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Evaluating p97 inhibitor analogues for their domain-selectivity and potency against the p97-p47 complex

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Abstract

We previously found that p97 ATPase inhibitors **ML240** and **ML241** specifically target the D2-domain of wild type p97. In addition, one of the major p97 cofactors, p47, decreases their potencies by ~50-fold. In contrast, **DBeQ** targets both the D1 and D2 domains and shows only a 4- to 6-fold decrease in potency against the p97-p47 complex. To elucidate structure-activity relationships of inhibitors, we screened 200 p97 inhibitor analogues for the ability to inhibit the ATPase activity of the D1 or D2 domains, or both, as well for effects on p47 potency. The selectivity of 29 of these compounds was further examined by 8-dose titrations. Four compounds showed modest selectivity to inhibit the ATPase activity of D1. Eleven compounds inhibited D2 with greater potencyies, and 4 compounds showed similar potencies against D1 and D2. p47 decreased the potencies of the majority of the compounds and increased the potencies of 5 compounds. These results highlight the possibility of developing domain-selective and complex-specific p97 inhibitors, in order to further elucidate physiological roles of p97 and its cofactors.

Keywords

p97; VCP; AAA ATPase; cancer; structure-activity relationships; ubiquitin proteasome system

The molecular chaperone p97 is a type II ATPase associated with various cellular activities (AAA), also known as valosin-containing protein (VCP) in *vertebrates* or Cdc48 in *S. cerevisiae*. It is conserved from archaebacteria and yeast to humans, and is abundant in the cytoplasm and nucleoplasm (~1% cell protein).[1,2] Critical cellular roles for p97 include taking part in ubiquitin proteasome-dependent degradation of cytosolic proteins,

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endoplasmic reticulum-associated degradation (ERAD), and autophagy processes.[3–10] The diverse functions of p97 are directed by the binding of specific p97 cofactors,[11] the most extensively studied being p47. Trimers of p47 enable p97 to participate in homotypic membrane fusion and have been shown to bind ubiquitinated proteins via the ubiquitin-associated domain in p47.[12] Considering the diverse functions of p97 related to cell proliferation and protein homeostasis, one could envision p97 as a potential therapeutic target for cancer and neurodegenerative diseases.

p97 is a barrel-like molecular machine comprising an N-terminal domain and two tandem ATPase domains (D1 and D2) (Figure 1A), which are stacked in a head-to-tail manner in a hexameric double ring.[13] Conformational transitions are necessary for p97 to function in disassembling protein complexes or segregating polypeptides from intracellular structures, such as the endoplasmic reticulum (ER).[14–16] When it binds to a substrate, p97 uses the chemical energy from ATP hydrolysis to stimulate major intrinsic conformational changes that are transmitted throughout the entire p97 molecule to the N-terminal domain.[13–16] Recent studies, including crystallographic structure analysis, have revealed that conformational communication between the rings and the mobility of the N-domain are important for ATPase activity.[13] The N-terminal domain of p97 recruits cofactor proteins, accounting in part for the broad range of cellular functions of p97, and D2 has been shown to be the dominant AAA domain.[11]

ATPase activity is essential for p97's biological functions. Therefore, if the ATPase activity could be efficiently suppressed, one may be able to uncover biological functions of p97 and relationships between ATPase activity and particular cellular processes. Moreover, development of effective and specific inhibitors would clarify whether p97 is a valid pharmacotherapeutic target for cancer.[17,18] For these purposes, potent and specific inhibitors of p97 ATPase activity have been identified via high-throughput screening (HTS), followed by chemical optimization.[19–23] These small molecule inhibitors fall into two classes: ATP-competitive inhibitors (**DBeQ**, **ML240**, and **ML241**) and noncompetitive inhibitors (**NMS-873**) (Figure S1).[17–21] In previous studies, we showed that **DBeQ** and **NMS-873** inhibit both the D1 and D2 ATPase domains, whereas **ML240** and **ML241** selectively inhibit D2. However, communication between the domains imposes limits on inhibitor effectiveness. For example, changes in the D1 active site due to mutation or the absence of nucleotide binding weaken the potency of **ML240** as a D2 specific inhibitor. Moreover, we have found that D1 ATPase activity alone is difficult to block.[19]

Besides domain selectivity, we also found that p47, acting as one of the main cofactors of p97, can decrease the potencies of **ML240** and **ML241** as ATP-competitive p97 inhibitors. The effect is consistent with the finding that p47 dramatically changes the conformation of p97 and lowers the maximal velocity of its ATPase activity.[12,24] Therefore, it may be possible to develop complex-specific p97 inhibitors for p47 and other cofactors, which could have important biological implications.

In this study we characterized 200 p97 inhibitor analogues,[23] in order to identify those that specifically inhibit the D1 ATPase domain or the p97-p47 complex. There are several possible ways in which small molecule inhibitors can suppress the ATPase activity of p97.

Some inhibitors may suppress activity at both the D1 and D2 domains, whereas others may selectively interact with only one of the domains (Figure 1B). Some inhibitors may be more potent for p97 alone, while others may be more potent for the p97-p47 complex (Figure 1C).

In the present work, we employed two Walker B (DExx box) motif mutants -- a D2-active mutant (E305Q) and a D1-active mutant (E578Q) -- to screen for D1- and D2-specific inhibition. These two mutants assemble into a ring structure similar to that of wild type p97, but they are unable to hydrolyze ATP at the D1 or D2 domains, respectively.[19] The 200 compounds were screened for their potential to suppress ATPase activity of wild-type p97, the D2-active mutant (E305Q), the D1-active mutant (E578Q), and the p97-p47 complex. Activities were measured in the presence of 200 μM ATP, which is the concentration that gave optimal signal to noise ratios [19,20,22,23]. **DBeQ** and **NMS-873** were used as positive controls in every experiment. The IC_{50} values of **DBeQ** increased 2.3-, 1.4-, and 2.4-fold against the p97-p47 complex (12.3 μM), the D2-active mutant (E305Q) (7.6 μM), and the D1-active mutant (E578Q) (13.1 μM), compared to WT p97 (5.4 μM) (Table S1 and Figures S1 and S2). **NMS-873** inhibited the ATPase activity of both the D1 and D2 domains (Table S1 and Figure S2).[17–19]

Inhibitors of interest from among the 200 compounds were classified into two groups: compounds that selectively suppressed the ATPase activity of the D1 domain and those that selectively inhibited the p97-p47 complex (Figure 2). The first round of screening revealed 139 compounds that inhibited either the D2-active mutant (E305Q) or the D1-active mutant (E578Q). Among these active compounds, 70 compounds showed highly selective inhibition against the D2-active mutant (E305Q). The IC_{50} of 19 compounds were 2- to 10- fold higher against the D1-active mutant (E578Q), compared to the D2-active mutant (E305Q). 28 compounds showed comparable inhibition of both mutants (1.9- to 0.6-fold), and 22 compounds showed modest selective inhibition against the D1-active mutant (E578Q) (<0.6-fold).

During the initial screens of ATPase activity of the 200 compounds, 171 displayed inhibition against wild-type p97 and the p97-p47 cofactor complex. In the presence of p47, the inhibitive ability of 149 compounds decreased. The degree of decrease was >10-fold for 123 compounds, and 2- to 9-fold for 26 compounds. 20 compounds did not show remarkable selectivity toward either p97 or the p97-p47 complex. Interestingly, 2 compounds were ~2-fold more potent against the p97-p47 complex, rendering the complex <0.6-fold less active than p97 (Figure 2). These findings are consistent with steady-state kinetic analyses of the p97-p47 complex, which show that p47 decreases the K_m of ATP and therefore makes ATP bind more tightly to p97 (Figure S3).

We selected 29 compounds for 8-dose titrations of ATPase inhibition. A total of 19 compounds were identified as active against the Walker B mutants (Figure 3 and Table S1). There were 7 compounds that selectively inhibited D1-E305Q, with an inhibition potential >10-fold higher than that for D2-E578Q. 13 of the 19 active compounds showed modest selectivity between D1 and D2, with IC_{50} ratios for the D1-active mutant (E578Q) over the D2-active mutant (E305Q) ranging from 10 to 0.7. The compounds **P1-F2**, **P1-E3**, **P1-B6**, and **P2-H11** displayed IC_{50} ratios <1.0 (for E578Q/E305Q), suggesting good potencies

against D2-E578Q. Among the 29 inhibitors tested by titration, 6 were selective against wild type p97, but were inactive against the p97-p47 complex (with IC_{50} values >10-fold higher) (Figure 3 and Tables S1 and S2). 8 compounds showed comparable inhibition of both wild type p97 and p97-p47. Interestingly, the IC_{50} of 5 compounds decreased slightly in the presence of p47.

The inhibitors were divided into several groups based on their structural similarities. Among compounds with halogen substitutions at the benzyl ring (Figure 3A), di-chloro substitution (**P1-F8**) produced the largest decrease in inhibition, which occurred against the D1-active mutant (E578Q) [among wild type p97, the p97-p47 complex, the D2-active mutant (E305Q), and the D1-active mutant (E578Q)]. Selectivity toward the D2 domain was also observed for compounds **P1-F7** and **P1-E4**.

Methoxyl substitution at the 4-position of the benzyl group (compound **P1-E11**, Figure 3B) also produced a large increase in IC_{50} toward the D1-active mutant (E578Q). Moreover, methylation at the 2-position (compound **P1-F4**) showed an even greater increase, which suggests that these compounds specifically inhibit the ATPase activity of the D2-domain. Furthermore, it appears that substitution of the benzyl group had almost no effect on the selectivity between wild type p97 and the p97-p47 complex (Figure 3A and 3B).

Of the substitutions on the quinazoline rings (Figure 3C), methoxy substitution (compound **P1-G9**) produced inhibition toward the D2-active mutant (E305Q) (IC_{50} ratio 1.2), with comparable inhibition of WT p97, the D1-active mutant (E578Q), and the p97-p47 complex. 8-fluoro (compound **P1-A11**) and 7-chloro (compound **P1-A5**) derivatives exhibited drastically reduced abilities to inhibit the p97-p47 complex and the D1-domain [not effective toward the D1-active mutant (E578Q)]. Interestingly, 4-chloro (compound **P1-B6**) and 7-Me (compound **P1-A9**) derivatives retained their ability to inhibit the p97-p47 complex and both D1 and D2 activities. These results suggest that these compounds (**P1-G9**, **P1-B6**, and **P1-A9**) may have the potential to be further developed into D1-selective inhibitors.

Replacement of the aminobenzyl group at the 2-position of the quinazoline core of **DBeQ** with aminophenyl, phenyl, *p*-tolyl, or another substitution (Figure 3D) was unfavorable for inhibiting the p97-p47 complex and the D1-active mutant (E578Q). Modification with a ring-constrained structure at the C4-amino N position on the quinazoline core (compound **P2-H7**) caused a selective decrease in the IC_{50} against the p97-p47 complex (17.9 μM), compared to WT p97 (29.1 μM). Therefore P2-H7 could be a starting point for developing p97-p47 selective inhibitors.

From this SAR analysis, we hypothesize that small molecule inhibitors that selectively target the D1 ATP-binding site of p97 can be developed. Moreover, DBeQ (N2,N4-dibenzylquinazoline-2,4-diamine) is a symmetrical quinazoline that showed a 3-fold selectivity toward the D2 domain. This suggests that D1 and D2 conformations are somewhat different, when p97 is not bound to its cofactors. The conformational change upon binding to the p47 cofactor seems to drastically reduce the ability of D2-selective inhibitors to bind to the p97-p47 complex. We hypothesize that this could be due to

enhanced ATP binding to the D2 domain, caused by p47 (Figure S3) or significant reorganization of the ATP binding site. Therefore, p97 alone and the p97-p47 complex are distinct entities.

Several compounds screened here showed selective inhibition against the D1-active Walker B mutant (E578Q), whereas others showed greater potential for blocking the ATPase activity of the p97-p47 complex (compared to p97 alone). Despite advances in the development of inhibitors against p97 ATPase, the current data are not sufficient for predicting which compounds may be effective and specific p97 inhibitors. Development of specific p97-p47 inhibitors is likely to be very important, in view of the many cellular functions of p47 upon binding to p97.

The present study identifies several potential starting points for future SAR analyses, to improve the potencies of D1-specific inhibitors and selective inhibitors of the p97-p47 complex. It also highlights the potential importance of context-based inhibition of p97 ATPase activity.[21] The important implications of this study are: (1) to improve SAR studies of p97 inhibitors *in vitro*, one needs to use D1 and D2 mutants to determine the extent to which compounds inhibit the D1 and D2 sites; (2) to correlate *in vitro*, cellular, and *in vivo* potencies of p97 inhibitors, one needs to consider the existence of different p97-cofactor complexes;[21] and (3) further work is needed to develop specific cell-based assays for specific p97-cofactor complexes, to facilitate the development of complex-specific inhibitors.

Experimental Section

The preparation and classification of the screened compounds have been previously described.[23]

ATPase assay

The detailed method was described previously.[19,22,23] Inhibition of human p97 (25 nM monomer) was carried out in Assay Buffer (50 mM Tris pH 7.4, 20 mM $MgCl_2$, 1 mM EDTA, 0.5 mM TCEP) containing 0.01% Triton X-100 and 200 μM ATP. The initial 4-dose titration was done in triplicate at the following compound concentrations: 30, 10, 3.3, and 1.1 μM; the 8-dose titration was performed at 30, 10, 3.3, 1.1, 0.37, 0.12, 0.04, and 0 μM. ATPase activity was determined by addition of Biomol Green Reagent (Enzo Life Sciences). p47 was added to a final concentration of 400 nM. NMS-873 was purchased from Xcess Biosciences Inc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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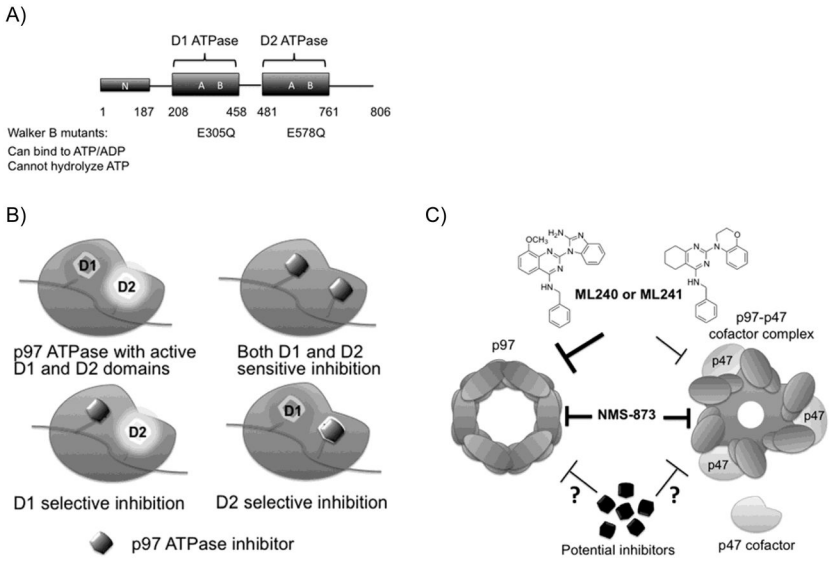

Figure 1.

Inhibition of the p97 AAA ATPase. A) p97 protein structure and domain mutants. The p97 AAA sequence contains an N-terminal (1-187) domain associated with cofactor binding, followed by two ATPase domains (D1 and D2) that contain conserved Walker A and B motifs. Walker B mutants of the D1 (E305Q) and D2 (E578Q) domains are able to bind to ATP/ADP, but are unable to catalyze hydrolysis. B) Possible modes of action of p97 inhibitors. Inhibition of p97 ATPase activity may occur through several possible combinations of effects. Inhibitors may affect enzymatic activity at the D1 or D2 domain, or both. C) The binding of cofactors changes the conformational structure of p97, which alone is ring-like. Electron microscopy demonstrated that p97 undergoes distinct conformational changes when bound to p47, including reduction of the diameter of the central channel of the p97 hexameric ring and a change in shape of the p97 ring from a hexagon to a pinwheel.[12] Molecular inhibitors ML240 and ML241 both showed less inhibition of the p97-p47 complex (right), compared to p97 alone (left). The allosteric inhibitor NMS-873 showed similar potencies toward both p97 alone and the complex. Potential inhibitors may have a combination of effects on the two p97 conformations.

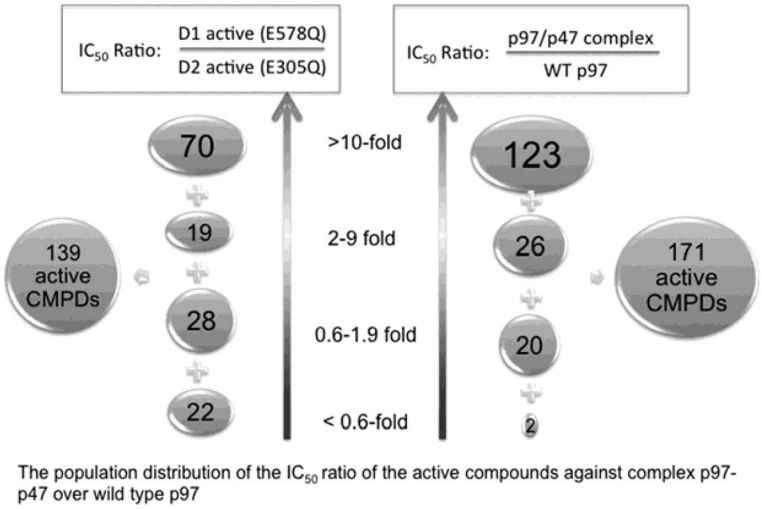

Figure 2.

Results of initial screening of 200 compounds. (Left) 139 of 200 compounds showed various levels of inhibition toward the D1-active mutant (E578Q) and the D2-active mutant (E305Q). Among the 139 compounds, 70 compounds showed >10-fold stronger inhibition against the D2-active mutant (E305Q), 19 were 2- to 10-fold more potent against the D2-active mutant (E305Q), 28 compounds had comparable abilities to inhibit the D2-active (E305Q) and the D1-active (E578Q) mutants, and 22 showed selective inhibition against the D1-active mutant (E578Q). (Right) Among the 200 compounds screened, 171 compounds were active toward wild type p97, the p97-p47complex, or both. IC_{50} values increased >10-fold in the presence of p47 for 123 compounds, ranged from 2- to 9-fold higher with p47 for 26 compounds, and had no discernible change for 20 compounds. There were 2 compounds that showed specific inhibition of the p97-p47 complex (with <0.6-fold lower IC_{50} values).

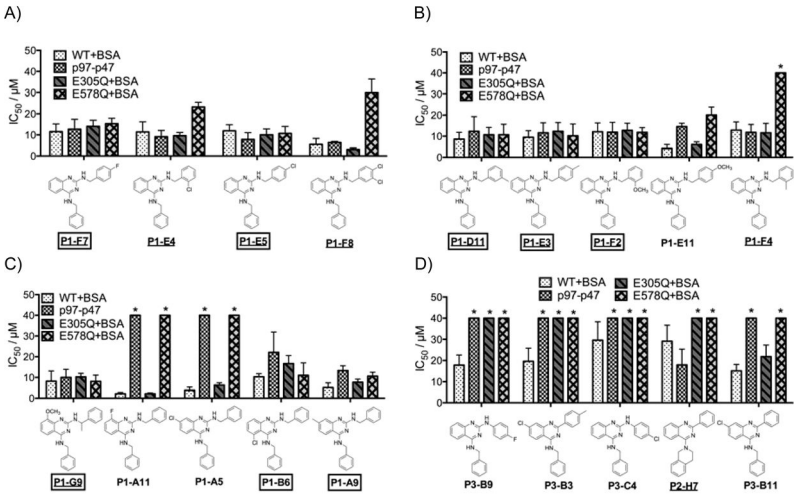

Figure 3.

IC_{50} values of p97 inhibitors against WT p97, the p97-p47 complex, D1-E305Q, and D2 E578Q, grouped by chemical structure. A) Halogen substitution at the benzyl ring. Two compounds (P1-E4 and P1-E8) had increased selectivity toward the D2-domain, whereas P1-F7 and P1-E5 exhibited equal potencies toward D1 and D2. B) Other substitutions of the benzyl ring. P1-E11 and P1-F4 were selective toward the D2-domain, whereas other compounds were equally potent toward D1 and D2. C) Substitutions at the quinazoline phenyl ring. Methoxy substitution (P1-G9), 4-chloro (P1-B6), and 7-Me (P1-A9) led to comparable levels of inhibition toward the D1-domian, D2-domain, WT p97, and the p97-p47 complex. D) Replacement of the aminobenzyl group at the 2-position of the quinazoline core of DBeQ. Modification with a ring-constrained structure at the C4-amino N position on the quinazoline core (P2-H7) caused a selective decrease in the IC_{50} against the p97-p47 complex, compared to WT p97.

*Column heights of 40 μM (marked with an asterisk (*) and shown with no error bars) are used for compounds that were not active toward the indicated form of p97. Compounds with boxes had equal or slightly better potencies toward the D1-domain. Compounds with underlined labels had equal or slightly better potencies toward the p97-p47 complex.