Zoledronic Acid Enhances the Chemotherapeutic Efficiency of 5-fluorouracil or Flutamide in Prostate Cancer Cells with Modulation of miR-382 and miR-18a Expression

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Abstract. Owing to a lack of appropriate therapeutic regimens, prostate cancer (PC) is a global health concern with a high incidence and mortality rate in elderly men. Combination treatment seems to have the highest clinical benefit and avoids unwanted side effects. The current study focused on the chemotherapeutic efficacy of Zoledronic acid (ZA) in combination with 5-fluorouracil (5-FU) or Flutamide on prostate cancer cells, as well as its effect on apoptosis. The MTT assay was used to determine the cytotoxic effect of Zoledronic acid (ZA), 5-FU, and flutamide on PC-3 and DU-145 cells, as well as the combined therapy of ZA with 5-FU or flutamide. Additionally, immunofluorescence staining analysis was used to assess changes in Bcl-2 and p53 expression. Furthermore, the western blotting method was extensively used to evaluate Bax, caspase 3, and cyclin D1. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) was applied to determine the relative expression of miRNA-382 (miR-382) and miRNA-18a (miR-18a). Instead of (13.47, 8.23, and 9.42 μM) for PC-3 or (38.77, 17.6, and 8.47 μM) for DU145 cells, the combination therapy improved cytotoxicity with doses approximately half of IC50 (6.74, 4.12, and 7.07 μM) in PC-3 and (19.38, 8.8, and 6.33 μM) in DU145 cells for ZA, 5-FU, and flutamide, respectively. When compared to a single therapy, the combination therapy significantly up-regulated the pro-apoptotic Bax, cleaved caspase 3 and p53 levels while down-regulated the cyclin D1 and Bcl-2 expression. In addition, the combination therapy was linked to changes in miR-382 and miR-18a expression. Our findings suggest that combining ZA with 5-FU or flutamide improves chemotherapeutic efficacy against prostate cancer cells, at least in part by encouraging apoptosis and modulating miRNA expression, especially miR-382 and miR-18a.

Keywords: 5-Fluorouracil (5-FU), Flutamide, miRNAs, PC-3, Prostate cancer, Zoledronic acid

1. Introduction
Prostate cancer (PC) a form of famous cancers in elder men, also act as the second prominent cause of cancer-related mortality [1]. Despite recent advances in cancer therapies, PC remains an incurable disease that relapses [2]. PC’s high prevalence, incurability, relapse, and mortality are attributed to a lack of adequate treatment, which is closely related to the genetics and epigenetics of the disease [3].

Zoledronic acid (ZA) is a highly active bisphosphonate containing nitrogen (BPs) and was approved by the FDA in 2002 for the treatment of solid tumor bone metastases [4, 5]. Furthermore, new evidence suggests that BPs treatment, especially zoledronate therapy, is linked to changes in miRNA profile expression [6]. In addition, BPs are often combined with other chemotherapeutic drugs to achieve a synergistic effect with minimal side effects [7].

Anti-androgens including flutamide and the thymidylate synthase inhibitor 5-fluorouracil (5-FU) are normal treatments for advanced PC, despite health concerns and efficacy issues [8-10].

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Combining two or more chemotherapeutic drugs, in particular, aims to target cancer-inducing or cell-assisting pathways, which are a keystone in cancer therapy. In addition, the combination procedure allows for the use of chemotherapy at low doses, which may reduce the side effects often associated with high doses [11, 12]. Consequently, new PC treatment combinations must be used, and novel synergistic, beneficial combinations must be created to improve PC management.

Bisphosphonates, such as ZA, were previously used in combination with other chemotherapeutic drugs to achieve a synergistic effect with few side effects; however, its potential role in the control of miRNAs is still not fully understood, its potential effect in the regulation of miRNAs is a goal to improve chemotherapeutic efficiency against PC.

MiRNAs are concerned to control the expression of upwards of 70% of the human genome, and dysregulation of miRNA has been linked to tumor development [13, 14]. MiRNAs have either oncogenic or tumor-suppressive effects by controlling genes involved in proliferation, differentiation, and apoptosis [15, 16].

MiRNAs such as miRNA-382 (miR-382) and miRNA-18a (miR-18a) are engaged in the control of PC [17]. PC cell proliferation, migration, and tumor growth are all inhibited by MiR-382 [18]. Several shreds of evidence suggested that miR-18a is an oncogene that plays a role in the progression of PC. When compared with normal people and patients with benign prostatic hyperplasia, MiR-18a is abundantly expressed in the blood patients of PC [19].

As a result, we sought to investigate how ZA affected the cytotoxic effect of 5-FU or flutamide on prostate cancer cells, as well as how it affected apoptosis.

2. Materials and methods

2.1. Chemicals

Drugs, reagents, and antibodies which used in this research were purchased from different companies, Flutamide was kindly provided from (Sigma Pharmaceutical Industry; Egypt), ZA was kindly provided from (Pharco Pharmaceuticals; Egypt), and 5-FU was purchased from (Sandoz Pharma; Canada). RPMI-1640 media complemented by L-glutamine, fetal bovine serum (FBS), trypsin EDTA, phosphate-buffered saline (PBS), and penicillin/streptomycin solution for PC-3 and DU-145 cell lines were acquired from Gibe (Thermo Fischer Scientific, Inc., USA). MTT reagent: 3′-(4, 5 dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, chloroform, phenol, isoamyl, Tris, and ethylenediaminetetraacetic acid (EDTA) were provided from (Sigma-Aldrich, Germany). Bcl-2 antibodies (catalogue no. sc-7382), p53 (catalogue no. sc-393031), cyclin D1 (catalog no. sc-8396), and Bax (B-9) (catalogue no. sc-7480) were acquired from (Santa Cruz Biotechnology, USA). While the caspase 3 (cleaved) (catalogue no. AB3623) from (Merck KGaA, Germany). Radio immune precipitation assay lysis buffer (RIPA buffer) [1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl pH 7.6, 0.1% SDS, 5 mM EDTA, 1% sodium deoxycholate, protease inhibitors] was provided from (Sigma-Aldrich, Milan, Italy). Most of the other substances were of the greatest analytical standard. Completed dilutions were prepared instantaneously before use, and current stock solutions for each experiment were prepared.

2.2. Cell culture

PC-3 and DU-145 human prostate cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, USA). Cells were grown in polystyrene flasks (75 cm²) as monolayer adherives in a serum-containing medium, RPMI 1640 supplemented with 1% penicillin-streptomycin, 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and incubated at 37°C in a humidified atmosphere with 5% CO₂. Morphological shape and the cell growth monitoring until they reached 90% of the confluence, after which the cells were easily passed.

2.3. Cell viability assay

MTT assay was used to evaluate the cytotoxic activity of ZA, 5-FU, Flutamide, ZA/5-FU and ZA/Flutamide in PC-3 and DU-145 cell lines [20]. We plated 5000 cells/well in a 96-well plate and were
grown for 24 h after being treated with media containing concentrations (0.1, 1, 10, 100 and 1000 μM) of ZA, 5-FU, and Flutamide, and incubated for 48 h at 37°C, 5% CO₂. In combination therapy, the fractional design was performed by combining/mixing different concentrations (¼, ½ and ¾ IC₅₀) for each drug (2.06, 4.12, and 6.17 μM) of 5-FU or (2.36, 4.71, and 7.07 μM) of flutamide with various concentrations of ZA (3.37, 6.74, and 10.1 μM) for PC-3 cells, while for DU-145 cells the fractional design was carried out by combining various concentrations (4.40, 8.80, and 13.20 μM) of 5-FU or (2.11, 4.23, and 6.33 μM) of flutamide with various concentrations of ZA (9.69, 19.38, and 29.07 μM) for 48 h. Upon treatment, the media was removed, and 100 μL of yellow MTT has been added to every well and incubated at room temperature for 4 h. To dissolve the subsequent formazan drug, 100 μL of dimethyl sulfoxide (DMSO) buffer was made, and absorbance was measured at 570 nm using the ELISA microplate reader (Epoc-2 C micro-plate reader, USA). The percentage of control cells is stated as a percentage of the total number of cells (100% of cell viability). The combination index (CI) for the level of interaction between ZA with either 5-FU or flutamide on PC-3 cells and DU-145 cells was also determined using CompuSyn software. CI < 1 means synergism, CI > 1 means antagonism, and CI=1 means additive.

After determination of IC₅₀ and calculation of CI, cells were divided into 6 groups:

<table>
<thead>
<tr>
<th>Groups</th>
<th>PC-3 Cells</th>
<th>DU-145 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Control untreated cells</td>
<td>Control untreated cells</td>
</tr>
<tr>
<td>Group 2</td>
<td>Treated cells with ZA (6.74 μM).</td>
<td>Treated cells with ZA (19.38 μM).</td>
</tr>
<tr>
<td>Group 3</td>
<td>Treated cells with 5-FU (4.12 μM).</td>
<td>Treated cells with 5-FU (8.80 μM).</td>
</tr>
<tr>
<td>Group 4</td>
<td>Treated cells with Flutamide (7.07μM).</td>
<td>Treated cells with Flutamide (6.33 μM)</td>
</tr>
<tr>
<td>Group 5</td>
<td>ZA (6.74 μM) + 5-FU (4.12 μM).</td>
<td>ZA (19.38 μM) + 5-FU (8.80 μM).</td>
</tr>
</tbody>
</table>

In combined therapy, PC-3 and DU-145 cells were treated with the suitable concentration of ZA drug firstly for one hour before obtaining the matching concentration of 5-FU or flutamide was placed and incubated for 2 days at 37 °C, 5% CO₂.

2.4. Western blot analysis

PC-3 and DU-145 cells were incubated in six-well plates (250×10³ cells/ well) for 24 h, and then treated with ZA for 1h before receiving the matching concentration of 5-FU or flutamide was placed and incubated for 48 h at °C, 5% CO₂. The cells were then trypsinized, separated by centrifugation, obtained as pellets, and rinsed three times with ice-cold PBS. After that, the cells pellet was lysed using RIPA lysis buffer on ice for 30 min. Then, the blend was sonicated, centrifugated, the supernatant was collected and stored at -80°C. The protein concentration of each cell lysate was determined by Bicurom method [21, 22]. Protein levels of cyclin D1, Bax, and active caspase 3 in PC-3 and DU-145 cell lysate were evaluated using the relevant mouse monoclonal and rabbit monoclonal antibodies following the previously published methods [23, 24]. In Brief, the proteins in each cell lysate were denatured at 95°C in 2× Laemmli buffer containing 5% β-mercaptoethanol for 10 min. Afterward, SDS–PAGE electrophoresis was performed by inserting 50 μg of denatured protein per lane at 100 V through stacking gel (6%) followed by 125 V through resolving gel (10%) for about 2 h and blotted to the PVDF membrane using cleaver scientific® semidy transfer unit for 25 min. Immunoblotting was accomplished by incubating the PVDF membrane in a TBS buffer that containing 0.1% Tween and 5% defatted milk (TBST) for 1 h at 4°C. After that, membrane was incubated with rabbit monoclonal anti-caspase 3 and mouse monoclonal anti-Bax, and anti-cyclin D1 as primary antibodies at 1:500 dilutions for 24 h at 4 °C. Then washed with TBST buffer and incubated at 37°C for 1 h with goat anti-rabbit and goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Novus Biologicals, LLC, USA) in a dilution of 1:5000. The incubated membrane was washed 4 times with TBST, then the membrane-bound antibody was detected using BCIP/NBT detection Kit (Genemed Biotechnologies, USA), and statistical analysis were performed using Image J/NIH quantification software (National Institute of mental health, USA). Approximately equal protein loading for every lane was revealed by stripping followed by re-blotting at
4°C for each membrane against anti-β-actin mouse monoclonal antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:500.

2.5. Immunofluorescence assay

The expression of the Bcl-2 protein and the p53 tumor suppressor were evaluated by immunofluorescence assay on PC-3 cells. Firstly, cells were seeded on cover slips 6-well plates and allowed to treat with chemotherapy drugs and combinations as indicated above, while control cells were incubated in drug-free media. After 2 days, the media were discarded, and the cells washed 3 times with Phosphate Buffer Saline (PBS). Cell fixation was done with 4% paraformaldehyde for 10 min and washed 3 times with PBS. Blocking cells for 15 min at room temperature with block buffer then incubated as a primary antibody with mouse monoclonal anti-Bcl-2 and anti-p53 (Santa Cruz Biotechnology, Inc., USA) at 1:500 dilutions for 2 h at 37°C. After the primary antibody, cells were rinsed 3 times with PBS and then incubated with secondary antibody goat anti-mouse Alexa flour 488 at room temperature in the dark for 1 h. After washing in PBS, nuclei were stained by 4', 6-Diamidino-2 Phenylindole, Dihydrochloride (DAPI) for 5 min, then excess DAPI was washed out using PBS. Slides were consistent in fluoromount G and coverslips were inverted over the mounted slides. Target antigen was visualized using LEICA fluorescence microscope (model: Leica DM5500 B from Leica Microsystems, USA) under red, blue, and green channels.

Fluorometric analysis: for statistical analysis, the microscopic field fluorometric intensity was measured using Image J/NIH software (National Institute of mental health, USA) and represented as a ratio green (Antibody fluorescence intensity) / blue (DAPI nucleus staining).

2.6. Assessment of miRNAs expression

2.6.1. Total RNA isolation

Standard TRIzol® Reagent Extraction Method has been utilized to isolate total RNA from prostate cancer PC-3 and DU-145 cells (cat#15596-026, Invitrogen, Germany). In a brief period, centrifugation was carried out to harvesting the cells and then homogenized in 1 mL of TRIzol® Reagent. After that, homogenized samples were brooded for 15 min. at 37°C, and 0.2 mL of chloroform per 1 mL of TRIzol® Reagent was added. After that, the samples vortexed and centrifuged repeatedly to produce three separated layers. The RNA remained alone during the aqueous phase. The RNA was precipitated by homogeneous mixing with isopropyl alcohol from this phase. 1 mL of 70% ethanol was used to wash the RNA pellets. The samples were mixed with vortexing and centrifuged at 4°C at 5 min. Then evacuated the supernatant and the pellets of RNA were air-dried for 15 min. RNA was dispersed in diethylpyrocarbonate-treated water bypassing the solution through the tip of the pipette. To complete the digestion of DNA residues, the total RNA must be treated with 1 U of RQ1 RNase-free DNase (Invitrogen, Germany), re-suspended in DEPC-treated water. Purity and the quantity of RNA were measured as optical density (OD) 260/280 by Thermo Scientific NanoDrop™ 1000 Spectrophotometer V3.7. Aliquots were used directly for reverse transcription (RT), otherwise stored at -80 °C.

2.6.2. Reverse transcription (RT) reaction

The overall RNA isolated from prostate cancer PC-3 and DU-145 cells was switch transcribed into the corresponding cDNA in a total volume of 20 μL using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). After that, incubation occurred at 25°C for 15 min, preceded by 1 h at 42°C and stopped by heating at 99°C for 5 min. Hence, tubes of the response containing RT arrangements were cooled directly in an ice apartment until they were utilized for DNA amplification by RT-PCR.

2.6.3. Quantitative real-time PCR (qRT-RPC)

The studied miRNAs expressions were assessed by using StepOne™ real-time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA). The miScript SYBR Green PCR
kit (Qiagen GmbH) was utilized to perform the amplifications. PCR conditions were adjusted as the following: starting enactment of the polymerase at 95°C for 15 min, taken after by 40 cycles of 94°C for 20 s, 55°C for 35 s and, 70°C for 35 s. RNU6B was used as a positive control gene for normalize our results. The comparative cycle threshold was used to evaluate the relative quantification (RQ) of miRNAs, with $RQ = 2^{-\Delta\Delta Ct}$.

Statistical analysis
All graphics, data, and calculations of IC₅₀ were done using GraphPad Prism version 8.0.1, 2018. Mean ± standard deviation ($\bar{x} \pm SD$) was used to express all data, of at least three independent repeats (n=3). The statistical significance variables among the various treated groups were assessed by One-way ANOVA continued by Tukey’s post hoc tests. $P < 0.05$ was considered significant.

3. Results and discussions
3.1. Results
3.1.1. Zoledronic acid, 5-FU and flutamide decreased PC-3 and DU-145 cell viability in a concentration-dependent manner
Treatment of PC-3 and DU-145 cells with ZA, 5-FU, and flutamide gradual concentrations (0.1, 1, 10, 100 and 1000 μM) resulted in decrease of cell viability in a concentration-dependent manner. IC₅₀ values in PC-3 cells were (13.47, 8.23, and 9.42 μM) and in DU-145 cells were (38.77, 17.6, and 8.47 μM) for ZA, 5-FU, and flutamide respectively (Figure 1).

![Figure 1](image-url)

Figure 1. In a concentration-dependent manner, zoledronic acid, 5-FU, and flutamide reduced PC-3 and DU-145 cell viability. Our researched prostate cancer cells were treated for 48 hours with increasing concentrations of ZA, 5-FU, and flutamide (0.1, 1, 10, 100, and 1000 μM), and cell viability was calculated using the MTT method. The results were expressed as $\bar{x} \pm SD$ of three separate repeats (n=3). To assess statistical significance, a one-way ANOVA was used, followed by Tukey’s post hoc multiple comparison tests. a: significant from control untreated group, b: significant from 0.1μM concentration, c: significant from 1μM concentration, d: significant from 10 μM concentration, e: significant from 100 μM concentration, at $P < 0.05$
3.1.2. Zoledronic acid synergistically enhanced the cytotoxic effects of 5-FU and flutamide against PC-3 and DU-145 cells

Combination therapy (½ IC₅₀ of ZA + ½ IC₅₀ of 5-FU and ½ IC₅₀ of ZA + ¾ IC₅₀ of flutamide) improved the cytotoxicity when compared to a single treatment. CI for the interactions between ZA either with 5-FU or flutamide on PC-3 and DU-145 cells were <1 indicating a synergistic interaction (Table 1).

Table 1. Combination index for the interaction after ZA co-treatment either with 5-FU or flutamide on PC-3 and DU-145 cell viability using non-constant ratio model

<table>
<thead>
<tr>
<th>PC-3 cells (µM)</th>
<th>5-FU</th>
<th>CI</th>
<th>ZA</th>
<th>Flutamide</th>
<th>CI</th>
<th>ZA</th>
<th>5-FU</th>
<th>CI</th>
<th>ZA</th>
<th>Flutamide</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.37</td>
<td>2.06</td>
<td>0.39</td>
<td>3.37</td>
<td>2.36</td>
<td>0.23</td>
<td>9.69</td>
<td>4.40</td>
<td>1.40</td>
<td>9.69</td>
<td>2.11</td>
<td>1.53</td>
</tr>
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<td>3.37</td>
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<td>0.32</td>
<td>3.37</td>
<td>4.71</td>
<td>0.20</td>
<td>9.69</td>
<td>8.80</td>
<td>0.92</td>
<td>9.69</td>
<td>4.23</td>
<td>0.75</td>
</tr>
<tr>
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<td>6.17</td>
<td>0.24</td>
<td>3.37</td>
<td>7.07</td>
<td>0.17</td>
<td>9.69</td>
<td>13.20</td>
<td>0.94</td>
<td>9.69</td>
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</tr>
<tr>
<td>6.74</td>
<td>2.06</td>
<td>0.32</td>
<td>6.74</td>
<td>2.36</td>
<td>0.20</td>
<td>19.38</td>
<td>4.40</td>
<td>0.92</td>
<td>19.38</td>
<td>2.11</td>
<td>0.61</td>
</tr>
<tr>
<td>6.74</td>
<td>4.12</td>
<td>0.20</td>
<td>6.74</td>
<td>4.71</td>
<td>0.16</td>
<td>19.38</td>
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<td>0.49</td>
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<tr>
<td>6.74</td>
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<td>0.17</td>
<td>6.74</td>
<td>7.07</td>
<td>0.09</td>
<td>19.38</td>
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<td>0.68</td>
<td>19.38</td>
<td>6.33</td>
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</tr>
<tr>
<td>10.1</td>
<td>2.06</td>
<td>0.16</td>
<td>10.1</td>
<td>2.36</td>
<td>0.15</td>
<td>29.07</td>
<td>4.40</td>
<td>0.46</td>
<td>29.07</td>
<td>2.11</td>
<td>0.44</td>
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<tr>
<td>10.1</td>
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<td>0.11</td>
<td>10.1</td>
<td>4.71</td>
<td>0.11</td>
<td>29.07</td>
<td>8.80</td>
<td>0.38</td>
<td>29.07</td>
<td>4.23</td>
<td>0.20</td>
</tr>
<tr>
<td>10.1</td>
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<td>0.06</td>
<td>10.1</td>
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<td>0.05</td>
<td>29.07</td>
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<td>0.28</td>
<td>29.07</td>
<td>6.33</td>
<td>0.02</td>
</tr>
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</table>

The most effective combination of lower CI was 4.12 µM of 5-FU or 7.07 µM of flutamide with 6.74 µM of ZA for PC-3 cells, while the most effective combinations with lower CI were 8.80 µM of 5-FU or 6.33 µM of flutamide with 19.38 µM of ZA for DU-145 cells. Therefore, these combinations were used in all further experiments (Figure 2, 3 and 4).

Figure 2. Using a non-constant ratio design, a combination index plotting of ZA combined therapy with 5-FU or flutamide on PC-3 and DU-145 cell viability was established. PC-3 cells were treated with a combination of (3.37, 6.74, 10.1 µM) of ZA either with (A) (2.06, 4.12, 6.17 µM) of 5-FU or (B) (2.36, 4.71, 7.07 µM) of flutamide, while DU-145 cells were treated with (9.69, 19.38, 29.07 µM) of ZA either with (C) (4.4, 8.8, 13.2 µM) of 5-FU or (D) (2.11, 4.23, 6.33 µM) of flutamide for 48 h. CompuSyn software was used to measure the combination index (CI) for the degree of interaction between ZA and 5-FU or flutamide on the previous cancer cells. CI for these results was < 1 suggesting a synergistic effect. CI: the combination index, Fa: the fractional effect.
Figure 3. The cytotoxic activity of 5-FU and flutamide instead of PC-3 were enhanced by zoledronic acid in a synergistic manner. PC-3 cells were treated for 48 hours by (3.37, 6.74, and 10.1 μM) of ZA either with (A) (2.06, 4.12, and 6.17 μM) of 5-FU or (B) with 4.12 μM of 5-FU, or (C) with (2.36, 4.71, and 7.07 μM) of flutamide or (D) with 7.07 μM of flutamide, then using MTT to assess the cell viability, and calculated the IC_{50} of each combination. Our results were expressed as χ¯± SD of three separate repeats (n=3). To assess statistical significance, a one-way ANOVA was used, followed by Tukey's post hoc multiple comparison tests. a: significant from the control untreated group, b: significant from 5-FU group alone or flutamide group alone, c: significant from the related concentration of ZA group at P < 0.05.
Figure 4. Zoledronic acid increased the cytotoxic activity of 5-FU and flutamide against DU-145 in a synergistic manner. DU-145 cells were treated for 48 hours by (9.69, 19.38, and 29.07 μM) of ZA either with (A) (4.40, 8.80, and 13.20 μM) of 5-FU or (B) with 8.80 μM of 5-FU, or (C) with (2.11, 4.23, and 6.33 μM) of flutamide or (D) with 6.33 μM of flutamide, then using MTT to assess the cell viability, and calculated the IC₅₀ of each combination. Our results were expressed as $\bar{x} \pm SD$ of three individual repeats (n=3). To assess statistical significance, a one-way ANOVA was used, followed by Tukey's post hoc multiple comparison tests. a: significant from the control untreated group, b: significant from 5-FU group alone or flutamide group alone, c: significant from the related concentration of ZA group at P < 0.05

3.1.3. Zoledronic acid co-treatment with 5-FU or flutamide increased caspase 3 and Bax expressions, and down-regulated the expression of cell cycle regulatory protein cyclin D1 on PC-3 and DU-145 cells

The protein expression of Bax, cleaved caspase-3 and cyclin D1 was evaluated by western blot analysis. Our results showed that combined therapy from both (ZA/5-FU and ZA/Flutamide) cooperatively increased pro-apoptotic proteins caspase 3 and Bax expressions, while decreased cell cycle regulatory protein cyclin D1 expression in compared to every single drug, highlighting the utility and synergistic value of ZA co-treatment (Figure 5 and 6).
**Figure 5.** In PC-3 cells, zoledronic acid co-treatment with 5-FU or flutamide increased Bax and caspase 3 expression levels while reducing the expression of cyclin D1. The previous proteins expressions were examined by western blotting technique in PC-3 cells of various groups. PC-3 cells were treated with ZA (6.74 μM), either with 5-FU (4.12 μM) or flutamide (7.07 μM), combined ZA either with 5-FU or flutamide for 48h. β-actin was used as the internal control. Graphical results expressed as $\bar{x} \pm$ SD of three independent repeats (n=3). Statistical significance evaluated by One-way ANOVA followed by Tukey's post hoc multiple comparison tests. a: significant from the control untreated group at $P < 0.05$. 

![Western Blot and Bar Graphs](image-url)
Figure 6. In DU-145 cells, zoledronic acid co-treatment with 5-FU or flutamide increased level of expressions of caspase 3 and Bax while reducing the expression of the cell cycle regulator cyclin D1. The previous proteins expressions were examined by western blotting technique in DU-145 cells of different forms. DU-145 cells were treated with ZA (19.38μM) either with 5-FU (8.80 μM) or flutamide (6.33 μM), combined ZA either with 5-FU or flutamide for 48h. β-actin act as the internal control. Graphical results expressed as χ± SD of three independent repeats (n=3). Statistical significance evaluated by One-way ANOVA followed by Tukey's post hoc multiple comparison tests. a: significant from the control untreated group at P < 0.05

3.1.4. Combined ZA either with 5-FU or flutamide on PC-3 reduced Bcl-2 and enhanced p53 expressions

The expression of Bcl-2 was reduced after treatment when compared to untreated control cells. Flutamide-treated cells showed a significantly lower difference in Bcl-2 expression compared to ZA and 5-FU treated cells. Both combinations showed significantly lower levels of Bcl-2 compared to single drug-treated cells, concluding a remarkable decrease in the antiapoptotic protein (Figure 7). On the otherwise, p53 levels were elevated after treatment and there were significant differences in ZA-treated cells as compared to 5-FU and Flutamide-treated cells, both combinations showed significantly higher levels of p53 compared to single drug-treated cells indicating an enhancement in the tumor suppressor level (Figure 7).
Figure 7. Immunofluorescence staining reveals the expression of Bcl2 (A) and p53 (B) in PC-3 with single and combined drugs. When compared to untreated control cells, Bcl-2 expression was decreased after treatment; however, both combinations showed elevated levels of p53 expression when compared to single drug-treated cells. Blue (DAPI): nucleus; green: Bcl2 or p53 respectively. a: significant from control untreated group b: significant from ZA. c: significant from 5-FU. d: significant from Flutamide at p < 0.05. Using mean ± standard deviation (x̄ ± SD) to expressed data

3.1.5. Combination of ZA with 5-FU and flutamide was associated with modulation in MiRNAs expressions in PC-3 cells and DU-145 cells

To evaluate the probable mechanism by which these enhancements in the cytotoxic effect of 5-FU and flutamide after co-treatment with ZA mediated in part through modulation of miRNAs expression, Oncogenic miR-18a and the tumor suppressor miR-382 expression levels were assessed by qRT-PCR using RNU6B as an endogenous control. In PC-3 cells, the oncogenic miR-18a was down-regulated after
treatment with ZA, 5-FU, Flutamide, ZA/5-FU, and ZA/Flutamide by 4.50, 4.17, 3.60, 2.90, and 2.40 folds respectively as compared to control untreated cells. At the level of the combination, both combinations ZA with 5-FU or flutamide showed significant downregulation in the oncogenic miR-18a expression when compared to single drug-treated cells. On the other hand, miR-382 was up-regulated after treatment with ZA, 5-FU, Flutamide, ZA/5-FU, and ZA/Flutamide by 1.92, 2.30, 2.83, 3.06, and 3.53 folds respectively when compared to control untreated cells. At the level of the combination, both combinations ZA with 5-FU or flutamide showed a significant increase in miR-382 expression when compared to single drug-treated cells (Figure 8). Also, in DU-145 cells we found down-regulation for miR-18a expression after treatment with ZA, 5-FU, Flutamide, ZA/5-FU, and ZA/Flutamide by 6.83, 6.18, 4.59, 3.14, and 2.89 folds respectively as compared to control cells. At the level of the combination, both combinations ZA with 5-FU or flutamide showed significant down-regulation in the oncogenic miR-18a expression when compared to single drug-treated cells. While miR-382 was up-regulated after treatment with ZA, 5-FU, Flutamide, ZA/5-FU, and ZA/Flutamide by 2.90, 3.29, 3.81, 5, and 5.50 folds respectively when compared to control cells. At the level of the combination, both combinations ZA with 5-FU or flutamide showed a significant increase in miR-382 expression when compared to single drug-treated cells (Figure 9). These results showed that enhancement in the cytotoxic effect of 5-FU and flutamide after co-treatment with ZA may be mediated at least in part through regulation of miRNAs expression. Particularly decrease in the expression level of miR-18a and increase in the expression of miR-382. Our results can be summarized as shown in (Figure 10).

Figure 8. Using RNU6B as an endogenous control, co-treatment of ZA with 5-FU or flutamide regulated MiRNA expression in PC-3 cells by qRT-PCR. Decreased expression of miR-18a and increased expression of miR-382 in (PC-3) cells after treatment with half maximal inhibitory concentration (IC$_{50}$) of ZA, 5-FU, Flutamide, and combined ZA with 5-FU or Flutamide. a: significant from control group and b: significant from ZA at p < 0.05. Our data expressed as ($\bar{x}$ ± SD)
Figure 9. Using RNU6B as an endogenous control, co-treatment of ZA with 5-FU or flutamide regulated MiRNA expression in DU-145 cells by qRT-PCR. Decreased of miR-18a and enhanced of miR-382 expressions in (DU-145) after treatment with half maximal inhibitory concentration (IC_{50}) of ZA, 5-FU, Flutamide, and combined ZA with 5-FU or Flutamide. a: significant from control untreated group and b: significant from ZA at p < 0.05. Our data expressed as (χ± SD).

Figure 10. The following is a diagram of our observations. Treatment of prostate cancer PC-3 and DU-145 cells with ZA and 5-FU or flutamide resulted in up-regulation of miR-18a and miR-382, which in turn may be incorporated in decreased cyclin D1 and Bcl-2 expression while increasing Bax, caspase 3, and p53 expression, triggering the arrest of cell cycle and increased apoptosis. As a result, cell proliferation was reduced while cell death was increased.
3.2. Discussions

MiRNAs regulate apoptosis, cell growth and proliferation, autophagy, invasion, metastasis, and the epithelial-to-mesenchymal transition (EMT) in PC, and thus play an important role in cancer progression. In PC, evidence suggests that various miRNAs play a role in cancer initiation, progression, and metastasis by functioning as anti-neoplastic tumor suppressors (down-regulated) or oncogenic oncomiRs (up-regulated) [25]. Furthermore, miRNAs play an indispensable role in therapy response. As a result, controlling miRNAs, which are deregulated in cancer, may be a promising strategy for enhancing the anti-androgen flutamide and the chemotherapeutic 5-FU’s chemotherapeutic efficacy [26, 27]. In PC therapy, innovative therapeutic techniques and/or novel adjuvant drugs are critically needed [11].

For clinical applications, a drug combination has been recommended that can not only enhance therapeutic effects but also minimize chemotherapy clinical doses, thus reducing toxic side effects [7]. To our knowledge, this is the first research to study the effects of combining ZA as a BPs that plays a role in miRNA profile expression with 5-FU or flutamide, two of the most widely used chemotherapeutic agents for PC. According to our findings, the viability of prostate cancer PC-3 and DU-145 cells is decreased by ZA, 5-FU, or flutamide in a dose-dependent manner.

This result was more pronounced in a synergistic manner after ZA was combined with 5-FU or flutamide, suggesting that ZA in combination with 5-FU or flutamide could be useful against PC. These findings corroborated a previous study that found that treating PC-3 cells with a combination of Thymoquinone and ZA resulted in substantial synergistic cytotoxic activity [28]. In PC-3 and DU-145 cells, a combination of ZA with the serine/threonine protein phosphatase inhibitors; calyculin A and okadaic acid had synergistic cytotoxic and apoptotic effects [29]. Furthermore, our previous cancer cells when treated with ZA in combination with 5-FU or flutamide resulted in lower expressions of Bcl-2 and cyclin D1, as well as higher expressions of pro-apoptotic caspase 3 and Bax than in single drug-treated and untreated cells.

These findings showed that after 5-FU or flutamide co-treatment with ZA, there was a substantial increase in chemotherapeutic efficiency and increased PC-3 and DU-145 cell death, as well as coupled with an increase in apoptotic protein markers and suppress in anti-apoptotic ones.

Co-treatment also increased the tumor suppressor p53 and decreased the cell cycle regulator cyclin D1, demonstrating the possible importance of these adjuvant therapies in cancer progression regulation. When compared to single-drug treatment of PC3 cells, findings from other studies showed that the combination of 5-FU and rutin significantly suppressed Bcl-2 protein and enhanced p53 expression [30]. In human colon cancer cells, the combination of rutin and hypersoide also decreased Bcl-2 expression [31].

Furthermore, we investigated the effect of chemotherapeutic drugs, ZA, 5-FU, flutamide alone and/or in combined forms on the expression of miR-382 and miR-18a in PC-3 and DU-145 cells to establish the probable mechanism by which the synergistic increase in the cytotoxic efficiency of 5-FU, flutamide after co-treatment with ZA against PC occurs. Our findings revealed that treatment with ZA, Flutamide, and 5-FU individually resulted in over-expression of miR-382, an effect that was exacerbated after co-treatment, demonstrating the synergistic effect of ZA with 5-FU or Flutamide. This is consistent with previous studies that found tumor suppressor microRNAs were up-regulated after chemotherapy in various cancer cells, such as miR-16 in colorectal cancer cells and MCF-7 after 5-FU and irinotecan treatment [32, 33]. On the other hand, the oncogenic miR 18a, a widely expressed miRNA in PC-3 and DU145, was down-expressed after treatment with single drugs and even more so after ZA co-treatment with 5-FU or flutamide. Other studies have found that oncogenic miRNA levels are decreased after chemotherapeutic treatment [32, 34]. These findings suggest that ZA enhances the cytotoxic activity of flutamide and 5-FU in PC in part due to its effect on altered miRNAs.

Despite this, there are some pitfalls in our study. For example, we should investigate the effects of our chosen combinations on various types of PC cells, more miRNAs, and the effectiveness of these combinations on cell cycle factors, as well as in vivo studies.
4. Conclusions

The enhancement in the cytotoxic effect of flutamide and 5-FU after combined treatment with ZA is mediated at least in part through regulation of the expression levels of altered miRNAs in PC. Particularly the oncogenic miR-18a and the tumor suppressor miR-382, their expression levels are affected by the chemotherapeutic co-treatment by down-regulation or up-regulation, respectively. Finally, we can conclude that when ZA is combined with 5-FU or Flutamide, the therapeutic results are synergistic and superior to single-drug therapy. Low doses and consequently low side effects make these combinations more effective. The increased cytotoxicity of 5-FU and Flutamide after co-treatment with ZA is mediated, at least in part, by the control of the expression levels altered miRNA expression levels in PC. Particularly, the expression levels of oncogenic miR-18a and tumor suppressor miR-382 are both affected by chemotherapeutic co-treatment, with down-regulation or up-regulation, respectively.

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