Endogenous Interleukin 6 Is a Resistance Factor for cis-Diaminedichloroplatinum and Etoposide-mediated Cytotoxicity of Human Prostate Carcinoma Cell Lines

Nicolò Borsellino, Arie Beldegrun, and Benjamin Bonavida

INTRODUCTION

The incidence of prostate cancer is steadily increasing and is becoming a public health problem in the United States. It is the most commonly diagnosed malignancy in men of all ages with over 244,000 new cases projected in 1995. Furthermore, after lung cancer, it is the second most common fatal cancer among United States males with 40,400 deaths from metastatic disease estimated for 1995 (1).

The coaddition of anti-IL-6 antisera and CDDP or VP-16 resulted in synergy in cytotoxicity in both cell lines, whereas the combination of antibody and ADR or suramin resulted only in additive effects. Sequential treatment revealed that anti-IL-6 antibody was required to achieve synergy, whereas either sequence of pretreatment resulted in synergy with anti-IL-6 and CDDP but not with VP-16. CDDP treatment of tumor cells down-regulated IL-6 mRNA expression and IL-6 secretion. The present findings demonstrate that IL-6 is an autocrine/paracrine growth factor for DU145 and PC-3 prostate lines. Additionally, the secretion of this cytokine protects the tumor cells against the cytotoxic effect of CDDP and VP-16 and its neutralization sensitizes the cells to cytotoxicity. Overall, the studies suggest that agents that can down-regulate or inhibit protective factors in tumors may overcome drug resistance.

ABSTRACT

Hormonal treatment of advanced prostatic cancer patients generally results in an initially beneficial response, but the treated patients develop hormonally resistant disease in which no curative therapy is currently available. Recent studies have revealed that interleukin 6 (IL-6) is a growth factor for myeloma, renal cell carcinoma, and certain T-cell lymphomas. Further, IL-6 has been shown to block apoptosis induced by p53, transforming growth factor β, and certain cancer chemotherapeutic compounds. The objective of the present study was to determine whether IL-6 is a growth factor for two human prostate cancer lines and whether it protects the tumor cells from drug-induced cell death. Two hormone-independent prostate cell lines were used in this study, namely PC-3 and DU145, and these have been shown to be relatively resistant to cis-diaminedichloroplatinum (CDDP), etoposide (VP-16), and adriamycin (ADR). Both cell lines express IL-6 mRNA and secrete IL-6 constitutively. The addition of anti-IL-6 antiserum to the cell lines resulted in a significant inhibition of cell growth up to day 2, and when additional antibody was added at day 2 the inhibition persisted for 4 days.

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The abbreviations used are: CDDP, cis-diaminedichloroplatinum; ADR, adriamycin; VP-16, etoposide; IL, interleukin; TNF-α, tumor necrosis factor α; NRS, nonimmune rabbit serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Reagents. ADR, CDDP, and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). After reconstitution in DMSO, they were stored as 1 mg/ml (AD) or 10 mg/ml (CDDP and VP-16) solutions. Suramin was kindly supplied by Dr. Beldegrun and was stored at 4°C. A polyclonal rabbit anti-IL-6 antibody was prepared in our laboratory by immunizing a rabbit with recombinant purified human IL-6 (Pepro Tech, Inc., Piscataway, NJ), and the antiserum was partially purified by ammonium sulfate precipitation. The antiserum was specific for IL-6 as assessed by ELISA and showed no reactivity to TNF-α, IL-1β, IL-4, IL-8, or IL-10.

RNA Extraction, First Strand cDNA Synthesis and PCR. Total cytoplasmic RNA was isolated from the tumor cell lines following the method of Chomczynski and Sacchi (21). Total RNA was used as template to make cDNA. Hexamer random primer ( Gibco) was used to prime cDNA synthesis with Moloney murine leukemia virus reverse transcriptase ( Gibco) and 1 mm dNTP (Pharacid Biotech, Piscataway, NJ) by incubation at 42°C for 15 min, then at 95°C for 5 min, and soaked at 5°C for 5 min. The cDNA amplification was done in a 30-cycle PCR reaction with denaturation at 94°C for 45 s, annealing at 42°C for 30 s, extension at 72°C for 1 min and 30 s, and final extension at 72°C for 10 min in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The sequence of the primers used were: 5'-ATGTTGCAACACACAGAG-3' for IL-6-5' and 5'-CATCTATCTTTTTAGCCAT3'- for IL-6-3', which generate a 171-bp fragment; and 5'-GAAGACATCATCAGCTGG-CTCTACTG-3' for G6PDH-5' and 5'-GGTGTGTCAGCAGAAAATTCCTGTTG-3' for G6PDH-3', which generate a 355-bp fragment. Amplified cDNA was electrophoresed on 2% agarose gel containing 100 μg/ml ethidium bromide ( Gibco BRL) and photographed with Polaroid type 667 film.

IL-6 ELISA. The presence of IL-6 protein in the supernatant of cultured tumor cells was quantitated by ELISA. Briefly, 1 × 10⁶ cells were incubated in RPMI for 12 h, the cells were pelleted, and the supernatants were collected. The IL-6 ELISA is based on a double-antibody sandwich technique and has been shown to be highly sensitive (lowest detectable level is about 10 pg/ml) and specific (not reactive with other cytokines). mAb mouse anti-IL-6 (Genzyme, Cambridge, MA) was used as the first coating antibody. For the secondary antibody, the polyclonal rabbit anti-IL-6 antibody was used. Wells of 96-well ELISA plates (Costar, Cambridge, MA) were coated with 50 μl of mAb at least for 1 day and then stored for up to 4 weeks at 4°C. To set up the assay, antibody-coated plates were washed three times and blocked with ELISA PBS containing 1% bovine serum albumin for 1 h. The plates were washed twice, and 50 μl of cell supernatant or the recombinant human IL-6 standard was added to the wells. After overnight incubation, the plates were washed three times, and 50 μl of polyclonal anti-IL-6 antibody was added to each well. After 1 h of incubation, alkaline phosphatase-conjugated goat anti-rabbit IgG (Caltag, South San Francisco, CA) was added to each well and incubated for an additional 1 h. Finally, the plates were washed and incubated with the substrate (Sigma 104). The plates were read 2 h later at 405 nm by using a Titertek Multiscan MCC/240 ELISA reader.

Cytotoxicity Assays. The antiproliferative effect of rabbit anti-IL-6 antibody alone or in combination with drugs was assessed by trypan blue dye exclusion and by MTT (Sigma). Briefly, PC-3 and DU145 prostatic cancer cell lines were resuspended in complete medium at a concentration of 1 × 10⁵ cells/ml after verifying their viability. One ml of cell suspension was distributed to each well of a 96-well flat-bottomed microtiter plate (Costar) and incubated at 37°C in a humidified 5% CO₂ atmosphere to allow the cells to adhere overnight. At time 0, the culture medium was replaced with 1 ml of medium containing the appropriate dilutions of anti-IL-6 antibody, and each plate was incubated for an additional 48 h. In other experiments, after 48-h incubation, the culture medium was replaced with 1 ml of the first medium containing anti-IL-6 antibody, and the plates were incubated for an additional 48 h. When CDDP, VP-16, ADR, and suramin were used in combination with the anti-IL-6 antibody, appropriate concentrations of these drugs in a volume of 10 μl were added at time 0. In all experiments, two different controls were used; namely in one set of wells the medium was replaced with medium alone, and in another set of wells the medium was replaced with medium together with NRS (prebleed) at a concentration comparable to the highest anti-IL-6 antibody concentration used. After incubation, the cells were harvested by treatment with trypsin-EDTA ( Gibco-BRL). Cell viability was determined by the ability of cells to exclude trypan blue. Dye exclusion was assessed visually by microscopic view, and viable cell number was calculated by subtracting the number of cells that stained positive from the total number of cells. Percentage of cytotoxicity was calculated as:

\[
\% \text{ of cytotoxicity} = 1 - \frac{\text{No. of experimental viable cells}}{\text{No. of control viable cells}} \times 100
\]

In the sequential treatment experiments, the MTT assay was used. Briefly, 100 μl of target cell suspension (1 × 10⁶ cells) were added to each well of a 96-well flat-bottomed microtiter plate (Costar), and each plate was incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. After incubation, 100 μl of the first agent or complete medium for control were added, and each plate was incubated for 3 h. After this period, the culture medium was removed, the cells were washed twice with PBS, and then they were incubated at 37°C with 200 μl of the second agent or complete medium for an additional 21 h. After incubation, 20 μl of MTT working solution (5 mg/ml; Sigma) were added to each well culture, and the plate was incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was removed from the wells and replaced with 100 μl isopropyl alcohol (Sigma) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Titertek Multispan MCC 340; Flow Laboratories) at 540 nm. Percentage cytotoxicity was calculated as:

\[
\% \text{ of cytotoxicity} = 1 - \frac{\text{(Absorbance of experimental wells)}}{\text{(Absorbance of control wells)}} \times 100
\]

Statistical Analysis. Statistical significance of the antiproliferative effect of the anti-IL-6 antibody treatment was determined by the Student t test. A P < 0.05 was considered significant. Calculations of synergistic cytotoxicity were determined using isobologram analysis as described by Berenbaum (22).

RESULTS

Expression and Secretion of IL-6 by PC-3 and DU145 Cell Lines. We first examined whether PC-3 and DU145 cell lines secrete IL-6 constitutively into the culture medium, using a sensitive ELISA system. Both cell lines secrete IL-6, and the level of secretion was higher for PC-3 than for DU145 (Fig. 1A). The time course kinetics of IL-6 accumulation into the medium revealed that IL-6 secretion is linear until 12 h after which it reached a plateau at IL-6 concentrations of 0.9 ng/ml for PC-3 and 0.6 ng/ml for DU145. PCR analysis confirmed the above findings by demonstrating IL-6 gene expression in both cell lines. Reverse transcriptase PCR revealed the presence of a 171-bp fragment specific for IL-6 mRNA both in PC-3 and DU145, thus, corroborating the constitutive production of IL-6 by the two prostatic cancer cell lines (Fig. 1B).

Effect of Anti-IL-6 Antibody Treatment on Cell Proliferation of PC-3 and DU145 Cell Lines. Since it has been reported that IL-6 can affect the cell growth of many tumors via an autocrine mechanism (7) and that both PC-3 and DU145 cell lines express IL-6 receptor mRNA (14), we studied the effect of addition of rabbit anti-IL-6 antibody on proliferation of these cell lines. As shown in Fig. 2, tumor cell growth in normal medium supplemented with NRS was not significantly different from cell growth obtained in normal medium alone. However, when the tumor cell lines were cultured for 48 h in the presence of various dilutions (between 1:500 and 1:62.5) of anti-IL-6 antibody, both cell lines were growth inhibited. Growth inhibition was dependent on the antibody concentration used and was maximal at the highest concentration used. Growth inhibition was greater in the DU145 cell line than in the PC-3 line, particularly when low concentrations of the antibody were used. The inhibition by anti-IL-6 was specific for IL-6 as rabbit anti-TNF-α had no effect (data not shown).

We also studied the effect of addition of various concentrations of anti-IL-6 antibody at time 0 over a 4-day period. A significant and concentration-dependent inhibition of cell growth was observed up to day 2, thereafter, the kinetics of proliferation of both cell lines reached the control levels. However, when additional antibody was added at 48 h, a significant concentration-dependent inhibition of cell growth persisted for 4 days (Fig. 3). However, there was no significant change in the percentage of cell viability in both cell lines (data not shown).
IL-6 RESISTANCE IS A RISK FACTOR IN PROSTATE CARCINOMA CELL LINES

IL-6 neutralization on PC-3 and DU145 sensitivity to CDDP, VP-16, ADR, and suramin. Various concentrations of drugs were used in combination with either NRS (1:250) or with two different concentrations of anti-IL-6 antibody (1:500 and 1:250), and the resulting cytotoxicity was measured after 48 h of culture. Clearly, there was a significant augmentation of cytotoxicity by CDDP and by VP-16 in the presence of anti-IL-6 antibody and synergy was documented by isobologram analysis (Ref. 22, Fig. 4). Noteworthy, the synergy was achieved at concentrations of each agent that were 8—100-fold less than those required to achieve similar cytotoxicity by either drug used alone. In contrast to the synergy obtained with CDDP and VP-16, the combination of anti-IL-6 and ADR or suramin produced only additive effects (data not shown). Altogether, these results show that the addition of anti-IL-6 antibody sensitizes the prostate tumor cell lines to cytotoxicity by CDDP and VP-16.

Mechanism of Tumor Cell Sensitization to CDDP and VP-16 by Anti-IL-6. We examined the effect of sequential treatment of anti-IL-6 and CDDP or VP-16 in both prostatic cancer cell lines. The cells were first treated for 3 h with one of the agents, washed, and then incubated for an additional 21 h with the other agent, and cytotoxicity was assessed by the MTT assay. The results in Table 2 indicate that pretreatment with anti-IL-6 antibody, followed by VP-16, resulted in a synergistic effect in both cell lines, whereas pretreatment with VP-16 resulted in an additive effect. With regard to CDDP, the synergy was observed irrespective of the sequence of treatment as each combination produced a positive interaction in both cell lines, although the level of synergy was slightly less than that seen when both agents were added simultaneously (Table 3).

We then studied by ELISA whether treatment with CDDP affected IL-6 secretion by the DU145 and PC-3 prostatic cell lines. The cells were incubated for 4 h with drug, washed, counted, and then incubated at 1 X 10⁶ cells/ml for an additional 12 h. As shown in Fig. 5, treatment with CDDP (0.1 μg/ml) was able to reduce IL-6 secretion in both cell lines, whereas treatment with VP-16 did not inhibit IL-6 secretion.

DISCUSSION

The present study demonstrates that antibody-mediated neutralization of IL-6 secretion by PC-3 and DU145 prostate cancer cell lines significantly inhibited the growth of both cell lines and sensitized the tumor cells to cytotoxicity by CDDP and VP-16. Additionally, a significant synergy was achieved with combination treatments. These studies demonstrate that neutralization of protective factors produced by the tumor cells reverses drug resistance of tumor cells.
IL-6 is a pleiotropic cytokine that regulates various aspects of host defense immune responses, the acute phase reaction, and hematopoiesis (7, 23). IL-6 is also a bifunctional regulator of cell growth because it can induce cell proliferation in several tumors (8–13) and it can also inhibit the growth of other tumors (24–26). Recently, IL-6 has been reported to be a viability factor for both normal and leukemic cells (27–29) and to block programmed cell death induced by wild-type p53, transforming growth factor-β1, and various drugs (19, 20, 30, 31). Because prostate cancer cells have been shown to express IL-6 and IL-6 receptors (14, 32), we reasoned that interfering with anti-IL-6 antibody in the IL-6/IL-6 receptor loop could affect the growth as well as the sensitivity of prostate tumor cells to chemotherapeutic drugs. Indeed, our findings support this hypothesis.

Several growth factors and their receptors have been characterized in prostate-derived cell lines and human prostate cancer specimens (33, 34). These included transforming growth factor α, epidermal growth factor, and insulin-like growth factors. These growth factors function via an autocrine mechanism in prostate cancer, and their secretion in normal and neoplastic hormone-responsive tumors are regulated by androgens (35, 36). The androgen-independent phenotype could emerge when cancer cells acquire ability to secrete constitutively their own mitogenic growth factors, thus, bypassing the need of androgens (37–39). Our present report suggested that IL-6 secretion by DU145 and PC-3 hormone-resistant prostate cancer cell lines might contribute to their growth advantage.

### Table 1 Resistance of DU145 and PC-3 to various chemotherapeutic drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>DU145 (μg/ml, RI)</th>
<th>PC-3 (μg/ml, RI)</th>
</tr>
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<tbody>
<tr>
<td>Adriamycin</td>
<td>222 (2.97)</td>
<td>105.9 (4.81)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>65.4 (2.97)</td>
<td>105.9 (4.81)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.55 (5.0)</td>
<td>0.58 (1.87)</td>
</tr>
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</table>

The values reported are the drug concentrations for ID₅₀ and were obtained by trypan blue exclusion test after 48-h exposure to the drug.

RI, resistance index. It was calculated as: ID₅₀ of the drug in prostate cell line/ID₅₀ of the drug in 222 cell line.
IL-6 resistance is a risk factor in prostate carcinoma cell lines. These findings corroborate those of Siegall et al. (14) who demonstrated that the addition of recombinant IL-6 to the same cell lines used in our study resulted in an increased DNA synthesis but not to the levels seen when cultured in medium containing normal amounts of FCS, suggesting that IL-6 is only one of the factors that contribute to the growth of prostate carcinoma.

Treatment of tumor cells with combination of anti-IL-6 antibody and CDDP or VP-16 resulted in a significant synergistic cytotoxic activity in both cell lines. Synergy was achieved at suboptimal concentrations of each agent, much lower than those required to achieve similar cytotoxicity by either drug used alone. Unlike CDDP and VP-16, the association of anti-IL-6 and ADR or suramin produced only additive effects. In the case of VP-16, pretreatment with anti-IL-6 antibody is crucial to get synergy, whereas both sequence treatment schedules produced a positive interaction between anti-IL-6 and CDDP. The latter observation can be explained, at least in part, by the down-modulation of IL-6 secretion observed after treatment with CDDP.

It has been proposed that resistance to CDDP, as well as to VP-16, could be explained by increased expression of the anti-apoptotic gene bcl-2 (40-42). The expression of bcl-2 has been documented in normal prostate tissue where it is confined in the basal cells of the glandular epithelium (43) that do not undergo apoptosis after androgen withdrawal (44). Furthermore, bcl-2 expression in prostatic tumor cells is enhanced by androgen ablation therapy and, thus, enhanced expression of bcl-2 may be responsible for the emergence of androgen-independent prostate cancer cell clones (45). The relationship between IL-6 and bcl-2 is complex and depends on the differential effect IL-6 has on the growth of particular tumor cells. For instance, IL-6 down-regulates bcl-2 in tumors in which it sensitizes for the induction of apoptosis (46, 47), whereas up-regulation of bcl-2 has been reported in tumor cell lines in which IL-6 is a viability or growth
factor (48, 49). Therefore, the block of autocrine IL-6 activity observed in DU145 and PC-3 cell lines by treatment with anti-IL-6 antibody could sensitize the cells to the cytotoxic effect of CDDP and VP-16 through down-regulation of the bcl-2 gene. Alternatively, IL-6 may be regulating apoptosis through up-regulation of cellular Bcl-x mRNA and Bcl-xL protein (50). Recently, Mizutani et al. (51) have reported that treatment of human renal carcinoma cells with CDDP in combination with anti-IL-6 antibody or anti-IL-6 receptor can overcome their CDDP resistance and that the downregulation of glutathione S-transferase expression by anti-IL-6 or anti-IL-6 receptor antibody may play a role in the enhanced cytotoxicity. However, the role of bcl-2 was not examined in their study. The down-modulation of glutathione S-transferase could explain also the synergy observed in our system between anti-IL-6 antibody and VP-16. In fact, the cytotoxicity of VP-16 is considered to be dependent on a dual mechanism of DNA cleavage via inhibition of DNA topoisomerase II enzyme and via direct DNA damage (52, 53). It has been reported that metabolic activation of etoposide is essential for its effect (54). Also, it has been shown that VP-16 oxidation products are important for its cytotoxicity, and it has been suggested that interaction of VP-16 phenoxyl radical with glutathione may be crucial in blocking VP-16 metabolic activation (55, 56).

Additional investigations are certainly needed to understand the precise mechanism(s) of the synergy observed between anti-IL-6 and CDDP or VP-16. With regard to the down-modulation of IL-6 secretion by treatment with CDDP, it has been reported previously that CDDP can down-regulate mRNA expression for other cytokines like TNF-α (57). It is possible that CDDP produces an increased expression of p53 (58), which is a transcriptional repressor for the IL-6 gene, both in wild type and to a lesser extent, with mutant p53 genes (59).

The development of a hormonal and drug-resistant disease in prostate cancer patients is currently one of the major obstacles in the treatment of this tumor. Thus, adenocarcinomas of the prostate remain a deadly factor (94). It is possible that CDDP produces an increased expression of p53 (58), which is a transcriptional repressor for the IL-6 gene, both in wild type and to a lesser extent, with mutant p53 genes (59).

Table 2 Effect of sequential treatment of anti-IL-6 and VP16 on cytotoxicity

<table>
<thead>
<tr>
<th>First treatment (3 h)</th>
<th>Second treatment (21 h)</th>
<th>% of cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IL-6</td>
<td>Anti-IL-6</td>
<td>9.2 ± 4.0</td>
</tr>
<tr>
<td>VP-16</td>
<td>VP-16</td>
<td>15.0 ± 1.8</td>
</tr>
<tr>
<td>Anti-IL-6 + VP-16</td>
<td>Anti-IL-6 + VP-16</td>
<td>39.4 ± 3.1</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium</td>
<td>3.7 ± 1.9</td>
</tr>
<tr>
<td>VP-16</td>
<td>VP-16</td>
<td>14.2 ± 3.0</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>Anti-IL-6</td>
<td>38.1 ± 2.7</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium</td>
<td>8.5 ± 1.9</td>
</tr>
<tr>
<td>VP-16</td>
<td>VP-16</td>
<td>6.3 ± 4.4</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>Anti-IL-6</td>
<td>14.1 ± 1.3</td>
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</table>

The cells were treated for 3 h with one of the agents, washed, and then incubated for an additional 21 h with the other agent and cytotoxicity was assessed by the MTT assay. Anti-IL-6 was used at the concentration of 1:250, CDDP at 0.1 µg/ml.

Table 3 Effect of sequential treatment of anti-IL-6 and CDDP on cytotoxicity

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<tr>
<td>Anti-IL-6</td>
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<td>8.5 ± 2.4</td>
</tr>
<tr>
<td>CDDP</td>
<td>CDDP</td>
<td>16.9 ± 1.5</td>
</tr>
<tr>
<td>Anti-IL-6 + CDDP</td>
<td>Anti-IL-6 + CDDP</td>
<td>45.0 ± 2.1</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>CDDP</td>
<td>CDDP</td>
<td>13.2 ± 3.2</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>Anti-IL-6</td>
<td>33.4 ± 3.7</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>CDDP</td>
<td>CDDP</td>
<td>7.0 ± 2.5</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>Anti-IL-6</td>
<td>31.1 ± 3.3</td>
</tr>
</tbody>
</table>

The cells were treated for 3 h with one of the agents, washed, and then incubated for an additional 21 h with the other agent and cytotoxicity was assessed by the MTT assay. Anti-IL-6 was used at the concentration of 1:250, CDDP at 0.1 µg/ml.

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The development of a hormonal and drug-resistant disease in prostate cancer patients is currently one of the major obstacles in the treatment of this tumor. Thus, adenocarcinomas of the prostate remain a deadly disease (1). In this study, we provided a new strategy to sensitize hormone- and drug-resistant prostate cancer cell lines to the cytotoxic action of chemotherapy by the use of anti-IL-6 antibody. These findings, if confirmed with freshly derived tumors, may have a potential clinical application in the treatment of hormone-independent prostate cancer.

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