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Potential Otubain1 Inhibitor, an Approach for a Treatment against Breast Cancer

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ABSTRACT

The develop of new anticancer drug continues worldwide and one of the new therapeutic targets to reach it is Otubain 1 (OTUB1), since OTUB1 has functions related to prognosis in a variety of tumors and is strongly related to tumor proliferation, migration, and apoptosis by their functions on deubiquitinating. This study uses OTUB1´s active site to develop a specific pharmacological treatment to regulate the OTUB1 functions. The aim of this research was to evaluate the effects of ten compounds (OT1 - OT10), that previously were selected by molecular docking to develop a new anticancer drug to decrease the OTUB1 functions in the cancer processes. We evaluated the cytotoxic effect of OT1 – OT10 compounds on MCF-7, BT474 and MDA-MB 231 cells by MTT assay, and we determined characteristics of apoptosis by western blot analysis. Then, the best compound (OT5) was analyzed by molecular docking, molecular dynamics and theoretical toxicity for describing the interactions of OT5 compound with the OTUB1´s active site. We proposed that the OT5 compound has a high probability to be selective against OTUB1, with an apoptosis (regulating caspase-8) and cytotoxic effect on some cancer lines; IC50 for MCF-7: 97 µM and MDA-MB 231:147 µM, as well as we described that this compound could have specific interactions in the catalytic domain of OTUB1, modifying this protein's activity, decreasing the OTUB1 functions, and probably safe for humans. These results show the high potential of this compound for promoting the development of this compound as a new drug against cancer.

Keywords: Apoptosis, caspase-8, MCF-7, MDA-MB 231, drug

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Introduction

The development of a new anticancer drug continues worldwide, we previously reported ten compounds (OT1-OT10) selected according to pharmacological characteristics and Lipinsky´s rules, and this could interact with the Otubain 1 (OTUB1) (1), this protein is a promising therapeutic target against cancer, due to the functional complexity of OTUB1 in autophagy and immune response, researchers need to be more careful when evaluating the prognostic value of OTUB1 in different cancers and for poor prognosis (2), it is a deubiquitinating enzyme that belongs to the OTU family of cysteine proteases (3,4), OTUB1 has multiple functions, this enzyme can decrease the ubiquitination state of a protein by removing ubiquitin (canonical activity) or prevent ubiquitin conjugation (non-canonical activity) (5,6). Examples of regulating processes are immune response, factors that regulate interferon and NF-κB (7,8), and regulating many cancer-associated signaling pathways including Mitogen‑activated protein kinase (MAPK), Epithelial-mesenchymal transition (EMT), Forkhead box protein M1 (FOXM1), Ras homologous A (RHOA), mechanistic target of rapamycin complex 1 (mTORC1), c-Maf, cellular inhibitor of apoptosis-1 (c-IAP1), and P53 to promote tumor cell survival, proliferation, invasiveness, prognosis, and therapeutic resistance (9-13). Furthermore, 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 and is known to inhibit the degradation of molecules that regulate cell proliferation due to the abovementioned (5, 9, 14, 15). Otherwise, there are studies over the inhibition of the OTUB1 (2,9), like in the c‑Maf axis by extracts that induces myeloma cell apoptosis, due to OTUB1 stabilizes c-Maf by preventing its polyubiquitination and enhances c-Maf transcriptional activity (13), as well as being identified that the expression of OTUB1 is very important for melanoma-cell survival (9).

On the other hand, OTUB1 could regulate apoptosis in cancer cell, and the regulation of the TNF-related apoptosis-inducing ligand (TRAIL) expression (9), thus for any development of anticancer drug is important to generate apoptosis (16), and the extrinsic apoptotic pathway could be activated by activating the members of the tumor necrosis factor alpha (TNFα) receptor super-family, which are usually called the death receptors (i.e., Fas, also called CD95/Apo-1, DR4 and DR5, TNF receptors, TRAIL) (17). OTUB1 is related with these receptors, for example, once that the level of TRAIL is increased, the oligomerization leading to the formation of pro-caspase-8 and activating the death-inducing signaling complex is identified (9). Thus, OTUB1 could regulate TRIAL, since it has been identified that inhibiting OTUB1 increases the function of TRAIL, which leads to the activation of caspase-8, which is related to regulating cell death, through the intrinsic or extrinsic pathway and that apoptosis-initiating caspases (2,8,9, and 10), and thus avoid necroptosis (18,19). The *caspase-8* may be at different levels of expression according to the type of cancer (20,21), for example in MCF-7 and Esophageal carcinoma (ESCA) with overexpression of *caspase-8*, in addition, are studies where it is reported that in MCF-7 the caspase-8 activation is an initiating event in DHA-induced apoptotic cell death (22), even other studies relating to an OTUB1 inhibitory effect (13,23), both studies using OTUB1 as therapeutic target (12) with promising results that demonstrated that OTUB1 might be a good therapeutic target.

In this study, we evaluated ten compounds reported (1), that could interact near the catalytic site of OTUB1 (Asp88, Cys91 and His265) (3,5,6), these compounds could decrease the deubiquitinating function

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of OTUB1 for developing a cancer drug (5,9,11,12,24,25), by molecular docking, molecular dynamics simulation, cytotoxicity, MTT, and western blot assays.

Materials and methods

Synthetic compounds

We use the compounds that previously reported OT1 – OT10 (1), these compounds were bought in Chembridge Corp. (26) (ID- Chembridge: OT1-7988434, OT2-7501037, OT3-5194547, OT4-7986805, OT5-7013154, OT6-7699311, OT7-5634266, OT8-7987933, OT9-5181121, and OT10-7661200). Purity of the compound reported by the manufacturer was greater than 95% checked by LC/MS, the stock solutions for compounds were prepared in DMSO (1-10 mM).

Cell Viability Assay

Cell culture reagents were purchased from Thermo-Fisher (Carlsbad, CA, U.S.A.), tissue culture plates and other plastic materials were obtained from Corning Inc. Cell lines BT-474, MDA-MB-231, and MCF-7 were obtained from American Type Culture Collection (ATCC). Cells were cultured following the provider's recommendations, and the cytotoxic effect was determined by MTT assay as described previously; MTT was obtained from Merck (Darmstadt, Germany) (27). Briefly, the cell lines were seeded (8 - 20 x 10^3 cells per well). First, we used 100 µM concentration of each compound (OT1 – OT10) in 0.4 % DMSO incubated for 48 h to determine the cytotoxic effect, and if some compound had cytotoxic effect was determined the IC50 using concentrations between 10, 20, 50, 100, and 170 µM in 0.4 % DMSO incubated for 24 h. After 24 h, the medium was aspirated, 20 µL of 5 mg/mL MTT reagent was added to each well and incubated for 4 h. The excessive liquid was gently removed and 100 µL of DMSO (0.4 %) were added to the wells. Then, the plate was read at 570 nm using the microtiter plate reader (µQuant, Bio-Tek Instrument, USA). The assays were performed in triplicate in three independent studies and the percentage of viability was calculated according to the formula: % viability= [mean Optical Density (O.D.) treated cells×100]/ (mean O.D. control cells). The concentration leading to 50% inhibition viability (IC50) was calculated by non-linear regression analysis (percentage of viability vs. log concentration) with GraphPad Prism software ver. 8.0 software (GraphPad, CA, USA).

Western blot to identify caspase-8 in Apoptosis effect

MCF-7 cells were seeded into a 6-well plate (400,000 cells per well) until they reached 90-95% confluence. DMEM supplemented medium was replaced with DMEM experimental medium for 2h. Later, cells were incubated with DMSO, 90 µM and 170 µM of OT5 compound for 24 h. Cells were washed with PBS and lysed with lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% Triton X- 100, 0.5% NP40, EDTA, 0.2 mM sodium orthovana-date, 0.3 µM aprotinin,130 µM bestatin, 14 µM E- 64, and 1 µM leupeptin). Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked at 37 ºC for 1h in TBS/0.1% tween 5% low fat milk. Anti-pro-caspase-8 (sc-56070) and anti-β actin (sc-47778) primary antibodies (Santa Cruz Biotechnology, USA). Anti-mouse secondary antibodies coupled to Horseradish peroxidase from Thermo-Fisher. Chemiluminescence detection was performed using Immobilon Western kit (Millipore, MA, U.S.A.) and X-ray film for some blots, for others Bio-Rad ChemiDoc XRS+ was used and a digital image was obtained.

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Description of the molecular interaction of cytotoxic compounds with OTUB1 by docking

Otubain 1 structure from the Protein Data Bank (28) (OTUB1, PDB code 3VON) was used for molecular interaction using Molecular Operating Environment (MOE) following procedures previously reported (29-31) and Protein-Ligand Interaction Profiler (29,32,33,34) to describe the interaction between compounds and OTUB1.

Molecular dynamics simulations of cytotoxic compounds with OTUB1

The complex of OTUB1 with compound OT5 generated by docking was used for molecular dynamics simulation (MD). It was performed using Dynamics – MOE and OPLS-AA forcefield. At the beginning, in two consecutives steps of 100 ps reaching a temperature of 300 K and a pressure of 1 bar. Finally, a 10 ns MD was carried out. Furthermore, the interactions were analyzed.

Statistical analysis

Data´s results were expressed as mean ± standard deviation (SD) of minimum three independent experiments. Averages and standard deviations were calculated in Excel (365 Microsoft, USA). IC50 for cytotoxicity of compounds were calculated using GraphPad Prism 8 Software (La Jolla, CA, USA).

Results

Cytotoxic effect of OT1 – OT10 compounds

In this study, we tested ten compounds (OT1 – OT10) that we reported previously to interact with OTUB1 (1). After the initial concentration at 100 µM of each compound, we determined only one compound (OT5 compound) with cytotoxic effect at MCF-7 (ER+ cells), MDA-MB 231 (TNBC cells), and BT-474 (HER2+ cells) (Figure 1).

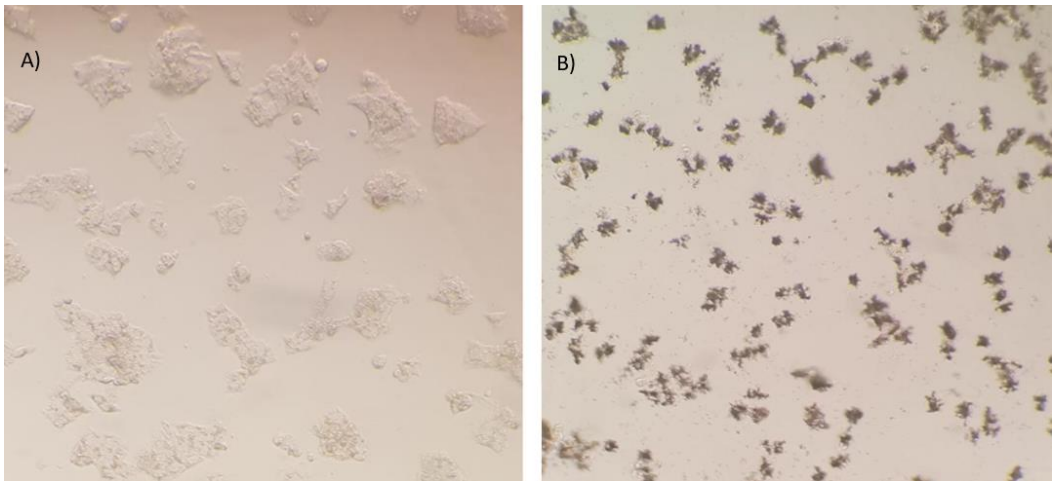

Fig.1. BT-474 cell line after 48 h of incubation. A) Group control with DMSO as vehicle, B) Group of OT5 compound with 100 µM.

IC50 of OT5 compound

We determined the IC50 using the results of the percentage of viability by MTT. Concentrations of OT5 compound between 10, 20, 50, 100, and 170 µM were used (Table 1). With these results, we determined an IC50 of 97.2 ± 1.13 µM (MCF-7 cells) and 147.4 ± 1.09 µM (MDA-MB-231 cells) for 24 h of incubation, using the Graph Pad Prism 8.

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Table 1. Concentrations used of OT5 compound and viability percentage in *in vitro* cultures of MCF-7 and MDA-MB-231 cells.

Concentration (µM)	Viability percentage of MCF-7	Viability percentage of MDA-MB-231
0	100	100
10	90	91
20	88	84
50	80	76
100	48	69
170	22	39

Apoptosis Assay

We made WB to determine pro-Caspase-8, and we identified that caspase-8 increased by OT5 compound with 90 µM (near of IC50), and with 170 µM was identified cleaved caspase-8 (Figure 2) after 24 h of incubation using MCF-7 cells. These results were related with the apoptosis effect by OT5 compound.

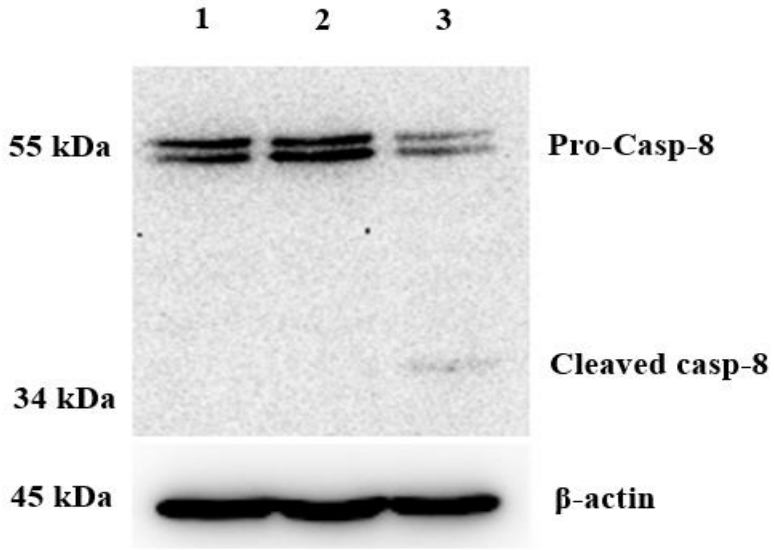

Fig.2. Western Blot assay for pro-caspase-8 and β-actin after 24 h of incubation in MCF-7 cells. Line 1: control with DMSO, line 2: 90 µM of OT5 compound and Line 3: 170 µM of OT5 compound.

Interaction of OT5 compound with OTUB1

According to the interactions report (1), OT5 compound could have interaction in the region between Lys84, Asp88, Cys91, and His265 amino acids (Figure 3). The main interactions are in Asn45, Lys84, Arg86, Pro87, Asp88, Gln165, Cys212, Arg262, Pro263, Gly264, and His265 in OTUB1. This reported particularly a greater interaction with Arg86, Pro263 and His265 (mainly hydrogen bonding interaction in the conformers analyzed), 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 with conformers of each compound are shown in the previous report (1).

Molecular dynamics simulation

To study in more detail the complex OTUB1-OT5, 10 ns of molecular dynamics simulation was performed. The root mean square deviation (RMSD) was analyzed, the data showed that the inhibitor formed

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a stable complex almost during simulation time, after 10 ns were stable in the main interactions, notably the interactions with Lys84, Arg86 and Pro286 (Figure 4).

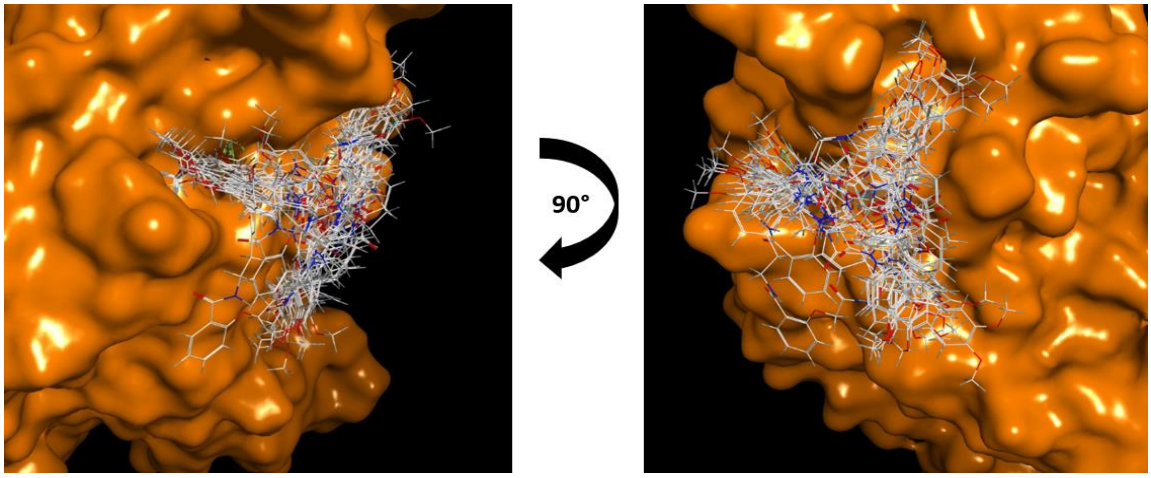

Fig.3. OTUB1 (orange) with 22 conformers of OT5 compound (white) in the active site.

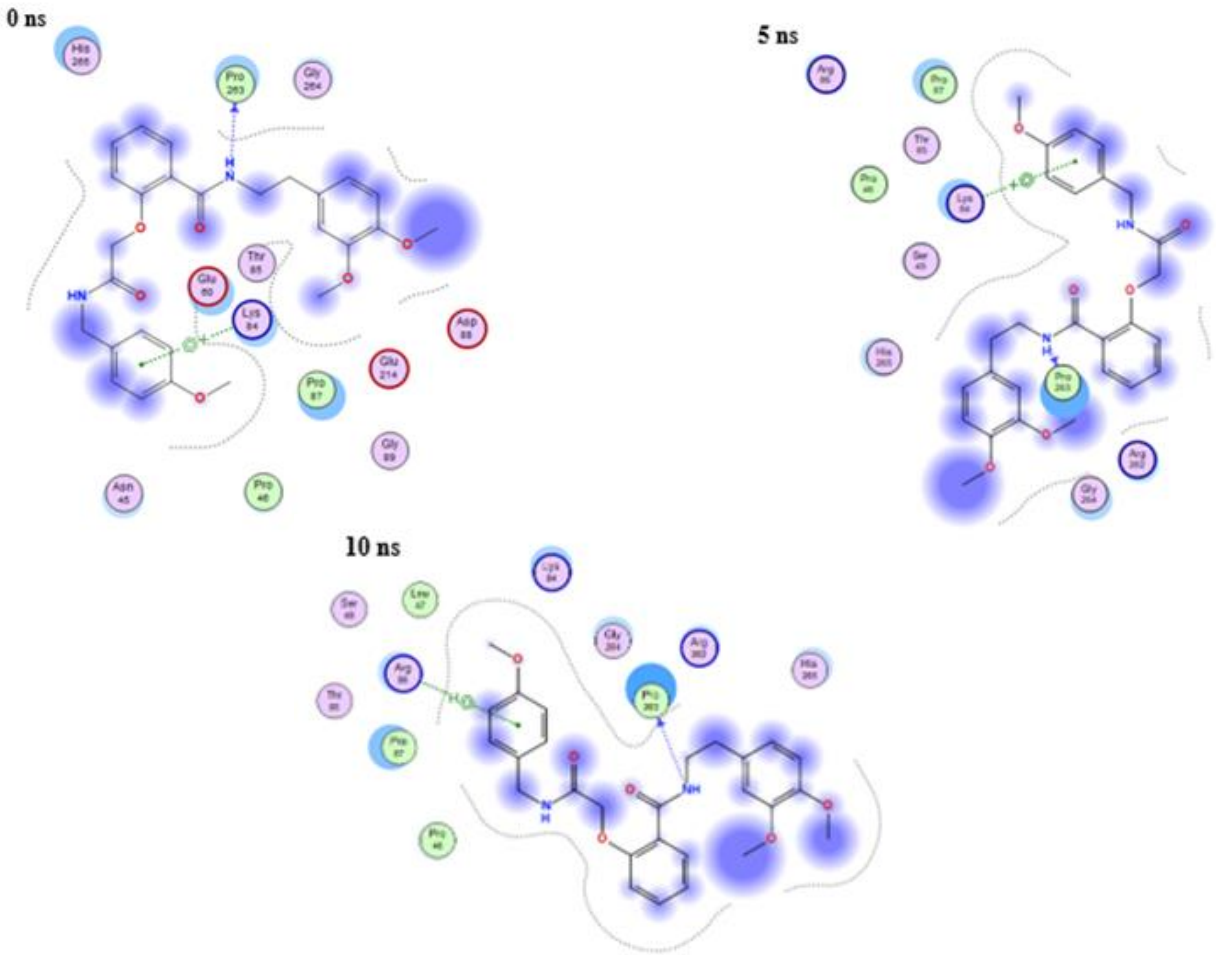

Fig.4. Interactions at 0, 5 and 10 ns frame from molecular dynamics simulation between OT5 compound and OTUB1.

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The overall RMSD after 10 ns was 1.518 A, and specifically in the amino acids with the main interactions, the RMSD were between 0.627 to 1.287 A (Table 2).

Table 2. Main interactions and RMSD of OT5 with OTUB1 between 0 – 10 ns.

Time	Ligand	Receptor (Residues in OTUB1)	Interaction	Distance	RMSD
0 ns	N	PRO263	H-donor	3.16	-
	6-ring	LYS84	pi-cation	3.77	-
5 ns	N	PRO263	H-donor	2.8	0.800
	6-ring	LYS84	pi-cation	3.6	0.886
		ARG86			0.795
10 ns	N	PRO263	H-donor	2.77	1.207
		LYS84			1.287
	6-ring	ARG86	pi-H	4.17	0.627
	6-ring	ARG86	pi-H	4.16	

Discussion

In this study, we evaluated ten compounds directed to the OTUB1´s active site to develop a specific pharmacological treatment to regulate the OTUB1 functions (mainly as deubiquitinating), someone of these related to cancer progression, and immune response (35), that have increased the development of specific inhibitors; for example, the 61 compounds with great results over lung cancer (36). Thus, our study focuses on determining the OTUB1 inhibitors effect over breast cancer. We determined that one compound (OT5) has results enough to continue the development of this anticancer drug. We propose that the OT5 compound could be regulating the deubiquitylating function of OTUB1 (3, 35).

The *in vitro* results showed that OT5 compound has a cytotoxic effect and an apoptosis effect related to regulate the caspase-8 determined by western blot. We determined the cytotoxic effect on different cell lines (MCF-7, MDA-MB 231, and BT474) using 100 µM of OT5 compound, and it generated signals of apoptosis and death cell, by loss of monolayer and changes morphological in cells (Figure 1) (9,37,38,39). As we had mentioned, it is important to generate apoptosis in the studies to develop new anticancer drugs, and the caspase-8 is a key protein of crosstalk signaling in a variety of cancers (40), and one of them, an anticancer drug reported an increase of caspase-8 level after 24 to 72 h incubation (41), and MCF-7 cells are related to the caspase-8 level increase with the apoptosis (19,42,43,44). Our results showed that OT5 compound has an effect over caspase-8 that could justify the apoptosis effect by this compound after 24 h (Figure 2). In this way, OT5 compound could be promoting the apoptosis, and this effect is related to an report in melanoma, where in this study was generating/promoting the apoptosis by decreasing OTUB1 functions, and it increased the expression of TRAIL and this increases apoptosis by caspase-8 (9), and it could be related to our results (Figure 2), where with a low concentration of OT5 compound increased the concentration of caspase-8 and identified as death cell, this effect could be related to an apoptotic effect of an anticancer compound, where there is an increase in the pro-caspase-8 (55 kDa) and active-caspase-8 in some cancer lines (9,45), for example in human bladder cancer (T24 cells) (46), colon cancer cells (DLDI) (47) and in MDA-MB 231 cells, so, in these studies, the caspase-8 concentration was maintained or increased, and all of them were

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related to apoptosis (38). On the other hand, OTUB1 has an important relation with c-MYC. Consequently, OTUB1 increases c-MYC protein levels, which increase glycolysis and tumor growth, and this is related in breast cancer cells (MCF-7 and MDA-MB 231) (48). Therefore, the activation of c-MYC contributes to the progression of multiple types of cancer (35).

To justify the cytotoxic and apoptosis effect, the *in silico* results of OT5 compounds showed that the main interactions between OT5 and OTUB1 are with amino acids in a region considered as potential therapeutic target (active site) (3,5,6) (Figures 3 and 4) that could regulate the c-Maf axis to generate apoptosis (12) (13). The results of the molecular dynamics simulation showed that the interactions between the OT5 compound with the active site (Figure 5) are stable, and we describe the main interactions of OT5 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), these interactions could block the accessibility to the catalytic site (Asp88, Cys91 and His265) and decreases some function of OTUB1. In this way, an example of OTUB1 inhibitor is the 61 compound tested in lung cancer, it interacts near the OTUB1´s active site (36). Thus, according to some functions of OTUB1, it is reported that Asp88 and Cys91 are necessary to OTUB1/c-Maf axis (12-13), and the OT5 compound could generate a disfunction of OTUB1, due to this region essential for the deubiquitinating functions of OTUB1. Thus, we related our results with other studies where OTUB1 was inhibited, it reduced their functions and the caspase-8 was activated, and all those studies with an effect on the control of apoptosis (18, 19, 22, 40, 41, 42, 43, 44).

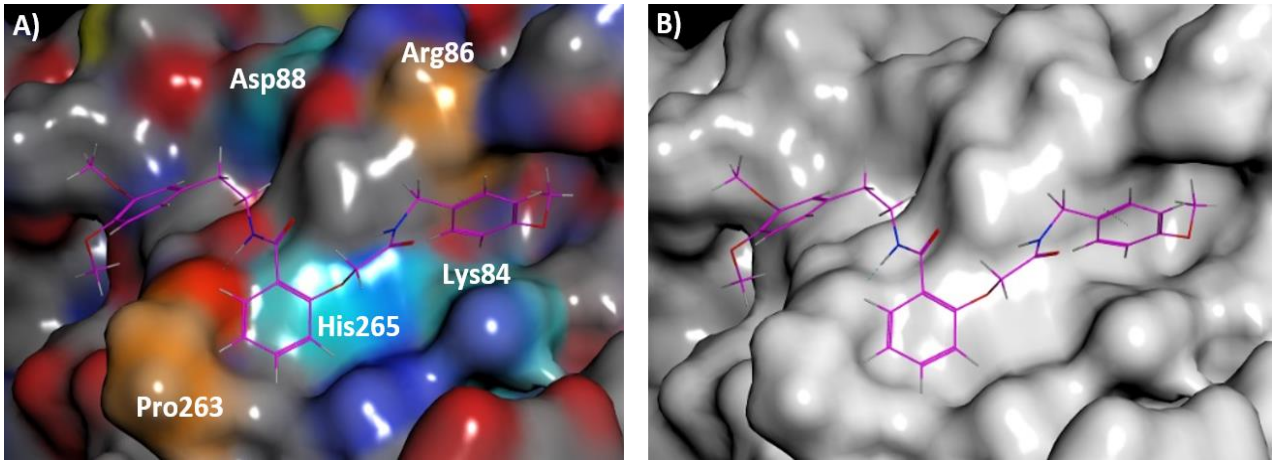

Fig.5. OTUB1 structure surface, the results of the molecular dynamics simulation after 10 ns. A) It shows the main amino acids' interactions between the OT5 compound and the active site are stables. B) It shows the region of active site (white) and the colocation of OT5 compound (pink), for blocking the access to active site.

Moreover, we expand the theoretical toxicity studies (49-51), and OT5 compound toxicity results are potentially secure to be used in humans, as well as to propose the OT5 compound for pharmacophore modeling studies (52-54), and reach to improve the anticancer effect of new molecules.

As conclusion, we propose the OT5 compound with high probability to be selective against OTUB1, with an apoptosis and cytotoxic effect on some cancer lines. We describe that this compound could have specific interactions in the catalytic domain of OTUB1, modifying this protein's activity, decreasing the OTUB1 functions, and probably safe for humans. Therefore, this study demonstrates the potential of OT5

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compound as anticancer drug, and other studies should be performed to continue the development of this new anticancer drug.

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