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Etoposide and topoisomerase II inhibition for aggressive prostate cancer: data from a translational study.

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Research

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Abstract

Background. Etoposide phosphate (VP-16) is a topoisomerase 2 (TOP2) inhibitor that demonstrated limited activity in patients with metastatic castration-resistant prostate cancer (mCRPC). In our study, we investigated the sensitivity of prostate cancer (PCa) cells (LNCaP, 22Rv1, PC3, DU145, PDB and MDB) to VP-16 and the possible relationship between VP-16 activity and TOP2 expression. We also compared the activity of VP-16 with that of docetaxel, enzalutamide and olaparib. In the datasets analysis, we explored the prevalence and clinical significance of TOP2 genetic and transcriptomic alterations in mCRPC.

Methods. Cell cultures and crystal violet cell proliferation assays were performed. Specific antibodies were used in western blots analyses of cell protein extracts. Large scale datasets were analyzed in cBioportal and STRING was used for the functional enrichment analysis.

Results. VP-16 was active in all PCa cell lines analyzed and this drug demonstrated increased activity in the aggressive PC3 and DU145 cells. VP-16 was more cytotoxic compared to the other treatments, except for LNCaP and 22Rv1, which were more sensitive to docetaxel. Of note, the maintainment of antiandrogen treatment in bicalutamide-resistant MDB and PDB increased sensitivity to VP-16, docetaxel and enzalutamide. Compared to LNCaP, TOP2A was found to be overexpressed in 22Rv1, DU145 and PC3, whereas TOP2B was overexpressed in 22Rv1 and PDB. In the mCRPC datasets analysis, TOP2A mRNA overexpression was associated with worse patients' prognosis, with the molecular features of neuroendocrine prostate cancer (NEPC) and with lower androgen receptor (AR) score. Patients overexpressing TOP2A mRNA were more likely to harbor RB1 loss.

Conclusions. Specific subpopulations of patients with aggressive variant prostate cancer (AVPC) could benefit from VP-16 treatment. TOP2A overexpression, rather than TOP2B, might be a good biomarker to predict response to VP-16. Further investigations are warranted to determine the role of TOP2A in mCRPC and to assess the efficacy of VP-16, alone or in combination with other agents, for the treatment of patients with mCRPC.

Key points:

· Etoposide is a chemotherapeutic agent that shows significant activity in preclinical models of prostate cancer and increased activity is observed in the most aggressive cells.

· Overexpression of topoisomerase II alfa and decreased functionality of the androgen receptor signaling seem to be associated with response to etoposide.

· Specific patients with castration-resistant prostate cancer could benefit from etoposide treatment, alone or in combination with other agents.

1. Introduction

Topoisomerase II (TOP2) inhibitors have a long history in the treatment of prostate cancer (PCa). Mitoxatrone was one of the first chemotherapeutic agents to be approved for the treatment of metastatic castration-resistant prostate cancer (mCRPC) in 1996. Tannock and colleagues published a phase 3 study that demonstrated its benefit on pain palliation[1]. However, this drug has never shown a survival advantage in patients with PCa and its use is currently reserved to selected patients without any further treatment option[2]. Same fate has occurred to etoposide phosphate, commonly known as VP-16. This TOP2 inhibitor in combination with estramustine phosphate was included in the National Comprehensive Cancer Network (NCCN) guidelines for the treatment of mCRPC since 1997[3]. However, given the inconvenient risk-benefit ratio, this combination has been progressively abandoned by the majority of the centers. The use of etoposide in PCa is currently limited to combination with platinum agents to treat the aggressive forms of neuroendocrine PCa (NEPC)[4].

Both isoforms of TOP2, TOP2A and TOP2B, are the targets of mitoxantrone and etoposide. TOP2A is tightly related to cell proliferation, whereas TOP2B is also expressed in non-dividing cells[5]. TOP2A is repressed by RB1 and TP53/DREAM pathway and it is upregulated in presence of RB1 or TP53 inactivation[6, 7]. This isoenzyme enhances androgen-receptor (AR) signalling and it has been associated with PCa aggressiveness, metastatic progression and reduced survival[8-12]. Haffner and colleagues have also revealed the strict relationship between AR and TOP2B, and this isoenzyme has been involved in the production of TMPRSS2-ERG fusions, which are frequently observed in patients with PCa[13].

Etoposide acts as a TOP2 poison through the trapping and stabilization of TOP2-DNA complexes, generating DNA breaks[5]. Mitoxantrone, similarly to doxorubicin, stabilizes these TOP2-DNA complexes less efficiently than etoposide and acts as a TOP2 catalytic inhibitor at high concentrations, rather than a TOP2 poison[14]. Given the dose-dependent stabilization of TOP2-DNA complexes induced by etoposide, this drug appears to be the best candidate to exert cytotoxic activity in PCa that overexpress TOP2. In addition, etoposide has been shown to potently inhibit AR ligand binding, further supporting its use in the context of PCa[15].

In the present study, we analyzed the effect of etoposide in different in vitro models of PCa compared to other standard treatments and the possible relationship between the expression of TOP2 isoforms and etoposide activity.

We also explored the association between TOP2 expression and clinical data from patients with mCRPC, in order to provide useful information for the selection of patients potentially candidates to etoposide treatment.

2. Materials And Methods

2.1. Cell cultures and chemicals

In vitro experiments were conducted using cell models that harbor distinct molecular alterations and mimic different phases of PCa progression (**Table S1**): LNCaP (hormone-sensitive), 22Rv1 (AR-V7

variant), PC3 and DU145 (mCRPC). PDB and MDB cells were obtained from LNCaP following the selective pressure of the antiandrogen bicalutamide, as previously described[16]. The human prostate cancer cell lines LNCaP, 22Rv1, DU145 and PC3 were purchased from American Type Culture Collection (CRL1740, CRL2505, HTB81, CRL1435) and cultured in RPMI 1640 medium added with 10% FBS, 2mM glutamine and 1% penicillin/streptomicin solution (all from Celbio, Milano, Italy). The medium for LNCaP and 22Rv1 was also supplemented with 0.25% glucose, 10 mM sodium pyruvate and 10 mM HEPES. Bicalutamide-resistant cell lines PDB and MDB were cultured in phenol red-free RPMI-1640 containing 10% charcoal stripped fetal bovine serum, 1% penicillin, 1% streptomycin, 1% glutamine, 10 μM of bicalutamide (Sigma), with or without 0.1 nM 5-α-dihydrotestosterone (DHT, Sigma) for PDB and MDB respectively. The mycoplasma testing in all cell lines was checked routinely. The STR analysis for authenticating cell lines was carried out in the Biological Bank of our institute.

Etoposide (341205) was from Merck-Millipore while enzalutamide (MDV3100), docetaxel (S1148) and olaparib (AZD2281) were from Sellechem. For each chemical, concentrate solution in DMSO was prepared, maintained at -20 °C and freshly diluted in appropriate medium in each experiment.

2.2. Cell proliferation assays

The crystal violet assay was performed to evaluate the treatment effects of chemotherapeutic agents on cell proliferation. Cells were seeded at different concentrations depending on their doubling time (**Table S1)** in 96-well plates and treated for 120h with various concentrations of etoposide (0.1-10 μM), docetaxel (0.1-10 nM), enzalutamide (0.1-20 μM) and olaparib (1-20 μM). In the middle of treatment fresh medium with drug was added. Data of cell proliferation were calculated as percentages of the absorbance value read for untreated cell (0.1% DMSO). At least three independent experiments, each performed 10 times, were done. Using ED50plus v1.0 software, we evaluate the drug concentration required for 50% growth inhibition of the cells (IC50). To compare etoposide treatment responses among PCa in vitro models, we evaluated for each cell line the drug concentration required for 50% viability inhibition (IC50) and its ratio to IC50 of LNCaP used as reference (Rln= IC50 cell line evaluated / IC50 LNCaP). If Rln<1, etoposide treatment was more effective than in LNCaP cell line, whereas if Rln>1 etoposide was less effective than in LNCaP. To test the effectiveness of etoposide treatment in comparison with other antineoplastic agents, we evaluate the ratio of cell growth inhibition of each drug to etoposide (Ret= IC50 drug evaluated/ IC50 etoposide). If Ret>1, the treatment was less effective than with etoposide, whereas if Ret<1 the drug was more cytotoxic than etoposide.

2.3. Protein extraction and Western blot analysis

Total protein extract from LNCaP, 22Rv1, DU145, PC3, PDB and MDB cell lines was obtained using RIPA buffer as previously reported[17]. All extraction procedures were performed at 4 °C in presence of protease inhibitors. Protein concentration was determined by Bio-Rad protein microassay (DC Protein Assay kit). Equal protein amount (8 μg) was resolved by SDS-PAGE gel electrophoresis, transferred to a Hybond-P membrane (GE Healthcare Life Sciences) and probed overnight at 4°C with rabbit anti-TOP2A (Proteintech, diluted 1:500), anti-TOP2B (Proteintech, diluted 1:500), anti-PARP1 (Santa Cruz, diluted

1:1000), anti-PSA (Cell Signaling Technology, diluted 1:2000) antibodies and mouse anti-AR (DAKO, diluted 1:600) antibody. After 1h incubation with anti-mouse or -rabbit immunoglobulins antibodies HPR-conjugated (Cell Signaling Technology, both diluted 1:2000), immunoreactive bands were revealed using Immobilon Western reagents (Millipore) and Hyperfilm ECL (GE Healthcare Life Sciences). As already reported[17] the conventional approach cannot be used for WB quantitative analyses using different PCa cell lines, therefore quantitative analysis of protein expression was evaluated in three different WB experiments (original image are reported in **Figure S1**) and normalized to total protein concentration evaluated on the corresponding Sypro Ruby stained gel (**Figure S2**).

2.4. Invasion assays

The cells were seeded in serum-free media with 0.1% DMSO as control or with 1μM etoposide (dose corresponding to the value of ≥IC60) and placed in Matrigel chambers (BD Bio Coat). Medium supplemented with 20% FBS was used as chemoattractant. The invasion assays were performed for 48h in three replicate experiments.

2.5. Statistical analysis

Two-tailed Student's t-test was used to calculate the statistical differences between two groups. Histograms reported data expressed as mean ± standard error (SE), p-values were considered statistically significant when $p < 0.05$..

2.6. TOP2 analysis in large-scale datasets

We explored the association of TOP2A and TOP2B with clinical features and outcomes of PCa patients using data available on the cBioPortal (http://cbioportal.org)[18, 19]. Differential analysis of TOP2A and TOP2B transcripts expression in various pathological conditions was carried out by GEO2R tool (http://www.ncbi.nlm.nih.gov/geo/geo2r/) on four GEO datasets (GSE70768, GSE32269, GSE101607 and GSE32967). STRING was used for the functional enrichment analysis.

3. Results

3.1. Evaluation of etoposide activity on in vitro PCa models with different AR functionality

In order to investigate etoposide clinical relevance in PCa treatment, we tested this drug in LNCaP, 22Rv1, PC3, DU145, PDB and MDB cell lines, which were selected among in vitro models that could reflect different PCa progression stages according to AR activity and androgen sensitivity (**Table S1**).

The treatment of PCa cells for 120h with scalar concentration of etoposide elicited significant dose-dependent inhibition of cell growth for all concentrations tested (**Figure 1A and 2B**). Etoposide had similar inhibitory effect on LNCaP and 22Rv1 (Rln ≈1), whereas increased cytotoxicity was observed in DU145, PC3, PDB and MDB (Rln <1) (**Table 1**). The invasion assay was performed in presence of 1mM etoposide

for 48h, and we observed that etoposide affected aggressiveness in all resistant cell lines, with an increased effect in the AR-negative PC3 and DU145 cell lines **(Figure 1C)**.

Subsequently, we compared the activity of etoposide to that of other antineoplastic agents used for PCa therapy. The above cell lines were treated with enzalutamide, docetaxel and the PARP1 inhibitor olaparib (**Figure 2A and 3B; Table 1**). The cytotoxic effect of etoposide was higher compared to the other treatments investigated in almost all cell lines, except for LNCaP and 22Rv1 that showed slightly higher sensitivity to docetaxel compared to etoposide (Ret value 0.92 and 0.77, respectively). Of note, olaparib showed activity irrespective of PARP1 cellular content, which was equally or more expressed in resistant cell lines compared to androgen-responsive LNCaP **(Figure 1B)**. Interestingly, etoposide show higher cytotoxicity compared to the others compounds tested (Ret >1) in DU145, PC3, PDB and MDB cell lines, which had impaired or absent AR (**Table S1**).

We also assessed the activity of etoposide and of other treatments in PDB and MDB, which are bicalutamide-resistant, LNCaP-derived, cell lines that show significant plasticity after antiandrogen withdrawal[16]. In addition to bicalutamide, the normal culture medium of PDB includes 0.1 nM DHT, whereas MDB grow in absence of DHT. In our experiment, PDB and MDB were grown in absence of bicalutamide and DHT (PDB w/o Bic and MDB w/o Bic, respectively) for 1 or 2 months. The WB analysis of protein extract from these cells after 1 month from withdrawal showed significant modulation of PSA and AR expression **(Figure 2A).** Both cell lines had increased PSA expression, suggesting augmented AR activity. However, PDB also showed downregulation of AR expression, revealing a possible compensative mechanism to maintain homeostasis following antiandrogen withdrawal.

Using the IC50 ratio of treatments under investigation (calculated as the ratio of IC50 in cells grown without bicalutamide compared to that of cells grown with bicalutamide) (Rbic, **Table S2**), we found that etoposide, docetaxel and enzalutamide treatments showed reduced cytotoxicity (Rbic<1) 1 month after bicalutamide withdrawal compared to parental cell lines grown in the antiandrogen regimen (**Figure 2B**).

Table 1. Evaluation of etoposide effect on cell viability in different in vitro PCa models in respect to LNCaP or in comparison with other antineoplastic drugs used for PCa therapy

	ETOPOSIDE				DOCETAXEL				ENZALUTAMIDE				OLAPARIB			
Cell Line	IC50* (mM)	se	Rln**	Ret***	IC50 (nM)	se	Rln	Ret	IC50 (mM)	se	Rln	Ret	IC50 (mM)	se	Rln	Ret
LNCaP	9.24	0.57	1.00	1.00	8.49	0.55	1.00	0.92	20.06	0.29	1.00	2.17	13.47	0.17	1.00	1.46
22RV1	10.31	0.82	1.12	1.00	7.96	0.30	0.94	0.77	nd	nd	nd	nd	24.62	3.81	1.83	2.39
DU145	5.05	0.51	0.55	1.00	8.47	0.03	1.00	1.68	56.06	0.95	2.79	11.10	16.14	1.37	1.20	3.20
PC3	7.04	0.36	0.76	1.00	11.86	0.52	1.40	1.69	74.61	9.13	3.72	10.60	15.10	0.91	1.12	2.15
PDB (with Bic)	5.96	0.17	0.65	1.00	8.89	1.45	1.05	1.49	22.24	1.43	1.11	3.73	16.94	2.65	1.26	2.84
MDB (with Bic)	7.48	0.99	0.81	1.00	26.59	4.47	3.13	3.55	31.86	2.41	1.59	4.26	16.25	1.77	1.21	2.17

* IC50 is the drug concentration required for 50% inhibition in vitro

** Rln =IC50 (Cell Line evaluated)/IC50 LNCaP; Rln<1 treatment more effective than in LNCaP cell line; Rln>1 treatment less effective than in LNCaP; Rln=1 effect equal to LNCaP

*** Ret = IC50 (drug evaluated)/IC50 etoposide; Ret<1 treatment more effective that with etoposide; Ret>1 treatment less effective that with etoposide treatment; Ret=1 effect equal to etoposide treatment

3.2. TOP2A and TOP2B expression in prostate cancer cell lines

Western blot analysis was performed on whole protein extract from LNCaP, 22Rv1, DU145, PC3, MDB and PDB, in order to investigate the expression of TOP2 isoforms in these cell lines (**Figure 3**). Although the isoform expression of TOP2A and TOP2B was different among these cell lines, the total content of TOP2 was comparable **(Figure S3)**. Full-length TOP2A (TOP2A-FL) was poorly expressed in LNCaP, PDB and MDB cell lines, whereas it was highly expressed in the castration-resistant 22Rv1, DU145 and PC3. Cell lines with very aggressive phenotype (DU145 and PC3) showed significant lower expression of TOP2A proteolytic fragments compared to LNCaP. Of note, etoposide induced significant inhibition of invasion on these cell lines (**Figure 1C**). A statistically significant overexpression of TOP2B was found in 22Rv1 and PDB compared to LNCAP

3.4. Consistency between TOP2 mRNA and protein expression

In order to investigate the consistency between mRNA and protein expression, we analyzed the data reported in Human Protein Atlas. We found that TOP2A and TOP2B expression profiles, evaluated by immunohistochemistry, were consistent with mRNA levels assessed in 36 and 44 normal tissues, respectively. This feature was also confirmed in the TCGA-Prostate Adenocarcinoma dataset of PCa tissues; despite significant data dispersion from both approaches, mRNA distribution analysis by GEPIA was consistent with immunohistochemistry data from HPA (**Figure S4**).

3.5. Relevance of TOP2A and TOP2B genetic alterations in mCRPC

Using the mutation and copy-number alterations data provided by cBioPortal, we found that TOP2A and TOP2B genetic alterations were rarely reported in primary PCa, whereas they were more frequently

observed in metastatic samples. TOP2A and TOP2B amplifications were the most common alterations and were found in 5% of patients included in the SU2C-Prostate Cancer Foundation (PCF) Dream Team metastatic dataset[20]. In the Neuroendocrine Prostate Cancer (NEPC) dataset, TOP2A and TOP2B amplifications were found in 19% and 20% of patients with adeno-mCRPC, respectively, and in 30% and 27% of those with neuroendocrine-mCRPC[21]. We investigated the relationship between TOP2A and TOP2B amplifications and copy-number variations in genes commonly involved in PCa biology and NEPC differentiation. We identified significant correlations between TOP2A/TOP2B amplifications and AURKA, MYCN, SRRM4, FOXA1 and FOXA2 amplifications **(Figure S5)**. Of note, concurrent TOP2A amplification was found in 3 of 4 NEPC patients with RB1 loss in the NEPC dataset. However, we did not identify any correlation or association between TOP2A or TOP2B amplifications and clinical data. We also found a significant correlation between these amplifications and genomic burden in both datasets[20, 21].

3.6. Relevance of TOP2A and TOP2B mRNA expression in mCRPC

We investigated possible association between TOP2A or TOP2B mRNA expression and clinical data. Insufficient mRNA data were available in the Neuroendocrine Prostate Cancer dataset[21]. Using the data on metastatic tumors from the SU2C/PCF dataset[20], we found a statistically significant association between high TOP2A mRNA expression and poor overall survival **(Figure 4A)**. In the same dataset, we identified a significant correlation between TOP2A mRNA expression and neuroendocrine features, as well as an inverse correlation with AR score **(Figure 4C)**. This observation was consistent with our observation of increased TOP2A expression in aggressive castration-resistant cell lines, such as PC3 and DU145 **(Figure 3)**. No correlation was identified in patient overexpressing TOP2B[20]. We investigated possible associations between TOP2A overexpression and presence of alterations in genes that are commonly altered in metastatic PCa and in NEPC. We identified that patients with higher TOP2A mRNA expression were significantly more likely to harbor RB1 homologous deletions compared to those with lower TOP2A mRNA expression (38.2% vs. 5.5%) **(Figure 4B)**. We also observed that patients with higher TOP2A mRNA expression were less likely to harbor AR amplifications (not statistically significant). In addition, we found that TOP2A mRNA was significantly overexpressed in the 43 metastatic patients with RB1 loss compared to the whole population (q value = 0.007), and RB1 mRNA was significantly underexpressed in TOP2A-overexpressed metastatic PCa (q value < 0.001).

Using GEO2R analysis tool, we observed that the TOP2A mRNA was significantly overexpressed in mCRPC compared to primary PCa and normal tissues (**Figure S6**). In addition, TOP2A mRNA expression was higher in non-AR driven PCa compared to AR-driven PCa, as well as in small-cell or neuroendocrine PCa compared to adeno-PCa. Regarding TOP2B mRNA values, no substantial differences between primary PCa and mCRPC were found; however, significant lower expression of TOP2B was observed in primary PCa compared to normal tissues and in small-cell or neuroendocrine PCa compared to adeno-PCa.

3.7. TOP2A interaction network and functional enrichment analysis

In order to identify potential interactors of TOP2A, we analyzed the mRNA enrichments in patients with high TOP2A mRNA expression both in primary and metastatic PCa datasets[20, 22]. Separately for each cohort of datasets, we selected the first 150 mRNA with the highest statistically significant enrichment in patients with high TOP2A mRNA compared to patients with lower TOP2A mRNA expression. In patients with TOP2A-overexpressed tumors, we identified an interaction network of 63 mRNA that were significantly co-expressed with TOP2A both in primary and metastatic datasets. This network was visualized by STRING database and showed low protein-protein interaction (PPI) enrichment p-value (<1.0e-16) **(Figure 5)**. The gene ontology enrichment analysis revealed a gene set tightly involved in the cell cycle and mitotic cell processes, such as AURKA, AURKB and Cell Division Cycle Associated gene family.

4. Discussion

In the '90, several studies assessed the role of etoposide as monotherapy or in combination with other agents for the treatment of mCRPC, and the regimen etoposide-estramustine demonstrated activity in PCa, becoming an option included in the international guidelines[3]. In a phase 2 clinical study that enrolled 42 chemo-naïve patients with mCRPC, oral estramustine plus oral etoposide demonstrated PSA decline ≥50% in 54.7% of patients and response in 6/13 patients with measurable disease[23]. Grade 3-4 neutropenia was reported in 13% of patients, but nausea or vomiting of any grade occurred in 76% of patients, showing an inconvenient toxicity profile[3]. Etoposide as a single oral agent, without estramustine, showed less toxicity, but also less antitumor activity. A old trial tested this single drug in 24 patients with mCRPC, showing 2 partial responses, 2 stable diseases and short-lived PSA drop (19-68%) in 7/24 patients[24]. In the more recent randomized GETUG-P02 study, different agents were tested in elderly and/or frail patients after chemotherapy with palliative purpose[25]. In the oral etoposide plus prednisone arm, 50% decline of PSA was observed in 4/29 patients and analgesic objective response in 43% of patients. Although these results are quite disappointing, they suggest that selected subpopulations of patients with mCRPC could benefit from treatment with this TOP2 inhibitor.

Currently, etoposide in combination with platinum agents is an option for patients with NEPC, based on few data on NEPC and on the known efficacy of this combination in patients with small-cell lung cancers[4, 26, 27]. TP53 and RB1 loss are characteristic alterations of NEPC and are associated with poor prognostic features[4, 28]. However, these alterations are also encountered in patients with primary and advanced prostate adenocarcinoma without NEPC features[29]. NEPC may arise de novo, but often occurs in the later stages of mCRPC as a mechanism of resistance[30-32]. In this context, the aggressive variant prostate cancer (AVPC) is emerging as an important clinical entity that affects the outcome of patients with mCRPC[33, 34]. AVPC includes AR-independent anaplastic forms of PCa that are characterized by a rapidly progressive clinical course[33]. A phase II trial evaluated the activity of first-line carboplatin-docetaxel, followed by second-line cisplatin-etoposide, in 113 patients with AVPC[35]. Two cycles of second-line cisplatin-etoposide induced partial responses in 30% (n=10) of patients who received this regimen and stable disease was observed in half of them (n=17). Notably, 7 patients (9.4%)

showed response to cisplatin-etoposide but not to carboplatin-docetaxel. This observation suggests that etoposide could be useful in the context of AVPC.

In our work, we investigated the activity of etoposide in PCa cell models and its possible relationship with the expression of TOP2A and TOP2B. For this purpose, we used the castration-sensitive LNCaP model, intermediate models of castration resistance (PDB, MDB)[16], and castration-resistant models (22RV1, PC3 and DU145). We found that etoposide was more active in castration-resistant cells (DU145, PC3, PDB and MDB) compared to castration-sensitive LNCaP **(Table 1**). We also found that TOP2A was significantly overexpressed in 22Rv1, DU145 and PC3 compared to LNCaP, whereas TOP2B expression was increased in 22Rv1 and PDB compared to LNCAP **(Figure 3)**. Etoposide exerted a marked inhibition of proliferation and invasion of PC3 and DU145, which showed high expression of full-length TOP2A and low expression of TOP2A fragments **(Figure 2C)**. Of note, it has been already reported that TOP2 integrity is associated with increased activity and functionality[36, 37]. Given that DU145 showed significant sensitivity to etoposide despite low levels of TOP2B, our data suggest that TOP2A overexpression, rather than TOP2B, might predict response to this agent.

Our results also support the notion that etoposide activity might be affected by a functional AR axis. PDB and MDB cells showed significant sensitivity to etoposide during treatment with bicalutamide, whereas the activity of etoposide was decreased after antiandrogen withdrawal **(Figure 2B)**. In addition, 22Rv1 cells harbor the constitutively active AR variant 7 that drives proliferation; although these cells showed high expression of TOP2A and TOP2B, etoposide activity in these cells was similar to LNCaP and lower compared to the other castration-resistant cells (**Figure 3D and Table 1**). It has been described that etoposide inhibit AR ligand binding, and an efficient or constitutively active AR axis might antagonize the activity of this drug[15].

We acknowledge that our cell models not only differ based on TOP2 expression and AR activity, but they are also characterized by distinct genetic alterations (**Table S1**). Therefore, etoposide might show increased activity based on the presence of specific alterations. DU145 cells harbor the RB1 K715* nonsense mutation, which causes RB1 loss of function and have also two missense mutations in TP53 (V274F, P223L), although they stain positively for this last gene. PC3 cells show high RB1 expression with wildtype RB1, but they harbor PTEN deep deletion and the TP53 K139Rfs*31 frame shift deletion, staining negatively for TP53[38, 39]. PC3 cells also share some characteristics of NEPC[40].

However, our preclinical data indicate that etoposide treatment could be a valid strategy for patients with AVPC, which are characterized by several molecular alterations and are more likely to develop androgen-independent pathways to sustain cell survival and proliferation[41].

In the dataset analysis, we explored the prevalence and relevance of TOP2A and TOP2B alterations, which represent the specific targets of etoposide, in patients with PCa. We found that TOP2A and TOP2B amplifications are the most common genetic alteration reported in the available databases, particularly in metastatic patients, but we did not identify correlations with clinical features. We observed that patients with TOP2A/TOP2B amplification were more likely to harbor amplifications in other genes that are

commonly involved in the neuroendocrine divergent differentiation **(Figure S5)**. However, we acknowledge that TOP2A amplification correlated with tumor mutational burden and the association of these alterations with copy-number variations burden might have biased this result.

We also explored possible associations between TOP2 isoforms transcriptional levels and clinical data. We identified a significant association between TOP2A mRNA overexpression and shorter overall survival in mCRPC, whereas no association was detected with TOP2B **(Figure 4A)**. We also found a significant correlation between TOP2A mRNA overexpression and NEPC score, as well as an inverse correlation with AR score **(Figure 4C)**. These results are consistent with our in vitro data and highlight that TOP2A overexpression is associated with the aggressive features of PCa and with the tendency towards a NEPC phenotype. More importantly, we found that patients with high TOP2A mRNA expression were more likely to harbor RB1 deep deletions **(Figure 4B)**. RB1 loss has been recently identified as one of the most relevant prognostic indicators in PCa and this alteration is involved in the development of NEPC and antiandrogen resistance[42, 43]. These data suggest that tumors with RB1 loss can show overexpression of TOP2A and that etoposide treatment might be beneficial in patients harboring this alteration.

Interestingly, our functional enrichment analysis uncovered a STRING protein-protein network of transcripts, upregulated both in primary and metastatic PCa, which were associated with TOP2A overexpression **(Figure 5)**. This network revealed significant interactions among proteins that were involved in cell cycle and mitotic cell processes, supporting the role of TOP2A in the proliferation mechanisms of PCa cells. Among these genes, AURKA overexpression is well known in the context of NEPC and this figure remarks the potential direct involvement of TOP2A in the development of NEPC.

Overall, our results suggest that the spectrum of patients with PCa who could benefit from etoposide treatment might be wider than that currently expected. Beyond patients with pathologically-defined NEPC, etoposide efficacy should be also investigated in patients with specific clinical or molecular features (i.e., TOP2A overexpression, reduced AR functionality, RB1 loss).

5. Conclusion

Our data showed that etoposide is active in castration-resistant models of PCa, suggesting that this topoisomerase inhibitor should be evaluated in clinical trials for patients with AVPC or with specific molecular alterations. TOP2A overexpression, rather than TOP2B, seems to have significant predictive and prognostic implications. Although more data are needed, our results also support a strict relationship between AR functionality and etoposide activity. Further investigations are warranted to determine the role of TOP2A in mCRPC and to assess the efficacy of etoposide, alone or in combination with other agents, for the treatment of patients with mCRPC.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets analysed during the current study are available in cBioportal repository (www.cbioportal.org). The other data generated during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they don't have any conflict of interest.

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Authors' contributions: FB and PB conceived the study. MC and PB performed the in vitro experiments. PB and CC analysed the datasets. CC, MC and PB were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Figures

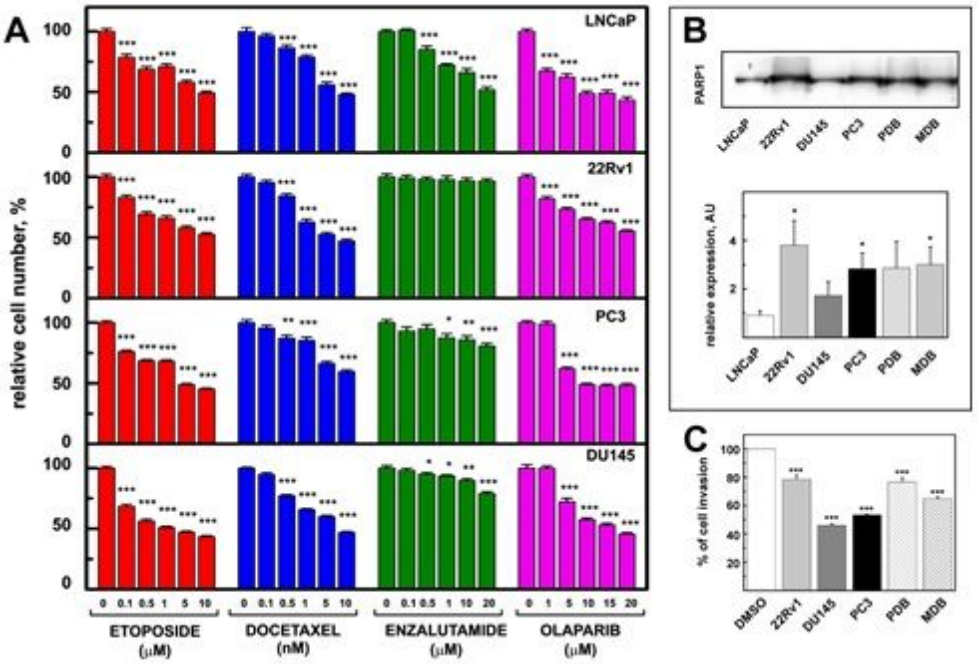

Figure 1

(A) Cell viability of LNCaP, 22Rv1, PC3 and DU145 cell lines treated for 120h with incremental concentration of etoposide, docetaxel, enzalutamide and olaparib. Mean ± SE of almost 3 experiments are reported. Asterisks indicate statistical significance in comparison to untreated cells (0.1% DMSO). (B) Representative WB analysis of PARP1 expression in total extracts from LNCaP, 22Rv1, DU145, PC3, PDB and MDB cell lines (top) and quantitative evaluation of three WB experiments (bottom). Asterisks indicate statistical significance in comparison to LNCaP. (C) Invasion assay of 22Rv1, DU145, PC3, PDB and MDB cell lines treated with 1μM etoposide for 48h. Histogram reports Mean ± SE of 3 experiments. Asterisks indicate statistical significance in comparison to untreated cells (0.1% DMSO) *p<0.05, **p<0.01, ***p<0.001.

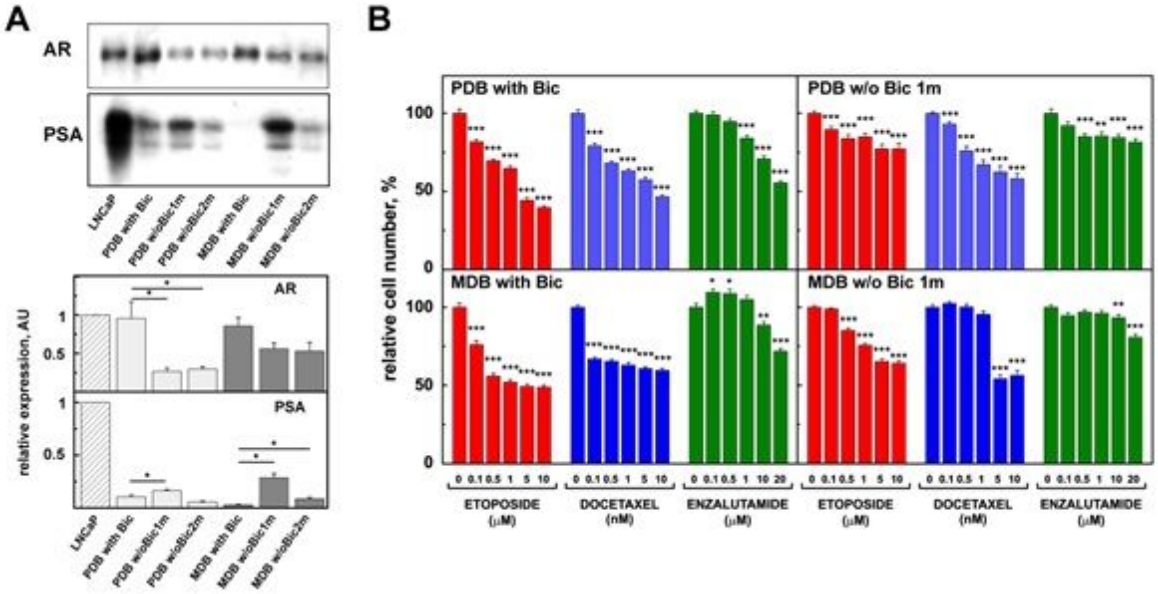

Figure 2

(A) WB analysis of whole protein extracts from PDB and MDB grown in normal conditions (with Bic) or without Bic and DHT (w/o Bic) for 1 or 2 months, probed with anti-AR and anti-PSA antibodies. LNCaP cells are used as positive control. Asterisks indicate statistical significance in comparison to cell lines with Bic (B) Cell viability of PDB and MDB cell lines grown in normal conditions (with Bic) or in absence of Bic and DHT for 1 months (w/o Bic 1m) treated for 120h with incremental concentration of etoposide, docetaxel and enzalutamide. Asterisks indicate statistical significance in comparison to untreated cells (0.1% DMSO). *p<0.05, **p<0.01, ***p<0.001. Mean ± SE of 3 experiments are reported.

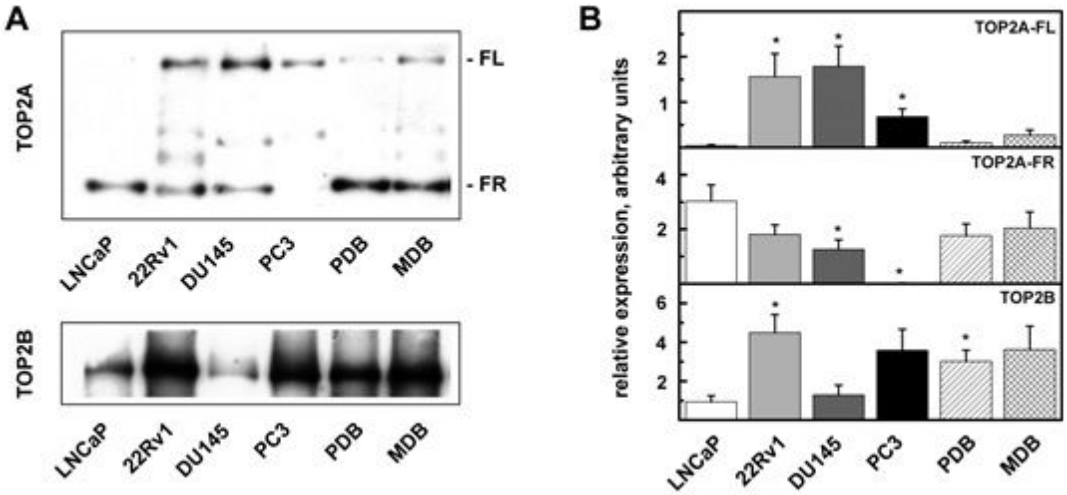

Figure 3

(A) Representative WB analysis of full length (FL) or fragmented (FR) TOP2A and TOP2B expression in total extracts from LNCaP, 22Rv1, DU145, PC3, PDB and MDB cell lines. (B) Quantitative evaluation of three WB experiments. Mean ± SE are reported. Asterisks indicate statistical significance in comparison to LNCaP *p<0.05.

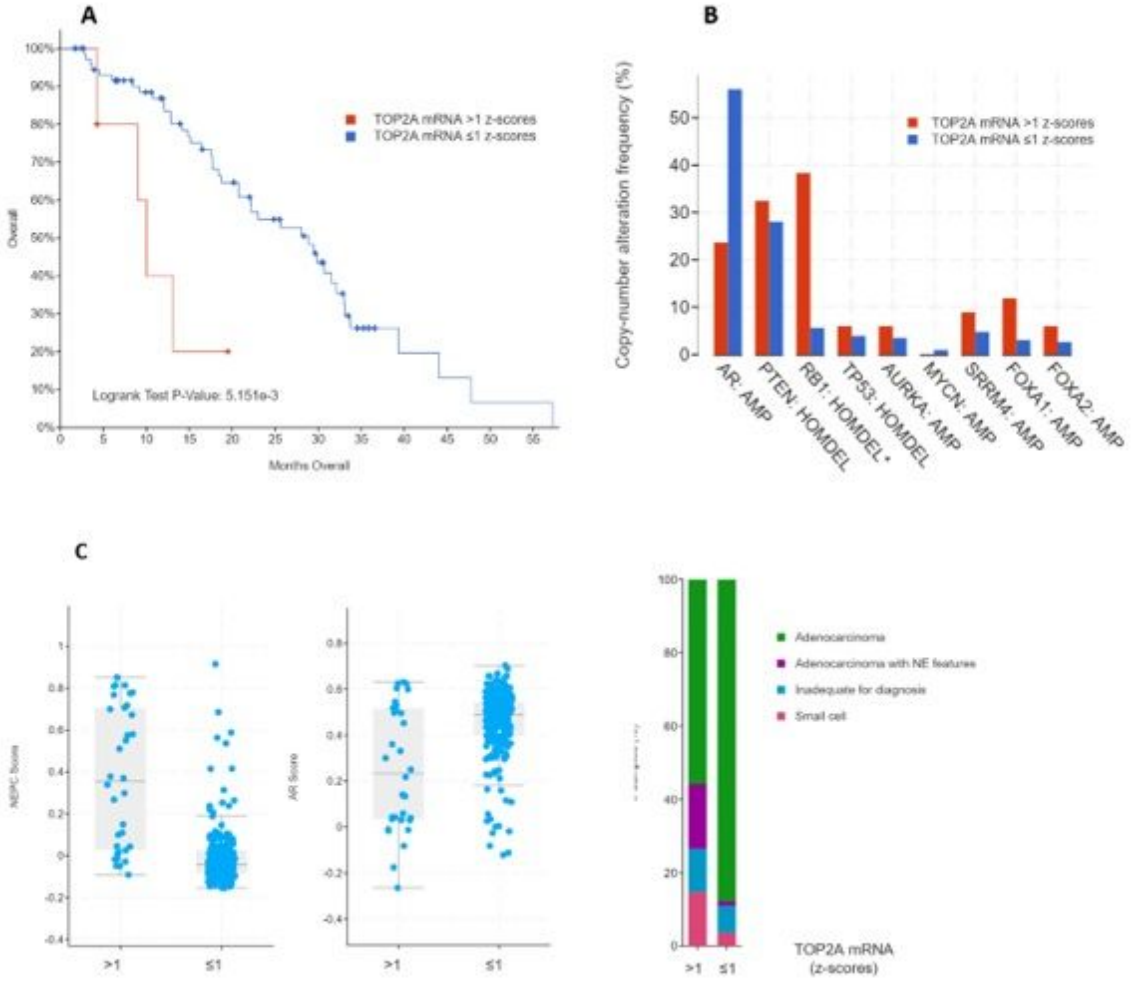

Figure 4

Association of TOP2A mRNA expression with clinical data in the metastatic PCa SU2C/PCF dataset. (A) Association between TOP2A mRNA expression and overall survival. (B) Frequency of copy-number alterations in patients with higher TOP2A mRNA expression compared to those with lower TOP2A expression. Patients with increased TOP2A mRNA expression (>1 z-scores) were more likely to harbor RB1 loss. AMP: amplification; HOMDEL: homologous deletion. *q-value statistically significant, Benjamini-Hochberg procedure. (C) Correlation between TOP2A mRNA expression and clinical and molecular features.

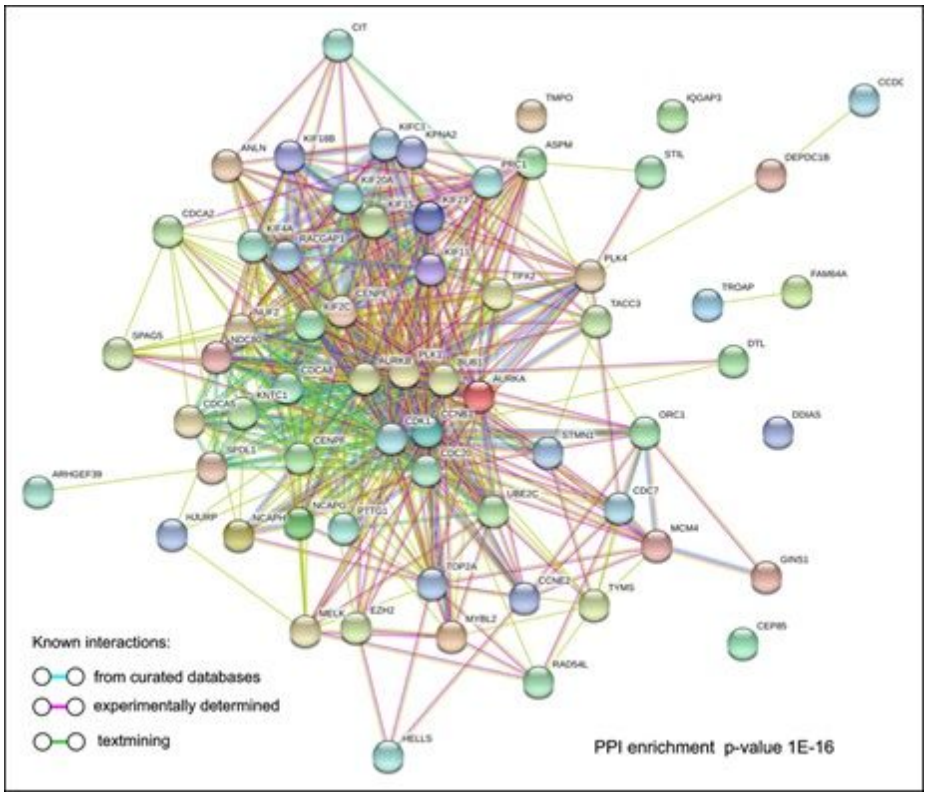

Figure 5

STRING interaction network of 63 proteins significantly co-expressed with TOP2A both in primary and metastatic PCa.

Supplementary Files

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