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Vitamin B12 Enhances Cisplatin Efficacy via Apoptosis and MAPK/ERK1-2, P38, PARP-1 Modulation in Prostate Cancer

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Abstract

Introduction: Prostate cancer (PC) is the most common malignancy among men and remains a major cause of cancer-related mortality worldwide. Cisplatin is a widely used chemotherapeutic agent in cancer treatment. Vitamin B12 has been shown to play a role in enhancing the efficacy of certain cancer drugs when used in combination therapies. This study investigates the antitumor effects and mechanisms of action of B12 and Cisplatin combination therapy in prostate cancer cells.

Materials and Methods: The clonogenic assay was used to determine the fraction of surviving cells after treatment. The MTS assay and flow cytometry were performed to assess the impact of B12 and Cisplatin on cell proliferation and apoptosis, while Western blot analysis was used to examine the expression of key signaling proteins involved in these processes.

Results: Our results revealed that the combination treatment of B12 and Cispalatin significantly inhibited the proliferation and viability of the PC cell line. Also, clonogenic assay indicated that B12 and Cisplatin combination treatment inhibited the colony formation. Moreover, the combined treatment showed a 2.3-fold increase in P38 and a 1.8-fold increase in PARP-1 protein expression compared to control. In addition, MAPK/ERK1-2 and Bcl-2 protein expression were significantly reduced by approximately 40% and 45% respectively in the combination treatment.

Conclusion: Our findings suggest that the combination of B12 and Cisplatin enhances the antitumor effects of Cisplatin by promoting apoptosis and modulating key signaling pathways, including P38, PARP-1, and MAPK/ERK1-2. These findings, supported by significant reductions in cell viability (up to 50%), suggest a promising role for B12 and Cisplatin combination therapy. Further in vivo and clinical studies are warranted to validate these preliminary in vitro findings.

Key words: Prostate cancer; vitamin B12; cisplatin; apoptosis.

Introduction

Globally, prostate cancer (PC) stands out as a common malignancy in men and a key factor in the increasing death rate, with high-risk disease diagnosed in 15% of cases (1). Despite recent advances in diagnosis and treatment strategies, PC remains incurable. Consequently, there is an imperative necessity to identify novel therapeutic molecules that are more efficacious and economically viable. The mitogen-activated protein kinase (MAPK) signaling pathway connects extracellular signals to intracellular reactions. The mammalian MAPK cascade comprises many proteins. The predominant subprotein families include ERK1/2, ERK5, JNK, and p38. The MAPK/ERK signaling system controls cell proliferation, differentiation, and stress responses(2). In addition, cell proliferation is initiated and apoptosis is inhibited by activation of the MAPK/ERK pathway. MAPK is a complex and interconnected signalling cascade that often plays a role in the development of cancer, in tumor cell proliferation and progression, invasion-metastasis, differentiation and in drug resistance. The MAPK cascade is also a critical pathway for the survival and proliferation of cancer cells and their resistance to drug therapy(3). Known for its role in transducing environmental stress signals, P38 plays an important role in regulating cell death, cell differentiation, growth, proliferation and survival(4). PARP1 is a nuclear enzyme that plays a crucial role in the detection and repair of DNA damage, as well as the regulation of cell survival and apoptosis(5). A structurally complex, non-polymeric biomolecule, vitamin B12 is also known as cobalamin. It plays an important role in the energy metabolism of cells and in the synthesis of DNA(6). There is a limited amount of research

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on the role of vitamin B12 in cancer cells and on its potential therapeutic effects. Information on the specific effects of vitamin B12 on PC is also lacking in the literature. Cisplatin, or cis-diamminedichloroplatinum, is a well-known and widely used agent for chemotherapy. It is a systematic compound preferred as a first-line against many solid tumors such as testicular cancer, cervical, ovarian, head and neck cancers due to its anticancer activity(7). In the vast majority of patients, despite initial response to treatment, the disease eventually relapses with the emergence of resistant cell populations. Therefore, combination therapies of cisplatin with other drugs are of great interest for the overcoming of drug resistance and the reduction of toxicity. The effects of vitamin B12 on the P38, PARP-1, MAPK/ERK1-2 pathways in PC have not been clearly established. Given its metabolic importance, vitamin B12 may potentially interact with several signaling pathways, including MAPK/ERK and PARP-1, which are critical for tumor survival and resistance. However, its role in PC remains largely unexplored. This study aims to evaluate the synergistic effects of B12 and Cisplatin on apoptosis and cancer-related signals in prostate cancer cells. Our study is the first in the literature to investigate the potential role of vitamin B12 and its possible synergistic effects with Cisplatin in the treatment of PC.

Materials and Methods

Cell line, culture conditions, and reagents: LNCaP cells were supplied by ATCC (Manassas, VA, USA). All experiments were performed with at least three independent biological replicates for each condition in the LNCaP prostate cancer cell line. Sigma (Cat. No. V6629, Sigma-Aldrich, USA) supplied vitamin B12, which was dissolved in distilled water at room temperature. Merck Millipore supplied cisplatin (232120-50MG). Cells were cultured in RPMI 1640 medium containing 10% FBS, 50 IU/mL penicillin, and 50 µg/mL streptomycin and incubated at 37°C with 5% CO2 in a humidified environment.

Cell proliferation assay: The MTS assay (Promega Corporation, USA) was used to assess LNCaP cell viability following B12 and cisplatin treatment. In 96-well plates, 3000 cells were exposed to B12 concentrations (100, 200, 300, 400, 800, 1000, 2000 and 3000 µM), cisplatin concentrations (1, 2, 5, 8, 10 and 12 µM) and B12 + cisplatin (3000 µM + 5 µM) for 24, 48 and 72 hours at 37°C in a humidified atmosphere with 5% CO2. The triplicate experiment gave the percentages relative to the control group.

Clonogenic assay: LNCaP cells were seeded in 12-well plates at a density of 500 cells. After the proliferation phase, the cells were treated with B12 (3000 µM), cisplatin (5 µM) and a combined treatment of B12 and cisplatin (3000 µM + 5 µM). After treatment, the cells were stained with 0.5% crystal violet solution (Cat. No. 50-180-6565, Sigma-Aldrich, USA), Colony numbers were analyzed and measured using the Image J software developed by NIH (Bethesda, MD, USA). Clonogenic survival was assessed through three independent experiments (8).

Annexin V-FITC based apoptosis detection assay: The Annexin V-FITC Apoptosis Detection Kit was used to assess the apoptotic state of the cells. At the designated time, cells were subjected to trypsinization, rinsed with PBS, and incubated with Annexin V-FITC and PI stains according to the manufacturer's guidelines. The percentage of apoptotic cells after labelling was determined by flow cytometry (9, 10).

Western blotting: LNCaP cells were injected into 6-well plates at a density of 2×10^5 cells per well. On the subsequent day, the cells were administered B12 (3000 µM), cisplatin (5 µM), or a combination of both (3000 + 5 µM). The medium was extracted into 15 mL Falcon tubes after a 72-hour period. Following trypsin dissociation, the cells were centrifuged at 1500 rpm for 5 minutes. Cell pellets were lysed in RIPA buffer (Thermo Fisher Scientific, Cat. No. 89900), and protein concentration was quantified using the DC™ Protein Assay Kit (Bio-Rad Laboratories, Cat. No. 5000112). SDS-PAGE was used to isolate the proteins. The proteins were then transferred to PVDF membranes (11). The membranes were obstructed with skim milk and incubated overnight at +4°C with the subsequent primary antibodies: Anti-Bcl-2, Anti-MAPK/ERK1/2, Anti-PARP-1, P38, Bax, and Beta actin. Subsequently, goat anti-rabbit (Abcam, Cat. No. ab6721) and goat anti-mouse secondary antibodies were used for incubation. Detection was performed with ECL Western Blotting Reagents and a ChemiDoc-It®2 Imager (UVP, Analytik Jena, USA). Densitometric analysis was performed using Image J (NIH, Bethesda, MD, USA, Version 1.51).

Statistical analysis: The Shapiro-Wilk test was used to confirm normality of distribution for continuous variables, and homogeneity of variances was confirmed by Levene's test prior to ANOVA analyses. Data are shown as mean ± standard deviation (SD) derived from three

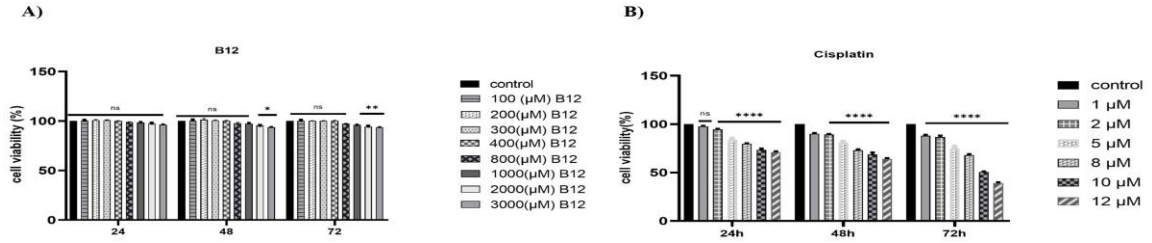

Figure 1: Cell viability analysis of LNCaP cells treated with different concentrations of B12 and Cisplatin over time.

A. The bar graph shows the percentage of cell viability in LNCaP cells treated with B12 at concentrations of 100, 200, 300, 400, 800, 1000, 2000, and 3000 μM, measured at 24, 48, and 72 hours. The control group (untreated) is shown for comparison. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post-hoc comparison tests.
B. LNCaP cells were treated with various concentrations (1 μM, 2 μM, 5 μM, 8 μM, 10 μM, 12 μM) of the compound for 24h, 48h, and 72h. Cell viability was measured using the MTS assay. The graph displays the percentage of viable cells relative to the control group across the three time points. Control group (black bars) represents untreated cells, maintaining near 100% viability at all time points. Bars represent mean ± SD, and statistical analysis was performed using two-way ANOVA followed by Bonferroni's post-hoc comparisons tests. Significance levels: *p < 0.05, **p < 0.01, ****p < 0.0001, ns: not significant.

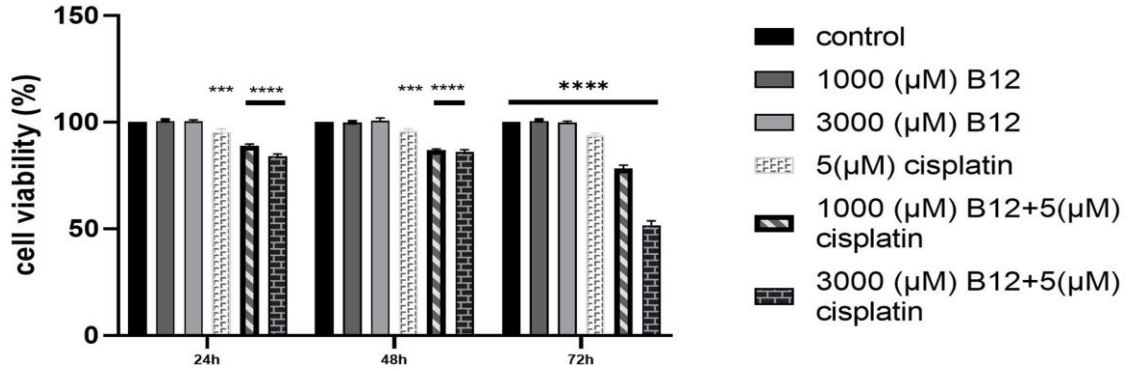

Figure 2: Cell viability analysis of LNCaP cells treated with combination of B12 and cisplatin over time. The bar graph shows the percentage of cell viability in LNCaP cells treated with different combinations of B12 and cisplatin over 24, 48, and 72 hours. Bars represent mean ± SD, and statistical analysis was performed using two-way ANOVA followed by Bonferroni's post-hoc comparison tests. ***p < 0.001, ****p < 0.0001

distinct experiments. The MTS assay data were analyzed using two-way ANOVA with multiple comparisons and Bonferroni's post-hoc test. Clonogenic assay data were evaluated with one-way ANOVA and Dunnett's multiple comparisons test. One-way ANOVA with Dunnett's test was employed for the analysis of Annexin V-FITC Apoptosis Detection Assay data. Differences in protein expression were evaluated using two-way ANOVA with Bonferroni correction. Statistical analyses were conducted utilizing Windows Office Excel and GraphPad Prism 8.0 (GraphPad Software Inc, San Diego, CA, USA). A p-value below 0.05 was deemed significant.

Results

B12 combined with cisplatin inhibited the proliferation of PC cells: Cell viability was assessed by MTS assay after 24, 48 and 72 hours of treatment with increasing doses of single and combined agents. The dose-response relationship of cell viability was analysed after treatment with B12, cisplatin and their combinations. The administration of vitamin B12 (1000, 3000 μM) on its own did not appear to have a significant effect on the viability of the cells (figure 1). However, at 48, 72 hours, compared to cisplatin alone (5 μM, 75 % cell viability) (figure 1) (p=0,0072, p=0,0065), treatment with cisplatin in

combination with B12 resulted in a greater decrease in LNCaP cell viability (50 % cell viability) (figure 2) The results indicated that, at the doses used, the combined treatment was more cytotoxic than cisplatin alone.

B12 and cisplatin downregulation on PC cells colony formation: To determine the effect of the combination of B12 (3000 µM) and Cisplatin (5 µM) on the long-term survival of PC cells, we performed clonogenic assay. We found that colony formation in PC cells was significantly suppressed after treatment with the combination of these agents for 24 hours (figure 3) (p=0,0001). In conclusion, these results show that the treatment of PC cells with B12 and Cisplatin has a synergistic effect on the survival of the cells.

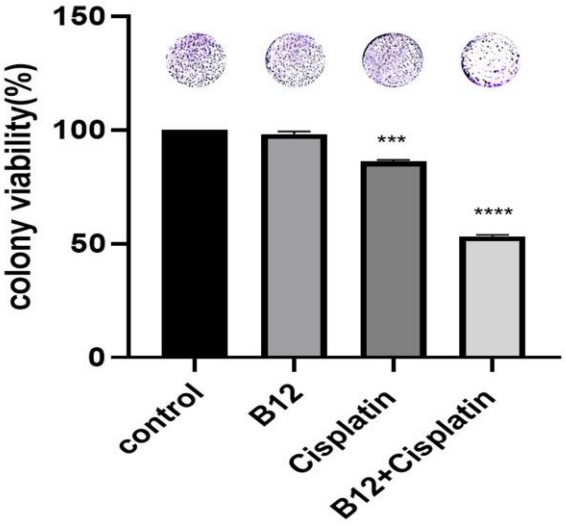

Figure 3: Colony viability assay of LNCaP cells treated with B12 and cisplatin. The bar graph represents the percentage of colony viability in LNCaP cells after treatment with B12, cisplatin (IC25), and their combination (B12 + Cisplatin), compared to non-treated (control) cells. Above the bars, representative images of the colonies are shown for each condition, highlighting the reduction in colony number and size across the treatment groups. Bars represent mean ± SD, and statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. ***p < 0.001, ****p < 0.0001.

Combination treatment with reduced dose of B12 and Cisplatin induces more apoptosis in LNCaP cells than treatment with either drug alone and modulates cell survival pathways: An increase in apoptosis is usually associated with the inhibition of cell growth in tumor cells. Flow cytometry analysis and stained with Annexin V-FITC and the PI double staining method was used to analyze the effect of B12 with Cisplatin alone and in combination on apoptosis. The results showed that the untreated sample had a healthy and viable cell count of 99.98% (Figure 4). A remarkable increase in cellular apoptosis was observed in the combination treatment where only 90.83% of the cells were found to be viable,

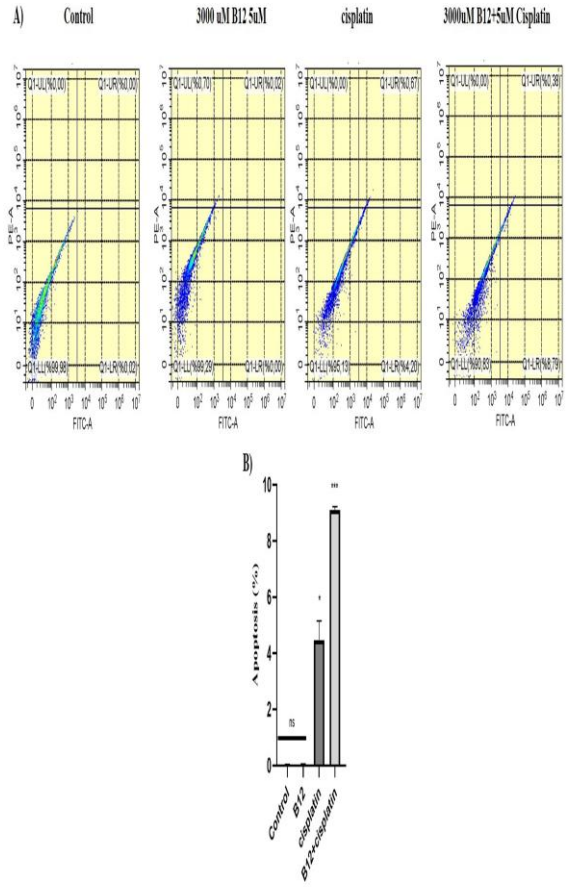

Figure 4: Annexin V/PI assay to assess apoptosis in LNCaP cells treated with B12 and cisplatin. Flow cytometry dot plots showing the distribution of cells in different stages of apoptosis after treatment with 3000 µM B12, 5 µM cisplatin, and their combination (3000 µM B12 + 5 µM cisplatin). The quadrants represent: Q1-LL (lower left): Live cells (Annexin V-/PI-),Q1-UL (upper left): Necrotic cells (Annexin V-/PI+), Q1-UR (upper right): Late apoptotic cells (Annexin V+/PI+), Q1-LR (lower right): Early apoptotic cells (Annexin V+/PI-). Bars represent mean ± SD, and statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. (*p < 0.05, ***p < 0.001).

8.79% were in early apoptosis and 0.38% were in late apoptosis. Very little apoptosis was seen with the single doses of B12 and cisplatin. In summary, low doses of cisplatin (IC25) given in combination with B12 were found to resulted in a higher rate of apoptosis in PC cells. (p= 0,0153, p= 0,0001)

B12 and cisplatin showed anti-tumor activity via the P38, PARP-1 and MAPK/ERK1-2 pathways: The production of an 89 kDa PARP-1 degradation product and 26 kDa Bcl-2 is

considered a good indicator of the onset of apoptosis, so we also analyzed the effect of cisplatin and B12 alone and in combination by Western blotting. We observed that the combination of B12 and Cisplatin significantly reduced Bcl-2 proteins expression and increased PARP-1proteins expression compared to alone.

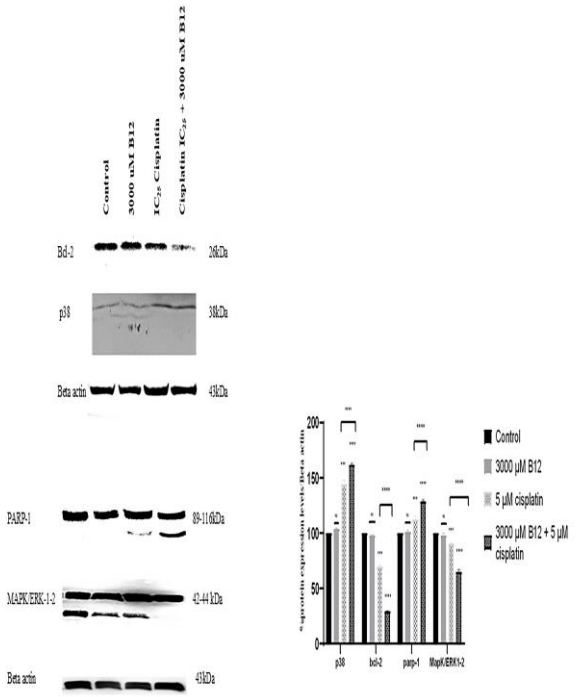

Figure 5: Western blot analysis of p38, Bcl-2, PARP-1 and MAPK/ERK1-2 proteins in LNCaP cells treated with B12, cisplatin and their combination. PARP-1 cleavage in LNCaP for 72 h with (10 nM cisplatin and B12) was measured using a monoclonal antibody against PARP-1 that recognises both the uncut form (116 kDa) and the apoptosis-specific cleaved fragment (89 kDa) of PARP-1. The relative amounts of the 116 and 85 kDa bands are shown. A) Western blot bands; B) Relative levels of p38, Bcl-2, PARP-1 and MAPK/ERK1-2. All experiments were carried out in triplicate and the data are presented as the mean ± SD. One-way ANOVA followed by Dunnett's multiple comparison test was used for statistical analysis. ***p < 0.001, ****p < 0.0001.

(Figure 5). In addition, our results demonstrated that the combination treatment importantly increased p38. This is an indication that the combination treatment strongly triggers the cellular stress response. Moreover, compared to all other groups, MAPK/ERK1-2 levels were significantly reduced in the combination group (Figure 5). This suggests that the signals for cell proliferation have been suppressed. Taken together, these data indicate that B12 and cisplatin combination therapy can induce apoptosis via the p38, Bcl-2, PARP-1 and MAPK/ERK1-2 pathways.

Discussion

This study showed that the combination of B12 and cisplatin significantly improved therapeutic effects on PC cells. PC is one of the most common malignant tumors affecting men worldwide and contributes significantly to cancer-related mortality (12, 13). Although various treatments such as hormone therapy, radiation and chemotherapy have been used to treat PC, their limited efficacy has led to a demand for more effective options. Clinical studies have shown that combining chemotherapy drugs can result in reduced toxicity, enhanced anti-tumor activity, and increased treatment efficacy (14). B12 is an important co-factor in a number of processes including DNA synthesis and mitochondrial metabolism, cellular reprogramming and the regeneration of tissues. Vitamin B12 is an essential co-factor in DNA synthesis, mitochondrial metabolism, and tissue regeneration. It has been reported to improve drug response in various cancers (15). Cisplatin, a commonly used chemotherapeutic agent, is effective across a wide range of tumors including prostate, lung, and ovarian cancers. Our findings suggest a synergistic effect of B12 with cisplatin in prostate cancer cells, which has not been reported previously. Apoptosis, a programmed form of cell death, is a crucial therapeutic target in cancer therapy (16, 17). The potential role of B12 in the treatment of PC and its synergistic effect with cisplatin has been investigated for the first time in our study. Cancer cell proliferation, migration and invasion are integral components of metastasis, the leading cause of death in cancer patients(18,19). In our study, the combination of B12 and cisplatin not only increased apoptotic markers (e.g., Annexin V positivity) but also modulated the expression of key apoptosis-related proteins. Overexpression of the anti-apoptotic protein Bcl-2 contributes to therapeutic resistance in several cancers, including prostate cancer (20, 21). We observed downregulation of Bcl-2 and upregulation of PARP-1, supporting the activation of apoptotic pathways. Furthermore, the MAPK signalling cascade plays a central role in cell fate decisions (22). While ERK1/2 signaling promotes proliferation and survival, p38 MAPK activation is typically associated with stress-induced apoptosis. Our data revealed upregulation of p38 and downregulation of ERK1/2 in the combination group, indicating a shift toward apoptotic signaling (23). These findings are in agreement

with literature. Solovieva et al. demonstrated that B12 derivatives enhanced chemotherapeutic cytotoxicity via paraptosis-like mechanisms involving severe ER stress and mitochondrial dysfunction (24). The researchers also confirmed increased apoptotic signaling through p38 activation in glioblastoma cells exposed to vitamin derivatives (25). B12-related modulation of deubiquitinases, such as USP3, indicates a potential mechanistic link between B12 and the regulation of stress signalling (26). In addition, B12 plays a critical role in one-carbon metabolism and DNA methylation. Garg and Miousse found that B12 supplementation rescued proliferation in methionine-deprived colorectal cancer cells by preventing activation of the integrated stress response (27). However, the clinical relationship between B12 and cancer is complex. A U-shaped association between B12 intake and cancer risk was reported by Ngoan Tran Le et al., suggesting that both deficiency and excess may affect risk differently across populations and cancer types (28). Similarly, Fanidi et al. observed that elevated serum B12 levels were associated with a higher risk of lung cancer (29). These insights highlight the importance of precise regulation and patient-specific consideration in B12 supplementation strategies, as also emphasised by Frost et al., who reviewed the dual role of vitamin B12 in tumorigenesis depending on dose, duration, and tumor context (30). Taken together, our study presents new insights into the synergistic anti-tumor activity of B12 and cisplatin in prostate cancer and supports further investigation through in vivo and clinical studies.

Study limitations: This study was conducted in vitro using the LNCaP cell line, which may not fully represent the complexity of prostate cancer in vivo. Additionally, further in vivo studies and clinical trials are needed to confirm the therapeutic potential and safety of the B12 and cisplatin combination in prostate cancer treatment.

Conclusion

In summary, the combination treatment of B12 and cisplatin showed a potent antitumor effect on PC by inducing cell death and DNA damage, possibly through decreased expression of the anti-apoptotic proteins Bcl-2, MAPK/ERK1- 2 and increased expression of P38 and PARP-1. Our findings demonstrate the potential of the combination of B12 and Cisplatin as an anticancer agent for the treatment of PC.

Ethical approval: This study was conducted in accordance with ethical research standards and Good Cell Culture Practice (GCCP) guidelines. Since it involved only in vitro cell culture experiments and did not include human participants or animal subjects, formal ethical approval was not required.

Conflict of interest: On behalf of all authors, the corresponding author confirms the absence of any conflicts of interest.

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Author contributions: Concept (GE, BO), Design (GE, BO), Data Collection and/or Processing (GE, BO, IT), Analysis and/or Interpretation (GE, BO, IT)

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