Review

The role of the ubiquitination-proteasome pathway in breast cancer

Applying drugs that affect the ubiquitin-proteasome pathway to the therapy of breast cancer

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Received: 17 June 2002 Revisions received: 23 July 2002 Accepted: 25 July 2002 Published: 14 August 2002

Breast Cancer Res 2003, **5**:1-7 (DOI 10.1186/bcr460)

Abstract

The ubiquitin-proteasome pathway is responsible for most eukaryotic intracellular protein degradation. This pathway has been validated as a target for antineoplastic therapy using both *in vitro* and preclinical models of human malignancies, and is influenced as part of the mechanism of action of certain chemotherapeutic agents. Drugs whose primary action involves modulation of ubiquitin-proteasome activity, most notably the proteasome inhibitor PS-341, are currently being evaluated in clinical trials, and have already been found to have significant antitumor efficacy. On the basis of the known mechanisms by which these agents work, and the available clinical data, they would seem to be well suited for the treatment of breast neoplasms. Such drugs, alone and especially in combination with current chemotherapeutics, may well represent important advances in the therapy of patients with breast cancer.

Keywords: chemotherapy, neoplasm, NF-κB, p44/42 MAPK, proteasome, PS-341, ubiquitin

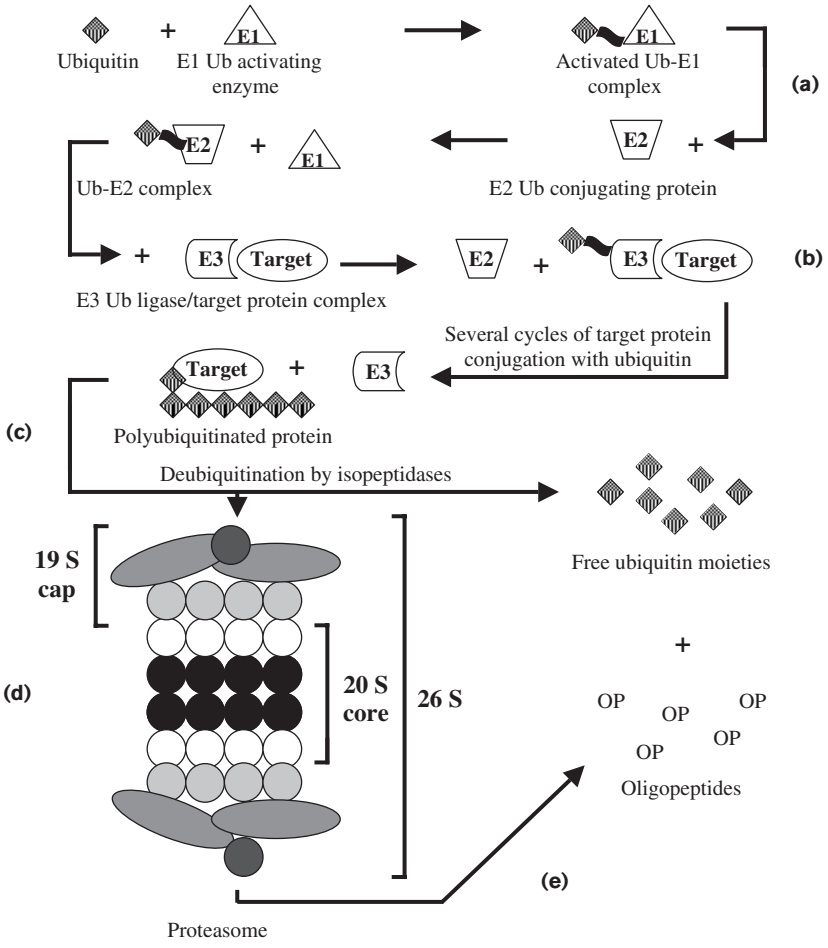
Introduction

Function of the ubiquitin-proteasome pathway is essential for many fundamental cellular processes, including the regulation of receptor signaling pathways and the timely degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors during mitosis. In addition, ubiquitin-proteasome activity is necessary for antigen processing, angiogenesis, and apoptosis and for processing and degradation of misfolded and short-lived regulatory proteins such as transcription factors. This pathway consists of the ubiquitin-conjugating machinery (including an E1 ubiquitin-activating enzyme) and many E2 and E3 ubiquitin-conjugating and ubiquitin-ligase proteins (Fig. 1). The latter are responsible for transferring the activated ubiquitin moieties from E1 to specific sets of proteins, which are thereby targeted for degradation. It is the 26S proteasome, which contains the proteins responsible for proteolysis in a 20S core, that is responsible for degradation of these ubiquitinated products. Recent studies have also identified an increasing number of proteins that are subject to degradation through the 20S proteasome without prior ubiquitination.

The possibility of targeting the ubiquitin-proteasome pathway therapeutically was met in the past with skepticism, because of concerns that this approach would be inimical to life itself because of the important role played by the proteasome in normal cellular homeostasis. With the first demonstration that proteasome inhibitors were well tolerated and had activity in models of human malignancies *in vivo* [1], however, and the use in Phase I safety trials of inhibitors (such as PS-341 [2]) that showed acceptable toxicity with significant clinical benefit [3], targeting the ubiquitin-proteasome pathway for cancer

HIV = human immunodeficiency virus; MAPK = mitogen-activated protein kinase; MDR = multidrug resistance; MKP = MAPK phosphatase; NF-κB = nuclear factor-κB; SUMO = small ubiquitin-like modifier 1; Top-1 = topoisomerase 1.

Figure 1



Protein degradation through the ubiquitin (Ub)-proteasome pathway. Most proteins that are destined for degradation through the Ub-proteasome pathway are first subjected to polyubiquitination. This is accomplished in several stages. **(a)** The E1 Ub-activating enzyme, in an ATP-dependent reaction, forms an activated complex with Ub and transfers it to the E2 Ub-conjugating protein. **(b)** The E2 Ub-conjugating protein then transfers Ub to an E3 Ub-ligase protein, which has formed a complex with the target protein. In some cases an E3 Ub-ligase may not be necessary. **(c)** After several cycles of ubiquitination, the polyubiquitinated target protein is recognized by the proteasomal cap proteins (shaded gray and labeled 19 S cap) through its ubiquitin moieties, which are cleaved off by isopeptidases and recycled. **(d)** In an ATP-dependent fashion the protein is then unwound and fed into the 20S core through an interior channel, where it is exposed to the active proteolytic enzymes (shaded black). **(e)** Oligopeptide digestion products (OP) are then released and degraded further to amino acids by oligopeptidases. Some proteins may be subject to proteasomal degradation without the need for prior ubiquitination. Please note that this schematic diagram does not represent the various components to scale. Interested readers are referred to several excellent recent reviews with more detailed descriptions of this pathway [43,44].

therapy has become an area of intense investigation. This pathway may already play a major role in the therapy of patients with breast cancer who receive anthracyclines. For example, doxorubicin (Adriamycin) binds to subunits of the 20S proteasome, which then translocates to the nucleus [4], thereby acting as a carrier for this drug to exert many of its cytotoxic effects. Several other agents, however, influence either ubiquitination or proteasome-mediated degradation (Table 1), and can be divided into those that act indirectly, at steps prior to this pathway, or directly on some pathway component. This article will review the current status of these drugs, with a focus on their potential application to clinical care of breast cancer.

Drugs with indirect effects

Increasing ubiquitin-proteasome function

Several drugs that stimulate ubiquitin-proteasome pathway mediated degradation of a target protein in another disease have been evaluated in breast cancer. All-*trans* retinoic acid, an important step forward in the therapy of acute promyelocytic leukemia, may in part work by redistributing the promyelocytic leukemia-retinoic acid receptor oncoprotein, accelerating its proteasome-mediated degradation [5]. All-*trans* retinoic acid has been studied in patients with metastatic breast cancer and found not to have significant activity, but in combination with tamoxifen some responses were noted [6]. Whether

Table 1

Drugs that influence ubiquitin-proteasome activity

Drug class	Action and mechanism
Chemotherapeutic agents	
Aclarubicin	Inhibits the chymotrypsin-like proteolytic activity of the proteasome
All-*trans* retinoic acid	May accelerate PML fusion protein degradation through the proteasome
Arsenic trioxide	Inhibits ubiquitination and degradation of IκB through effects on the IκB kinase
Camptothecin	Stimulate ubiquitination and degradation of topoisomerase 1
Geldanamycin	Inhibits HSP90 ATPase, stimulating proteasomal degradation of client proteins
PS-341/LDP-341/MLN-341	Inhibits the chymotrypsin-like activity of the proteasome
Vinblastine, Vincristine	Inhibit the chymotrypsin-like, trypsin-like- and peptidyl-glutamyl peptide hydrolyzing proteasome activities
Immunosuppressive agents	
Cyclosporine A	Uncompetitive inhibitor of the proteasomal chymotrypsin-like activity
Rapamycin	Inhibits proteasome function by inhibiting the proteasome activator PA28
Miscellaneous agents	
Fulvestrant	Stimulates proteasome-dependent proteolysis of ERα
Tannic acid	Inhibits the chymotrypsin-like activity of the proteasome
Lovastatin	Mechanism unknown, but appears structurally similar to the proteasome inhibitor lactacystin
Anti-retroviral drugs	Inhibit the chymotrypsin-like and trypsin-like proteasome activities

ER, estrogen receptor; HSP, heat shock protein; PML, promyelocytic leukemia.

these effects in breast cancer are mediated through an impact on the proteasome, however, is not known.

More clearly proteasome-related is the anticancer effect of the camptothecins, which block the religation step of the topoisomerase-1 (Top-1) reaction, and stimulate ubiquitination and subsequent proteasomal Top-1 degradation [7]. Several camptothecin derivatives have been studied in Phase I trials, and occasional responses in breast cancer patients have been noted. Although Phase II results have been generally disappointing, a recent study of irinotecan in patients with refractory metastatic breast cancer showed a 29% response rate, and tolerable toxicity [8].

Several interesting compounds under development are based on geldanamycin, which inhibits the ATPase activity of the heat shock chaperone protein HSP90. This leads to the degradation of client proteins via the ubiquitin-proteasome pathway, and since these include the c-*erb*B-2 (HER-2/neu) receptor protein-tyrosine kinase [9], their potential application to breast cancer therapy is clear. Analogues such as 17-allylamino-17-demethoxygeldanamycin are now in Phase I clinical trials.

Another agent in this category is the pure estrogen antagonist fulvestrant (Faslodex®), which has been approved for use by postmenopausal patients with estrogen-receptor-positive breast cancer who have progressed following other anti-estrogen therapy (reviewed in [10]). This drug appears to work in part by enhancing proteasome-dependent degradation of estrogen receptor α [11]. Since some estrogen agonists appear to have a similar activity with respect to estrogen receptor α [11], it would be interesting to determine if part of the well-known activity of tamoxifen and other hormonal agents is also due to a similar impact on the proteasome.

Inhibition of ubiquitin-proteasome function

Arsenic trioxide is an example of a drug that acts indirectly on the ubiquitin-proteasome pathway. It modifies a critical cysteine residue in the activation loop of the IκB kinase, preventing IκB phosphorylation. Subsequent IκB degradation is prevented, because degradation through the ubiquitin-proteasome pathway normally follows phosphorylation. Arsenic, therefore, indirectly inhibits NF-κB activation [12]. As detailed below, activation of NF-κB by chemotherapeutic agents and radiation is anti-apoptotic. In addition, arsenic has been reported to specifically inhibit expression and signaling through the estrogen receptor pathway [13]. Arsenic trioxide, therefore, may warrant further study in breast cancer either alone, or in combination with other agents, and a variety of Phase I and Phase II trials are underway.

Drugs with direct ubiquitin-proteasome effects

Drugs with targets other than the proteasome

All of the agents that have been noted to have a direct impact on ubiquitin- and proteasome-mediated proteolysis have been proteasome inhibitors. Since some of these were originally directed against other targets, they will be discussed separately from those which were designed to specifically inhibit the proteasome. In the former category are dietary compounds such as tannic acid [14], antiretroviral agents including the HIV protease inhibitors [15,16], and lipid-lowering agents, such as lovastatin [17], that inhibit the proteasome, although possible applications to breast cancer have not been investigated.

The immunosuppressive agent cyclosporine A is an uncompetitive proteasome inhibitor [18], but in the breast cancer setting it has been used predominantly to block cytochrome-P450-mediated drug resistance, or to induce graft-versus-host disease when patients have undergone high dose chemotherapy, followed by autologous bone marrow or peripheral blood stem cell rescue. Perhaps more interesting is another immunosuppressive, rapamycin, which inhibits expression of the proteasome activator PA28, and thereby inhibits proteasome function [19]. Since rapamycin blocks the estrogen-driven transition of breast cancer cells from the G_1 to S phases of the cell cycle [20], further studies in breast cancer may be warranted.

Chemotherapeutic agents have been identified which inhibit the proteasome, including aclarubicin (aclacinomycin A) [21], and vinblastine and vincristine [22], though it is unclear if, in the case of aclarubicin, this occurs at clinically relevant drug concentrations. Aclarubicin, an anthracycline derivative, has been evaluated in several Phase I and Phase II trials with generally disappointing results, though none were targeted towards breast cancer patients. The vinca alkaloid vinorelbine (Navelbine®), however, has well-documented activity in breast cancer [23], and it would be interesting to determine if this activity is a result of proteasome inhibition.

Proteasome-targeted drugs

Inhibitors of the proteasome were first synthesized two decades ago, and were initially used as laboratory tools to probe the proteolytic activities of this complex (reviewed in [24]) and its role in cellular processes. Subsequent investigations indicating these inhibitors were able to activate programmed cell death in a variety of human tumor-derived cell lines (reviewed in [25]) raised interest in such agents as possible cancer chemotherapeutics. Several lines of evidence suggest that proteasome inhibitors would be active agents in patients with breast cancer. From a mechanistic perspective, the transcription factor NF-κB, an important regulator of apoptosis, can be constitutively activated in several cancers, including some breast cancers (reviewed in [26]). As mentioned above, proteasome inhibitors work in part by blocking degradation of the inhibitory protein IκB, thereby decreasing NF-κB nuclear translocation [25]. Therefore, malignancies with high levels of activated NF-κB, such as breast cancer, should be especially sensitive to interruption of this pathway, which would induce tumor cell death.

A second, recently elucidated, mechanism by which proteasome inhibitors effect apoptosis is by decreased signaling through the p44/42 mitogen-activated protein kinase (MAPK) pathway [27]. High levels of expression of c-*erb*B-2 (HER-2/neu), and the homologous c-*erb*B-1, is a poor prognostic sign, and signaling from these receptors occurs in part through p44/42 MAPK. Furthermore, elevated p44/42 MAPK activity alone has been suggested to have prognostic significance for disease-free survival (reviewed in [28]), and therefore interruption of such signaling, such as by proteasome inhibition, would seem to hold promise for breast cancer therapy.

Proteasome inhibitors may also be effective in breast cancer treatment by helping to overcome some of the major pathways by which cancer cells resist the action of chemotherapy. Two of these have already been referred to above, in that both signaling through NF-κB and p44/42 MAPK can be anti-apoptotic. Chemotherapeutic agents such as taxanes and anthracyclines have been shown to activate one or both of these pathways, potentially limiting their own ability to induce tumor cell death. Since proteasome inhibitors block these pathways, they may be able to not only activate apoptosis, but also enhance the antitumor activity of drugs such as paclitaxel and doxorubicin.

Another important mechanism of resistance to chemotherapy is the expression by cancer cells of the P-glycoprotein, a membrane pump that promotes the efflux of xenobiotics such as chemotherapy drugs, decreasing their intracellular concentration and effectiveness. Proteasome function is necessary for normal maturation of P-glycoprotein. Proteasome inhibition could decrease the accumulation of P-glycoprotein in the membranes of cancer cells, thereby preventing it from ridding these cells of chemotherapy drugs, resulting in increased tumor killing.

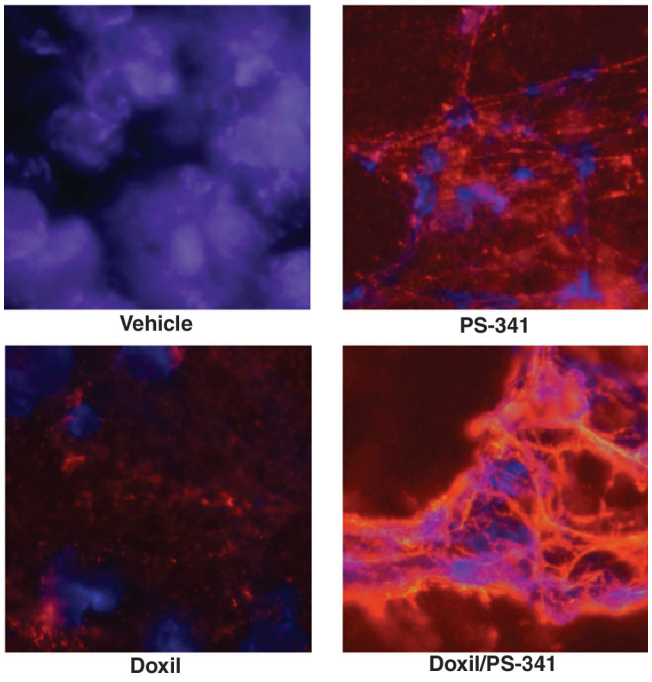
Preclinical studies

Because of the promising rationale described above, a variety of proteasome inhibitors, most commonly based on short peptides, have been synthesized and evaluated using *in vitro* and *in vivo* model systems. The best studied of these in models of breast cancer, and in clinical trials as described below, has been Millennium Pharmaceuticals' bortezomib (Velcade™; previously known as PS-341, LDP-341, and MLN-341). This drug decreased the survival of both cultured MCF-7 cells derived from human breast

cancer and of EMT-6/Parent mouse mammary carcinoma xenograft tumors in a dose-dependent fashion. PS-341 also increased the ability of radiation or cyclophosphamide to kill tumor cells in this model system [29].

In our laboratory we have been interested in combinations of PS-341 with anthracyclines, given the prominent role of the latter group of agents in breast cancer therapy. We have especially focused on liposomal doxorubicin, or Doxil®, because of this drug's activity in refractory breast cancer, its ease of administration (with dosing once every three to four weeks), and its favorable toxicity profile. Using a BT-474-based xenograft model of human breast cancer, we have found that the combination of PS-341 and Doxil® results in enhanced antitumor efficacy, and increased apoptosis when compared with that obtained using either agent alone (Fig. 2).

Figure 2



The combination of PS-341 and Doxil® induces enhanced apoptosis *in vivo*. The impact of vehicle, PS-341 alone, Doxil® alone, or the combination, was studied in a murine xenograft model of human breast cancer established using BT-474 breast carcinoma cells. Apoptosis was evaluated in tumor sections 24 hours after the indicated treatments by detection of single stranded DNA fragmentation using the murine monoclonal antibody Mab 3299 [45] (Chemicon International, Temecula, CA, USA). Single stranded DNA associated with programmed cell death (red) is shown, along with total nuclear DNA (blue), the latter detected using 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Slides were visualized using an ultraviolet Zeiss Axioplan fluorescence microscope (Carl Zeiss Optical, Inc., Chester, VA, USA). Separate photographs were taken with appropriate filters for blue nuclear staining and red single-stranded-DNA staining, overlayed using Adobe Photoshop software, and displayed as a fusion image at 10× magnification.

Clinical trials

More than 400 patients in the United States have been treated in Phase I and Phase II clinical trials of PS-341, which is given by intravenous push once or twice weekly. In the twice weekly for two weeks out of three schedule which has been used most often, the maximum tolerated dose in patients with solid tumors has been defined to be 1.3 mg/m^2 [30]. Because of significant activity against multiple myeloma seen in Phase I trials [3], Phase II [31] and Phase III studies of PS-341 are being pursued or planned for use against multiple myeloma. Preclinical data in chronic lymphocytic leukemia have also been encouraging, and Phase II trials of PS-341 are being pursued to treat this disease as well.

In Phase I studies of PS-341 as a single agent in patients with solid tumors, rare responses have been seen in cancers of the prostate, kidney, head and neck, and lung. Given its potential to enhance chemosensitivity, however, PS-341 is being combined with conventional agents in several ongoing Phase I studies. Some of these combination regimens hold promise for breast cancer treatment. For example, given the preclinical data supporting a Doxil®/PS-341 combination discussed above, a Phase I clinical trial of this combination is being conducted at the University of North Carolina at Chapel Hill. Similarly, a Phase I study of the combination of doxorubicin and PS-341 is ongoing at the University of Wisconsin [32]. The combination of paclitaxel and PS-341 is being studied at the Ohio State University (C Shapiro, personal communication). There are also currently ongoing Phase I trials of PS-341 in combination with 5-fluorouracil [33], irinotecan [34], and gemcitabine [35]. Preliminary data from these trial centers suggest that their respective combinations have been tolerated well so far. While all of these are Phase I studies that will enroll a variety of solid-tumor patients, at least some of the sites plan to focus on breast cancer patients, particularly once the maximum tolerated dose has been identified. This should enable preliminary evidence of antitumor activity to be obtained in this patient population in preparation for Phase II efficacy studies.

Future directions

Currently available drugs that most specifically target the ubiquitin-proteasome pathway, such as PS-341, focus predominantly on the proteasome itself. Research into the machinery responsible for ubiquitination has lagged somewhat in the past, but interest in this area has recently grown greatly. Inhibition of the E1 ubiquitin-activating enzyme would have effects on normal and neoplastic cells that would, in some ways, be even more broad-ranging than proteasome inhibitors. Drugs that would inhibit or stimulate specific E3 ubiquitin ligases, however, could

have an impact upon a much more restricted set of proteins, and could be more specifically targeted and better tolerated clinically. One interesting potential target would be MDM2, which is overexpressed in some human breast tumors [36]. MDM2 is an E3 protein responsible for p53 degradation. Inhibition of MDM2 should result in increased levels of p53, prompting cell-cycle arrest, apoptosis, and possibly enhanced chemosensitivity in breast tumors with wild-type p53. Inhibitors such as these are currently being actively sought, and hopefully will soon be available for preclinical and clinical trials.

A second interesting target in this same light would be the F-box protein FWD-1, which mediates ubiquitination of the IκB α, β, and ε proteins [37]. Inhibitors of this component of the SCF(FWD1) complex would provide a more specific means of inhibiting NF-κB, and might sensitize cells to chemotherapy, as described earlier.

Finally, $p27^{Kip1}$ could also be targeted. This cyclin-dependent kinase inhibitor is present at low levels in aggressive carcinomas. Its expression level, therefore, may have prognostic significance in breast cancer (reviewed in [38]). Since this protein is ubiquitinated by SCF(Skp2) in at least some phases of the cell cycle [39,40], inhibition of this complex could result in accumulation of p27 and consequent cell-cycle arrest and apoptosis.

Ubiquitination could also be influenced by impacting upon related pathways, such as protein modification by the small ubiquitin-like modifier-1 (SUMO-1). SUMOlation of IκBα prevents its subsequent ubiquitination, thereby stabilizing its association with NF-κB [41]. Thus, stimulation of SUMOlation of IκBα could provide another mechanism of inhibiting nuclear NF-κB translocation and enhancing chemosensitivity. Interestingly, inhibition of SUMOlation may have some benefits as well, especially in combination with Top-1 inhibitors. Treatment of cells with camptothecin results in conjugation of Top-1 with SUMO-1, which is a possible repair response to topoisomerase-mediated DNA damage [42]. Thus, inhibitors of this repair mechanism may enhance sensitivity to agents such as irinotecan.

Conclusions

The ubiquitin-proteasome pathway is just beginning to be exploited as a target for cancer therapy. Nonetheless, given the available molecular biological, preclinical, and clinical data, there is very good reason to be optimistic that current drugs and future candidates will contribute significantly to the care of patients with breast cancer. Agents such as the proteasome inhibitor PS-341 are already undergoing clinical trials, and data concerning the Phase I safety and Phase II efficacy of combinations with other antineoplastic agents will be forthcoming over the next several years. This period should prove to be an exciting era for this field of research.

This article is the first in a review series on *The role of the ubiquitination-proteasome pathway in breast cancer*, edited by Nancy Hynes.

Other articles in the series can be found at http://breast-cancer-research.com/articles/series.asp?rqs=hynes2

Acknowledgements

The authors would like to acknowledge support from the Department of Defense Breast Cancer Research Program, BC991049, the Leukemia and Lymphoma Society, R6206-02 (to RZO), from the National Cancer Institute SPORE in Breast Cancer (to RZO and ECD), and from the Doris Duke Charitable Foundation (to ECD).

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