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·Original Article·

Dexamethasone suppresses DU145 cell proliferation and cell cycle through inhibition of the extracellular signal-regulated kinase 1/2 pathway and cyclin D1 expression

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Abstract

Aim: To determine the mechanisms of glucocorticoids in inhibiting advanced prostate cancer growth. **Methods:** The cell proliferation and cell cycle of prostate cancer DU145 cells following dexamethasone treatment were determined by proliferation assay and fluorescence-activated cell sorter. Western blot analysis was carried out to evaluate the effects of dexamethasone on phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and expression of cyclin D1 in DU145 cells with or without glucocorticoid receptor (GR) antagonist RU486. Reverse transcription–polymerase chain reaction verified the expression of GR mRNA in DU145 cells. **Results:** Dexamethasone significantly inhibited DU145 cell proliferation at the G_0/G_1 phase. Western blot analysis showed a dramatic reduction of ERK1/2 activity and cyclin D1 expression in dexamethasone-treated cells. The decreased phosphorylation of ERK1/2 in dexamethasone-treated cells was attenuated by GR blockade. Additionally, the effects of dexamethasone in inhibiting cyclin D1 expression were altered by GR blockade. **Conclusion:** Dexamethasone suppresses DU145 cell proliferation and cell cycle, and the underlying mechanisms are through the inhibition of phosphorylation of ERK1/2 and cyclin D1 expression. The inhibition of ERK1/2 phosphorylation and cyclin D1 expression is attenuated by GR blockade, suggesting that GR regulates ERK1/2 and cyclin D1 pathways. These observations suggest that dexamethasone has a potential clinical application in prostate cancer therapy. *(Asian J Androl 2008 Jul; 10: 635–641)*

Keywords: dexamethasone; prostate cancer; extracellular signal-regulated kinase 1/2; cell cycle

1 Introduction

The growth of prostate cancer during its initial phase is androgen-dependent. However, the progress of cancer from androgen-dependent to androgen-independent has been observed in prostate cancer patients [1]. Indeed, patients with relapsed cancer are refractory to current hormonal therapy, and the outcome is usually poor due to the lack of effective therapies [2].

The growth of cells is regulated by multiple intracellular signaling pathways [3, 4]. Mitogen-activated protein kinases play an important role in regulating cell responses, and this superfamily includes extracellular sig-

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nal-regulated kinase (ERK)1/2, c-Jun NH2-terminal kinase, and the p38 protein kinase [5]. The phosphorylation of ERK1/2 followed by translocation to the nucleus is critical for initiation of cells from the G_1 to S phase resulting in cell growth/proliferation [6]. Other studies have suggested that the activation of ERK1/2 results in cyclin D1 upregulation [7], and shown the links between overexpression of cyclin D1 and primary or tumor-derived prostate cancer cells [8]. The activity of ERK1/2 pathway plays important roles during prostate cancer development. The ERK1/2 activation is directly related to poor histological and prognostic features [9], and constitutive activation of the ERK1/2 pathway can promote androgen hypersensitivity in prostate cancer cells [10].

Glucocorticoids have been used to treat cancer due to their overall palliative effects. Recent studies have observed benefits in combined use of glucocorticoids with calcitriol, somatostatin analog and ^{131}I in advanced prostate cancer patients [11–13]. *In vivo* studies have shown growth inhibition by glucocorticoids is through anti-angiogenetic effects or inhibition of lymphangiogenesis [14, 15]. Earlier studies suggested a possible role of nuclear factor-κB/interleukin-6 signaling and transforming growth factor (TGF)-β in the growth inhibitory effects of dexamethasone [16, 17]. Considering ERK1/2 effects and its high activity during the progress of prostate cancer, we have hypothesized that glucocorticoids might inhibit growth of prostate cancer cells by altering the activity of the ERK1/2 pathway and cyclin D1 expression. To test this hypothesis, we have chosen an advanced prostate cancer cell line, DU145, that has high ERK1/2 pathway activity, and used dexamethasone to interfere with cell growth. We detected the cell proliferation, cell cycle, ERK1/2 pathway activity, and cyclin D1 expression after dexamethasone treatment.

2 Materials and methods

2.1 Reagents and antibodies

Dexamethasone and RU486 were purchased from Sigma (St. Louis, MO, USA), and dissolved in 100% ethanol. Rabbit polyclonal antibodies specific to human phospho-ERK1/2 and total ERK1/2, and monoclonal antibodies specific for cyclin D1 and β-actin, and isotype control immunoglobulin (Ig) were obtained from R&D Systems (Minneapolis, MN, USA). Secondary goat anti-rabbit IgG antibody and isotype Ig were obtained from Shenergy BioScience (Shanghai, China).

2.2 Cell culture

Prostate cancer cell line DU145, characterized with an androgen-independent phenotype, was obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). Primary DU145 cultures and subcultures were carried out in Ham's F12K culture medium (Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum and antibiotics.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR)

The glucocorticoid receptor (GR) protein expression in DU145 cells has been shown in earlier reports [18]. In this study, RT-PCR was carried out to verify the expression of GR mRNA in DU145 cells. Briefly, normal untreated DU145 cells were collected from cultures, and mRNA was isolated by the acid guanidine-phenol-chloroform method. Complementary DNA (cDNA) was synthesized using an RT-PCR kit (Shenergy BioScience, Shanghai, CHina). RT-PCR was carried out using GR-specific primers (sense 5'-TCCCTTTCTCAACAGCAGGAT-3' and antisense 5'-CAATCATTCCTTCCAGCACAT-3'). The 371 bp cDNA product was separated on a 2% agarose gel and visualized by staining with ethidium bromide. A cell line of JAR was used as a negative control.

2.4 Cell proliferation assays

DU145 cells were collected from cultures and diluted to 10^4 cells/mL with culture medium. A total of 10^4 cells was added into 100 mm culture dishes and incubated overnight until cells adhered to the bottom of the dishes. Cells were washed to remove non-adhered cells then treated with dexamethasone at different concentrations. To determine whether the inhibitory effects were GR dependent, RU486, a GR antagonist, was used to block GR. RU486 was added to dexamethasone-treated cells at different concentrations to measure its antagonist effects. An equal volume of ethanol vehicle was added to cells and used as a control. Proliferative cell numbers were determined by a hemacytometer. All experiments were repeated at least three times with equivalent results.

2.5 Cell cycle analysis

DU145 cells were harvested after a 3-day incubation with ethanol carrier or dexamethasone (2×10^{-7} mol/L) with or without RU486 (2×10^{-7} mol/L). Cells were stained with staining buffer containing propidium iodide

(50 μg/mL), RNAse A (100 Kunitz units/mL), then washed twice with phosphate-buffered saline. The cells were re-suspended in buffer, and the DNA content was detected by a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA, USA) to determine DNA state after final wash. ModFit software version 5.11 (Verity Software House Inc., Topsham, ME, USA) was used to evaluate cell cycle.

2.6 Western blot analysis

Cells were collected from cultures with or without treatment, and whole cell lysates were prepared using lysis buffer (0.5% NP-40, 10% glycerol, 50 mmol/L Tris-HCl [pH 7.5], 0.3 mmol/L sodium orthovanadate, 100 mmol/L NaCl, and 1 mmol/L dithiothreitol). Protein concentrations were determined by bicinchoninic acid assay. Equal amounts of protein lysates (50 μg) were loaded and electrophoretically separated on sodium dodecylsulfate-polyacrylamide gels. Proteins were transferred electrophoretically from gels to nitrocellulose membranes. Membranes were washed with Tris-Buffered Saline Tween-20 (TBST), and incubated with TBST containing 5% non-fat dry milk to block non-specific antibody binding followed by incubation with various primary antibodies at 4ºC for 12 h. Membranes were washed with TBST then immunoblotted with secondary antibodies at room temperature for 60 min. Detection of ERK1/2 and cyclin D1 proteins was carried out in the dark using enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the instructions provided by the manufacturer. The semiquantification of protein expression was determined by an imaging system (Bio-Rad, Hercules, CA, USA).

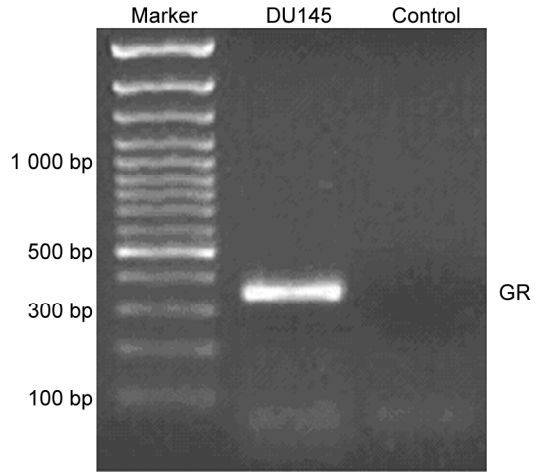

Figure 1. Glucocorticoid receptor (GR) mRNA expression in the DU145 prostate cancer cell line. JAR cells were used as the negative control. Total RNA was isolated from proliferating cells and reverse transcribed to cDNA. GR mRNA expression was detected by reverse transcription-polymerase chain reaction, which amplifies a 371 bp fragment.

2.7 Statistical analysis

The statistical significance of the results was analyzed by paired *t*-test, and $P < 0.05$ was considered to be statistically significant. All statistical analyses were carried out with the use of SPSS software version 11.0 (SPSS, Chicago, IL, USA). All statistical tests were two-sided.

3 Results

3.1 GR mRNA expression in DU145 cells

We have hypothesized that the effects of dexamethasone in inhibiting prostate cancer cell proliferation are GR dependent. The initial focus of this *in vitro* study was to verify the expression of the GR gene in DU145 cells. As shown in Figure 1, RT-PCR showed the expression of GR-specific mRNA in prostate cancer cell DU145.

3.2 Dexamethasone inhibits DU145 cell proliferation

The proliferation of DU145 cells with or without dexamethasone was determined by a cell proliferation assay. Cells without treatment or treated with control ethanol carrier showed dramatic proliferation beginning after 2 days of incubation, and reached high levels after 6 days of culture. The proliferation of DU145 cells in the presence of dexamethasone was dramatically inhibited in a dose-dependent manner after incubation for 6 days when compared to control groups (Figure 2A). But the cell viability evaluated by Trypan Blue exclusion method did not indicate increased cell death when compared to untreated cells. Cells treated with both dexamethasone and RU486 showed that GR blockade dramatically reduced the inhibitory effects of dexamethasone in preventing cancer cell proliferation (Figure 2).

3.3 Dexamethasone induces cell cycle arrest

To determine the effects of dexamethasone in inhibiting the cell cycle during proliferation, cells treated with or without dexamethasone were collected from 3-day cultures, and the cellular DNA content was measured by fluorescence-activated cell sorting. Table 1 summarizes

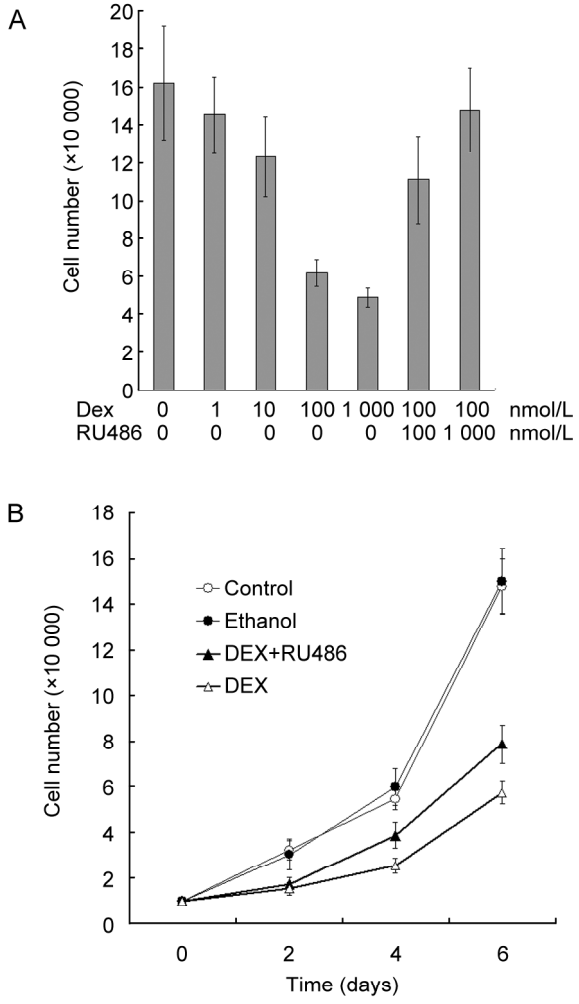

Figure 2. Prostate cancer DU145 cells were treated at different concentrations of dexamethasone and RU486 and the cells were collected at post-treatment day 6. Dexamethasone inhibits DU145 cell proliferation in a dose-dependent manner; RU486 counteracted this effect. When cells were treated with a high concentration of RU486, the inhibitory effect of dexamethasone was completely abolished (A). In order to show a time effect of treatment, cells were divided to four groups: cultured without any treatment; with vehicle only; with 2×10^{-7} mol/L dexamethasone; or with 2×10^{-7} mol/L dexamethasone plus 2×10^{-7} mol/L RU486. Cells were collected and counted on days 0, 2, 4, and 6 (B).

the inhibitory effects of dexamethasone on the DU145 cell cycle. The inhibitory effects of dexamethasone on the cell cycle were blocked by GR antagonist RU486.

3.4 Dexamethasone inhibits phosphorylation of ERK1/2 and expression

To study the underlying mechanisms, the total and phospho-ERK1/2 in DU145 cells treated with or without dexamethasone were evaluated by Western blot analysis. Untreated cells indicated expression of ERK1/2 proteins and constant phosphorylation of ERK1/2 between 1 and 3 days of cultures. Dexamethasone gradually diminished ERK1/2 activity at 24, 48, and 72 h after treatment (Figure 3). The most important finding was that, although the ERK1/2 pathway was consistent active and total ERK1/2 expression was not affected by dexamethasone treatment (Figure 4A), phosphorylation of ERK1/2 in cells was dramatically suppressed after 3 days of incubation (Figure 4B). The semiquantification of ERK1/2 phosphorylation showed a 55.1% ± 10.7% reduction in cells treated with dexamethasone when compared with untreated cells

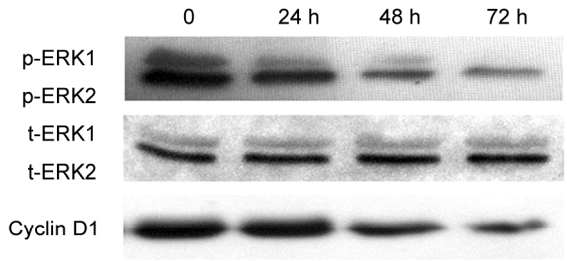

Figure 3. The effects of dexamethasone on extracellular signal-regulated kinase (ERK)1/2 and cyclin D1 expression and ERK1/2 activity of prostate cancer DU145 cells at 24, 48 and 72 h after treatment. DU145 cells were cultured in the absence or presence of 2×10^{-7} mol/L dexamethasone and collected at 0, 24, 48 and 72 h after treatment. The whole cell lysates were prepared using lysis buffer. The proteins were resolved on sodium dodecylsulfate–12% polyacrylamide gels and transferred to nitrocellulose. The blots were probed with antibodies directed against total and phospho-ERK1/2 and cyclin D1. p-, phospho-; t-, total-.

Table 1. Dexamethasone (Dex) induces cell cycle arrest. $^{*}P < 0.001$, compared with control; $^{**}P < 0.001$, compared with Dex.

Cell cycle phase	Control (%)	Ethanol (%)	Dex (%)	Dex + RU486 (%)
G_0/G_1	51.3 ± 6.1	53.5 ± 7.1	86.1 ± 5.9	77.2 ± 8.4
S	32.3 ± 5.8	31.5 ± 4.5	7.6 ± 1.3*	14.6 ± 3.2**
G_2/M	16.4 ± 2.4	15.0 ± 3.7	6.3 ± 1.4	8.2 ± 1.7

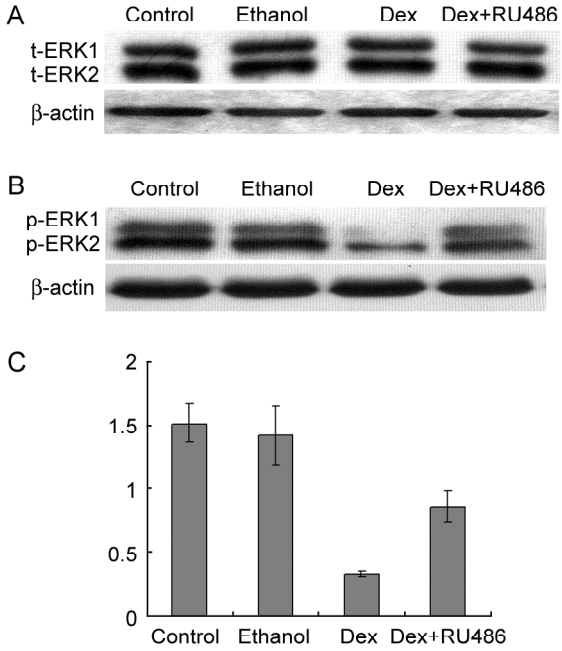

Figure 4. Expression of total and phospho-extracellular signal-regulated kinase (ERK)1/2 proteins in response to dexamethasone. Prostate cancer DU145 cells were cultured in the absence or presence 2×10^{-7} mol/L dexamethasone or cultured with 2×10^{-7} mol/L dexamethasone plus 2×10^{-7} mol/L RU486 for 3 days. β-Actin blot was used to show equal protein loading. The protein of total ERK1/2 in DU145 cells remained unchanged after treatment (A). The activity of ERK1/2 in DU145 cells was suppressed by dexamethasone treatment. RU486 can attenuate the suppressive effects of dexamethasone (B). The semiquantification of ERK1/2 activity was expressed as the ratio of scanning density units of phospho-ERK1/2 *vs.* β-actin (C). t-, total-; p-, phospho-.

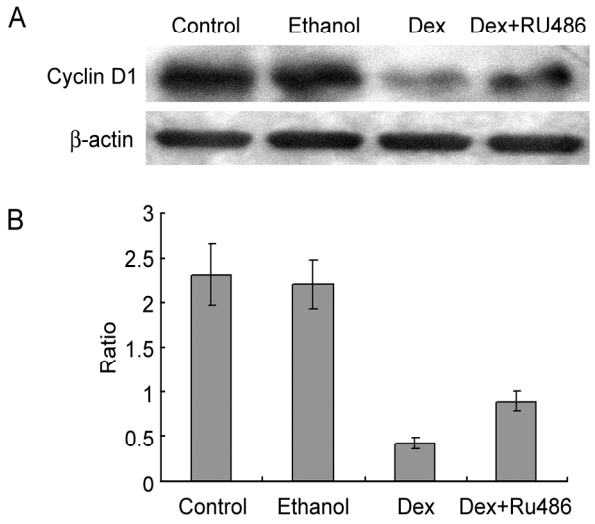

Figure 5. Expression of cyclin D1 protein in response to dexamethasone. Prostate cancer DU145 cells were cultured in the absence or presence of 2×10^{-7} mol/L dexamethasone or cultured with 2×10^{-7} mol/L dexamethasone plus 2×10^{-7} mol/L RU486 for 3 days. Western blot analysis was carried out to determine the cyclin D1 protein expression. The blots were detected by antibodies directly against cyclin D1 protein. β-Actin was used to verify equal protein loading (A). The data for semiquantification were expressed as the ratio of scanning density units of cyclin D1 *vs.* β-actin (B).

(Figure 4C). Additionally, GR blockade attenuated the effects of dexamethasone in inhibiting phosphorylation of ERK1/2 in DU145 cells (Figure 4B, C).

3.5 Dexamethasone inhibits cyclin D1 expression

We first examined the expression of cyclin D1 protein during DU145 cancer proliferation, and a persistent expression of cyclin D1 protein in DU145 cells was observed. Dexamethasone gradually diminished cyclin D1 expression at 24, 48, and 72 h after treatment (Figure 3). As shown in Figure 5, dexamethasone dramatically reduced cyclin D1 expression in DU145 cells after 3 days of culture. The cyclin D1 expression as determined by semiquantification was decreased by 82.6% ± 5.5% when compared with untreated cells. Additionally, GR blockade altered the effects of dexamethasone on DU145 cells, and the reduction of cyclin D1 protein expression was attenuated in dexamethasone-treated cells.

4 Discussion

One very limited report has shown that cortisol and cortisone promote growth activation through mutant androgen receptor in some patients [19]. However, increasing evidence has suggested that dexamethasone can provide therapeutic effects in patients with prostate cancer, particularly hormone refractory carcinoma [11–13]. Indeed, DU145 cells lack androgen receptors. Nevertheless, the mechanisms of dexamethasone that block cancer cell proliferation were unclear. The phosphorylation of ERK1/2 followed by translocation to the nucleus has been shown to be critical for initiation of cells from the G_1 to S phase resulting in cell growth/proliferation [8]. Activation of ERK1/2 leads to upregulation of cyclin D1 [7]. We have used the advanced prostate cancer cell line DU145 to determine the effects of dexamethasone in blocking cancer proliferation, and to deter-

mine whether the inhibitory effects are ERK1/2 pathway dependent. Our data shows that dexamethasone can dramatically inhibit DU145 cell proliferation without increased cell death, and the majority of DU145 cells remained at the G_0/G_1 phase in the presence of dexamethasone, suggesting its direct antiproliferative effects to prostate cancer cells.

We carried out a mechanistic study to determine the mechanisms of dexamethasone in inhibiting the ERK1/2 pathway. The phosphorylation of ERK1/2 protein and total protein was evaluated following treatment with dexamethasone. We found that the activated form of ERK1/2 was markedly reduced after dexamethasone treatment and the levels of total ERK1/2 remained unchanged, suggesting that dexamethasone alters phosphorylation of ERK1/2 protein without suppressing total ERK1/2 protein expression. Additionally, cyclin D1 expression in DU145 cells treated with dexamethasone is also dramatically reduced. These findings suggest that the inhibitory effects of dexamethasone on DU145 cancer cells are associated with ERK and cyclin D1 pathways.

We also investigated whether the effects of dexamethasone in inhibiting prostate cancer cell proliferation are GR-dependent. The DU145 cells express the GR gene, and the ligation of GR leads to the suppression of phosphorylation of ERK1/2 proteins. The GR blockade with GR antagonist RU486 dramatically attenuates the inhibitory effects of dexamethasone not only on cell proliferation but also phosphorylation of ERK1/2 proteins. These observations indicate that the inhibitory effects of dexamethasone on phosphorylation of ERK1/2 proteins are through the GR pathway. One interesting observation is that there is a marked reduction of cyclin D1 expression in dexamethasone-treated cancer cells. Additionally, GR blockade displays a dramatic effect in altering the inhibitory effects of dexamethasone on cyclin D1 expression in DU145 cells. These findings suggest that pathways for the inhibition of DU145 cell proliferation by dexamethasone are dependent on its interaction with GR followed by suppression of ERK1/2 phosphorylation and decreased cyclin D1 expression leading to inhibition of cell proliferation.

It has been elucidated that glucocorticoid has genomic and non-genomic actions with different features. Non-genomic action occurs in seconds or minutes, whereas genomic action occurs in hours or days. Both genomic and non-genomic actions can affect phosphorylation of ERK1/2 and expression of cyclin D1. Our study found that ERK1/2 activity and cyclin D1 expression gradually diminished over 3 days following treatment with dexamethasone. This finding suggests that dexamethasone suppresses DU145 cell proliferation through genomic action.

It is believed that intracellular signaling pathways, such as the ERK1/2 pathway, and cyclin D1 expression play critical roles in regulating cell proliferation/apoptosis and in the development of prostate cancer. This study clearly shows that dexamethasone suppresses phosphorylation of ERK1/2 and cyclin D1 expression in DU145 cells, leading to reduced cell proliferation. Both ERK1/2 and cyclin D1 pathways contribute to the effect of dexamethasone in inhibiting cancer cell proliferation, and the inhibition of the ERK1/2 pathway and cyclin D1 expression by dexamethasone is GR-dependent. These observations suggest that dexamethasone might have a potential clinical application in prostate cancer therapy.

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