CDK Inhibitors Upregulate BH3-Only Proteins to Sensitize Human Myeloma Cells to BH3 Mimetic Therapies

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Abstract

BH3 mimic drugs induce cell death by antagonizing the activity of antiapoptotic Bcl-2 family proteins. Cyclin-dependent kinase (CDK) inhibitors that function as transcriptional repressors downregulate the Bcl-2 family member Mcl-1 and increase the activity of selective BH3 mimetics that fail to target this protein. In this study, we determined whether CDK inhibitors potentiate the activity of pan-BH3 mimetics directly neutralizing Mcl-1. Specifically, we evaluated interactions between the prototypical pan-CDK inhibitor flavopiridol and the pan-BH3 mimetic obatoclax in multiple myeloma (MM) cells in which Mcl-1 is critical for survival. Coadministration of flavopiridol and obatoclax synergistically triggered apoptosis in both drug-naïve and drug-resistant MM cells. Mechanistic investigations revealed that flavopiridol inhibited Mcl-1 transcription but increased transcription of Bim and its binding to Bcl-2/Bcl-xL. Obatoclax prevented Mcl-1 recovery and caused release of Bim from Bcl-2/Bcl-xL and Mcl-1, accompanied by activation of Bax/Bak. Whether administered singly or in combination with obatoclax, flavopiridol also induced upregulation of multiple BH3-only proteins, including BimEL, BimL, Noxa, and Bik/NBK. Notably, short hairpin RNA knockdown of Bim or Noxa abrogated lethality triggered by the flavopiridol/obatoclax combination in vitro and in vivo. Together, our findings show that CDK inhibition potentiates pan-BH3 mimetic activity through a cooperative mechanism involving upregulation of BH3-only proteins with coordinate downregulation of their antiapoptotic counterparts. These findings have immediate implications for the clinical trial design of BH3 mimetic-based therapies that are presently being studied intensively for the treatment of diverse hematopoietic malignancies, including lethal multiple myeloma. Cancer Res. 72(16): 4225–37. ©2012 AACR.

Introduction

Multiple myeloma (MM) is an incurable accumulative disease of plasma cells characterized by dysregulation of Bcl family members (1). These apoptosis regulatory proteins are divided into pro- and antiapoptotic groups. The former consists of multidomain proteins (e.g., Bak and Bax) and BH3-only proteins (e.g., Bim, Bid, Puma, Noxa, Bad, Bik, Bmf, and Hrk, etc.). The latter includes multidomain proteins, for example, Bcl-2, Bcl-xL, and Mcl-1 (2). Although Bak and Bax are absolutely required for apoptosis, BH3-only proteins, which convert noxious stimuli into death signals, consist of "activators" (e.g., Bim) and "sensitizers/derepressors" (e.g., Noxa, Bik; ref. 2). Evidence implicating BH3-only proteins in anticancer agent-induced apoptosis (3, 4) prompted the development of BH3 mimetics that bind to and inactivate antiapoptotic Bcl proteins (5).

One such agent, ABT-737, binds avidly to Bcl-2/Bcl-xL, but not Mcl-1 (6). Consequently, relative levels of Bcl-2/Bcl-xL versus Mcl-1 determine susceptibility to this agent (7). Mcl-1 is highly expressed in MM (e.g., in 51% patients at diagnosis and 81% at relapse), and high Mcl-1 expression correlates with poor clinical outcome (8). Mcl-1 also plays an important role in resistance to agents such as bortezomib (9). Recently, the novel pan-BH3 mimetic obatoclax has been developed, which in addition to other antiapoptotic proteins, antagonizes the activity of Mcl-1 in various tumors types (10, 11), including hematologic malignancies such as MM. Preclinical in vitro studies in MM showed single-agent activity and additivity with other agents, but limited in vivo bioactivity when administered alone (12).

Cyclin-dependent kinases (CDK) regulate cell-cycle progression and transcription (13). Pan-Cdk inhibitors such as...
flavopiridol (alvocidib) act in part by inhibiting Cdk9, a kinase involved in RNA polymerase II (Pol II)-mediated transcription elongation (13). Consequently, Cdk inhibitors block gene transcription and downregulate short-lived proteins including Mcl-1, promoting apoptosis (14, 15). Clinical trials have suggested activity for flavopiridol singly or in combination with other agents in patients with hematopoietic malignancies, including MM (16–18). Recently, several new-generation pan-Cdk inhibitors (e.g., CYC202, SCH727965), which also target Cdk9, have entered clinical trials (13).

Although pan-Cdk inhibitors have been shown to potentiate ABT-737 lethality in transformed cells by downregulating Mcl-1 (7), it is unknown whether synergistic interactions would occur with pan-BH3 mimetics such as obatoclax, which bind to/inactivate Mcl-1 (10). To address this question, we examined interactions between the pan-Cdk inhibitor flavopiridol and obatoclax in human MM cells. Here we report that flavopiridol synergistically increases obatoclax lethality in diverse MM cells, including those resistant to novel agents, in the presence of stromal cell factors, and in primary CD138<sup>+</sup> MM samples, but not in their normal counterparts. Significantly, obatoclax/flavopiridol coadministration, in sharp contrast to obatoclax alone, displays marked in vivo activity and increases survival in multiple murine systems. From a mechanistic standpoint, the unexpected upregulation of multiple BH3-only proteins, including BimEL, BimL, Noxa, and Bik/NBk, cooperates with downregulation of antiapoptotic proteins (e.g., Mcl-1, Bcl-xl) to play a significant functional role in lethality. Collectively, these findings provide proof-of-principle for a novel anti-MM strategy in which pan-Cdk inhibitors are combined with pan-BH3 mimetics and highlight the critical importance of interplay between pro- and antiapoptotic proteins in synergistic interactions between such agents.

Materials and Methods

Cells and reagents

Human MM U266 and RPMI826 cells were obtained from American Type Culture Collection (ATCC) and maintained as before (19). Both were authenticated (Basic STR Profiling Service, ATCC 135-X) by ATCC immediately after this study was completed. Bortezomib-resistant cells (PS-R) were generated by continuously culturing U266 cells in increasing concentrations of bortezomib (beginning at 0.5 nmol/L) and increasing in stepwise increments of 0.2 nmol/L until 20 nmol/L, and maintained in medium containing 15 nmol/L bortezomib. A revlimid-resistant RPMI826 (R10R) cell line was similarly established and maintained in 10 μmol/L revlimid (20). Dexamethasone-sensitive (MM.1S) and -resistant (MM.1R) cell lines were provided by Dr Steven T. Rosen (Northwestern University, Chicago, IL). U266/Mcl-1 and RPMI826/Bcl-xl cells were established by stably transfecting full-length human Mcl-1 and Bcl-xl cDNA, respectively (19). All experiments used logarithmically growing cells (3–5 × 10<sup>5</sup> cells/mL). MycoAlert (Lonza) assays were carried out, showing that all cell lines were free of Mycoplasma contamination.

Bone marrow samples were obtained with informed consent according to the Declaration of Helsinki and Virginia Commonwealth University Institutional Review Board approval from 4 patients with MM undergoing routine diagnostic aspirations. CD138<sup>+</sup> cells were separated using a MACS magnetic separation technique (Miltenyi Biotech). Normal CD34<sup>+</sup> hematopoietic progenitor cells were isolated from 2 cord blood (CB) samples; purity and viability were >90%, by flow cytometry and trypan blue exclusion, respectively.

Obatoclax, flavopiridol, and SCH 727965 were generously provided by GeminX Pharmaceuticals, Sanofi-Aventis, and Merck, respectively, and by the National Cancer Institute, NCI. Cycloheximide (CHX) and MG-132 were purchased from Sigma and Calbiochem respectively, dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20°C. In all experiments, final DMSO concentrations did not exceed 0.1%. Recombinant human interleukin (IL)-6, IGF-1, BAFF, and APRIL were obtained from PeproTech.

Procedures for in vitro studies

For procedures related to flow cytometry, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, quantitative real-time PCR (qRT-PCR), immunoblot, communoprecipitation, subcellular fractionation, Bak and Bax conformational change, and RNA interference see Supplementary Materials and Methods (7).

Animal studies

Animal studies were approved by the Virginia Commonwealth University The Institutional Animal Care and Use Committee, and carried out in accordance with the U.S. Department of Agriculture and Department of Health and Human Services, and the NIH. Three mouse models were used in this study.

Model #1—intravenous (i.v.) flank murine model. Athymic NCr-nu/nu mice (NCI) were s.c. inoculated in the right rear flank with 5 × 10<sup>6</sup> RPMI826 cells stably transfected with a construct encoding luciferase. Treatment was administered after luciferase activity was detected.

Model #2—intraperitoneal (i.p.) orthotopic murine model. NOD/SCID–γ mice (Jackson Laboratories) were s.c. inoculated in 2 opposite flanks with 1 × 10<sup>7</sup> U266 cells stably transfected with constructs encoding short hairpin RNA (shRNA) targeting either Bim (shBim, left flank) or scrambled sequence negative control (shNC, right flank).

Model #3—intravenous (i.v.) orthotopic murine model. NOD/SCID–γ mice (Jackson Laboratories) were i.v. injected with 5 × 10<sup>6</sup> U266 cells stably transfected with constructs encoding luciferase.

Obatoclax mesylate (GX15-070MS) was freshly reconstituted with 5% dextrose for injection (USP) and administered via intramuscular (i.m.) or intraperitoneal (i.p.) injection. Flavopiridol in DMSO was diluted in 0.9% saline and administered via i.p. injection. Control animals were injected with equal volumes of vehicle. Mice were monitored for tumor growth every other day visually or with the use of an IVIS 200 imaging system (Xenogen Corporation). Measurement of animal body weight was carried out every other day throughout the study to monitor toxicity. Tumor volumes were calculated using the formula (L × W<sup>2</sup>)/2, with L and W representing length and width respectively, and when tumor size reached 2,000 mm<sup>3</sup>, mice were euthanized in accordance with institutional guidelines.

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Statistical analysis

Values represent the means ± SD for at least 3 independent experiments carried out in triplicate. The significance of differences between experimental variables was determined using the Student t test or 1-way ANOVA with Tukey–Kramer multiple comparisons test. The significance of P values was <0.05 (**), <0.01 (***), or <0.001 (****) wherever indicated. Analysis of synergy was carried out according to median dose effect analysis using the software CalcuSyn (Biosoft). Kaplan–Meier analysis of mouse survival or hind-leg paralysis was carried out using IBM SPSS Statistics software.

Results

Cdk inhibitors synergistically potentiate BH3 mimic lethality by engaging the mitochondria-related apoptotic cascade

Coadministration (24 hours) of minimally toxic concentrations of obatoclax and flavopiridol sharply reduced mitochondrial membrane potential and increased 7-AAD uptake in U266 cells (Fig. 1A), accompanied by a pronounced increase in both early (annexin V+/PI−) and late (annexin V+/PI+) apoptosis (Fig. 1B). Interactions occurred at obatoclax concentrations as low as 300 nmol/L (data not shown). Median dose effect analysis revealed CI values less than 1.0, indicating synergism (Fig. 1C). Although individual treatment had only modest effects, combined exposure markedly increased AIF and cytochrome c release (Fig. 1D, left), and caspase-3, -8, -9, and PARP cleavage (right). Apoptosis was confirmed by TUNEL staining (data not shown). Concordant results were observed in other human MM lines (Fig. 5C). Similar interactions were observed when other pan-Cdk inhibitors (e.g., SCH727965) and pan-Bcl-2 antagonists (e.g., HA14-1) were used (Supplementary Fig. S1A).

Cdk inhibitor/BH3 mimic interactions are associated with Mcl-1 and Bcl-xL downregulation

Immunoblot analysis revealed Mcl-1 downregulation 6 hours after flavopiridol exposure, with recovery at 16 hours
(Fig. 2A) despite the fact that inhibition of Pol II CTD phosphorylation (serine-2) persisted (Supplementary Fig. S1B), a phenomenon observed in an earlier study (14). Obatoclax alone clearly decreased Mcl-1 levels in a dose- and time-dependent manner (21). Notably, flavopiridol/obatoclax coadministration prevented recovery from Mcl-1 downregulation at later intervals (e.g., 16–48 hours; Fig. 2A). Treatment with flavopiridol with or without obatoclax markedly diminished serine-2 phosphorylation of Pol II CTD at 6 hours (Fig. 2B, left) and 16 hours (Supplementary Fig. S1B), indicating Cdk9/cyclin T (P-TEFb) inhibition. Moreover, in flavopiridol/obatoclax-treated cells, coadministration of the translation inhibitor CHX further reduced Mcl-1 levels (Fig. 2B, middle), whereas the proteasome inhibitor MG-132 failed to restore Mcl-1 expression (Fig. 2B, right), arguing against translational or posttranslational mechanisms of Mcl-1 downregulation.

Figure 2. Flavopiridol (FP)/obatoclax downregulates Mcl-1. U266 cells were treated as follows: A, 300 nmol/L to 1.5 μmol/L obatoclax (Obat) with or without 100 nmol/L flavopiridol for 6, 16, 24, and 48 hours. B, 100 nmol/L flavopiridol with or without 500 nmol/L obatoclax for 6 hours (left); 100 nmol/L FP + 500 nmol/L obatoclax, in the presence or absence of 1 μmol/L CHX, for 16 hours (middle); 100 nmol/L FP + 500 nmol/L obatoclax, with or without the addition of 300 nmol/L MG-132, for 16 hours (right). After drug treatment, immunoblot analysis was carried out to monitor Mcl-1 protein levels and PARP cleavage, or total and phosphorylated (serine-2, CTD) RNA Pol II. C, U266 cells were incubated with 500 nmol/L obatoclax with or without 75 to 100 nmol/L flavopiridol for 16 hours, after which Mcl-1 mRNA levels were assessed by qRT-PCR using GAPDH as control (see Supplementary Materials and Methods). D, U266 cells were stably transfected with human full-length Mcl-1 or empty vector (EV). Cells were exposed (24 or 48 hours) to 100 nmol/L flavopiridol with or without 500 nmol/L obatoclax, or 4 nmol/L bortezomib (btzmb) for comparison, and then subjected to flow cytometry (left) or immunoblot analysis (right). UT, untreated.
cells overexpressing Mcl-1 were substantially resistant to bortezomib at both 24 and 48 hours (Fig. 2D), consistent with previous reports (9). Bcl-xL overexpression partially but significantly protected cells from flavopiridol/obatoclax lethality at both 24 and 48 hours (Supplementary Fig. S2E). Together, these findings suggest that Mcl-1 and Bcl-xL downregulation plays a significant but limited functional role in flavopiridol/obatoclax lethality.

Upregulation of BH3-only proteins in MM cells exposed to Cdk inhibitor/BH3 mimetic

Because Mcl-1 or Bcl-xL overexpression only partially protected cells from flavopiridol/obatoclax, effects of the regimen were then examined in relation to expression of proapoptotic BH3-only proteins using a BH3-only detection kit. Unexpectedly, 24-hour exposure of MM cells to flavopiridol with or without obatoclax resulted in marked upregulation of several BH3-only proteins, including Bim (EL and L isoforms; Fig. 3A), Bik/NBK (Supplementary Fig. S3A), and Noxa (Supplementary Fig. S3B).

Consistent with results in other tumor cell types (21), obatoclax played a major role in Noxa upregulation. Time course analysis of U266 cells revealed that obatoclax alone sharply increased Noxa levels as early as 6 hours after exposure, but this effect was no longer apparent after 16 hours (data not shown). Notably, obatoclax-induced Noxa upregulation was sustained for longer intervals (e.g., at least 24 hours) in the presence of flavopiridol (Supplementary Fig. S3B, top). Similar events occurred in RPMI8226 cells (Supplementary Fig. S3B, bottom). Importantly, Noxa shRNA dramatically blocked apoptosis induced by either bortezomib (23) or flavopiridol/obatoclax (Supplementary Fig. S3C), arguing that upregulation of the BH3-only protein Noxa plays a significant functional role in flavopiridol/obatoclax lethality.
Upregulation of Bim at the transcriptional level plays a significant functional role in Cdk inhibitor/BIM mimetic interactions

The functional significance of upregulation of the "direct activator" Bim (24) was then examined. flavopiridol induced Bim expression (EL and L isofoms), with or without obatoclax, in U266 (Fig. 3A, left) and RPMI8226 cells (right). Immunoblot analysis confirmed increased expression of both Bim isofoms after flavopiridol treatment alone (Fig. 3B, left) or in combination with obatoclax (right), events occurring at 6 hours and sustained for at least 24 hours after treatment. In contrast, obatoclax alone did not upregulate Bim (data not shown). flavopiridol-induced Bim induction was largely blocked by CHX (Fig. 3C), suggesting a requirement for de novo protein synthesis. Moreover, qRT-PCR showed significant increases in Bim mRNA levels at 3, 6, and 16 hours after flavopiridol treatment with or without obatoclax in both U266 and RPMI8226 cells (Fig. 3D and Supplementary Fig. S3D), arguing that Bim upregulation by flavopiridol occurs at the transcriptional level.

Coimmunoprecipitation was carried out to examine interactions between Bim and antiapoptotic Bcl proteins. Although bortezomib increased BimEL rather than BimL bound to Bcl-2 and particularly to Bcl-xL, flavopiridol alone clearly increased Bim (both EL and L) binding to Bcl-2 and Bcl-xL (Fig. 4A). The latter events were markedly attenuated by obatoclax. Interestingly, flavopiridol or obatoclax alone modestly diminished BimEL bound to Mcl-1, an effect only slightly enhanced with the combination (Fig. 4A). In flavopiridol-treated cells, unleashing of Bim from both Bcl-2 and Bcl-xL by obatoclax was associated with conformational activation of Bax and to a lesser extent Bak (Fig. 4B, left), as well as Bax mitochondrial translocation (right), triggering mitochondrial outer membrane permeabilization, caspase activation, and pronounced apoptosis (Fig. 4D). Moreover, transient transfection of a construct encoding Bax shRNA significantly diminished flavopiridol/obatoclax lethality (Supplementary Fig. S3E).

To define the functional role of Bim upregulation, U266 cells were stably transfected with a construct encoding shRNA targeting Bim (shBim). In these cells, both BimEL and BimL were substantially knocked down, compared with shNC (Fig. 4C, left). Notably, shBim essentially abrogated flavopiridol/obatoclax-mediated lethality (Fig. 4C, right), analogous to its ability to protect cells from bortezomib (25). Consistent with these findings, shBim prevented Bax activation, and pronounced apoptosis (Fig. 1D). Moreover, transient transfection of a construct encoding Bax shRNA significantly diminished flavopiridol/obatoclax lethality (Supplementary Fig. S3E).
conformational change and translocation (Supplementary Fig. S3F), caspase activation, and PARP cleavage (Fig. 4D) induced by flavopiridol/obatoclax. However, coexposure to flavopiridol/obatoclax induced nearly equivalent Mcl-1 downregulation in both shNC and shBim cells (Fig. 4D). Analogous results were obtained when another Bim shRNA was used (Supplementary Fig. S3C). Together, these findings raise the possibility that unleashing of upregulated Bim from antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, and Mcl-1) by obatoclax contributes to synergistic interactions. They also argue that in the setting of downregulation of antiapoptotic proteins, upregulation of BH3-only proteins such as Bim play a critical functional role in lethality.

The Cdk inhibitor/BH3 mimetic regimen is active against MM cells displaying conventional or novel forms of drug resistance, as well as primary MM cells.

In addition to drug resistance because of Bcl family dysregulation, microenvironmental factors also confer resistance in MM (26). To address these issues, U266 cells were cultured in the presence of HS-5 cells (a human bone marrow stromal cell line; ref. 27), HS-5-conditional medium (CM), or both. Although CM slightly reduced Bim levels, coculture with HS-5 markedly downregulated Bim (Fig. 5A). Notably, HS-5 plus CM essentially abolished Bim expression, raising the possibility that Bim downregulation represents a mechanism underlying stromal cell–mediated drug resistance (28). Importantly, neither HS-5 with or without CM prevented flavopiridol/obatoclax lethality (P > 0.05, Fig. 5B, left). Furthermore, addition of IL-6, BAFF, APRIL, or IGF-1 also failed to attenuate flavopiridol/obatoclax lethality (P > 0.05, Fig. 5B, right), suggesting that the flavopiridol/obatoclax regimen overcomes drug resistance related to microenvironmental factors (e.g., stromal cells, cytokines, and growth factors).

Dexamethasone-resistant (MM.1R) and revlimid-resistant (R10R) cells exhibited roughly equivalent sensitivity to flavopiridol/obatoclax, compared with their drug-naïve counterparts (MM.1S and RPMI8226) respectively (Fig. 5C). Interestingly, bortezomib-resistant U266 cells (PS-R), which were resistant to 20 nmol/L bortezomib (top), suggested a clear increase in Mcl-1 levels, accompanied by a dramatic increase in Mcl-1 expression (24), particularly the EL isoform (top). Significantly, PS-R cells exhibited no cross-resistance to flavopiridol/obatoclax, compared with parental cells (Fig. 5D, bottom), suggesting that MM cells exhibiting either conventional or novel forms of drug resistance remain fully susceptible to this regimen.

Although sensitivity to individual agents varied between primary CD138+ MM specimens isolated from different patients, cotreatment with flavopiridol/obatoclax sharply increased cell death (Fig. 5E). However, toxicity toward CD138+ bone marrow cells was minimal in all samples. Interestingly, despite differences between samples, CD138+ cells displayed relatively higher levels of Bim compared with their CD138− counterparts (data not shown; ref. 29). Furthermore, flavopiridol or obatoclax alone displayed only modest toxicity toward normal CB CD34+ cells, whereas combined treatment did not increase lethality (Fig. 5E), suggesting that this regimen is active against and relatively selective toward primary MM cells.

The Cdk inhibitor/BH3 mimetic regimen displays marked in vivo antitumor activity through a Bim-dependent mechanism.

Obatoclax reportedly lacked in vivo single-agent bioactivity in mice bearing s.c. KMS12PE human MM tumors (12). To determine whether the flavopiridol/obatoclax regimen exhibits in vivo activity, athymic nude mice were inoculated in the flank with RPMI8226 cells carrying a luciferase gene. When tumors became visible, mice were treated with flavopiridol (5 mg/kg, i.p.) with or without obatoclax (3 mg/kg, i.m.). Consistent with previous reports (12), obatoclax (3 mg/kg) alone had no effect on tumor growth, manifested by luciferase activity (Fig. 6A, left). However, whereas flavopiridol (5 mg/kg) alone exerted modest but discernible effects, combined treatment substantially suppressed tumor growth. The size of tumors excised from mice confirmed pronounced tumor growth suppression with combined treatment (Fig. 6A, right). Moreover, tumor size measurements yielded concordant results (P = 0.0002; Fig. 6B). Interestingly, immunoblot analysis of tumor tissues revealed that flavopiridol/obatoclax coadministration downregulated Mcl-1 and upregulated Bim (particularly the L isoform) and Noxa, accompanied by caspase-3 activation and PARP cleavage (Fig. 6C), consistent with in vitro observations. To determine whether BH3-only protein upregulation (e.g., Bim) plays a significant functional role in flavopiridol/obatoclax lethality in vivo, NOD/SCID-γ mice were inoculated s.c. with U266 cells stably transfected with shBim or shNC respectively (Fig. 4C) in each flank, after which flavopiridol (3 mg/kg, i.p.) with or without obatoclax (3 mg/kg, i.p.) was administered. Flavopiridol/obatoclax coadministration markedly suppressed growth of shNC tumors (Fig. 6D, right flank), analogous to results observed in the previously described flank model (Fig. 6A–C). Notably, whereas a slight reduction in tumor size was observed in the obatoclax group, no obvious growth suppression was observed by flavopiridol alone or with obatoclax in shBim tumors (Fig. 6D, left flank), showing an important functional role for Bim in flavopiridol/obatoclax lethality in vivo.

The bone marrow microenvironment plays a critical role in survival, growth, and drug resistance of MM cells (26). The activity of the flavopiridol/obatoclax regimen was therefore assessed in an animal model in which human MM cells form bone marrow lesions, leading to bone disease at late intervals. In this orthotopic murine model, NOD/SCID-γ mice were i.v. injected with U266 cells stably transfected with a luciferase gene, after which homing and growth of tumor cells were dynamically monitored by imaging luciferase activity. Notably, U266 cells homed to bone marrow and then formed lesions at skeletal sites (Fig. 7A), without detectable lesions in other organs, findings confirmed by H&E staining for human CD138+ cells (data not shown). At later intervals (9–13 weeks after cell injection), inoculated mice displayed hind-leg paralysis (Fig. 7B, right), a classic indicator of bone disease. After luciferase signals were visible, flavopiridol (5 mg/kg, i.p.) with or without obatoclax (3 mg/kg, i.p.) was administered daily for 5 days, published online first June 12, 2012; DOI: 10.1158/0008-5472.CAN-12-1118.
followed by flavopiridol (3 mg/kg) with or without obatoclax (3 mg/kg) twice every 3 days. Tumor growth was monitored every 2 days. As shown in Fig. 7A, combined treatment substantially reduced tumor burden compared with agents administered individually. Kaplan–Meier hazard analysis showed that although obatoclax alone clearly delayed the appearance of hind-leg paralysis, flavopiridol alone had only minimal effects (Fig. 7B). Notably, hind-leg paralysis was not seen in any mice in the flavopiridol/obatoclax group throughout the entire 4-month observation period ($P$ = 0.0268; Fig. 7B, Supplementary Video). Moreover, Kaplan–Meier survival analysis revealed more prolonged survival of mice receiving combined treatment compared with mice treated with flavopiridol or obatoclax alone ($P$ = 0.0161; Fig. 7C). Finally, significant
neurologic toxicity (e.g., onset of agitation and hyperactivity), which has been noted immediately after a rapid i.v. injection of obatoclax (4 mg/kg; ref. 12), was not observed in mice after either i.m. or i.p. obatoclax (3 mg/kg) alone or in combination with flavopiridol, similar to results with i.v. injection of 2 mg/kg obatoclax in mice bearing solid tumors (11). Moreover, there was no significant loss of body weight (Fig. 7D) or other signs of toxicity observed after flavopiridol and obatoclax administered alone or in combination in these murine systems. Together, these findings argue that an anti-MM regimen combining a pan-Cdk inhibitor with a pan-BH3 mimetic is active in vivo, and suggest that mechanisms identified in vitro (e.g., upregulation of BH3-only proteins such as Bim, Mcl-1 downregulation) may be operative in vivo.
Discussion

Tumor cells are commonly characterized by overexpression of antiapoptotic Bcl proteins (e.g., Bcl-2, Bcl-xL, or Mcl-1), which contribute to drug resistance by disabling the death machinery (30, 31). Transformed cells also frequently express proapoptotic Bcl proteins including multidomain (e.g., Bax and Bak) and some, if not all, BH3-only proteins such as Bim (3, 4). Although tumor cells may be fully equipped with anti- and proapoptotic machinery, the latter are often inactivated through binding to their highly expressed antiapoptotic counterparts (2). Because of the balance between anti- and prodeath Bcl proteins, tumor cells may be "primed" for cell death (32) by disrupting this balance, for example, by upregulating or mimicking proapoptotic protein actions, or by downregulating/antagonizing the function of antiapoptotic proteins (33, 34). The present findings show that pan-Cdk inhibitors markedly potentiates the lethality of pan-BH3 mimetics (e.g., obatoclax) in vitro and in vivo in MM cells, including those displaying conventional or novel forms of drug resistance. They also reveal that these agents interact synergistically by interfering with both arms of the apoptosis-regulatory balance, that is, downregulating/disabling Bcl-2, Bcl-xL, and Mcl-1, and upregulating BH3-only proteins including Bim, Noxa, and Bik/NBK.

Pan-Cdk inhibitors like flavopiridol act as transcriptional repressors by inhibiting Cdk9, a catalytic subunit of the cyclin T/Cdk9 complex (P-TEFb), preventing serine-2 phosphorylation of RNA Pol II CTD, thereby blocking transcription elongation (13) and inducing downregulation of short-lived proteins such as Mcl-1 (14), an essential survival factor for MM cells (35). The present results show that flavopiridol downregulates Mcl-1 at early intervals (e.g., 6 hours) in association with diminished CTD serine-2 phosphorylation. However, Mcl-1 expression recovered after 16 hours, indicating that flavopiridol rapidly but only transiently inhibits Mcl-1 transcription in cells (14). Recently, obatoclax was shown to downregulate Mcl-1 through induction of Noxa, which binds to Mcl-1 and triggers its degradation, whereas qRT-PCR revealed more than a 5-fold increase in Mcl-1 mRNA levels after obatoclax exposure (6 hrs; ref. 21). Similar events were observed in this study, but mRNA
upregulation was largely blocked by flavopiridol. Although the mechanism underlying these phenomena is uncertain, increases in Mcl-1 mRNA may represent a compensatory response to downregulation of the protein. Importantly, obatoclax strikingly blocked recovery from Mcl-1 protein downregulation after prolonged flavopiridol exposure (e.g., 16–48 hours), suggesting that persistent Mcl-1 downregulation following flavopiridol/obatoclax coexposure may reflect 2 separate but cooperative mechanisms: (i) Mcl-1 mRNA transcription inhibition by flavopiridol; and (ii) promotion of Mcl-1 protein degradation through Noxa induction by obatoclax. However, Mcl-1 overexpression only partially attenuated flavopiridol/obatoclax-mediated apoptosis at 24 hours, but not at intervals ≥48 hours, raising the possibility that the flavopiridol/obatoclax regimen may circumvent Mcl-1-related drug resistance.

MM cells exposed to flavopiridol with or without obatoclax exhibited sharp increases in expression of BH3-only proteins including Bim (EL and L isoforms), Noxa, and Bik/NBK. Unlike Bax/Bak, protein levels of which are relatively stable (2), and which must be activated to trigger apoptosis, expression of BH3-only proteins is tightly regulated, and thus represents a candidate target (3, 36). Many novel agents induce expression (e.g., HDAC inhibitors; ref. 19) or prevent degradation (e.g., proteasome inhibitors; ref. 37) of BH3-only proteins such as Noxa, Puma, and Bik/Bak, thereby directly or indirectly activating Bax/Bak (2). Obatoclax antagonizes Mcl-1 antiapoptotic functions by unleashing Bim or Bak from Mcl-1, and also downregulates Mcl-1 via induction of Noxa (21). This finding shows that obatoclax induced Noxa upregulation at early intervals (e.g., 6 hours), and that this event was enhanced and rendered more sustained by flavopiridol. Functionally, both Noxa and Bim shRNA profoundly diminished flavopiridol/obatoclax lethality. Thus, although downregulation of antiapoptotic proteins (e.g., Mcl-1 and Bcl-xL) may contribute to the lethality of this regimen to a limited extent, upregulation of BH3-only proteins (e.g., Bim and Noxa) clearly plays a critical role in CdK inhibitor/pan-BH3 mimetic interactions.

The marked increase in Bim (both EL and L isoforms) by flavopiridol with or without obatoclax has, to the best of our knowledge, not been described previously. Transcriptional regulation of Bim represents a major mechanism of apoptosis regulation (38). Several findings argue that flavopiridol upregulates Bim at the transcriptional level. First, transcription inhibition by CHX largely blocked Bim upregulation induced by flavopiridol. Second, qRT-PCR indicated that flavopiridol induced a marked increase (e.g., 3- to 5-fold) in Bim mRNA levels. In this context, transcriptional regulation of Bim is complex and multifactorial. For example, growth factor withdrawal-induced Bim upregulation requires JNK activation in neurons, although it depends on theforkhead transcription factor FKHR-L1 in hematopoietic cells (3). Moreover, the bim promoter is regulated by stress-related transcriptional factors such as FOXO and AP-1 family members (38). The mechanism by which a pan-Cdk inhibitor transcriptionally that upregulates BH3-only proteins such as Bim is not intuitively obvious. One possibility is that flavopiridol may activate JNK that upregulates BH3-only proteins to sensitize human myeloma cells to Bcl-2 antagonists (e.g., HA14-1; ref. 39). Another possibility is that Cdk2 inhibition by pan-Cdk inhibitors may activate FOXO1 (40), which induces Bim transcription (41). In addition to transcriptional induction, Bim also exhibits multifactorial regulation at the levels of mRNA stability, post-translational modifications, proteasomal degradation, and cellular localization (38, 42). Consequently, the possibility that additional mechanisms contribute to Cdk inhibitor-mediated Bim upregulation cannot be excluded.

The observation that Bim shRNA knockdown essentially abrogated apoptosis indicates a critical functional role for Bim upregulation in CdK inhibitor/pan-BH3 mimetic lethality. Mechanisms of Bim-mediated cell death may reflect direct actions following displacement/derespression of antiapoptotic proteins by BH3-only "sensitzers," leading to Bax/Bak activation. Alternatively, in the neutralization model, Bim binds to and neutralizes/inactivates all antiapoptotic Bcl proteins that repress constitutively active Bax/Bak. Obatoclax releases Bim from Bcl-2, Bcl-xL, and Mcl-1 binding, thereby promoting apoptosis (10, 12). Interestingly, flavopiridol upregulated expression of Bim but increased its binding to Bcl-2 and Bcl-xL, suggesting that upregulated Bim may "prime" cells for death (43, 44). Indeed, obatoclax unleashed Bim from Bcl-2, Bcl-xL, and Mcl-1, leading to Bax/Bak activation and cell death in Cdk inhibitor-treated cells. Significantly, the latter event (i.e., Bax/Bak activation) did not occur in cells in which Bim was knocked down by shRNA. Notably, in a dual-sided flank murine model that circumvents problems in interpretation related to mouse-to-mouse variability (45), flavopiridol/obatoclax failed to suppress the growth of tumors carrying Bim shRNA, showing a functional in vivo role for Bim in lethality. Finally, the observation that obatoclax released Bim from multiple antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, and Mcl-1) suggests that as observed in the case of BH3 mimetics (e.g., ABT-737) administered alone (43), interactions between Bcl family members, rather than simply their expression profiles, may be critical predictors of sensitivity to the Cdk inhibitor/pan-BH3 mimetic regimen in MM.

MM cells interact with the bone marrow microenvironment to promote cell proliferation, survival, migration, and drug resistance. Genetic profiling studies revealed that Bcl family members represent microenvironment-specific chemotherapeutic response determinants in malignant hematopoietic cells, and that BH3-only “activators” are necessary for cell death (45). IL-6 and IGF-1, which are critical for MM cell/microenvironment interactions, promote MM cell survival and confer drug resistance through (i) Mcl-1 induction (46, 47) and (ii) Bim downregulation (34, 48). The latter involves at least 2 mechanisms, for example, posttranslational phosphorylations and/or transcription (48). Notably, flavopiridol/obatoclax was fully active in the presence of these cytokines or other autocrine MM survival/growth factors (e.g., BAFF and APRIL; ref. 49). Direct MM cell–stroma contact also contributes to MM drug-resistance (28). Interestingly, HS-5 coculture and conditioned medium largely abrogated Bim expression in MM cells, implicating this phenomenon as a mechanism of stromal cell–mediated drug resistance. Significantly, flavopiridol/obatoclax lethality...
although pan-BH3 mimetics such as obatoclax may not exceed the apoptotic threshold. In summary, the present pan-Cdk inhibitors may trigger a constellation of events that collectively, these findings argue that those resistance mechanisms fail to protect MM cells from Cdk inhibitor/pan-BH3 mimetic regimens, which coordinate target both pro- and antiapoptotic Bcl family members.

The marked lethality of flavopiridol/obatoclax in primary CD138⁺ MM specimens, with minimal toxicity toward their normal CD138⁻ counterparts or CD34⁺ hematopoietic cells, raises the possibility that MM cells may be particularly susceptible to this strategy. Significantly, in marked contrast to obatoclax administered alone, combined treatment produced marked suppression of tumor growth in vivo in both s.c. flank and i.v. orthotopic models. Particularly in the latter, in which MM cells selectively homed to the bone marrow and produced MM-specific bone disease, coadministration of flavopiridol/obatoclax markedly improved outcomes for these animals, for example, significantly preventing hind-leg paralysis and prolonging survival. Such findings argue that although pan-BH3 mimetics such as obatoclax may not be sufficient by themselves to induce cell death in vitro or in vivo, additional perturbations (e.g., prolongation of antiapoptotic protein downregulation in conjunction with upregulation of proapoptotic BH3-only proteins) induced by pan-Cdk inhibitors may trigger a constellation of events that exceed the apoptotic threshold. In summary, the present findings provide a preclinical justification for combining pan-Cdk inhibitors, possibly including new-generation Cdk inhibitors that have recently entered the clinic (13), with pan-BH3 mimetics (e.g., obatoclax; ref. 50) in MM and other hematologic malignancies. They also highlight the critical importance of coordinate disruption of pro- and antiapoptotic arms of the apoptotic regulatory machinery in promoting transformed cell death.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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