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Mifepristone Has Limited Activity to Enhance the *In Vivo* Efficacy of Docetaxel and Enzalutamide Against Bone Metastatic and Castration-Resistant Prostate Cancer

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Abstract. *Background: Mifepristone has gained great interest in its potential as a novel agent against human cancers, including prostate cancer (PCa). However, recent clinical trials using mifepristone in PCa and other cancers have been disappointing. We evaluated the in vitro and in vivo activities of mifepristone, in combination with docetaxel and enzalutamide, against bone metastatic castration-resistant PCa. Materials and Methods: The effects of mifepristone, alone or in combination with docetaxel or enzalutamide, on PCa cell viability, in vitro, were determined by the colorimetric assay. Intratibial model of C4-2-Luc tumors in athymic nude mice was used to evaluate the in vivo efficacy of mifepristone alone or in combination with docetaxel or enzalutamide. Tumor growth in mouse bone was assessed by serum prostate-specific antigen (PSA) levels and radiography. Results: Although mifepristone exhibits a certain degree of synergism with docetaxel or enzalutamide in cell culture, statistical analyses showed that combination regimens fail to demonstrate effectiveness in suppressing the skeletal growth of PCa and enhancing the in vivo efficacy of docetaxel or enzalutamide in athymic nude mice ($p>0.05$). Conclusion: These results provide the first pre-clinical evidence suggesting that mifepristone may not effectively inhibit bone metastatic PCa, either as a single agent or combined with standard chemotherapy and androgen-deprivation therapy. This report may raise concerns over the clinical use of mifepristone in the management of advanced PCa.*

Bone is the most common site for metastasis in prostate cancer (PCa). Autopsy findings show development of skeletal metastasis in more than 85% of PCa cases. The quality of life of patients can be significantly compromised by skeletal complications (1-3). Recent years have seen an expansion of treatment options for PCa bone metastasis, including chemotherapy (docetaxel and cabazitaxel), androgen-deprivation therapy (ADT; enzalutamide and abiraterone), immunotherapy (Sipuleucel-T) and radium-223 dichloride. Unfortunately, these new therapies only modestly prolong overall survival by 2-5 months, and patients generally relapse and develop extremely resistant tumors within 1 year (4-6). Clearly, it is imperative to develop novel strategies to overcome therapeutic resistance and improve clinical outcomes in PCa patients (7).

Numerous trials are in progress to identify optimal treatment combinations among various standard therapies, as well as combinations with other biological agents in order to treat metastatic castration-resistant PCa (CRPC). Recently, mifepristone (RU486) has gained great interest regarding its potential use as an anti-cancer agent (8-11). As an

Abbreviations: ANOVA: Analysis of variance; AR: androgen receptor; CI: combination index; CYP17A1: steroid 17α-hydroxylase; CRPC: castration-resistant prostate cancer; DHT: dihydrotestosterone; GR: glucocorticoid receptor; IC_{50}: half maximal inhibitory concentration; IP injection: intraperitoneal injection; PCa: prostate cancer; PSA: prostate-specific antigen.

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abortifacient, mifepristone was approved in France and China in 1988 and in the United States in 2000 (12). Mifepristone is safe and effective when used to terminate a pregnancy and has been on the World Health Organization Model List of Essential Medicines. A number of pre-clinical studies demonstrated that mifepristone has significant growth inhibition and antitumor effects on various human cancer cells, including PCa (8, 13). At least ten clinical trials have been conducted, and more are being performed to evaluate the efficacy of mifepristone in treating several solid tumors (14-17). Some patients in these trials had complete or partial response (for example, in refractory ovarian cancer), whereas other patients had no response (8). In a non-randomized phase II study, mifepristone failed to demonstrate any benefits in producing a prostate-specific antigen (PSA) response in patients with CRPC (18). Surprisingly, mifepristone treatment markedly increased adrenal androgens, testosterone, and dihydrotestosterone (DHT). These results indicated that as a single agent, mifepristone has limited clinical activity against CRPC. A reasonable speculation is that mifepristone may demonstrate better efficacy when combined with standard therapies. Indeed, a randomized, open-label phase I/II study is being conducted to test whether the combination of mifepristone and enzalutamide can extend PSA progression-free survival in CRPC patients (NCT02012296).

Using an intratibial model of CRPC, we evaluated the *in vivo* efficacy of the combination of mifepristone with two major treatments for bone metastatic CRPC, docetaxel and enzalutamide. Herein we report that both combinations fail to effectively suppress the skeletal growth of CRPC in mouse models.

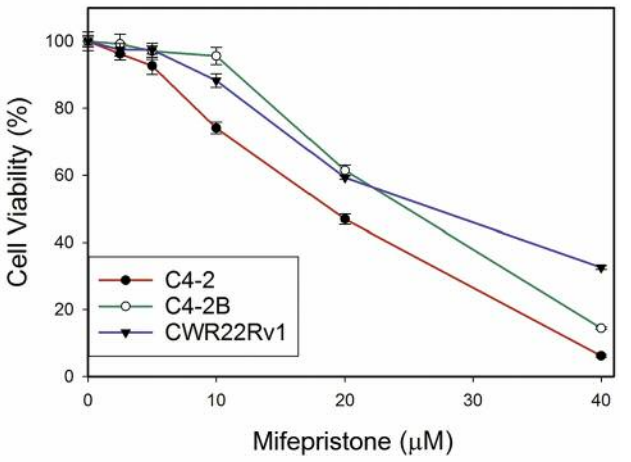


Figure 1. *In vitro cytotoxicity of mifepristone in CRPC cells. AR-positive C4-2, C4-2B, and CWR-22Rv1 cells were cultured in varying concentrations of mifepristone for 72 h. Cell viability was determined by MTS or CCK-8 assays. AR, Androgen receptor; CRPC, castration-resistant prostate cancer; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); CCK-8, cell counting kit-8 (Dojindo).*

Materials and Methods

Cell culture and reagents. Human PCa cell lines C4-2, C4-2-Luc, and C4-2B were provided by Dr. Leland WK Chung (Cedars Sinai Medical Center, Los Angeles, CA, USA), and CWR-22Rv1 cells were provided by Dr. Jindan Yu (Northwestern University, Chicago, IL, USA). Cells were routinely maintained at 37˚C, 5% CO_2 and 95% humidity in T-medium (Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA) or RPMI1640 medium (Corning, Corning, NY, USA) supplemented with 10% FBS. A final concentration of 400 μg/ml of G418 (Invitrogen, Carlsbad, CA, USA) was added to maintain luciferase expression in C4-2-Luc cells. Mifepristone was obtained from Sigma Aldrich (St. Louis, MO, USA). Docetaxel was obtained from LC Laboratories (Woburn, MA, USA) and enzalutamide (MDV3100) from Selleckchem (Houston, TX, USA).

Cell proliferation assay. Cell proliferation was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation (MTS) Assay kit (Promega, Madison, WI, USA) or Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. For the cell viability assay, 4×10^3 cells per well were seeded in 96-well plates overnight and treated with mifepristone, docetaxel, enzalutamide, combination of mifepristone and docetaxel, combination of mifepristone and enzalutamide, or vehicle control at the indicated concentrations for 72 h. A microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) was used to determine cell viability, which was expressed as relative survival compared to controls recorded as 100%. Combination index (CI) between mifepristone and docetaxel or enzalutamide was determined using the CompuSyn software (ComboSyn, Inc).

In vivo treatment with the combination of mifepristone and docetaxel in the intratibial model of C4-2-Luc cells. All animal procedures were approved by the Augusta University Institutional Animal Care and Use Committee (IACUC). Athymic male nude mice (Hsd: athymic nude-nu; 5-week-old) were purchased from Harlan Laboratories (Indianapolis, IN, USA). For each mouse, a total of 2.0×10^6 C4-2-Luc cells were inoculated into the bilateral tibia as we described previously (19). Blood specimens were obtained from the facial veins every week for serum PSA determination using an ELISA kit from United Biotech, Inc (Mountain View, CA, USA). With the confirmation of positive PSA levels, a total of 35 tumor-bearing mice were randomly divided into 6 groups, and received the following injection *via* the intraperitoneal (IP) route for the indicated time: vehicle control group (n=5): 100% DMSO, 3 times per week; docetaxel group (n=5): 5 mg/kg body weight, once per week; mifepristone: 30 mg/kg (n=5) or 60 mg/kg (n=6), 3 times per week; or combination group: 5 mg/kg of docetaxel, once per week, and 30 mg/kg (n=7) or 60 mg/kg (n=7) of mifepristone, 3 times per week. Mice were weighed twice a week, and tumor growth in bilateral tibia was followed by serum PSA measurements. X-ray analyses were performed at a time close to endpoint with Faxitron MX20 digital radiography system (Faxitron Bioptics, LLC; Tucson, AZ, USA).

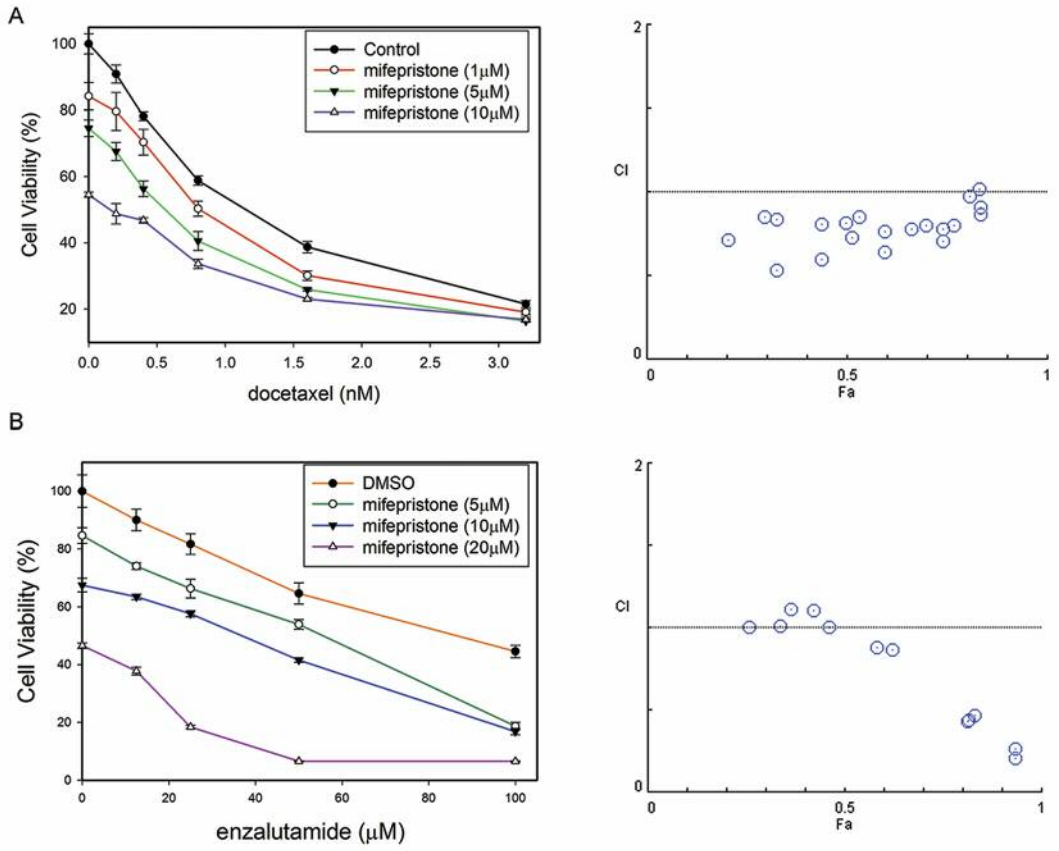


Figure 2. *In vitro effects of the combinations of mifepristone with docetaxel or enzalutamide in CRPC cells. C4-2 cells were treated with varying concentrations of mifepristone, in combination with docetaxel (A) or enzalutamide (B), for 72 h. CI was calculated using the CompuSyn software that provides quantitative definition of synergism (CI<1), additive effect (CI=1), and antagonism (CI>1) between two or more drugs. CRPC, Castration-resistant prostate cancer; CI, Combination index.*

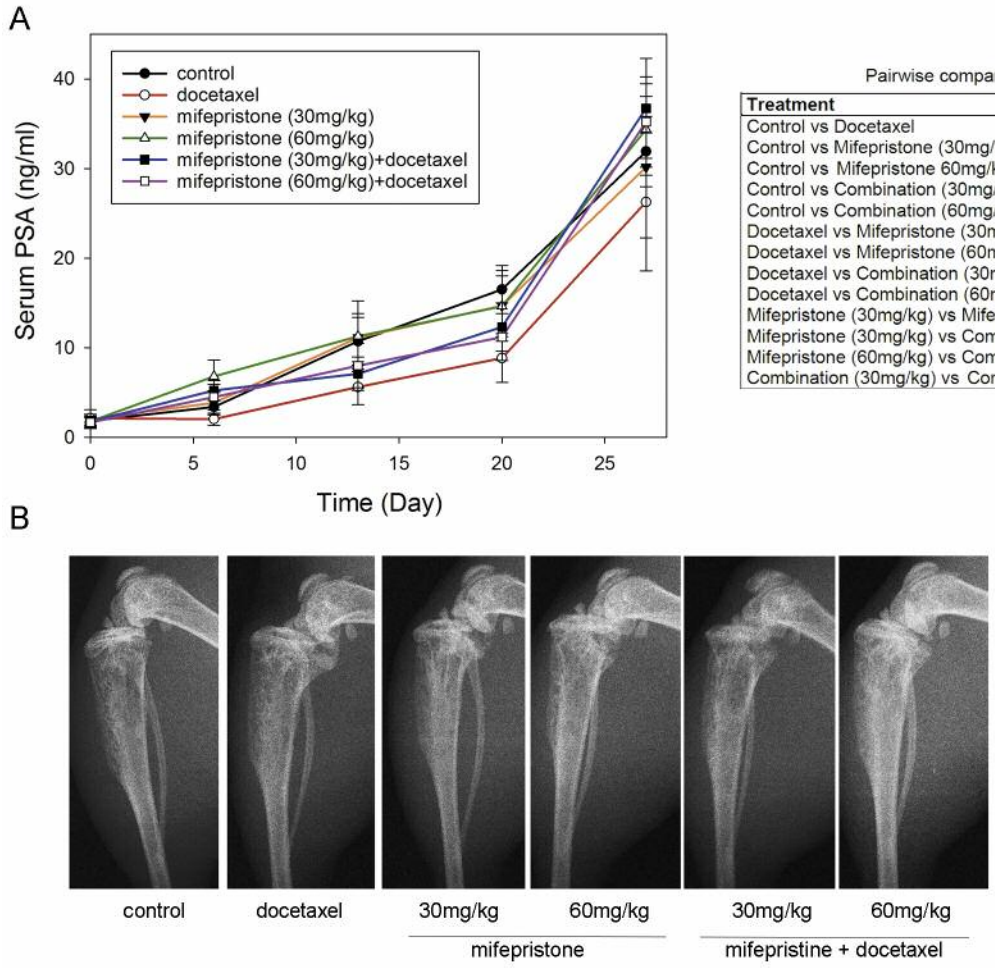
In vivo treatment with the combination of mifepristone and enzalutamide in the intratibial model of C4-2-Luc cells. C4-2-Luc tumor inoculation was performed as described above. A total of 20 tumor-bearing mice were randomly divided into four groups and received the following injection *via* the IP route for the indicated time: vehicle control group (n=5): 100% DMSO, 3 times per week; enzalutamide group (n=5): 30 mg/kg body weight, 3 times per week; mifepristone (n=5): 30 mg/kg, 3 times per week; or combination group (n=5): 30 mg/kg of enzalutamide and 30 mg/kg of mifepristone, 3 times per week. Mice were weighed twice a week, and tumor growth in bilateral tibia was followed by weekly serum PSA assays. X-ray radiography was performed at a time close to endpoint.

Statistical analysis. A two-way analysis of variance (ANOVA) model was used to assess the longitudinal effect of treatment between each pair of groups during the whole study period. The significance levels were set at 0.05 for all tests. The SigmaPlot 13.0 package (Systat Software, Inc., San Jose, CA) was used for data management and analysis.

Results

In vitro cytotoxicity of mifepristone in CRPC cells. Previous studies have demonstrated that mifepristone inhibits the growth of human cancer cells originating from various sites, including brain, breast, prostate, ovary and bone (8). We evaluated the *in vitro* cytotoxicity of mifepristone in a panel of androgen receptor (AR)-positive CRPC cell lines, which closely mimic the aggressive phenotypes of clinical CRPC. As shown in Figure 1, a 72-h treatment with mifepristone significantly inhibited the viability of these cells ($p<0.05$), with the 50% inhibitory concentration (IC_{50}) of 19.23 μM, 23.96 μM and 26.73 μM in C4-2, C4-2B and CWR-22Rv1 cells, respectively. These data indicate that mifepristone exhibits modest cytotoxicity in CRPC cells, *in vitro*.

In vitro cytotoxicity of the combination of mifepristone and docetaxel or enzalutamide in CRPC cells. We examined the



Pairwise comparison (2-Way ANOVA)

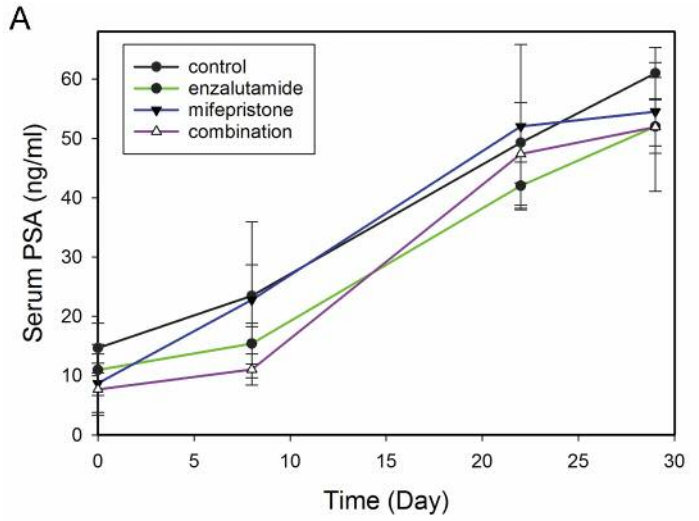
Treatment	*p*-value
Control vs Docetaxel	0.057
Control vs Mifepristone (30mg/kg)	0.796
Control vs Mifepristone 60mg/kg	0.671
Control vs Combination (30mg/kg)	0.891
Control vs Combination (60mg/kg)	0.605
Docetaxel vs Mifepristone (30mg/kg)	0.187
Docetaxel vs Mifepristone (60mg/kg)	0.039
Docetaxel vs Combination (30mg/kg)	0.077
Docetaxel vs Combination (60mg/kg)	0.116
Mifepristone (30mg/kg) vs Mifepristone (60mg/kg)	0.375
Mifepristone (30mg/kg) vs Combination (30mg/kg)	0.906
Mifepristone (60mg/kg) vs Combination (60mg/kg)	0.375
Combination (30mg/kg) vs Combination (60mg/kg)	0.755

Figure 3. *In vivo effects of mifepristone and its combination with docetaxel in C4-2-Luc intratibial model. (A) Left panel: Mifepristone alone or combined with docetaxel does not significantly reduce serum PSA levels in athymic nude mice bearing C4-2-Luc tumors; Right panel: statistical analysis on the in vivo effect of mifepristone and its combination with docetaxel. (B) X-ray radiography of C4-2-Luc-bearing tibias receiving drug treatments as indicated. PSA, Prostate-specific antigen.*

combined effects of mifepristone and docetaxel, a first-line chemotherapeutic drug for CRPC, in C4-2 cells. As shown in Figure 2A, the addition of mifepristone increased the *in vitro* cytotoxicity of docetaxel. Quantitative analysis of dose-effect relationships using the CompuSyn software indicated there is weak to modest synergism between the two drugs, with the combination index (CI) ranging from 0.53 to 1.02. The best synergism occured when mifepristone was used at 2.0 μM and docetaxel at 0.2 μM.

We further tested the combined effects of mifepristone with enzalutamide, a new-generation AR antagonist. Interestingly, synergism was only observed when both mifepristone and enzalutamide were present at relatively high doses. The lowest CI occurs when mifepristone is used at 20 μM and enzalutamide is used at 50 μM. At other doses, the combined effects of mifepristone and enzalutamide are mostly additive between the two drugs.

In vivo efficacy of combined treatment with mifepristone and docetaxel against the skeletal growth of C4-2 tumors, in vivo. We examined the *in vivo* efficacy of mifepristone against the skeletal growth of CRPC in an intratibial model of C4-2 cells. After the confirmation of successful tumor inoculation, athymic nude mice were treated with vehicle control, docetaxel, mifepristone or the combination of mifepristone and docetaxel. Based on our previous observations, docetaxel was administered at 5 mg/kg body weight, once per week, *via* IP route. Mifepristone was administered at 2 escalating doses, *i.e.*, 30 and 60 mg/kg body weight, 3 times per week, IP. Following 4 weeks of treatment, the average PSA level in each group at the endpoint



Pairwise comparison (2-Way ANOVA)

Treatment	*p*-value
Control vs Enzalutamide	0.043
Control vs Mifepristone	0.644
Control vs Combination	0.104
Enzalutamide vs Combination	0.891
Mifepristone vs Combination	0.424
Enzalutamide vs Mifepristone	0.410

Figure 4. *In vivo effects of mifepristone and its combination with enzalutamide in C4-2-Luc intratibial model. (A) Left panel: Mifepristone alone or combined with enzalutamide does not significantly reduce serum PSA levels in athymic nude mice bearing C4-2-Luc tumors; Right panel: statistical analysis on the in vivo effect of mifepristone and its combination with enzalutamide. (B) X-ray radiography of C4-2-Luc-bearing tibias receiving drug treatments as indicated. PSA, Prostate-specific antigen.*

was 31.92±3.92 ng/ml (control), 26.28±7.69 ng/ml (docetaxel), 30.17±7.90 ng/ml (mifepristone, 30 mg/kg), 34.38±5.09 ng/ml (mifepristone, 60 mg/kg), 36.74±5.57 ng/ml (mifepristone, 30 mg/kg, and docetaxel), and 35.21±5.04 ng/ml (mifepristone, 60 mg/kg and docetaxel) (Figure 3A). Statistical analysis using a two-way ANOVA model found no significant difference in the longitudinal PSA values between any two treatment groups ($p>0.05$), except that between the docetaxel and mifepristone group ($p=0.039$). X-ray radiography showed that C4-2 tumor-bearing bone treated with either mifepristone, docetaxel or the combination regimen displayed similar skeletal architectures with multiple osteoblastic and osteolytic lesions, compared with the control group (Figure 3B). These results indicated that mifepristone has limited or no effects in suppressing C4-2 tumors in mouse bones and in enhancing docetaxel efficacy.

In vivo efficacy of the combined treatment with mifepristone and enzalutamide against the skeletal growth of C4-2 tumors. We also tested the *in vivo* efficacy of mifepristone and enzalutamide in the intratibial model of C4-2 cells. A single dose (30 mg/kg body weight) of mifepristone was used in this experiment. At this dose, mifepristone appears to exhibit similar (or better, although statistically not significant) effects on C4-2 tumor growth as the dose of 60 mg/kg. Enzalutamide was administered at 30 mg/kg body weight, 3 times per week, *via* the IP route. As shown in Figure 4A, the average PSA level in each group at the endpoint was 61.01±4.31 ng/ml (control), 52.02±4.53 ng/ml (enzalutamide), 54.53±5.78 ng/ml (mifepristone), and 51.95±10.86 ng/ml (combination). Statistical analysis showed that there was no significant difference in the

longitudinal PSA values between any two treatment groups ($p>0.05$), except that between the control and enzalutamide groups ($p=0.043$). X-ray radiography showed that, compared with the control group, C4-2 tumor-bearing bone treated with either mifepristone, enzalutamide or the combination displayed similar skeletal architectures with multiple osteoblastic and osteolytic lesions (Figure 4B). Similarly to mifepristone and docetaxel results, mifepristone had limited or no effect in suppressing C4-2 tumors in mouse bones and in enhancing the *in vivo* efficacy of enzalutamide.

Discussion

A reversible and potent glucocorticoid receptor (GR) antagonist, mifepristone has gained considerable attention for its potential as an antineoplastic agent, particularly in hormone-associated cancers. Most evidence came from pre-clinical studies that showed that mifepristone can induce growth arrest, apoptotic lethality, and the reversal of multidrug resistance in human cancer cells from various origins (8). At the molecular level, mifepristone can affect multiple genes involved in regulation of the cell cycle (such as cyclin-dependent kinase 2/cdk2), apoptosis (such as caspase-3, B-cell lymphoma 2/Bcl-2, B-cell lymphoma-extra large/Bcl-XL, transforming growth factor β1), and acquired therapeutic resistance (p-glycoprotein). In triple-negative breast cancer cells, mifepristone alone has no significant effect on cell viability, but markedly increases the effects of dexamethasone/paclitaxel treatment, presumably *via* the inhibition of glucocorticoid-inducible protein kinase-1 and mitogen-activated protein kinase (20). Mifepristone may also specifically inhibit the triple-negative breast cancer stem cell population by targeting Krüppel-like factor 5 (21).

The inhibitory effect of mifepristone on PCa cells seem to be independent of AR status, since the drug exhibits similar activity against the proliferation of both AR-positive PCa cells (such as LNCaP, C4, C4-2, CWR22Rv1) and AR-negative cells (such as PC-3, DU-145) (13, 22-25). Subcutaneous injection of mifepristone at a dose of 50 mg/kg/day for 28 days has been shown to inhibit tumor growth by 50% in the LNCaP, C4 and C4-2 xenografts (22). Another study reported that IP injection of mifepristone at 12 mg/kg/day was associated with an approximately 33% prolongation of time to tumor progression compared to vehicle control, in castration-resistant CWR-22Rv1 cells (23). These studies suggested that mifepristone might have clinical benefits in improving the overall outcomes in PCa patients. Indeed, Taplin *et al*. have conducted a phase II trial using mifepristone for the treatment of patients with CRPC. Unfortunately, it was found that mifepristone had limited activity in these patients and caused a significant increase in testosterone, adrenal androgens, and DHT (18). These disappointing results suggested that mifepristone alone may not be effective in treating CRPC. Currently, clinical efficacy of the combination regimens of mifepristone with standard CRPC therapy is being tested, including an ongoing randomized, open-label phase I/II trial that investigates the safety and activity of the combination of mifepristone and enzalutamide in CRPC patients with rising PSA.

In the current study, we sought to evaluate the potential of mifepristone to enhance standard CRPC treatments using xenograft models of bone metastatic CRPC. Our results presented here show that mifepristone fails to demonstrate synergy in enhancing the efficacy of either docetaxel or enzalutamide in bone metastatic CRPC treatment. These data point to a more complex scenario than we expected regarding the mechanism of mifepristone action in PCa cells and its incorporation with standard of care for the treatment of CRPC.

PCa initially progresses in an androgen-dependent manner (26). In the setting of surgical or chemical castration, additional suppression of adrenal androgen synthesis can be achieved with glucocorticoid therapy. Glucocorticoids inhibit the release of corticotropin-releasing hormone from the hypothalamus and thereby suppress adrenal androgen steroidogenesis, which leads to the inhibition of androgen-dependent proliferation of PCa cells (27). Therefore, synthetic glucocorticoids (such as prednisone and dexamethasone) are frequently incorporated with standard treatment regimens for advanced PCa, particularly in combination with chemotherapy (docetaxel and cabazitaxel) and/or radiotherapy (28, 29). Indeed, a number of retrospective studies and phase II trials have evaluated the effectiveness of glucocorticoids as single agents in CRPC patients and demonstrated certain degrees of serum PSA response (30). Due to their anti-inflammatory properties and ability to reduce toxic adverse effects of cytotoxic chemotherapy, the inclusion of glucocorticoids may help to improve the quality of life of patients, such as reducing bone pain and increasing appetite (31-33). More recently, glucocorticoids (such as prednisone) have been combined with abiraterone, a potent inhibitor of steroid 17α-hydroxylase (CYP17A1) and androgen synthesis (34). The incorporation of glucocorticoids in the standard PCa treatment can partially counteract a reduction of circulating cortisol and a compensatory stimulation of adrenocorticotropic hormone in response to abiraterone treatment, thereby reducing side effects from CYP17A1 inhibition (35-37). Despite these clinical benefits of glucocorticoids, post-hoc analyses on several completed trials indicated that glucocorticoids have adverse effects on the overall outcomes (30). For example, the use of glucocorticoids is associated with inferior overall survival and higher rate of adverse effects in the AFFIRM study, a phase II trial of enzalutamide in CRPC patients (38). Similarly, in the COU-AA-301 phase III study that compared prednisone alone with its combination with abiraterone in chemoresistant CRPC, the prednisone arm had inferior overall

survival on univariate analysis and exhibited worse baseline disease characteristics (38, 39). These unexpected negative effects suggest a complicated mechanism of action of glucocorticoids in PCa patients, particularly those at late stages and having a long-term use of glucocorticoids (30).

Accumulating evidence indicated that increased GR expression is a common feature of drug-resistant CRPC (23, 40). An elegant study from Arora *et al*. showed that GR can actually substitute for the AR to activate a similar but distinguishable set of target genes and is required for the maintenance of enzalutamide resistance. Furthermore, acute AR inhibition leads to GR upregulation due to relief of AR-mediated feedback repression of GR expression, thereby conferring therapeutic resistance. Conversely, treatment with a GR antagonist, compound 15, effectively restored enzalutamide sensitivity (41). In a recent study, Kroon *et al*. found that GR is also significantly upregulated in docetaxel-treated primary PCa tissues when compared with chemonaïve tumors. Consistently, GR is increased in cultured PCa cell lines with acquired docetaxel resistance regardless of their AR status, and the treatment with GR antagonists (mifepristone and cyproterone acetate) reversed docetaxel resistance in these cells (42). Although it remains unclear whether GR upregulation in PCa cells significantly contributes to the observed adverse effects of glucocorticoids in clinical settings, these studies provided a rationale to exploit GR antagonism to re-sensitize CRPC cells to docetaxel and enzalutamide treatments. Given its promising pre-clinical activity against PCa growth and favorable safety profile, mifepristone was pursued as an ideal agent to antagonize GR signaling in CRPC and enhance the efficacy of docetaxel and/or enzalutamide. However, in contrast to previous observations in other models (22-24, 41, 42), our results indicate that mifepristone has very limited activity against the skeletal growth of PCa cells, either used alone or in combination with docetaxel or enzalutamide. This controversy is further highlighted by other studies, where AR signaling was taken into account. In various pre-clinical models, it appears that GR exerts opposite effects on different populations of PCa cells (23); it inhibits growth of PCa cells with intact AR signaling (43, 44), and promotes growth of PCa cells lacking AR and expressing high levels of GR (13, 45, 46). Therefore, it is plausible that GR antagonism with mifepristone in C4-2 cells, which express fully functional AR (47), may relieve the suppressive effects of GR on PCa cell proliferation, thereby attenuating the inhibitory efficacy of docetaxel or enzalutamide. In addition, it has been shown that mifepristone, when bound to AR mutant AR-E897A, exhibits comparable to or better agonist activity than that seen with AR agonists, DHT and R1881 (48). Mifepristone can also promote the interaction between AR and one of its coactivators, ARA70, in a dose-dependent manner, which may subsequently enhance AR transcriptional activity (49). The agonist activity of mifepristone on AR signaling may be largely context-dependent, that could further complicate the overall outcomes when mifepristone is used to treat CRPC, due to the high heterogeneity of CRPC.

Historically, cell line-derived xenograft models have been repeatedly shown to be valuable in triaging investigational antineoplastic agents (50). Taken together, our data could raise questions regarding the use of mifepristone for the treatments of patients with advanced PCa. It would be interesting to follow-up and review clinical results from the ongoing phase I/II trial NCT02012296.

Conflict of Interest

The Authors declare no conflict of interest.

Acknowledgements

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