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Boc₃Arg-linked ligands induce degradation by localizing target proteins to the 20S proteasome

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Abstract

Targeted protein degradation is a promising strategy for drug design and functional assessment. Several small molecule approaches have been developed that localize target proteins to ubiquitin ligases, inducing ubiquitination and subsequent degradation by the 26S proteasome. We discovered that the degradation of a target protein can also be induced by a recognition ligand linked to tert-butyl carbamate (Boc₃)-protected arginine (B₃A). Here we show that this process requires the proteasome, but does not involve ubiquitination of the target protein. B₃A does not perturb the structure of the target protein; instead a B₃A-ligand stabilizes its target protein. B₃A ligands stimulate activity of purified 20S proteasome, demonstrating that the tag binds directly to the 20S proteasome. Moreover, purified 20S proteasome is sufficient to degrade target proteins in the presence of their respective B₃A-linked recognition ligands. These observations suggest a simple model for B₃A-mediated degradation wherein the B₃A tag localizes target proteins directly to the 20S proteasome. Thus B₃A ligands are the first example of a ubiquitin-free strategy for targeted protein degradation.

Graphical abstract

Targeted protein degradation is an emerging strategy in drug design ^{1, 2}. Several drugs, most notably fulvestrant and other selective estrogen receptor downregulators (SERDs), induce degradation of their target protein. SERDs change the conformation of the estrogen receptor, exposing a hydrophobic surface that triggers ubiquitination and subsequent degradation ³. Other examples of this phenomenon include a CaCCinh-A01 calcium-activated chloride channel inhibitor ⁴, the ErbB2/HER2 ligand CL-1033 ⁵, the androgen receptor antagonist bicalutamide ⁶ and RNA polymerase inhibitor BMH-21 ⁷. All of these compounds were discovered serendipitously.

Several laboratories have recently developed targeted degradation methods by linking a recognition ligand for a target protein to a ligand for an E3 ubiquitin ligase, thus inducing ubiquitination and degradation of the target ⁸⁻¹¹. Crews and colleagues have also devised a hydrophobic tagging strategy by attaching a recognition ligand to adamantyl and other hydrophobic moieties (e.g., HyT13; Figure 1; ¹²⁻¹⁷). Adamantyl-tagged recognition ligands destabilize their target proteins and recruit Hsp70, inducing ubiquitination and eventual degradation via the proteasome ^{12, 14}. Adamantyl-tagged recognition ligands can also induce the unfolded protein response characterized by phosphorylation of eIF2 and increased expression of XBP1s ¹⁸.

We serendipitously discovered that tert-butyl carbamate (Boc₃)-protected arginine (hereafter B₃A) acts as a small molecule chemical degron to elicit targeted protein degradation ¹⁹. We attached B₃A to ethacrynic acid (EA-B₃A), a covalent inhibitor of glutathione transferases (GST), and EA-B₃A induced the degradation of GST- α and GST- α -EGFP fusion proteins as well as endogenous GST- π in cells and lysates. Similarly, trimethoprim-linked B₃A (TMP-B₃A) induced the degradation of its target, *E. coli* DHFR (eDHFR), as well as eDHFR-EGFP fusion proteins, demonstrating that degradation does not require covalent attachment of the tag to the target protein. Importantly, B₃A ligands decreased the half-lives of their respective target proteins in the presence of the translation inhibitor cycloheximide, confirming that the B₃A tag induced degradation.

At first glance, B₃A appears to be another example of hydrophobic tagging described by the Crews laboratory. However, B₃A does not appear to induce ubiquitination of the target protein ¹⁹, nor does it induce phosphorylation of eIF2 ²⁰, indicating that the mechanism of B₃A induced degradation is distinct from that of adamantyl-tagged recognition ligands. Here we show that B₃A ligands activate the 20S proteasome, and the 20S proteasome degrades

target proteins in a B₃A-dependent manner. Thus we propose that B₃A binding localizes the target protein to the 20S proteasome, resulting in degradation.

RESULTS AND DISCUSSION

B₃A does not induce ubiquitination

Previously reported methods to induce targeted protein degradation rely on ubiquitination of the target protein^{1, 8, 10, 11}. However, we failed to observe the high molecular weight bands indicative of ubiquitin conjugation when B₃A-induced degradation was blocked by the addition of proteasome inhibitors¹⁹. We performed several additional experiments to more rigorously probe for ubiquitination of the target protein.

HA-ubiquitin (HA-Ub) pulldown experiments are perhaps the most sensitive method to detect ubiquitination²¹. Therefore we probed the ubiquitination of endogenous GST- π in HEK293T cells transiently expressing HA-Ub. These cells were treated with either ethacrynic acid (EA) or EA-B₃A in the presence of the proteasome inhibitor bortezomib, and lysates were prepared in the presence of the pan-deubiquitinating enzyme inhibitor PR-619. No high molecular weight GST- π was observed, indicating that EA-B₃A did not induce ubiquitination (Figure 2a). Similar results were observed in Cos1 cells (Supplementary Figure S1). These experiments strongly suggest that EA-B₃A does not induce ubiquitination of GST- π .

We also investigated the ubiquitination of an HA and FLAG-tagged fusion protein eDHFR-HA-GFP-FLAG. Cos1 cells transiently expressing eDHFR-HA-GFP-FLAG were treated with bortezomib followed by TMP or TMP-B₃A. Cells were lysed in the presence of 0.3% SDS and the irreversible deubiquitinating enzyme (DUB) inhibitor WP1130²². eDHFR-HA-GFP-FLAG was immunoprecipitated with anti-FLAG antibody and analyzed (Figure 2b). No high molecular weight bands were observed in an anti-HA blot in either the TMP or TMP-B₃A treated samples. Furthermore, less K48-linked ubiquitin was immunoprecipitated in the TMP-B₃A-treated cells than in TMP treated cells. These data provide further evidence that ubiquitination is not involved in B₃A-mediated protein degradation.

B₃A-induced degradation does not require UAE1

Ubiquitin-activating enzyme 1 (UAE1) is required for the majority of cellular ubiquitination²³. PYR-41 is a cell permeable small molecule inhibitor of UAE1²⁴. A significant decrease in K48-linked high molecular weight ubiquitin conjugates was observed when HEK293T cells were treated with the proteasome inhibitor MG132 followed by PYR-41 (Supplementary Figure S2a), although no effect was observed in the absence of MG132. These observations are consistent with reports from other laboratories²⁵. PYR-41 also inhibits DUBs, which stabilizes ubiquitination and can obscure the effect of UAE1 inhibition, explaining these relatively modest effects²⁶. Having confirmed that PYR-41 inhibited ubiquitination in HEK293T cells, we then tested the effect of PYR-41 on B₃A-induced degradation. Cells were pre-treated with cycloheximide to inhibit protein biosynthesis, then treated with both EA-B₃A and PYR-41. No effect on the degradation of

GST- π was observed (Figure 2c, d), further suggesting that B₃A-induced degradation does not involve ubiquitination.

B₃A-induced degradation does not involve the 26S

B₃A-induced degradation is blocked by proteasome inhibitors such as bortezomib and MG132¹⁹. However, these inhibitors do not distinguish the core 20S proteasome from the 26S proteasome that is responsible for the degradation of ubiquitinated proteins. The 26S proteasome contains the 19S regulatory complex, which includes the DUBs USP14 and UCH37. Inhibition of these DUBs by the small molecule b-AP15 leads to inhibition of 26S proteasome²⁷. Therefore we investigated the degradation of eDHFR in Cos1 cell lysates treated with b-AP15 to determine if B₃A-induced degradation required the 26S proteasome. Inhibition of UCH37 was confirmed by activity profiling (Supplementary Figure S2b). TMP-B₃A induced the degradation of eDHFR similarly in both untreated and b-AP15 treated lysates (Figure 2e). No accumulation of polyubiquitinated eDHFR was observed. These observations strongly suggest that B₃A-mediated eDHFR degradation does not involve the ubiquitin-26S proteasome pathway.

B₃A does not destabilize the target protein

Hydrophobic tagging is proposed to induce protein degradation by destabilizing the target protein¹⁴. Therefore we investigated whether the B₃A tag destabilized the eDHFR. The structure and folding of this small monomeric protein has been extensively characterized by other laboratories²⁸. The melting temperature for eDHFR was 52.4 ± 0.4 °C as measured by circular dichroism spectroscopy, in good agreement with previous reports (literature $T_m = 49\text{--}51$ °C; ^{29, 30}). The presence of TMP increased the melting point of eDHFR by 14 degrees ($T_m = 66.7 \pm 1.8$ °C; Supplementary Figure S3). Such stabilization is commonly observed when ligands bind to proteins. A similar increase in the value of T_m was observed in the presence of TMP-B₃A ($T_m = 64.6 \pm 1.0$ °C). Thus TMP-B₃A does not destabilize its target protein.

It is possible that the B₃A tag might disrupt the structure of eDHFR, perhaps by embedding into the protein. Such a process would likely require a significant rearrangement of the protein structure. Therefore we analyzed the structure of the eDHFR•TMP-B₃A complex using NMR spectroscopy. The ¹H-¹⁵N HSQC NMR spectra of the eDHFR•TMP and eDHFR•TMP-B₃A complexes are almost identical, showing that the B₃A tag does not embed into the target protein (Figure 3).

SAR of B₃A-induced degradation

A series of EA-B₃A derivatives were synthesized to explore the structure activity relationship (SAR) of B₃A induced protein degradation (Figure 1d). As reported previously, degradation of GST- α 1 modified with EA-B₃A was observed in HeLa cell lysates within 2 h (Figure 4a–c). No degradation was observed when GST- α 1 was modified with EA. Shorter and rigid linkers are expected to limit the interactions between B₃A and the target protein, but replacement of the six carbon diamine linker with cyclohexane-1,4-diamine (EA-CY-B₃A, **2**) or ethylenediamine (EA-C2-B₃A, **3**) had no effect on degradation (Figure 4a). In contrast, no degradation was observed when B₃A was replaced with di-butyl carbamate-

protected ornithine (B₂O, **4**) or butyl carbamate-protected-protected glycine (B₁G, **5**) (Figure 4b). This observation suggests that all three boc groups are required for B₃A-induced degradation. Lastly, NH₂-B₃A, which consists of B₃A and the 6 carbon diamine linker but no recognition ligand, inhibits the EA-B₃A induced degradation of GST- α 1 in lysates (Figure 4c). Collectively these observations further indicate that the B₃A tag interacts with another protein factor distinct from the target protein.

B₃A ligands activate the 20S proteasome

Although proteasome inhibitors block B₃A-induced degradation, the above experiments suggest that the 26S proteasome is not involved. Therefore B₃A-induced degradation may instead utilize the 20S proteasome. Hydrophobic peptides are known to activate the 20S proteasome^{31, 32}, so we hypothesized that B₃A might similarly activate the 20S proteasome, thereby docking the target protein to the 20S proteasome. Therefore we investigated the effect of various B₃A-containing ligands on the activities of the 20S proteasome (Figure 5). All five B₃A ligands activated the trypsin-like subunits by 2–16-fold (Figure 5a and S4a). Activation of the trypsin-like subunits was also observed with EA-B₁G and EA-B₂O. No activation of the chymotrypsin-like activity was observed under these conditions. The activity of the 20S proteasome varies with different monovalent cations³², so we also tried different assay buffers, and a modest stimulation (1.4–1.7-fold) of the chymotrypsin-like activity was observed with three B₃A ligands, NH₂-B₃A, Ac-B₃A and TMP-B₃A when the reaction was performed in sodium chloride (Figure 5b and Supplementary S4b). EA-B₃A and Cbz-B₃A failed to activate the chymotrypsin-like activity and EA-B₁G and EA-B₂O inhibited. No activation of the postglutamyl activity was observed (Supplementary Figure S4c). In contrast to the 20S proteasome, none of the activities of the 26S proteasome were increased by the presence of NH₂-B₃A (Supplementary Figure S4d). In addition, the adamantyl ligand Ad-NH₂ failed to stimulate the 20S proteasome (Supplementary Figure S5a, b), which further demonstrates that there are significant mechanistic differences between the adamantyl system and B₃A.

We characterized the activation of the 20S proteasome by NH₂-B₃A in more detail (Figure 5d and Supplementary Table S1). The values of k_{cat}/K_m increased for both the trypsin-like and chymotrypsin-like activities, albeit with different underlying causes. For the trypsin-like activity, the values of k_{cat} increased with increasing B₃A ligand while K_m was unchanged. In contrast, the value of K_m decreased with increasing B₃A ligand while the value of k_{cat}/K_m was unchanged for the chymotrypsin-like proteasome activity. For the postglutamyl activity, the value of k_{cat}/K_m decreased with increasing NH₂-B₃A due to an increase in the values of K_m . These observations indicate that B₃A ligands allosterically modulate all of the proteasome active sites.

B₃A interacts noncovalently with 20S

We performed a dilution experiment to determine if proteasome activation involved covalent or noncovalent interactions with B₃A. Proteasome was activated by incubation with substrate and NH₂-B₃A, then diluted into substrate (Figure 5e). The nonlinear behavior shows that activation is not stable to dilution, as expected if B₃A dissociates from the 20S proteasome.

B₃A has no effect on other 20S activators

The 20S proteasome is also regulated by PA28 α , which accelerates peptide degradation in an ATP-independent manner^{33, 34}. We measured the effects of NH₂-B₃A on PA28 α stimulated 20S proteasome (Figure 5e). PA28 α alone stimulated the trypsin-like activity of 20S proteasome by 10 – 15 fold at a concentration of 400 nM. NH₂-B₃A does not affect the stimulatory effect of PA28 α nor further activate the 20S proteasome. This observation suggests that these two activators likely bind to different sites.

Betulinic acid is a well recognized small molecule activator of the 20S proteasome³⁵. Consistent with previous reports, betulinic acid stimulated the trypsin-like activities of the 20S proteasome by 8-fold (Figure 5f). Addition NH₂-B₃A to saturating concentrations of betulinic acid elicited further stimulation of the proteasome, indicating that these molecules bind to different sites on the proteasome.

20S is sufficient for B₃A-induced degradation

We characterized the degradation of eDHFR and GST- α 1 to determine if the 20S proteasome was sufficient to degrade proteins in a B₃A-dependent manner. eDHFR was a poor substrate of the 20S proteasome (Figure 6a). TMP protected eDHFR from degradation as expected given that TMP stabilizes eDHFR. Equimolar NH₂-B₃A did not significantly increase degradation of eDHFR, also as expected since this concentration of B₃A ligand is not sufficient to activate the 20S proteasome. In contrast, degradation increased in the presence of equimolar TMP-B₃A. No degradation was observed with a mixture of equimolar NH₂-B₃A and TMP, confirming that B₃A must be directly linked to the recognition ligand. Importantly, excess NH₂-B₃A inhibited TMP-B₃A-induced degradation. These observations are consistent with a model that the B₃A ligands bind to a site on the proteasome, localizing the target protein for degradation.

In principle, degradation should release the B₃A ligand, allowing it to bind and degrade another target protein. However, substoichiometric TMP-B₃A did not accelerate the degradation of eDHFR, indicating that the B₃A ligand does not act catalytically under these conditions.

Likewise, little degradation of GST- α 1 was observed in the absence of B₃A ligands or in the presence of EA (Figure 6b). EA-B₃A induced the degradation of GST- α 1, but no degradation was observed with EA-B₂O or EA-B₁G. Thus the purified 20S proteasome displays the same SAR as GST- α 1 degradation in lysates. These observations strongly suggest that the 20S proteasome is responsible for B₃A-mediated degradation in cells.

In contrast to the above observations, TMP-B₃A and EA-B₃A protected their respective target proteins from degradation by the 26S proteasome, further indicating that the 20S proteasome is responsible for B₃A-mediated degradation (Figure 6c).

Collectively these observations suggest degradation is mediated by a ternary complex between target protein, B₃A-linked recognition ligand and 20S proteasome. This model predicts that excess recognition ligand will inhibit degradation by blocking access to the proteasome. A bell-shaped dose dependence is indeed observed for both the TMP-B₃A

mediated degradation of eDHFR and the EA-B₃A mediate degradation of GST- α 1 (Figure 6d, e). These observations demonstrate that localization of the target protein to the 20S proteasome, rather than the stimulation of proteasome activity, is the critical feature of B₃A-induced degradation.

Selectivity of B₃A-induced degradation

We monitored the reaction of mixtures of eDHFR and GST- α 1 with the 20S proteasome and their respective recognition ligands in order to test the selectivity of degradation. The addition of EA-B₃A caused the selective degradation of its target GST- α 1 as expected (Supplementary Figure S6). However, the addition of TMP-B₃A caused the degradation of both its target eDHFR and the non-target protein GST- α 1 (Figure 6f). No GST- α 1 degradation was observed in the absence of eDHFR, indicating that degradation did not result from a promiscuous interaction between TMP-B₃A and GST- α 1. This observation indicates that the 20S proteasome complex with eDHFR•TMP-B₃A can promiscuously degrade other proteins, which suggests that off-target protein degradation may be an issue for some B₃A ligands/target protein pairs.

B₃A ligands activate the proteasome in living cells

We used the luminogenic proteasome substrate Z-Leu-Arg-Arg-aminoluciferin to assay the ability of B₃A ligands to activate the proteasome in living cells³⁶. A strong luminescent signal was observed when HELA cells were treated with Z-Leu-Arg-Arg-aminoluciferin. This signal was reduced by 40% when cells were treated with the proteasome inhibitor bortezomib, indicating that at least 40% of the activity can be attributed to the proteasome. Addition of Cbz-B₃A and TMP-B₃A increased luminescence in the absence of bortezomib, but had no effect on luminescence in the presence of bortezomib (Figure 7). These observations indicate that both Cbz-B₃A and TMP-B₃A activate the proteasome in cells. NH₂-B₃A did not activate the proteasome in cells, possibly because it was not permeable.

The above assay does not distinguish between the 20S and 26S proteasomes. Therefore we performed the Ub^{G76V}-GFP assay to monitor 26S proteasome activity³⁷. The G76V mutation prevents the cleavage of ubiquitin from GFP, leading to its degradation by the 26S proteasome. Inhibition of the proteasome by bortezomib treatment causes an increase of Ub^{G76V}-GFP as expected^{38, 39}. An approximately 15% decrease in Ub^{G76V}-GFP was observed when cells were treated with Cbz-B₃A, suggesting that B₃A ligands do not activate the 26S proteasome in cells.

B₃A ligands localize their targets to 20S

The experiments described above indicate that, unlike other methods, B₃A does not induce ubiquitination of the target protein. Instead, our experiments suggest that B₃A localizes the target protein to the 20S proteasome (Figure 8). B₃A activates purified 20S proteasome, demonstrating that it binds to the 20S proteasome. We have also found that proteasome subunits α 7 and β 7 were strongly enriched in the pool of proteins bound to B₃A-linked resin (Supplementary Table S2)²⁰, further substantiating an interaction between B₃A and the proteasome. While it is possible that other protein factors are involved in B₃A-mediated degradation within a cell, purified 20S proteasome is sufficient for the B₃A-mediated

degradation of a target protein in vitro. Thus B₃A ligands provide proof of concept for a new strategy of targeted protein degradation. Intriguingly, this strategy could be applied to other AAA⁺ proteases such as ClpXP or FtsH⁴¹. These proteases are present in virtually every organism, and so this strategy has the potential to extend beyond the reach of ubiquitination targeting methods.

It's noteworthy that TMP-B₃A induces the degradation of eDHFR even as it stabilizes the protein fold. The 20S proteasome degrades intrinsically unfolded proteins, and can latch onto unfolded segments to degrade folded proteins^{42, 43}. Both eDHFR and GST- α contain flexible segments that could be vulnerable. We propose that the B₃A ligand localizes the target protein to 20S proteasome, greatly increasing the probability that a flexible segment enters the barrel. This model predicts that the efficiency of degradation will depend on the interaction between the recognition ligand and target and the lifetime of the ternary complex. Optimization of these parameters should improve the efficiency of degradation. For example, the efficiency of eDHFR degradation might improve with a lower affinity recognition ligand that does not stabilize the folded structure to the extent observed with TMP.

Potential of B₃A-induced degradation in drug discovery

It's important to recognize that functional assessment and drug discovery have fundamentally different objectives and requirements. Ideally, functional assessment requires rapid and surgical removal of the target protein so that the resulting phenotypes can be confidently assigned. The methods that rely on E3 ubiquitin ligases appear to satisfy both of these criteria¹⁰. Hydrophobic tagging with adamantyl ligands also induces rapid degradation, but can cause a transient unfolded protein response that may complicate the functional assessment of target proteins¹⁸. B₃A does not induce the unfolded protein response²⁰, but can activate the 20S proteasome and induce the degradation of non-target proteins. In addition, some B₃A-ligands inhibit protein translation²⁰. Both phenomena depend on the specific B₃A-ligand and target protein, which will complicate the use of B₃A induced degradation in functional assignment.

In contrast, the goal of a drug is to elicit a clinically beneficial response. The great advantage of targeted protein degradation for drug discovery is the potential to uncouple pharmacokinetics from pharmacodynamics. The effects of a conventional inhibitor usually depend on its concentration in plasma, and therefore wane as the drug is excreted or metabolized⁴⁴. In contrast, the effect of targeted degradation will endure until the cell resynthesizes the target protein, which may be long after the drug has cleared the patient. Selectivity is not as important in this context. In fact many drugs are successful precisely because they interact with many targets⁴⁵. In the case of B₃A ligands, the ability to inhibit translation may further enhance the effects of target degradation.

To our knowledge, B₃A ligands are the first small molecules to induce protein degradation by localization to the proteasome. Though the importance of the 26S proteasome in cellular protein degradation is widely recognized, the substantial contribution of the 20S proteasome is often overlooked⁴⁶. The 20S proteasome is typically 2–3-fold more abundant than the 26S proteasome^{47–50}. The 20S proteasome degrades misfolded and oxidatively damaged

proteins, as well as proteins with large regions of disorder in an ubiquitin-independent and ATP-independent manner⁵¹. Localization of a target protein to the 26S proteasome has long been known to be sufficient for degradation⁵², but the potential of the 20S proteasome for targeted protein degradation has been largely overlooked. Intriguingly, B₃A binding appears to be independent of other activators such as PA28 α and betulinic acid, suggesting that the 20S proteasome may harbor several potential binding sites that can be exploited with localization ligands. Thus proteasome docking is a promising new strategy for targeted protein

METHODS

Detailed Methods are available in the Supplementary Material.

Proteasome catalytic activity assays

20S Proteasome purified from human erythrocytes (catalog #E-360), 26S proteasome (catalog #E-365) and PA28 α (catalog #E-381) was purified from transfected HEK cells were purchased from BostonBiochem. For peptide AMC substrates using a BioTek plate reader. For protein degradation assays, the target protein (eDHFR or GST) was preincubated with its respective inhibitor (TMP-B₃A or EA-B₃A) then mixed with proteasome. Aliquots were quenched with 3.5 μ L of 5X SDS loading buffer at the desired time intervals and analyzed by SDS-PAGE and immunoblotting. Protein was visualized with the appropriate primary and secondary antibodies using ECL Plus (GE, Bucks, UK) after transfer. Densitometry was carried out using software IMAGE-J V 1.44p.

DUB activity profiling

Cos1 cell pellets were lysed on ice by vortexing with glass beads and crude lysate was centrifuged for 5 min. The lysate (1.5 mg/ml) was incubated with b-API5 (or 1% DMSO control) for 20 min, then HA-ubiquitin-VME (250 nM) was added and incubated for 25 min. The reaction mixture (20 μ l) was removed and quenched in 2X (final concentration) reducing SDS-PAGE loading buffer.

In cell proteasome activity

Proteasome activity in cells was measured using Proteasome-GloTM Cell-Based Assay (Promega)³⁶ using Z-leucine-arginine-arginine-aminoluciferin. For the ubiquitin^{G76V}-GFP assay, Cos1 cells expressing ubiquitin^{G76V}-GFP were treated with compound for 8 h. Cells were harvested and analyzed on a Beckman FACS-Calibur. All data were analyzed using FlowJo V10, from TreeStar (Ashland, OR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mechanisms of small molecule targeted protein degradation

A) Several methods, e.g. PROTACS and SNIPERS, localize the target protein to an E3 ubiquitin ligase via a chimeric ligand, causing ubiquitination and subsequent degradation through the 26S proteasome ¹. B) Mechanism of hydrophobic tag induced degradation, e.g., HaloTag/HyT ^{14, 15}. Ligand binds to Halo protein. Halo protein unfolds, causing ubiquitination and degradation via the 26S proteasome. C) Possible mechanisms of Boc3Arg mediated degradation. D) Compounds used in this work.

Figure 2. EA-B₃A does not increase the ubiquitination of target proteins

A) Endogenous GST- π . HEK293T cells expressing HA-Ub were treated with bortezomib (1 μ M) followed by EA or EA-B₃A (40 μ M) for 2 h. Lysates (5 mg/ml) were prepared in the presence of the pan-DUB inhibitor PR-619 and ubiquitinated proteins were isolated with anti-HA beads. Protein was analyzed by western blot using anti-GST- π ; anti-HA (HA-ubiquitin) and anti-tubulin. B) Cos1 cells were transfected with DHFR-HA-GFP-FLAG and treated with either TMP (80 μ M) or TMP-B₃A (80 μ M) for 3 h in the presence of bortezomib (1 μ M). Lysates were prepared in 0.3% SDS containing buffer supplemented with irreversible DUB inhibitor WP1130 and then immunoprecipitated with anti-FLAG. Protein analyzed by western blot using anti-HA (DHFR-HA-GFP-FLAG) and anti-K48-linked ubiquitin. C) PYR-41 does not block the B₃A-induced degradation of endogenous GST- π . HEK293T cells were preincubated with cycloheximide (CHX) (50 μ g/ml) for 30 min, then treated with EA-B₃A (40 μ M), PYR-41 (40 μ M), DMSO (vehicle only), or EA-B₃A (40 μ M) with PYR-41 (40 μ M) and MG132 (10 μ M) for 3 h and blotted for K48 ubiquitination. Same condition without MG132 was used when blotting GST- π . D) Plots show mean $\pm$ s.d. for experiments as in panel D, (N=3). E) b-AP15 does not block B₃A-induced degradation of eDHFR. A Cos1 cell lysate (1 mg/ml) was preincubated with b-AP15 (20 μ M) for 20 min before addition of eDHFR (800nM) and TMP-B₃A (1.6 μ M) were added to the assay.

Figure 3. TMP-B₃A does not destabilize eDHFR

2D ^1H - ^{15}N HSQC spectra of eDHFR•TMP (blue) and eDHFR•TMP-B₃A (red). NMR samples contained 0.1 mM DHFR and 0.14 mM of ligands in 25 mM Phosphate (pH 7.6), 100 mM KCl, 1 mM DTT and 10% D₂O.

Figure 4. SAR of B₃A-mediated degradation

a) GST-α1 was modified with EA derivatives and added to a HeLa cell lysate. b) SAR of EA-B₁G, EA-B₂O and EA-B₃A. c) NH₂-B₃A inhibits degradation. All plots show mean ± s.e.m.

Figure 6. The 20S proteasome is sufficient to induce B₃A-dependent degradation

a) TMP-B₃A induces degradation of eDHFR by the 20S proteasome. eDHFR (800 nM) was incubated with purified 20S proteasome (5 nM) at 37 °C for 2 h (N=3). b) EA-B₃A induces degradation of GST-α1 by the 20S proteasome. GST-α1 (800 nM) was incubated with purified 20S proteasome (5 nM) at 37 °C for 2 h (N=3). c) Degradation of eDHFR and GST by 26S proteasome in vitro. eDHFR (1.6 μM) or GST-α1 (1.6 μM) in the absence and presence of their respective recognition ligands were incubated with 26S proteasome (1 nM) at 37 °C. d) eDHFR (800 nM) was incubated with TMP-B₃A and 20S proteasome (5 nM) at 37 °C for 2 h. e) GST-α1 (800 nM) was incubated with EA-B₃A and 20S proteasome (5 nM) at 37 °C for 1 h. f) TMP-B₃A induces the degradation of both GST-α1 and eDHFR. A mixture of eDHFR and GST-α1 (both 160 nM) were incubated with 20S proteasome (5 nM) in the absence and presence of TMP-B₃A (1.6 μM).

Figure 7. B₃A ligands activate the 20S proteasome in cells

a) HELA cells were treated with the luminogenic proteasome substrate Z-Leu-Arg-Arg-aminoluciferin and the indicated activators (80 μ M) in the absence (solid bars) and presence of bortezomib (striped bars, 20 μ M). b) Effect of B₃A ligands on stability of G76V-Ub-GFP. Cos1 cells expressing G76V-Ub-GFP were treated with 100 μ M B₃A ligands for 8 h. GFP was quantified by FACS analysis (N=4).

Figure 8.
Model for B₃A-mediated protein degradation.