Synergistic Interactions between DMAG and Mitogen-Activated Protein Kinase Kinase 1/2 Inhibitors in Bcr/abl+ Leukemia Cells Sensitive and Resistant to Imatinib Mesylate

Tri K. Nguyen,1 Mohamed Rahmani,1 Ning Gao,1 Lora Kramer,1 Amie S. Corbin,4 Brian J. Druker,4 Paul Dent,2 and Steven Grant1,2,3

Abstract

Purpose: To characterize interactions between the heat shock protein 90 antagonist 17-dimethylaminoethylamino-17-demethoxygeldanamycin (DMAG) and the mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase 1/2 inhibitor PD184352 in Bcr/abl+ leukemia cells sensitive and resistant to imatinib mesylate.

Experimental Design: K562 and LAMA 84 cells were exposed to varying concentrations of DMAG and PD184352 for 48 hours; after which, mitochondrial integrity, caspase activation, and apoptosis were monitored. Parallel studies were done in imatinib mesylate–resistant cells, including BaF3 cells transfected with plasmids encoding clinically relevant Bcr/abl mutations conferring imatinib mesylate resistance (e.g., E255K, M351T, and T315I) and primary CD34+ bone marrow cells from patients refractory to imatinib mesylate.

Results: Cotreatment of Bcr/abl+ cells with minimally toxic concentrations of DMAG and PD184352 resulted in synergistic induction of mitochondrial injury (cytochrome c release and Bax conformational change), events associated with the pronounced and sustained inactivation of ERK1/2 accompanied by down-regulation of Bcl-xL. Conversely, cells ectopically expressing Bcl-xL displayed significant protection against PD184352/DMAG–mediated lethality. This regimen effectively induced apoptosis in K562 cells overexpressing Bcr/abl, in BaF3 cells expressing various clinically relevant Bcr/abl mutations, and in primary CD34+ cells from patients resistant to imatinib mesylate, but was relatively sparing of normal CD34+ bone marrow cells.

Conclusions: A regimen combining the heat shock protein 90 antagonist DMAG and the mitogen-activated protein kinase/ERK kinase 1/2 inhibitor potently induces apoptosis in Bcr/abl+ cells, including those resistant to imatinib mesylate through various mechanisms including Bcr/abl kinase mutations, through a process that may involve sustained ERK1/2 inactivation and Bcl-xL down-regulation. This strategy warrants further attention in Bcr/abl+ hematopoietic malignancies, particularly those resistant to Bcr/abl kinase inhibitors.

The Raf/mitogen-activated protein kinase kinase 1/2 (MEK1/2)/extracellular signal–regulated kinase 1/2 (ERK1/2) pathway is frequently dysregulated in neoplastic transformation, particularly in hematologic malignancies such as leukemia (1–4). The MEK1/2/ERK1/2 module comprises, along with stress-activated protein kinase/c-Jun NH2-terminal kinase and p38-mitogen-activated protein kinase, members of the mitogen-activated protein kinase family (5). These kinases are involved in responses to diverse environmental stresses, including DNA damage, osmotic stress, and hypoxia, among others, and have also been implicated in multiple cellular functions, including proliferation, differentiation, and apoptosis (5). Although exceptions exist, activation of MEK1/2/ERK1/2 is associated with cell survival whereas induction of c-Jun NH2-terminal kinase and p38-mitogen-activated protein kinase generally signal apoptosis (5). There is also evidence that the net output between the cytoprotective MEK1/2/ERK1/2 and the stress-related c-Jun NH2-terminal kinase pathways determines whether a cell lives or dies following various results (5). Although the mechanism(s) by which ERK1/2 activation promotes survival is not known with certainty, several antiapoptotic activities have been identified, including inactivation of caspase-9 (6), induction of cyclic AMP-responsive element binding protein (5), induction of Bcl-xL (7), and interference with the function of several proapoptotic proteins such as Bad (8, 9) and Bim (10). In view of the importance of the MEK1/2/ERK1/2 pathway in neoplastic cell survival, the

Cancer Therapy: Preclinical
development of MEK1/2 inhibitors has been the focus of intense interest (11) and one of these, PD184352 (CI-1040), has recently entered the clinical arena (12).

Heat shock protein 90 (Hsp90) is a chaperone protein involved in the proper folding and intracellular disposition of multiple proteins involved in cell signaling and survival (13). Disruption of Hsp90 function in tumor cells (e.g., by ansamycin antibiotics such as geldanamycin) has been shown to induce misfolding of diverse proteins, including Raf, Akt, Bcr/abl, and Erb2, among numerous others, culminating in proteasomal degradation (14, 15). These events can induce apoptosis or, alternatively, increase the susceptibility of tumor cells to established cytotoxic agents (16). Such considerations have led to the development of clinically relevant Hsp90 antagonists, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), which has superior pharmacokinetic characteristics compared with geldanamycin (17). More recently, a 17-AAG derivative, DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin), has been developed, which is more soluble than 17-AAG and is more potent on a molar basis (18). Clinical trials involving DMAG have recently been initiated (19, 20).

The (9:22) translocation, encoding the chimeric constitutive-active Bcr/abl kinase, represents the characteristic lesion of chronic myelogenous leukemia (CML). It is responsible for activation of multiple antiapoptotic signaling pathways that collectively confer a survival advantage to CML progenitor cells (21). Treatment of CML has been revolutionized by the development of Bcr/abl kinase inhibitors, such as imatinib mesylate (22), and recently by more potent second-generation kinase inhibitors, such as AMN107 and BMS 354825 (23, 24). However, an alternative and potentially complementary approach to the eradication of Bcr/abl+ cells by kinase inhibitors involves the use of agents that interrupt survival signaling pathways downstream of the Bcr/abl kinase. Such strategies have employed both MEK1/2 inhibitors and Hsp90 antagonists. For example, MEK1/2 inhibitors have been shown to induce apoptosis in Bcr/abl+ leukemia cells (25) and to potentiate the activity of imatinib mesylate (26). In addition, Hsp90 antagonists such as 17-AAG have been shown to induce CML cell apoptosis in association with Bcr/abl down-regulation (27, 28). In this context, it is noteworthy that mutant proteins such as Bcr/abl may be particularly susceptible to degradation arising from interference with Hsp90 function (29).

In view of the capacity of MEK inhibitors to potentiate the antileukemic activity of various other targeted agents (e.g., protein kinase C and the Chk1 inhibitor UCN-01; ref. 30), as well as the preclinical evidence of activity of Hsp90 antagonists in Bcr/abl+ hematopoietic malignancies (27, 28, 31), the possibility arose that these agents might cooperate to trigger apoptosis in Bcr/abl+ leukemia cells. The goal of the present studies was to determine whether and by what mechanism the clinically relevant MEK1/2 inhibitor PD184352 might enhance the activity of DMAG against Bcr/abl+ leukemia cells. Our results indicate that PD184352 interacts synergistically with DMAG to induce Bax transformation and mitochondrial injury in Bcr/abl+ leukemia cells in association with a pronounced and sustained inactivation of ERK1/2, accompanied by diminished expression of the antiapoptotic protein Bcl-xL. Furthermore, this strategy is effective against cells expressing imatinib mesylate–resistant Bcr/abl mutants refractory to second-generation Bcr/abl kinase inhibitors.

Materials and Methods

Cells. LAMA 84 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Parental K562 cells were kindly provided by Dr. L. Varticovski (Tufts University School of Medicine, Boston, MA). Imatinib mesylate–resistant K562 cells exhibiting increase in Bcr/abl protein expression were employed as previously described in detail (26). Cells were cultured in RPMI 1640 supplemented with sodium pyruvate, non-essential amino acid, i-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (HyClone, Logan, UT). BaF3 cells expressing wild-type and mutant forms of Bcr/abl (e.g., E255K, M351T, and T315I) were obtained from Dr. Brian Druker (Oregon Health Sciences Center, Beaverton, OR) and maintained as previously outlined (32).

Bone marrow was obtained with informed consent from two patients with chronic phase CML, who were undergoing a routine diagnostic bone marrow aspiration and who had become refractory to imatinib mesylate. Mononuclear cells were isolated by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient separation and enriched for CD34+ cells using a Miltenyi microbead separation system (Miltenyi BioTech, Auburn, CA) according to the protocol of the manufacturer. Granulocyte colony-stimulating factor mobilized CD34+ cells from the peripheral blood of a normal subject were purchased from Cambrex Bioproducts (Walkersville, MD). CD34+ cells were diluted into RPMI medium containing 10% FCS at a concentration of 4 × 10^6 cells/ml and exposed to drugs.

Reagents. 17-AAG and 17-DMAG were provided by the Cancer Treatment and Evaluation Program, National Cancer Institute (Bethesda, MD). U0126 was purchased from Alexis Biochemicals (San Diego, CA). The MEK inhibitor PD184352 was from Upstate Biotechnology (Lake Placid, NY). Materials were dissolved in sterile DMSO and stored frozen under light-protected conditions at −20°C. Z-VAD-fmk was purchased from MP Biomedicals (Irvine, CA).

Annexin V/propidium iodide assays for apoptosis. Briefly, 1 × 10^6 cells were washed twice with PBS and then stained with 5 μL of Annexin V-FITC (BD Pharmingen, San Diego, CA) and 10 μL of propidium iodide (50 μg/mL) in 1× binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaOH, 2.5 mmol/L CaCl_2] for 20 minutes at room temperature in dark. The apoptotic cells were determined using a Becton Dickenson FACScan cytoflurometer (Mansfield, MA). Both early apoptotic cells (Annexin V positive, propidium iodide negative) and late apoptotic cells (Annexin V positive and propidium iodide positive) were included in cell death determinations.

Cell growth and viability. Cell growth and viability were assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) compound. Briefly, after exposure of cells to the indicated drugs for various intervals, cells were seeded into 96-well plates (100 μL/well) in the presence of 20 μL of MTS solution (Promega, Madison, WI). Cells were incubated for an additional 2 to 4 hours, after which, absorbance, reflecting reduction of MTS by viable cells, was determined at 490 nm using a microplate reader. Values were expressed as a percentage relative to those obtained in untreated controls.

Western blot analysis. Western blot analysis was done using the NuPAGE Bis-Tris electrophoresis system (Invitrogen, Carlsbad, CA). Total cellular samples were washed twice with cold PBS and lysed by sonication in 1× Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and 50 mmol/L DTT (Fisher BioTech, Pittsburgh, PA). The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Lysed protein was prepared in a desired final concentration in 1× NuPAGE LDS sample buffer (Invitrogen). Equal amounts of protein (20-25 μg) were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane in 20 mmol/L Tris-HCl (pH 8.0) containing 150 mmol/L glycine and 20% (v/v) methanol. The blots were blocked with 5% milk in TBS-Tween 20 (0.05%; TBS-T) at room temperature for 1 hour and probed with the appropriate dilution of

Materials and Methods
primary antibody overnight at 4°C. The blots were washed 3 × 15 minutes in TBS-T and then incubated with a 1:2,000 dilution of horseradish peroxidase–conjugated secondary antibody (Kirkegaard & Perry, Gaithersburg, MD) in 5% milk/TBS-T at room temperature for 1 hour. After washing 3 × 15 minutes in TBS-T, the proteins were visualized by enhanced chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA). The following antibodies were used as primary antibodies: Bcr, phospho-Bcr (Tyr177), Bcl-xL, cleaved caspase-3, caspase-3, Hsp27, p-STAT5 (Tyr694), Lyn, phospho-Lyn (Tyr507) (Cell Signaling Technology); total and phospho-ERK1/2 (Tyr202), phospho-Akt (Ser473), Raf-1, Hsp90, Hsp70, Mcl-1, Bid, p-Bad, STAT5, Ab1 (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal Bax (PharMingen); caspase-8 (Alexis); poly(ADP-ribose) polymerase (Biomol Research Laboratories, Plymouth Meeting, PA); α-tubulin (Calbiochem); and actin (Sigma). The blots were stripped and reprobed with actin or tubulin antibodies to ensure equal loading and transfer of proteins.

**Analysis of cytotoxic cytchrome c.** Cells (4 × 10^6) were lysed by incubating in digitonin lysis buffer (75 mmol/L NaCl, 8 mmol/L Na_2HPO_4, 1 mmol/L NaH_2PO_4, 1 mmol/L EDTA, and 350 μg/mL digitonin) for 3 minutes. The lysates were centrifuged at 12,000 × g for 3 minutes. Then, the supernatant was collected, quantified, and prepared in a final concentration in 1× NuPAGE LDS sample buffer (Invitrogen) and subjected to Western blot as described above. Cytochrome c and apoptosis-inducing factor antibodies (Santa Cruz Biotechnology) were used as primary antibodies.

**Bax conformational change.** Cells were lysed in buffer containing 150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), anti-protectases, and 1% CHAPS or 1% Triton X-100. Subsequently, 500 μg of protein lysates were subjected to immunoprecipitation using anti-Bax monoclonal antibody 6A7 (Sigma-Aldrich), which recognizes only Bax protein that has undergone a conformational change (33). Immunoprecipitates were then subjected to Western blot analysis with anti-Bax polyclonal antibody (BD Biosciences PharMingen).

**Generation of stably transfected cell lines.** Bcl-2 and Bcl-xL constructs were cloned into a pUSEamp and pSFFV vectors containing a neomycin selection marker. Transfection of K562 cells was carried out using an Amaxa Nucleofector apparatus (Cologne, Germany) according to the protocol of the manufacturer. Briefly, 2 million cells were pelleted by centrifugation and resuspended in 100 μL of transfection reagent of kit V; after which, 2.5 μg of DNA were added. Resuspended cells containing DNA were transferred to cuvettes and transfected using the Nucleofector using a cell-specific optimized protocol (T-16). Transfected cells were immediately transferred to regular medium. Cells were seeded and DNA were transferred to cuvettes and transfected using the Nucleofector using a cell-specific optimized protocol (T-16). Transfected cells were immediately transferred to regular medium. Cells were seeded and selected in regular medium in the presence of G418 (700 μg/mL) and G418 plus G418 (700 μg/mL). Cells were treated with PD184352 for 48 hours; after which, the percentage of apoptotic cells was determined by Annexin V staining. C, K562 cells were exposed for 48 hours to the designated concentration of DMAG alone or in combination with 10 μmol/L PD184352 (PD) or 75 nmol/L DMAG alone or in combination, for 48 hours; after which, the percentage of apoptotic cells was determined by Annexin V staining. D, K562 cells were exposed to the designated concentration of PD184352, alone or in combination with 75 nmol/L DMAG, for 48 hours; after which, apoptosis was determined as above. E, K562 cells were treated with PD184352 (10 μmol/L) or DMAG (75 nmol/L) individually or in combination for the indicated intervals; after which, the extent of cell death was evaluated using an MTS assay as described in Materials and Methods. G, median dose effect analysis of apoptosis induction by PD184352 and DMAG. K562 cells were exposed to varying concentrations of PD184352 and DMAG at a fixed ratio (100:1). Combination index (CI) values were determined in relation to the fractional effect using a commercially available software program as described in Materials and Methods. Combination index values <1.0 correspond to a synergistic interaction.
by itself exerted minimal toxicity, significantly increased the lethality of DMAG concentrations as low as 25 nmol/L, with maximal potentiation occurring at DMAG concentrations ≥75 nmol/L (Fig. 1C). Parallel PD184352 dose-response studies revealed potentiation of 75 nmol/L DMAG lethality by PD184352 concentrations as low as 2.5 μmol/L, with modest further increases occurring at higher concentrations (Fig. 1D).

Time-course studies revealed that an increase in lethality was first noted after 24-hour exposure to PD184352 + DMAG, with near-maximal lethality occurring after 48-hour incubation (Fig. 1E). These results were confirmed by MTS assay, which reflects both inhibitions of cell proliferation as well as viability (Fig. 1F). Median dose effect analysis, in which K562 cells were exposed to a range of PD184352 and DMAG administered at a fixed concentration (100:1), yielded combination index values significantly <1.0, indicating a high synergistic interaction (Fig. 1G).

Parallel studies were done in another Bcr/abl+ leukemic cell line (LAMA 84). Combined exposure (48 hours) to DMAG and PD184352 also resulted in combination index values <1.0, corresponding to a synergistic interaction (range 0.3-0.75; data not shown). In addition, coadministration of a marginally toxic concentration of PD184352 (10 μmol/L) with a modestly toxic concentration of 17-AAG (0.4 μmol/L) in K562 cells resulted in a significant increase in lethality (i.e., from 25% to 65%; data not shown), indicating that this phenomenon was not restricted to DMAG. Finally, a modestly toxic concentration of the MEK1/2 inhibitor U0126 (25 μmol/L) also substantially increased DMAG lethality in K562 cells, although results were not quite as striking as in the case of PD184352 (data not shown).

The effects of DMAG and PD184352, alone and in combination, were then examined in relation to mitochondrial injury, activation of the caspase cascade, expression of various Bcl-2 family members, and expression/activation of various signaling proteins, including ERK1/2. Exposure of cells to DMAG or PD184352 alone for 18 hours, an interval before the induction of a substantial degree of apoptosis (as shown in Fig. 1E), or for 30 hours had little effect on release of cytochrome c into the cytosol, whereas combined treatment had a pronounced effect, particularly at the latter interval (Fig. 2A). Comparable results were obtained when Bax conformational change, a known antecedent of mitochondrial injury (34, 35), was monitored (Fig. 2B). Notably, evidence of activation of the caspase cascade [e.g., caspase-9 and poly(ADP-ribose) polymerase cleavage] was slightly discernible in cells exposed to the combination of DMAG and 17-AAG at 18 hours, and, along with caspase-8 and caspase-3 cleavage, considerably more marked at 30 hours (Fig. 2B).

Expression of Mcl-1 was minimally affected by PD184352 alone for 18 hours, an interval before the induction of apoptosis was minimal (Fig. 2C). On the other hand, combined treatment resulted in a marked decline in expression of these proteins at 30 hours, when apoptosis was more marked. In contrast to these results, expression of Bcl-xL was strikingly reduced in PD184352/DMAG–treated cells at 18 hours and virtually absent at 30 hours (Fig. 2C). Basal expression of Bcl-2 was minimal in these cells and not altered by any treatment (data not shown). A comparable reduction in Bcl-xL expression following an 18-hour exposure to the PD184352/DMAG regimen was also noted in LAMA 84 cells (data not shown). Together, these findings suggest that down-regulation of Bcl-xL represents a relatively early event in PD184352/DMAG–mediated lethality and is particularly pronounced in the case of combined drug exposure.

Interactions between these agents were then examined with respect to various signaling proteins. Individual treatment with 10 μmol/L PD184352 or 75 nmol/L DMAG for 18 or 30 hours had little effect on total levels of Hsp90 in K562 cells, whereas with combined treatment, a cleavage product was noted, particularly at the later interval (Fig. 3A). A slight but discernable increase in Hsp70 levels was observed in cells exposed to DMAG alone or DMAG + PD184352 at both intervals, a phenomenon that has previously been observed in cells exposed to Hsp90 antagonists (17). Notably, expression of both Bcr/abl and phospho-Bcr/abl was diminished by DMAG alone at 18 and 30 hours whereas coadministration of PD184352 resulted in only a minimal further decline. Lastly, total Stat5 levels did not change with any treatment whereas a decrease in phospho-Stat5 expression was observed at 30 hours with DMAG alone or
with combined treatment (Fig. 3A). Similarly, expressions of both total and phospho-Akt were diminished by DMAG at both intervals and minimal further changes were observed when PD184352 was added. Comparable results were noted with expression of Raf-1 and the phosphorylated form of the Src kinase Lyn (Fig. 3B).

However, a significantly different response pattern was observed when expression of phospho-ERK was monitored. For example, whereas both DMAG and PD184352 alone modestly reduced expression of phospho-ERK1/2 in K562 cells, combined treatment resulted in the virtual abrogation of phospho-ERK1/2 (Fig. 3B). A more detailed time-course study was then carried out. Treatment of K562 cells with PD184352 alone resulted in a marked decline in phospho-ERK1/2 expression by 2 hours, followed by a partial but clearly discernible recovery over the ensuing 18 hours (Fig. 3C). Total ERK1/2 levels were minimally affected by any treatment. On the other hand, DMAG reduced phospho-ERK1/2 expression following an 18- to 30-hour incubation interval, although a marked decline was noted at 48 hours. In striking contrast to these findings, combined treatment with DMAG and PD184352 resulted in a pronounced and sustained decrease in expression of phospho-ERK1/2 over the entire 48-hour treatment interval. Very similar results were noted in LAMA 84 cells (data not shown). Taken together, these findings suggest that DMAG and PD184352 cooperate to inactivate ERK1/2 and prevent its recovery in K562 cells.

To determine whether any of these perturbations were secondary to caspase-mediated degradation, K562 cells were incubated with 10 μmol/L PD184352 + 75 nmol/L DMAG in the presence or absence of the broad-spectrum caspase inhibitor Z-VAD-fmk 20 μmol/L. Addition of Z-VAD failed to protect cells from PD184352/DMAG–mediated cytochrome c release and Bax conformational change (Fig. 4A), suggesting that these events do not simply reflect activation of the caspase cascade. Similarly, Z-VAD-fmk did not attenuate PD184352/DMAG–mediated down-regulation of Bcl-xL, phospho-ERK1/2, and Raf-1 expression, nor did it reverse the modest decline in Bcr/abl levels (Fig. 4B). Essentially equivalent results were obtained in apoptosis-resistant K562 cells ectopically expressing Bcl-2 (data not shown). These findings suggest that PD184352/DMAG–induced changes in signaling proteins in all likelihood do not represent a consequence of cell death.

To assess the functional significance of early and pronounced Bcl-xL down-regulation in PD184352/DMAG–mediated lethality, parallel studies were done with K562 cells ectopically expressing Bcl-xL. To this end, two clones (cl-11 and cl-12) overexpressing Bcl-xL (Fig. 5A, inset) were exposed to PD184352 and DMAG, and cell death was compared with empty-vector controls. Although protection was not complete, cells ectopically expressing Bcl-xL were significantly more resistant to PD184352/DMAG–mediated lethality compared with controls (P < 0.01 in each case; Fig. 5A). Ectopic expression of Bcl-xL also substantially protected cells from PD184352/DMAG–mediated cytochrome c release and Bax conformational change (Fig. 5B). Finally, increased Bcl-xL expression substantially attenuated PD184352/DMAG–induced caspase-3 and caspase-9 activation and poly(ADP-ribose) polymerase degradation but had little effect on Bcr/abl and phospho-ERK1/2 down-regulation (Fig. 5C). Collectively, these findings are consistent with the notion that decreased Bcl-xL expression contributes functionally to PD184352/DMAG–mediated lethality and suggest that the observed perturbations in signaling proteins occur upstream of Bcl-xL down-regulation.

Finally, the effects of the PD184352/DMAG regimen were examined in Bcr/abl+ cells resistant to imatinib mesylate through disparate mechanisms. As shown in Fig. 6A, combined treatment with PD184352 and DMAG resulted in a marked increase in apoptosis in imatinib mesylate–resistant K562 cells displaying increased expression of Bcr/abl (K562/R; ref. 26). Furthermore, the extent of apoptosis with combined treatment was equivalent to that observed in wild-type cells (Fig. 1B). Parallel studies were then done in BaF3 cells transfected with Bcr/abl proteins exhibiting several clinically relevant mutations conferring a high degree of resistance to imatinib mesylate (i.e., M351T, E255K, and T315I; ref. 22). Notably, DMAG was more potent on a molar basis than 17-AAG in wild-type BaF3 cells (Fig. 6B), analogous to findings in K562 cells. Furthermore, the dose-response curves of each of the mutant cell lines to DMAG were virtually superimposable with that of wild-type cells (Fig. 6C), indicating that cells expressing mutant proteins are not cross-resistant to DMAG.
Consistent with these results, the PD184352/DMAG regimen induced similar degrees of apoptosis in wild-type and each of the mutant Bcr/abl-expressing cell lines (Fig. 6D). Median dose effect analysis revealed that cotreatment of imatinib mesylate–resistant T315I mutant cells with PD184352/DMAG yielded combination index values considerably <1, indicating a synergistic interaction comparable to that observed in K562 cells (Fig. 6D, inset). Similar interactions were noted in other mutant cell lines (data not shown). Together, these findings indicate that a regimen combining DMAG with a MEK1/2 inhibitor effectively induces apoptosis in Bcr/abl + cells exhibiting imatinib mesylate resistance through several disparate mechanisms, including clinically relevant mutations in the Bcr/abl kinase.

Lastly, attempts were made to determine whether the combination of DMAG and PD184352 resulted in enhanced toxicity toward primary CML cells. To this end, CD34+ bone marrow cells were obtained from two patients with chronic phase CML, who had become refractory to imatinib mesylate. As shown in Fig. 6E, exposure of these cells to PD184352 (5 μmol/L) or DMAG (100 nmol/L) individually for 48 hours resulted in relatively little toxicity whereas combined exposure was associated with a marked increase in cell death in both cases. In contrast to these findings, the PD184352/DMAG regimen induced relatively little apoptosis in normal bone marrow CD34+ cells (Fig. 6E).

Discussion

Hsp90 antagonists, of which the ansamycin analogue geldanamycin and its less toxic derivative, 17-AAG, represent the prototypes, have become a focus of considerable interest as antineoplastic agents, and clinical trials involving 17-AAG have been initiated (20). These agents act by disrupting the chaperone function of Hsp90, leading to degradation of diverse proteins implicated in the neoplastic cell survival, including Raf, Akt, hypoxia-inducible factor 1α, and Erb2, among numerous others (13, 14). In fact, mutant proteins, including Bcr/abl, seem to be particularly susceptible to agents that disrupt Hsp90 function (17), and 17-AAG has been shown to act in CML cells, at least in part, by diminishing expression of the Bcr/abl protein (27, 28). Whereas the basis for selectivity of such agents is not known with certainty, there is evidence that Hsp90 derived from tumor cells has a markedly increased affinity for agents, such as 17-AAG, compared with protein obtained from normal cells (36).
Synergistic Interaction of Hsp90 and Mek Inhibitors

One barrier to the development of 17-AAG has been its limited water solubility (18). Recently, an analogue of 17-AAG, DMAG, has been described, which is considerably more water-soluble than 17-AAG (37). In addition, it seems to be more potent on a molar basis than 17-AAG in both solid tumors (e.g., MDA-MB-231) and leukemic (e.g., HL-60) preclinical whole-body xenograft models (37). This report extends these findings by showing that DMAG is considerably more potent than 17-AAG against Bcr/abl+ human leukemia cells, including those expressing several mutations conferring a high degree of resistance to the Bcr/abl kinase inhibitor imatinib mesylate. The observations that (a) Hsp90 antagonists selectively target neoplastic cells; (b) mutant proteins may be particularly sensitive to Hsp90 antagonists; and (c) DMAG potently induces apoptosis in cells expressing wild-type and mutant forms of Bcr/abl suggest that this agent may play a useful therapeutic role in Bcr/abl+ hematologic malignancies.

The Raf/MEK1/2/ERK1/2 pathway is known to exert cytoprotective actions in diverse neoplastic cell types, particularly those of hematopoietic origin (4). This has prompted the development of several pharmacologic inhibitors, including the multikinase and Raf inhibitor Bay 43-9006 (38) and the MEK1/2 inhibitor PD184352 (12). Recently, PD184352, or CI-1040, has undergone clinical evaluation in phase I trials involving patients with advanced malignancies (39). Significantly, inhibition of ERK1/2 phosphorylation in tumor tissues and peripheral blood mononuclear cells was observed at higher CI-1040 doses (39), indicating that achieving desired pharmacodynamic effects in vivo is feasible. In addition, a more potent MEK1/2 inhibitor with superior pharmacokinetic characteristics (PD0325901) is currently undergoing clinical evaluation (40). In this context, it is notable that several laboratories, including our own, have shown that MEK1/2 inhibitors such as PD184352 potentiate the lethality of various other targeted agents, including the small-molecule Bcl-2 antagonist HA14-1 (41), the Chk1 inhibitor UCN-01 (30), and, in Bcr/abl+ leukemia cells, Bcr/abl kinase inhibitors such as imatinib mesylate (26) as well as histone deacetylase inhibitors (42). Because Hsp90 antagonists can themselves down-regulate the Raf/MEK1/2/ERK1/2 pathway (43), it is not intuitively obvious why a MEK1/2 inhibitor should lower the apoptotic threshold for an agent such as DMAG. However, it is noteworthy that when administered to K562 cells at low, marginally toxic concentrations, DMAG had only a modest, and delayed effect on ERK1/2 inactivation. Furthermore, administration of low concentrations of PD184352 in these cells resulted in an initial abrogation of ERK activation, followed by a gradual recovery. On the other hand, coadministration of PD184352 with DMAG resulted in the profound and sustained inactivation of ERK1/2 throughout the entire exposure interval. In view of evidence that the duration of MEK/ERK signaling plays a critical role in the biological consequences of activation of this pathway (1, 5), it is tempting to speculate that sustained inactivation of ERK1/2 signaling partially contributes to the lethality of the PD184352/DMAG regimen in these cells.

The findings that (a) combined treatment with PD184352 and DMAG markedly down-regulated Bcl-xL expression and (b) ectopic expression of Bcl-xL significantly protected Bcr/abl+ cells from lethality argue that down-regulation of Bcl-xL plays a

---

**Fig. 6.** Effect of PD184352 ± DMAG on various imatinib-resistant Bcr/abl+ leukemia cells. A, K562R imatinib mesylate-resistant cells displaying increased expression of Bcr/abl (26) were treated with 75 nmol/L DMAG ± 10 μmol/L PD184352 for 48 hours; after which, apoptosis was determined by Annexin V staining as described in Materials and Methods. B, BaF3/wild-type cells were exposed to increasing concentrations of 17-AAG (▲) or DMAG (■) for 30 hours; after which, the extent of apoptosis was determined as above. C, BaF3/wild-type (WT), BaF3/E255K, BaF3/T315I, and BaF3/M315T were exposed to increasing concentrations of DMAG, after which, the percentage of apoptotic cells was determined by Annexin V staining after 30 hours of drug exposure. D, wild-type and mutant BaF3 cells were exposed to 4 μmol/L PD184352 and 150 nmol/L DMAG, alone or in combination, for 30 hours; after which, the percentage of apoptotic cells was determined as above. E, CD34+ cells isolated from the bone marrow of two patients with chronic-phase CML, who had become refractory to imatinib mesylate, were isolated and exposed to the indicated concentrations of PD184352 and DMAG, alone and in combination, for 48 hours. At the end of this period, the percentage of apoptotic cells was determined by Annexin V propidium iodide staining. The extent of apoptosis in normal bone marrow CD34+ cells exposed to the same concentrations of PD184352 and DMAG is shown for comparison. A to D, columns, mean of triplicate determinations done on three separate occasions; bars, SD.
significant functional role in synergistic interactions between these agents. Bcl-xL is a multidomain Bcl-2 family member that acts like Bcl-2 to block perturbations in proapoptotic BH3-only proteins such as Bax (e.g., conformational change), resulting in inhibition of release of proapoptotic mitochondrial proteins (e.g., cytochrome c) into the cytoplasm (35). In this context, increased expression of Bcl-xL has specifically been implicated in the antiapoptotic actions of constitutively activated Bcr/abl through a Bcl-2-independent process (44). Furthermore, this phenomenon has been shown to act, at least in some cases, through a Stat5-related mechanism (45). Collectively, these observations suggest that Bcl-xL up-regulation plays a particularly important role in the prosurvival functions of Bcr/abl. However, it is important to note that whereas DMAG by itself diminished expression of total and phosphorylated Bcr/abl, consistent with previous results involving 17-AAG (27, 28), diminished expression of total and phosphorylated Bcr/abl, or Stat5. On the other hand, there is evidence that ERK1/2 plays a critical role in the regulation of Bcl-xL expression in malignant hematopoietic cells and that interference with this pathway can lead to Bcl-xL down-regulation (46). Consequently, the possibility that the sustained inactivation of ERK1/2 observed in PD184352/DMAG–treated cells partially contributed to the pronounced down-regulation of Bcl-xL and, as a consequence, enhanced mitochondrial injury and apoptosis, seems plausible. This notion is supported by the finding that ectopic expression of Bcl-xL significantly diminished cell lethality but not ERK1/2 inactivation induced by the PD184352/DMAG regimen in Bcr/abl leukemia cells.

In addition to down-regulation/inactivation of Bcr/abl and Stat5, cells exposed to DMAG and PD184352 exhibited several additional perturbations in survival signaling pathways, including down-regulation/inactivation of Raf, Akt, and Lyn, and reduced expression of Mcl-1, which is known to play an important role in the survival of malignant hematopoietic cells (47). However, in the case of Raf, Akt, and Lyn down-regulation, effects were similar in cells exposed to DMAG alone versus the combination of DMAG and PD184352, arguing against a major contribution to synergistic interactions. In the case of Mcl-1, down-regulation seemed to be a relatively late event, in contrast to the early reduction in Bcl-xL expression, suggesting that the former represents a secondary phenomenon. Nevertheless, the possibility that down-regulation/inactivation of these proteins may amplify the apoptotic process cannot be completely excluded.

It is significant that Bcr/abl+ hematopoietic cells expressing various mutations conferring high degrees of imatinib mesylate resistance (22) remained sensitive to DMAG and were particularly vulnerable to the PD184352/DMAG regimen. The former finding is consistent with evidence that cells bearing mutant oncogenic tyrosine kinases are highly dependent on Hsp90 chaperone function for survival (29). The observation that the PD184352/DMAG regimen synergistically induced cell death in cells expressing the E255K, M351T, and T315I Bcr/abl mutations, which interfere with imatinib mesylate activity through various mechanisms, suggests that a strategy bypassing or acting downstream of Bcr/abl may be effective in overcoming drug resistance. For example, the E255K mutation destabilizes the P-loop, the M351T mutation induces a conformation change in the activation loop, and the T315I mutation results in a steric clash between imatinib mesylate and a side chain (22, 48). In each case, the net effect is interference with the ability of imatinib mesylate to stabilize Bcr/abl in its inactive conformation. In this context, recent studies with newer generation Bcr/abl kinase inhibitors, such as AMN107 and BMS354825, indicate that these agents are active in imatinib mesylate–resistant cells expressing certain Bcr/abl mutations (23, 24). However, mutations such as T315I seem to confer resistance to these agents as well as to imatinib mesylate (23). The present findings, which indicate that the PD184352/DMAG regimen effectively induces apoptosis in Bcr/abl+ cells bearing the T315I mutation, as well as in some primary Bcr/abl leukemia cells, raise the possibility that this approach may complement or provide an alternative to agents such as BMS-354825 or AMN107. Accordingly, preclinical studies designed to test this hypothesis are currently under development.

References
42. Yu C, Dasmahapatra G, Dent P, Grant S. Synergistic interactions between MEK(1,2) and histone deacetylase inhibitors in BCR/ABL+ human leukemia cells. Leukemia 2005;19:1579–89.
Synergistic Interactions between DMAG and Mitogen-Activated Protein Kinase Kinase 1/2 Inhibitors in Bcr/abl⁺ Leukemia Cells Sensitive and Resistant to Imatinib Mesylate

Tri K. Nguyen, Mohamed Rahmani, Ning Gao, et al.