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ARTICLE

Nicardipine is a putative EED inhibitor and has high selectivity and potency against chemoresistant prostate cancer in preclinical models

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BACKGROUND: It is imperative to develop novel therapeutics to overcome chemoresistance, a significant obstacle in the clinical management of prostate cancer (PCa) and other cancers.
METHODS: A phenotypic screen was performed to identify novel inhibitors of chemoresistant PCa cells. The mechanism of action of potential candidate(s) was investigated using in silico docking, and molecular and cellular assays in chemoresistant PCa cells. The in vivo efficacy was evaluated in mouse xenograft models of chemoresistant PCa.
RESULTS: Nicardipine exhibited high selectivity and potency against chemoresistant PCa cells via inducing apoptosis and cell cycle arrest. Computational, molecular, and cellular studies identified nicardipine as a putative inhibitor of embryonic ectoderm development (EED) protein, and the results are consistent with a proposed mechanism of action that nicardipine destabilised enhancer of zeste homologue 2 (EZH2) and inhibited key components of noncanonical EZH2 signalling, including transducer and activator of transcription 3, S-phase kinase-associated protein 2, ATP binding cassette B1, and survivin. As a monotherapy, nicardipine effectively inhibited the skeletal growth of chemoresistant C4-2B-TaxR tumours. As a combination regimen, nicardipine synergistically enhanced the in vivo efficacy of docetaxel against C4-2 xenografts.
CONCLUSION: Our findings provided the first preclinical evidence supporting nicardipine as a novel EED inhibitor that has the potential to be promptly tested in PCa patients to overcome chemoresistance and improve clinical outcomes.

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BACKGROUND

Prostate cancer (PCa) is the most common type of cancer and the second leading cause of cancer-related death in American men. In 2023, an estimated 288,300 new cases will be diagnosed, and 34,700 patients will die [1]. Docetaxel was initially approved in 2004 as the first-line chemotherapy for bone metastatic, castration-resistant PCa, but only had limited survival benefits of 3–4 months [2]. Several large-scale trials, including CHAARTED, GETUG-AFU, LATITUDE, and STAMPEDE, demonstrated that the combination of docetaxel with androgen deprivation therapy (ADT) significantly improved the overall survival in patients with high-volume, hormone-sensitive metastatic PCa [3, 4]. These recent studies are changing the treatment landscape of metastatic PCa and indicated that docetaxel would remain a standard of care for this lethal disease [5, 6]. While the upfront use of docetaxel increases, chemoresistance eventually occurs, posing a significant challenge to the clinical management of metastatic PCa [7]. It is imperative to uncover the underlying mechanism of chemoresistance and develop novel therapeutics to overcome it.

Drug repositioning or repurposing is an attractive strategy for discovering effective cancer treatments. It can facilitate the translation from bench research to bedside use due to its potential lower overall development costs and shorter timelines [8, 9]. However, a major technical hurdle in drug repurposing is the lack of clinically relevant screens that efficiently identify lead compounds for potential new therapeutic targets in different diseases. Therefore, it is unsurprising that recent years have seen a renewed interest in phenotypic screens in drug discovery that could better address the poorly understood complexity of human cancers and hold the promise of identifying first-in-class drugs [10].

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Moffat et al. introduced the concept of 'mechanism-informed phenotypic drug discovery (MIPDD)', which conducts phenotypic assays for specific molecular pathways/targets and determines causal relationships between target inhibition and phenotypic effects. Compared with traditional phenotypic screens, MIPDD can provide a more efficient approach to discovering new drugs with optimal mechanisms of action for treating advanced and therapeutically resistant cancers [11].

Polycomb repressive complex 2 (PRC2) is essential in transcriptional repression via mono-, di-, and tri-methylation of histone H3 at lysine 27 (H3K27). The core subunits of PRC2 include enhancer of zeste homologue 1 or 2 (EZH1 or EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 (SUZ12) [12, 13]. EZH2 is a histone methyltransferase (HMT) and acts as the catalytic 'writer' subunit of PRC2 in the transcriptional repression of genes [12]. Aberrant overexpression and activation of EZH2 have been associated with clinical progression and poor prognosis of PCa and other cancer types [14–17]. However, the role of EZH2 signalling in chemoresistance remains largely unknown.

We established a sequential phenotypic screen using two independent PCa models with distinct molecular characteristics and mechanisms of chemoresistance, i.e., the ARCaP$_E$-shEPLIN (inherent chemoresistance) and C4-2B-TaxR (acquired chemoresistance) cells [18]. We proposed that these models could closely mimic the complex biology and high heterogeneity of chemoresistant PCa. Recently, we demonstrated that the activation of noncanonical EZH2 signalling represents a novel mechanism of chemoresistance in PCa cells. Specifically, phosphorylation of EZH2 at serine 21 (p-EZH2[S21]) activates a survival signalling pathway consisting of signal transducer and activator of transcription 3 (Stat3), S-phase kinase-associated protein 2 (SKP2), ATP binding cassette B 1 (ABCB1, p-glycoprotein) and survivin, thereby conferring chemoresistance. Furthermore, we developed a small-molecule compound, namely LG1980, that effectively interrupts the physical interaction between EED and EZH2, disassembles PRC2, and promotes the degradation of its core components, thereby inhibiting p-EZH2(S21) and suppressing the expression of its downstream effectors. Significantly, LG1980 demonstrated high specificity and potent efficacy against the in vitro and in vivo growth of chemoresistant PCa cells [18]. These results indicated that the ARCaP$_E$-shEPLIN and C4-2B-TaxR cells could be exploited as a MIPDD platform for the discovery of novel inhibitors of chemoresistant PCa. Using this platform, we have identified several FDA-approved, non-oncology drugs that selectively and effectively inhibit the in vitro and in vivo growth of chemoresistant PCa cells. In this report, we have described an anti-hypertensive drug, nicardipine [19], as a specific and potent inhibitor of chemoresistant PCa in preclinical models. Intriguingly, our mechanistic studies demonstrated that nicardipine might function as an EED inhibitor that disrupts the noncanonical EZH2 signalling and confers its anticancer activities in chemoresistant PCa cells.

MATERIALS AND METHODS

Cell culture and reagents

Human PCa ARCaP$_E$ cells stably expressing human EPLIN short hairpin RNA (shRNA) (ARCaP$_E$-shEPLIN) or control shRNA (ARCaP$_E$-shCtrl) were established and cultured as we described in [18, 20]. C4-2B and its docetaxel-resistant derivative C4-2B-TaxR (provided by Dr Allen C. Gao at the University of California Davis, USA) were cultured following the procedures described in [21], with the modification that C4-2B-TaxR cells were maintained in the presence of 100 nM docetaxel (LC Laboratories, Woburn, MA). The final concentration of docetaxel in the culture medium was reduced to 5 nM before experimental assays [18]. C4-2 and C4-2-Luc cells were provided by Dr Leland WK Chung (Cedars-Sinai Medical Center, Los Angeles, CA, USA). C4-2 cells were routinely cultured in T-medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA) and penicillin-streptomycin (Corning Inc, Corning, NY, USA). C4-2-Luc cells were cultured in the same media as C4-2 with additional G418 (Thermo Fisher Scientific, Waltham, MA) at 400 μg/mL. Human PC-3 cells were routinely maintained in RPMI 1640 medium (Corning Inc) supplemented with 10% FBS and penicillin-streptomycin. Human CWR22Rv1 cells were provided by Dr Jin-Tang Dong (Emory University, Atlanta, GA, USA) and maintained in RPMI 1640 medium containing 2% L-glutamine, 10% FBS, penicillin-streptomycin, 1.5 g/L sodium bicarbonate, 10 mmol/L HEPES, 4.5 g/L glucose, and 10 mmol/L sodium pyruvate. All cell lines were regularly tested for mycoplasma contamination. Cells were counted using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) following the manufacturer's instructions. The half-minimal inhibitory concentrations (IC_{50}) of the specified agent were calculated with the SigmaPlot program (Systat Software Inc., San Jose, CA, USA). Cycloheximide (CHX), dimethyl sulfoxide (DMSO), nicardipine hydrochloride, and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Molecular docking and binding energy calculation

The three-dimensional (3D) structures of tested compounds (LG1980, nicardipine, losartan, EED226, MAK683, gallopamil, NNC 55-0396, verapamil, and metformin) were retrieved from PubChem and built using the Maestro program (Schrödinger, New York, NY, USA), as we described previously [18]. All the tested compounds were prepared using Ligprep in Maestro 12.4. The structure of EED protein (PDB ID: 5WUK) was retrieved from RCSB's Protein Data Bank [22]. Using the Protein Preparation Wizard in Maestro, the protein structure was prepared through three steps: preprocessing, optimisation, and minimisation [23]. Preprocessing includes assigning bond orders, adding hydrogens, creating disulfide, and generating het states using Epik [24, 25]. The process of optimisation optimises hydrogen bonds by using PROPKA [26]. The step of minimisation is performed by using the OPLS3e force field [27]. A receptor grid box was generated based on the five residues (Phe97, Tyr148, Trp364, Tyr365, Arg367) around the binding site. The size of the receptor grid box was set as default (20 Å). Ligand-protein docking was performed in extra-precision (XP) mode using the Ligand Docking panel. After molecular docking, the binding energies were calculated using Prime MM-GBSA (molecular mechanics generalised Born surface area) in Maestro Program.

Cellular thermal shift assay (CETSA)

CETSA was performed following a modified procedure described in [18]. C4-2B-TaxR cells were incubated for 1 h in the presence of DMSO or nicardipine (50 μM). A MyCycler™ thermal cycler system with a gradient option (Bio-Rad Laboratories, Hercules, CA, USA) was used to incubate live cells at varying temperatures.

RNA-seq analysis

RNA samples were collected from C4-2B-TaxR cells treated with DMSO or nicardipine (2.1 μM) for 24 h in triplicates. RNA-seq analyses were performed by Omega Bioservices (Norcross, GA, USA). Data were analysed by Rosalind® (Rosalind, Inc., San Diego, CA, USA), Ingenuity Pathway Analysis (IPA, Qiagen, Germantown, MD, USA), and Gene Set Enrichment Analysis (GSEA, University of California San Diego and Broad Institute, USA).

In vivo efficacy studies

All animal procedures performed in this study were approved by Augusta University Institutional Animal Care and Use Committee (IACUC) and followed the National Institutes of Health guidelines. A total of 2×10^6 C4-2B-TaxR or C4-2-Luc cells suspended in 20 μL PBS were injected into the bilateral tibia of male athymic nude mice (Hsd: athymic nude-nu; 5 weeks old; Envigo RMS, Inc, Indianapolis, IN). Tumour establishment in mouse bones was confirmed by rising serum levels of human prostate-specific antigen (PSA) using an enzyme-linked immunosorbent assay kit (United Biotech, Inc, Mountain View, CA, USA), and only mice with successful tumour inoculation were included in the studies. For the C4-2B-TaxR xenograft model, tumour-bearing mice were randomly divided into three groups and treated with vehicle control (DMSO), docetaxel, or nicardipine, respectively, at the indicated doses and schedule via intraperitoneal (i.p.) injection. For the C4-2-Luc xenograft model, an additional group of mice was treated with the combination of docetaxel and nicardipine at the indicated doses and schedules via i.p. injection. The vehicle control and docetaxel treatment groups in the C4-2B-TaxR and C4-2-Luc xenografts were the same as those described in a previous study [18]. Body weights were monitored twice a week. Intratibial growth of tumours was followed by weekly PSA measurements. Investigators were not blinded to the group allocation during the experiments and/or when assessing the outcomes.

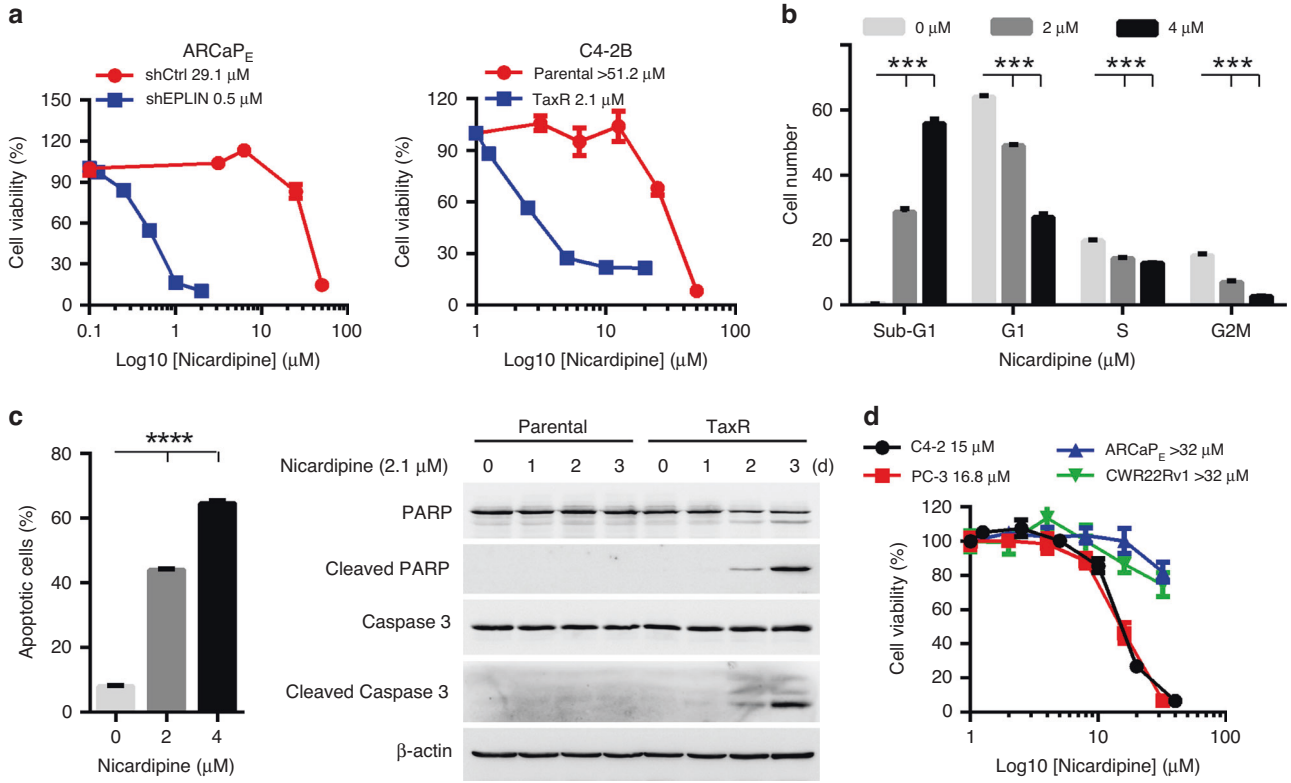

Fig. 1 Nicardipine selectively and potently inhibits chemoresistant PCa cells. a In vitro cytotoxicity of nicardipine in the ARCaP$_E$ and C4-2B models (72 h). **b** Flow cytometry results on cell cycle in C4-2B-TaxR cells treated with nicardipine at the indicated concentrations (48 h). *** $p < 0.001$ for all pairwise comparisons between the percentages of cells from the control and nicardipine treatment groups in each cell cycle. **c** Left: flow cytometry analysis on Annexin V staining in C4-2B-TaxR cells treated with nicardipine at the indicated concentrations (72 h). **** $p < 0.0001$ for all pairwise comparisons between the control and nicardipine treatment groups; right: western blot analysis on the expression of apoptotic markers in C4-2B and C4-2B-TaxR cells treated with nicardipine (2.1 μM) at the indicated time points. β-actin was used as the loading control. **d** In vitro cytotoxicity of nicardipine in C4-2, PC-3, ARCaP$_E$, and CWR22Rv1 cells (72 h).

Statistical analysis

For molecular and cellular studies, all samples were prepared in at least triplicates, and all experiments were repeated at least three times. For animal experiments, a sample size of ≥ three mice per group was used based on our previous observations, which could detect the differences in the pairwise comparison of average PSA levels among different treatments [18]. The unpaired *t*-test was performed to examine the significant difference between the means of any two groups. A two-way analysis of variance (ANOVA) was performed to measure the significant difference by comparing the means between groups affected by two independent factors. $p < 0.05$ represents statistical significance.

RESULTS

Nicardipine has high selectivity and potency against chemoresistant PCa

Recently, we established a two-tier phenotypic screening system to identify selective inhibitors of chemoresistant PCa cells [18]. In this platform, the primary screening was based on our original discovery of epithelial protein lost in neoplasm (EPLIN) as a molecular regulator of metastasis and chemoresistance [20, 28]. ARCaP$_E$-shEPLIN cells stably expressing EPLIN shRNA are highly resistant to docetaxel compared with ARCaP$_E$-shCtrl cells, thus representing the characteristics of intrinsic chemoresistance [20, 28]. Primary screening was performed to identify small-molecule compounds that selectively inhibit ARCaP$_E$-shEPLIN, but not ARCaP$_E$-shCtrl cells. Primary hits were further validated in a second (orthogonal) assay for their high potency against C4-2B-TaxR cells, a cellular model representing acquired chemoresistance [21], but not in docetaxel-sensitive parental C4-2B cells.

Using the ARCaP$_E$/C4-2B screen platform, nicardipine, an antihypertensive drug, was identified as a potential inhibitor of chemoresistant PCa cells. When the compound was tested at a single concentration of 12.3 μM, nicardipine exhibited a high selectivity index (SI) of 8.9, where the SI was defined as the ratio of the percentage of inhibition on viability in ARCaP$_E$-shEPLIN cells and that in ARCaP$_E$-shCtrl cells [18]; only 1.1% of chemoresistant ARCaP$_E$-shEPLIN cells survived following the treatment. To confirm the selectivity of nicardipine in chemoresistant PCa cells, we determined its half-minimal inhibitory concentration (IC_{50}) in the ARCaP$_E$-shEPLIN/ARCaP$_E$-shCtrl and C4-2B-TaxR/C4-2B pairs. Nicardipine had an IC_{50} of 29.1 μM in ARCaP$_E$-shCtrl cells and 0.5 μM in ARCaP$_E$-shEPLIN cells, with a fold of difference of 58.2 (Fig. 1a, left). Consistently, nicardipine demonstrated higher cytotoxicity in C4-2B-TaxR cells ($IC_{50} = 2.1$ μM) than in C4-2B cells ($IC_{50} > 51.2$ μM), with a fold of difference of > 24.4 (Fig. 1a, right). Flow cytometry analyses showed that compared with vehicle control, nicardipine treatment at 2.0 and 4.0 μM significantly induced cell cycle arrest at both G_1-S and G_2-M checkpoints with an accumulation of a sub-G1 population representing apoptotic cells (Fig. 1b). Nicardipine treatment also significantly induced apoptosis dose-dependently, as demonstrated by increased surface staining of Annexin V, a marker of apoptosis (Fig. 1c, left). Western blotting analyses confirmed that nicardipine induced the cleavage of poly (ADP-ribose) polymerase (PARP) and caspase-3 in C4-2B-TaxR cells but not in C4-2B cells (Fig. 1c, right and Supplementary Table S1).

The in vitro cytotoxicity of nicardipine was further determined in several commonly used PCa lines, i.e., ARCaP$_E$, C4-2, CW22Rv1, and PC-3. These cell lines have distinct genetic backgrounds and represent different aspects of PCa progression, but are relatively

Table 1. In vitro cytotoxicity of docetaxel and nicardipine in several established PCa cell lines (72 h).

Cell line	Molecular characteristics	IC_{50} of docetaxel (nM)	IC_{50} of nicardipine (μM)
$ARCaP_E$	Androgen-repressive	4.2	> 32.0
C4-2	AR-positive, androgen-independent	0.7 (refer to Fig. 6a)	15.0
CWR22Rv1	AR/AR-V7-positive, androgen-independent	3.9	> 32.0
PC-3	AR-negative, androgen-independent	4.2	16.8

sensitive to docetaxel treatment (Table 1). Interestingly, nicardipine had low potency in these chemosensitive PCa cells, with its IC_{50} values ranging from 15.0 to >32.0 μM (Fig. 1d). Nicardipine also exhibited very low cytotoxicity ($IC_{50} = 79.5$ μM) in BPH1 cells, a benign human prostatic epithelial cell line (Supplementary Fig. S1). These results indicated that nicardipine had high selectivity and potency against chemoresistant PCa cells.

Nicardipine is a putative EED inhibitor

EED is the 'reader' component of the PRC2 complex that binds trimethylated H3K27 (H3K27me3) and activates the HMT function of EZH2. As a classical WD40 repeat (WDR)-containing protein, EED also serves as a scaffolding protein to interact with EZH2 and SUZ12 and maintain the integrity of the PRC2 complex [29–31]. Current EED inhibitors, including EED226, A-395, and BR-001, target the histone-binding central pocket, or the 'aromatic cage', formed by the seven WDRs in EED and prevent allosteric activation of the catalytic activity of PRC2 [32]. Our previous studies have identified LG1980 as a novel EED inhibitor that effectively blocks noncanonical EZH2 survival signalling and selectively targets chemoresistant PCa cells [18]. To determine whether nicardipine exerts its anticancer effect via a similar mechanism of action, the following experiments were performed: (1) molecular docking analyses demonstrated that nicardipine bound the 'aromatic cage' of EED, interacting with Phe97 and Tyr365 through Pi-cation interactions, and with Arg414 by forming a salt bridge. In addition, a hydrogen bond was formed between nicardipine and Arg414 and Trp364, respectively (Fig. 2a). The binding energy between nicardipine and EED was calculated as −65.25 kcal/mol, suggesting that nicardipine had a higher EED affinity than known EED inhibitors such as EED226 (−49.01 kcal/mol) and MAK683 (−56.15 kcal/mol). Nicardipine also had a higher predicted EED affinity than the other examined calcium channel modulators, losartan, and metformin. As the positive control, LG1980 had the highest binding affinity to EED with the calculated energy of −73.62 kcal/mol (Table 2). (2) CETSA was performed to determine the intracellular binding of nicardipine and EED protein in live C4-2B-TaxR cells [18]. Compared with vehicle control, nicardipine treatment shifted the melting temperature (*Tm*) of EED protein from 49.8 °C to 50.4 °C, indicating that nicardipine could specifically bind and stabilise EED protein in live cancer cells (Fig. 2b). Taken together, these computational and experimental studies indicated that nicardipine could be a novel EED inhibitor.

Nicardipine inhibits noncanonical EZH2-Stat3-SKP2-ABCB1/survivin signalling in chemoresistant PCa cells

The integrity and function of the PRC2 complex rely on the presence of EED and SUZ12 [12, 13]. Consistent with our published results [18], EED and p-EZH2(S21) were significantly upregulated in C4-2B-TaxR cells compared with parental C4-2B cells. In contrast, the basal levels of EZH2 and SUZ12 were similar between the two cell lines. Treatment with nicardipine at 2.1 μM effectively downregulated EZH2, p-EZH2(S21), EED and SUZ12 in a time-dependent manner in C4-2B-TaxR cells but not in parental C4-2B cells (Fig. 3a and Supplementary Table S1). We further determined whether nicardipine could affect EZH2 protein stability in chemoresistant PCa cells, thereby reducing the expression of EZH2 and p-EZH2(S21). A CHX chase experiment showed that in the presence of nicardipine, the half-life ($T_{½}$) of EZH2 protein was significantly shortened from ≥ 48 h to 14.8 h (Fig. 3b). This result indicated that nicardipine might facilitate EZH2 degradation via a proteasome-mediated mechanism.

Table 2. Predicted binding energies (kcal/mol) between the tested compounds/drugs and human EED protein (PDB ID: 5WUK).

Compound	Binding energy (kcal/mol)
LG1980	−73.62
Nicardipine	−65.25
EED226	−49.01
MAK683	−56.15
Losartan	−47.31
Metformin	−17.87

We evaluated whether nicardipine affects the canonical function of EZH2 on histone methylation. Consistent with our previous findings [18], there was no significant difference in the basal expression of H3K27me3 between C4-2B and C4-2B-TaxR cells, indicating that canonical EZH2 signalling may not play a dominant role in PCa chemoresistance. Treatment with nicardipine at 2.1 μM did not affect the tri-methylation of H3K27 in either C4-2B or C4-2B-TaxR cells during a 72 h period. In comparison, nicardipine significantly inhibited the mono-methylation of H3K27 in a time-dependent manner, starting at 24 h, and reduced H3K27 di-methylation after 48 h in C4-2B-TaxR cells but not in C4-2B cells (Fig. 3c and Supplementary Table S1). These results indicated that the anticancer activity of nicardipine in chemoresistant PCa cells might be independent of the canonical HMT function of EZH2.

We further determined the effect of nicardipine on the expression of core components of a novel noncanonical EZH2 signalling pathway in chemoresistant PCa cells [18]. Supporting our published results, p-EZH2(S21), p-Stat3(S727), SKP2, ABCB1 and survivin were upregulated in C4-2B-TaxR cells compared with parental C4-2B cells. Nicardipine selectively and effectively suppressed the expression of p-EZH2(S21), p-Stat3(S727), SKP2, ABCB1, and survivin in chemoresistant C4-2B-TaxR cells but not in C4-2B cells (Fig. 3d and Supplementary Table S1). These results suggested that nicardipine may effectively target noncanonical EZH2-Stat3-SKP2-ABCB1/survivin signalling and inhibit the proliferation and viability of chemoresistant PCa cells.

Nicardipine facilitates cellular uptake of chemotherapeutics in chemoresistant PCa cells

Overexpression of ABCB1 (p-glycoprotein) has been recognised as a central molecular mechanism in multidrug resistance [33]. Consistently, our previous studies have shown that ABCB1 depletion effectively increased the intracellular presence of chemotherapeutics in chemoresistant PCa cells [18]. Since nicardipine significantly reduced ABCB1 protein levels in chemoresistant C4-2B-TaxR cells, we examined whether nicardipine could facilitate the uptake of Oregon Green 488-conjugated paclitaxel. As shown in Fig. 3e, nicardipine pre-treatment resulted in a rapid (within 15 min) accumulation of fluorescent paclitaxel in C4-2B-TaxR cells. In comparison, there was no paclitaxel uptake until 30 min in control cells (Fig. 3e). These results indicated that

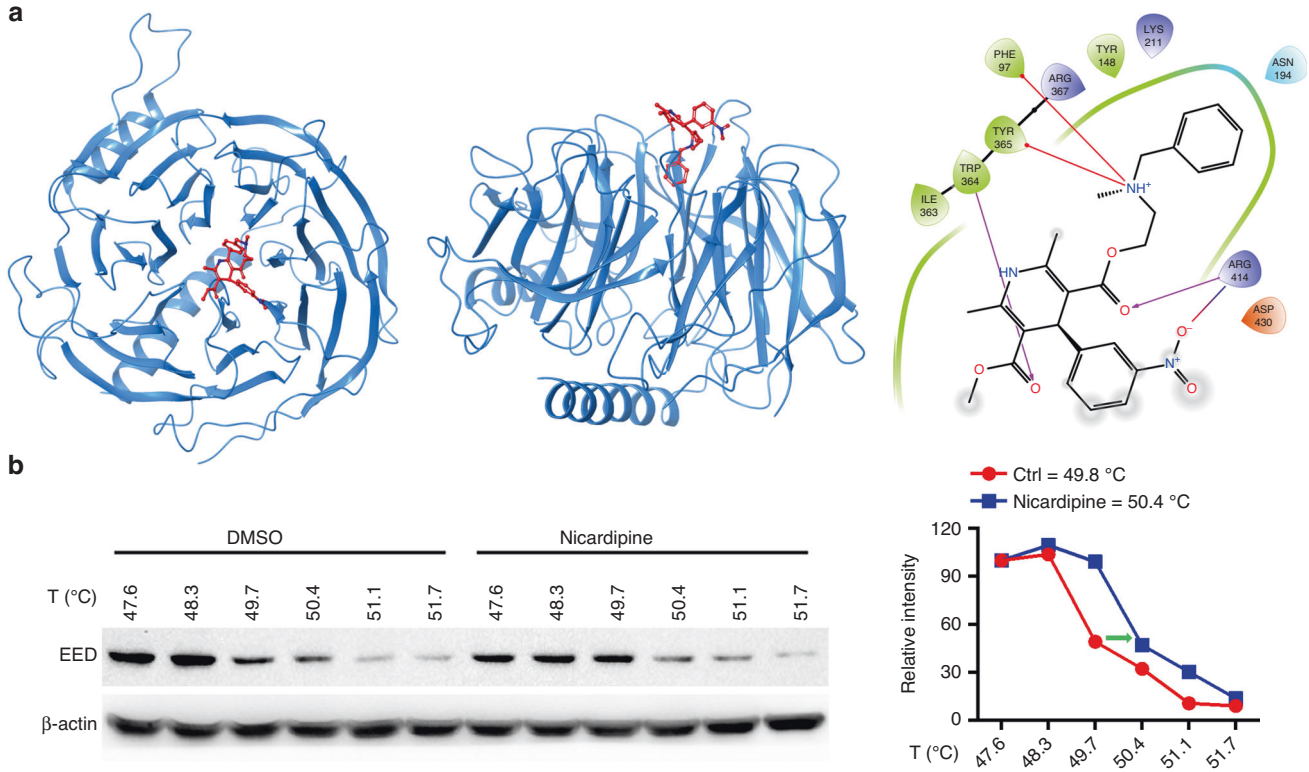

Fig. 2 Nicardipine is a putative EED inhibitor. a Left and middle: docking poses of nicardipine and EED; right: two-dimensional ligand-protein interaction diagram of nicardipine in the binding site of human EED protein (PDB ID: 5WUK). The pink arrow indicates the hydrogen bond; the blue-red line indicates the salt bridge; the red line represents pi-cation. **b** Left: CETSA analysis of EED expression in C4-2B-TaxR cells treated with DMSO or nicardipine (50 μM, 1 h). β-actin was used as the loading control; right: melting temperature curves of EED protein in C4-2B-TaxR cells treated with DMSO or nicardipine.

nicardipine-mediated ABCB1 downregulation could contribute to the increased uptake and retention of chemotherapeutics in chemoresistant PCa cells.

Taken together, these molecular and cellular results are consistent with a working model that nicardipine may bind EED and induce protein degradation of EZH2, thereby reducing p-EZH2 and suppressing Stat3/SKP2/ABCB1/survivin survival signals in chemoresistant PCa cells (Fig. 3f).

Nicardipine affects multiple genes implicated in the control of the cell cycle in chemoresistant PCa cells

To obtain an unbiased view of the mechanism of action of nicardipine in chemoresistant PCa cells, we performed RNA-seq analyses and compared gene expression in C4-2B-TaxR cells treated with vehicle control or nicardipine (2.1 μM, 24 h). When an adjusted p value (p-adj) < 0.05 was used, 336 unique genes were upregulated, and 259 unique genes were downregulated significantly following nicardipine treatment (Fig. 4a). IPA profiling found that the top canonical pathways affected by nicardipine included cell cycle control and DNA damage responses, which were in line with the known functions of PRC2 in cancer cells [34] (Table 3). Among the significant signalling nodes affected by nicardipine treatment, CDKN1A (p21)-, TP53- and RB1-related genes were activated, whereas E2F-regulated genes were suppressed (Fig. 4b). GSEA studies confirmed the inhibitory effect of nicardipine on EED- and cell cycle-related genes ($p = 0.026$ and 0.015, respectively). Nicardipine appeared to activate EZH2-repressed genes, although with low statistical significance ($p = 0.05$) (Fig. 4c). These results supported a mechanism of action that nicardipine induces cell cycle arrest and apoptosis in chemoresistant PCa cells via PRC2-mediated signalling.

Nicardipine inhibits the skeletal growth of chemoresistant C4-2B-TaxR tumours

A notable feature of nicardipine was that as a single agent, it demonstrated high selectivity and potency in chemoresistant PCa cells, with an IC_{50} of 0.5 μM in $ARCaP_E$-shEPLIN cells and $IC_{50} = 2.1$ μM in C4-2B-TaxR cells, respectively (Fig. 1a). We investigated the in vivo efficacy of nicardipine against the skeletal growth of C4-2B-TaxR cells, a model closely mimicking the clinicopathology of AR-positive, chemoresistant and bone metastatic PCa [21]. Serum levels of human PSA were measured as the primary indicator of xenograft growth in mouse bones (Fig. 5a). At the endpoint, the average PSA level of each group was determined as 43.47 ± 15.62 ng/mL (control), 36.63 ± 19.75 ng/mL (docetaxel, 5 mg/kg, once per week) and 28.78 ± 11.60 ng/mL (nicardipine, 5 mg/kg, three times per week). Compared with vehicle control ($p = 0.020$) or docetaxel ($p = 0.039$), i.p. injection of nicardipine significantly inhibited the growth of C4-2B-TaxR tumours in mouse tibias. On the other hand, docetaxel treatment did not significantly affect the in vivo growth of PCa cells ($p = 0.741$). Nicardipine treatment was not associated with obvious in vivo toxicity or reduced body weights of mice (Fig. 5b). These results indicated that as a monotherapy, nicardipine could effectively suppress the in vivo growth of chemoresistant PCa xenografts in mouse bones with a good safety profile.

Nicardipine enhances the in vivo efficacy of docetaxel and inhibits the skeletal growth of C4-2 xenografts

Compared with an IC_{50} at the low-micromolar range (2.1 μM) in C4-2B-TaxR cells, nicardipine had relatively weak cytotoxicity in C4-2 cells ($IC_{50} = 15.0$ μM) that exhibited typical phenotypes of docetaxel-responsive PCa ($IC_{50} = 0.72$ μM; Fig. 6a). Interestingly, when C4-2 cells were treated with a combination of nicardipine and docetaxel, the two drugs demonstrated a synergistic inhibitory

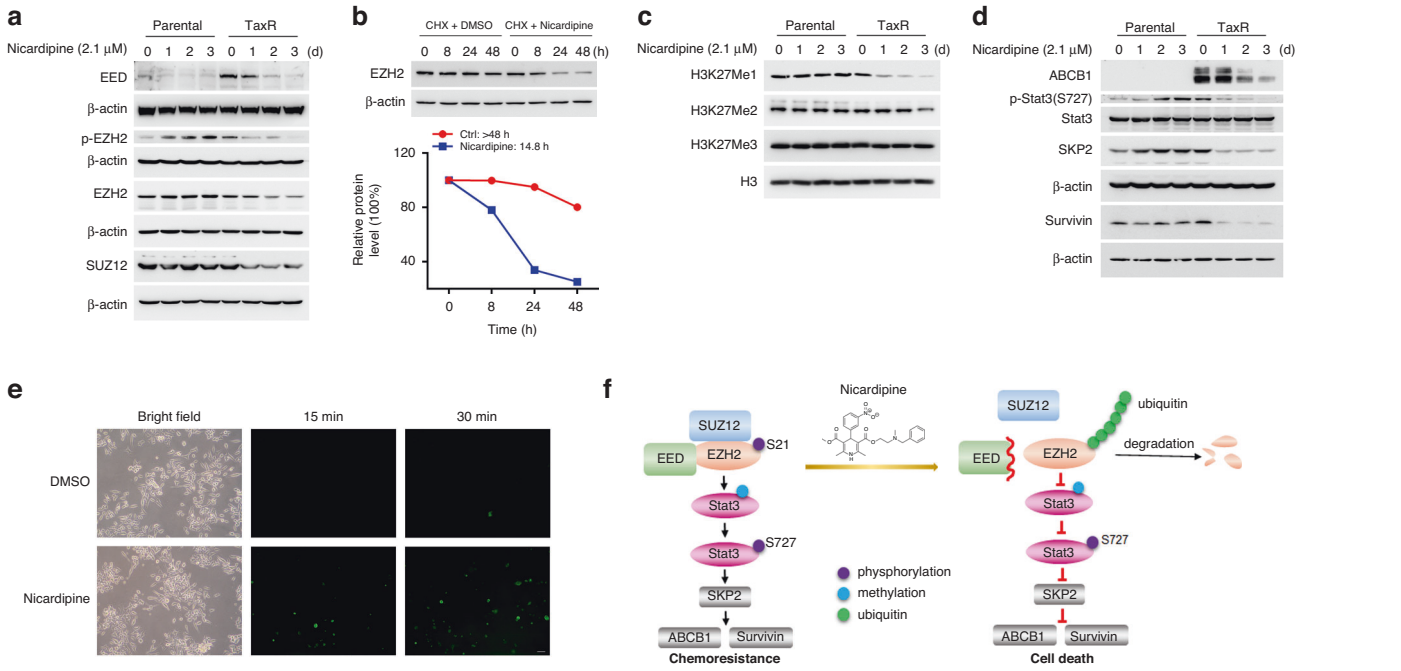

Fig. 3 Nicardipine targets noncanonical EZH2 survival signalling in chemoresistant PCa cells. a Western blot analysis on the expression of p-EZH2, EZH2, EED and SUZ12 in C4-2B and C4-2B-TaxR cells treated with nicardipine (2.1 μM) at the indicated time points. **b** Upper: EZH2 expression in C4-2B-TaxR cells treated with DMSO or nicardipine (2.1 μM) in the presence of CHX (50 μg/mL); bottom: the calculated half-life of EZH2 protein in C4-2B-TaxR cells treated with DMSO or nicardipine (2.1 μM). **c** Western blot analysis on the expression of H3K27 methylation in C4-2B and C4-2B-TaxR cells treated with nicardipine (2.1 μM) at the indicated time points. **d** Western blot analysis on the expression of p-Stat3, Stat3, SKP2, ABCB1, and survivin in C4-2B and C4-2B-TaxR cells treated with nicardipine (2.1 μM) at the indicated time points. **e** Fluorescence microscopy images of cellular uptake of Oregon Green 488-paclitaxel at the indicated time points in C4-2B-TaxR cells treated with DMSO or nicardipine (2.1 μM) for 72 h before paclitaxel incubation. Scale bar: 50 μm. **f** Schematic depiction of the proposed mechanism of action of nicardipine in chemoresistant PCa cells.

effect on the in vitro proliferation of C4-2 cells, which was reflected by the combination indexes lower than 1.0 in isobologram analyses using the CompuSyn program (Fig. 6a, right and Supplementary Table S3). These in vitro results suggested that nicardipine may be effective in enhancing the anticancer effect of docetaxel chemotherapy. To test this hypothesis, C4-2 tumours were inoculated into the tibiae of male athymic nude mice. Tumour-bearing mice were treated with vehicle control, docetaxel, nicardipine, and the combination of docetaxel and nicardipine, respectively. At the endpoint, the average PSA level of each group was determined as 79.17 ± 17.92 ng/mL (control), 49.04 ± 14.92 ng/mL (docetaxel, 5 mg/kg, once per week), 42.80 ± 9.29 ng/mL (nicardipine, 10 mg/kg, three times per week), and 23.23 ± 6.27 ng/mL (docetaxel and nicardipine). Compared with the vehicle control, docetaxel could moderately retard the in vivo growth of C4-2 tumours ($p = 0.048$), and nicardipine monotherapy was ineffective in suppressing tumour growth compared with the vehicle control ($p = 0.090$) or docetaxel ($p = 0.706$). However, the combination of nicardipine and docetaxel significantly decreased serum PSA levels compared to the treatment with vehicle control ($p < 0.0001$), docetaxel ($p = 0.0025$), or nicardipine ($p = 0.0020$) (Fig. 6b). Compared with vehicle control, docetaxel treatment significantly decreased the body weight, whereas the combination of nicardipine and docetaxel increased the body weight compared to the docetaxel group (Fig. 6c). These results indicated that as an adjunct agent, nicardipine could be effective in enhancing the in vivo efficacy of docetaxel against the skeletal growth of C4-2 tumours.

DISCUSSION

EZH2 overexpression and mutation, as well as aberrant EZH2 signalling, have been associated with advanced stages and poor clinical outcomes in various types of cancer [15–17]. Numerous EZH2 inhibitors have been developed, most of which target the catalytic SET domain via competition with methyl-donating S-adenosylmethionine (SAM). One of these inhibitors, tazemetostat (EPZ-6438), was approved in 2020 for locally advanced or metastatic epithelioid sarcoma [35, 36]. Unfortunately, EZH2 inhibitors have not demonstrated satisfactory clinical outcomes in other solid tumours [37–39]. The limited success of current EZH2 inhibitors in clinical settings indicated that blocking the catalytic activity of EZH2 alone is insufficient and highlighted a need for novel PRC2-targeting strategies. An alternative approach is to develop small-molecule compounds that specifically bind the H3K27me3-interacting 'aromatic cage' in EED and allosterically affect EZH2 enzymatic activity, thereby leading to the loss of PRC2 functions [32]. Interestingly, several allosteric EED inhibitors could also alter physical interactions between EED and other core components of PRCs (mainly EZH2 and SUZ12) and destabilise these proteins [40–44]. Compared with SAM-competitive EZH2 inhibitors, EED inhibitors could achieve a general and more efficient blockade of the PRC2 oncogenic signalling in highly heterogeneous cancer cells. An EED inhibitor (MAK683) developed by Novartis has entered Phase I/II trials in patients with advanced malignancies (NCT02900651) [45].

Our recent studies revealed an essential role of noncanonical EED-EZH2 signalling in chemoresistant PCa cells. We have developed a small molecule, LG1980, as an EED inhibitor with promising anticancer activity in chemoresistant PCa cells and xenograft models [18]. These results indicated that pharmacological targeting of EED could be a promising strategy to overcome chemoresistance and validated our phenotypic screen as a novel MIPDD platform for discovering effective EED modulators. In a recent screening using the ARCaP$_E$/C4-2B-based platform, we identified several potential inhibitors of chemoresistant PCa, including nicardipine. Here, we presented experimental evidence that nicardipine is highly specific

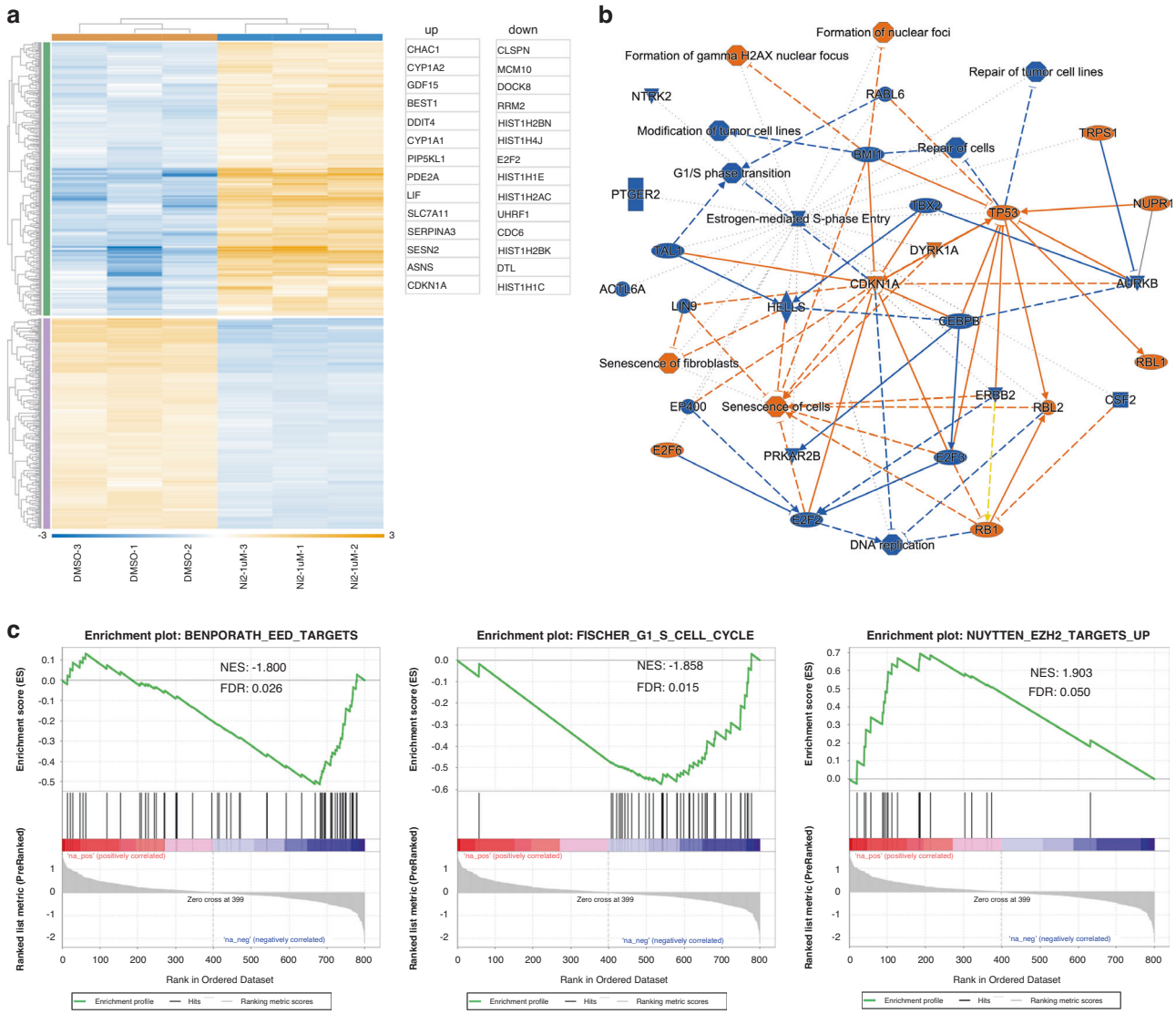

Fig. 4 RNA-seq analysis of potential target genes of nicardipine in C4-2B-TaxR cells. a Left: heatmap of genes in C4-2B-TaxR cells treated with vehicle control (DMSO) or nicardipine (2.1 μM; 24 h); right: the top genes affected by nicardipine treatment in C4-2B-TaxR cells. **b** IPA analyses of major gene clusters affected by nicardipine treatment in C4-2B-TaxR cells. **c** GESA of EED-, G1/S checkpoint-, and EZH2-associated gene signatures in C4-2B-TaxR cells treated with nicardipine. Positive (red) and negative (blue) enrichment scores indicate enrichment in vehicle-and nicardipine-treated cells, respectively. Normalised enrichment score (NES) and false discovery rate (FDR) are indicated for each gene set. *Y*-axes indicate enrichment scores (top) and ranked list metric (bottom). *X*-axis bars represent individual genes of the indicated gene sets.

and effective against chemoresistant PCa cells in both cellular and animal models. We also provided molecular and cellular evidence supporting a proposed mechanism of action that nicardipine acts as a putative EED inhibitor and inhibits noncanonical EED-EZH2 signalling in chemoresistant PCa cells (Fig. 3f). These preclinical studies revealed an unexpected function and mechanism of action of nicardipine in chemoresistant cancer cells and could have a significant translational implication.

Nicardipine is an approved drug for treating hypertension, angina, and related cerebrovascular diseases [19, 46]. As a second-generation dihydropyridine class of calcium channel blockers (CCBs), nicardipine inhibits the transmembrane influx of calcium into cardiac and smooth muscle without changing serum calcium levels. Given the wide use of CCBs in the management of cardiovascular diseases as well as the well-recognised role of calcium signalling in cancer progression [47, 48], there has been a longtime interest in the possible effects of CCBs on the clinical outcomes in cancer patients. Since the 1980s, several groups have investigated the potential anticancer activities of nicardipine and other CCBs in preclinical and clinical settings. It appeared that nicardipine could enhance the in vitro and in vivo effects of certain chemotherapeutics, such as vincristine, carmofur, and nimustine, in experimental models of PCa, oesophageal cancer, gastric cancer, glioma, and leukaemia; however, it was not clear whether the observed effects of nicardipine in human cancer cells were associated with its function as a CCB [49–54]. In a recent study, Shi et al. found that nicardipine could enhance the toxic effect of temozolomide and promote apoptosis in glioma stem cells, probably through the upregulation of mTOR and inhibition of autophagy, which is a protective response in glioma cells during chemotherapy [55].

Only a few studies have been published regarding the clinical benefits of nicardipine in cancer patients, and the results are inconclusive and sometimes conflicting. For example, in three patients with relapsed and chemoresistant non-Hodgkin's lymphoma, nicardipine was found to be capable of increasing the

efficacy of vinca alkaloids [56]; in contrast, nicardipine failed to improve adriamycin and vinca alkaloid in seventeen patients with solid tumours or haematologic malignancy [57]. Although these studies were largely observational in very small patient cohorts, they suggested that there is no straightforward strategy for using nicardipine or other CCBs for cancer treatment in general patient populations. Supporting this notion, network meta-analyses and trial sequential analyses of 324,168 participants from randomised trials found no significant differences in the risk of cancer or cancer-related death with CCBs or other individual classes of anti-hypertensive drugs [58].

Among nineteen calcium channel modulators included in a primary screen on the $ARCaP_E$-shCtrl/$ARCaP_E$-shEPLIN platform, only four (gallopamil, nicardipine, NNC 55-0396, and verapamil) exhibited an SI ≥ 6.0 when all compounds were used at the final concentration of 12.3 μM) (Supplementary Table S4). Molecular docking studies found that verapamil and NNC 55-0396 had similar EED-binding energies to nicardipine, while gallopamil exhibited a higher affinity at the 'aromatic cage' than nicardipine (Supplementary Table S5). Although these results were derived from a limited set of experiments, and target validation using molecular and cellular approaches is still needed, it appeared that the EED docking scores of these compounds might be associated with their selectivity and potency in chemoresistant PCa cells. The in silico models used in our study could be integrated into the current EED-focused MIPDD and provide a more efficient screen for identifying potential EED inhibitors.

The translational potential of these putative EED inhibitors was our primary criterion for selecting nicardipine and evaluating its anticancer activities in preclinical models of chemoresistant PCa. As an approved drug for treating chronic cardiovascular diseases, nicardipine exhibits excellent long-term safety profiles in humans [19]. This drug also has favourable pharmacokinetics and metabolism in terms of its complete absorption and nonlinear accumulation in the circulation following oral administration. The approved schedule of oral administration (i.e., every 8 h) and a readily measured effect (e.g., change in blood pressure) from nicardipine treatment could allow rapid and convenient adjustments in human trials if any severe adverse effects appear. These pharmacological and physiological features of nicardipine suggested that this drug might have a high potential for further clinical development. In comparison, other approved drugs with high EED-binding affinities (i.e., gallopamil, verapamil) have limited clinical potential, mainly due to their relatively higher toxicities in human subjects. For example, the antiarrhythmic drug gallopamil was withdrawn in 2001 for causing excessive hypotension, bradycardia, or impaired

Table 3. Top canonical pathways affected by nicardipine in C4-2B-TaxR cells.

Name	*p* value	Overlap
Cell cycle control of chromosomal replication	2.76E–19	39.9% (22/56)
Role of BRCA1 in DNA damage response	9.75E–18	30.0% (24/80)
NER (nucleotide excision repair, enhanced pathway)	5.31E–16	24.3% (25/103)
Hereditary breast cancer signalling	1.83E–13	18.3% (26/142)
Role of CHK proteins in cell cycle checkpoint control	8.83E–12	28.1% (16/57)

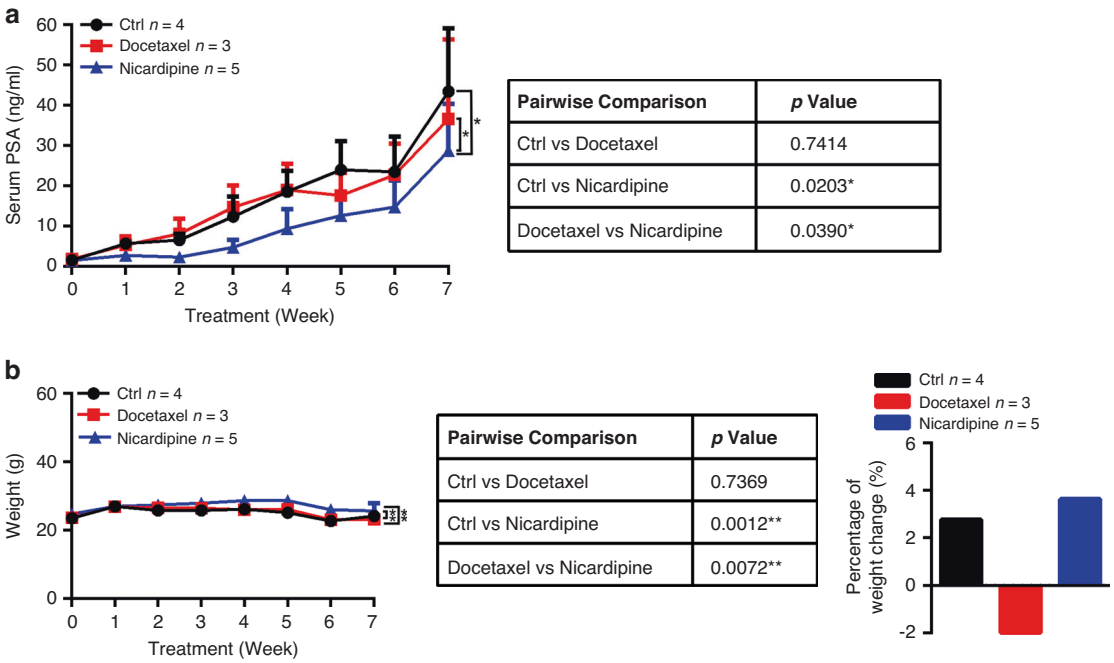

Fig. 5 Nicardipine monotherapy inhibits the skeletal growth of chemoresistant PCa in male athymic nude mice. a Left: serum PSA values of C4-2B-TaxR tumour-bearing mice treated with vehicle control ($n = 4$), docetaxel (5 mg/kg, i.p., once per week; $n = 3$), or nicardipine (5 mg/kg, i.p., three times per week; $n = 5$); right: two-way ANOVA analysis of the PSA values between different treatment groups. * $p < 0.05$. **b** Left: average body weights of C4-2B-TaxR tumour-bearing mice in different treatment groups; middle: pairwise comparison of the body weights between different treatment groups; right: percentage of body weight change of C4-2B-TaxR tumour-bearing mice in different treatment groups. ** $p < 0.01$.

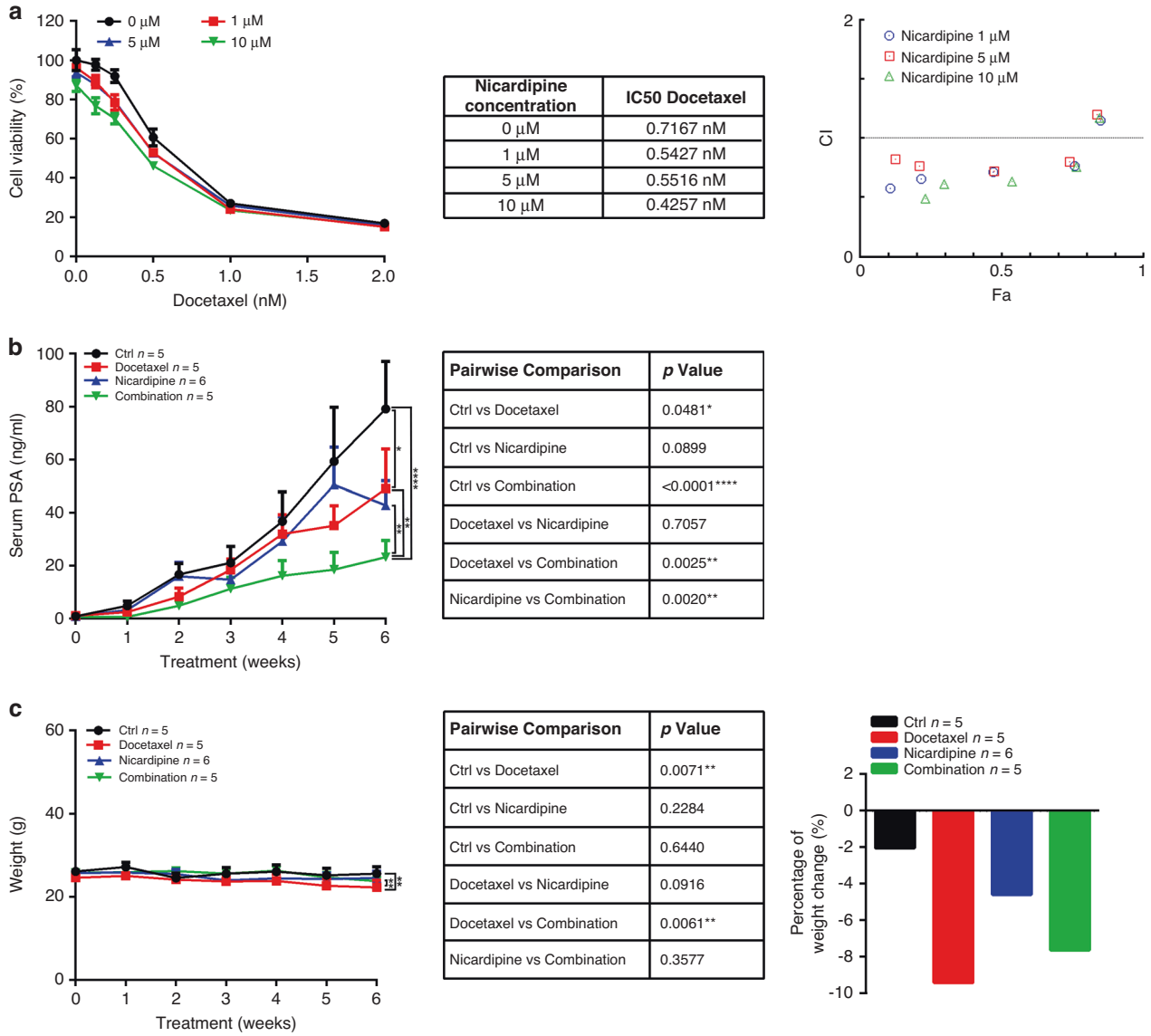

Fig. 6 Nicardipine synergistically enhances the in vivo efficacy of docetaxel against the skeletal growth of C4-2 tumours in male athymic nude mice. a Left: in vitro cytotoxicity of docetaxel in C4-2 cells in varying concentrations of nicardipine (72 h); right: CompuSyn analyses of the synergistic effect between docetaxel and nicardipine in C4-2 cells. Fa fraction affected, CI combination index. **b** Left: serum PSA values of C4-2-Luc tumour-bearing mice treated with vehicle control ($n = 5$), docetaxel (5 mg/kg, i.p., once per week; $n = 5$), nicardipine (10 mg/kg, i.p., three times per week; $n = 6$), or the combination of docetaxel and nicardipine ($n = 5$); right: two-way ANOVA analysis of the PSA values between different treatment groups. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. **c** Left: average body weights of C4-2-Luc tumour-bearing mice in different treatment groups; middle: pairwise comparison of the body weights between different treatment groups; right: percentage of body weight change of C4-2-Luc tumour-bearing mice in different treatment groups. ** $p < 0.01$.

cardiac performance when combined with β-adrenoceptor blockers [59]. Verapamil, another hypertension drug, was thought to be a functional inhibitor of ABCB1 (p-glycoprotein) that could re-sensitise cancer cells to chemotherapeutics. However, verapamil failed to demonstrate clinical benefits in lung cancer patients, mainly due to its poor pharmacokinetics, high dose-limiting toxicity, and low therapeutic window [60].

Our studies provided the first preclinical evidence supporting the promise of nicardipine as a targeted agent for cancer treatment. The translational potential of our work could be two-fold. First, the discovery of nicardipine as a putative EED inhibitor and a potent compound against chemoresistant PCa provided a solid rationale for designing biomarker-based, subtype-specific trials to test the clinical efficacy of nicardipine in PCa patients. For example, the expression profile of major components of the noncanonical EED-EZH2 signalling axis, including EED, p-EZH2(S21), SKP2, ABCB1 and survivin, can be evaluated in localised tumours from patients with high-volume, high-risk PCa. Nicardipine can be offered to patients with active noncanonical EED-EZH2 signalling as an adjunct therapy in combination with docetaxel and/or ADT, with the expectation of eliminating chemoresistant PCa cells and enhancing the efficacy of standard treatments. Given its excellent pharmacological properties and safety profiles as a common anti-hypertensive, nicardipine could be promptly tested in human trials and integrated with the standard of care for chemoresistant PCa. Second, nicardipine could be modified and optimised to develop more specific EED inhibitors. Nicardipine-derived analogues could represent novel first-in-class EED inhibitors with distinct chemical structures from current ones investigated in academic and pharmaceutical laboratories.

DATA AVAILABILITY

All raw data supporting the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualisation: YD, HF, OK, DW. Methodology and investigation: XL, YC, YY, LB, RZ, YW, Z-RX, JMW, NJB, AD, NC, DL, MQ, YD. Data curation: XL, Z-RX, NJB, YD, DW. Formal analysis: XL, Z-RX, YD, DW. Funding acquisition: DW. Manuscript preparation: XL, YW, Z-RX, NJB, YD, HF, AOO, OK, DW.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Institutional Animal Care and Use Committee (IACUC) at Augusta University approved all animal protocols used in this study. All animal procedures were subjected to National Institutes of Health guidelines.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41416-023-02359-y.

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