Synergistic Solutions: Exploring Clotrimazole’s Potential in Prostate and Bladder Cancer Cell Lines

Mariana Pereira 1,2,3 and Nuno Vale 1,2,4,*

1 PerMed Research Group, Center for Health Technology and Services Research (CINTESIS), Rua Doutor Plácido da Costa, 4200-450 Porto, Portugal; mariana.m.pereira2097@gmail.com
2 CINTESISRISE, Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal
3 ICBAS—School of Medicine and Biomedical Sciences, University of Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
4 Department of Community Medicine, Health Information and Decision (MEDCIDS), Faculty of Medicine, University of Porto, Rua Doutor Plácido da Costa, 4200-450 Porto, Portugal
* Correspondence: nunovale@med.up.pt; Tel.: +351-220426537

Abstract: Clotrimazole (CLZ), traditionally an antifungal agent, unveils promising avenues in cancer therapy, particularly in addressing bladder and prostate cancers. In vitro assessments underscore its remarkable efficacy as a standalone treatment, significantly diminishing cancer cell viability. Mechanistically, CLZ operates through multifaceted pathways, including the inhibition of Ca²⁺-dependent K⁺ channels, suppression of glycolysis-related enzymes, and modulation of the ERK-p65 signaling cascade, thus underscoring its potential as a versatile therapeutic agent. Our investigation sheds light on intriguing observations regarding the resilience of UM-UC-5 bladder cancer cells against high doses of paclitaxel (PTX), potentially attributed to heightened levels of the apoptosis-regulating protein Mcl-1. However, synergistic studies demonstrate that the combination of Doxorubicin (DOXO) and CLZ emerges as particularly potent, especially in prostate cancer contexts. This effectiveness could be associated with the inhibition of drug efflux mediated by multidrug resistance-associated protein 1 (MRP1), underscoring the importance of exploring combination therapies in cancer treatment paradigms. In essence, our findings shed light on the anticancer potential of CLZ, emphasizing the significance of tailored approaches considering specific cancer types and molecular pathways in drug repurposing endeavors. While further validation and clinical exploration are warranted, the insights gleaned from this study offer promising prospects for enhancing cancer therapy efficacy.

Keywords: bladder cancer; prostate cancer; in vitro evaluation; clotrimazole; drug repurposing; drug combination

1. Introduction

Clotrimazole (CLZ) is a broad-spectrum antifungal drug used to treat candidiasis and various dermatophyte diseases (Figure 1a). It comes in several forms, including pessaries, creams, and troche formulations (slow-dissolving pills) for topical or vaginal applications. Since its discovery in the late 1960s, CLZ, an azole antifungal, has been classified as an imidazole [1]. When used against Candida albicans and other species of Candida in vitro, CLZ has fungistatic activity up to 20 mcg/mL and potentially fungicidal activity at greater doses [2]. Regrettfully, CLZ resistance, uncommon in the past, is now widespread in many patient populations [1].

The main way that CLZ works is by breaking down the fungal cytoplasmic membrane’s permeability barrier. By preventing the demethylation of 14 alpha lanosterol, CLZ suppresses the production of ergosterol in a concentration-dependent manner. The cell can no longer assemble an entire and functioning cell membrane when ergosterol production is blocked. Because ergosterol also directly stimulates fungal cell development...
in a manner akin to hormones, the quick commencement of the processes results in a dose-dependent suppression of fungal growth [3,4]. Other pharmacological targets of CLZ include sarcoplasmic reticulum Ca\(^{2+}\) ATPase inhibition, intracellular calcium depletion, and calcium-dependent potassium and voltage-dependent calcium channels blockage [5].

Besides its prescribed use, CLZ has been shown to have effects on other diseases. Due to its inhibition of potassium (K\(^{+}\)) channels dependent on calcium (Ca\(^{2+}\)), this drug effectively prevents the red cell dehydration of sickle erythrocytes in patients with sickle cell anemia [6,7]. Growth inhibition of the malaria parasite *Plasmodium falciparum* by CLZ was also previously demonstrated, through the disturbance of its hemoglobin catabolism, which is this parasite’s major source of amino acids [8].

The other big area where this drug has been tested, and which is the concern and basis for this study, is cancer therapeutics. Research on human melanoma and glioblastoma cells in vivo revealed that CLZ had an inhibitory impact on cancer cell proliferation and tumor formation. As a calmodulin inhibitor, clotrimazole causes cells’ calcium reserves to be depleted, which is thought to be the cause of its anti-cancer effects [9,10]. Furthermore, clotrimazole inhibits glycolysis in a variety of cancer cell lines, including MCF-7, MCF10A, MDA-MB-231, HeLa, B16-F10, and Lewis lung carcinoma. It does this by targeting important glycolytic enzymes, such as hexokinase, phosphofructokinase, and aldolase [11,12]. It has also been determined that CLZ’s triphenylmethyl pharmacophore is essential to its anti-tumor effects, since analogs with similar structures have strong anticancer effects as well. They cause cell cycle arrest without influencing tubulin polymerization, which lessens adverse effects when compared to other chemotherapeutic medications [13,14]. A study from our research team has also demonstrated the effects of CLZ on PC-3 prostate cancer and HT-29 colon cancer cells, alone and in combination with antineoplastic drugs [15].

Taking all the above information, this study set out to further explore the potential of CLZ in prostate cancer, continuing the work started in Duarte et al. [16] and bladder cancer. The focus of this work is the combination of CLZ with the anticancer drugs Doxorubicin (DOXO, Figure 1b) and paclitaxel (PTX, Figure 1c), which are widespread in the treatment of various cancers [17,18]. Since it has been shown that CLZ works in inhibiting cancer cells, the purpose here is to understand if its combination with antineoplastic drugs increases the anticancer effect of both drugs alone.

![Chemical structures of clotrimazole (a), Doxorubicin (b), and paclitaxel (c). All structures were obtained using ChemDraw software (version 12.0, PerkinElmer, Inc., Waltham, MA, USA).](image)

**Figure 1.** Chemical structures of clotrimazole (a), Doxorubicin (b), and paclitaxel (c). All structures were obtained using ChemDraw software (version 12.0, PerkinElmer, Inc., Waltham, MA, USA). Molecule with blue color is a repurposing drug. Red, are antineoplastic agents or anticancer drugs.
2. Results  
2.1. PC-3 Cell Line

DOXO and CLZ were tested in PC-3 cells alone and in combinations at concentrations of 0.25×, 0.5×, 1×, 2×, and 4× the IC_{50} of the drugs, which is 8 μM [19] and 14.08 μM [15], respectively. This translates to tested concentrations of 2, 4, 8, 16, and 32 μM of DOXO, and 3.52, 7.04, 14.08, 28.16, and 56.32 μM of CLZ. Figures 2 and 3 demonstrates the cell viability results obtained, and Figure 4 the morphological images. In addition, a cell viability bar graph of the combination of PTX and CLZ is also shown in Figure 3 that was the result of the previous study performed in our investigation group that is already published [15]. The IC_{50} values used were 0.01 μM for PTX and 14.08 μM for CLZ, and the graph is shown here to better compare the results obtained.

![Figure 2. Results of PC-3 cell cytotoxicity following exposure to a single drug and a combination of DOXO and CLZ for 48 h. Both drugs were added at the same time and at concentrations of 0.25×, 0.5×, 1×, 2×, and 4× the IC_{50} of the drugs. To the controls cells, 0.1% DMSO (vehicle) was added. The MTT test was used to determine cell viability, and the findings are shown as mean ± SEM (n = 3). **** Indicate p < 0.0001 when compared to the control. **** Statistically significant vs. drug alone at p < 0.0001.](image1)

![Figure 3. Results of PC-3 cell cytotoxicity following exposure to a single drug and a combination of PTX and CLZ for 48 h. Both drugs were added at the same time and at concentrations of 0.25×, 0.5×, 1×, 2×, and 4× the IC_{50} of the drugs. To the controls cells, 0.1% DMSO (vehicle) was added. The MTT test was used to determine cell viability, and the findings are shown as mean ± SEM (n = 3). * Statistically significant vs. drug alone at p < 0.05; *** statistically significant vs. drug alone at p < 0.001; **** statistically significant vs. drug alone at p < 0.0001.](image2)
Figure 4. Morphological evaluation of PC-3 cells after exposure to a single drug and a combination of DOXO and CLZ for 48 h. Both drugs were added at the same time and at concentrations of 0.25 ×, 0.5 ×, 1 ×, 2 ×, and 4 × the IC₅₀ of the drugs. To the control cells, 0.1% DMSO (vehicle) was added. Three separate experiments are represented by these pictures. The scale bar is 200 µm.

The results demonstrate that DOXO and CLZ in combination at 0.25 × IC₅₀ (2 µM DOXO and 3.52 µM CLZ) and 0.5 × IC₅₀ (4 µM DOXO and 7.04 µM CLZ) concentrations significantly reduce cell viability compared to control and individual treatments. It was also found that DOXO and CLZ alone or in combination at higher concentrations (1 × IC₅₀ to 4 × IC₅₀) did not significantly reduce cell viability compared to the control, but it is important to note that CLZ alone compared with DOXO alone had similar effects for
higher concentrations. Although the middle concentrations used here were of IC_{50}, the decrease in cell viability was higher than only 50%. However, since the values used were from other studies, this slight change is normal and can be attributed to differences in experiment circumstances. The results suggest that DOXO and CLZ in combination at lower concentrations may have potential as treatments for prostate cancer. When compared with the combination of PTX and CLZ presented in Figure 3 and adapted from [15], DOXO and CLZ lead to an overall higher decrease in cell viability, showing that these drugs are more effective for prostate cancer than PTX and CLZ.

2.2. UM-UC-5 Cell Line
2.2.1. Drugs Alone

To assess cell viability and the IC50 values of DOXO, PTX, and CLZ in UM-UC-5 bladder cancer cells, several concentrations of these drugs were tested for 48 h. These were 0.01, 0.1, 1, 10, 25, and 50 µM of DOXO; 0.01, 0.1, 1, 10, and 25 µM of PTX; and 0.01, 0.1, 1, 10, 25, 50, and 100 µM of CLZ. DOXO and PTX concentration ranges were lower since these are known antineoplastics with high efficacy in decreasing cancer cell viability. The results are demonstrated in a cell graph in Figure 5, as well as through cell morphology images in Figures 6–8.

![Cell viability graphs](image)

**Figure 5.** Results of UM-UC-5 cell viability following exposure to DOXO (a), PTX (b), and CLZ (c) at escalating concentrations for 48 h. To the control cells, 0.01% DMSO was applied (vehicle). The MTT test was used to determine cell viability, and the findings are shown as mean ± SEM (n = 3). **** Indicate p < 0.0001 when compared to the control. **** Statistically significant vs. control (vehicle) at p < 0.0001.
Figure 6. UM-UC-5 morphology after exposure to DOXO for 48 h at concentrations of 0.01, 0.1, 1, 10, 25, and 50 µM (n = 3). Control cells received the vehicle treatment (0.01% DMSO). The scale bar is 200 µm.

Figure 7. UM-UC-5 morphology after exposure to PTX for 48 h at concentrations of 0.01, 0.1, 1, 10, and 25 µM (n = 3). Control cells received the vehicle treatment (0.01% DMSO). The scale bar is 200 µm.
PTX was the most potent drug, with instant decreases in cell viability from 0.01 µM (Figure 5b). However, after 10 µM, the cell viability increases, which could be a signal that this drug can induce a stress response in cells that leads to increased adaptive survival mechanisms of bladder cancer cells to PTX at higher concentrations. The graph also indicates that, when the DOXO concentration rises, cell viability significantly decreases. Nearly no viable cells are left at a dose of 10 µM, suggesting that DOXO is very effective in lowering UM-UC-5 cell viability (Figure 5a). Lastly, the reduction in cell viability with increasing concentrations of CLZ is less drastic compared to DOXO and PTX, as it would be expected since these drugs are directly targeted toward cancer treatment. However, there is still a noticeable decrease in cell viability at higher concentrations of 25 µM and above (Figure 5c).

Table 1 shows a summary of all the IC50 values obtained in this study (in UM-UC-5 cells) as well as in previous studies (for PC-3). It is important to note here that the IC50 value of PTX for UM-UC-5 cells was not obtained in the study due to the increase in cell viability, and the authors decided to use the same value as that previously obtained for PC-3 cells [15].

### Table 1. Summary of the IC50 values of DOXO, PTX, and CLZ in PC-3 and UM-UC-5.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>PC-3</th>
<th>UM-UC-5</th>
</tr>
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<tbody>
<tr>
<td>DOXO</td>
<td>8.00 µM</td>
<td>0.202 µM</td>
</tr>
<tr>
<td>PTX</td>
<td>0.01 µM</td>
<td>0.01 µM</td>
</tr>
<tr>
<td>CLZ</td>
<td>14.08 µM</td>
<td>20.24 µM</td>
</tr>
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Comparing the results of DOXO shows a markedly decreased IC50 for bladder cancer cells (8 µM vs. 0.2016 µM), while this was contrary to what was obtained for CLZ, where there was a slight increase in the IC50 value from prostate to bladder cancer cells (14.08 µM vs. 20.24 µM).
2.2.2. Drug Combination

DOXO and CLZ were tested in UM-UC-5 cells alone and in combinations at concentrations of $0.25\times$, $0.5\times$, $1\times$, $2\times$, and $4\times$ the IC$_{50}$ of the drugs, which were 0.2016 µM and 20.24 µM, respectively. This translates to tested concentrations of 0.054, 0.1008, 0.2016, 0.4032, and 0.8064 µM of DOXO, and 5.06, 10.12, 20.24, 40.48, and 80.96 µM of CLZ. Figure 9a demonstrates the cell viability results obtained, and Figure 10 the morphological images. Similarly, PTX and CLZ were also tested in UM-UC-5 cells alone and in combination in the same model using IC$_{50}$ values of 0.01 µM and 20.24 µM, respectively. This translates to tested concentrations of 0.0025, 0.005, 0.01, 0.02, and 0.04 µM of PTX, and 5.06, 10.12, 20.24, 40.48, and 80.96 µM of CLZ. Figure 9b demonstrates the cell viability results obtained, and Figure 11 the morphological images.

![Figure 9. Results of UM-UC-5 cell cytotoxicity following exposure to a single drug and combinations of DOXO and CLZ (a) and PTX and CLZ (b) for 48 h. Both drugs were added at the same time and at concentrations of $0.25\times$, $0.5\times$, $1\times$, $2\times$, and $4\times$ the IC$_{50}$ of the drugs. To the controls cells, 0.1% DMSO (vehicle) was added. The MTT test was used to determine cell viability, and the findings are shown as mean ± SEM ($n = 3$). *, ** and **** indicate $p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively, when compared to the control. **** Statistically significant vs. drug alone at $p < 0.0001$.](image-url)
Figure 10. Morphological evaluation of UM-UC-5 cells after exposure to a single drug and a combination of DOXO and CLZ for 48 h. Both drugs were added at the same time and at concentrations of 0.25×, 0.5×, 1×, 2×, and 4× the IC_{50} of the drugs. To the controls cells, 0.1% DMSO (vehicle) was added. Three separate experiments are represented by these pictures. The scale bar is 200 µm.
Figure 11. Morphological evaluation of UM-UC-5 cells after exposure to a single drug and combination of PTX and CLZ for 48 h. Both drugs were added at the same time and at concentrations of 0.25×, 0.5×, 1×, 2×, and 4× the IC\textsubscript{50} of the drugs. To the controls cells, 0.1% DMSO (vehicle) was added. Three separate experiments are represented by these pictures. The scale bar is 200 µm.

The results show little relation between the drugs alone and combined for both DOXO and PTX, and while, in some instances, the decrease is clear, like for 0.25, 0.5, 1, and 4× IC\textsubscript{50} DOXO + CLZ, it is not statistically significant, only for the 4× IC\textsubscript{50} DOXO with 4× IC\textsubscript{50} of DOXO + CLZ (Figure 9a). The results for PTX with CLZ appear to be better,
but it is shown that the combination is never statistically better than PTX alone, with some instances where it is worse, as in $2 \times \text{IC}_{50}$ (0.02 $\mu$M PTX + 40.48 $\mu$M CLZ), which indicates that the effect on decreasing cell viability is mostly related to PTX and that CLZ can even work antagonistically with this drug (Figure 9b).

3. Materials and Methods

3.1. Cell Culture and Reagents

The UM-UC-5 squamous cell cancer cell line and PC-3 human prostate carcinoma cell line were utilized to evaluate the combined toxicity of DOXO, PTX, and CLZ. The American Type Culture Collection (ATCC, Manassas, VA, USA) provided these cell lines, while Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) provided the drugs. All cell reagents were acquired from Millipore Sigma (Merck KGaA, Darmstadt, Germany), and cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution at 37 °C and 5% CO$_2$. Confluent cells were trypsinized using a 0.25% trypsin-EDTA solution (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for maintenance. This was followed by subculturing the cells in fresh DMEM medium and renewing it every 96 h. In the studies, 3000 PC-3 and 5000 UM-UC-5 cells per well were planted onto 96-well plates and allowed to adhere for the whole night.

3.2. Drug Treatment

Cytotoxicity levels in PC-3 cells of DOXO and CLZ were evaluated alone and in combination after 48 h using concentrations of $0.25 \times$, $0.5 \times$, $1 \times$, $2 \times$, and $4 \times$ the IC$_{50}$ of the drugs, 8 $\mu$M and 14.08 $\mu$M, respectively, as described in previous articles [15,19].

For the UM-UC-5 cell line, no studies exist, so the drugs DOXO, PTX, and CLZ were first tested alone for 48 h to assess the IC$_{50}$ of these drugs. DOXO was tested at concentrations of 0.01, 0.1, 1, 10, 25, and 50 $\mu$M; PTX was tested at concentrations of 0.01, 0.1, 1, 10, and 25 $\mu$M; and finally CLZ was tested at concentrations of 0.01, 0.1, 1, 10, 25, 50, and 100 $\mu$M. For the combination studies, the same method used in the PC-3 cells was applied to assess the combination of DOXO and CLZ, as well as PTX and CLZ.

In all cases, the control cells were treated with 0.1% of the vehicle dimethyl sulfoxide (DMSO) in which the drugs were dissolved.

3.3. Morphological Analysis

Cell morphology was assessed using a Leica DMI 6000B microscope fitted with a Leica DFC350 FX camera (Leica Microsystems, Wetzlar, Germany) after the drug incubation period. Leica LAS X imaging software (v3.7.4) (Leica Microsystems, Wetzlar, Germany) was then used to analyze the acquired images.

3.4. MTT Assay

The colorimetric assay known as MTT (thiazolyl blue tetrazolium bromide) was used to assess the toxicity of the drugs being tested. Following 48 h, 100 $\mu$L of an MTT solution (0.5 mg/mL) in PBS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the cell culture medium after aspirating the cell medium. After incubating the cells for two hours at 37 °C and 5% CO$_2$ in complete darkness, the MTT solution was aspirated, and the purple formazan crystals that had formed were dissolved using 100 $\mu$L of DMSO. An automated microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) was used to measure the absorbance at 570 nm. Cell viability was determined by comparing the absorbance values of the experimental and control groups.

3.5. Statistical Analysis

The cell viability mean ± SEM is displayed in the cell viability graphs, which were generated using the GraphPad Prism 9 program (GraphPad Software Inc., San Diego, CA, USA). Dunnett’s multiple comparisons between the control and experimental groups
of drugs alone were evaluated using one-way ANOVA tests. A two-way ANOVA was used for combination experiments, and the viability outcomes of the combinations were contrasted with the viability outcomes of each drug at the appropriate concentration. At \( p \) values < 0.05, statistical significance was established. All data sets passed the normality test with \( p \)-values greater than 0.05, indicating no significant deviation from normality. We also used Levene’s test to assess homogeneity of variances and confirmed that this assumption was met as well.

The viability findings were first normalized using the viability of the control group and displayed using the logarithmized drug concentrations using a non-linear regression test to create the dose–response curves.

4. Discussion

In this work, the aim was to evaluate the effects of CLZ, a widely used antifungal drug, alone and in combination with DOXO and PTX, two anticancer reference drugs, in PC-3 prostate cancer and UM-UC-5 bladder cancer cells, to evaluate if the repurposing of this drug could be useful for cancer therapy. Repurposing drugs is a quicker and less expensive method of discovering novel, potentially curative treatments for cancer. Repurposed drugs are simpler to approve for another application than new drugs since they have already been authorized for other disorders and have established clinical characteristics. Additionally, drug combination techniques enable a decrease in the drug concentrations required to provide therapeutic effects [20].

CLZ demonstrated interesting effects on decreasing the cancer cell viability of PC-3 and UM-UC-5 cancer cells when used alone. These effects on cancer cells could be related to \( \text{Ca}^{2+} \)-dependent K\(^+\) channels, specifically KCa3.1 channels from the KCNN4 gene, known as IK, which are commonly present in the membranes of cancer cells [21]. The high expression of this channel has been correlated to the increased aggressiveness of cancer [16,22] and progression of cell cycle and migration activities of cancer cells [23]. As said above, CLZ has been known to block these channels [6]; hence, this pathway could be a potential mechanism of action of CLZ in cancer. Other pathways could involve the inhibition of enzymes associated with glycolysis, which is an often activated in cancer cells as a new adaptation mechanism that allows proliferation and tumor progression [24]. Examples of such enzymes are hexokinases, which, when highly bound to mitochondria, are associated with decreased apoptosis and the protection of cancer cells [25], and CLZ has been shown to detach this enzyme from the mitochondria and increase the apoptosis of melanoma and breast cancer cells [12,26]. Another such example is the detachment of aldolase and phosphofructokinase-1 (PFK-1) from the cytoskeleton by CLZ, which impedes glycolysis and decreases cell viability [12,27]. Lastly, of interest, it is known that CLZ regulates levels of the ERK-p65 signaling pathway, involved in epithelial mesenchymal transition and a key regulator of cell proliferation, survival, and migration, and its dysregulation is often associated with cancer progression. CLZ has shown a decrease in this enzyme and an associated invasion and migration of hepatocellular carcinoma cells [28]. This pathway is known to be relevant in both bladder [29] and prostate [30] cancers.

A result also worth mentioning is the study of PTX alone in UM-UC-5 cells. At PTX concentrations above 10 \( \mu \text{M} \), bladder cancer cells developed resistance to this drug that increased cellular viability (Figure 5b). This resistance at high concentrations was further confirmed in combination studies, since there was a decrease in cell viability with an increased concentration, but these concentrations never exceeded 0.04 \( \mu \text{M} \) of PTX (Figure 9b). One of the main mechanisms of PTX resistance in bladder cancer cells is the overexpression of a protein called Mcl-1, which is involved in regulating apoptosis. These cells can increase their expression of Mcl-1 to prevent apoptosis induced by PTX, effectively protecting themselves against this drug [31].

In this study, the combination of the three drugs was also tested. The concentrations chosen were around the IC\(_{50}\) of each drug, which is a common approach in pharmacological studies, since the concentrations would all fall around the effective and most relevant
range of these drugs, making it directly relevant to the drug’s therapeutic use. Studying concentrations around the \( IC_{50} \) also allows for a better understanding of the dose–response relationship, which is often nonlinear. Furthermore, using the \( IC_{50} \) as a reference point allows for a comparison between different drugs or different cell lines, standardizing for potency (Figure 12).

![Combination model](image)

**Figure 12.** Combination model used in this study of concentrations around the \( IC_{50} \) and its benefits.

The findings overall demonstrate that the superior combination tested is DOXO and CLZ, and especially in prostate cancer. This could be related to altered expression levels of the multidrug resistance-associated protein 1 (MRP1), which is a membrane-associated protein that functions as an ATP-dependent efflux pump for various substrates, including drugs and organic anions, like glutathione disulfide. MRP1 contributes to multidrug resistance in cancer cells by exporting chemotherapeutic agents out of the cells, reducing their intracellular concentrations and, thus, their efficacy [32]. The levels of this transporter in prostate cancer often rise with the disease’s stage and degree of invasiveness. Moreover, prostate cancer cell lines treated to the DNA intercalating drug DOXO exhibit chemoresistance due to MRP1 overexpression [33]. CLZ was shown to inhibit drug efflux mediated by MRP1 in a concentration-dependent way in two MRP1-expressing cell lines in a comparable manner to known MRP1 inhibitors, potentially due to the interference with either the binding or the hydrolysis of ATP, which is crucial for MRP1 functioning since it is ATP-dependent [34]. Hence, CLZ could potentially block MRP1 channels and lead to a higher intracellular concentration of DOXO, increasing its effect.

On the other hand, the findings of this current work regarding the PTX and CLZ combination were not as expected (when compared with isolated \( IC_{50} \) values added shortly afterwards), since very similar concentrations of PTX and CLZ (0.0125 and 25 \( \mu \)M, respectively) were shown in another study to synergistically act against breast cancer cells by inducing genotoxicity and decreasing glucose uptake through nitrogen and oxidative stress [35]. This could be related to alterations in many factors associated with different cancers, such as in drug resistance mechanisms or drug targets. Prostate cancer is also a widely heterogeneous disease, with a highly variable genetic makeup and mutations that often lead to resistance to existing therapy. This increases the need for other treatments, such as using repurposed drugs, but is also important to take into account when performing experiments, since different cancer cell lines or even the same line after several passages can provide different results [36].
In the future, mechanistic studies will be developed to understand if the purposed mechanisms of action of CLZ alone and in combination mentioned throughout the discussion are valid, to better understand the potential of this drug.

5. Conclusions

This study aimed to evaluate the potential repurposing of CLZ, an antifungal drug, for cancer therapy, particularly in PC-3 prostate cancer and UM-UC-5 bladder cancer cells. The results indicate that CLZ demonstrates significant effects in reducing the viability of cancer cells when used alone. The potential mechanisms of action could involve the inhibition of Ca\(^{2+}\)-dependent K\(^{+}\) channels, specifically KCa3.1 channels, the inhibition of enzymes related to glycolysis, and regulation of the ERK-p65 signaling pathway.

The study also highlighted the resistance of UM-UC-5 bladder cancer cells to PTX at high concentrations, which could be attributed to the overexpression of the apoptosis-regulating protein Mcl-1. In combination studies, the most effective combination was found to be DOXO and CLZ, particularly in prostate cancer. This efficacy could be linked to the inhibition of drug efflux mediated by MRP1, which is a protein associated with cancer cell protection. Interestingly, the expected synergistic effects of the PTX and CLZ combination, as observed in breast cancer cells, were not replicated in these cells. The discrepancies may be attributed to differences in cancer types, drug resistance mechanisms, or specific drug targets.

In summary, the study provides valuable insights into the potential anticancer effects of CLZ, either alone or in combination with other drugs, and underscores the importance of considering specific cancer types and associated molecular pathways in drug repurposing efforts. Further research is warranted to explore the clinical relevance and applicability of these findings in cancer therapy.

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References
13. Almeida, A.; Bolaños, J.P.; Moncada, S. E3 ubiquitin ligase APC/C-Cdh1 accounts for the Warburg effect by linking glycolysis to cancer cell proliferation, viability and glycolysis. PLoS ONE 2012, 7, e30462. [CrossRef] [PubMed]
18. Liu, X.; Gao, J.; Sun, Y.; Zhang, F.; Guo, W.; Zhang, S. Clotrimazole Inhibits HCC Migration and Invasion by Modulating the ERK-p65 Signaling Pathway. PloS ONE 2022, 17, e0266710. [CrossRef] [PubMed]


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