

Enoxacin inhibits growth of prostate cancer cells and effectively restores microRNA processing

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Keywords: enoxacin, microRNAs, prostate cancer, therapy, TRBP

Abbreviations: CRPC, castration-resistant prostate cancer; DAB, 3,3-diaminobenzidine; DMSO, dimethyl sulfoxide; HDAC1, histone deacetylase 1; miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; PAGE, polyacrylamide gel electrophoresis; PCa, prostate cancer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RISC, RNA-induced silencing complex; RNAi, RNA interference; SIRT1, silent information regulator 1; TARBP2, Trans-activator RNA-binding protein 2

Prostate cancer (PCa) is one of the most incident malignancies worldwide. Although efficient therapy is available for early-stage PCa, treatment of advanced disease is mainly ineffective and remains a clinical challenge. microRNA (miRNA) dysregulation is associated with PCa development and progression. In fact, several studies have reported a widespread downregulation of miRNAs in PCa, which highlights the importance of studying compounds capable of restoring the global miRNA expression. The main aim of this study was to define the usefulness of enoxacin as an anti-tumoral agent in PCa, due to its ability to induce miRNA biogenesis in a TRBP-mediated manner. Using a panel of five PCa cell lines, we observed that all of them were wild type for the *TARBP2* gene and expressed TRBP protein. Furthermore, primary prostate carcinomas displayed normal levels of TRBP protein. Remarkably, enoxacin was able to decrease cell viability, induce apoptosis, cause cell cycle arrest, and inhibit the invasiveness of cell lines. Enoxacin was also effective in restoring the global expression of miRNAs. This study is the first to show that PCa cells are highly responsive to the anti-tumoral effects of enoxacin. Therefore, enoxacin constitutes a promising therapeutic agent for PCa.

Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies worldwide and a leading cause of cancer-related deaths among men.¹ Although most cases are clinically indolent, a variable proportion of patients develop castration-resistant PCa (CRPC), an aggressive and lethal form of disease, associated with widespread metastatic disease.² Currently, most therapeutic strategies are largely ineffective and only allow for a small increase in survival.³ Therefore, new therapeutic strategies, ideally based on the understanding of the biology of this disease, are urgently needed.

PCa is a complex and heterogeneous disease that arises through the acquisition of several genetic and epigenetic alterations.^{4,5} Among the latter, dysregulation of microRNA (miRNA) expression has been recently emphasized as a critical mechanism in PCa development and progression.⁶ miRNAs are small non-coding RNAs that posttranscriptionally regulate gene expression by inducing cleavage of their target mRNA or by inhibiting their translation.⁷ These molecules are produced through a multi-step

process that involves the RNase III enzymes DROSHA and DICER, resulting in the production of mature miRNAs of ~22 nucleotides, which are incorporated into the RNA-induced silencing complex (RISC) through the action of the DICER-TRBP (Trans-activator RNA-binding protein) complex.⁸ miRNAs play a crucial role in the regulation of almost every biological process, including differentiation, apoptosis, cell cycle, development and metabolism.^{9,10} Moreover, dysregulation of any of these processes due to abnormal expression of miRNAs or alterations in their machinery of biogenesis has been implicated in cancer, including PCa.^{11,12} Although miRNAs have been proposed to function as either oncogenes or tumor-suppressor genes,¹³ most human tumors are characterized by a general defect in miRNA production that results in global downregulation of miRNA expression.¹⁴⁻¹⁶ Thus, compounds with the ability to restore the global miRNA expression might be an excellent therapeutic option for cancer.

Enoxacin, an antibacterial compound based on a fluoroquinolone skeleton, was shown to be effective in the inhibition of tumor cell growth in vitro and in vivo by enhancing

the production of miRNAs with tumor suppressor functions.¹⁷ Remarkably, the drug did not affect normal cells and was not associated with toxicity in mice models.¹⁷ Moreover, it has been reported that enoxacin promotes RNA interference (RNAi) and miRNA biogenesis by facilitating the interaction between TRBP, encoded by the *Trans-activator RNA-binding protein 2 (TARBP2)* gene, and miRNA precursors.^{17,18} Therefore, it has been recently demonstrated that *TARBP2*-mutant cells are less responsive to enoxacin.^{17,19} Although those results are promising for cancer therapeutics, no studies are available concerning the effect of enoxacin on PCa. Thus, we aimed to investigate the effectiveness of enoxacin as a tumor growth inhibitor in PCa. Furthermore, we evaluated the alterations in miRNA expression induced by this compound in PCa cells.

Results

PCa cells do not harbor *TARBP2* mutations and retain TRBP protein expression. In view of the fact that cell lines harboring *TARBP2* mutations are less responsive to enoxacin,^{17,19} five PCa cell lines (LNCaP, 22Rv1, VCaP, DU145 and PC-3) were screened for the presence of mutations in all the exonic mononucleotide repeats localized in the coding sequences of *TARBP2*. Co115, a *TARBP2*-mutant colorectal cancer cell line,^{17,19} was used as positive control. No *TARBP2* mutations were found in any of the tested PCa cell lines. Subsequently, we analyzed TRBP protein expression in PCa cell lines by western blot. As expected, all PCa cell lines expressed higher protein levels of TRBP than Co115 cells, which display very low expression levels (Fig. 1A). Since DICER acts in complex with TRBP,⁸ we also assessed DICER protein expression in PCa cell lines, and we verified that all PCa cell lines tested expressed DICER (Fig. 1A).

Primary PCa tumors are *TARBP2* wild type and express TRBP. To investigate the putative clinical usefulness of enoxacin for PCa therapy, we first assessed the *TARBP2* mutational status of 25 primary PCa tumors, and only wild type sequences were detected. Furthermore, using immunohistochemistry, TRBP expression was evaluated in a series of 50 primary PCa tumors, including the same cases analyzed for *TARBP2* mutational status. No differences in immunoreactivity for TRBP were apparent between normal and tumorous prostatic tissues representing different histopathological grades (Fig. 1B).

Enoxacin reverts neoplastic features of PCa cell lines. The half-maximal effective concentration (EC_{50}) of enoxacin was calculated in LNCaP and DU145 prostate cancer cell lines at 72 h. The drug presented an EC_{50} of 105 μ M in LNCaP and 141 μ M in DU145. Thus, to evaluate the effects of enoxacin, five human PCa cell lines were continuously exposed for 5 d to 124 μ M (40 μ g/mL) of enoxacin. As expected, enoxacin did not alter the expression of both TRBP and DICER proteins in any of the analyzed cell lines (Fig. 2A).

Importantly, a significant decrease in the number of viable cells was observed after exposure to the drug when compared with the vehicle, DMSO (Fig. 2B). For LNCaP and 22Rv1 cell lines, the effect was observed from day 1, whereas a significant decrease in the number of viable cells in VCaP, DU145 and PC-3 was found

after 2 d of drug exposure. The reduction in the percentage of viable cells at day 5 ranged between 17 and 59%, with LNCaP being the most responsive cell line (Fig. 2B).

To determine whether enoxacin was capable of inducing significant cell death, an apoptosis assay was performed. Indeed, a significant increase in apoptosis was apparent in all tested cell lines at days 2 and 5 (Fig. 3A). After 5 d of exposure to enoxacin, LNCaP and DU145 displayed the highest levels of apoptotic cells (Fig. 3A).

Apoptosis was also confirmed at molecular level, through the evaluation of mRNA expression of *CASP3*. Although all cell lines showed an increase in *CASP3* expression levels, statistically significant differences were depicted only for LNCaP, 22Rv1 and DU145 (Fig. 3B). Furthermore, cleaved PARP was analyzed after enoxacin exposure. 22Rv1, VCaP and DU145 presented increased protein levels of cleaved PARP after exposure to the drug (Fig. 3C).

Cell cycle distribution was evaluated by flow cytometry. Interestingly, 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, showed cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited a significant increase in the percentage of cells in late S and G2/M transition (Fig. 4; Fig. S2).

Moreover, the percentage of cells in sub-G1 phase, which is an indirect measure of cell death, increased significantly after enoxacin exposure (Table 1; Fig. S2). Thus, both the increase of apoptotic cells and cell cycle arrest support a growth inhibitory effect of enoxacin on PCa cells.

The effect of enoxacin on the invasiveness of PCa cells was only assessed in LNCaP and DU145 cell lines because, as described in the previous experiments, these cells were the most responsive to enoxacin. We decided to extend enoxacin exposure from 5 to 8 d in order to allow the vehicle cells to invade. In contrast to DU145, LNCaP cells did not show invasive potential in this system. Remarkably, enoxacin significantly reduced the invasiveness of DU145 cells (Fig. 5).

Enoxacin restores miRNAs expression. The impact of enoxacin exposure on the expression profile of miRNAs was analyzed in LNCaP and DU145 cell lines, using a panel of 742 miRNAs. miRNA analysis demonstrated that enoxacin induced a global upregulation of miRNA expression in both cell lines. Among miRNAs differentially expressed, upregulation was observed in 53% of the miRNAs (65 of 122) for LNCaP cells (Fig. 6A; Table S1) and in 60% (147 of 247) for DU145 (Fig. 6A; Table S2).

Remarkably, enoxacin was able to alter the expression of several miRNAs that have been previously associated with prostate carcinogenesis. Concerning tumor-suppressor miRNAs reported in PCa, miR-17*, miRNA-29b, miR-34a, miR-132, miR-146a and miR-449a showed increased expression levels following enoxacin exposure. Furthermore, decreased expression of some oncogenic miRNAs was also observed, including miR-141 and miR-191 (Table 2).

To confirm the impact of enoxacin on miRNA targets, protein expression levels of HDAC1, a miR-449a target oncoprotein, and SIRT1, a miR-34a target oncoprotein, were assessed by Western Blot. Both cell lines after drug exposure displayed decreased protein levels of HDAC1 and SIRT1 (Fig. 6B).

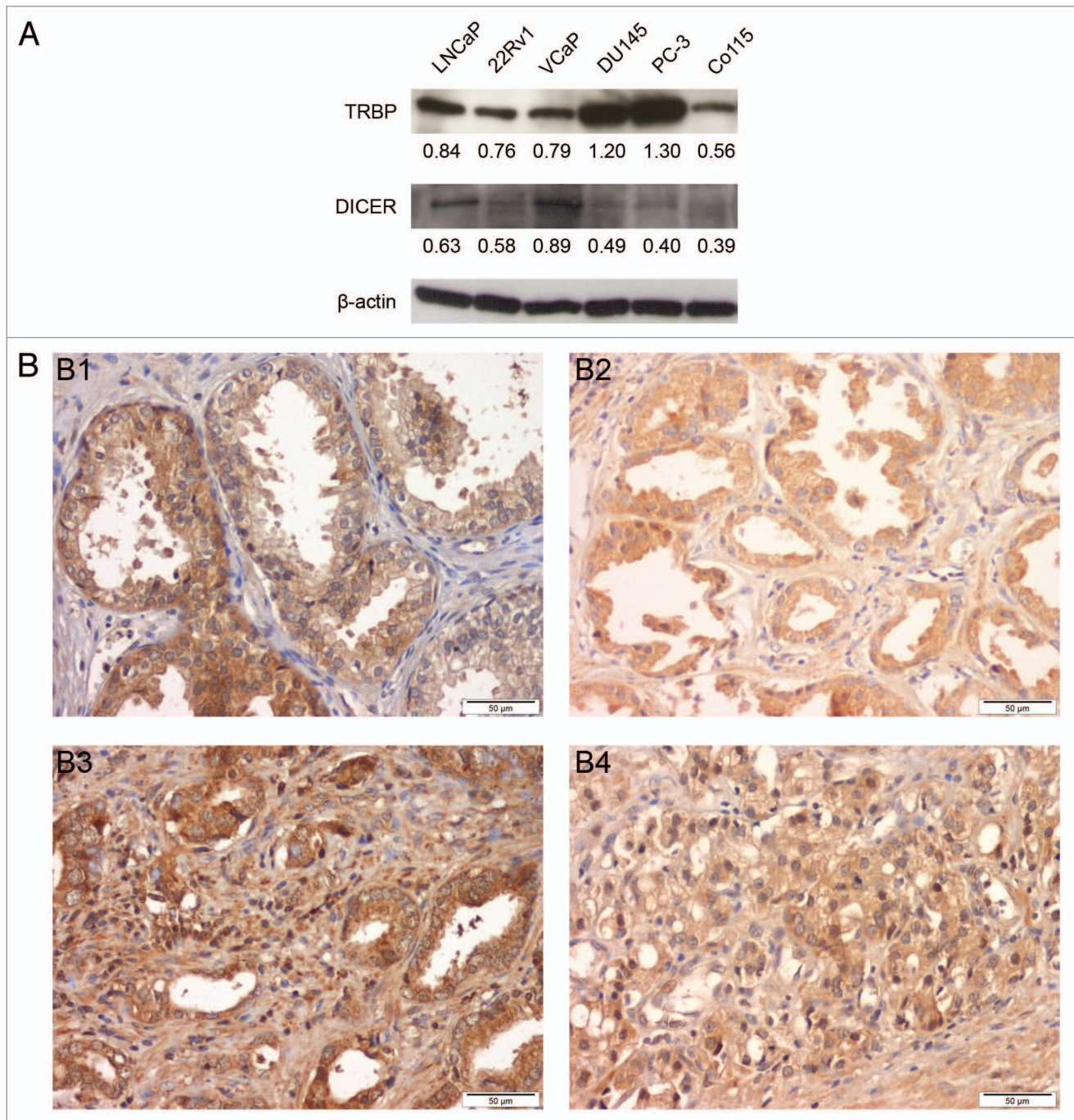


Figure 1. TRBP and DICER expression in PCa. **(A)** TRBP and DICER expression was assessed by Western Blot in PCa cell lines. The picture is representative of three independent experiments. β -actin was used as a loading control and the relative density of bands was densitometrically quantified. Mean quantitation values are shown. Co115, a *TARBP2*-mutant colon carcinoma-derived cell line, was used as positive control. **(B)** Immunohistochemical stain for TRBP expression in prostatic tissue. Protein immunoexpression in tumor cells was similar to that of normal epithelial cells. Normal glands **(B1)**, and tumorous tissue: Gleason score 6 **(B2)**, Gleason score 7 **(B3)**, and Gleason score 8 **(B4)**.

Discussion

PCa is one of the leading causes of cancer-related deaths worldwide,¹ and almost all of those deaths occur as a result of the emergence of castration-resistant disease.²⁰ Although PCa patients initially respond to androgen-deprivation therapy, about 18–24 months after treatment initiation, most patients develop CRPC,

which results in progressive clinical deterioration and, ultimately, death.^{21,22} For patients with CRPC, there are limited treatment options with proven survival benefit.^{23–26}

Recently, several miRNA microarray profiles demonstrated that miRNAs are commonly dysregulated in PCa when compared with normal prostate tissue and that they are also differentially expressed in different stages of PCa.^{15,16} Hence, miRNAs

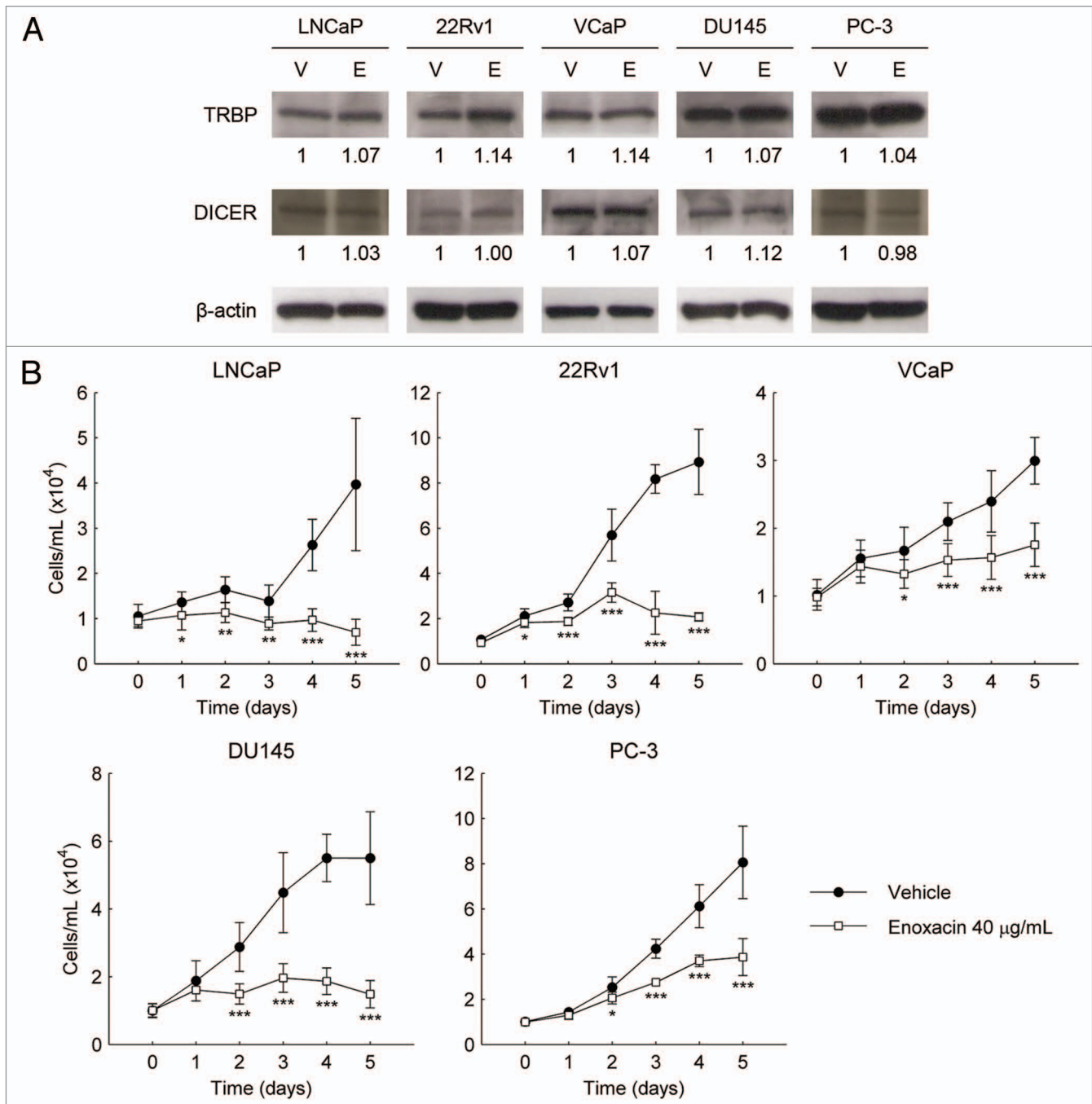


Figure 2. (A) Effect of enoxacin on the expression of TRBP and DICER. Protein expression of TRBP and DICER was analyzed by Western Blot in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μ g/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β -actin was used as a loading control and the relative density of bands was densitometrically quantified. **(B)** Effect of enoxacin on PCa cell viability. Cell viability was evaluated by MTT assay in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μ g/mL or DMSO (vehicle) for 5 d. The number of cells/mL is shown as mean of three independent experiments performed in triplicates \pm SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test * p < 0.05, ** p < 0.01, *** p < 0.001, compared with vehicle group.

might be used not only as diagnostic and prognostic biomarkers but also as therapeutic targets in PCa. In recent years, efforts have been made to find effective miRNA-based therapeutic strategies for cancer. Indeed, artificial miRNAs that might act as potential anti-tumoral agents are the most studied so far, with most reports focusing on oncogenic miRNAs inhibition.^{27,28}

Until now, few studies have reported the use of these synthetic miRNAs as tumor suppressors and, additionally, the effective technology for delivery of these oligonucleotide-based therapies remains a problem.²⁸ Because most human cancers exhibit global miRNA downregulation,¹⁴⁻¹⁶ the search for compounds able to globally restore the expression of tumor-suppressor miRNAs

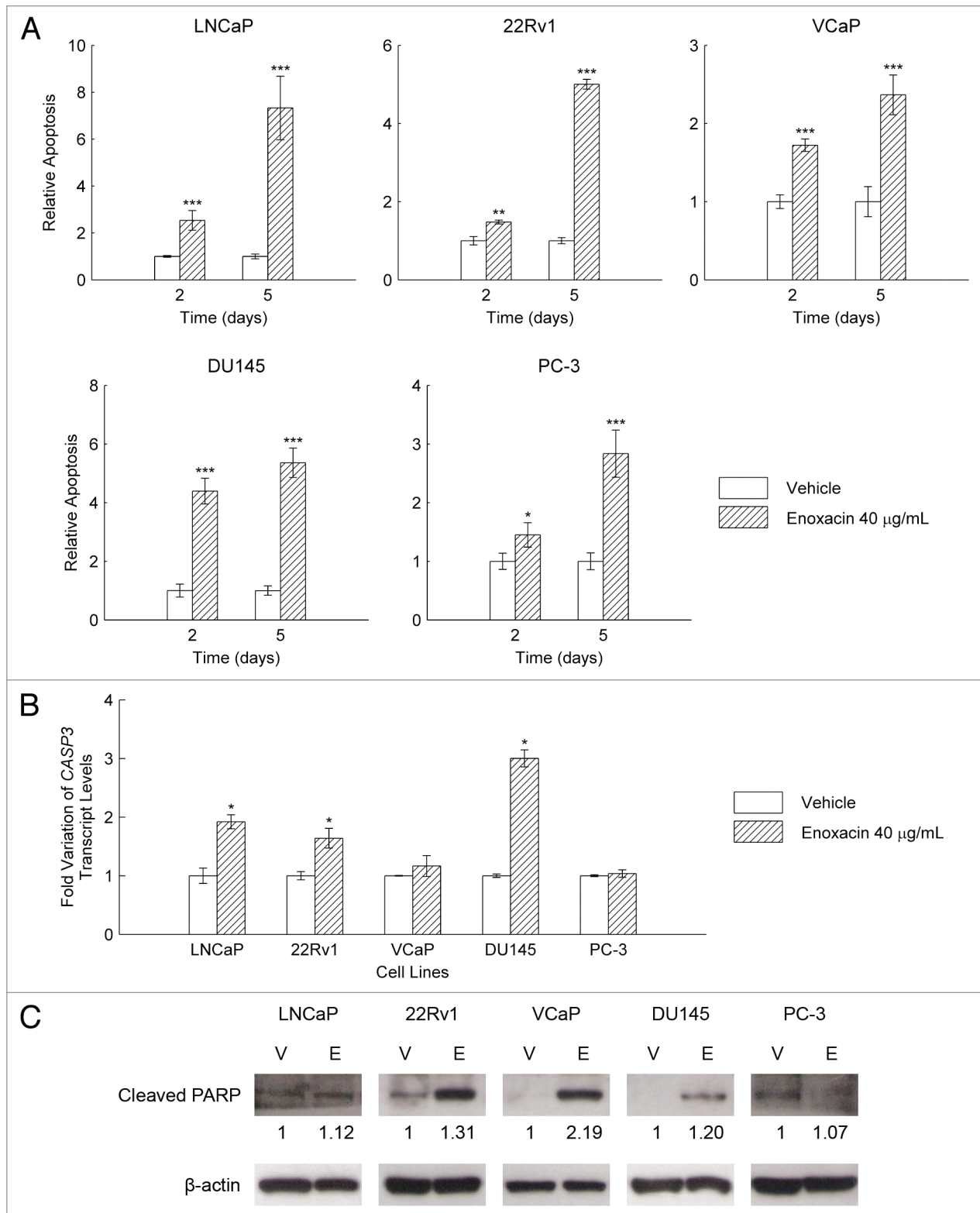


Figure 3. Effect of enoxacin on PCa cell apoptosis. **(A)** Apoptosis was analyzed by APOPercentage assay at days two and five in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at days two and five. **(B)** CASP3 mRNA expression was evaluated by qRT-PCR in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. Data are presented as mean of three independent experiments performed in duplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle group. **(C)** Cleaved PARP was analyzed by Western Blot in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified.

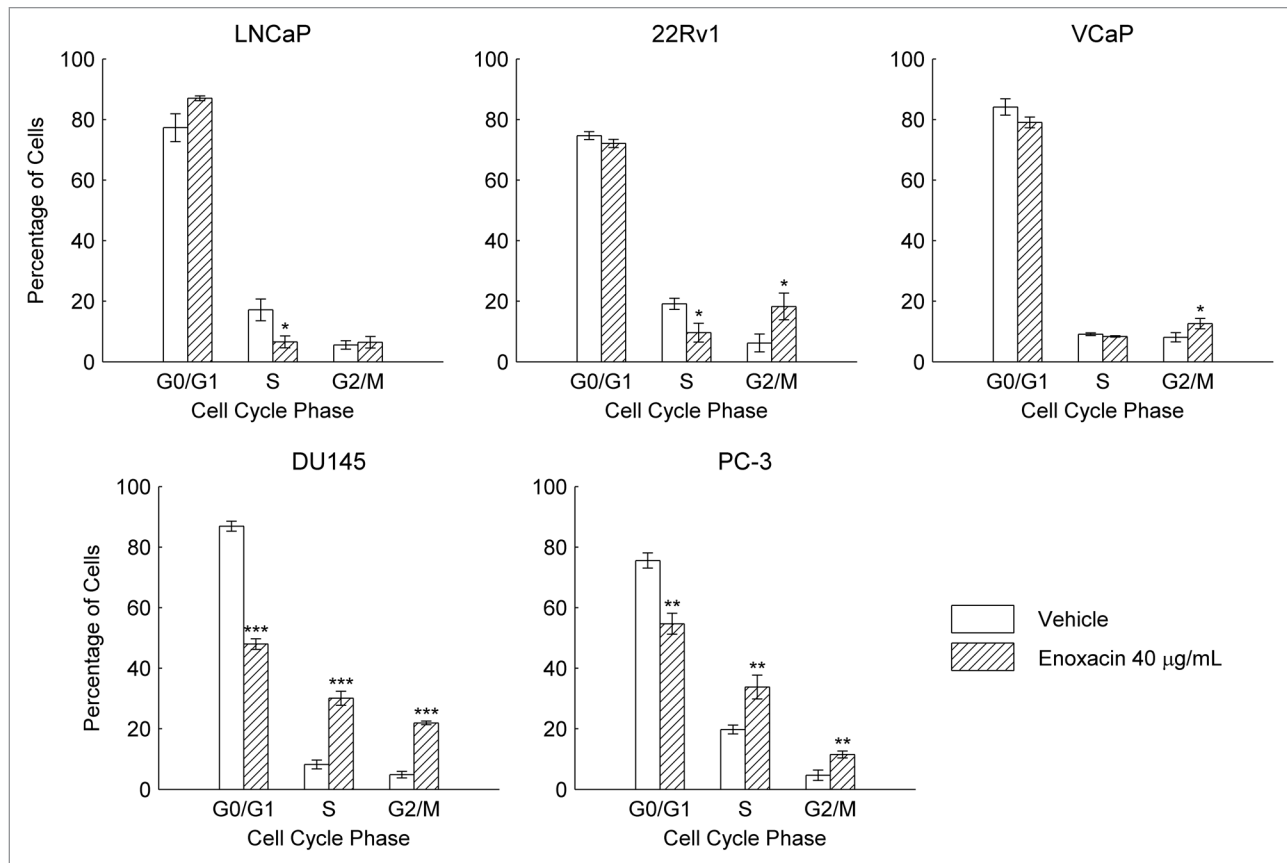


Figure 4. Effect of enoxacin on PCa cell cycle distribution. Cell cycle distribution was assessed by flow cytometry in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. The percentage of cells is shown as mean of three independent experiments ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle group.

Table 1. Impact of enoxacin on the percentage of cells in Sub-G1 phase assessed by flow cytometry

Cell Line	Cells in Sub-G1 phase (%)		p value
	Vehicle	Enoxacin	
LNCaP	1.7	2.0	ns
22Rv1	1.8	13.1	< 0.001
VCaP	2.5	7.3	0.002
DU145	1.7	7.5	< 0.001
PC-3	1.0	3.6	0.004

Ns, non-significant.

remains a priority in miRNA research. Herein, we report for the first time the anti-cancer effect of enoxacin, one of such compounds, on PCa cell lines.

Enoxacin, which has been used as a broad-spectrum antibiotic to treat bacterial infections (e.g., urinary tract infections),^{29,30} was recently reported as being capable of enhancing RNAi and consequently induce miRNA expression.^{17,18} However, the mechanism of action of enoxacin is dependent of TRBP as it has been shown that this compound is less effective in cells harboring alterations in this protein caused by *TARBP2* gene

mutations.^{17,19} Thus, we initially screened PCa cell lines for *TARBP2* mutations and none was found, although we were able to confirm a *TARBP2* mutation in the control, colorectal cancer cell line Co115, as previously reported.^{17,19} To further validate our results, we performed western blot for TRBP and confirmed that all PCa cell lines tested displayed higher protein levels than Co115, in agreement with previous studies.^{17,19} The *TARBP2* mutational status was also assessed in primary PCa tumors and only wild type sequences were detected. We then interrogated primary PCa cases using an immunohistochemistry assay for TRBP and we found that protein immunorexpression in tumor cells was similar to that of normal epithelial cells. Thus, we may infer that primary prostate carcinomas do not harbor deleterious mutations at the *TARBP2* locus and display normal levels of TRBP protein, rendering them sensitive to restoration of normal miRNA biogenesis by enoxacin.

To demonstrate the growth-inhibitory effect of enoxacin on PCa cell lines, we assessed cell viability, apoptosis and cell cycle characteristics following five-day exposure. Remarkably, in all tested cell lines, exposure to enoxacin resulted in a significant decrease in cell viability and induction of cell death by apoptosis, as previously demonstrated for other cancer cell lines.¹⁷ These results were further confirmed at the molecular level

through the observed statistically significant increase in *CASP3* mRNA expression for three of the five cell lines analyzed (LNCaP, 22Rv1 and DU145). Regarding protein expression of cleaved PARP, we also observed an increase after exposure to the drug in three cell lines (22Rv1, VCaP and DU145). Although the percentage of cells in Sub-G1 differs among the tested cell lines, globally there is an increase after exposure to enoxacin. With some variations, *CASP3* mRNA, and cleaved PARP protein expression follow the same trend. The percentage of cells in Sub-G1 may also reflect cell death due to necrosis or other mechanisms, but the results of the remaining three parameters (*CASP3* mRNA levels, APOPercentage and cleaved PARP) strongly indicate that apoptosis is, indeed, the main mechanism underlying cell death in this study. Concerning cell cycle distribution, it was observed that 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, presented cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited an increase in the percentage of cells at late S and G2/M transition.

Furthermore, we have shown for the first time that enoxacin significantly reduce the invasive potential of PCa cells. As metastasis is the major cause of morbidity and mortality in PCa patients,⁴ the development of new treatment regimens that would reduce tumor dissemination is extremely important for PCa therapy. It could be argued that the impact of enoxacin on cell invasion might be the result of reduced cell viability. However, it should be pointed out that it is difficult to dissociate one feature from the other, as both are likely to act in concert. Indeed, if tumor cell viability is reduced, it is expected that the potential to invade is also impaired. Irrespective of the underlying cause, however, our results demonstrate that a reduction in invasive potential does occur after exposure to enoxacin.

The ability to disrupt pathways of cancer cell survival has been already reported for a broad spectrum of cancer cells, both in vitro and in vivo, through the enhancement of the miRNA-processing machinery.¹⁷

In this study, we also demonstrated that enoxacin was effective in globally restoring the expression of miRNAs. Nevertheless, we found a decrease in the expression of a significant number of miRNAs, which is not totally in agreement with the mechanism of action purposed for enoxacin.^{17,19} According to previous studies, the presence of enoxacin increases the binding affinity of TRBP for miRNA precursors promoting miRNA biogenesis.^{17,19} Hence, it is tempting to speculate that besides this mechanism there might be other pathways through which enoxacin exerts its action.

Importantly, upregulation of several tumor-suppressor miRNAs known to be involved in PCa development and progression was observed, including miR-29b,^{31,32} miR-449a,³³ miR-146a,³⁴ miR-17*³⁵ and miR-34a.^{36,37} For instance, miRNA-29b was reported to be a negative regulator of PCa cell growth by modulating the expression of multiple proteins implicated in metastasis formation, including MMP2, E-cadherin, N-cadherin, Snail and Twist.^{31,32} miR-146a is downregulated in CRPC cell lines, and targets ROCK1 and EGFR, which are implicated in the development of CRPC.³⁴ miR-17* also suppresses tumorigenicity

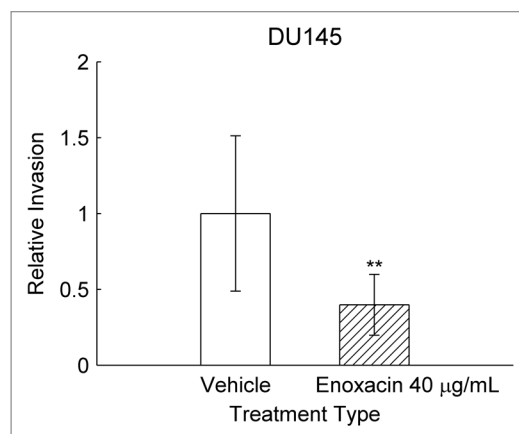


Figure 5. Effect of enoxacin on the invasive potential of PCa cells. Relative invasion was evaluated by Oris™ Cell Invasion Assay in DU145 cell line after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day eight. Data are presented as mean of three independent experiments performed in triplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test. **p < 0.01, compared with vehicle group.

of PCa cells through inhibition of mitochondrial antioxidant enzymes.³⁵ On the other hand, miR-34a, which presents tumor-suppressor functions, inhibits silent information regulator 1 (SIRT1), a gene that suppresses p53-dependent apoptosis.^{36,37} Finally, miR-449a, which is underexpressed in PCa, regulates cell growth and viability by repressing HDAC1.³³ Remarkably, in LNCaP and DU145 cell lines, upregulation of miR-449 by enoxacin resulted in the downregulation of HDAC1, an oncoprotein expressed at significantly higher levels in PCa than in normal prostate.^{38,39} Moreover, in LNCaP cells, upregulation of miR-34a by enoxacin induced the downregulation of SIRT1, which is overexpressed in PCa.⁴⁰ On the contrary, in DU145, SIRT1 downregulation cannot be attributed to miR-34a, as this was not found to be upregulated following enoxacin exposure. However, because SIRT1 may be regulated by other miRNAs, this hypothesis may not be ruled out completely at this point. In spite of globally upregulating the expression of miRNAs, enoxacin also caused a decrease in the expression of some oncogenic miRNAs, including miR-141⁴¹ and miR-191,⁴² reported as oncomiRs in PCa. miR-141 is a target of androgen regulation and it has been suggested that its upregulation may enhance the growth of CRPC cells.⁴¹ Finally, although less studied, miR-191 has also been reported as being overexpressed in PCa.⁴² Thus, the simultaneous upregulation of tumor-suppressor miRNAs and downregulation of oncomiRs by enoxacin in PCa cells highlights the therapeutic relevance of this drug in PCa. Notwithstanding, enoxacin also affected the expression levels of several other miRNAs, which play a role in different types of cancer, but with an unknown function in PCa. Thus, further studies are mandatory to disclose the biological function of these miRNAs in PCa.

In conclusion, enoxacin constitutes a promising therapeutic agent and in vivo studies should be conducted to further support the potential of enoxacin for therapy of PCa patients.

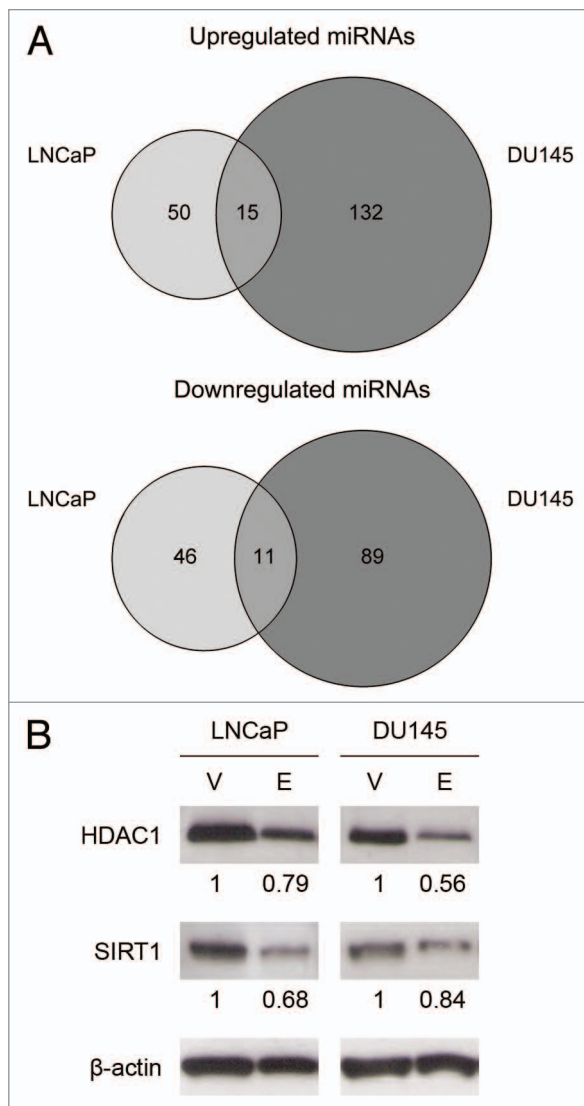


Figure 6. Enoxacin impact on miRNA. **(A)** Venn diagrams depicting the number of differentially expressed miRNAs after enoxacin exposure compared with the vehicle group. Top, miRNAs with increased expression (fold change ≥ 1.5); bottom, miRNAs with decreased expression (fold change ≤ -1.5). **(B)** Protein expression of two miRNA targets (HDAC1 and SIRT1) was analyzed by Western Blot in LNCaP and DU145 cell lines after exposure to enoxacin 40 $\mu\text{g}/\text{mL}$ or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β -actin was used as a loading control and the relative density of bands was densitometrically quantified.

Materials and Methods

Cell lines, drug preparation and exposure. DU145 was obtained from the American Type Culture Collection (ATCC), whereas LNCaP, VCaP and PC-3 were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at The Institute for Cancer Research (Oslo, Norway) and 22Rv1 by Dr. David Sidransky from the Johns Hopkins University School of Medicine (Baltimore, MD). For control purposes, we used the human colon carcinoma-derived cell line Co115, which was kindly provided by Prof. Fátima Baltazar from the Life and

Health Sciences Research Institute at the University of Minho (Braga, Portugal). All cell lines were cultured in the recommended medium, supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (GIBCO). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. All PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). Enoxacin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20°C until further use. PCa cells were continuously exposed to 40 $\mu\text{g}/\text{mL}$ (124 μM) enoxacin, for 5 d. For control purposes, cell lines were exposed to the vehicle of the drug (DMSO) only.

Direct sequencing. Genomic DNA was extracted from cell lines and prostatic cancer tissue using a standard technique comprising digestion with proteinase K (20 mg/mL) in the presence of 10% SDS at 55°C, followed by phenol-chloroform extraction and precipitation with 100% ethanol.⁴³ *TARBP2* was screened for mutations using the primers previously described by Melo et al.¹⁹ Direct sequencing was performed in an ABI PRISM 310 automatic sequencer using Big Dye Terminator Chemistry (Applied Biosystems), according to the manufacturer's recommendations.

Western blot. Protein extraction from whole-cell lysates was obtained using RIPA lysis buffer (Santa Cruz Biotechnology). Protein concentrations were determined using a Pierce BCA assay (Thermo Scientific Inc.), according to the manufacturer's instructions. Briefly, 30 μg of protein from each sample were separated using 10% Mini-PROTEAN[®] TGX[™] Precast Gel (Bio-Rad) at 200 V and subsequently blotted onto Protran nitrocellulose transfer membranes (Whatman) using Trans-Blot[®] Turbo[™] Transfer System (Bio-Rad). Membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies were polyclonal rabbit against TRBP (1:1,000, Abcam), polyclonal rabbit against DICER (1:500, Santa Cruz Biotechnology), monoclonal mouse against cleaved PARP (1:500, Cell Signaling), monoclonal mouse antibody against HDAC1 (1:1,000, Sigma-Aldrich) and polyclonal rabbit against SIRT1 (1:1000, Abcam). The membranes were developed using Immuno-Star WesternC Chemiluminescent kit (Bio-Rad) and exposed to Amersham Hyperfilm (GE Healthcare). To ascertain equal loading of protein, the membranes were stripped and reprobed with a monoclonal mouse antibody against β -Actin (1:8,000, Sigma-Aldrich). To relate the protein band intensity with the loading control (β -Actin), protein band intensities were determined using Quantity One software (Bio-Rad).

Patients and sample collection. Fifty prostate tumor samples from patients with clinically localized PCa (clinical stage II: T1cN0M0 or T2N0M0, according to the TNM staging system) consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute Porto, Portugal, were prospectively collected from 2001 to 2006. Tumor tissue was routinely fixed in buffered formalin and paraffin-embedded. All patients were enrolled after informed consent. This study was approved by the institutional review board (Comissão de Ética do IPO Porto).

Immunohistochemistry. TRBP expression in tumor tissue samples was assessed by immunohistochemistry using the Novolink™ Polymer Detection System (Novocastra). Deparaffinized tissue sections were submitted to antigen retrieval in a 700-W microwave oven, in 1x EDTA buffer solution. Endogenous peroxidase activity was blocked by incubating the slides with Peroxidase Block (Novocastra) for 5 min. The slides were incubated with Protein Block (Novocastra) for 5 min, and after incubation, primary antibody against TRBP (1:100, Abcam) was applied for 1 h in a humid chamber, at room temperature. The slides were then incubated with Post Primary Block (Novocastra) for 30 min followed by incubation for 30 min with the NovoLink Polymer (Novocastra). After incubation in 3,3-diaminobenzidine (DAB; Sigma-Aldrich) in a solution of 50 mL PBS/0.05% mL H₂O₂ for 7 min, the slides were counterstained with hematoxylin (Harris Modified Hematoxylin Stain; Fisher Scientific) for 20 sec and mounted with Entellann (Merck KGaA). Colorectal cancer tissues showing intense immunoreactivity for TRBP protein were used as positive control. The negative control consisted on the omission of the primary antibody. The assessment of immunostaining results was performed by an experienced pathologist and was expressed in a semiquantitative way according to the estimated percentage of positive tumor cells. Immunostaining of more than 10% of the tumor cells was required for scoring a case as positive.

Cell viability assay. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. In brief, PCa cells were seeded onto 96-well plates (Sarstedt) at 2×10^3 cells per well and allowed to adhere overnight. After exposure to the drug, cell viability was measured at 1, 2, 3, 4 and 5 d. Briefly, 200 μ L of 0.5 mg/mL MTT (Sigma-Aldrich) were added to each well and incubated at 37°C and 5% CO₂ for 3 h. Formazan crystals were solubilized with 100 μ L of DMSO. The absorbance was measured using a microplate reader (Fluostar Omega) at a wavelength of 540 nm with background subtraction at 630 nm. The number of cells was calculated using the formula: [(OD experiment \times Number of cells at day 0)/Mean OD at day 0].

Apoptosis assay. Apoptosis evaluation was performed using the APOPercentage apoptosis assay kit (Bicolor Ltd.) according to the manufacturer's instructions. This assay is based on phosphatidylserine transmembrane movements, which results in the uptake of the APOPercentage dye by apoptosis-committed cells. Cells were seeded onto 24-well plates (Sarstedt) at 5×10^4 cells per well and apoptosis levels were assessed at days 2 and 5. The absorbance was determined using a microplate reader (Fluostar Omega) at a wavelength of 550 nm with background subtraction at 620 nm. To normalize the OD measured in the apoptosis test, according to the cell number, the OD of the apoptosis assay was divided by the OD of the cell viability assay. The results were expressed as the ratio of the OD of the cells exposed to enoxacin to that of vehicle cells (set as 1).

Cell cycle analysis. Cell cycle distribution was determined by flow cytometry. Briefly, 5×10^5 harvested cells were fixed with 70% cold ethanol overnight at 4°C. After washing with cold PBS, cells were resuspended in staining Propidium Iodide Solution (Cytognos S.L.) and incubated at room temperature for 30 min

Table 2. Effect of enoxacin on the expression of several miRNAs already implicated in PCA

Cell Line	miRNAs	Fold change	p value
LNCaP	miR-29b	2.9	0.023
	miR-449a	1.8	0.012
	miR-34a	2.3	0.014
	miR-191	-2.3	0.023
DU145	miR-449a	2.2	0.011
	miR-146a	1.8	0.008
	miR-29b	1.7	0.004
	miR-132	1.7	0.003
	miR-17*	1.6	0.008
	miR-141	-1.7	0.002

in the dark. Cell cycle data were collected using Cytomics FC500 flow cytometer (Beckman Coulter) and analyzed with Modfit LT (Verity Software House Inc.).

Cell invasion assay. Cell invasion was evaluated using the Oris™ Cell Invasion Assay (Platypus Technologies) according to the manufacturer's instructions. After starvation for 18 h in a serum-free medium, cells were seeded at 5×10^4 cells per well and exposed to 40 μ g/mL of enoxacin or DMSO for 8 d. At this time point, cells were labeled with Calcein AM (AnaSpec) at a final concentration of 0.5 μ g/mL. A detection mask was attached to the bottom of the plate to block from view all cells except those that had invaded into the center zone and fluorescence was measured at excitation and emission wavelengths of 492 nm and 530 nm, respectively, using a microplate reader (Fluostar Omega). The results were expressed as the ratio of the fluorescence index (FI) of the cells exposed to enoxacin to that of vehicle cells (set as 1).

Quantitative reverse transcription PCR (qRT-PCR). RNA was extracted from all cell lines using TRIzol® (Invitrogen) according to manufacturer's instructions and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *CASP3* mRNA expression levels were assessed using TaqMan gene expression assay, purchased as pre-developed assays from Applied Biosystems. The mean quantity of *CASP3* expression levels of cell lines samples was normalized against mean quantity of the respective endogenous control (*GUSB*) expression levels.

miRNA expression assessment by miRCURY LNA™ array. cDNA synthesis and real-time qRT-PCR was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instructions. In brief, the cDNA products were transferred to the Ready-to-use microRNA PCR Human Panels (I + II) and quantified using SYBR green based real-time PCR and LNA enhanced miRNA specific primers. Based on miRBase 11.0 annotation, a total of 757 human miRNA probes were interrogated in this platform. Three independent experiments were performed. The data were imported into GenEX software (MultiD Analyses AB) for subsequent microarray analysis. Global mean normalization was used as a normalization factor for miRNA.⁴⁴ miRNAs with fold change above 1.5 or below -1.5 were considered up or downregulated, respectively.

Statistical analysis. Two-tailed Student's t-test was used to assess differences between the results obtained after exposure to enoxacin and after exposure to vehicle. Prior to application of the test, all data were transformed to logarithmic scale. Analysis was performed with the aid of SPSS software for Windows, version 20.0 (IBM-SPSS Inc.), and the statistical significance level was set at $p < 0.05$. Graphics were built using MATLAB 7.10.0 r2010a software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by Liga Portuguesa Contra o Cancro—Núcleo Regional do Norte and Research Center of the Portuguese

Oncology Institute—Porto [CI-IPOP 4-2008]. E.S. was supported by a grant from Liga Portuguesa Contra o Cancro—Núcleo Regional do Norte. I.G. and F.V. were supported by grants from FCT—Fundação para a Ciência e a Tecnologia [SFRH/BD/64082/2009 and SFRH/BD/70564/2010, respectively]. The authors would like to express gratitude to the Laboratory of Flow Cytometry at the Department of Hematology of Portuguese Oncology Institute—Porto.

Supplemental Materials

Supplemental material may be found here:

<http://www.landesbioscience.com/journals/epigenetics/article/24519>

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