

Inhibitor Mediated Protein Degradation

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SUMMARY

The discovery of drugs that cause the degradation of their target proteins has been largely serendipitous. Here we report that the *tert*-butyl carbamate-protected arginine (Boc₃Arg) moiety provides a general strategy for the design of degradation-inducing inhibitors. The covalent inactivators ethacrynic acid and thiobenzofurazan cause the specific degradation of glutathione-S-transferase when linked to Boc₃Arg. Similarly, the degradation of dihydrofolate reductase is induced when cells are treated with the noncovalent inhibitor trimethoprim linked to Boc₃Arg. Degradation is rapid and robust, with 30%–80% of these abundant target proteins consumed within 1.3–5 hr. The proteasome is required for Boc₃Arg-mediated degradation, but ATP is not necessary and the ubiquitin pathways do not appear to be involved. These results suggest that the Boc₃Arg moiety may provide a general strategy to construct inhibitors that induce targeted protein degradation.

INTRODUCTION

Several authors have suggested that the ideal drug dissociates slowly from its target protein, so that new target synthesis must occur to restore function (Copeland et al., 2006; Swinney, 2004; Zhang and Monsma, 2009). Such perfection usually requires very high affinity binding or covalent modification, which accounts for the current resurgence of interest in covalent drugs (Singh et al., 2011). Intriguingly, several drugs meet this metric by inducing the degradation of their targets. Selective estrogen receptor downregulators (SERDs) such as fulvestrant are the best-characterized examples of this phenomenon (Howell, 2006). These compounds induce degradation of the estrogen receptor via the ubiquitin/26S proteasome pathways. In cell culture, the level of estrogen receptor is decreased by 45%–83% in 4 hr (Wittmann et al., 2007). Importantly, tamoxifen-resistant breast cancer cells remain sensitive to fulvestrant and other SERDs, illustrating the advantage of this strategy. Similar degradation of the androgen receptor is observed with bicalutamide treatment (Waller et al., 2000), and the success of the retinoic acid/arsenic trioxide combination in acute promyelocytic

leukemia therapy derives from its ability to induce the degradation of the oncogenic fusion protein PML-RARA (Nasr et al., 2009). These examples illustrate the therapeutic potential of small molecules that induce protein degradation. In addition, such compounds could have broad utility as reagents in protein knockdown experiments. A robust, broadly applicable strategy for the design of degradation-inducing small molecule inhibitors has yet to emerge. Given that inhibitor binding generally stabilizes protein structure, the principles of such design are not obvious.

One approach to this problem is proteolysis targeting chimeric molecules (PROTACs) developed by Crews and colleagues (Raina and Crews, 2010). PROTACs contain a ligand that recognizes the target protein linked to a ligand that binds to a specific E3 ubiquitin ligase. Degradation of methionine aminopeptidase, androgen receptor, estrogen receptor, and the aryl hydrocarbon receptor has been reported (Lee et al., 2007; Rodriguez-Gonzalez et al., 2008; Sakamoto et al., 2001). In many cases, the E3 ligase-targeting ligand is a peptide, which can limit therapeutic use. More importantly, the ubiquitin pathways are extremely complicated and poorly understood (Clague and Urbé, 2010; Finley, 2009). The details of tissue expression, cellular localization, and regulation are known in only a tiny fraction of the over 600 putative E3 ligases in humans (Deshaies and Joazeiro, 2009). Such observations suggest that the reliable manipulation of these pathways may prove challenging.

Conditional protein knockdown is an invaluable tool for delineating protein function. In addition to RNAi, several methods have been developed that rely on small molecule ligands (Raina and Crews, 2010). The most common strategy involves expression of fusion proteins that couple the target to an unstable “degron” domain. The degron domain is usually stabilized by the presence of a ligand; removal of the ligand induces degradation (Banaszynski et al., 2006; Dohmen et al., 1994; Iwamoto et al., 2010; Lévy et al., 1999; Pratt et al., 2007; Stankunas et al., 2003). These techniques have proven to be very useful, but are limited by the requirement for the constant presence of ligand to maintain protein levels. Several systems have been developed to address this problem. One method uses a small molecule to localize a target fusion protein directly to the proteasome (Janse et al., 2004). Importantly, the success of this strategy demonstrates that proteasome localization is sufficient to induce degradation. A similar strategy has been used to target protein degradation in bacteria (Davis et al., 2009). In another clever variation, a small molecule has been used to unmask a cryptic degron (Bonger et al., 2011). Recently, a system based on the covalent attachment of hydrophobic

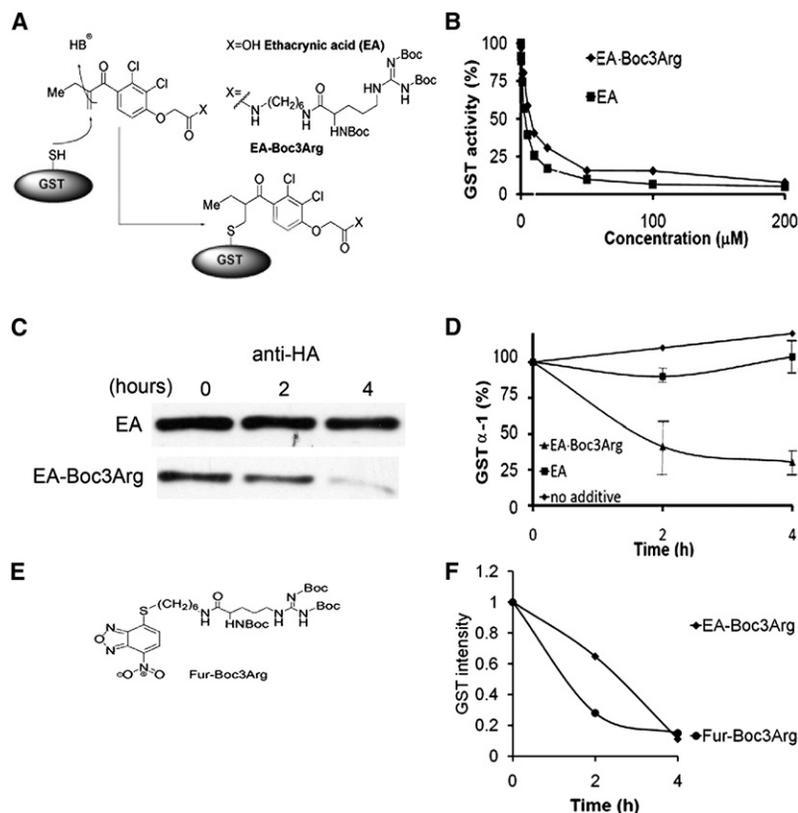


Figure 1. Degradation of HA-Tagged GST- α 1

(A) Structures of EA and EA-Boc₃Arg and the reaction of GST with EA derivatives.

(B) EA and EA-Boc₃Arg inactivate GST- α 1 with similar potency. Purified recombinant GST- α 1 (200 nM) was incubated with EA or EA-Boc₃Arg and assayed for activity. (C and D) The degradation of EA-Boc₃Arg-modified GST- α 1. Purified recombinant C-terminally HA-tagged GST- α 1 (0.2 mg/ml) was inactivated by EA or EA-Boc₃Arg (80 μ M) and diluted 50-fold into HeLa cell lysates supplemented with an ATP regenerating system. Samples were analyzed by immunoblotting for the HA tag. Inosine monophosphate dehydrogenase (IMPDH) was used as a loading control. (C) A representative immunoblot. (D) Average of four experiments. Error bars show standard errors.

(E) Structure of Fur-Boc₃Arg.

(F) Degradation of GST- α 1 by Fur-Boc₃Arg. Purified C-terminally HA-tagged GST- α 1 was modified with Fur-Boc₃Arg (40 μ M) and added to NIH 3T3 cell lysates supplemented with an ATP regenerating system.

tags to dehalogenase fusion proteins has also been reported (Neklesa et al., 2011). However, although these systems can effectively modulate levels of transgenic fusion proteins, they cannot be used to reduce the levels of endogenous proteins.

We have discovered that inhibitors linked to a *tert*-butyl carbamate-protected arginine (Boc₃Arg) moiety induce selective degradation of their target proteins. Remarkably, this process appears to be ATP- and ubiquitin-independent. We propose that Boc₃Arg-induced degradation provides a strategy to induce protein degradation for both conditional protein knockdown and chemotherapeutic applications.

RESULTS

EA-Boc₃Arg Induces the Degradation of GST- α 1 in Cell Lysates

GST isozymes, especially GST- π , are potential targets for anti-cancer chemotherapy (Laborde, 2010; Sau et al., 2010). These proteins are well characterized with several readily modified inhibitors such as ethacrynic acid (EA) and thiobenzofurazan (Fur) (Ploemen et al., 1993; Ricci et al., 2005). Therefore, we initiated an investigation of protein degradation using C-terminally hemagglutinin (HA)-tagged glutathione-S-transferase α 1 (GST- α 1) as the target protein. HA-tagged proteins are widely used reagents because horseradish peroxidase-coupled anti-HA antibody provides a sensitive assay with a dynamic range of >100. Our plan was to link EA to an arginine residue, reasoning that such an inhibitor would induce protein degradation via the N-end rule pathways (Tasaki and Kwon, 2007). Some indication

that the N-end rule pathways can be exploited in this manner is present in the patent literature (Kenten and Roberts, 2001). EA alkylates the active site Cys residue in GST (Figure 1A); the inhibitor's carboxylate moiety can be readily modified without perturbing this reaction (Figure 1B) (Shi et al., 2006). One of the compounds produced during the synthetic route was EA-Boc₃Arg (Figure 1A), which contains *N,N,N*-triBoc-protected arginine moiety linked to EA via a 1,6-diaminohexane linker. Unfortunately, removal of the Boc groups to produce the unprotected arginine derivative was unsuccessful. Surprisingly, EA-Boc₃Arg-modified GST- α 1 was readily degraded in HeLa cell lysates (Figures 1C and 1D). No degradation was observed in the absence of EA-Boc₃Arg, with unmodified EA (Figure 1D) or with EA linker (data not shown). Similar degradation of EA-Boc₃Arg-modified GST- α 1 was observed in NIH 3T3 cell lysates. These results suggested that the Boc₃Arg moiety targets the protein for degradation.

Fur-Boc₃Arg Induces the Degradation of GST- α 1 in Cell Lysates

To test the generality of this degradation phenomenon, we synthesized a second Boc₃Arg-linked GST inactivator, Fur-Boc₃Arg (Figure 1E). Fur also forms a covalent adduct with GST; this molecule can be modified at the indicated positions with retention of activity (Ricci et al., 2005). Fur-Boc₃Arg-modified GST- α 1 was readily degraded in lysates from NIH 3T3 cells (Figure 1F). These results demonstrate that Boc₃Arg-induced degradation does not depend on the nature of the ligand interacting with GST- α 1.

TMP-Boc₃Arg Induces the Degradation of eDHFR in Cell Lysates

To determine if Boc₃Arg-dependent degradation is a unique property of GST- α 1, we turned to the *Escherichia coli* dihydrofolate reductase/trimethoprim (eDHFR/TMP) system developed by Cornish (Calloway et al., 2007). TMP is a specific inhibitor of

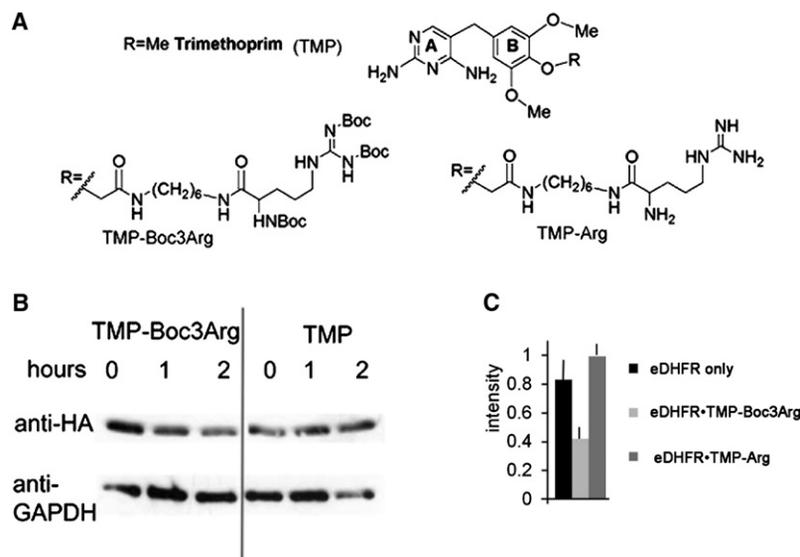


Figure 2. TMP-Boc₃Arg Induced Degradation of HA-Tagged eDHFR in Cell Lysates

(A) Structures of TMP and derivatives. (B and C) Purified recombinant eDHFR-HA (0.2 mg/ml) was incubated with TMP derivatives (80 μ M) and diluted 50-fold into Cos-1 cell lysates supplemented with an ATP regenerating system. (B) Representative immunoblot showing degradation of eDHFR-HA in the presence of TMP and TMP-Boc₃Arg. (C) Combined data from two experiments after 4 hr incubation. Bar graphs show the average and the range of values.

eDHFR with much lower affinity for mammalian DHFRs ($K_d = 20$ pM versus 4 μ M for bacterial and mammalian DHFRs, respectively). Importantly, the interaction between eDHFR and TMP is noncovalent, although dissociation is very slow ($T_{1/2} \sim 20$ min) (Dunn and King, 1980). TMP can be modified at the 4 position of the B ring with retention of potency and selectivity (Figure 2A) (Calloway et al., 2007). This strategy has been used to fluorescently label eDHFR fusion proteins in lysates and whole cells (Calloway et al., 2007).

We synthesized a Boc₃Arg derivative of TMP using modified published methods (Figure 2A; see Experimental Procedures for synthetic procedures) (Calloway et al., 2007; Long et al., 2011). As above, eDHFR contained a C-terminal HA-tag to facilitate detection. While eDHFR degraded slowly in Cos-1 cell lysates, the eDHFR-TMP complex was stable, as expected (Figures 2B and 2C). The deprotected compound TMP-Arg also stabilized eDHFR (Figure 2C). In contrast, the DHFR-TMP-Boc₃Arg complex was readily degraded. Similar TMP-Boc₃Arg-dependent degradation of eDHFR was observed in HeLa and NIH 3T3 cell lysates (data not shown). Importantly, these observations demonstrated that the Boc₃Arg moiety does not need to be covalently attached to the target protein to induce degradation.

Boc₃Arg Induces Protein Degradation in Whole Cells

We constructed a variety of GST and eDHFR fusion proteins to determine the efficacy of Boc₃Arg-mediated protein degradation in mammalian tissue culture cells. Importantly, TMP-Boc₃Arg has no effect on cell viability at concentrations up to 135 μ M over the course of these experiments (Figures S1A and S1B available online). Likewise, no toxicity was observed when Cos-1 cells were treated with EA-Boc₃Arg (100 μ M), although HeLa cells displayed some sensitivity (Figures S1C and S1D). Endogenous GST isozymes are abundant proteins, so we chose to focus our initial efforts on ectopically expressed eDHFR fusion proteins. As noted above, the eDHFR/TMP system is used to selectively label proteins in cells (Calloway et al., 2007), which suggested that the TMP-Boc₃Arg would

induce the degradation of eDHFR fusion proteins in whole cells.

Boc₃Arg-initiated protein degradation was monitored using a modified global protein stability (GPS) assay (Yen et al., 2008). GFP-fusion proteins are co-expressed with *Discosoma* sp. red fluorescent protein (RFP) in a bicistronic construct under control of a CMV

promoter, with GFP-fusion protein translation occurring at the internal ribosome binding site. Thus, a single mRNA is responsible for the production of both RFP and GFP-fusion proteins, so that both proteins are produced in a constant ratio in all transfected cells (Yen et al., 2008). Changes in the relative intensities of red and green fluorescence are measured by flow cytometry, providing a facile and quantitative measure of the protein stability in living cells and real time. This assay has been used to monitor the stability of ~ 8000 human proteins to identify substrates of the proteasome and E3 ligases (Yen and Elledge, 2008; Yen et al., 2008).

In the present application, an eDHFR-HA-GFP fusion protein is expressed with translation occurring at the internal ribosome binding site. The ratio of GFP/RFP does not change in the presence of TMP (Figure 3A). In contrast, the GFP/RFP ratio decreases in the presence of TMP-Boc₃Arg (Figures 3B and 3C). We confirmed the degradation of eDHFR-HA-GFP with both anti-HA and anti-GFP blotting (Figure 3D). Approximately 30%–40% of the GFP signal was lost over 5 hr. Similar decreases in GFP/RFP ratios were observed in MCF-7 cells. These experiments demonstrate that Boc₃Arg-induced degradation is sufficiently robust to compete with CMV-driven protein expression.

In the context of a whole cell, depletion of target protein levels will be a function of the rate of protein synthesis, the rate of degradation, and the rate of uptake of the Boc₃Arg ligand. To further gauge the contribution of new protein synthesis to the extent of protein knockdown, we monitored TMP-Boc₃Arg-induced degradation in the presence of cycloheximide, which blocks the synthesis of new proteins. Under these conditions, eDHFR-HA-GFP levels are reduced to $\sim 10\%$ within 3 hr in cells treated with TMP-Boc₃Arg (Figure 4). No degradation was observed when cells were treated with dimethyl sulfoxide (DMSO) alone or with TMP, demonstrating that degradation is induced by the Boc₃Arg degron. Given that eDHFR-HA-GFP expression is driven by a CMV promoter, and that GFP is a very stable protein, these observations suggest that Boc₃Arg-induced degradation will be sufficiently efficient to knock down most proteins.

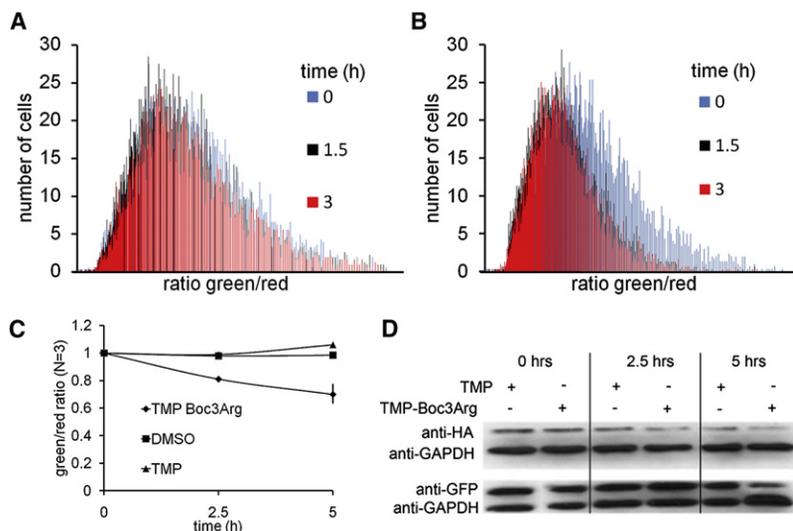


Figure 3. Degradation of eDHFR Fusion Proteins in Whole Cells

(A and B) Global protein stability assay. HeLa cells co-express RFP and eDHFR-HA-GFP from a bicistronic construct. Red and green fluorescence was measured by flow cytometry. (A) TMP (80 μ M). (B) TMP-Boc₃Arg (80 μ M).

(C) Quantitation of four independent GPS experiments. Bar graphs show the average and the standard deviation. (D) Degradation of eDHFR-GFP was confirmed by anti-HA and anti-GFP immunoblotting.

See also Figure S1.

EA-Boc₃Arg Induces Degradation of Endogenous GST- π

Encouraged by the above results, we investigated whether EA-Boc₃Arg could cause the degradation of *endogenous* GST- π . This protein is abundant; its concentration can be as high as 50 μ M in cancer cells (Ricci et al., 2005). Moreover, alkylators induce the expression of GST (Hu et al., 2006a; Thimmulappa et al., 2002), so this is another demanding test of the efficacy of Boc₃Arg-mediated degradation. Cos-1 cells were incubated with EA or EA-Boc₃Arg for 3 hr. EA treatment increased the level of endogenous GST- π by 30% relative to the DMSO control (Figure 5A). In contrast, EA-Boc₃Arg treatment reduced the levels of endogenous GST- π by 30% relative to the DMSO control and by 50% relative to EA treatment (Figure 5A). GST- π is a bona fide anticancer target (Raj et al., 2011; Sau et al., 2010), so this experiment provides proof of concept with a pharmaceutically relevant protein.

EA-Boc₃Arg Induces Degradation of Ectopic Proteins in Whole Cells

Compounds such as EA are known to amplify the expression of GST- π (Higgins and Hayes, 2011), so it is possible that the rate of GST- π synthesis was increasing over the course of the above experiment. Therefore, we ectopically expressed GST- α 1-fusion proteins under control of a CMV promoter to ensure that the target protein would be expressed at a constant rate. Gratifyingly, treatment with EA-Boc₃Arg caused the rapid and robust degradation of eDHFR-HA-GST- α 1. Fusion protein levels decreased by \sim 70% within 2 hr (Figures 5B and 5C). As observed with GST- π , eDHFR-HA-GST- α 1 levels increased when cells were treated with EA alone, suggesting that the unmodified inhibitor stabilized the protein. Degradation was dose-dependent, with 10 μ M causing \sim 50% degradation in 1.2 hr (Figure 5D). Preincubation of cells with EA protected eDHFR-HA-GST- α 1 against degradation, confirming the specificity of EA-Boc₃Arg (Figure S2).

The eDHFR-HA-GST- α 1 fusion protein allows the direct comparison of degradation induced by EA-Boc₃Arg and TMP-Boc₃Arg. While 80 μ M EA-Boc₃Arg induces nearly complete degradation of the fusion protein in under 2 hr, only \sim 25% degra-

degradation is observed in 5 hr when cells are treated with the same concentration of TMP-Boc₃Arg (data not shown). Although it is possible that the superior efficacy of EA-Boc₃Arg derives from better cellular uptake, these results may also indicate that a covalent attachment between the target protein and ligand is the preferred design strategy.

Degradation Requires the Proteasome, but Not Ubiquitylation

The ubiquitin-proteasome system is the major route for intracellular protein degradation (Xie, 2010). SERD-induced degradation of the estrogen receptor utilizes these pathways (Howell, 2006), and the degradation of dehalogenase fusion proteins with hydrophobic tagging also has been shown to require the proteasome (Neklesa et al., 2011). Therefore, we investigated whether Boc₃Arg-induced degradation also utilized the ubiquitin-proteasome system. The specific proteasome inhibitor bortezomib blocks the Boc₃Arg-mediated degradation of eDHFR and endogenous GST- α 1-GFP in whole cells (Figures 4A and 5A). Lactacystin, a second specific proteasome inhibitor, blocks Boc₃Arg-induced degradation in Cos-1 cell lysates (Figure 6A), as does the less specific inhibitor MG132 (Figure 6D). However, we failed to observe the accumulation of higher molecular weight forms of any of the proteins in the presence of proteasome inhibitors, even when deubiquitylating enzyme inhibitors were included with proteasome inhibitors. The experiment with ubiquitin aldehyde addition is shown Figure 6C; similar results were obtained with IU1, an inhibitor of USP14 (data not shown; Lee

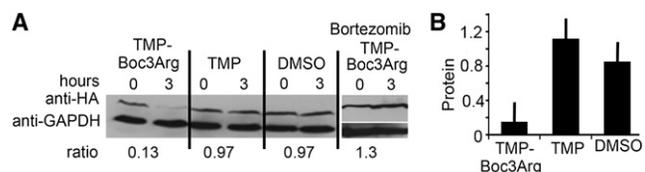


Figure 4. Degradation of eDHFR Fusion Proteins in Cycloheximide-Treated Cells

(A) Experiment as in Figure 3, but HeLa cells were incubated with 0.2 mg/ml cycloheximide for 20 min prior to TMP-Boc₃Arg treatment. The rightmost panel is an independent blot.

(B) Quantitation of three independent experiments. Bar graphs show the average and the standard deviation.

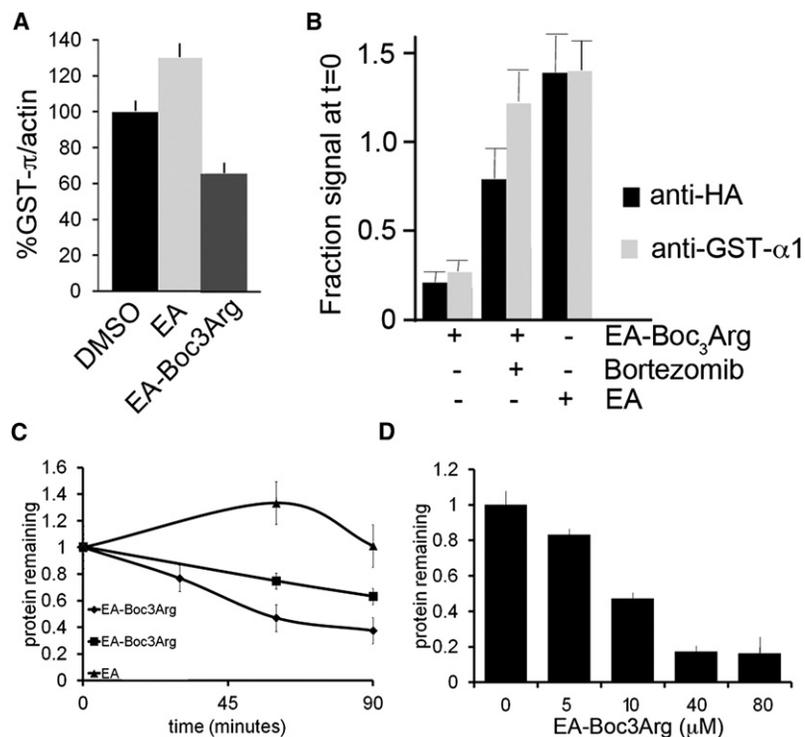


Figure 5. EA-Boc₃Arg-Induced Degradation of Endogenous and Ectopic GST in Whole Cells

(A) Degradation of endogenous GST- π when Cos-1 cells are treated with EA and EA-Boc₃Arg for 3 hr ($n = 4$). Bar graphs show the average and the standard deviation.

(B) Degradation of eDHFR-HA-GST- α 1 in HeLa cells. Cells expressing eDHFR-HA-GST- α 1 were treated with EA-Boc₃Arg (80 μ M) over 1.8 hr. Protein was measured by both anti-GST and anti-HA immunoblotting ($n \geq 3$). Bar graphs show the average and the standard deviation.

(C) HeLa cells expressing eDHFR-HA-GST- α 1 were treated with: triangles, EA (80 μ M); squares, EA-Boc₃Arg (8 μ M); and diamonds, EA-Boc₃Arg (80 μ M). At the indicated time points, the eDHFR-HA-GST- α 1 was quantitated and normalized to GAPDH ($n = 2$). Bar graphs show the average and the range of values.

(D) HeLa cells expressing eDHFR-HA-GST- α 1 were treated with a range of concentrations of EA-Boc₃Arg or just dimethyl sulfoxide. After 1.2 hr, the amount of eDHFR-HA-GST- α 1 was quantitated ($n = 2$). Bar graphs show the average and the range of values.

See also Figure S2.

et al., 2010). ATP is required both for ubiquitylation and for the degradation of ubiquitylated proteins by the 26S proteasome, but omission of the ATP regenerating system did not inhibit Boc₃Arg-induced degradation (Figure 6B). Although it can often be difficult to detect ubiquitylation (Jariel-Encontre et al., 2008), these observations suggest that the Boc₃Arg group induces degradation of the target protein by a mechanism that is independent of the ubiquitin pathways.

DISCUSSION

The Boc₃Arg Moiety Provides a General Strategy for the Design of Degradation-Inducing Inhibitors

The use of small molecules to stabilize misfolded proteins is a promising approach for the treatment of several inherited diseases (Bernier et al., 2004; Ringe and Petsko, 2009). Such chemical/pharmacological chaperones exploit the common ability of inhibitor binding to stabilize protein structure. In contrast, the experiments described above demonstrate that the Boc₃Arg moiety provides a general strategy for the construction of small molecules that induce protein degradation. Importantly, a covalent interaction between the inhibitor and target is not required, which suggests that the Boc₃Arg-induced degradation design may be broadly applicable. EA is a diuretic, GST- π has an essential role in the development of colon cancer (Dang et al., 2005), TMP is a widely used antibiotic, and DHFR is an important target for cancer, immunosuppressive, and antimicrobial chemotherapy, so this work demonstrates proof of concept with therapeutically relevant inhibitors and targets.

Boc₃Arg-induced degradation can be rapid and robust: our work demonstrates that as much as 30%–80% of an abundant protein can be consumed in 2–5 hr. Haploinsufficiency is

a frequent cause of autosomal dominant disorders, which suggests that such levels of protein knockdown should be sufficient to observe a phenotype. Further, similar levels of estrogen receptor knockdown are observed when tissue culture cells are treated with SERDs (Wittmann et al., 2007), demonstrating that Boc₃Arg-induced degradation should be sufficient to make a pharmacological impact. Boc₃Arg-induced protein knockdown is more rapid than RNAi, which typically requires several days to deplete the target mRNA. Of course, the extent of protein knockdown will depend on the rate of synthesis and the intrinsic structural stability of the protein target, but these issues also determine the efficacy of RNAi.

The structure-activity profile of Boc₃Arg-induced degradation has not been defined, and our work toward this end is ongoing. The Boc₃Arg and linker together have a molecular weight approaching 600 Da, with a polar surface area of more than 150 Å². Although the linker requirements are likely to be target-specific, more effective degrons may be obtained with an optimization program that seeks to reduce the size of the Boc₃Arg moiety. The properties of the recognition ligand must also be defined. We expect that Boc₃Arg-induced degradation will have a bell-shaped dependence on the affinity of the recognition ligand. If this interaction is too weak, the association will not be long enough for the target to transit to the proteasome. If the interaction is too strong, the target will not unfold and enter the proteolytic chamber. It seems reasonable to expect that more effective examples of inhibitor-mediated degradation will be realized.

The Pharmacological Potential of Boc₃Arg-Mediated Degradation

Drugs that induce protein degradation are likely to have different pharmacological effects than those that simply obstruct a binding site. For example, inhibition of the kinase activity of the Bcr-Abl kinase is not sufficient to block activation of its downstream signaling pathways (Hu et al., 2006b); obviously,

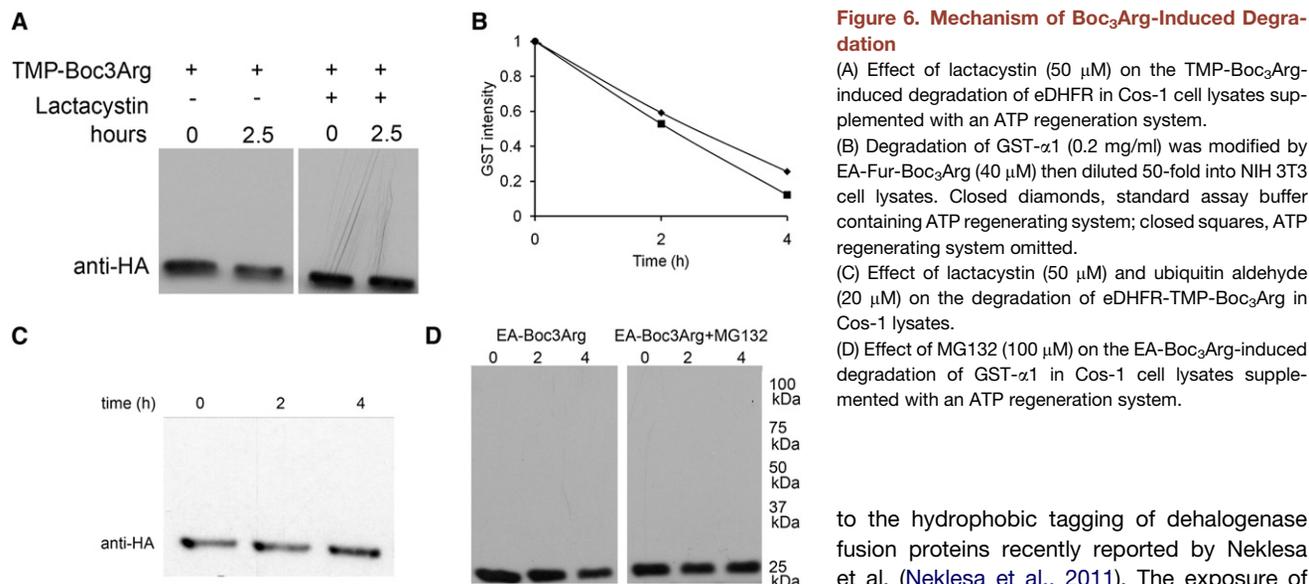


Figure 6. Mechanism of Boc₃Arg-Induced Degradation

(A) Effect of lactacystin (50 μ M) on the TMP-Boc₃Arg-induced degradation of eDHFR in Cos-1 cell lysates supplemented with an ATP regeneration system.

(B) Degradation of GST- α 1 (0.2 mg/ml) was modified by EA-Fur-Boc₃Arg (40 μ M) then diluted 50-fold into NIH 3T3 cell lysates. Closed diamonds, standard assay buffer containing ATP regenerating system; closed squares, ATP regenerating system omitted.

(C) Effect of lactacystin (50 μ M) and ubiquitin aldehyde (20 μ M) on the degradation of eDHFR-TMP-Boc₃Arg in Cos-1 lysates.

(D) Effect of MG132 (100 μ M) on the EA-Boc₃Arg-induced degradation of GST- α 1 in Cos-1 cell lysates supplemented with an ATP regeneration system.

such kinase-independent signaling would be eliminated if Bcr-Abl was degraded. Treatment of botulinum toxin (BoNT) intoxication highlights another advantage of this strategy. BoNT has a half-life of 90 days, so patients must be kept on respirators for many months (Foran et al., 2003); a small molecule inhibitor of BoNT would also have to be administered for many months, but an agent that could induce the degradation of BoNT would require a much-reduced treatment schedule. The degradation of aberrant proteins also provides a novel and much-needed strategy for treating many hereditary diseases. For example, mutations in *IMPDH1* cause retinitis pigmentosa, an inherited blindness resulting from the degeneration of rod photoreceptors. *IMPDH1* knockout mice display only a mild retinopathy (Aherne et al., 2004), suggesting that the removal of the mutant protein will prevent disease.

The knockdown of GST- π illustrates the potential of inhibitor mediated protein degradation. EA increased the level of GST- π relative to the DMSO-treated control. In contrast, EA-Boc₃Arg decreased the levels of GST- π , so that only 50% of the protein is present relative to EA-treated cells. In both cases, the active site of GST- π is blocked, disrupting phase-II detoxification and protein glutathionylation reactions. However, GST- π has additional functions that appear to be independent of the active site (Tew and Townsend, 2011), including a "ligandin" activity that binds large planar aromatic anions outside the active site. This ligandin activity may serve to prevent the generation of reactive oxygen species. GST- π also forms a complex with c-Jun N-terminal kinase 1 to negatively regulate MAP kinase signaling. Lastly, GST- π forms higher-level oligomers under conditions of cellular stress, although the functional consequences of oligomerization are not known. These processes are likely to be regulated by the levels of GST- π , which suggests that the pharmacology of EA and EA-Boc₃Arg treatment will be distinct.

Mechanism of Boc₃Arg-Induced Degradation

The three *tert*-butyl groups of Boc₃Arg create a large hydrophobic surface that may cause degradation in a similar manner

to the hydrophobic tagging of dehalogenase fusion proteins recently reported by Neklesa et al. (Neklesa et al., 2011). The exposure of hydrophobic surfaces is also believed to be the underlying cause of SERD-induced degradation

of the estrogen receptor, oxidized protein degradation, and unfolded protein degradation (Breusing and Grune, 2008; Howell, 2006). Although the characterization of these processes is far from complete, it is already clear that multiple pathways are at work. SERD-induced degradation involves additional estrogen receptor cofactors such as SRC3/AIB1 and requires polyubiquitylation and the 26S proteasome (Calligé and Richard-Foy, 2006). Further, each SERD exposes a distinct hydrophobic surface (Wittmann et al., 2007; Wu et al., 2005), suggesting that different sets of cofactors/ubiquitylation enzymes could be utilized in each case. The degradation of oxidatively damaged proteins is similarly complex. The ubiquitin pathways are needed when damage occurs to newly synthesized proteins (Medicherla and Goldberg, 2008), while the 20S proteasome degrades the majority of oxidized proteins in an ubiquitin- and ATP-independent process (Jung et al., 2009; Shringarpure et al., 2001). Unfolded proteins are also substrates for the 20S proteasome (Asher et al., 2006; Breusing and Grune, 2008). In addition, protein cofactors such as HSP90 can act in concert with the 20S proteasome to promote degradation (Whittier et al., 2004).

Boc₃Arg-mediated degradation requires the proteasome, but appears to be ubiquitin- and ATP-independent. These observations suggest that the 20S proteasome may be involved. The 20S proteasome accounts for approximately 40% of the total proteasome pool in cells (Tanahashi et al., 2000), so a large proteolytic capacity is available for ubiquitin-independent degradation. Proteasome inhibitors also block the hydrophobic tagging/dehalogenase degradation system (Neklesa et al., 2011), although it is not yet known if this is a ubiquitin- and ATP-independent process. Enzyme-inhibitor complexes are generally more stable than unliganded enzymes, which makes degradation all the more curious. One possibility is that the Boc₃Arg moiety might enter the proteasome and drag the rest of the protein into the proteolytic chamber. Although such a process is easily envisioned with a covalently attached inhibitor, it is difficult to understand how this mechanism could work with

noncovalent interaction. Another possibility is that the Boc₃Arg group intercalates into the target protein, exposing a hydrophobic surface that then interacts with the 20S proteasome, or perhaps Boc₃Arg interacts with other protein factors such as HSP90. Which of these mechanisms is operable has important implications for the optimization of Boc₃Arg-induced degradation. How the Boc₃Arg moiety targets proteins to the proteasome remains to be elucidated.

SIGNIFICANCE

Drugs such as fulvestrant cause the degradation of their target proteins. Such compounds generally display superior pharmacodynamic properties relative to analogs that do not induce degradation. However, the discovery of compounds with this property has been largely serendipitous and a robust general strategy for the design of small molecules that cause protein degradation has yet to emerge. Given that inhibitor binding generally stabilizes protein structure, the principles of such a design are not obvious. We have discovered that Boc₃Arg-linked inhibitors induce degradation of their target proteins in mammalian tissue culture cells. Robust degradation was observed with the Boc₃Arg moiety in the context of three different inhibitor scaffolds and with three different target proteins, including an abundant endogenous protein. Importantly, the inhibitors can be covalent or noncovalent. Thus, Boc₃Arg provides a general strategy for the design of small molecules that induce protein degradation that will be useful in the design of drugs and chemical tools.

EXPERIMENTAL PROCEDURES

Materials

All reagents were of the highest commercial grade. Monoclonal rat anti-hemagglutinin (HA) conjugated to horseradish peroxidase (anti-HA-HRP, clone 3F10) was from Roche Diagnostics (Indianapolis, IN). Mouse monoclonal anti-GAPDH conjugated to HRP (anti-GAPDH-HRP), mouse anti-tubulin, rabbit anti-actin, and MG132 were from Sigma Aldrich (St. Louis, MO). Rabbit anti-human IMPDH and secondary antibody HRP conjugates were purchased from Abcam (Cambridge, MA). Rabbit anti-GST- α 1 and anti-GST- π were from Oxford Biomedical (Oxford, UK). Rabbit anti-GFP was from Chemicon (Billerica, MA). Purified 20S proteasome from HeLa, immunoproteasome from mouse spleen, lactacystin, and IU1 were from Boston Biochem (Cambridge, MA). Bortezomib was from Sellek Chemicals LLC (Boston, MA). We thank Charles Morrow (Wake Forest University) for the GST- α 1 expression plasmid pOXO4- α 1 and the Elledge Laboratory for the PMSCV-vector containing the DSRED_IRES_eGFP cassette. Recombinant GST- α 1 and eDHFR proteins were purified by affinity chromatography as described in [Supplemental Information](#). Compound synthesis and characterization is also described in [Supplemental Information](#).

Cell Culture

For lysate experiments, cells were grown to confluence in DMEM with 10% FBS, and then cultured without FBS for 2 days. Lysates were prepared in buffer A (300 mM phosphate, pH 7.8, 200 mM sucrose, 42 mM MgCl₂, 10 mM NaCl). Protein content was determined using the Bradford assay with IgG as a standard (BioRad, Hercules, CA).

In Vitro Degradation Assays

Lysates were validated for proteolytic activity using a fluorescence-based proteasome assay (Sigma Aldrich, St. Louis, MO). Addition of MG132 (100 μ M) or lactacystin (50 μ M) inhibited this reaction, demonstrating the presence of

active proteasomes. An optimized concentration (1 mg/ml) lysate was used in degradation assays. Unless otherwise stated, the lysate was supplemented with: 10 mM ATP, 63 mM MgCl₂, 100 mM creatine phosphate, and 0.01 mg/ml creatine kinase. The target enzyme (0.2 mg/ml, 8.3 μ M) was preincubated with the respective inhibitor (typically 80 μ M) for 20 min at 37°C in 50 mM Tris-Cl buffer, pH 8.0. Then the inhibitor-enzyme complex was diluted 50-fold into lysate, overlaid with Chill-out wax (Biorad), and incubated at 37°C. Aliquots (15 μ l) were added to 10 μ l of 5X SDS loading buffer at the appropriate time intervals. Protein was visualized with the appropriate primary and secondary antibodies using ECL Plus (GE, Bucks, UK). Densitometry was carried out using IMAGE-J V 1.44p.

In Cell Degradation Assays

Cells were grown to approximately 60% confluence, and then transfected with the required plasmid using Transit2020 (except HeLa, for which Transit HeLaMonster was used) (Mirus, Pittsburgh, PA). Fresh media containing compound was added to initiate the assay. Cells were incubated at 37°C and harvested by trypsinization and lysis at the appropriate time intervals. For Fluorescence Activated Cell Sorting (FACS), cells were collected by centrifugation, and then resuspended in FACS buffer (1% FBS in PBS). FACS was carried out on a Becton Dickinson FACScalibur instrument. Details of analysis are included in Supporting Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.chembiol.2012.04.008](https://doi.org/10.1016/j.chembiol.2012.04.008).

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