Increasing Melanoma Cell Death Using Inhibitors of Protein Disulfide Isomerases to Abrogate Survival Responses to Endoplasmic Reticulum Stress

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Abstract
Exploiting vulnerabilities in the intracellular signaling pathways of tumor cells is a key strategy for the development of new drugs. The activation of cellular stress responses mediated by the endoplasmic reticulum (ER) allows cancer cells to survive outside their normal environment. Many proteins that protect cells against ER stress are active as protein disulfide isomerases (PDI) and the aim of this study was to test the hypothesis that apoptosis in response to ER stress can be increased by inhibiting PDI activity. We show that the novel chemotherapeutic drugs fenretinide and velcade induce ER stress–mediated apoptosis in melanoma cells. Both stress response and apoptosis were enhanced by the PDI inhibitor bacitracin. Overexpression of the main cellular PDI, procollagen-proline, 2-oxoglutarate–4-dioxgenase β subunit (P4HB), resulted in increased PDI activity and abrogated the apoptosis-enhancing effect of bacitracin. In contrast, overexpression of a mutant P4HB lacking PDI activity did not increase cellular PDI activity or block the effects of bacitracin. These results show that inhibition of PDI activity increases apoptosis in response to agents which induce ER stress and suggest that the development of potent, small-molecule PDI inhibitors has significant potential as a powerful tool for enhancing the efficacy of chemotherapy in melanoma. [Cancer Res 2008;68(13):5363–9]

Introduction
Exploiting vulnerabilities in the intracellular signaling pathways of tumor cells is a key strategy for the development of new drugs. Agents that disrupt normal signaling pathways may induce homeostatic responses to restore normal function. The endoplasmic reticulum (ER) is responsible for the regulation of intracellular calcium (Ca2+) and the synthesis of cell surface or secretory proteins. Disruption of ER function induces a stress response characterized by the up-regulation of ER chaperones and a cascade of transcriptional regulation allowing the cell to adapt and focus resources for damage repair. The unfolded protein response is an important ER stress response which rescues the cell by removing unfolded or misfolded proteins (1). However, ER stress will induce apoptotic death if homeostatic mechanisms are insufficient to protect or repair the cell. The ability of ER stress to drive apoptosis could be harnessed to increase the effectiveness of cancer treatment if homeostatic responses can be attenuated with appropriate drugs. Recent studies in which elements of the ER stress response have been down-regulated or blocked have shown that this can shift the balance towards apoptosis in cells treated with ER stress–inducing agents (2, 3).

Cancer cell types differ in their susceptibility to chemotherapy, and malignant melanoma, one of the most difficult cancers to treat, is largely unresponsive to conventional chemotherapy, resulting in low 5-year survival rates (4). Melanoma cells have extensive repertoires of molecular defenses against immunologic and cytotoxic attack (5), resulting in defective apoptotic signaling. Increased expression of ER stress chaperones can be an early event in tumor initiation (6), and targeting the ER stress responses of melanoma cells is a novel therapeutic approach (7). Many ER stress–response chaperones have protein disulfide isomerase (PDI) activity or PDI-like domains (8, 9), and blocking this activity may be a way to attenuate ER stress responses and tip the balance towards apoptosis in stressed cells. The aim of the present study was to test the hypothesis that apoptosis in response to ER stress can be increased using a PDI inhibitor to attenuate homeostatic mechanisms.

Materials and Methods

Cell culture, transfection, and measurement of apoptosis. Melanoma cell lines CHL-1, A375, and WM266-4, obtained from the American Type Culture Collection, were cultured as described previously (3). Melanocytes were obtained from human foreskin keratinocytes (10) by selective trypsinization, confirmed by immunostaining for the melanocyte differentiation antigen Melan-A (antibody from Abcam), and cultured in medium 254 supplemented with human melanocyte growth supplement-2 as described by the manufacturers (Invitrogen). For the overexpression of PDI, plasmids containing constructs for a wild-type and an inactive mutant procollagen-proline, 2-oxoglutarate–4-dioxgenase β subunit (P4HB), the main cellular PDI, were generous gifts from Ou and Silver (11). The transient transfection of 3 μg of wild-type P4HB, mutant P4HB, or pCMVSPORT-β-galactosidase (Invitrogen) as an unrelated control construct was done using Lipofectamine 2000 (Invitrogen) as previously described (12). Flow cytometry of propidium iodide–stained cells was used to estimate the level of cell death or apoptosis by measuring the percentage of cells in the sub-G1 fraction (3). Cell viability was measured using the MTS assay (CellTiter 96 AQueous Assay; Promega) according to the manufacturer’s protocol.

Quantitative PCR. Primers were designed using Primer Express 1.0 software (Applied Biosystems). ATF4 and GADD34 mRNA were quantified
relative to the ribosomal protein L34 as an internal control using the comparative Ct method. PCR amplification was performed using a LightCycler with DNA Master SYBER Green I kit (Roche Applied Science) according to the manufacturer's instructions as previously described (3). Primers were GTGGCAGAAGCCTCAAAACC and CCCCCGAAGG-GCATCCTC for ATF4, ACACCCGAGTTTGTCCAG and TGGGCAAGA-CACCCAAGTG for GADD34, and GTCCCCAAACCCCCGTGTAATAGA and GGGGCTGCGACATTTTCTT for L34.

PCR for XBP-1 splicing. The human XBP-1 sequence was amplified by PCR using the primer pair AAACAGAGTAGCAGCTCAGACTGC and CCTTCTGGGTAGACCTCTGGGAG as previously described (3).

Western blotting. Total protein was extracted from cell pellets, separated by electrophoresis through 4% to 20% SDS-PAGE gels (25 μg per track) and blotted onto polyvinylidene difluoride membranes as previously described (13). Antibodies used to probe the blots were a GADD153 antibody (B-3, Santa Cruz Biotechnology) diluted 1:500, a P4HB antibody from Cell Signaling Technology (New England Biolabs) diluted 1:1,000, and as a loading control, a β-actin antibody (Sigma) diluted 1:5,000. The binding of primary antibodies was detected with secondary peroxidase-conjugated antibodies (Upstate Biotechnology) diluted 1:2,000 and visualized using the enhanced chemiluminescence system (Amersham Biosciences).

PDI activity assay. Assays for PDI activity were carried out according to Smith and colleagues (14) on cell extracts in Tris-HCl buffer containing 25 mmol/L of KCl and 5 mmol/L of MgCl₂ (15). Protein extracts were reacted with 80 mmol/L of bovine insulin and PDI activity monitored as increased turbidity at 650 nm; the reaction was linear after 30 min and up to 50 to 80 min, and turbidity at 50 min was used as the end point. Turbidity was linear in proportion to log₂, 5 to 100 μg bovine liver PDI standard (Sigma) and up to 80 μg of A375 cell-extract protein (data not shown). PDI activity of cell extracts and the bovine liver PDI standard was abolished by heat denaturation (data not shown).

Statistical analysis. For the analysis of synergy or additivity, the CalcuSyn program (Biosoft) was used to derive parameter estimates for the median-effect equation with single drug treatments (fenretinide, velcade, bacitracin) and combination indices (CI) for treatments with bacitracin and fenretinide or velcade. CI values for effective doses at 50% (ED₅₀), 75% (ED₇₅), and 90% (ED₉₀) were calculated. Data for log₂, relative mRNA levels and PDI activity in cells transfected with P4HB constructs were analyzed by one-way ANOVA with post hoc Dunnett’s test and data for apoptosis in cells transfected with mutant or wild-type PDI were analyzed by two-way ANOVA. SPSS v.15.01 (SPSS, Inc.) and Systat v. 10.01 (SPSS Inc.) were used for statistical analysis. For the PDI activity assays, a transformed ordinate scale (OD650 nm raised to the power e) was used for graphical presentation.
of results because turbidity in this scale was linear with respect to PDI quantity, but statistical analysis was done in the original scale as the variance of these data were independent of the mean.

Results

Induction of ER stress in melanoma cells. Fenretinide, a synthetic derivative of retinoic acid, and the 26S-proteosome inhibitor velcade (bortezomib) both induce apoptosis of melanoma cells by inducing ER stress (3, 16). The induction of ER stress in human melanoma cell lines CHL-1, A375, and WM266-4 (17) in response to fenretinide or velcade at clinically achievable concentrations (18, 19) was confirmed by the marked induction of mRNA for GADD34 (protein phosphatase 1, regulatory subunit 15A) and the stress transcription factor ATF4 (Fig. 1A and B; refs. 1, 3), and the splicing of XBP-1 mRNA (Fig. 1C). For ATF4 and GADD34 in CHL1 and A375 cells, all treatments were significantly different from control ($P < 0.001$). For WM266-4 cells, fenretinide, velcade at 6 and 18 h, and thapsigargin significantly induced ATF4 mRNA ($P \leq 0.002$, $P \leq 0.001$, and $P < 0.001$, respectively), but only velcade (6 and 8 h) and thapsigargin significantly induced GADD34 mRNA ($P < 0.03$ and $P < 0.001$, respectively) at the drug concentrations used. The A375 and WM266-4 cell lines carry B-Raf mutations (17, 20), resulting in constitutive activation of the Ras/Raf/MEK/ERK pathway and increased resistance to apoptosis (21); these cell lines showed lower inductions of ATF4 and GADD34 than the B-Raf wild-type CHL-1 cells (22) at the drug concentrations used and differed in the time course of responses, particularly with respect to velcade (Fig. 1).

Increasing the consequences of stress using PDI inhibitors. We have previously shown that the small interfering RNA–mediated down-regulation of the ER stress chaperones Erdj5 or Erp57 in A375 cells increases apoptosis induced by fenretinide or velcade (3). Both these proteins have PDI domains (23, 24) and this raises the possibility that the inhibition of PDI activity may also increase apoptosis in response to ER stress. To test this hypothesis, CHL-1, A375, or WM266-4 melanoma cells were treated for 24 h with 10 $\mu$mol/L of fenretinide or 30 nmol/L of velcade in the presence or absence of fenretinide (FenR, 10 $\mu$mol/L) or velcade (Vel, 33 nmol/L).
there was an increase in apoptosis with CI ranging from 0.096 at ED50 to 0.002 at ED90, indicating a high degree of synergy (CI < 1; Fig. 3A; ref. 30). There was also an increase in apoptosis when velcade was combined with bacitracin at a dose ratio of f1:15,000; CI at ED50 and ED75 were 0.833 and 1.076, respectively, indicating additivity (30) but 1.4 at ED90 suggesting some inhibition at high doses. IC50 values for bacitracin with fenretinide or velcade were estimated to be 446 and 256 μmol/L, respectively (Fig. 3A); these are comparable to the reported 500 μmol/L IC50 value for PDI inhibition by bacitracin (29). Similar responses were obtained with respect to cell viability (Fig. 3B). Measurement of PDI activity in A375 cells confirmed that bacitracin was acting as an inhibitor of PDI activity under these conditions (Fig. 3C). Therefore, these results are evidence that inhibition of PDI activity enhances apoptotic responses to ER stress–inducing agents in melanoma cells. To ask if the effect of bacitracin in enhancing apoptosis in response to fenretinide or velcade was specific to melanoma cells, normal human melanocytes were treated with 2 to 5 μmol/L of fenretinide or 7 to 17 nmol/L of velcade in the presence or absence of 100 to 250 μmol/L of bacitracin (dose ratios 1:50 for fenretinide/ bacitracin and 1:15,000 for velcade/bacitracin). Under these conditions, there was no evidence for an effect of bacitracin at increasing apoptosis in normal melanocytes (Fig. 3D).

**Bacitracin increases apoptosis by PDI inhibition.** Commercial preparations of bacitracin may be contaminated with other activities (31); therefore, in the context of this study, it was important to obtain evidence that bacitracin enhances apoptosis as a result of inhibiting PDI activity. Because many ER stress chaperones also have PDI activity, it is not feasible to test the involvement of PDI inhibition in ER stress abrogation by targeted knockdown of all proteins with PDI activity. However, we predicted that overexpression of a protein with PDI activity would reduce
The main cellular PDI is P4HB, which catalyzes the formation, isomerization, and removal of disulfide bonds (8). Therefore, wild-type P4HB and an inactive mutant with mutations in both active PDI sites (11) were overexpressed in A375 cells by transient transfection, as confirmed by Western blotting using an antibody to P4HB (Fig. 4A). To verify the activity of the transfected constructs, PDI activity was measured in untransfected A375 cells and A375 cells transfected with an unrelated control construct, wild-type P4HB or the inactive mutant P4HB (one-way ANOVA, $F_{2,11} = 10.85; P = 0.003$; Fig. 4B). Transfection with the control construct or the mutant P4HB had no effect on PDI activity relative to untransfected cells (Dunnett’s test, $P = 0.765$ and $P = 0.468$, respectively), whereas mean PDI activity was significantly increased 3-fold in cells transfected with wild-type P4HB (Dunnett’s test, $P = 0.008$; Fig. 4B).

The effect of transient overexpression of control construct, mutant P4HB or wild-type P4HB on apoptosis of A375 cells in response to fenretinide or velcade in the presence and absence of bacitracin, compared with control construct–transfected and untreated (control) cells is shown in Fig. 4C. Statistical analysis was by two-way ANOVA for transfection plasmid and treatment (bacitracin, fenretinide, velcade, bacitracin + fenretinide, bacitracin + velcade). Both main effects and the interaction terms were statistically significant ($F_{1,30} > 71, P < 0.00001$; $x = 2, 4, 8$); hypothesis tests on the effect of transfection plasmid showed no overall difference between the control construct and mutant P4HB ($F_{1,30} = 1.29, P = 0.27$), whereas transfection of wild-type P4HB significantly decreased apoptosis compared with the control construct or mutant P4HB ($F_{1,30} > 497, P < 0.00001$). For cells treated with either fenretinide or velcade alone, transfection with wild-type P4HB decreased apoptosis compared with the control construct or mutant P4HB, indicative of a protective effect of P4HB with functional PDI activity (hypthesis tests, $F_{1,30} > 10; P < 0.003$). The mutant P4HB did not increase apoptosis and therefore was not acting in a dominant-negative manner with respect to endogenous PDI activity. In cells treated with bacitracin and fenretinide, or bacitracin and velcade, levels of apoptosis in control-transfected cells or cells overexpressing mutant P4HB were substantially greater than in cells treated with fenretinide or velcade alone, showing that the expression of these constructs did not reduce the ability of bacitracin to enhance apoptosis in response to these ER stress inducers. Conversely, in cells overexpressing wild-type P4HB, levels of apoptosis in response to bacitracin in combination with either drug were substantially lower and only marginally more than cells treated with fenretinide or velcade alone. These data suggest that overexpression of the wild-type P4HB reduced the availability of bacitracin to inhibit endogenous proteins with PDI activity, thus protecting the cells against ER stress–induced apoptosis.

Discussion

These results show that fenretinide and velcade induce ER stress in melanoma cells, that the antibiotic PDI inhibitor bacitracin increases cell death as a consequence of ER stress and that these effects of bacitracin result from its ability to inhibit PDI activity. Although both fenretinide and velcade induced similar ER stress responses, the mechanism of action of these drugs is very different. Fenretinide induces stress via an oxidative stress mechanism (3), which has, thus far, not been characterized. Conversely, velcade is a specific inhibitor of the 26S-proteasome; however, the central role of the proteasome in most cellular functions gives velcade the ability to inhibit cellular function on a broad scale resulting in ER stress, but with the time course and end result, in terms of cell arrest or apoptosis, varying depending on cell type (32). Cancer cells, particularly melanoma and leukemia, are more sensitive to
velcade than normal cells (16, 32, 33). Although leukemia cells are also more sensitive than normal cells to fenretinide (34), this is not true with respect to ovarian cancer/normal ovarian epithelial cells (35), and the normal melanocytes used in this study were relatively sensitive to fenretinide; preliminary data for the normal melanocytes suggested that these cells accumulated fenretinide more readily than melanoma cells,\(^5\) and such differences in uptake is one mechanism that could explain cell type–specific variability in fenretinide sensitivity.

Despite the sensitivity of normal melanocytes to fenretinide, bacitracin treatment did not increase the response to fenretinide or to velcade. This is in clear contrast to the melanoma cells in which bacitracin produced a dose-dependent increase in cell death in response to fenretinide or velcade, which was synergistic with fenretinide and additive with velcade across a broad dose-range. Therefore, these results suggest that inhibiting PDI activity may offer a novel mechanism to increase the therapeutic benefit of chemotherapeutic drugs for treating metastatic melanoma, particularly in the context of ER stress–inducing agents. The potential for enhancing the activity of velcade is particularly significant as this drug has good clinical activity in myeloma and is being investigated for use in a range of other cancers (36). Furthermore, velcade has shown promise in combination with existing chemotherapeutic drugs (32) and enhances the responses of melanoma cells to temozolomide in vitro (37). The ability to increase the cancer cell–specific effects of velcade with PDI inhibitors would be a powerful addition to the chemotherapeutic arsenal. However, because the clinical use of bacitracin is limited by its nephrotoxicity (38) and low membrane permeability (31, 39), it is important to develop safer and more effective alternatives. This can only be done with adequate knowledge of how bacitracin achieves its effects. Bacitracin did indeed reduce PDI activity in A375 cells; however, bacitracin can have PDI-independent effects (40), has metal-binding activity, and the commercial product can comprise a mixture of bacitracins differing in amino acid sequence and NH₂-terminal structure (27).

In the absence of purified bacitracins and data to link PDI inhibition to specific bacitracins, it is critical to verify that the ability of ‘bacitracin’ to increase velcade or fenretinide-induced cell death is a result of PDI inhibition. As predicted, overexpression of P4HB in melanoma cells increased PDI activity and substantially reduced the ability of bacitracin to increase apoptosis in combination with either fenretinide or velcade, presumably by competing with endogenous PDIs for binding to the PDI inhibition function of bacitracin. The mutant P4HB used as a negative control for this experiment lacked PDI activity as a result of cysteine to serine mutations in both active PDI sites (11); the fact that this mutant P4HB construct, despite being overexpressed to similar levels as wild-type P4HB, did not abrogate the potentiation of fenretinide- or velcade-induced apoptosis by bacitracin is strong evidence that inhibiting PDI activity reduces the ability of cells to survive ER stress. These results also show the importance of PDI activity in facilitating the survival of cells exposed to ER stress. In melanoma cells, PDIs have been reported to be expressed at a higher level than normal melanocytes (41, 42) and the expression of PDI family proteins correlates with cancer invasion, metastasis, and drug resistance in other tumor types (43–45). Although recent studies indicate that the sensitivity of melanoma cells to tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis may be increased by modulation of the unfolded protein response (46), the present results suggest that PDI inhibition can be used as a more general mechanism to down-regulate homeostatic stress responses and drive apoptosis.

The results of this study provide proof-of-principle for the concept that ER stress–induced apoptosis of melanoma cells can be enhanced using PDI inhibitors. Furthermore, the data imply that potent, small-molecule PDI inhibitors designed to bind to the CXXC motif of the PDI active site may have significant potential as powerful tools for enhancing the efficacy of chemotherapy in a wide range of cancers.

**Disclosure of Potential Conflicts of Interest**

C.P.E. Redfern, P.E. Lovat, and M.A. Birch-Machin are coapplicants on a U.K. patent on the concept of combining PDI inhibitors with anticancer drugs. The other authors disclosed no potential conflicts of interest.

**Acknowledgments**

Received 1/4/2008; revised 3/27/2008; accepted 4/12/2008.

**Grant support:** Cancer Research U.K., Newcastle Healthcare Charity, Ricerca Corrente e Finalizzata del Ministero della Salute, COFIN 2007, Associazione Italiana Ricerca sul Cancro, Progetto Europeo Health-F4-2007-200767, and Teletfon, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The authors thank Drs. J. Silver and Wu Ou, National Institute of Allergy and Disease, NIH, for the generous gift of mutant and wild-type P4HB constructs, and Ross Flockhart, Dermatological Sciences, Newcastle University, for immunostaining the melanocytes.

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