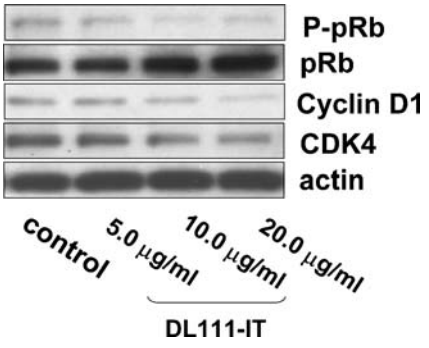

Fig. 5 Protein expression of pRb, hyperphosphorylated pRb, Cyclin D1 and CDK4. HO-8910 cells were treated with DL111-IT (5.0–20.0 μg/ml) for 24 h. Levels of pRb, hyperphosphorylated pRb, Cyclin D1 and CDK4 proteins were detected by western blotting. Overexpression of pRb, downregulated level of hyperphosphorylated pRb, and reduction of Cyclin D1 and CDK4 expression mediated by DL111-IT in a dose-response pattern were observed

DL111-IT. Contrary to expectation, DL111-IT restrained HO-8910 cell proliferation without causing apoptosis, but induced an arrest in G0/G1 phase. The result of p53 protein expression test also did not exhibit obvious change induced by DL111-IT in HO-8910 cells for 48 h (Data not shown). These data suggested that DL111-IT exhibited anticancer activity via cell cycle regulation pathway.

Cancer is frequently considered to be a disease of the cell cycle; alterations in different families of cell cycle regulators cooperate in tumor development. Cancer cell division is precisely regulated in a timely manner by a family of protein kinases—the CDKs, a group of serine/threonine kinases that form active heterodimeric complexes after binding to cyclins, their regulatory subunits. A succession of kinases (CDK4, CDK6, CDK2, and CDC2) are expressed along with a succession of cyclins (cyclins D, E, A and B) as cells go from G1 to S to G2 to M phase [11]. G1 regulator includes cyclin D1, CDK4 and pRb, etc. Cyclin D1 functions by activating the CDK4 and CDK6, which in turn phosphorylate the product of the retinoblastoma tumor-suppressor gene (pRb), resulting in the loss of pRb grip on 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 [12, 13]. Overexpression of CDK4 has been found in 14–15% of a relatively large number of ovarian tumors on mRNA and protein levels [11]. CDK4 overexpression has been reported to be associated with an increased expression of cyclin D1; Sui et al. [14] described a significant increase of CDK4 activity in malignant ovarian tumors in contrast with benign tumors ($P < 0.01$), suggesting that CDK4 activity may play an important role in ovarian carcinogenesis. Our data showed that DL111-IT could increase the percentage of G1 phase in a dose-response pattern, with an elevated expression of pRb, a downregulated expression of Cyclin D1 and CDK4, and a decreased level of hyperphosphorylated pRb. Thus, DL111-IT-mediated accumulation of hypophosphorylated pRb, through downregulated Cyclin D1 and CDK4, caused G0/G1 arrest, inhibited the HO-8910 cell proliferation, and finally exerted the anticancer activity. Previous literatures reported that CDK2 and CDK6 were involved in G0/G1 arrest [11]; further experiments will be conducted to evaluate the effect of DL111-IT on the CDK2 and CDK6 protein expression.

In conclusion, as a lead compound, Dl111-IT exhibited a high anticancer proliferation activity both in vitro

and in vivo mainly via cell cycle regulation pathway, and implicated that triazole series compounds were promising in gynecologic cancer therapeutics.

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