Synthesis and In Vitro Evaluation of 2-[3-(2-Aminoethyl)-1H-indol-1-yl]-N-benzylquinazolin-4-amine as a Novel p97/VCP Inhibitor Lead Capable of Inducing Apoptosis in Cancer Cells

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ABSTRACT: P97/VCP, an endoplasmic reticulum associated protein, belongs to AAA ATPase family, ubiquitous ATPases associated with various cellular activities. Recent research has elucidated the roles of p97/VCP and evaluated its potential as a therapeutic target for some kinds of cancer diseases. We screened the small molecule compounds from a previously established library and found promise in the compound 2-[3-(2-aminoethyl)-1H-indol-1-yl]-N-benzylquinazolin-4-amine (FQ393). Data from docking simulation indicates FQ393 acts as an ATP competitor, and ATPase activity assays showed FQ393 was an inhibitor of p97/VCP. Furthermore, in vitro FQ393 is able to promote apoptosis and prohibit proliferation in a variety of cancer cell lines. Using comparative proteomic profiling of HCT-116 cells, we found significantly different canonical KEGG pathways, which revealed that the protein changes in FQ393 groups were associated with p97/VCP or tumor-related pathways. The present data suggests that FQ393 exerts antitumor activity, at least in part through p97/VCP inhibition.

1. INTRODUCTION

An evolutionarily conserved type II AAA ATPase, valosin-containing protein p97/VCP, acts as a segregase to extract target protein organelle membranes and protein or DNA complexes, thereby promoting their recycling, refolding, relocation, or degradation.1–3 A VCP forms a double-ring-shaped homohexameric complex to perform its ATPase activity. In this complex, the C terminal and N terminal are responsible for interacting with the cofactors, the D1 domain is in charge of oligomerization, and the D2 domain catalyzes the ATP hydrolysis.4,5 By combining with more than 40 cofactors, the VCP is recruited to various subcellular fractions to participate in different cellular processes, such as, autophagy, endocytosis, membrane fusion, apoptosis, DNA repair, cell cycle progression, and endoplasmic reticulum associated protein degradation (ERAD).6,7 The VCP regulates these processes through the ubiquitin–proteasome system, which regulates intracellular levels of all proteins by tagging the proteins with ubiquitin. Then, these tagged proteins are transported to the proteasome and degraded.8–10

Because of its important role in regulating various physiological responses, p97/VCP has gradually become a potential therapeutic target.11,12 Autosomal dominant mutations in p97/VCP lead to a multisystem degenerative disease called IBMPFD/ALS that can manifest in patients with any combination of the following phenotypes: frontotemporal dementia (FTD), Paget’s disease of bone (PDB), inclusion body myopathy (IBM), and amyotrophic lateral sclerosis (ALS).1,13–15 Elevated p97/VCP expression has been found in several different tumors and is associated with poor clinical outcomes.16–19 Some labs have previously shown that increased levels of p97/VCP in cancer cells allow the cancer cells to proliferate and metastasize.8–10,20 Inhibiting the function of this protein has shown to reduce cancerous cellular growth by inducing apoptosis, as well as inhibiting the migration and arresting cell cycle.8,9,20–23 Since p97/VCP plays an significant role in the malignant progress of tumor cells, some research groups have been looking for p97/VCP inhibitors with a view to eventually developing drugs for cancer treatment. DBeQ (Figure 1) is a selective, reversible ATP-competitive small molecule inhibitor of p97/VCP that inhibits the proliferation of cancer cells by impairing both autophagic protein clearance pathways and ubiquitin-independent degradation.22 By modifying the structure of DBeQ, researchers found more potent p97/VCP inhibitors, such as ML240 and...
From these molecules, the most advanced p97/VCP inhibitor CB-5083 was subsequently developed by Cleave Biosciences. Due to retention of endoplasmic reticulum-associated degradation (ERAD) substrates, CB-5083 induces apoptosis of cancer cells, which has been indicated to be a novel approach of cancer treatment. CB-5083 entered two phase I clinical trials in 2015. However, when toxicities due to off-target effects of the compound were found, the trials were halted. Recently, a crystal structure combining CB-5083 with D1-D2 p97/VCP (lacking a N domain) was solved, which revealed the molecular basis of selective inhibition of CB-5083 on the D2 domain of p97/VCP.

Since p97/VCP is identified as a potential target in cancer cells and there is no inhibitor of p97/VCP clinically available for this target, finding new p97/VCP inhibitors is worth investigating. We screened small molecule compounds from the compound library in our laboratory to exploit novel small compounds with anticancer cell proliferation activity for discovering p97/VCP inhibitors. Among those compounds, FQ393 showed a relative activity. We found that FQ393 has similar structural units with DBeQ, both of which contain quinazoline and phenylmethanamine. The difference between the two compounds is that FQ393 replaces benzylamine with tryptamine. Perhaps because of their structural similarity, FQ393 has similar p97-inhibiting activity. In the present study, we show inhibitory activity of FQ393 through p97/VCP inhibition in an ATP-competitive manner and scores on the docking with the p97/VCP protein. FQ393 inhibited hydrolysis of ATP in a dose-dependent method, and it also has a favorable binding score of $-7.9 \text{ kJ/mol}$, which is similar to ATP (a natural ligand of p97/VCP) with a binding score of...
−8.3 kJ/mol. These encouraged the present study to synthesize FQ393 and evaluate its in vitro activities.

2. RESULTS AND DISCUSSION

2.1. Synthesis of FQ393. FQ393 was prepared via a four-step reaction based on Scheme 1. The yields of the four-step reaction were 92.6, 83.9, 55.6, and 92.8%, respectively. The details and related data are provided in the Experimental Section. The synthetic route is suitable for preparation of FQ393 with appropriate conditions and a simple procedure.

2.2. FQ393 Inhibits ATPase Activity of p97/VCP. Since FQ393 has a similar structure to DBeQ, a known p97/VCP inhibitor, we investigated whether FQ393 has inhibitory activity against p97/VCP. Using structural data of active sites in the p97/VCP catalytic domain, in silico flexible docking simulations were conducted. The total binding scores and 3D docking poses between FQ393 and p97/VCP are shown in Figure 2A. The intermolecular interaction between the active pocket of p97/VCP and FQ393 mainly consists of van der Waals and electrostatic interactions in the force field representation. Van der Waals were formed by the quinazoline of FQ393 and the side chain of Ile656 of the active pocket, the phenylmethanamine of FQ393 and the side chain of Asp478 of the active pocket, and the amidogen of FQ393 and the side chain of Ala685 of the active pocket. Electrostatic interactions were formed by the quinazoline of FQ393 and the side chain of Ile656, Leu526, and Gly684 of the active pocket; and the indole of FQ393 and the side chain of Ala685 and Leu526.

FQ393 has a favorable binding score of −7.9 kJ/mol, which is similar to ATP (a natural ligand of p97/VCP) with a binding score of −8.3 kJ/mol. The results indicate that FQ393 may have inhibitory activity against p97/VCP in cancer cells.

Then, we tested the performance of FQ393 as a viable inhibitor of p97/VCP using a cell-free assay. A purified p97/VCP protein was used for p97/VCP inhibition assays. FQ393 inhibited ATP hydrolysis in a dose-dependent method (Figure 2B, IC50 = 0.90 ± 0.11 μmol/L). These data encouraged us to evaluate its other in vitro antitumor activities.

2.3. FQ393 against Proliferation of Various Carcinoma Cells. The in vitro antiproliferation of FQ393 against lung adenocarcinoma cells (H1975, H1299, H1792, H460, A549, and 95D), colorectal carcinoma cells (HCT-116, DLD-1, and HCT-8), breast cancer cells (MDA-MB-231, MCF-7), and pancreatic cancer cells (MA116, H696, and BxPC-3) was assessed. FQ393, at various concentrations, inhibited cell growth in a dose-dependent manner (Figure 2C). The IC50 values for FQ393 against these carcinoma cells are shown in Table 1. FQ393 exhibited significant inhibitory activity against all carcinoma cell lines, with IC50 values ranging from 0.54 to 6.05 μmol/L. These data suggest that FQ393 has potential as a novel antitumor agent.

Figure 3. IC50 of FQ393 against (A) lung adenocarcinoma cells, (B) colorectal cancer cells, (C) breast cancer cells, (D) pancreatic cancer cells, and (E) other cancer cells, n = 4. The exact mean and SD are shown in (F).
HCT-8), breast carcinoma cells (MCF-7 and MDA-MB-231), pancreatic carcinoma cells (PNAC-1 and BxPC-3), and other cancer cells (human myeloma MM1S, human cervical carcinoma HeLa, human skin cancer cell line A431, and human hepatoma cell line HepG2) were evaluated by the MTT method and represented with an IC_{50} value. Figure 3 shows that when the cancer cells are exposed to serial concentrations (1.6−50 μM) of FQ393, the IC_{50} values range from 1.37 to 10.80 μM; therefore, FQ393 efficiently inhibits the proliferation of 17 carcinoma cell lines. Figure 3 also shows

Figure 4. (A) Apoptosis assay of HCT-116 and A549 cells treated with FQ393. The cells were treated for 12 h, and cell apoptosis was detected by PI/annexin V staining and flow cytometry. Percentages of apoptotic (B) HCT-116 and (C) A549 cells after FQ393 treatment are shown. *p < 0.01 compared with the control group.

Figure 5. Proteomic analysis of HCT-116 cells incubated with FQ393. (A) Volcano plot of all proteins (red and blue points represent increased and decreased expression respectively). (B) PPI network of 252 differently expressed proteins that were performed with String database. Nodes represent proteins. Lines represent protein−protein associations. (C) Significantly enriched pathways identified by KEGG pathway analysis (p < 0.05).
that when exposed to FQ393, cell lines 95D, HCT-116, H460, and A549 are more sensitive than MCF-7 and HeLa cells to apoptosis.

2.4. Apoptosis Activity of FQ393 In Vitro. An inhibitor lead of p97/VCP, FQ393, was confirmed as an apoptosis inducer by the flow cytometry assay, and suspensions of HCT-116 and A549 cells were used in this assay. The cells were exposed to 5 μM FQ393, and the resulting data is presented in Figure 4. The data revealed that at 12 h, the total percentage of apoptosis for HCT-116 an A549 cells were 15.8 and 16.8%, respectively, indicating that FQ393 is an apoptosis inducer. We discovered that the p97/VCP inhibitor lead FQ393 can inhibit carcinoma cells proliferation and effectively induce apoptosis, and these findings provide a potential avenue for the development of tumor therapies.

2.5. Mass Spectrometric Analysis of Proteomic Profiling after FQ393 Treatment. Using proteomic profiling of HCT-116 cells from DMSO and FQ393 groups, 4960 proteins were detected with at least one unique peptide and a 1% false discovery rate (FDR). Figure 5A shows a volcanic plot of all the proteins. Compared with the DMSO group, proteins with p values < 0.05 and 1.5-fold changes (>1.50 or < 0.66) for the proteomic analysis were finally considered to be differentially expressed and chosen for further analysis. Based on the criteria, 301 differentially expressed proteins were identified and selected for further analysis. Among them, 149 proteins showed increased expression, while the remaining 152 experienced a decrease. A total of 252 altered proteins were annotated by the "String" database. Figure 5B shows the protein–protein interaction network of these proteins. The significantly different canonical Kyoto Encyclopedia of Genes and Genomics (KEGG) pathways are displayed in Figure 5C. Based on differentially expressed proteins, the KEGG pathway analysis revealed ALS signaling, protein processing in endoplasmic reticulum signaling, cell cycle signaling, endocytosis signaling, ribosome signaling, autophagy signaling, ubiquitin-mediated proteolysis signaling, and nucleotide excision repair signaling, which have previously been associated with p97/VCP.29 Also, the KEGG pathway analysis revealed other signaling, such as pathways in cancer, proteoglycans in cancer signaling, viral carcinogenesis signaling, MAPK signaling pathway, colorectal cancer signaling, hepatocellular carcinoma signaling, PI3K-Akt signaling pathway, breast cancer signaling, mTOR signaling pathway, gastric cancer signaling, microRNAs in cancer signaling, and pancreatic cancer signaling, which are recognized tumor-related pathways.28 The data above indicate that FQ393, as a potential inhibitor of p97/VCP, may play a role in inhibiting cancer cells by inhibiting the main function of p97/VCP.

P97/VCP plays a significant role in protein homeostasis and cancer cell-dependent protein quality control mechanisms, which makes its modulation an attractive target.29 Evidence is accumulating that the expression level of p97/VCP is markedly elevated in non-small-cell lung carcinoma, colorectal carcinomas, multiple myeloma, breast carcinoma, hepatocellular carcinoma, pancreatic endocrine neoplasms, gastric carcinoma, esophageal squamous cell carcinoma, gingival squamous cell carcinoma, follicular thyroid cancer, and prostate cancer and has been found to be associated with poor prognosis.30,31

Clearly, targeting p97/VCP has potential as a therapeutic strategy for the treatment of cancer. The current study showed that FQ393 was active against many kinds of cancer cells, in which p97/VCP was overexpressed in cancer cells. The results are consistent with those reported in the literature.

Traditional heterocyclic compounds represented by DBeQ, ML-240, ML-241, NMS-873, CB-5339, and UPCDC30245 show potent effects targeting p97/VCP.29,33 In 2015, CB-5339 entered phase I clinical trials for multiple myeloma and advanced solid tumors. The first drugs targeting p97 are expected to play a role in oncology, with the potential for future trials in solid cancers and acute myeloid leukemia.29 However, clinical development was terminated due to visual loss, a consequence of off-target inhibition of phosphodiesterase-6.34 As a potent and selective, second-generation, oral small molecule inhibitor of p97/VCP, CB-5339 have entered phase I clinical trials for acute myeloid leukemia or myelodysplastic syndrome reported in the ClinicalTrials.gov (NCT04402541). Despite p97/VCP offering therapeutic opportunities, the development of effective and selective therapeutic agents for p97/VCP remains challenging. There is still an urgent need for more chemotypes of p97/VCP inhibitors. Based on unmet clinical needs, we screened the compound library, the internal library of our laboratory, including heterocyclic small molecules, small molecule peptides, and their conjugates, totaling about 3000 compounds. Interestingly, FQ393 is the most active compound, and the structure of FQ393 is similar to DBeQ. We preliminarily verified the antitumor activity of FQ393 in vitro, and FQ393 showed a relative activity. At present, the activity of FQ393 is not satisfactory, and FQ393 will be used as the lead compound for structural modification in the future, hoping to obtain candidate compounds with high activity, good selectivity, and low off-target effect.

3. CONCLUSIONS

In summary, p97/VCP is a ubiquitous protein involved in many biological processes, and p97/VCP inhibition is a promising strategy to treat cancer patients. We screened the small molecule compounds in our compound library to exploit a novel small compound, FQ393. The data from in silico docking simulations and ATPase activity assays indicate that FQ393 acts as both an ATP competitor and inhibitor of p97/VCP. Furthermore, in vitro FQ393 is able to promote the apoptosis of carcinoma cells and inhibit the proliferation of carcinoma cells. Using proteomic profiling of HCT-116 cells from DMSO and FQ393 groups, the significantly different canonical KEGG pathways revealed that the protein changes in FQ393 groups were associated with p97/VCP or a tumor-related pathway. FQ393 is thus considered a promising lead compound as an inhibitor of p97/VCP for chemotherapy.

4. EXPERIMENTAL SECTION

4.1. General. Organic solvents were dried and purified when necessary by standard methods. All commercially available reagents were purchased from Sigma (St Louis, MO, U.S.). Silica gel of 200–300 mesh was used for column chromatography. The purities of the intermediates were identified with thin-layer chromatography (TLC). The purities of products were identified with both TLC and high-performance liquid chromatography (HPLC, Waters Corporation, U.S.A., C18 column 4.6 × 150 mm) and were higher than 97%. Reactions were monitored by TLC (silica gel coated with a fluorescent indicator F 254). The melting point of FQ393 was measured on an XTS hot stage apparatus (uncorrected, Beijing Keyi Electro-Optic Factory, China).
With tetramethylsilane as the internal standard, proton nuclear magnetic resonance (1H NMR) and 13C nuclear magnetic resonance (13C NMR) spectra were recorded on a Bruker Avance 300 (300 and 75 MHz, respectively). Electrospray ionization mass spectrometry (ESI-MS) was measured on a ZQ 2000 (Waters Corp).

The data was analyzed by GraphPad Prism (version 7.0), and the results of assays are presented with mean ± standard deviation. A p value less than 0.05 was considered to have statistical significance.

4.2. Synthesis of FQ393. The preparation of FQ393 was carried out according to Scheme 1. After the four-step reaction, the target compound FQ393 is obtained in a 40.1% overall yield. Processes for the preparation and the physicochemical data of FQ393 and intermediates are given as follows.

4.2.1. Preparing N-Benzyl-2-chloroquinazolin-4-amine (1). At 0 °C, to the solution of 2.00 g (10 mmol) of 2,4-dichloroquinazoline in 30 mL of acetonitrile (ACN), 30 mL of benzylamine was added. Then, the reaction mixture was stirred for 12 h at room temperature. The temperature of the reaction mixture was stirred for 0.5 h and bubble removal was performed. The reaction mixture was filtered, and the residue was washed with ACN. The obtained residue was dried to give the title compound as a colorless powder (yield = 83.9%). ESI-MS (m/z): 283.30 [M + Na]+. 1H NMR (300 MHz, DMSO-δ6): 8.32 (d, J = 8.1 Hz, 1H), 7.33 (d, J = 7.8 Hz, 1H), 7.82 (t, J = 7.5 Hz, 1H), 7.64 (d, J = 8.1 Hz, 1H), 7.55 (t, J = 7.5 Hz, 1H), 7.34−7.26 (m, 9H), 4.76 (d, J = 5.4 Hz, 2H). 13C NMR (75 MHz, DMSO-δ6): 161.58, 157.37, 150.84, 138.97, 134.20, 128.86, 127.88, 127.50, 127.15, 126.68, 123.57, 113.96, 112.32, 77.95, 44.81, 28.73, 25.57.

4.2.2. Preparing tert-Butyl [2-(1H-indol-3-yl)ethyl]carbamate (2). To the solution of 2.00 g (10 mmol) of tryptamine in 5 mL of distilled water, 10 mL of aqueous NaOH (2 N) was added dropwise. In 15 mL of anhydrous 1,4-dioxiane, 2.66 g (12 mmol) of (Boc)2O was dissolved and added to the reaction solution. The reaction mixture was stirred for 0.5 h and bubble removal was performed. The reaction mixture was stirred for 12 h at room temperature, and its pH is kept at 8 by adding 2 N aqueous NaOH. TLC detection (CH2Cl2:MeOH, 20:1) indicated the raw material tryptamine was disappeared. The pH of the solution was adjusted to 4.5 by adding saturated aqueous KHSO4; then, the reaction solution was evaporated under vacuum. The residue is washed with ethyl acetate and evaporated under vacuum. This procedure was repeated for three times so as to remove the excess hydrogen chloride thoroughly. The residue is reconstituted with ethyl acetate and washed with saturated NaHCO3 to neutrality; then, the combined organic layers were washed with saturated aqueous NaCl and dried over anhydrous Na2SO4 for 2 h. The solution was filtered, and the residual material disappeared. The reaction mixture was reconstituted with ethyl acetate and washed with saturated NaHCO3 to neutrality; then, the combined organic layers were washed with saturated aqueous NaCl and dried over anhydrous Na2SO4 for 2 h. The solution was filtered and evaporated under reduced pressure to give the title compound (0.13 g, 92.8%) as a colorless powder. ESI-MS (m/z): 394.42 [M + H]+. Mp: 248 °C.

4.3. Determining IC50 Values of FQ393 in ATPase Assays. The detailed method has been reported in the literature. Briefly, inhibition of human p97/VCP (25 nM monomer) was carried out in the assay buffer (50 mM Tris, pH 7.4; 1 mM EDTA; 20 mM MgCl2; 0.5 mM TCEP) containing 200 μM ATP and 0.01% Triton X-100. The IC50 value of FQ393 in blocking ATPase activity was determined by eight-dose titration through the addition of Biomol Green reagent (Enzo Life Sciences, Farmingdale, NY, U.S.A.).
The cells were treated with FQ393 (final concentrations: 1.6, 3.1, 6.3, 12.5, 25, and 50 μM) for 48 h; MTT reagent (25 μL, 5 mg/mL) was added to each well. After a 4 h incubation at 37 °C, the supernatant was replaced by 100 μL DMSO. The optical density (O.D) was measured at 492 nm using a Spectra Max M3 microplate reader (BioTek, Winooski, VT, U.S.).

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c04478

Notes
The authors declare no competing financial interest.

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ASSOCIATED CONTENT
Supporting Information
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