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Pim-1 Kinase Protects P-Glycoprotein from Degradation and Enables Its Glycosylation and Cell Surface Expression

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ABSTRACT

The oncogenic serine/threonine kinase Pim-1 phosphorylates and activates the ATP-binding cassette transporter breast cancer resistance protein (ABCG2). The ABC transporter P-glycoprotein (Pgp; ABCB1) also contains a Pim-1 phosphorylation consensus sequence, and we hypothesized that Pim-1 also regulates Pgp. Pgp is exported from the endoplasmic reticulum (ER) as a 150-kDa species that is glycosylated to 170-kDa Pgp, translocates to the cell surface, and mediates drug efflux; alternatively, 150-kDa Pgp is cleaved to a 130-kDa proteolytic product by ER proteases or undergoes ubiquitination and proteasomal degradation. Pim-1 and Pgp interaction was studied in GST pull-down and phosphorylation in in vitro kinase assays. Pim-1 knockdown and inhibition effects on Pgp expression were studied by immunoblotting and flow cytometry and on Pgp stability by immunoblotting after cycloheximide treatment. Pim-1 directly interacted with and phosphorylated Pgp in intact cells and in vitro. Pim-1 knockdown or inhibition decreased cellular and cell surface 170-kDa Pgp, in association with both transient increase in 130-kDa Pgp and increased Pgp ubiquitination and proteasomal degradation. Pim-1 inhibition also decreased expression of 150-kDa Pgp in the presence of the glycosylation inhibitor 2-deoxy-D-glucose. Finally, Pim-1 inhibition sensitized Pgp-overexpressing cells to doxorubicin. Thus, Pim-1 regulates Pgp expression by protecting 150-kDa Pgp from proteolytic and proteasomal degradation and enabling Pgp glycosylation and cell surface translocation and thus Pgp-mediated drug efflux. Pim-1 inhibitors are entering clinical trials and may provide a novel approach to abrogating drug resistance.

The serine/threonine protein kinase Pim-1, encoded by a proto-oncogene originally identified as the *p*roviral *i*ntegration site in Moloney *m*urine leukemia virus lymphomagenesis, is overexpressed in diverse malignances, including acute myeloid leukemia, acute lymphoblastic leukemia, prostate cancer, and gastric cancer (Amson et al., 1989; Dhanasekaran et al., 2001; Chen et al., 2005). Pim-1 has two protein isoforms, 33-kDa Pim-1S and 44-kDa Pim-1L, that are synthesized with alternative translation initiation sites (Saris et al., 1991) and differ in cellular localization, in that 33-kDa Pim-1S is primarily intracellular, whereas 44-kDa Pim-1L is primarily localized to the plasma membrane (Xie et al., 2006). Pim-1 substrates include the pro-apoptotic protein BAD (Aho et al., 2004); the cell cycle regulatory proteins p21 (Zhang et al., 2007), p27 (Morishita et al., 2008), Cdc25A (Mochizuki et al., 1999), and Cdc25C (Bachmann et al., 2006); and the transcription factors SOCS-1 (Chen et al., 2002), RUNX3 (Kim et al., 2008), and c-myc (Zhang et al., 2008). Pim-1 also regulates drug resistance, in that we recently demonstrated that it phosphorylates the G-subfamily ATP-binding cassette (ABC) transporter breast cancer resistance protein (BCRP; ABCG2) at Thr362, thereby promoting its multimerization and cell surface translocation (Xie et al., 2008).

Like BCRP, P-glycoprotein (Pgp), a B-subfamily ABC transporter (ABCB1) that is strongly associated with clinical drug resistance (Mahadevan and Shirahatti, 2005), also has a Pim-1 phosphorylation consensus sequence (Palaty et al., 1997; Bullock et al., 2005), QDRKLS, located at Ser683, between nucleotide binding domain 1 and transmembrane domain 1. We therefore hypothesized that Pim-1 might also be implicated in regulating drug resistance mediated by Pgp.

Regulation of Pgp expression is post-translational as

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ABBREVIATIONS: BCRP, breast cancer resistance protein; ABC, ATP-binding cassette; ER, endoplasmic reticulum; Pgp, P-glycoprotein; PKC, protein kinase C; FCS, fetal calf serum; shRNA, small hairpin RNA; siRNA, small interfering RNA; GST, glutathione transferase; 2-DG, 2-deoxy-D-glucose; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; CHX, cycloheximide; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PSC-833, valspodar.

well as transcriptional. Pgp is exported from the endoplasmic reticulum (ER) as a 150-kDa core-glycosylated intermediate species that is then glycosylated in the Golgi apparatus to yield the mature 170-kDa form that translocates to the cell surface and mediates cellular drug efflux (Gribar et al., 2000). Alternatively, 150-kDa Pgp may be cleaved by ER proteases to form a 130-kDa proteolytic product (Loo and Clarke, 1998a) or ubiquitinated and thereby tagged for proteasomal degradation after export from the ER (Zhang et al., 2004). Pgp is phosphorylated by protein kinase C (PKC) at serines 661, 667, and 671 and by protein kinase A at serines 667, 671, and 683 (Chambers et al., 1994), the latter of which is also the Pim-1 phosphorylation consensus site. Based on studies of phosphorylation-defective Pgp mutants, phosphorylation does not directly regulate Pgp transport function (Germann et al., 1996; Goodfellow et al., 1996), but the effect of phosphorylation on Pgp degradation and cell surface trafficking has not been characterized, and signaling pathways regulating these processes remain to be elucidated.

We demonstrate here that Pim-1 kinase phosphorylates Pgp and thereby protects core-glycosylated Pgp from proteolytic and proteasomal degradation and enables Pgp glycosylation and cell surface trafficking of glycosylated Pgp. Pim-1 is thus a novel signaling pathway regulating cellular and cell surface Pgp expression, and inhibiting Pim-1 kinase is a novel approach to abrogating Pgp-mediated drug resistance by promoting degradation of core-glycosylated Pgp and thereby inhibiting its glycosylation and cell surface expression.

Materials and Methods

Cells. HL60/VCR leukemia cells (Ogretmen and Safa, 2000), 8226/Dox6 myeloma cells (Hazlehurst et al., 1999), and MCF7/AdrR ovarian carcinoma cells, re-designated NCI/ADR-RES, OVCAR-8-Pgp (Liscovitch and Ravid, 2007), all with Pgp overexpression and Pgp-mediated multidrug resistance, were obtained from Dr. Ahmad R. Safa (Indiana University, Indianapolis, IN), Dr. William S. Dalton (Moffitt Cancer Center, Tampa, FL), and Dr. Erasmus Schneider (Wadsworth Center, New York State Department of Health, Albany, NY), respectively. HL60/VCR cells were maintained in drug-free RPMI 1640 medium with 10% fetal calf serum (FCS), 8226/Dox6 cells in RPMI 1640 medium with 10% FCS and 60 nM doxorubicin, and MCF7/AdrR cells in RPMI 1640 medium with 10% FCS and 10 μM doxorubicin.

Pim-1 Gene Knockdown. Cells were infected with lentivirus containing Pim-1 small hairpin RNA (shRNA) or nontarget control according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). In brief, 2.5×10^5 cells were mixed with lentivirus in a 12-well plate and then cultured for 72 h after infection. As a control, cells were also infected with equal amounts of plasmid-expressing green fluorescent protein (Sigma-Aldrich). As another approach to Pim-1 knockdown, cells were infected with lentivirus containing Pim-1 small interfering RNA (siRNA) and scrambled siRNA control under identical conditions, as described previously (Xie et al., 2006; Xie et al., 2008). Three days after infection with Pim-1 shRNA or siRNA or controls, Pim-1 expression was measured by immunoblotting, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control, to confirm the expected effect or lack of effect on Pim-1 gene expression.

Immunoblotting. Cells were lysed in buffer with protease (Roche Applied Science, Indianapolis, IN) and phosphatase (Pierce, Rockford, IL) inhibitor cocktails, as described previously (Xie et al., 2006, 2008), and immunoblotting was performed as described previously (Kim et al., 2004). In brief, blots were incubated with primary antibodies, including 1:100 dilution of anti-phosphoserine (Calbiochem, San Diego, CA), 1:100 dilution of anti-Pim-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:2000 dilution of anti-GAPDH (Calbiochem), 1:200 dilution of polyclonal anti-Pgp (H241, Santa Cruz), 1:200 dilution of monoclonal anti-Pgp (3C3.2; Millipore Bioscience Research Reagents, Temecula, CA), and 1:200 dilution of monoclonal anti-ubiquitin (Santa Cruz) for 1 h at room temperature or overnight at 4°C, followed by detection with horseradish peroxidase-conjugated secondary antibody.

Pgp Cell Surface Expression. Pgp cell surface expression was measured with MRK16 antibody (Kamiya Biomedical Company, Seattle, WA) and IgG2a isotype control (Kamiya), detected with phycoerythrin-labeled goat anti-mouse antibody (Caltag), and measured by flow cytometry (Minderman et al., 2004; Qadir et al., 2005).

Immunoprecipitation. Cells were lysed in lysis buffer, as described above. Antibodies or control immunoglobulins were added to the lysates, followed by incubation at 4°C for 16 h. Antibodies used for immunoprecipitation included 1 μg of monoclonal anti-Pim-1, polyclonal anti-Pgp, and monoclonal anti-Pgp. Immunocomplexes were collected using protein A- or protein G-Sepharose beads, and the beads were then washed extensively with lysis buffer three times at 4°C.

Glutathione Transferase Pull-Down Assay. The glutathione transferase (GST) pull-down assay was performed as described previously (Xie et al., 2006, 2008) with minor modifications. In brief, GST-tagged Pim-1 fusion protein or control GST (Cell Signaling Technology Inc., Danvers, MA) was pulled down by glutathione beads for 1 h at 4°C and then washed three times with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100) with protease inhibitor cocktail. The immobilized GST fusion protein or control GST was incubated with HL60/VCR cell lysates for 1 h at 4°C. The beads were washed with the lysis buffer four times, and the protein complexes were then loaded in a NuPage 4 to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA), followed by immunoblotting with anti-Pgp antibody. Pgp and GST-Pim-1 fusion proteins in the reactions were also measured with anti-Pgp, anti-GST (Sigma-Aldrich), and anti-Pim-1 antibodies, respectively.

In Vitro Kinase Assay. An in vitro kinase assay of Pgp phosphorylation by Pim-1 was carried out as described previously (Xie et al., 2006, 2008). In brief, immunoprecipitation was performed using anti-Pim-1. The protein A-immunoprecipitate complex was washed twice with 1× kinase buffer (Kim et al., 2006) and resuspended in 40 μl of 1× kinase buffer supplemented with 500 μM ATP and Pgp substrate protein (Millipore, Billerica, MA). To prepare the substrate, crude membrane protein (100 μg) extracted from High Five insect cells overexpressing human Pgp (Kerr et al., 2001) was resuspended in lysis buffer (Xie et al., 2006, 2008) with protease inhibitor cocktail (Roche Applied Science). Insoluble material was removed by centrifugation, and 1 μg of monoclonal anti-Pgp antibody or control immunoglobulin was added to lysates, followed by incubation at 4°C for 16 h. Immunocomplexes were collected using protein G-Sepharose beads, and the beads were then washed extensively with lysis buffer three times at 4°C and resuspended in 40 μl of 1× kinase buffer (Kim et al., 2006) with 0.5 μg of soluble Pim-1 protein kinase (Cell Signaling Technology). After 30-min incubation at 30°C, the reaction was terminated by adding an equal volume of 2× SDS sample buffer followed by immunoblotting with phosphoserine, Pgp, and GST (GE Healthcare Life Sciences, Chalfont St. Giles, Buckinghamshire, UK) antibodies.

Inhibition of Pim-1 Kinase. Pim-1 kinase was inhibited by incubation with the Pim-1-selective kinase inhibitor SGI-1776 (Chen et al., 2009, Mumenthaler et al., 2009), generously provided by SuperGen, Inc. (Dublin, CA). SGI-1776 was used at 1 μM in 0.1% DMSO because it inhibits Pim-1 at a concentration of 7 + 1.8 nM and is selective for Pim-1, Pim-2, Pim-3, FLT3, and haspin in this concentration range but is more than 95% bound to human plasma

protein (Chen et al., 2009). Pim-1 inhibition was confirmed by measurement of phospho-BAD at Ser112 (Aho et al., 2004).

Inhibition of New Protein Synthesis. New protein synthesis was inhibited by culturing cells with 100 μg/ml cycloheximide (CHX; Sigma-Aldrich), and cellular protein was then measured at serial time points by immunoblotting.

Inhibition of Glycosylation. Glycosylation inhibition was carried out by treating cells with 50 mM 2-deoxy-D-glucose (2-DG; Sigma-Aldrich), a nonmetabolizable glucose analog that inhibits glycosylation.

Proteasome Inhibition. To evaluate the effect of proteasomal degradation on Pgp turnover, cells were incubated in the presence and absence of the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132; Calbiochem) at 1 μM.

Colony Assay. To study sensitization of drug-resistant cells by Pim-1 inhibition, Pgp-overexpressing multidrug resistant MCF7/AdrR, or OVCAR-8-Pgp cells were seeded in a six-well plate at 10^5 cells/well with or without 10 μM doxorubicin combined with 1 μM SGI-1776 or DMSO control. After 48 h, the cells were gently washed three times and then cultured again in fresh medium with or without drug(s). After 120 h, colonies were visualized by Coomassie Blue staining, and densitometric scanning was performed.

Statistical Analyses. All experiments were repeated at least three times. Cell staining with MRK16 and isotype control was

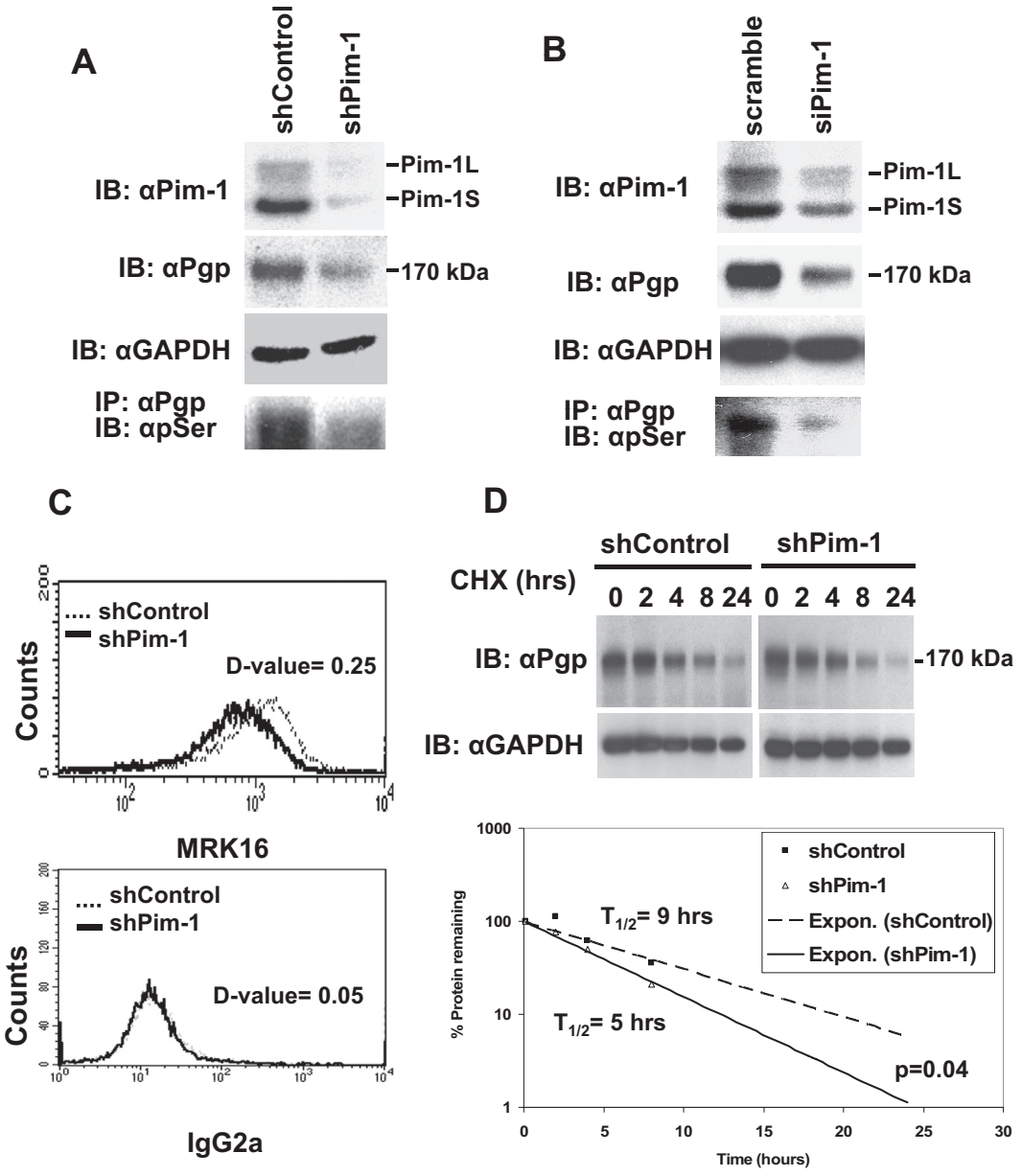

Fig. 1. Pim-1 knockdown decreases both cellular and cell surface Pgp expression and decreases Pgp stability. HL60/VCR cells were infected with lentivirus containing Pim-1 shRNA or nontarget shRNA control (A) or Pim-1 siRNA or Pim-1 scrambled siRNA control (B). Three days after infection, expression of Pim-1, measured by immunoblotting (IB), decreased in cells treated with Pim-1 shRNA or siRNA in relation to controls, as expected, and expression of 170-kDa mature Pgp also decreased, with GAPDH expression shown as a control. A decrease in serine-phosphorylated Pgp, measured by immunoprecipitation and immunoblotting, was also seen and was commensurate with the decrease in Pgp. C, cell surface expression of Pgp, studied by flow cytometry with the MRK16 antibody, was decreased 6 days after infection in cells treated with shRNA in relation to nontarget shRNA control. Labeling with IgG2a isotype control is also shown. D, HL60/VCR cells were infected with lentivirus containing Pim-1 shRNA or nontarget shRNA control for 72 h, and expression of Pgp and GAPDH, as a loading control, was measured by immunoblotting after CHX treatment for the times indicated, demonstrating decreased stability of 170-kDa Pgp in cells after Pim-1 knockdown, in relation to control. The results of densitometric scanning are also shown graphically, with Pgp signal normalized to GAPDH signal. Pgp half-lives were 5 versus 9 h (p = 0.04; Wilcoxon rank test).

compared by the Kolmogorov-Smirnov statistic, which generates D values ranging from 0 (no difference) to 1 (no overlap) (Qadir et al., 2005). Other comparisons were performed with the Wilcoxon rank test and the Student's t test, two-tailed.

Results

Pim-1 Knockdown Decreases Pgp Expression. We first tested the effect of Pim-1 on Pgp expression in Pim-1 knockdown experiments. HL60/VCR cells were infected with lentivirus containing Pim-1 shRNA or nontarget shRNA control (Fig. 1A), or Pim-1 siRNA or Pim-1 scrambled siRNA control (Fig. 1B). Three days after infection, expression of Pim-1, measured by immunoblotting, decreased in cells treated with Pim-1 shRNA or siRNA in relation to controls, as expected, and the expression of 170-kDa Pgp also decreased. A decrease in serine-phosphorylated Pgp, measured by immunoprecipitation and immunoblotting, was also seen and was commensurate with the decrease in Pgp. Cell surface expression of Pgp, studied by flow cytometry with the MRK16 antibody, also decreased 6 days after infection with shRNA, in relation to control, although to a lesser degree than cellular Pgp expression. Thus, Pim-1 promotes cellular and cell surface expression of 170-kDa Pgp. The lesser effect on cell surface, in relation to cellular, Pgp expression probably reflects the greater stability of cell surface, in relation to intracellular, Pgp (Pétriz et al., 2004).

Pim-1 Stabilizes Pgp. Based on Pim-1 enhancement of the stability of other substrate proteins, we hypothesized that Pim-1 might increase Pgp stability as a mechanism for increasing its expression. To test this hypothesis, HL60/VCR cells infected with lentivirus containing shPim-1 or nontarget control for 72 h were treated with 100 μg/ml CHX to block new protein synthesis, and expression of Pgp was measured at serial time points by immunoblotting. Compared with treatment with shRNA control, Pim-1 knockdown resulted in a significantly decreased half-life of total cellular Pgp (Fig. 1D) of 5 versus 9 h ($p = 0.04$; Wilcoxon rank test).

Pim-1 Interacts with and Phosphorylates Pgp. To determine the mechanism by which Pim-1 promotes Pgp expression, we tested whether Pim-1 physically interacts with Pgp using a coimmunoprecipitation assay. Lysates of drug-resistant HL60/VCR, 8226/Dox6, and OVCAR-8-Pgp cells overexpressing Pgp were immunoprecipitated with anti-Pim-1 antibody and then immunoblotted with anti-Pgp antibody or were immunoprecipitated with anti-Pgp antibody and then immunoblotted with anti-Pim-1 antibody. The Pim-1 antibody used recognizes both the 33-kDa isoform of Pim-1, also known as Pim-1S, which is primarily intracellular (Xie et al., 2006), and the 44-kDa isoform, also known as Pim-1L, which is primarily localized to the plasma membrane. Interaction between Pim-1 and Pgp was demonstrated in HL60/VCR, 8226/DOX6, and OVCAR-8-Pgp cells (Fig. 2A). It is noteworthy that Pim-1 interacted predominantly with 150-kDa Pgp in 8226/Dox6 cells and OVCAR-8-Pgp cells but interacted primarily with 170-kDa Pgp in HL60/VCR cells, possibly reflecting the fact that Pgp expression requires ongoing drug exposure in 8226/DOX6 and OVCAR-8-Pgp, whereas 170-kDa Pgp is expressed in the absence of ongoing drug exposure in HL60/VCR cells.

To test whether Pim-1 directly interacts with Pgp, a GST

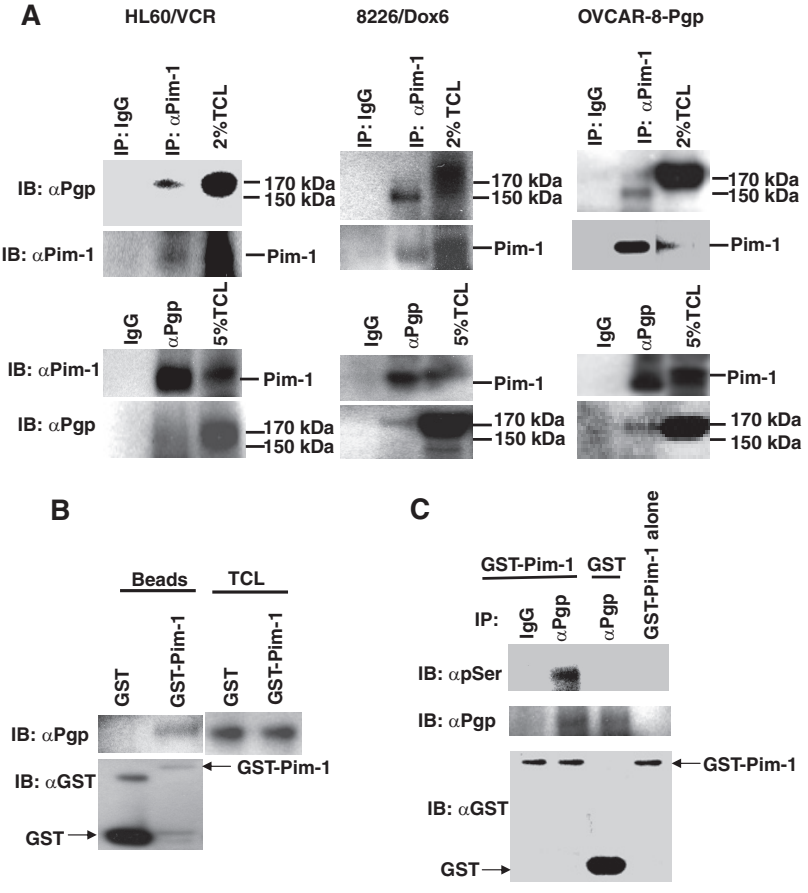

Fig. 2. Pim-1 interacts with and phosphorylates Pgp. A, to test for Pim-1 interaction with Pgp, lysates of drug-resistant HL60/VCR, 8226/Dox6, and OVCAR-8-Pgp cells overexpressing Pgp were immunoprecipitated with anti-Pim-1 antibody, then immunoblotted (IB) with anti-Pgp antibody (row 1) or immunoprecipitated with anti-Pgp antibody, and then immunoblotted with anti-Pim-1 antibody (row 3). Levels of Pgp and Pim-1 expression were measured both in total cell lysates (rows 1 and 3) and by immunoblotting of immunoprecipitates (rows 2 and 4) as controls. Pim-1 interaction with Pgp was demonstrated in all three cell lines. It is noteworthy that Pim-1 interacted predominantly with 150-kDa Pgp in 8226/Dox6 cells and OVCAR-8-Pgp cells but interacted primarily with 170-kDa Pgp in HL60/VCR cells, possibly reflecting the fact that Pgp expression requires ongoing drug exposure in 8226/DOX6 and OVCAR-8-Pgp, whereas 170-kDa Pgp is expressed in the absence of ongoing drug exposure in HL60/VCR cells. B, to test for direct interaction between Pim-1 and Pgp, purified GST-tagged recombinant Pim-1 (GST-Pim-1) protein or GST control pulled down by glutathione beads was incubated with lysates of HL60/VCR cells, which overexpress Pgp, and immunoblotting demonstrated specific and direct interaction of Pim-1 and Pgp (Row 1, left). Pgp expression was measured in total cell lysates (TCL) as a control (Row 1, right). In addition, GST-Pim-1 and GST in the reaction mixture were also measured by immunoblotting with anti-GST as controls (row 2). Direct interaction of Pim-1 with Pgp was demonstrated in the GST pull-down assay. C, to test whether Pim-1 directly phosphorylates Pgp, Pgp immunoprecipitated from High Five insect cell membranes (100 μg) expressing Pgp was incubated with GST-tagged recombinant Pim-1 (GST-Pim-1) protein in an in vitro kinase assay, and Pgp phosphorylation was measured by immunoblotting with anti-phosphoserine (pSer) (row 1). Pgp, GST-Pim-1, and GST in the reaction mixtures were measured by immunoblotting with anti-Pgp and anti-GST as controls (rows 2 and 3). Direct phosphorylation of Pgp by Pim-1 was demonstrated in vitro.

pull-down assay was then performed. Purified GST-tagged recombinant Pim-1 protein or GST control pulled down by glutathione beads was incubated with lysates of HL60/VCR cells, which overexpress Pgp. Immunoblotting demonstrated specific and direct interaction of Pim-1 and Pgp (Fig. 2B).

Finally, to test whether Pim-1 directly phosphorylates Pgp, Pgp immunoprecipitated from High Five insect cell membranes (100 μg) expressing Pgp was incubated with GST-tagged recombinant Pim-1 (GST-Pim-1) protein in an in vitro kinase assay, and Pgp phosphorylation was measured by immunoblotting with anti-phosphoserine. Pim-1 phosphorylation of Pgp was demonstrated (Fig. 2C).

Pim-1 Protects Pgp from Proteolysis. We sought to determine whether Pim-1 binding stabilizes Pgp at least in part by inhibiting its proteolytic degradation. To address this question, we measured the effect of Pim-1 knockdown on the 130-kDa Pgp proteolytic species by immunoblotting with anti-Pgp after Pim-1 knockdown with prolonged exposure to detect the proteolytic species. In addition to causing decreased expression of 170-kDa mature Pgp, Pim-1, knockdown also caused an increase in the 130-kDa proteolytic Pgp species (Fig. 3A), suggesting that Pim-1 enhances the expression of 170-kDa mature Pgp in association with protecting it from degradation by ER proteases to form 130-kDa Pgp.

We then investigated the dynamic effects of Pim-1 inhibition by the Pim-1-selective inhibitor SGI-1776 on the expression of 170-kDa Pgp and its 130-kDa proteolytic product in Pgp-overexpressing cells. It is expected that the effects of Pim-1 knockdown and Pim-1 inhibition will be different, because Pim-1 knockdown affects cell signaling by a genomic pathway and represents a stable cellular signaling change, whereas Pim-1 inhibition reflects a transient signaling change by a nongenomic effect. OVCAR-8-Pgp cells were treated with 1 μM SGI-1776, and expression of Pgp was measured by immunoblotting after 15 and 30 min and 1, 3, 8, 16, and 24 h. SGI-1776 treatment resulted in a transient increase in 130-kDa Pgp, followed by a decrease in the expression of 170-kDa Pgp (Fig. 3B), consistent with Pim-1 protection of Pgp from proteolysis as a mechanism of Pim-1-mediated stabilization of Pgp.

Pim-1 Protects Pgp from Ubiquitination and Proteasomal Degradation. We sought to determine whether Pim-1 also protects Pgp from proteasomal degradation. To this end, Pgp stability was studied by measuring its expression by immunoblotting at serial time points in HL60/VCR cells cultured with CHX to inhibit new protein synthesis with and without SGI-1776 in the presence and absence of the proteasome inhibitor MG-132. Similar to the effect of Pim-1

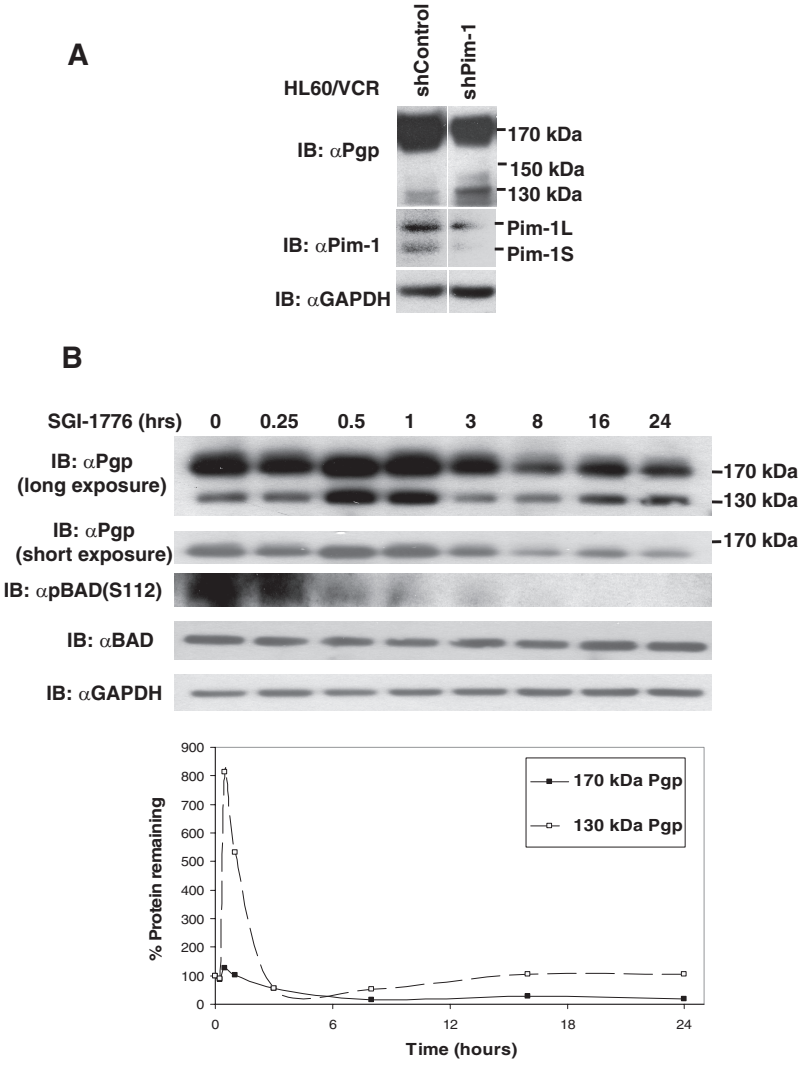

Fig. 3. Pim-1 protects Pgp from proteolytic degradation. A, to determine whether Pim-1 binding stabilizes Pgp in part by inhibiting its proteolytic degradation, the effect of Pim-1 knockdown on the 130-kDa Pgp proteolytic species was measured by immunoblotting with anti-Pgp after Pim-1 knockdown, with prolonged exposure to detect 130-kDa Pgp. HL60/VCR cells were infected with lentivirus containing Pim-1 shRNA or nontarget control, and Pim-1 expression was measured 6 days after infection. Infection with Pim-1 shRNA, in relation to control, resulted in an increase in 130-kDa proteolytic Pgp product, in conjunction with decreased expression of 170-kDa Pgp. B, to investigate the dynamic effects of Pim-1 inhibition on 130- and 170-kDa Pgp, OVCAR-8-Pgp cells were treated with the Pim-1-selective inhibitor SGI-1776 (1 μM) for the times indicated, and expression of Pgp was measured by immunoblotting. A long exposure was used to visualize the 130-kDa Pgp proteolytic product, whereas changes in the expression of 170-kDa Pgp were more sensitively demonstrated with a shorter exposure. BAD phosphorylation at Ser112 was also measured to demonstrate Pim-1 kinase inhibition, and GAPDH expression levels were measured as controls. The results are also shown graphically. Pim-1 inhibition, demonstrated by decreased BAD phosphorylation at Ser112, resulted in a transient increase in the 130-kDa proteolytic Pgp product, with subsequent decreased expression of the 170-kDa mature Pgp.

knockdown in HL60/VCR cells, Pim-1 inhibition by SGI-1776 decreased the half-life of cellular 170-kDa Pgp to 1 versus 9 h ($p = 0.03$; Wilcoxon rank test) (Fig. 4A). It is noteworthy that Fig. 4 shows Pgp expression in the presence of cycloheximide, and thus the marked decrease or absence of Pgp expression in Fig. 4 reflects the effect of Pim-1 inhibition in the absence of new protein synthesis. In contrast, Fig. 3 shows decreased but persistent Pgp expression when Pim-1 is inhibited without inhibiting new protein synthesis. Measurement of Pgp stability in the presence of the proteasome inhibitor MG-132 demonstrated that inhibition of proteasomal degradation overcame the effect of SGI-1776 on Pgp stability (Fig. 4A). Thus inhibition of Pim-1 by SGI-1776 promotes proteasomal degradation of Pgp, indicating that Pim-1 protects Pgp from proteasomal degradation.

To further test whether Pim-1 protects Pgp from ubiquitination, we additionally measured the effects of Pim-1 knockdown on Pgp ubiquitination. Ubiquitinated Pgp was measured by immunoprecipitation with anti-Pgp followed by immunoblotting with anti-ubiquitin. The level of ubiquitinated 150-kDa Pgp was found to be increased in HL60/VCR cells after Pim-1 knockdown (Fig. 4B), consistent with ubiquitination and degradation of core-glycosylated Pgp as a mechanism for decreased expression of 170-kDa Pgp protein in cells after Pim-1 knockdown and thus with Pim-1 protection of core-glycosylated 150-kDa Pgp from ubiquitination and proteasomal degradation.

Pim-1 Stabilization of Pgp Enables Its Glycosylation. It is noteworthy that total cellular Pgp did not decrease in cells incubated with both SGI-1776 and MG-132 (Fig. 4A, bottom) despite inhibition of synthesis of new protein by cycloheximide. This observation suggested that inhibition of proteasomal degradation might allow maturation of previously synthesized immature Pgp. Thus, we hypothesized that Pim-1 stabilization of immature Pgp might enable its glycosylation. To test this hypothesis, HL60/VCR cells were treated with the glycosylation inhibitor 2-DG with and without 1 μM SGI-1776 for 24 h, and expression of Pgp was examined by immunoblotting (Fig. 5). BAD phosphorylation at Ser112 was also measured to demonstrate Pim-1 kinase inhibition, and BAD and GAPDH expression levels were measured as controls. SGI-1776, which inhibited Pim-1 kinase, as evidenced by decreased BAD phosphorylation at Ser112, was found to decrease expression of the 150-kDa Pgp that accumulates when glycosylation is inhibited by 2-DG, consistent with the hypothesis that Pim-1 stabilizes 150-kDa

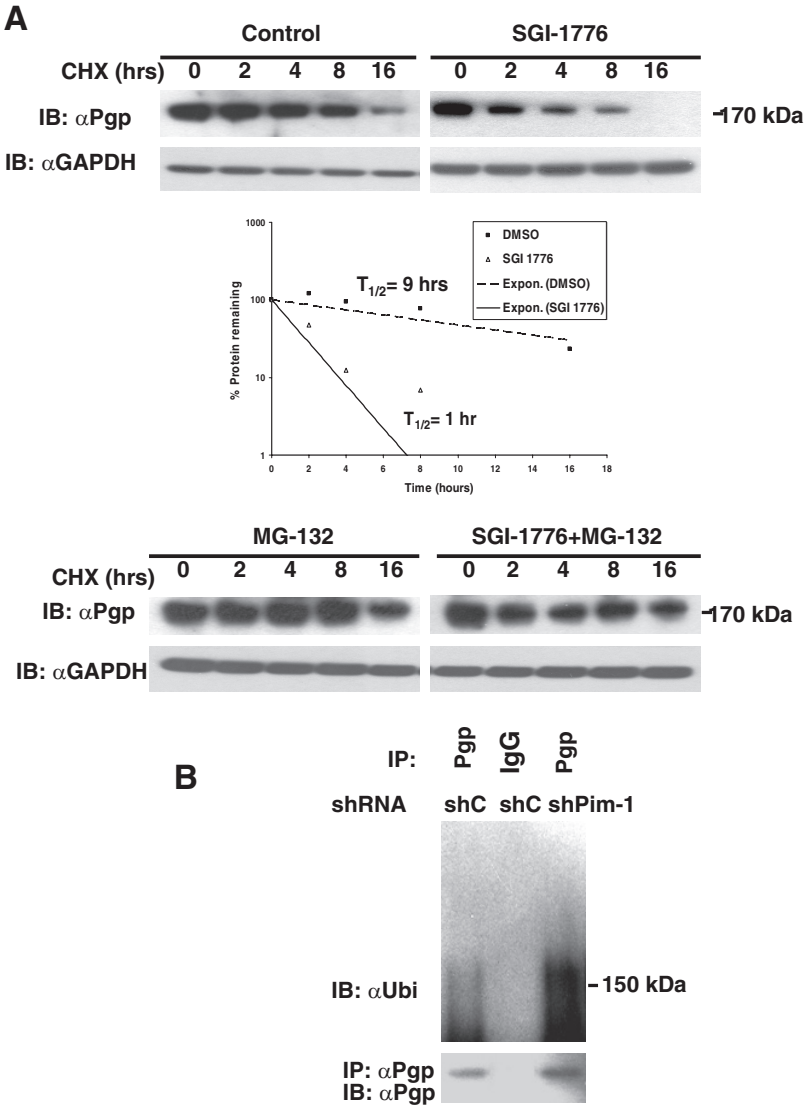

Fig. 4. Pim-1 protects Pgp from proteasomal degradation. A, to determine whether Pim-1 also protects Pgp from proteasomal degradation, Pgp turnover was studied by measuring its expression by immunoblotting at serial time points in HL60/VCR cells cultured with cycloheximide (CHX) to inhibit new protein synthesis with and without the selective inhibitor SGI-1776 (1 μM) in the presence and absence of the proteasome inhibitor MG-132. Expression of Pgp and GAPDH, as a loading control, was measured by immunoblotting, demonstrating decreased stability of 170-kDa Pgp after Pim-1 inhibition by SGI-1776 compared with control, also shown graphically, demonstrating Pgp half-lives of 9 and 1 h ($p = 0.03$). In addition, the effect of SGI-1776 was inhibited in the presence of the proteasome inhibitor MG-132. Thus, Pim-1 inhibition decreases Pgp stability, and proteasome inhibition overcomes this effect, consistent with Pim-1 protecting Pgp from proteasomal degradation. B, to further test whether Pim-1 protects Pgp from ubquitination, the effects of Pim-1 knockdown on Pgp ubiquitination were measured. Pgp ubiquitination, demonstrated by immunoprecipitation followed by immunoblotting with anti-ubiquitin (αUbi), was increased in cells treated with shRNA, in relation to nontarget shRNA control, for 3 days, and ubiquitination of 150-kDa immature Pgp was seen predominantly, consistent with ubiquitination and degradation of core-glycosylated Pgp as a mechanism for decreased expression of 170-kDa Pgp protein in cells after Pim-1 knockdown.

Pgp and thereby enables its glycosylation and subsequent cell surface translocation.

Pim-1 Inhibition Sensitizes Pgp-Overexpressing Cells to Doxorubicin. To test whether Pgp phosphorylation by Pim-1 plays a role in Pgp-mediated drug resistance, Pgp-overexpressing multidrug-resistant OVCAR-8-Pgp cells were seeded in a six-well plate at 10^5 cells/well with and without 10 μM doxorubicin combined with 1 μM SGI-1776 or DMSO control. Colonies visualized by Coomassie Blue staining were quantified by densitometric scanning. Results of triplicate experiments are shown in Fig. 6A. Although SGI-1776 at 1 μM was not cytotoxic in relation to DMSO control, culture in the presence of SGI-1776 markedly sensitized OVCAR-8-Pgp cells to doxorubicin (p = 0.00008).

To determine the range of concentrations at which SGI-1776 sensitizes Pgp-overexpressing cells to a Pgp substrate chemotherapy drug, we incubated OVCAR-8-Pgp cells as described above with and without 10 μM doxorubicin combined with SGI-1776 at 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, and 10 μM or DMSO control (Fig. 6B). SGI-1776 was not cytotoxic to OVCAR-8-Pgp cells in concentrations up to 1 μM but showed some cytotoxicity at 2.5 μM and marked cytotoxicity at 5 and 10 μM. Sensitization to doxorubicin increased progressively from 0.1 to 0.25 to 0.5 μM and was maximal at 0.5, 0.75, and 0.1 μM in the absence of SGI-1776 cytotoxicity.

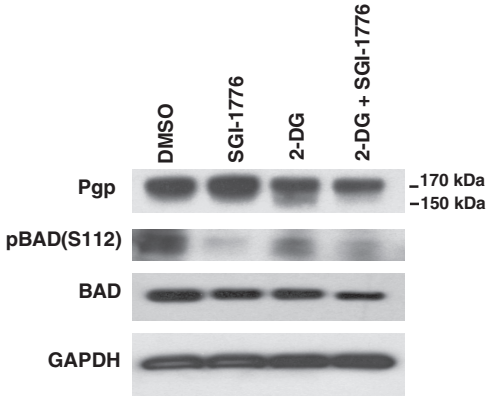

Fig. 5. Pim-1 stabilization of 150-kDa Pgp enables its glycosylation. To test whether Pim-1 stabilization of immature Pgp enables its glycosylation, HL60/VCR cells were treated with the glycosylation inhibitor 2-DG at 50 mM with and without the Pim-1-selective inhibitor SGI-1776 (1 μM) for 24 h, and expression of Pgp was examined by immunoblotting. BAD phosphorylation at Ser112 was also measured to demonstrate Pim-1 kinase inhibition, and BAD and GAPDH expression levels were measured as controls. SGI-1776, which inhibited Pim-1 kinase, as evidenced by decreased BAD phosphorylation at Ser112, was found to decrease the expression of the 150-kDa Pgp that accumulates when glycosylation is inhibited by 2-DG, consistent with the hypothesis that Pim-1 stabilizes 150-kDa Pgp and thereby enables its glycosylation and subsequent cell surface translocation.

Discussion

The serine/threonine protein kinase Pim-1 has been found to be frequently overexpressed in the malignancies in which it has been studied, including acute myeloid and lymphoblastic leukemia and prostate and gastric cancer (Amson et al., 1989; Dhanasekaran et al., 2001; Chen et al., 2005). The multidrug resistance-associated ABC protein Pgp is also fre-

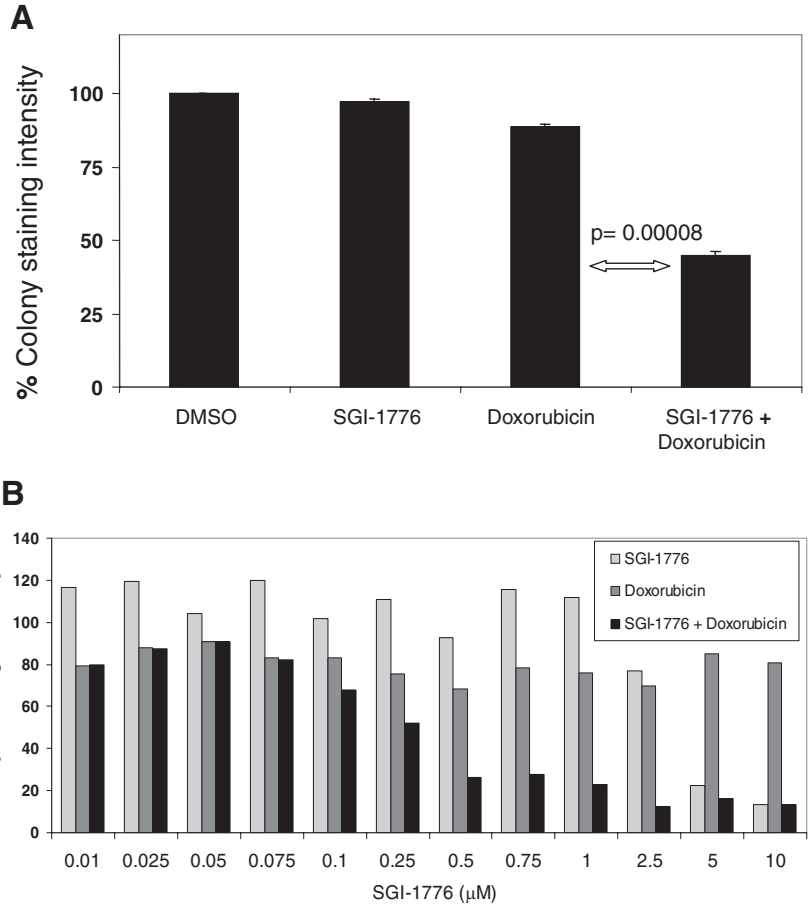

Fig. 6. Pim-1 inhibition sensitizes Pgp-overexpressing cells to doxorubicin. A, to test whether Pgp phosphorylation by Pim-1 plays a role in Pgp-mediated drug resistance, Pgp-overexpressing multidrug-resistant OVCAR-8-Pgp cells were seeded in a six-well plate at 10^5 cells/well with and without 10 μM doxorubicin in the presence of 1 μM SGI-1776 or DMSO control. After 48 h, cells were gently washed three times and then cultured again in fresh medium with or without drug(s). Colonies visualized by Coomassie Blue staining after 120 h were quantified by densitometric scanning. The results of densitometric scanning of triplicate experiments are shown graphically and are normalized to DMSO control signal. Although SGI-1776 at 1 μM was not cytotoxic in relation to DMSO control, culture in the presence of SGI-1776 markedly sensitized OVCAR-8-Pgp cells to doxorubicin (p = 0.00008). B, to determine the range of concentrations at which SGI-1776 sensitizes Pgp-overexpressing cells to a Pgp substrate chemotherapy drug, we incubated OVCAR-8-Pgp cells as above with and without 10 μM doxorubicin combined with SGI-1776 at 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, and 10 μM or DMSO control. SGI-1776 was not cytotoxic to OVCAR-8-Pgp cells in concentrations up to 1 μM. Sensitization to doxorubicin increased progressively from 0.1 to 0.25 to 0.5 μM and was maximal at 0.5, 0.75, and 0.1 μM in the absence of SGI-1776 cytotoxicity. A representative experiment among triplicate experiments is shown.

quently overexpressed in diverse malignancies (Mahadevan and Shirahatti, 2005). Pgp functions as an efflux pump for a number of natural product chemotherapy drugs, including anthracyclines, epipodophylloxins, taxanes, and vinca alkaloids, and Pgp overexpression and drug efflux mediated by Pgp are strongly associated with clinical drug resistance (Mahadevan and Shirahatti, 2005). We have demonstrated here that Pim-1 phosphorylates Pgp, protects it from proteolytic degradation and from ubiquitination and proteasomal degradation, and enables its glycosylation and thus its cell surface expression. The use of Pim-1 knockdown, in addition to Pim-1 kinase inhibition, in our studies served to demonstrate the specific role of Pim-1. Pim-1 is thus a regulator of Pgp-mediated drug resistance, and inhibiting Pim-1 provides a novel approach to abrogating Pgp overexpression and thus drug resistance mediated by Pgp.

Turnover of 170-kDa Pgp reflects both stability of 170-kDa protein and genesis of new 170-kDa protein by glycosylation of 150-kDa protein. We have demonstrated that Pim-1 both protects 170-kDa Pgp from proteolytic and proteasomal degradation and promotes glycosylation of 150-kDa Pgp, probably also by stabilizing it. It is unclear whether Pim-1 protects 170-kDa Pgp from proteolytic and proteasomal degradation by maintaining it in its glycosylated form.

Pim-1 has been shown to regulate the stability of a number of important cellular proteins, including p21 (Zhang et al., 2007), SOCS-1 (Chen et al., 2002), RUNX3 (Kim et al., 2008), and c-myc (Zhang et al., 2008). Our data further support the role of Pim-1 in regulating protein expression by increasing protein stability. We have demonstrated a role for Pim-1 in stabilizing and preventing proteolytic and proteasomal degradation of a substrate core-glycosylated protein, thus enabling its glycosylation. Thus, Pim-1-mediated increased protein half-life may be due to stabilization and thereby enhanced maturation of immature protein and to stabilization of mature protein.

We demonstrated previously that Pim-1 promotes cell surface expression of BCRP, another multidrug resistance-associated ABC protein (Xie et al., 2008). Pim-1 thus promotes cell surface expression of two ABC proteins that confer multidrug resistance, Pgp and BCRP, in cells in which these proteins are expressed. However, Pim-1 seems to have different effects on Pgp and BCRP. BCRP is a half-transporter that requires multimerization for function, and Pim-1 phosphorylates BCRP at Thr362, promoting BCRP multimerization and consequent plasma membrane trafficking and function (Xie et al., 2008). In contrast, Pgp is a full transporter that does not require multimerization, and the effect of Pim-1 on Pgp is to inhibit its proteolytic and proteasomal degradation and enable its glycosylation. Pim-1 does not seem to directly regulate the drug efflux function of either protein.

Pgp phosphorylation by PKC was demonstrated previously not to affect Pgp ubiquitination and proteasomal degradation based on study of NIH3T3 cells transfected with PKC phosphorylation site Pgp mutants, because substituting Pgp PKC phosphorylation sites by nonphosphorylatable residues did not affect Pgp ubiquitination (Gribar et al., 2000). Our demonstration that Pim-1 phosphorylation of Pgp affects Pgp ubiquitination may therefore a priori be unexpected, because studies of Pgp phosphorylation mutants did not implicate phosphorylation in the regulation of ubiquitination. However transfecting Pgp phosphorylation site mutants into cells that do not otherwise overexpress Pgp may yield different results from our approach of studying cells that overexpress Pgp. It is also possible that a selection of the transfectants with drug substrate (vincristine) resulted in the stabilization of the mutant Pgps, as demonstrated in in vitro studies of Clarke's group (Loo and Clarke, 1997, 1998b).

Inhibition of Pgp maturation has been proposed previously as a novel approach to inhibiting Pgp-mediated multidrug resistance (Loo and Clarke, 1999). Most clinical strategies for inhibiting Pgp-mediated multidrug resistance to date have focused on administering competitive inhibitors of Pgp function, or Pgp modulators, in conjunction with chemotherapy (Mahadevan and Shirahatti, 2005). Although Pgp functions as an efflux pump for many clinically used chemotherapy drugs and Pgp overexpression and function in patients' tumor cells are associated with inferior treatment outcome, clinical results of incorporation of Pgp modulators into treatment have generally been disappointing (Mahadevan and Shirahatti, 2005). Valspodar (PSC-833), a structural analog of cyclosporin A that is a potent inhibitor of Pgp-mediated drug resistance in vitro and can be administered at doses yielding concentrations that inhibit drug efflux in vivo, has not improved treatment outcome (Baer et al., 2002; van der Holt et al., 2005). Toxicity due to interactions with chemotherapy drugs has compromised outcome in some trials (Baer et al., 2002), but benefit has also not been seen in other trials in which outcome was not compromised by toxicity (van der Holt et al., 2005). Pgp overexpression and function remain strong adverse prognostic factors in acute myeloid leukemia (van der Holt et al., 2005) and other malignancies (Mahadevan and Shirahatti, 2005), and it is possible that inhibiting Pgp maturation as a novel approach to inhibiting Pgp-mediated multidrug resistance might show clinical benefit as a strategy for inhibiting resistance to chemotherapy. Based on our data presented here, inhibiting Pim-1 may represent a viable strategy for inhibiting Pgp maturation. Inhibiting Pim-1 also has the potential advantage of inhibiting drug resistance mediated by both Pgp and BCRP. It is noteworthy that our data on SGI-1776 resensitization of Pgp-overexpressing cells to a Pgp substrate drug are consistent with recently published data demonstrating SGI-1776 resensitization of 22Rv1 prostate cancer cells to paclitaxel (Mumenthaler et al., 2009). Both that study and ours focused on cancer cells, and it is not yet known whether Pim-1 inhibition also affects Pgp levels in nonmalignant cells, such as $CD56^{+}$ lymphocytes.

Efforts are ongoing to identify inhibitors of Pim-1 kinase (Cheney et al., 2007; Holder et al., 2007; Pogacic et al., 2007), and SGI-1776 (Chen et al., 2009) has entered clinical trials. Our data suggest that clinically applicable Pim-1 inhibitors may have promise as a novel approach to reversing clinical multidrug resistance.

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