Inhibitory Effect of Jatrorrhizine-Platinum(II) Complex on Prostate Cancer Cells via PI3K/AKT and STA3/JAK2 Phosphorylation Downregulation

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Background: Prostate cancer is common in men worldwide and its incidence in China has increased over the last 2 decades. The present study assessed the cytotoxicity of jatrorrhizine-platinum(II) complex [JR-P(II)] against prostate cancer and investigated the associated mechanism.

Material/Methods: MTT assay was used to assess the anti-proliferative potential and flow cytometry was used to assess apoptosis induction ability of JR-P(II). The protein expression was determined using Western blot assay. JR-P(II)-induced changes in Akt mRNA were assessed by RT-PCR assay and MMP was evaluated by flow cytometry using Rhodamine 123 staining.

Results: JR-P(II) inhibited 22Rv1 cell and LNCaP cell viability by 17% and 24%, respectively, after treatment with 16 µM JR-P(II). In JR-P(II)-treated 22Rv1 cells and LNCaP cells, the levels of cleaved-PARP and caspase-3 were elevated by 4.0, 8.0, and 16 µM JR-P(II). JR-P(II) treatment increased 22Rv1 and LNCaP cell populations in S phase, with reduction of G1/G0 and G2/M phase cell count. Treatment of 22Rv1 and LNCaP cells with JR-P(II) caused reduction of cyclin E1/A1/D1, pRb, and E2F1 proteins. Moreover, JR-P(II) treatment elevated p53 expression in 22Rv1 and LNCaP cells. JR-P(II) treatment raised ROS level and suppressed MMP in 22Rv1 and LNCaP cells. JR-P(II) treatment increased cytochrome c and Bax expression, and reduced Bcl-2 expression in 22Rv1 and LNCaP cells. In JR-P(II)-treated 22Rv1 and LNCaP cells, PI3K/AKT/ERK activation was downregulated relative to the control group. JAK2 and STAT3 phosphorylation gradually decreased with increased JR-P(II) concentration, from 4.0 to 16 µM.


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Background

Prostate cancer falls in the same category of malignancy as other systemic cancers. At the time of diagnosis, the morbidity of prostate carcinoma patients is associated with ethnicity, age, and family history [2]. It is reported that susceptibility to developing prostate cancer is determined by the genetic variation in an individual [3]. More than 30 sites have been detected during analysis of whole-genomes of various ethnic groups throughout world, which are susceptible to prostate carcinoma development [4]. Studies investigating associations of genetic predisposition with prostate cancer development have produced important results [4,5].

Aptosis is stimulated by stress due to endogenous and exogenous factors [6], induced mainly because of aggregation of unfolded proteins in the endoplasmic reticulum (ER), failure of ER to regulate release and ingestion of calcium, and improper processing of some proteins [6]. Apoptosis induction is associated with sustained stress in the ER [7]. Endoplasmic reticulum stress influences the release of caspase-3, which activates the apoptotic pathway [8]. Prostate cancer, on becoming androgen-independent, shows resistance to chemotherapeutics and secondary endocrine therapy [9,10]. Interleukin-6, which plays a prominent role in malignant tumor growth, is increased in serum of patients with prostate cancer [11,12]. It is stimulated by stress due to endogenous and exogenous factors [6], induced mainly because of aggregation of unfolded proteins in the endoplasmic reticulum (ER), failure of ER to regulate release and ingestion of calcium, and improper processing of some proteins [6]. Apoptosis induction is associated with sustained stress in the ER [7]. Endoplasmic reticulum stress influences the release of caspase-3, which activates the apoptotic pathway [8]. Prostate cancer, on becoming androgen-independent, shows resistance to chemotherapeutics and secondary endocrine therapy [9,10]. Interleukin-6, which plays a prominent role in malignant tumor growth, is increased in serum of patients with prostate cancer [11,12].

The complexes of cisplatin and various other similar complexes such as oxaliplatin and carboplatin are accompanied by adverse effects in cancer patients [15,16]. Studies have been performed to investigate complexes of platinum for cancer treatment to avoid the adverse effects associated with cisplatin [15,17]. Recently, chemists have synthesized novel compounds by linking natural products to metal atoms, and these metal complexes have shown satisfactory results for cancer treatment [18,19]. Jatrorrhizine, a natural compound isolated from Tinospora capillipes Gagnep., shows anti-tumor potential and other activities [20,21]. The present study evaluated JR-P(II) as an anti-proliferative and apoptosis-inducing agent for treatment of prostate cancer and investigated the associated mechanism.

Material and Methods

Chemicals and reagents

All chemicals used were obtained from Sigma-Aldrich. The purity of JR-P(II) was confirmed to be 98% using HPLC.

Cell culture

The 22Rv1 and LNCaP cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). DMEM mixed with 10% FBS and antibiotics was used for culturing the cells. The cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Cell viability assay

The 22Rv1 and LNCaP cells were distributed in 96-well plates at 2×10⁴ cells per well density and then were incubated for 24 h in DMEM mixed with 1% FBS. The cells were then incubated for 24, 48, and 72 h in medium containing 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16 μM of JR-P(II). Following treatment with JR-P(II), the cells were incubated for 4 h with a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). DMSO solvent (150 μl) was added to each well after the medium in plates was discarded. A Multimode Reader (Model: Varioskan Flash; Thermo Fisher Scientific, Inc.) was used for measurement of absorbance for each well 3 times independently at 568 nm.

Apoptosis analysis

To assess apoptosis in 22Rv1 and LNCaP cell cultures, Annexin V/PI-labeled cells were subjected to flow cytometry. In 6-well plates, 22Rv1 and LNCaP cells were seeded at 2×10⁵ cells per well concentration and incubated for 72 h in DMEM mixed with 0 (control), 4.0, 8.0, and 16 μM of JR-P(II). The cell rinsed in PBS were subjected to Annexin V-FITC (5 μl) and PI (10 μl) staining for 20 min in complete darkness. Detection of cell apoptosis was performed by flow cytometry (Beckman Coulter, Inc., Miami, FL, USA).

Cell cycle assay

The DNA content distribution in 22Rv1 and LNCaP cells treated with JR-P(II) was detected by flow cytometry. In 6-well plates, 22Rv1 and LNCaP cells at 2×10⁵ cells per well concentration were incubated with 0 (control), 4.0, 8.0, and 16 μM of JR-P(II) for 72 h. Then, PBS washing of the cells 3 times was followed by fixing for 2.5 h in 70% ethyl alcohol. Cellular centrifugation at 70 x g for 5 min and washing in PBS was followed by resuspending in 350 μl buffer mixed with RNase (10 μl) and PI (25 μl). Incubation of cells was continued for 20 min in the dark at room temperature. A flow cytometer (Beckman Coulter, Inc.) was used for determination of DNA content distribution in cells.

Detection of ROS

Production of ROS was determined using a previously described method [22]. The 22Rv1 and LNCaP cells at 2×10⁵ cells per well were incubated with 0 (control), 4.0, 8.0, and 16 μM of JR-P(II) for 4 h with a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). DMSO solvent (150 μl) was added to each well after the medium in plates was discarded. A Multimode Reader (Model: Varioskan Flash; Thermo Fisher Scientific, Inc.) was used for measurement of absorbance for each well 3 times independently at 568 nm.

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concentration were incubated with 0 (control), 4.0, 8.0, and 16 µM of JR-P(II) in the presence or absence of NAC for 72 h. The collected cells were centrifuged at 70 g for 5 min before washing and re-suspending in DCFH-DA (10 µM). Incubation of cells for 20 min was performed in the dark at room temperature. The PBS-washed cells were detected by flow cytometry (Cytomics FC 500; Beckman Coulter, Inc.) to observe ROS levels.

**Determination of MMP**

The MMP was determined using a previously described method [22]. The 22Rv1 and LNCaP cells at 2×10^5 cells per well concentration were incubated in 6-well plates with 0 (control), 4.0, 8.0, and 16 µM of JR-P(II) in the presence or absence of NAC for 72 h. After incubation, staining was performed at 37°C for 20 min with Rhodamine 123, and changes in MMP were analyzed by flow cytometry (Beckman Coulter, Inc.).

**Western blot analysis**

The 22Rv1 and LNCaP cells at 2×10^5 cells per well concentration in 6-well plates were subjected to incubation with 0 (control), 4.0, 8.0, and 16 µM of JR-P(II). The PBS-washed cells were prepared for protein extraction by use of the ProteoJET protein extraction kit (Thermo Fisher Scientific, Inc.). The measurement of protein concentrations was performed using the BCA kit (Thermo Fisher Scientific, Inc.) and samples (25 µg) were separated using 8–15% SDS-PAGE and then electrophoretically transferred to the PVDF membrane. The filters were blocked on treatment with non-fat milk (5%) plus TBST at 37°C. The probing of proteins was made by incubation overnight with antibodies against anti-RAC-a, anti-p(p)-Akt, antibodies against p-PI3 kinase (1: 1,000; cat. no. 4249), anti-p-Akt (1: 1,000; cat. no. 9272), anti-JAK2 (1: 1,000, cat. no. 3230), anti-p-JAk2 (1: 1,000; cat. no. 3771), anti-p-ERK (1: 2,000; cat. no. 4370), anti-p38 (1: 500; cat. no. 8690), STAT3 (1: 1,000; cat. no. 12640), p-STAT3 (1: 500; cat. no. 9145), anti-Bcl-2 (1: 1,000; cat. no. 2872), and Bax (1: 1,000; cat. no. 2774; all from Cell Signaling Technology, Inc. Danvers, MA, USA). Then, membranes were washed in PBS followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) for 2 h. The blots were analyzed by enhanced chemiluminescence development (ECL) reagent (Pierce; Thermo Fisher Scientific, Inc.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

The RNA samples from 22Rv1 and LNCaP cells were subjected to extraction by TRIZOL (Thermo Fisher Scientific, Inc.). The
reverse transcription of 1 µg total RNA content was followed by synthesis of first-stand cDNA. The amplification of platinum complexes was measured using SYBR-Green qPCR Super Mix-UDG (Thermo Fisher Scientific, Inc.). The PCR reactions sequence consisted of 8 min at 94°C, 42 cycles at 93°C for 28 s, 570°C for 2 min, and 70°C for 28 s. The primers used were: forward: 5’-TCT ATG GCG CTG AGA TTG TG-3’ and backward: 5’-CTT AAT GTG CCC GTC CTT GT-3’. The relative level of gene expression was normalized to β-actin.

Statistical analysis

The data are shown as the mean±SD of 3 measurements. SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. The statistically significant differences between multiple groups were analyzed using one-way ANOVA and Bonferroni post hoc test. Differences were considered significant at P<0.05.

Results

Inhibitory effect of JR-P(II) on 22Rv1 and LNCaP cells

The viability changes in 22Rv1 and LNCaP cells by 0.25–16 µM JR-P(II) were measured by MTT assay following 24, 48, and 72 h of incubation (Figure 1). The suppression of 22Rv1 and LNCaP cell viability increased with increased JR-P(II) concentration, from 0.25 to 16 µM. The IC50 of JR-P(II) for 22Rv1 and LNCaP cells was 4 and 8 µM, respectively, after treatment for 72 h. The JR-P(II) treatment at 0.25 µM reduced 22Rv1 and LNCaP cell viability to 84% and 91%, respectively. On the other hand, 22Rv1 and LNCaP cell viability was reduced to 17% and 24%, respectively, after treatment with 16 µM JR-P(II). The cytotoxicity of JR-P(II) for 22Rv1 and LNCaP cells also increased with increased incubation time, from 24 h to 72 h (Figure 1B, 1C).

Figure 2. JR-P(II) activated 22Rv1 and LNCaP cell apoptosis. (A, B) Treatment with 4.0, 8.0, and 16 µM JR-P(II) for 72 h was followed by Annexin V/PI labeling and flow cytometry to detect apoptosis. (C) The cleaved-PARP and caspase-3 level in JR-P(II)-treated protein extract was assessed by Western blot assay. * P<0.05 and ** P<0.01 vs. control.
**JR-P(II) acts as an apoptosis-inducing agent in 22Rv1 and LNCaP cells**

The apoptotic role of JR-P(II) at 4.0, 8.0, and 16 µM in 22Rv1 and LNCaP cells was determined by flow cytometry after Annexin V/PI labeling (Figure 2A, 2B). The apoptosis-inducing role of JR-P(II) in 22Rv1 and LNCaP cells was also concentration-dependent. In 22Rv1 cells, JR-P(II) induced apoptosis in 34.42%, 53.89%, and 79.20% of cells, respectively, at 4.0, 8.0, and 16 µM. Apoptosis induction by 4.0, 8.0, and 16 µM JR-P(II) was observed in 28.78%, 49.21%, and 73.37% of cells, respectively. The JR-P(II)-mediated changes in apoptosis-associated proteins in 22Rv1 and LNCaP cells were assessed by Western blotting (Figure 2C). In JR-P(II)-treated 22Rv1 and LNCaP cells, the levels of cleaved-PARP and caspase-3 were elevated by 4.0, 8.0, and 16 µM.

**JR-P(II) arrested 22Rv1 and LNCaP cell cycle**

The JR-P(II) treatment for 72 h increased 22Rv1 and the LNCaP cell population in S phase (Figure 3A). The increase in JR-P(II) concentration from 4.0 to 16 µM significantly enhanced the S phase cell cycle proportion in 22Rv1 and LNCaP cells. The cellular count in G1/G0 and G2/M phases was decreased significantly by treatment with JR-P(II). Treatment of 22Rv1 and LNCaP cells with JR-P(II) caused reduction of cyclin E1/A1/D1, pRb, and E2F1 proteins (Figure 3B). Moreover, JR-P(II) treatment elevated p53 expression in 22Rv1 and LNCaP cells in a concentration-dependent manner.

**JR-P(II) increased ROS and reduced MMP in 22Rv1 and LNCaP cells**

The ROS-producing activity of JR-P(II) in 22Rv1 and LNCaP cells was analyzed at 4.0, 8.0, and 16 µM in the presence or absence of the ROS inhibitor NAC (Figure 4A). The JR-P(II) treatment raised ROS levels to 27.65±2.09, 42.55±2.86, and 72.31±3.43%.

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**Figure 3.** Effect of JR-P(II) on 22Rv1 and LNCaP cell cycle. (A) The DNA content of cells after 72 h of JR-P(II) treatment was detected by flow cytometry following PI staining. (B) The JR-P(II)-treated cells were subjected to cyclin E1/A1/D1, pRb, and E2F1 protein assessment by Western blotting. * P<0.05 and ** P<0.01 vs control.
at 4.0, 8.0, and 16 µM, respectively, in 22Rv1 cells. Treatment with 4.0, 8.0, and 16 µM JR-P(II) elevated ROS to 21.14±1.43, 38.52±2.27, and 64.66±4.34%, respectively, in LNCaP cells. However, JR-P(II) treatment did not induce ROS generation in the presence of NAC in 22Rv1 and LNCaP cells.

The MMP disturbance by JR-P(II) in 22Rv1 and LNCaP cells was detected at 72 h using Rhodamine 123 staining (Figure 4B). The JR-P(II) treatment of 22Rv1 cells with 4.0, 8.0, and 16 µM decreased MMP to 78.47±3.87, 64.43±2.89, and 38.21±2.72%, respectively. MMP was suppressed to 83.76±4.12, 68.15±3.34, and 42.62±2.45% by treatment with 4.0, 8.0, and 16 µM, respectively, JR-P(II). In the presence of NAC, no change in MMP was detected in 22Rv1 and LNCaP cells after treatment with JR-P(II).

Moreover, JR-P(II) treatment increased cytochrome c and Bax expression in 22Rv1 and LNCaP cells (Figure 4C). The Bcl-2 expression in 22Rv1 and LNCaP cells was suppressed by JR-P(II) treatment.

**JR-P(II) suppressed PI3K/Akt/ERK and NF-κB and promoted p38**

In JR-P(II)-treated 22Rv1 and LNCaP cells, PI3K/Akt/ERK activation was downregulated relative to control (Figure 5). The PI3K/Akt/ERK phosphorylation downregulation in 22Rv1 and LNCaP cells by JR-P(II) was at a maximum at 16 µM. JR-P(II) treatment promoted p38 expression in 22Rv1 and LNCaP cells in a dose-dependent manner.
Figure 5. Effect of JR-P(II) on PI3K/Akt/ERK activation. The 22Rv1 and LNCaP cells treated with 4.0, 8.0, and 16 µM JR-P(II) were assessed for PI3K/Akt/ERK activation by Western blot assay.

**JR-P(II) suppressed JAK2/STAT3 activation in 22Rv1 and LNCaP cells**

The JAK2 and STAT3 phosphorylation in 22Rv1 and LNCaP cells was downregulated by JR-P(II) treatment (Figure 6). The level of JAK2 and STAT3 in JR-P(II)-treated 22Rv1 and LNCaP cells remained unchanged. JAK2 and STAT3 phosphorylation gradually decreased with increased JR-P(II) concentration, from 4.0 to 16 µM.

**Discussion**

We found that STAT3 and Akt levels were elevated in the cells possessing anti-apoptotic potential [23,24]. Targeting PI3K leads to inhibition of Akt, which consequently suppresses STAT3 in carcinoma cells [24]. In a similar pattern, Akt downregulation via targeting JAK2 inhibits STAT3 activation [25]. Therefore, studies suggest that STAT3 and Akt-NF-κB are connected via a positive trans-activation loop to each other [23]. The phosphorylation of STAT3 tyrosine is influenced by the PI3K/Akt pathway via Src tyrosine kinase, which is associated with PI3K and JAK2 through cytokine receptors. In tumor cells, negative feedback mechanism between Akt and STAT3 via the involvement of JAK2 has been observed [26,27]. Increased Akt suppresses JAK2, which then inhibits Akt and STAT3 levels [25,26]. The ERK activation in tumor cells is induced by phosphorylation of Akt [28]. It was reported that ERK and Akt inhibitors act as anti-proliferative agents for prostate cancer cells [29]. The present study evaluated JR-P(II)-induced changes in PI3K/Akt/ERK phosphorylation in 22Rv1 and LNCaP cells. The data showed that JR-P(II) treatment of 22Rv1 and LNCaP cells downregulated
PI3K/Akt/ERK phosphorylation. The JR-P(II)-treated cells also showed lower levels of Akt mRNA relative to the control 22Rv1 and LNCaP cells. The JAK2 and STAT3 levels were suppressed in 22Rv1 and LNCaP cells after treatment with JR-P(II). These findings indicate that JR-P(II) inhibits the anti-apoptotic activity of 22Rv1 and LNCaP cells by downregulation of PI3K/Akt/ERK activation. During cellular transition from G0/G1 to S-phase of the cell cycle, synchronization via cyclinE-CDK2 is involved [30]. The pRb activation promotes E2F1, which is associated with S phase to G2/M transition [31]. The present study investigated JR-P(II)-induced changes in progression of the cell cycle in 22Rv1 and LNCaP cells. The JR-P(II) treatment increased S phase cell count and reduced G1/G0 and G2/M phase cell count in 22Rv1 and LNCaP cells. The cyclin E1/A1/D1, pRb, and E2F1 protein levels in 22Rv1 and LNCaP cells were suppressed by JR-P(II) treatment. Thus, JR-P(II) targeted cyclin proteins and caused arrest of 22Rv1 and LNCaP cell cycle in S phase.

Conclusions

The present study shows the anti-proliferative and apoptotic potential of JR-P(II) for prostate cancer cells. The JR-P(II) targeted cyclins, downregulated JAK2/STAT3 activity, and inhibited PI3K/Akt/ERK phosphorylation. Therefore, JR-P(II) can be used to inhibit prostate cancer growth.

References:


