Endoplasmic Reticulum Vacuolization and Valosin-Containing Protein Relocalization Result from Simultaneous Hsp90 Inhibition by Geldanamycin and Proteasome Inhibition by Velcade

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Abstract

Geldanamycin and Velcade, new anticancer drugs with novel mechanisms of action, are currently undergoing extensive clinical trials. Geldanamycin interrupts Hsp90 chaperone activity and causes down-regulation of its many client proteins by the ubiquitin-proteasome pathway; Velcade is a specific proteasome inhibitor. Misfolded Hsp90 clients within the endoplasmic reticulum (ER) lumen are cleared by ER-associated protein degradation, a sequential process requiring valosin-containing protein (VCP)—dependent retrotranslocation followed by ubiquitination and proteasomal proteolysis. Cotreatment of cells with geldanamycin and Velcade prevents destruction of destabilized, ubiquitinated Hsp90 client proteins, causing them to accumulate. Here, we report that misfolded protein accumulation within the ER resulting from geldanamycin and Velcade exposure overpowers the ability of the VCP-centered machine to maintain the ER secretory pathway, causing the ER to distend into conspicuous vacuoles. Overexpression of dominant-negative VCP or the “small VCP-interacting protein” exactly recapitulated the vacuolated phenotype provoked by the drugs, associating loss of VCP function with ER vacuolization. In cells transfected with a VCP-enhanced yellow fluorescent protein fluorescent construct, geldanamycin plus Velcade treatment redistributed VCP-enhanced yellow fluorescent protein from the cytoplasm and ER into perinuclear aggresomes. In further support of the view that compromise of VCP function is responsible for ER vacuolization, small interfering RNA interference of VCP expression induced ER vacuolization that was markedly increased by Velcade. VCP knockdown by small interfering RNA eventually deconstructed both the ER and Golgi and interdicted protein trafficking through the secretory pathway to the plasma membrane. Thus, simultaneous geldanamycin and Velcade treatment has far-reaching secondary cytotoxic consequences that likely contribute to the cytotoxic activity of this anticancer drug combination.

(Mol Cancer Res 2006;4(9):667–81)

Introduction

All organisms have evolved complex mechanisms to protect themselves from the deleterious consequences of mutated, oxidized, or otherwise improperly folded proteins. In particular, the heat shock proteins Hsp90 and Hsp70, also called chaperones or stress proteins, critically assist newly synthesized polypeptides (clients) in achieving their functional conformation, localization, activation, and even oligomerization (1, 2). Currently, >100 Hsp90 client proteins have been described (see website maintained by Dr. D. Picard),1 including a myriad of transcription factors, signal-transducing receptor kinases, cell cycle regulators, and steroid hormone receptors, most of which are essential for cell proliferation and survival (3-5). Unfortunately, Hsp90 may be a double-edged sword because it also stabilizes mutated tumor suppressors and inappropriately activated oncoproteins that contribute to the initiation, growth, and metastasis of human cancers (6-11).

Geldanamycin and its clinically active analogue, 17-allylamino-17-demethoxygeldanamycin (17-AAG), specifically bind to and profoundly inhibit the protein chaperone function of Hsp90 (12-14), inducing ubiquitination and proteasomal destruction of its numerous client proteins (15-17). When cells are simultaneously exposed to geldanamycin and a proteasome inhibitor, the degradation of geldanamycin-destabilized Hsp90 clients is unproductive; consequently, ubiquitinated misfolded proteins accumulate and usually coalesce into highly insoluble, perinuclear inclusion bodies known as aggresomes (18, 19). In preclinical experiments, the combination of geldanamycin and the proteasome inhibitor, Velcade, exhibits selectivity for transformed cells, inhibits proliferation, and is more cytotoxic to tumor cells than either drug alone (20, 21). These observations prompted us to hypothesize that a buildup of...

Received 1/19/06; revised 6/19/06; accepted 6/29/06.

Grant support: Intramural Research Program of the Center for Cancer Research, NIH, National Cancer Institute.

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doi:10.1158/1541-7786.MCR-06-0019

misfolded, nondegradable Hsp90 client proteins might be responsible for the enhanced cytotoxicity of this drug combination (20). Both 17-AAG (5, 10) and Velcade (22-25) show promising clinical activity against various human cancers, and these drugs are currently in multiple advanced combinatory trials with standard first-line anticancer drugs (9, 26). Recently, a phase I clinical trial of 17-AAG combined with Velcade was initiated at several cancer research centers.

When protein misfolding occurs within the endoplasmic reticulum (ER)-Golgi secretory pathway, the stress-recognizing proteins, Ire1p, PERK, and ATF6, signal for augmented synthesis of ER residing chaperones to facilitate correction of protein misfolding and also initiate transcriptional repression of essentially all other proteins (27, 28). This signaling cascade specifically responding to protein misfolding in the ER is called the unfolded protein response (29), and both geldanamycin (30) and Velcade (31) stimulate powerful unfolded protein responses. When protein misfolding in the ER fails to be corrected by the unfolded protein response, cells activate a secondary defensive mechanism called ER-associated protein degradation (ERAD; refs. 32, 33). ERAD unblocks the ER-Golgi secretory pathway of misfolded polypeptides by retro-transporting them from the ER into the cytoplasm, where they undergo ubiquitination and proteasomal degradation (34, 35). This complex yet seamless mechanism of moving misfolded polypeptides across the ER membrane to undergo destruction requires the participation of the p97 ATPase, valosin-containing protein (VCP; Cdc48 in yeast; ref. 36). The hexameric, VCP-centered molecular machine, complemented with Ufd1p and Npl4p adapter proteins, mechanically ratchets misfolded polypeptides across the ER membrane at the expense of ATP (37-39). ERAD-specific ubiquitin ligases recruited to the extraction complex quickly ubiquitinate the emerging polypeptides (40-42) that are then escorted by ubiquitin-binding proteins to nearby cytosolic proteasomes for disassembly (37, 38, 43). Consequently, when geldanamycin or 17-AAG binds to Hsp90, destabilized clients, such as ErbB2 tyrosine kinase (15, 44), the cystic fibrosis transmembrane conductance regulator (16), or the mutated epidermal growth factor receptor (45), which are then degraded by the 26S proteasome (46), are instead rapidly down-regulated by ERAD.

In addition to initiating profound ER stress, activating the unfolded protein response and ERAD, and promoting massive protein ubiquitination, we have noted that simultaneous geldanamycin and Velcade treatment causes distortion of ER cisternae into numerous conspicuous vacuoles (20). Newly acquired data now indicate that geldanamycin plus Velcade—initiated ER vacuolization occurs as a consequence of overwhelming the ability of the VCP-centered molecular machine to extract misfolded proteins from the ER. We report here that VCP overexpression significantly mitigates geldanamycin plus Velcade—induced ER vacuolization. Conversely, transient transfections of dominant-negative VCP (dnVCP) or the interfering “small VCP-interacting protein” (SVIP) recapitulates the drug-induced vacuole phenotype. Knockdown of VCP by small interfering RNA (siRNA) interference induces ER vacuolization that is exacerbated by Velcade cotreatment and demoliishes ER and Golgi architectures, derailing ErbB2 trafficking to the plasma membrane. Compro-

mising the ERAD function of VCP by a massive accumulation of ubiquitinated and misfolded proteins is a heretofore unforeseen secondary consequence of combining geldanamycin with Velcade.

**Results**

**Geldanamycin plus Velcade Cotreatment Initiates Cytosolic and ER Stress Responses and Greatly Enhances Protein Ubiquitination**

The binding of geldanamycin to Hsp90 indirectly activates heat shock factor-1 (47, 48) and Ire1α (49) stress sensor proteins, which then enter the nucleus and activate multiple stress response genes. Similarly, intracellular accumulation of misfolded proteins resulting from proteasome inhibition by Velcade is known to activate cytosolic and ER stress responses (50, 51). When COS-7 cells were simultaneously exposed to geldanamycin and Velcade, the cytotoxic heat shock proteins Hsp70 and Hsp90 and the ER protein chaperone, BiP/GRP78 (30, 52), were elevated as much as 40-fold (Fig. 1A). Geldanamycin or Velcade alone induced only more modest cytosolic and ER stress responses (data not shown). Excessive protein misfolding in the ER initiates compensatory activation of the ERAD pathway, which depends on the mechanical action of the VCP-centered heterocomplex molecular machine to extract denatured polypeptides from the ER lumen (53-56). The crucial participation of VCP in this elegant mechanism prompted us to examine whether overexpression of VCP would mitigate the ER cellular stress response initiated by geldanamycin and Velcade. Surprisingly, we found that greatly elevated levels of VCP achieved by transient transfection diminished neither ER nor cytosolic cellular stress responses resulting from simultaneous Hsp90 and proteasome inhibition (Fig. 1A, lanes 3 and 4).

The amount of endogenous soluble VCP in COS-7 lysates was essentially unaltered by geldanamycin, Velcade, or the combination of the two inhibitors. However, interestingly, a substantial portion of endogenous VCP was detected in the detergent-insoluble, pellet fraction (Fig. 1B) from cells treated with geldanamycin plus Velcade. Velcade alone only slightly elevated the quantity of detergent-insoluble VCP, and geldanamycin alone had no effect. In cells in which the level of VCP was augmented by transient transfections, more than half of the overexpressed VCP protein was found in the detergent-insoluble fraction of cell lysates following geldanamycin plus Velcade treatment, whereas only a minuscule quantity of VCP partitioned into the pellet fraction from VCP-transfected cells not exposed to the drugs (Fig. 1C, top).

Protein ubiquitination in the soluble lysate fraction from COS-7 cells treated with geldanamycin plus Velcade was only slightly increased, but in the detergent-insoluble pellet fraction, protein ubiquitination was extensive (Fig. 1C). Transient transfection and overexpression of VCP neither attenuated nor augmented the extent of protein ubiquitination in either fraction derived from the drug-treated cells. Given that VCP itself, as well as its ERAD-specific adaptor proteins, Ufd1p and Npl4p, bind to polyubiquitin chains (57-59), it is probable that the large quantity of VCP in the detergent-insoluble pellet fraction from geldanamycin plus Velcade—treated cells cosedimented with aggregated insoluble ubiquitinated proteins.
Destabilization of Hsp90 by Geldanamycin Augments Velcade-Caused Vacuolization of COS-7 Cells

When used at 10 nmol/L, a concentration half-maximally inhibiting proteasome activity in intact cells within 1 hour (20), Velcade alone induced cytoplasmic vacuolation in only a small proportion of COS-7 cells (~2%) within 24 hours, but when cells were exposed to 200 nmol/L Velcade for 24 hours, vacuolation was extensive, occurring in as many as 75% of cells (Fig. 2A). When we examined the concentration dependency of Velcade-induced vacuolation in COS-7 cells, we found a linear relationship between Velcade concentration and cell vacuolation, up to ~50 nmol/L Velcade (Fig. 2B, left). Previous experiments in our laboratory indicated that 50 nmol/L Velcade inhibited proteasome activity in MCF-7 cells by ~90% (20), corresponding reasonably well with this outcome. Geldanamycin alone at 50 nmol/L (Fig. 2A) or even at 3 µmol/L (data not shown) was incapable of promoting vacuolation in COS-7 cells (Fig. 2A). However, the incidence of vacuolation in cells exposed to the low 10 nmol/L concentration of Velcade was enhanced ~2-fold by including 50 nmol/L geldanamycin (Fig. 2A, top right and C). Longer incubations with both drugs at these concentrations (up to 48 hours) increased the proportion of vacuolated cells to as much as 75%. But by then, most cells rounded up, released from the plastic, and developed surface blebbing indicative of apoptosis (results not shown). Interestingly, pretreatment of cells with geldanamycin for 24 hours followed by washout and overnight exposure to 10 nmol/L Velcade also effectively increased the incidence of vacuolization. The ability of geldanamycin to synergize with Velcade to induce cell vacuolation, even when the drugs are used sequentially, illustrates the durability of Hsp90 inhibition and suggests that client protein destabilization persists well after the drug is removed from the medium.

Geldanamycin plus Velcade–Induced Vacuolization of COS-7 Cells Results from ER Dilation

Cytoplasmic vacuolation of various types of cells treated with proteasome inhibitors has been ascribed to result from extensive dilation of ER cisternae (20, 59, 60). To verify that vacuoles induced by Hsp90 and proteasome inhibition in COS-7 cells originated from the ER, we transfected those cells with a plasmid vector expressing a red fluorescent protein fused to the KDEL ER localization sequence of calreticulin 48 hours before exposing them to geldanamycin and Velcade. In control cells, the fluorescent ER marker visualized the typical membranous ER architecture, which was concentrated near the nucleus and dispersed net-like throughout the cytoplasm, extending outward to the plasma membrane (Fig. 3A). When COS-7 cells were treated with geldanamycin plus Velcade, the fluorescent ER marker was strongly localized exclusively within the lumen of the drug-induced vacuoles, and essentially none of the normal reticulated ER structure remained (Fig. 3B). Beginning...
at ~16 hours, small vacuoles first originated at or near the nuclear envelope, then expanded outward, and became more numerous as they further distorted the normal ER architecture. In some cells, especially at later times, several vacuoles seem to have fused together. Most commonly, the distribution of cytoplasmic vacuoles appeared random, but occasionally in some cells they seemed to radiate outward linearly from the nucleus (Fig. 3B, top left). In nonresponding geldanamycin plus Velcade–treated cells that failed to form cytoplasmic vacuoles, the fluorescent ER marker visualized only the normal ER architecture (data not shown). Thus, we conclude that the geldanamycin and Velcade–induced vacuolization of COS-7 cells resulted from extensive dilation of the ER.

Tunicamycin-Caused Protein Misfolding in the ER Induces Vacuolization in Proteasome-Inhibited COS-7 Cells

We reasoned that if geldanamycin plus Velcade induced cell vacuolization by promoting protein misfolding and retention in the ER lumen, then exposing cells to tunicamycin, a protein glycosylation inhibitor known to induce substantial ER protein misfolding and the unfolded protein response (61), should recapitulate the geldanamycin plus Velcade–induced vacuolated phenotype. Whereas exposure to tunicamycin alone promoted no COS-7 cell vacuolization, when tunicamycin was combined with as little as 5 nmol/L Velcade, extensive vacuolization (>90% of cells) occurred within as little as 16 hours (Fig. 4A). Although the dynamics of vacuole development was more rapid, the overall appearance of the vacuoles induced by tunicamycin combined with Velcade was identical to the vacuolization induced by geldanamycin plus Velcade, including the initial perinuclear emergence, size distribution, and eventual congestion of the entire cytoplasm. These common traits reinforce the notion that geldanamycin plus Velcade–caused vacuoles are a predictable consequence of excessive protein misfolding within the ER. We have noted previously that incubating cells at 43°C in the presence of Velcade, but not heat shock alone, likewise caused cytoplasmic vacuolization (20), an outcome consistent with the accumulation of heat-denatured, misfolded proteins within the ER compartment (62, 63).

FIGURE 2. Geldanamycin greatly increases the ability of Velcade to induce vacuolization of cells. A. Cells were treated with either Velcade alone at 10 and 200 nmol/L, geldanamycin alone at 50 nmol/L, or 10 nmol/L Velcade + 50 nmol/L geldanamycin simultaneously for 24 hours. The low concentration of Velcade induced vacuolization in ~5% to 10% of COS-7 cells, whereas Velcade at 200 nmol/L prompted ~90% of cells to become heavily vacuolated. The extent of vacuolization caused by 10 nmol/L Velcade (a concentration that inhibits proteasome activity by ~50%) was greatly enhanced by either the simultaneous or sequential inclusion of geldanamycin. B. Concentration-dependent induction of cell vacuolization caused by Velcade alone. Following Velcade exposure for 24 hours, cells were photographed and the numbers of cells having numerous vacuoles (arbitrarily set at eight or more per cell) were counted in four separate microscopic fields. Points, mean of four separate determinations. C. Percentage of vacuolated cells induced by Velcade at 10 nmol/L for 22 hours was more than doubled by the simultaneous inclusion of 50 nmol/L geldanamycin. Longer exposure times, up to 48 hours, increased the percentage of vacuolated cells, but beyond 48 hours, most of the cells rounded up and released from the plastic surface.


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A vacuolated cellular phenotype has been shown to occur when endogenous wild-type VCP is compromised by overexpression of mutated, ATPase-inactivated dnVCP (64). Consistent with this observation, transient transfections of COS-7 cells with a plasmid vector expressing mutated dnVCP induced a vacuolated phenotype that was morphologically identical to that induced by geldanamycin and Velcade (Fig. 4B). Importantly, this result shows that specific interdiction of VCP function alone is sufficient to promote ER-derived cellular vacuolization.

Similarly, overexpression of SVIP protein interferes with VCP function and causes cells to become vacuolated (65). Within 48 hours after transfections with a plasmid expressing SVIP, COS-7 cells developed prominent vacuoles resembling those induced by geldanamycin plus Velcade treatment (Fig. 5A, top). When SVIP-transfected cells were cotreated with geldanamycin, twice to thrice as many cells became vacuolated (Fig. 5A, bottom), consistent with protein misfolding as a consequence of geldanamycin inhibition of Hsp90 chaperone function. SVIP was expressed as a FLAG fusion protein and anti-FLAG immunocytofluorescence revealed that the SVIP fusion protein was strongly localized on the drug-induced vacuoles, where it may have bound to VCP (Fig. 5B, top and bottom). Collectively, these data support the concept that misfolded polypeptide accumulation within the ER of Hsp90- and proteasome-inhibited cells necessarily occurs when the polypeptide extraction function of VCP is compromised by overexpressed dnVCP or SVIP or when VCP is overwhelmed by misfolded nondegradable proteins.

VCP is a relatively abundant protein compromising as much as 1% of total cellular proteins (66). However, the much lower quantity of VCP localized on ER membranes that participate in ERAD might become saturated with ubiquitinated proteins after cells are treated with geldanamycin and Velcade. Alternatively, VCP might even be depleted from the ER, given that a substantial quantity of VCP is relocated to the detergent-insoluble pellet fraction of cells in response to these two drugs. If ER vacuolization resulting from geldanamycin and Velcade exposure results from interference with the ERAD function of VCP, then supplemental VCP would be expected to attenuate the vacuolated phenotype induced by these two drugs. To explore this possibility, we transfected COS-7 cells with a vector expressing wild-type VCP-GFP fusion protein, waited 48 hours to permit the overexpressed VCP protein to accumulate, and then exposed cells to geldanamycin plus Velcade for an additional 24 hours. In those cells expressing abundant VCP-GFP protein (green in the overlaid images), exposure to the drugs did not induce ER vacuolization (Fig. 6, left and right). Although small percentage of drug-treated cells developed vacuoles, those cells uniformly failed to express detectable levels of the VCP-GFP fusion protein (Fig. 6, left and right). There were no obvious phenotypic changes in cells transfected with the VCP-expressing plasmid but not exposed to the drugs (data not shown). These data showing protection against ER vacuolization by exogenous VCP, especially when juxtaposed with the contrasting dnVCP and SVIP results, argue strongly that the ERAD function of VCP was susceptible to inhibition by geldanamycin plus Velcade–dependent protein misfolding.

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**FIGURE 3.** Cytoplasmic vacuoles in COS-7 cells induced by geldanamycin + Velcade treatment are ER derived. COS-7 cells were transiently transfected with a plasmid vector expressing the KDEL sequence of the ER resident protein, calreticulin, fused to red fluorescent protein before being treated with 10 nmol/L Velcade + 50 nmol/L geldanamycin for 24 hours. **A,** Fluorescent ER marker visualized the normal ER architecture in control cells. **B,** Reticulated ER structure was dramatically altered in cells exposed to the two drugs. Note that the fluorescent calreticulin-KDEL sequence-derived ER marker was localized inside the vacuoles, suggesting dilation of the ER lumenal space as a consequence of the accumulation of misfolded proteins following Hsp90 and proteasome inhibition. At early times, vacuolization was predominantly perinuclear, but eventually the vacuoles fill the entire cytoplasmic space.
Geldanamycin plus Velcade Redistributes VCP Away from the ER and Cytoplasm into Aggresomes

Because Hsp90 and proteasome inhibition redistributed both endogenous and transfected VCP into the detergent-insoluble fraction of COS-7 cells (Fig. 1B and C), we visually examined the consequences of geldanamycin plus Velcade treatment on the disposition of a transiently transfected VCP-GFP fusion protein. In controls, VCP-GFP-derived fluorescence was predominantly cytoplasmic (Fig. 7A), although a few cells exhibited faint nuclear fluorescence (data not shown). VCP is known to associate with the ER and Golgi apparatus membranes (66, 67), but we were unable to observe VCP-GFP on membranous structures because of its overpowering cytoplasmic fluorescence. When VCP-GFP-transfected COS-7 cells were exposed to geldanamycin plus Velcade for 24 hours, we observed that VCP-GFP had been completely redistributed into highly condensed, perinuclear bodies resembling aggresomes (Fig. 7B; refs. 18, 19, 26). Longer image capture (exposure) revealed that essentially all of the VCP-GFP fusion protein had been depleted from the cytoplasm, and no residual VCP-GFP fluorescence remained on ER membranes or on drug-induced vacuoles (data not shown). A previous report described condensation of VCP-GFP into perinuclear aggresomes after cells were treated with a proteasome inhibitor (59). VCP may have redistributed from the cytoplasm, ER, and Golgi, as a consequence of Hsp90 and proteasome inhibition, because the VCP-centered extraction complex remained bound to ubiquitinated proteins that could not undergo proteolysis by inactive proteasomes (57). When stabilized by proteasome inhibition, ubiquitinated ER transiting and cytosolic Hsp90 client proteins frequently aggregate and accumulate in large insoluble aggresomes (18, 68). Given that ubiquitinated proteins and VCP were both greatly increased in the detergent-insoluble fraction of geldanamycin plus Velcade–treated cells (Fig. 1C), we suggest that VCP-GFP fusion protein in the drug-treated cells was segregated into aggresomes.

Targeting VCP with siRNA Greatly Increases Vacuolization of Velcade-Treated Cells

COS-7 cells were exposed to VCP siRNA sequences for 72 hours, and separate dishes of cells were also exposed to the drugs for an additional 22 hours. Although siRNA decreased VCP protein by >90%, as estimated by anti-VCP immunoblotting (shown in Fig. 8C), VCP knockdown by siRNA induced vacuolization in only a few cells (Fig. 9, middle). In contrast, when cells treated with both VCP siRNA and Velcade (Fig. 9, right), ER vacuolization was increased as much as 10-fold when compared with cells treated with Velcade alone (Fig. 9, left). Neither irrelevant siRNA nor the si-Importer transfecting reagent induced any vacuoles in cells (data not shown). This observation of synergistic induction of cell vacuolization as a consequence of VCP knockdown and proteasome inhibition by Velcade recapitulates the observations of Wojcik et al. (69). Those authors reported that proteasome inhibition by MG-132 greatly enhanced HeLa cell vacuolization in cells pretreated with VCP siRNA. To our surprise, treating siRNA-VCP knockdown cells with geldanamycin did not further increase the proportion of cells displaying vacuoles, suggesting that sufficient residual VCP was capable of delivering misfolded ER Hsp90 clients to proteasomes (data not shown).

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siRNA Knockdown of VCP Markedly Changes the Architecture of Both ER and Golgi Apparatus

To more precisely determine how VCP participated in the ER morphologic alterations brought about by geldanamycin plus Velcade treatment, we treated COS-7 cells with siRNA directed against VCP and then transfected cells with vectors expressing either fluorescent ER or Golgi apparatus markers. The ER and Golgi in control cells were normal in appearance (70); however, in cells in which VCP had been depleted by siRNA, the structures of the ER and Golgi were dramatically changed (Fig. 10A-D). Instead of its normal interconnected, net-like ER structure, in VCP knockdown cells, the fluorescent ER marker instead revealed a punctate pattern of small irregular bodies (Fig. 10B). In addition to its role in ERAD, and in conjunction with its p47 and VCIP135 effectors, VCP regulates homotypic ER and Golgi membrane fusion, which reconstitutes the membranes of these organelles following their disassembly during cell division (71-73). In the absence of VCP following cell division, ER transition vesicles may have been unable to fuse to reconstruct normal ER architecture (74). Similarly, in cells exposed to siRNA-VCP, the fluorescent Golgi marker revealed dispersed small bodies instead of the normal condensed perinuclear Golgi architecture that was seen in the control cells (Fig. 9C and D). Finally, depletion of VCP by siRNA interference increased overall protein ubiquitination 3- to 4-fold (data not shown) as has been observed previously (69). Further experiments are required to determine whether there is a relationship between the greatly enhanced protein ubiquitination and the profound morphologic changes to the ER and Golgi structures in VCP knockdown cells.

FIGURE 5. SVIP causes vacuolization that is enhanced by Hsp90 inhibition. Recently discovered, a protein known as SVIP is thought to specifically regulate the ERAD function of VCP. SVIP binding to VCP interferes with its ability to extract polypeptides from the ER and promotes cell vacuolization (64). We therefore transfected COS-7 cells with a plasmid expressing SVIP and 24 hours later examined them for the appearance of vacuoles. SVIP overexpression caused vacuolization in a small percentage of cells (A), but interestingly, when SVIP-overexpressing cells were cotreated with 50 nmol/L geldanamycin, the incidence of vacuolization was enhanced ~4-fold. Because SVIP was expressed as a FLAG fusion protein, it was visualized by anti-FLAG/Cy3 immunofluorescence; (B) in SVIP-transfected, geldanamycin-treated cells, SVIP was localized in the cytoplasm and concentrated on the membranes surrounding vacuoles where presumably it was VCP bound.

FIGURE 6. Overexpression of VCP diminishes geldanamycin + Velcade–induced vacuolization. We reasoned that overexpression of wild-type VCP might augment the ability of cells to remove misfolded proteins from the ER compartment. Therefore, cells were transiently transfected with a plasmid vector expressing a fluorescent VCP-GFP fusion protein and 24 hours later were exposed to 10 nmol/L Velcade + 50 nmol/L geldanamycin for an additional 24 hours. Fluorescent images layered over phase-contrast photographs revealed that cells expressing high levels of exogenous VCP-GFP failed to form vacuoles. Conversely, cells with drug-induced vacuoles expressed little or no VCP-GFP-derived fluorescence.
Geldanamycin and Velcade Treatment Depletes ErbB2 from the Plasma Membrane and Relocates the Bulk of ErbB2 into Aggresomes

The ErbB2 plasma membrane–residing tyrosine kinase is a Hsp90 client protein known to be rapidly down-regulated by ubiquitin-dependent proteasomal degradation in cells treated with geldanamycin (15, 44, 75). As it is translated at ER membrane-bound ribosomes, immature ErbB2 protein is inserted directly into the ER membrane as a transmembrane polypeptide. Hsp90 binds to its cytoplasmic moiety and regulates the trafficking of nascent ErbB2 (76). The ER lumenal portion of ErbB2 becomes glycosylated and the molecule moves on to the Golgi apparatus where glycosylation trimming takes place. Finally, mature ErbB2 traffics to the plasma membrane, where it functions as a transmembrane signal transducer (76, 77). We reasoned that a fluorescent ErbB2-enhanced yellow fluorescent protein (EYFP) fusion protein would be useful to visualize changes in ER architecture promoted by geldanamycin plus Velcade treatment and to document consequences of the drug treatment on protein transit through the secretory pathway.

In control cells, ErbB2-EYFP was localized predominantly on the plasma membrane, where it was frequently highly concentrated in membrane folds and projections (Fig. 8A). It was impossible to visualize ErbB2-EYFP on ER or Golgi apparatus membranes because the intense cell surface fluorescence of the fusion protein obscured these organelles. However, in cells treated with geldanamycin plus Velcade, where the quantity of ErbB2-EYFP residing on the plasma membrane was substantially diminished, residual ErbB2-EYFP could be seen on the ER membranes, especially in cells containing prominent vacuoles (Fig. 8B). Whereas the fluorescent calreticulin-derived ER fusion protein marker shown in Fig. 3 filled the lumen of the geldanamycin plus Velcade–induced vacuoles, ErbB2-EYFP clearly was localized on the membranes outlining the vacuoles (Fig. 8B). Note the frequent and easily observed characteristic tripartite intersections of ER membranes in the drug-treated vacuolated cells. Visualization by the fluorescent ErbB2-EYFP fusion protein on intact and interconnected ER membranes surrounding the drug-induced vacuoles supports an interpretation that the drug-induced vacuoles were formed by dilation of ER, rather than from a swelling and pinching-off process, as we had hypothesized previously (20).

In the majority of cells treated with geldanamycin plus Velcade, however, ErbB2-EYFP-derived fluorescence was nearly absent from both the plasma membrane and ER, and the bulk of ErbB2-EYFP was highly concentrated in what appear to be perinuclear aggresomes (Fig. 8C). It is noteworthy that although inhibition of proteasome activity by Velcade prevented ErbB2 down-regulation and stabilized the cellular content of ErbB2, the ability of ErbB2 to function as a plasma membrane signal-transducing partner in geldanamycin plus Velcade–treated cells would be prevented by its relocalization to perinuclear aggresomes.

siRNA Knockdown of VCP Also Prevents ErbB2 from Trafficking to the Cell Surface

Given that VCP provides the ATP-driven mechanical force to eliminate misfolded polypeptides from the ER by retrograde translocation, we wondered whether depletion of VCP would promote accumulation of proteins within the ER. To examine this possibility, we first depleted the cellular level of VCP by siRNA interference and then transiently transfected cells with
ErbB2-EYFP. Control cells were treated with irrelevant siRNA (Fig. 11A). Instead of accumulating at the plasma membrane or within the ER or even the Golgi, VCP knockdown caused the fluorescent ErbB2-EYFP fusion protein to localize in numerous punctate, perinuclear structures (Fig. 11B). This abnormal and unexpected ErbB2-EYFP distribution may have resulted from the cells’ inability to perform homotypic membrane fusion because of depletion of VCP (72, 78). Notably, the profound ErbB2-EYFP relocalization brought about by VCP siRNA recapitulated the pattern shown by the ER fluorescent marker in VCP knockdown cells (Fig. 9). When siRNA-VCP knockdown cells were treated with geldanamycin plus Velcade for an additional 24 hours, the ErbB2-EYFP signal became extremely weak, and in most cells, residual ErbB2-EYPF was found only in punctate bodies surrounding the nucleus (data not shown). Estimated by immunoblotting, VCP siRNA decreased the VCP content of cells by >90% (Fig. 11C). VCP siRNA also greatly diminished the cellular content of ErbB2 (Fig. 11C) most likely because ErbB2 synthesis was inhibited by the deconstruction of the ER (Fig. 10). As a control, when cells were treated with an irrelevant siRNA, neither VCP nor ErbB2 levels were altered (Fig. 11C). Thus, knockdown of VCP not only disassembled the normal architecture of the ER and Golgi apparatus but also prevented ErbB2 trafficking through the ER secretory pathway to its normal residence on the plasma membrane.

**Discussion**

Why do cells form vacuoles in response to geldanamycin plus Velcade? The appearance of membrane-bound, ER-derived cytoplasmic vacuoles and the relocalization of VCP from its normal cytoplasmic and ER membrane locations into dense, compact aggresomes are major consequences of abnormal protein misfolding and ubiquitination resulting from inhibition of Hsp90 and exacerbated by proteasome inhibition. Several lines of evidence reported here provide insights into and support for this concept. First, geldanamycin plus Velcade–dependent activation of the cellular stress response, signaled by the marked increase in Hsp70 and Hsp90 in the cytoplasm and BiP/GRP78 within the ER, indicates this drug combination triggered profound cytoplasmic and ER stress, consistent with an abnormal amount of protein misfolding (28, 29, 50, 61, 79). Second, the tremendous increase in

**FIGURE 8.** Geldanamycin + Velcade treatment depletes ErbB2 from the plasma membrane and redirects ErbB2 into aggresomes. To monitor the disposition of a Hsp90 client protein that normally transits the ER secretory pathway to its plasma membrane residence following geldanamycin + Velcade treatment, we created an ErbB2-EYFP fluorescent construct and overexpressed this fusion protein in COS-7 cells before treating them with 25 nmol/L Velcade + 50 nmol/L geldanamycin for 24 hours. In control cells (A, top to bottom), ErbB2-EYFP-derived fluorescence was predominantly localized homogeneously on the cell surface or concentrated in surface patches and membrane folds. In cells treated with geldanamycin + Velcade (B, top to bottom), most of the ErbB2-EYFP fusion protein was depleted from the cell surface, although Velcade blocked its proteasomal degradation, and ErbB2-EYFP was observed on membranes surrounding the drug-induced vacuoles and in large perinuclear aggresomes. Finally, in geldanamycin + Velcade–treated cells devoid of vacuoles, ErbB2-EYFP fluorescence was strongly concentrated in perinuclear aggresomes (C, top to bottom). We have reported previously that the ErbB2 kinase in cells treated with geldanamycin + Velcade becomes extensively ubiquitinated before it is degraded by proteasomes (15). It is interesting to compare the localization of VCP-GFP following geldanamycin + Velcade shown in Fig. 7 with the localization of ErbB2-EYFP in the drug-treated cells shown here.
protein ubiquitination specifically in the detergent-insoluble fraction of lysates from geldanamycin plus Velcade–treated cells indicates a catastrophic amount of protein misfolding, aggregation, and precipitation had occurred. Third, inducing protein misfolding uniquely within the ER compartment with the protein glycosylation inhibitor, tunicamycin, exactly reproduced the vacuolated phenotype induced by geldanamycin plus Velcade but only when proteasomal proteolysis was inhibited by Velcade. Collectively, these observations point to a massive buildup of misfolded and ubiquitinated proteins within the ER lumen as the common factor in ER vacuolization resulting from both geldanamycin plus Velcade and tunicamycin plus Velcade treatments. It seems likely that accumulated misfolded proteins trapped within the ER would exert an osmotic force, inducing an influx of water from the cytoplasm and distending the ER lumenal space into vacuoles.

Additional evidence specifically implicates interference with the ERAD function of VCP as the crucial biochemical event responsible for initiating geldanamycin and Velcade–promoted ER vacuolization. As reported previously (59, 65), we observed that transient transfection of vectors overexpressing either dnVCP or the VCP-inhibiting SVIP protein, treatments that specifically interfere with VCP function, precisely recapitulated the vacuolated phenotype induced by Hsp90 and proteasome inhibition. The demonstrated redistribution of VCP into the detergent-insoluble fraction of cell lysates and its coactivation into aggresomes in geldanamycin plus Velcade–treated cells indicates that the bulk of the cellular VCP would be unavailable to facilitate the extraction of misfolded proteins from the ER, to deliver misfolded proteins to proteasomes, or to participate in its other multiple functions. Perhaps most convincingly, overexpression of the ATPase-active, wild-type VCP ameliorated the ER vacuolization induced by geldanamycin plus Velcade treatment. Cells expressing a high level of VCP protein were refractory to vacuolization, whereas cells containing an impoverished level of exogenous VCP remained susceptible to extensive vacuolation when challenged by geldanamycin plus Velcade exposure. This outcome most likely resulted from the enhanced ability of supplemental VCP to extract misfolded proteins from the ER lumen, although they could not be degraded by Velcade-inhibited proteasomes. In this context, by remaining bound to extracted polypeptides, the high level of exogenously supplemented VCP would function as a misfolded ubiquitinated protein chaperone as has been suggested previously in other circumstances by others (43, 66, 80).

Notwithstanding the robust induction of ER vacuolization following transient transfections of mutated dnVCP, we observed that knockdown of VCP by siRNA interference induced vacuolization in only a small percentage of COS-7 cells. Although immunoblotting indicated that siRNA knockdown depleted VCP protein levels by ~90%, it is possible that the small quantity of residual VCP was nevertheless sufficient to participate in ERAD at a level that, in the absence of unusual protein misfolding stress, was sufficient to prevent vacuolization. Upsetting this balance by treating VCP knockdown cells with Velcade to stabilize ubiquitinated proteins synergistically increased the incidence of cells generating vacuoles. These observations are consistent with a scenario where ubiquitinated protein accumulation in proteasome-inhibited cells overwhelms the diminished ability of siRNA-depleted VCP to export misfolded polypeptides from the ER.

At first glance, one might conclude that the geldanamycin plus Velcade–induced ER vacuolization is simply a toxic manifestation—a phenotypic consequence of a serious biochemical malfunction. Alternatively, expansion of the ER compartment in the face of massive protein misfolding within the ER lumen might be considered to be a stopgap mechanism of sequestering misfolded, potentially toxic proteins, thereby enabling the ER secretory pathway to remain at least partially operational. In this scenario, ER vacuolization could provide temporary survival advantage in circumstances where the ERAD machinery of cells becomes

![FIGURE 9. Knockdown of VCP with siRNA combined with Velcade induces extensive ER vacuolization. COS-7 cells were exposed to 100 nmol/L siRNA sequences targeting VCP for 3 days, 25 nmol/L Velcade alone for 22 hours, or the combination of siRNA with Velcade and then examined for vacuolization by phase-contrast microscopy. As expected, Velcade induced vacuolization in a small proportion of cells (left, arrows) as did siRNA-VCP treatment (middle, arrows). However, the incidence of ER vacuolization was markedly increased by the combination of VCP knockdown and proteasome inhibition by Velcade (right), illustrating how the ERAD function of VCP is linked to proteasomal protein degradation.](mcr.aacrjournals.org)
impaired. We know that drug-induced ER vacuolization is a reversible process; if geldanamycin and Velcade are removed from the medium bathing the cells before 24 hours, proteasomes become reactivated within 16 to 18 hours and ER vacuolization diminishes, presumably because ERAD function is regained and misfolded proteins can be cleared. On the other hand, continuous exposure of cells to the drug combination for 48 hours is extremely cytotoxic and essentially all cells, including those with vacuoles, rapidly undergo apoptosis (81, 82). Although additional molecular details of ER vacuolization are necessary to fully understand the genesis of this dramatic phenotypic, we propose that ER vacuolization, like aggresome formation, may very well be a defensive cellular response to an overwhelming quantity of aberrant misfolded proteins.

It should be pointed out that our interpretation of the experiments using VCP siRNA to modulate VCP gene expression are confounded by the fact that VCP performs multiple, hierarchical cellular functions in addition to participating in ERAD (66, 74). VCP is required for the disassembly of the mitotic spindle proteins preceding cell division (83), and VCP function is crucial during homotypic fusion of transient vesicles into membranes that reassemble into the ER and Golgi apparatus following cell division (73, 78, 84, 85). VCP also participates in cell cycle (86) and transcription factor regulation (87), and it even plays a role in apoptosis (69) and DNA repair (88, 89). Consequently, in situations of extreme cellular stress, such as occurs during Hsp90 and proteasome inhibition, when VCP becomes relocalized from its constitutive sites of action into insoluble aggresomes, all of the functions of VCP would be compromised. Thus, a loss of VCP-dependent ERAD function at the ER brought about by siRNA may be relatively unimportant to cells in which the entire structure of the ER and Golgi has been disassembled. On the other hand, it is possible that loss of ERAD function actually contributes to the severe morphologic alterations brought about by siRNA knockdown of VCP, and future experimentation will address this possibility in greater detail.

FIGURE 10. RNA interference of VCP restructures the architecture of the ER and Golgi apparatus. To more thoroughly study the participation of VCP in misfolded protein-induced cell vacuolization, we used siRNA interference to deplete VCP to an inconspicuous level. We then transfected siRNA-treated cells with vectors expressing either the calreticulin-derived ER fluorescent marker or a Golgi apparatus fluorescent marker to visualize those membranous structures. A, Top and bottom, ER architecture in control cells was completely normal; however, in cells treated with siRNA-VCP alone, the reticulated ER structure was completely destroyed and the remaining very faint fluorescence originating from the ER marker was localized in small, perinuclear, punctate structures (B, left and right, top and bottom). Visualization of the punctate bodies required 10- to 15-fold longer camera exposure time, indicating how little of the ER was left (note the extent of VCP knockdown by siRNA in COS-7 cells shown in the immunoblot in Fig. 8C). Additionally, the normal structure of the Golgi apparatus (C, top and bottom) was similarly disassembled by VCP knockdown, and the fluorescent Golgi marker visualized small, punctate structures similar to those visualized by the ER marker (D, left and right, top and bottom).
targeted with siRNA was diminished by irrelevant siRNA were unchanged from untreated control cells, VCP with drug-induced vacuoles. Although the VCP level in cells exposed to an ER structure in most cells and actually diminished the proportion of cells VCP before geldanamycin + Velcade treatment dramatically altered the ER vacuolization in Hsp90- and proteasome-inhibited cells, 

\[ \text{FIGURE 11. RNA interference of VCP expression depletes ErbB2 from the plasma membrane and causes the remaining ErbB2 to concentrate in small perinuclear bodies. To verify that siRNA knockdown of VCP interferes with the protein secretory pathway, COS-7 cells were treated with siRNA-VCP for 24 hours and then transfected with a plasmid vector expressing the ErbB2-EYFP fusion protein. Other siRNA-VCP-treated, ErbB2-EYFP-transfected cells were also exposed to 25 nmol/L Velcade and 100 nmol/L geldanamycin for an additional 24 hours. A, ErbB2-EYFP fluorescence in control cells was localized on the plasma membrane. Strikingly, siRNA-VCP nearly completely depleted ErbB2-EYFP from the cell surface and redistributed the fusion protein into numerous, perinuclear, punctate bodies (B) reminiscent of the distribution pattern of the fluorescent ER marker in VCP knockdown cells (shown in Fig. 9B). It should be noted that the cytofluorescence photomicrographs of the siRNA-VCP-treated cells required at least a 10-fold longer exposure time and computer enhancement because of the extremely low level of ErbB2-EYFP fusion protein in the VCP knockdown cells (B and C). Knockdown of VCP before geldanamycin + Velcade treatment dramatically altered the ER structure in most cells and actually diminished the proportion of cells with drug-induced vacuoles. Although the VCP level in cells exposed to an irrelevant siRNA were unchanged from untreated control cells, VCP targeted with siRNA was diminished by ~90% (C, top) when evaluated by immunoblotting. Geldanamycin + Velcade treatment in either the absence or presence of siRNA-VCP did not further alter the VCP status of cells. The near elimination of constitutive ErbB2 by siRNA-VCP observed in the cytofluorescence photographs was verified by immunoblotting (C, bottom). Although ErbB2 protein is stabilized in cells treated with Velcade and geldanamycin (C, bottom, lanes 2 and 4), in VCP knockdown cells, Velcade did not stabilize ErbB2 protein (lane 6), suggesting that perhaps its synthesis was inhibited by the devastating effects of VCP depletion on the ER architecture.}

\[ \text{In conclusion, although further experimentation is called for to fully elucidate the very complex interactions between protein misfolding, ubiquitination, VCP localization and function, and ER vacuolization in Hsp90- and proteasome-inhibited cells, based on the observations reported herein, it seems plausible that overwhelming the ERAD function of VCP may be the proximal cause of ER vacuolization in response to geldanamycin plus Velcade.}

\[ \text{Materials and Methods}

\[ \text{Cell Culture and Drug Treatments}

\[ \text{Mycoplasma-free, SV40-transformed COS-7 monkey kidney cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, and 10 mmol/L HEPES (pH 7.5) under standard tissue culture conditions of 5% humidified CO}_2\text{ and 37\degree C. COS-7 cells were}

\[ \text{We also suspect that VCP knockdown probably interferes with protein synthesis from ribosomes attached to the rough ER membranes. Indeed, even in the presence of Velcade to inhibit proteasomal degradation, the level of ErbB2 in cells exposed to siRNA-VCP was nearly undetectable by both cytofluorescence and direct immunoblotting of the ErbB2 protein. These observations are consistent with the possibility that protein synthesis at ER membranes was inhibited after VCP depletion most likely because of the deconstruction of the normal ER architecture. Thus, VCP knockdown would be expected to have a significant effect on both protein synthesis at the ER membrane and protein trafficking through the ER to the cell surface through the secretory pathway.}

\[ \text{The redistribution of VCP away from the ER and cytoplasm into aggresome-like bodies following geldanamycin plus Velcade treatment is an unexpected and provocative observation. VCP levels are reported to be elevated in a variety of different human solid tumors (90, 91), and the increased expression of VCP in these tumors seems to correlate with disease progression (90, 92) and a poor prognosis (93). Although the exact role VCP plays in human cancers is currently unknown, it is possible that VCP is elevated in solid tumors because it is needed to clear mutated misfolded proteins that are abundantly expressed in tumor cells (94). In this context, VCP should be considered as a credible molecular target for anticancer chemotherapy, especially because either depletion or inactivation of VCP is ultimately incompatible with cell viability (59, 69). We have reported previously that the combination of geldanamycin and Velcade potently inhibits MCF-7 tumor cell proliferation (20), and most recently, 17-AAG, the clinically useful analogue of geldanamycin, was shown by Mitsiades et al. (21) to sensitize multiple myeloma cells to Velcade. In this regard, the combination of 17-AAG and Velcade is currently undergoing National Cancer Institute--sponsored clinical evaluation at several cancer research centers. Based on results presented here, at least part of the cytotoxicity produced by the geldanamycin plus Velcade combination may result from the redistribution of VCP into an insoluble subcellular compartment.}

selected for this investigation because we observed previously they were especially sensitive to vacuolization induced by Hsp90 and proteasome inhibition and because they are efficiently transfected with plasmid DNA. Subconfluent, exponentially growing cells were exposed to Velcade alone (Millennium Pharmaceuticals, Boston, MA), geldanamycin alone (Developmental Therapeutics Program, National Cancer Institute, NIH, Bethesda, MD), or the combination of both agents at several concentrations and times; control COS-7 cells were exposed to equivalent amounts of DMSO solvent (±0.05%, v/v) in complete medium.

**Transfections of Cells with Expression Vectors**

COS-7 cells were transiently transfected with plasmids expressing either the Living Colors ER marker fused to HeRed fluorescent protein or plasmids expressing a Golgi apparatus marker fused to EYFP (Clontech, Palo Alto, CA) using FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN) precisely according to the manufacturer’s instructions. Control cells were transfected with a plasmid vector expressing only unmodified EYFP. In other experiments, cells were transfected with plasmids expressing wild-type or mutated VCP-GFP fusion proteins (Dr. Akira Kakizuka, Kyoto, Japan). Flag-tagged SVIP (a gift from Dr. M. Tagaya, Tokyo, Japan), or ErbB2-EYFP (constructed in our laboratory by fusing the EYFP sequence to the COOH terminus of the full-length ErbB2 sequence). Following transfections, multiple fields of cells were examined with an IX50-FLA fluorescence microscope (Olympus, Melville, NY) equipped with a Spot Junior digital camera and appropriate wavelength filters. Images illustrating the organelle markers and phenotypic changes (×400 and ×1,000) were captured and enhanced when necessary using Adobe Photoshop software on a Mackintosh G4 computer.

**Immunoreagents and Chemicals**

The antibodies used in this study were rabbit polyclonal anti-ubiquitin (Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-Hsp70 (StressGen BioReagents, Victoria, British Columbia, Canada), mouse anti-Hsp90 (StressGen BioReagents), rabbit anti-BiP/GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-VCP (Research Diagnostics, Concord, MA and Affinity BioReagents, Golden, CO), mouse anti-ErbB2 (Oncogene Research, San Diego, CA), anti-FLAG M2 and Cy3-conjugated secondary anti-mouse antibodies (Sigma-Aldrich), horseradish peroxidase–conjugated rabbit anti-mouse IgG1 (Cappel, Durham, NC), horseradish peroxidase–conjugated sheep anti-mouse, and horseradish peroxidase–conjugated donkey anti-rabbit (Amersham Life Sciences, Arlington Heights, IL). All other reagent chemicals and biochemicals used in this study were purchased from NIH stockroom or Sigma-Aldrich.

**Clarified Cell Lysate and Detergent-Insoluble Fractions**

Exponentially growing COS-7 cells were washed twice with ice-cold PBS and then lysed on ice for 20 minutes into TNESV lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 1% NP40 detergent, 2 mmol/L EDTA, 100 mmol/L NaCl, 10 mmol/L orthovanadate] supplemented with Complete protease inhibitor cocktail tablets (Roche Diagnostics, Penzberg, Germany). Lysates were centrifuged at 14,000 × g for 20 minutes at 4°C, the supernatant fraction was transferred to fresh tubes, and the NP40 detergent-insoluble fraction was resuspended in TNESV lysis buffer by brief sonication (10 seconds at 50 W) while immersed in ice. Protein concentrations of both fractions were determined by the microtiter plate bicinchoninic acid method using bovine serum albumin as the standard (95). Samples were diluted with 5 × reducing SDS loading buffer [100 mmol/L DTT, 50 mmol/L Tris-HCl (pH 6.8), 10% (v/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue tracking dye; ref. 96] before loading identical known protein quantities onto gels.

**Immunoblot Analysis**

After fractionating samples by 10% Tris-HCl SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA), proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by the wet transfer method. All membranes to be probed by anti-ubiquitin immunoblotting were first autoclaved while immersed in deionized water in a glass tray for 40 minutes to denature the adherent ubiquitinated proteins, which exposes latent ubiquitin epitopes and greatly enhances sensitivity (17, 97, 98). After blocking nonspecific binding sites on the membranes with 5% fat-free dry milk dissolved in 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 2.5 mmol/L EDTA buffer, various antigens were immunodetected with appropriately diluted antibodies followed by horseradish peroxidase–linked secondary antibodies. Visualization of protein bands was by enhanced chemiluminescence (99) using a luminol-based commercial kit (Pierce, Rockford, IL). Exposed XOMAT AR films (Kodak, Rochester, NY) were developed and scanned (Microtek Scannaker III, Carson, CA) and the images were captured and processed with a Macintosh G4 computer using Adobe Photoshop 5.0 and Microsoft PowerPoint software.

**RNA Interference**

COS-7 cells in 35-mm dishes were exposed to 100 mmol/L siRNA targeted against wild-type VCP (a mixture of equal amounts of two siRNA duplexes complementing the DNA target sequences: ACCCTGATTGCTCGAGCTGTA and AAGAACCGTCCCAATCGGTTA; Qiagen, Valencia, CA) according to the manufacturer’s instructions. Following a 48-hour incubation, siRNA-treated cells were transiently transfected with either ER or Golgi apparatus fluorescent marker expression vectors or plasmids expressing an ErbB2-EYFP fusion protein. Control cells were exposed to irrelevant, nonmammalian siRNA (Qiagen). On the third day of siRNA-VCP exposure, cells were cotreated with 50 mmol/L geldanamycin plus 25 mmol/L Velcade for an additional 24 hours and then examined for morphologic changes by phase-contrast and fluorescent microscopy. Satellite plates of cells treated in exactly the same way with siRNA and the two drugs were lysed, and after separation of proteins by SDS-PAGE and transfer to nitrocellulose membranes, VCP and ErbB2 protein levels were evaluated by immunoblotting.
References


60. Wojcik C. An inhibitor of the chymotrypsin-like activity of the proteasome (PSI) induces similar morphological changes in various cell lines. Folia Histochim Cytochim 1997;35:211–4.


77. Asai T, Tomita Y, Nakatsuka S, et al. VCP (p97) regulates NF-κB signaling pathway, which is important for metastasis of osteosarcoma cell line. Jpn J Cancer Res 2002;93:296–304.


Endoplasmic Reticulum Vacuolization and Valosin-Containing Protein Relocalization Result from Simultaneous Hsp90 Inhibition by Geldanamycin and Proteasome Inhibition by Velcade

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