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Potential Otubain 1 (OTUB1) Ligand, Advances for a Treatment Against Prostate Cancer

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Abstract: Advances in developing new anticancer drugs continue, and one of the new therapeutic targets to reach is Otubain 1 (OTUB1) because OTUB1 has functions related to prognosis in a variety of tumors, and this protein is strongly related to tumor proliferation, migration, and apoptosis by its deubiquitinating function. This study evaluated a potential OTUB1 ligand, the OT3 compound, which our research group previously proposed as a compound directed to interact with OTUB1´s active site to develop a drug to regulate OTUB1´s functions. The aim of this research is to evaluate the effects of the OT3 compound on OTUB1´s functions in prostate cancer (PC3) by *in vitro* cultures. We determined the cytotoxic effect of the OT3 compound on PC3 cells by MTT assays and examined the regulation of p-FAK and Caspase-3, which are involved in decreasing the viability of the cultures and regulating apoptosis, as determined by western blot analysis. Then, the OT3 compound was analyzed using molecular dynamics to describe the interactions of the OT3 compound with OTUB1´s active site. We propose that the OT3 compound has a high probability of being selective against OTUB1, with apoptosis (regulating caspase-3) and cytotoxic effect on prostate cancer lines; IC50 140 µM, as well as we describe that this compound could have specific interactions in the OTUB1´s active site, decreasing the OTUB1 functions, and is probably safe for humans. These results show the high potential of this compound as a new drug against prostate cancer.

Keywords: apoptosis; caspase-3; PC3; drug.

1. Introduction

The development of new anticancer drugs against new therapeutic targets is increasing worldwide. Our research group reported ten compounds (OT1 - OT10) that were selected by molecular docking [1]. It was performed using the OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) as a receptor because this protein is a promising therapeutic target against cancer, highlighting functions related to the prognosis in some cancer types [2-4]. Particularly in prostate cancer, OTUB1 promotes invasion and tumorigenesis by its functions on Rho-A [5] and Cyclin E1 [4], and some studies show OTUB1 regulating P53 to promote tumor cell survival, proliferation, invasiveness, prognosis, and therapeutic resistance [6-8]. Thus, all of this further strengthens the role of OTUB1 in the progression and development of prostate cancer [4].

OTUB1 has been reported to be present in many cancer tissues and has been negatively associated with the poor condition of cancer patients because OTUB1 is highly expressed in

many cancers [6,9,10]. Otherwise, there are studies on the inhibition of the OTUB1 [3,6]. Recently, we tested some compounds in breast cancer cell lines, and we proposed a cytotoxic effect due to the regulation of OTUB1 by the OT5 compound [11].

In this study, we tested the OT3 compound with prostate cancer cells (PC3) *in vitro*. Using PC3 is promising because it has important messengers to reach and promote the viability of this cancer type; some of these messengers are FAK, Rho-A, and Cyclin E1 [4, 12-16], where OTUB1 could have functions. Also, OTUB1 could regulate apoptosis in cancer cells; thus, any development of an anticancer drug is important to generate apoptosis [17]. There are studies related to an OTUB1 inhibitory effect [11,18,19]. These studies used OTUB1 as a therapeutic target [20] with promising results that demonstrated its potential therapeutic value.

In this study, we evaluated the OT3 compound that we previously reported [1]. It could interact with OTUB1 (Asp88, Cys91, and His265) [9,21,22], and in this way, this compound could regulate OTUB1´s function related to the prognosis of cancer [4,5]. It was determined by molecular dynamics simulation, cytotoxicity, MTT, and western blot assays.

2. Materials and Methods

2.1. Reagents.

Cell culture reagents were purchased from Thermo-Fisher (Carlsbad, CA, USA), and tissue culture plates and other plastic materials were obtained from Corning Inc. The following primary antibodies against p-FAK (Tyr397), Caspase-3, and β-actin were used from Santa Cruz Biotechnology, Inc. (TX, USA). Secondary antibodies conjugated with horseradish peroxidase were used from Sigma-Aldrich (MO, USA), and the OT3 compound previously reported [1] was bought in Chembridge Corp [23] (ID-Chembridge: OT3: 5194547; PubChem CID: 2833016). The purity of the compound reported by the manufacturer was greater than 95%, as checked by LC/MS. The stock solutions for compounds were prepared in DMSO (1-10 mM), and MTT was obtained from Merck (Darmstadt, Germany).

2.2. Cell culture.

The hepatocyte cells (C9) and prostate cancer (PC3) cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in 100 mm Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3.7 g/L sodium bicarbonate, 5% (PC3) and 10% (C9) fetal bovine serum (FBS), and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3. Cytotoxicity assay in PC3 and C9 cell cultures determined by MTT.

PC3 cells were cultured in 96-well microplates following the provider's recommendations, and the cytotoxic effect was determined by MTT assay as described previously [24,25]. Briefly, cells were seeded (10,000 cells per well) and treated for 48 h with 50, 100, and 150 µM concentrations of OT3 compound in 0.4 % DMSO to determine the cytotoxic effect. After treatment, the media were replaced with a medium containing MTT (0.5 mg/mL), and cells were incubated for an additional 4 h with lysis buffer. The medium was removed, and formazan crystals were dissolved in DMSO. Then, the plate was read at 570 nm by using the iMark™ Microplate Absorbance Reader (Bio-Rad, USA).

On the other hand, for C9 cells, the MTT viability assay was performed as described previously [24,25]. C9 cells in 96-well microplates (10,000 cells per well) were treated for 48 h at concentrations between 10 to 400 µM of OT3 compound, and DMSO at a final concentration of 0.4% was used as vehicle control, following the protocol mentioned [24,25].

The assays were performed in triplicate in three independent experiments, and the percentage of viability was calculated according to the formula:

$$\% \text{ viability} = [\text{mean Optical Density (O. D.) treated cells} \times 100]/ (\text{mean O. D. control cells}) \quad (1)$$

The concentration leading to 50% inhibition of viability (IC_{50}) was calculated by non-linear regression analysis (percentage of viability vs. log concentration) with GraphPad Prism software ver. 8.0 software (GraphPad, CA, USA).

2.4. Western blot analysis.

Plates of six wells were used; 150,000 cells per well were seeded in 1.5 mL of supplemented DMEM medium for 48 h, incubated at 37°C, and 5% CO_2 allowed to reach 90 % confluence. The DMEM-supplemented medium was replaced with the DMEM experimental medium, and cells were incubated at 37°C for 18 h. The medium was removed, and 1 mL of experimental DMEM medium was placed under the OT3 compound at a concentration of 50 µM. Treatments were maintained for 48 hours. Later, treatments were removed, and the cell monolayer was washed once with PBS. Total protein extraction was performed using the RIPA buffer system (ChemCruz sc-24948). For protein separation, 12% SDS-polyacrylamide gels were used to characterize proteins. Proteins were transferred overnight to PVDF membranes at 4°C, 90 mA. Membranes were blocked with 5% low-fat milk/TBST 0.1% and later incubated with the primary antibody overnight at 4°C under gentle agitation. We evaluated these targets: p-FAK (sc-81493), Caspase-3 (sc-7272), and β-actin (sc-8432). The β-actin was used as a loading control—anti-mouse secondary antibodies coupled to Horseradish peroxidase from Thermo-Fisher. Chemiluminescence detection was performed using an Immobilon Western kit (Millipore, MA, USA) and X-ray film for some blots. For others, Bio-Rad ChemiDoc XRS+ was used, and a digital image was obtained.

2.5. Molecular docking of OT3 compound with OTUB1.

Conformers of the OT3 compound and the Otubain 1 structure from the Protein Data Bank [26] (OTUB1, PDB code 3VON) were used to determine the molecular interactions by molecular docking using Molecular Operating Environment (MOE 2024.06.02) following procedures previously reported [27-29].

2.6. Molecular dynamics simulations of the OT3 compound with OTUB1.

The best complex of OTUB1 with compound OT3 generated by molecular docking was used for molecular dynamics simulation (MD). It was performed using Dynamics – MOE and OPLS-AA forcefield. At the beginning, in two consecutive steps of 100 ps, the temperature reached 300 K and a pressure of 1 bar. Finally, a 10 ns MD was carried out. Furthermore, the interactions were analyzed.

2.7. Statistical analysis.

Data results were expressed as mean ± standard deviation (SD) of a minimum of three independent experiments. Averages and standard deviations were calculated in Excel (Microsoft 365, USA). IC_{50} for cytotoxicity of compounds was calculated using GraphPad Prism 8 Software (La Jolla, CA, USA). 3. Results and Discussion

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, and the conclusions that can be drawn.

3. Results and Discussion

3.1. Cytotoxic effect of OT3 compound on PC3 cells cultures.

The OT3 compound that we reported previously to interact with OTUB1 [1] demonstrated a cytotoxic effect at 48 h *in vitro* PC3 cell cultures (Figure 1). After exposure to the C3 compound for 48 h at 100 µM, a cytotoxic effect, loss of monolayer, and very low viability of the culture (Figure 1) were observed.

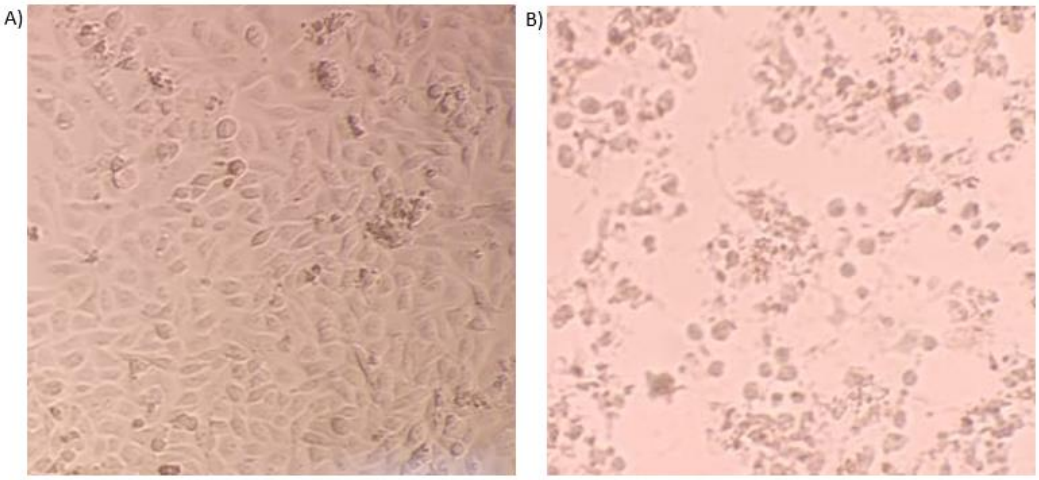


Figure 1. PC3 cell line after 48h of incubation. **(A)** Control with DMSO as a vehicle; **(B)** OT3 compound at 100 µM.

3.2. IC_{50} of OT3 compound.

We determined the IC_{50} using the results of the percentage of viability by MTT. Concentrations of the OT3 compound between 50, 100, and 150 µM were used in PC3 cell cultures (Table 1). With these results, we determined an IC_{50} of 140 ± 1.09 µM for 48 h of incubation using GraphPad Prism 8.

Table 1. Concentrations were used on the OT3 compound, and the viability percentage *in vitro* cultures of PC3 cells was determined after 48 h (n=3).

Concentration (µM)	Averages of viability (percentage)
0	99 ± 3
50	82 ± 4
100	64 ± 4
150	43 ± 6

3.3. Messengers related to the viability of cellular.

We determined p-FAK and Caspase-3 by western blot assays due to these proteins being important to the proliferation [12-14] and in the apoptosis process [30]. We identified that Caspase-3 was increased by OT3 compound at 50 µM, and p-FAK was decreased after 48 h of incubation with PC3 cell cultures (Figure 2). These results were related to the apoptosis effect and less proliferation generated by the OT3 compound in all assays tested (Figure 1).

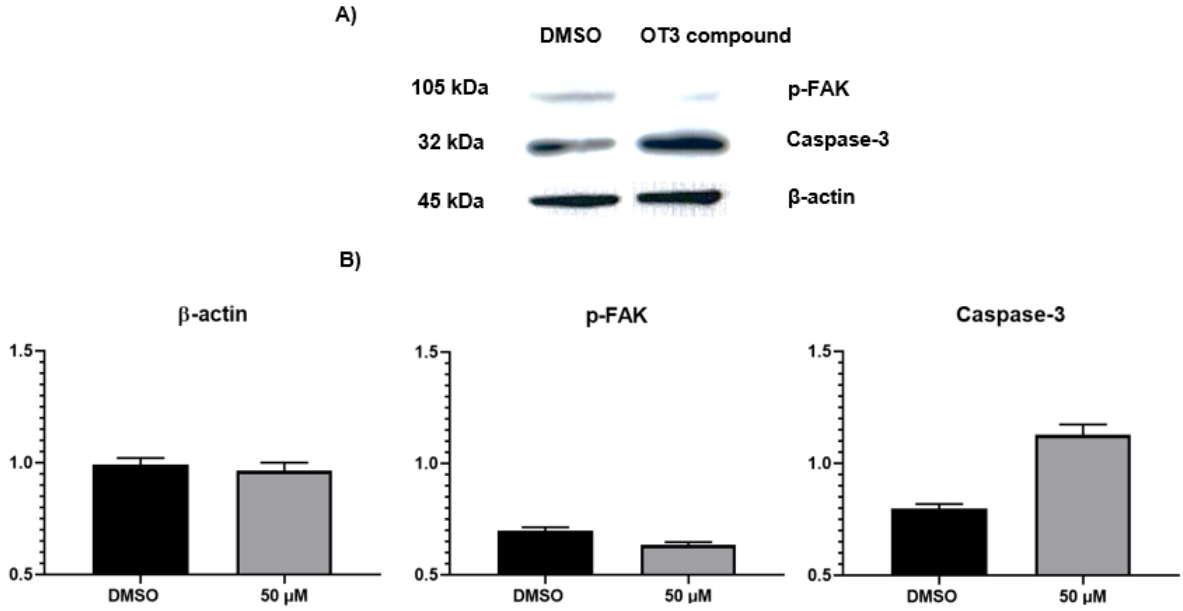


Figure 2. Effects of OT3 compound in PC3 cells. Cells were seeded with a seeding density of $1.5x10^5$ cells and were treated with the OT3 compound at 50 µM for 48 h. The control cells were left untreated (DMSO). The whole cell protein lysate was prepared, and 20 µg of protein was resolved in SDS-polyacrylamide gels. **(A)** Western blot analysis of p-FAK, Caspase-3, and β-actin proteins; **(B)** Densitometry analysis of the data in Western blot analysis by ImageJ software. The values were normalized against β-actin, and they are represented as mean ± SD (n = 3).

3.4. Interaction of OT3 compound with OTUB1.

According to the interactions report [1], we studied the interactions by molecular docking, and the OT3 compound could have interactions in the region between Glu60, Lys84, Asp88, Cys91, and His265 amino acids (Figure 3).

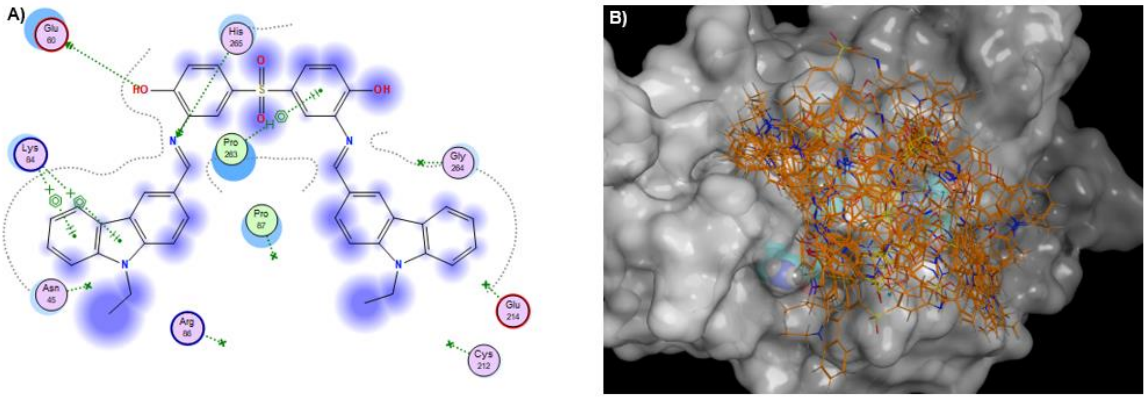


Figure 3. Results of molecular docking: **(A)** The best interaction of OT3 compound in the OTUB1¨s active site; **(B)** OTUB1 (white) with 22 conformers of OT3 compound (orange) in the active site.

The main interactions are in Asn45, Glu60, Lys84, Arg86, Pro87, Glu214, Pro263, Gly264, and His265 in OTUB1; particularly, the greater interactions are with Glu60, Arg86, and His265 amino acids (mainly hydrogen bonding interaction in the conformers analyzed)

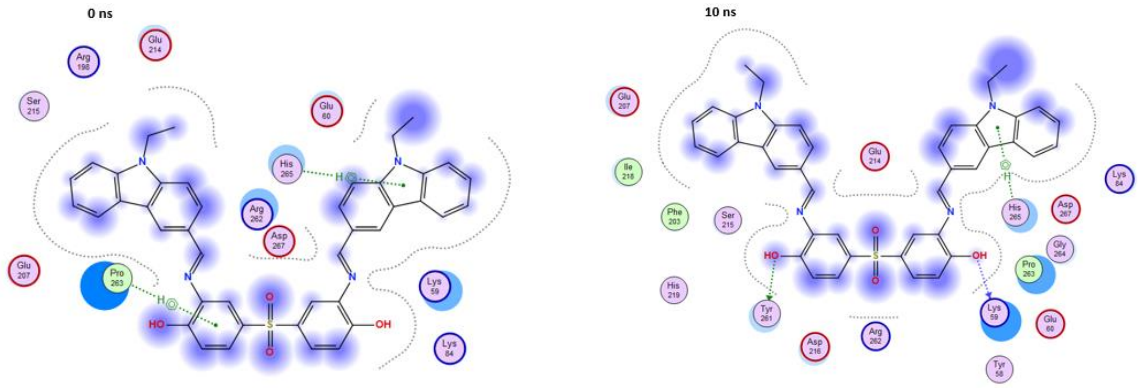
(Figure 3), with these results, the catalytic site could be blocked, which is essential for the deubiquitinating functions of OTUB1. The details of the interaction between OTUB1 and conformers of each compound are shown in the supplementary material (Table S1).

3.5. Molecular dynamics simulation.

To study in more detail the complex OT3-OTUB1, 10 ns of molecular dynamics simulation was performed. The root mean square deviation (RMSD) was analyzed, and the data showed that the inhibitor formed a stable complex almost throughout the simulation, and after 10 ns, the main interactions, notably interactions with Lys59 and His265 (Figure 4). The overall RMSD after 10 ns was 1.405 A, and specifically in the amino acids with the main interactions, the RMSD was between 1.34 and 2.88 A (Table 2).

Table 2. Main interactions and RMSD of OT3 with OTUB1 between 0 – 10 ns.

Time	Ligand	Receptor (Residues in OTUB1)	Interaction	Distance	RMSD (A)
0 ns	O	GLU60	H-donor	2.78	-
	6-ring	PRO263	pi-H	3.83	-
	5-ring	HIS265	pi-H	4.32	
5 ns	O	LYS59	H-donor	2.57	1.34
	5-ring	ARG262	pi-cation	3.90	1.58
10 ns	O	LYS59	H-donor	2.73	2.12
	O	TYR261	H-donor	2.74	2.88
	5-ring	HIS265	pi-H	3.55	1.86



(a) **(b)**

Figure 4. Interactions at **(a)** 0; **(b)** 10 ns from molecular dynamics simulation between OT3 compound and OTUB1.

3.6. Cytotoxicity assay in C9 cell cultures determined by MTT.

We determined the cytotoxic effect of the OT3 compound with hepatocyte cell cultures (C9) up to 400 µM of concentration, and all results were favorable: the OT3 compound had no effect on C9 cells *in vitro* cultures for 48 h. OT3 compound results were similar to the negative control and vehicle/DMSO, and these results are related to in silico results previously reported [1].

3.7. Discussion.

In this study, we evaluated one compound previously reported [1] to develop a specific pharmacological treatment to regulate the OTUB1 functions (mainly as deubiquitinating), mainly these functions related to cancer progression and immune response [4,21,31], and there

are studies to develop specific inhibitors; for example, against lung cancer [32] and breast cancer [11]. Thus, our study focuses on determining the effect of the OT3 compound on prostate cancer (PC3 cells). Prostate cancer is promising because it is reported that important messengers regulate the viability of this cancer type; some of these messengers are FAK, Rho-A [15], and Cyclin E1 [4]. In addition, by *in vitro* assays using prostate cells, the relation between OTUB1 and cyclin E1 for proliferation was demonstrated [10]. Therefore, OTUB1 could generate a regulation for Cyclin E1, Rho-A, and FAK [16] to generate apoptosis (increasing Caspase-3) and, in this way, to decrease the viability of prostate cancer cells.

Results showed that the OT3 compound has a cytotoxic effect caused by apoptosis, and it is important to justify this. We determined an IC_{50} of 140 µM from MTT results (Table 1), and then we determined the decreasing expression of p-FAK (Figure 2), which is related to the regulation of pathogenesis and progression of cancer [12,13,16]. In addition, this result is related to reports that OTUB1 has control over proliferation, and Cyclin E1/Rho-A is regulated by OTUB1 [4,10]. On the other hand, the increasing expression of Caspase-3 was determined (Figure 2). The *in vitro* results showed that the OT3 compound has a cytotoxic effect and an apoptosis effect related to regulating the Caspase-3, as determined by western blot (Figure 2). In addition, we showed changes in morphology and loss of monolayer in cultures *in vitro* (using 100 µM of OT3 compound) that are related to the cytotoxic effect on PC3 cells (Figure 1) and after generating apoptosis [6,11,33-35].

Some studies show the relevance of generating apoptosis by increasing caspases [36-39]. Our results showed that the OT3 compound affects Caspase-3, which could justify the apoptosis effect of this compound after 48 h (Figure 2). Thus, the OT3 compound increased the expression of Caspase-3, and this effect could be related to an apoptotic effect of an anticancer compound [30,33,34,40]. Therefore, we propose that the OT3 compound decreases OTUB1´s function (over Cyclin E1/Rho-A), and in this way, it decreases the viability of the culture that generates apoptosis.

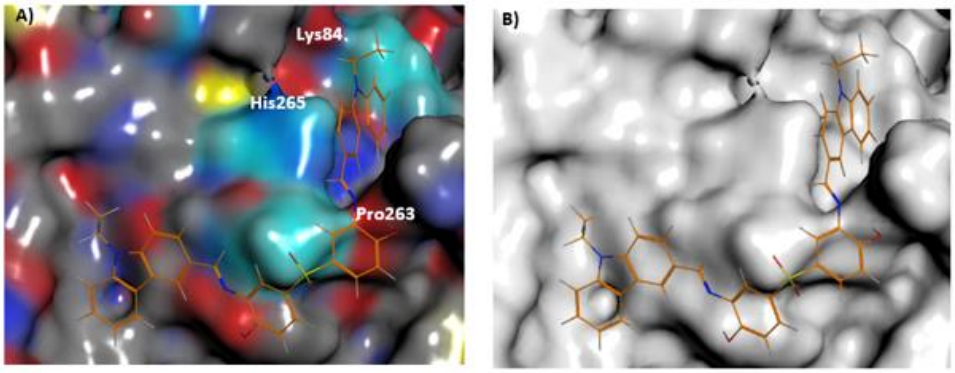


Figure 5. OTUB1 structure surface, the results of the molecular dynamics simulation after 10 ns. **(A)** It shows the main amino acids, the interactions between the OT3 compound, and the active site are stables; **(B)** It shows the region of the active site (white) and the colocation of OT3 compound (orange), for blocking the access to the active site.

To justify the cytotoxic and apoptosis effect by in silico results, first, results of molecular docking showed that the OT3 compound showed that the main interactions between OT3 and OTUB1 are with amino acids in a region considered as a potential therapeutic target (active site) [9,21,22] (Figure 3) that could regulate the OTUB1/Cyclin E1/Rho-A axis to generate proliferation/migration [4,10], besides the results of the molecular dynamics simulation showed that the interactions between the OT3 compound with the active site (Figure 4 and 5) are stable, and we describe the main interactions of OT3 compound with OTUB1, the

interactions are very similar either in the molecular docking and in the molecular dynamics simulation; in the OTUB1´s active site (Table 2), and decreases some function of OTUB1 where studies reported this region to generate the inhibition of this protein [11,18,20,32].

Therefore, the OT3 compound could generate a dysfunction of OTUB1 due to this region being essential for the deubiquitinating functions of OTUB1 [4,5,10,41]. Moreover, the OT3 compound is potentially safe to use in humans because, in this study, we widened the cytotoxic assays with normal cells, and these results were favorable. With these results, the OT3 compound has more results to continue the development of a new anticancer drug.

4. Conclusions

We propose the OT3 compound to develop a new anticancer drug against prostate cancer due to its cytotoxic and apoptosis-inducing effects. We describe that the OT3 compound could regulate OTUB1 by regulating the expression of proteins related to the proliferation of cancer cells (p-FAK and Caspase-3 proteins). Finally, the OT3 compound could have specific interactions in the catalytic domain of OTUB1, decreasing the OTUB1 functions and probably being safe for humans. Therefore, this study demonstrates the potential of the OT3 compound as an anticancer drug, and other studies should be performed to continue the development of the new anticancer drug.

Author Contributions

Conceptualization, J.L.V.S. and V.G.G.; methodology, J.L.V.S. and V.G.G.; software, J.L.V.S.; validation, J.L.V.S. and A.N.P.; formal analysis, V.G.G. and A.N.P.; investigation, J.L.V.S. and A.N.P.; resources, J.L.V.S. and V.G.G.; writing—original draft preparation, J.L.V.S. and V.G.G.; writing—review and editing, J.L.V.S.; project administration, J.L.V.S. and V.G.G.; funding acquisition, J.L.V.S.. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Material

The supplementary material provides a table of interactions between OTUB1-OT3, detailing the interaction per amino acid, which supports the information given in the results and discussion.

Table S1. Interaction report of each conformer of the OT3 compound. Number of conformers, number of atoms of the compound, amino acid in OTUB1, type of interaction, and distance in angstroms.

Conformer	Ligand	Residues in OTUB1		Interaction	Distance
1	6-ring	ARG	86	pi-H	4.42
	5-ring	ARG	86	pi-H	4.1
2	6-ring	ARG	86	pi-H	4.49
	5-ring	ARG	86	pi-H	3.98
	6-ring	CYS	212	pi-H	4.55
	5-ring	HIS	265	pi-H	3.59
	6-ring	HIS	265	pi-H	4.33
3	N	HIS	265	H-acceptor	3.44
	6-ring	LYS	84	pi-cation	3.57
	6-ring	GLU	214	pi-H	4.34
	6-ring	PRO	263	pi-H	4.35
	6-ring	GLY	264	pi-H	4.13
4	O	GLU	60	H-donor	2.92
	6-ring	GLU	60	pi-H	4.52
	6-ring	HIS	265	pi-H	4.68
5	O	GLU	60	H-donor	2.94
	O	LYS	84	H-acceptor	3.06
6	O	PRO	263	H-donor	3.03
	N	HIS	265	H-acceptor	3.25
	6-ring	ASN	45	pi-H	4.23
	5-ring	ASN	45	pi-H	4.09
7	O	GLU	60	H-donor	3.41
	N	HIS	265	H-acceptor	3.56
	6-ring	LYS	84	pi-cation	3.66
	5-ring	LYS	84	pi-cation	4.15
	6-ring	PRO	263	pi-H	4.36
8	O	GLU	60	H-donor	2.9
	6-ring	GLU	60	pi-H	4.21
	6-ring	ARG	86	pi-H	3.83
	6-ring	PRO	87	pi-H	4.26
9	5-ring	ARG	86	pi-H	4.06
10	6-ring	PRO	263	pi-H	3.97
11	6-ring	GLU	214	pi-H	4.5
	5-ring	HIS	265	pi-H	3.67
12	O	ASN	45	H-acceptor	3.28
	6-ring	GLY	264	pi-H	4.56
13	6-ring	PRO	263	pi-H	3.93
14	O	GLU	60	H-donor	3.04
	6-ring	ARG	86	pi-H	3.73
	6-ring	HIS	265	pi-H	3.94
15	O	GLU	60	H-donor	3.13
	6-ring	HIS	265	pi-H	3.89
16	6-ring	HIS	265	pi-H	3.92
17	5-ring	GLU	214	pi-H	4.06
18	6-ring	GLU	60	pi-H	4.4
	6-ring	ARG	86	pi-H	3.94
19	5-ring	GLY	264	pi-H	3.75
	6-ring	GLY	264	pi-H	4.66
20	O	PRO	87	H-donor	2.93

Conformer	Ligand	Residues in OTUB1		Interaction	Distance
	6-ring	ARG	86	pi-H	3.9
	5-ring	ARG	86	pi-H	4.1
	6-ring	PRO	87	pi-H	4.65
21	O	PRO	87	H-donor	3