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

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Abstract

Exploiting vulnerabilities in the intracellular signalling 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 the stress response and apoptosis were enhanced by the PDI inhibitor bacitracin. Over-expression of the main cellular PDI, procollagen-proline, 2-oxoglutarate-4-dioxygenase beta subunit (P4HB), resulted in increased PDI activity and abrogated the apoptosis-enhancing effect of bacitracin. In contrast, over-expression 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.

Introduction

Exploiting vulnerabilities in the intracellular signalling pathways of tumor cells is a key strategy for the development of new drugs. Agents that disrupt normal signalling pathways may induce homeostatic responses to restore normal function. The endoplasmic reticulum (ER) is responsible for regulation of intracellular calcium (Ca^{2+}) and the synthesis of cell-surface or secretory proteins. Disruption of ER function induces a stress response characterised 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 (UPR) is an important ER stress response which rescues the cell by removing unfolded or misfolded proteins (1). However, ER stress will induce apoptotic

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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 defences against immunological and cytotoxic attack (5) resulting in defective apoptotic signalling. Increased expression of ER stress chaperones can be an early event in tumour 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 WM266-4m, obtained from the American Type Culture Collection (Teddington, UK), 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, Cambridge, UK) and cultured in Medium 254 supplemented with Human Melanocyte Growth Supplement-2 as described by the manufacturers (Invitrogen, Paisley, UK). For the over-expression of PDI, plasmids containing constructs for wild-type and an inactive mutant procollagen-proline, 2-oxoglutarate-4-dioxygenase beta subunit (P4HB), the main cellular PDI, were generous gifts from J. Silver and W. Ou (11). The transient transfection of 3 µg of wild type P4HB, mutant P4HB or pCMVSPORT-β-galactosidase (Invitrogen, Paisley, UK) 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, Southampton, UK) according to the manufacturer's protocol.

Quantitative Polymerase Chain Reaction (qPCR)

Primers were designed using Primer Express 1.0 software (Applied Biosystems, Warrington, UK). ATF4 and GADD34 mRNA were quantified relative to the ribosomal protein L34 as an internal control using the comparative C_t method. PCR amplification was performed using a LightCycler with DNA Master SYBER Green I kit (Roche Applied Science, Monza, Italy) according to manufacturer's instructions as previously described (3). Primers were GTGGCCAAGCACTTCAAACC and CCCGGAGAAGGCATCCTC for ATF4, ACACCGGGAGTGTTGTCCAG and TGGGCAAGACACCCAAGTG for GADD 34 and GTCCCGAACCCCTGGTAATAGA and GGCCCTGCTGACATGTTTCTT 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 4-20% SDS-PAGE gels (10-25 µg per track) and blotted onto PVDF membranes as previously described (13). Antibodies used to probe the blots were a GADD153 antibody (B-3, Santa Cruz Biotechnology, Autogen Bioclear, Calne, UK) diluted 1:500, a P4HB antibody from Cell Signalling Technology (New England Biolabs Ltd, UK) diluted 1:1000, and, as a loading control, β-actin antibody (Sigma) diluted 1:5000. The binding of primary antibodies was detected with secondary peroxidase-conjugated antibodies (Upstate Biotechnology, UK) diluted 1:2000 and visualized using the ECL system (Amersham Biosciences, UK).

PDI activity assay

Assays for PDI activity were carried out according to Smith *et al* (14) on cell extracts in Tris/HCl buffer containing 25 mM KCl and 5 mM MgCl₂ (15). Protein extract was reacted with 80 mM bovine insulin and PDI activity monitored as increased turbidity at 650 nm; the reaction was linear after 30 min and up to 50-80 min and turbidity at 50 min was used as the end point. Turbidity was linear in proportion to log_e 5-100 µg bovine liver PDI standard (Sigma) and up to 80 µg 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, Cambridge, UK) 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 (ED) at 50 (ED₅₀), 75 and 90% were calculated. Data for log_e relative mRNA levels and PDI activity in cells transfected with P4HB constructs were analysed by one-way ANOVA with post-hoc Dunnett's test and data for apoptosis in cells transfected with mutant or wild-type PDI were analysed by 2-way ANOVA. SPSS v.15.01 (SPSS Inc., Chicago, IL) 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 since 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-proteasome 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 GADD 34 (Protein Phosphatase 1) and the stress transcription factor ATF4 (1, 3) (Fig. 1, A & B), and the splicing of XBP-1 mRNA (Fig. 1, C). 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 ($P = 0.002$, $P = 0.001$ and $P < 0.001$, respectively), but only velcade (6 and 8 h) and thapsigargin significantly induced GADD 34 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 induction 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 siRNA-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 μM fenretinide or 30 nM velcade in the presence or absence of the PDI inhibitor bacitracin, a macrocyclic dodecapeptide antibiotic (25-27); bacitracin was used at a concentration of 500 μM and the response measured with respect to a decrease in cell viability or the induction of apoptosis. Bacitracin on its own did not induce apoptosis or affect cell viability, but significantly increased apoptosis or decreased viability in response to fenretinide or velcade in these melanoma cell lines (Fig. 2A, B). Increased expression of GADD153 is widely accepted as a marker of apoptosis resulting from ER stress (28). GADD153 was induced in all three melanoma lines in response to velcade and in CHL1 cells in response to fenretinide (Fig. 2C). However, in the presence of bacitracin, GADD153 was induced by fenretinide in all three melanoma cell lines. Furthermore, velcade-induced GADD153, or fenretinide-induced GAD153 in CHL1 cells, was increased in the presence of bacitracin in all cell lines (Fig. 2C).

Fixed dose-ratio experiments with A375 cells were carried out combining fenretinide or velcade with the PDI inhibitor bacitracin (29). At a fixed dose ratio of 1:50 for fenretinide with bacitracin, there was an increase in apoptosis with combination indices (CI) ranging from 0.096 at ED_{50} to 0.002 at ED_{90} , indicating a high degree of synergy [$\text{CI} < 1$; (30)] (Fig. 3A). There was also an increase in apoptosis when velcade was combined with bacitracin at a dose ratio of approximately 1:15,000; combination indices at ED_{50} and ED_{75} were 0.833 and 1.076, respectively, indicating additivity (30) but 1.4 at ED_{90} suggesting some inhibition at high doses. IC_{50} values for bacitracin with fenretinide or velcade were estimated to be 446 and 512 μM , respectively (Fig. 3A); these are comparable to the reported 500 μM IC_{50} 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 2-5 μM fenretinide or 7-17 nM velcade in the presence or absence of 100-250 μM 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. Since 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 over-expression of a protein with PDI activity would reduce the ability of bacitracin to enhance ER-stress-induced apoptosis by competing with endogenous PDI activity for the inhibitor. The main cellular PDI is procollagen-proline, 2-oxoglutarate-4-dioxygenase, beta subunit (P4HB) which catalyses the formation, isomerisation and removal of disulphide bonds (8). Therefore, wild-type P4HB and an inactive mutant with mutations in both active PDI sites (11) were over-expressed 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 (Fig. 4B; one-way ANOVA, $F_{3,11}=10.85$, $P=0.003$). 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 over-expression 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 to control-construct transfected and untreated (control) cells, is shown in Fig. 4C. Statistical analysis was by 2-way ANOVA for transfection plasmid and treatment (bacitracin, fenretinide, velcade, bacitracin + fenretinide, bacitracin + velcade). Both main effects and the interaction term was significant ($F_{x,30}>71$, $P<0.00001$; $x=2,4$ or 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 to 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 to the control construct or mutant P4HB, indicative of a protective effect of P4HB with functional PDI activity (Hypothesis 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 over-expressing 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 over-expressing 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 over-expression 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, so far, has not been characterised. 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 leukaemia, are more sensitive to velcade than normal cells (16, 32, 33). Although leukaemia 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 (Armstrong, unpublished data) 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 responses of melanoma cells to temozolomide *in vitro* (37). The ability to increase cancer-cell-specific effects of velcade with PDI inhibitors would be a powerful addition to the chemotherapeutic arsenal. However, since 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 N-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, over-expression 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 over-expressed 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 TRAIL-induced apoptosis may be increased by modulation of the UPR (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 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.

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Abbreviations

ER	endoplasmic reticulum
PDI	protein disulfide isomerase
UPR	unfolded protein response
P4HB	procollagen-proline, 2-oxoglutarate-4-dioxygenase, beta subunit

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Figure 1.

Fenretinide and velcade induced markers of ER stress in melanoma cells. Induction of (A) ATF4 or (B) GADD34 mRNA as measured by real-time quantitative PCR, relative to the ribosomal protein L34 as an internal control, in CHL-1, A375 and WM266-4 human melanoma cells after 6, 18 or 24 h treatment with 10 μ M fenretinide (FenR), 30 nM velcade (Vel; obtained from Janssen-Cilag, High Wycombe, UK), or 24 h treatment with control vehicle or, as a positive control, 7.5 μ M thapsigargin (Thap). Each bar is the mean of 3 replicates $\pm$ 95% CI. C, induction of XBP-1 splicing in CHL-1, A375 and WM266-4 melanoma cells in response to 6, 18 or 24 h treatment with fenretinide (FenR; 10 μ M for CHL-1 cells and 15 μ M for A375 and WM266-4 cells) or velcade (Vel; 15 nM for CHL-1 cells and 30 nM for A375 cells) or in response to 24 h treatment with 7.5 μ M thapsigargin (Thap). Positions of the PCR products from the unspliced (U) and spliced (S) XBP-1 transcripts are marked with arrows.

Figure 2.

The PDI inhibitor bacitracin increased ER stress-induced apoptosis in melanoma cells. Relative viability (A) or apoptosis (B) of CHL-1, A375 or WM266-4 cells in response to 24h treatment with 500 μ M bacitracin (Bac) in the presence or absence of 10 μ M fenretinide (FenR) or 30 nM velcade (Vel). Control cells were treated with vehicle control for 24 h in each experiment. Each point is the mean of 8 (viability) or 3 (apoptosis) replicates $\pm$ 95% CI. Panel C shows Western blots for GADD153 expression in CHL-1, A375 and WM266-4 melanoma cells in response to 8 h (CHL-1 and WM266-4) or 12 h (A375) treatment with bacitracin (Bac, 500 μ M) in the presence or absence of fenretinide (FenR, 10 μ M) or velcade (Vel, 33 nM).

Figure 3.

Synergistic and additive effects of bacitracin with fenretinide or velcade. *A*, Apoptosis of A375 cells in response to 24 h treatment with bacitracin (Bac) or fenretinide (FenR) or both agents combined at a fixed dose ratio of 50:1 (left-hand graph), or in response to bacitracin or velcade or both agents combined at a fixed dose ratio of 15,000:1 (right-hand graph). Inserts in are dose-response curves at 24 h for bacitracin (Sigma, Poole, UK) in the presence of fixed concentrations of fenretinide (15 μM) or velcade (33 nM): abscissa, log₁₀ bacitracin dose; ordinate, % apoptosis. IC₅₀ values were estimated from 4-parameter sigmoidal curves and were 450 μM for bacitracin with fenretinide and 250 μM for bacitracin with velcade. Each point is the mean of 3 replicates ± 95% CI. *B*, Relative viability of A375 cells after 24 h treatment with bacitracin (Bac) or fenretinide (FenR) or both agents together at a fixed dose ratio of 50:1 (left-hand graph), or in response to bacitracin or velcade or both agents together at a fixed dose ratio of 15,000:1 (right-hand graph). Each point is the mean of 3 replicates ± 95% CI. *C*, PDI activity in A375 cells (cell extract, 40 μg protein) in the presence or absence of 500 μM bacitracin (Bac); bar heights are means + range of duplicate samples and the ordinate scale is OD_{650 nm} raised to the power *e* for linearity with PDI quantity. *D*, apoptosis (mean % + 95% CI) in normal human melanocytes in response to 2 or 5 μM fenretinide, or 7 or 17 nM velcade, with and without 100 μM or 250 μM bacitracin, respectively.

Figure 4.

Over-expression of PDI abrogates enhancement of fenretinide or velcade-induced apoptosis. *A*, over-expression of PDI in A375 cells: Western blot for P4HB or β -actin (loading control) in control (C) A375 cells or after transient transfection of wild type (PDI) or mutant (mPDI) P4HB (11) or p_{CMV} Sport- β -galactosidase control construct (V). *B*, PDI activity in A375 cells (cell extract, 80 μ g protein) after transient transfection for 24 h with control vector construct (V), wild type (PDI) or mutant (mPDI) P4HB and expressed as activity relative to mean PDI activity (transformed scale: OD650 nm to the power e) in control, untransfected cells in the same experiment. Bar heights represent the mean fold induction + upper 95% confidence limit. *, $P < 0.01$ compared to untransfected cells (see text). For these data, statistical analysis was done in the original scale (see text). *C*, Apoptosis of A375 cells in response to transient transfection for 24 h with either control vector construct (white bars), mutant P4HB (gray bars) or wild type (black bars) P4HB, and subsequent treatment for 24 h with 500 μ M bacitracin (Bac), 10 μ M fenretinide (FenR), 30 nM velcade (Vel) or 500 μ M bacitracin combined with 10 μ M fenretinide or 30 nM velcade. Apoptosis is expressed relative to cells transfected with the control construct without drug treatment and each point is the mean + 95% confidence limit of three independent experiments. ***, $P < 0.00001$ for the comparisons marked.