Up-Regulation of c-Jun Inhibits Proliferation and Induces Apoptosis via Caspase-Triggered c-Abl Cleavage in Human Multiple Myeloma

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Abstract

Here we show the antimyeloma cytotoxicity of adaphostin and carried out expression profiling of adaphostin-treated multiple myeloma (MM) cells to identify its molecular targets. Surprisingly, c-Jun was the most up-regulated gene even at the earliest point of analysis (2 h). We also observed adaphostin-induced c-Abl cleavage in immunoblot analysis. Proteasome inhibitor bortezomib, but not melphalan or dexamethasone, induced similar effects, indicating unique agent-dependent mechanisms. Using caspase inhibitors, as well as caspase-resistant mutants of c-Abl (TM-c-Abl and D565A-Abl), we then showed that c-Abl cleavage in MM cells requires caspase activity. Importantly, both overexpression of the c-Abl fragment or c-Jun and knockdown of c-Abl and c-Jun expression by small interfering RNA confirmed that adaphostin-induced c-Jun up-regulation triggers downstream caspase-mediated c-Abl cleavage, inhibition of MM cell growth, and induction of apoptosis. Finally, our data suggest that this mechanism may not only be restricted to MM but may also be important in a broad range of malignancies including erythroleukemia and solid tumors. [Cancer Res 2007;67(4):1680–8]

Introduction

Multiple myeloma (MM), the second most common blood cancer in adults, is characterized by bone marrow plasmacytosis, monoclonal protein in blood and/or urine, bone lesions, renal compromise, and immunodeficiency. Despite recent advances in our understanding of disease pathogenesis and treatment, median overall survival of MM remains at only 3 to 5 years. The identification and validation of targets for novel therapeutic regimens is therefore urgently needed.

The tyrophostin adaphostin (NSC 680410), first identified as an alternative to the 2-phenylaminopyrimidine derivative imatinib mesylate (1), achieved responses in patients with chronic myelocytic leukemia (CML), including Bcr-Abl–positive, Bcr-Abl–negative, and Bcr-Abl T315I mutant tumor cells resistant to both imatinib mesylate and second-generation BMS354825 and AMN107. It also shows cytotoxicity against chronic lymphocytic leukemia (CLL) and acute myelocytic leukemia (AML) cells (2–8). In contrast to imatinib mesylate, adaphostin induces significantly greater apoptosis at IC_{50} of 5 to 10 μmol/L, associated with significant inhibition of Bcr-Abl phosphorylation (5). Several mechanisms have been proposed as a basis for its robust antitumor activity including generation and release of reactive oxygen species, cytochrome *c*, and apoptosis-inhibiting factor; caspase cleavage; c-jun NH_2-terminal kinase (JNK) activation; as well as inactivation of Raf-1, signal transducers and activators of transcription (Stat)-3, and Stat5 (6, 7, 9). As these adaphostin-targeted pathways are relevant to MM pathogenesis (10), we here sought to determine the potential molecular sequelae and therapeutic promise of adaphostin in MM. Our present study shows that adaphostin treatment of MM cells causes marked and acute up-regulation of c-Jun. c-Jun is a central component of the activating protein-1 family of transcription factors, which consist of homodimers or heterodimers with either Fos (v-Fos, c-Fos, FosB, FosL1, and FosL2) or activating transcription factor. In addition, c-Jun also interacts with Stat3 and p53, molecules that play an important role in MM pathogenesis. Although mechanisms regulating the transcriptional activity of c-Jun have been investigated in great detail, its exact role in mediating cell differentiation, growth, survival, and apoptosis is not fully understood (11, 12). Importantly, proapoptotic functions of c-Jun have been reported in neurons, fibroblasts, and endothelial cells, suggesting that cell death is induced both indirectly through its transcriptional regulation of survival/death genes (e.g., Fas ligand) and directly via activation of the caspase cascade, resulting in the cleavage of numerous molecules [e.g., fodrin, poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, and protein kinase C; refs. 13–16]. To date, however, the role of c-Jun in mediating growth inhibition or apoptosis in MM cells is unknown.

c-Abl (140 kDa) was originally identified as the cellular homologue of the transforming gene of Abelson murine leukemia virus and encodes a ubiquitously expressed nonreceptor tyrosine kinase similar to the Src family of tyrosine kinases. c-Abl has been linked to diverse cellular processes including DNA replication, recombination, and repair, as well as cell cycle and cell survival control. As for c-Jun, the regulation of the diverse activities of c-Abl is controlled at the levels of c-Abl phosphorylation and its interactions with a variety of proteins, including retinoblastoma 1, ataxia telangiectasia mutated, p73, breast cancer 1, RNA polymerase II, Rad51, protein kinase C, DNA-dependent protein kinase, and p53 (17–19). Although the oncogenic potential of the Bcr-Abl hybrid protein (210 kDa) has been extensively studied in CML, the

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pathophysiologic relevance of aberrant c-Abl activity in other diseases is less well studied (19). Specifically, c-Abl is expressed in many MM cell lines including MM.1S, MM144, and interleukin 6 (IL-6)–dependent XG-6 MM (20, 21); moreover, c-Abl overexpression together with c-Myc and Bcl-X_L can promote MM in mice (22). On the other hand, c-Abl may play only a minor role in MM pathogenesis because tyrosine phosphorylation of c-Abl in MM cells is low (21) and imatinib mesylate was inactive in a phase II clinical trial in patients with refractory/relapsed MM (23). Whether adaphostin mediates its anti-MM activity via c-Abl is not yet studied.

In this study, we show that adaphostin exhibits robust anti-MM cytotoxicity against MM cells in both cell culture and bone marrow microenvironment models. These biological activities are associated with a new molecular mechanism in which c-Jun up-regulation triggers caspase-mediated c-Abl cleavage, and the resultant cleaved 60-kDa c-Abl product both inhibits MM growth and induces cell death. As for adaphostin, proteasome inhibitor bortezomib triggers these molecular sequelae, which also occur in leukemic cells. These studies not only delineate a novel mechanism of tumor cell cytotoxicity triggered by adaphostin and bortezomib but also provide the preclinical framework to initiate design of clinical trials.

Materials and Methods

Materials. Adaphostin (NSC 680410), the adamatyl ester analogue of tyrophostin AG957, was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). Anti–c-Abl monoclonal antibody was obtained from Dr. Ravi Salgia (University of Chicago Hospitals, Chicago, IL) and anti–phospho-tyrosine (4G10) monoclonal antibody from Dr. T. Roberts (Dana-Farber Cancer Institute, Boston, MA). Antibodies directed against actin, caspase-8, caspase-9, phospho-c-Jun, phospho-JNK, and PARP were obtained from Cell Signaling Technology (Beverly, MA); c-Jun, extracellular signal–regulated kinase-2 (ERK2), and caspase inhibitors z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), z-IETD-fmk, and z-LEDH-fmk were obtained from Calbiochem (San Diego, CA).

Cells and cell culture. All human MM [MM.1S, MM.1R, RPMI, RPMI-Dox40 (Dox40), OPM2, U266] cell lines, primary patient MM cells, CML line K562, and erythroleukemia cell line HEL were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Harlan, Indianapolis, IN), 100 units/mL penicillin, 10 g/mL streptomycin, and 2 mmol/L L-glutamine (Cellgro, Herndon, VA). INA-6 cells were cultured in 20% heat-inactivated FBS (Harlan), 100 units/mL penicillin, 10 g/mL streptomycin, and 2 mmol/L L-glutamine (Cellgro) with 2 ng/mL recombinant human IL-6. Before stimulation with IL-6 and insulin-like growth factor-I, MM cells were incubated overnight in RPMI 1640 with 2% FBS, followed by an additional 3 h in RPMI 1640 without FBS.

Isolation of patient's tumor cells. After appropriate informed consent, MM patient cells (96% $CD38^+CD45RA^-$) were obtained from patient bone marrow samples as previously described. The purity of MM cells was 95% (24).

DNA synthesis and cell proliferation assay. Cell growth was assessed by measuring [^{3}H]thymidine uptake, as in prior studies (24).

Microarray assay. Total RNA was isolated from adaphostin-pretreated or untreated MM.1S cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Affymetrix U133A 2.0 arrays were hybridized with biotinylated *in vitro* transcription products (10 g/chip), per manufacturer's instructions, within the DFCI Microarray Core Facility. The DNA chips were then analyzed with a Gene Array Scanner (Affymetrix). CEL files were obtained using Affymetrix Microarray Suite 5.0 software. The DNA Chip Analyzer (DChip; ref. 25) was used to normalize all CEL files to a baseline array with overall median intensity, and the model-based expression (perfect match minus mismatch) was used to compute the expression values. Probes showing at least 1.5-fold difference between control and adaphostin-treated cells at 2, 6, and 12 h were included in the analysis. The same data were also analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems). Cell viability, as assessed by trypan blue exclusion, was 85% in all adaphostin-treated cells during the times indicated.

Cell lysis, immunoprecipitation, and Western blotting. Cell lysis, immunoprecipitation, and Western blot analysis were done as previously described (24).

DNA fragmentation assay. Cell Death Detection ELISAplus (Roche Applied Sciences, Indianapolis, IN) was used to quantitate DNA fragmentation per manufacturer's instructions.

Plasmids and transfections. Wild-type pSGT-Abl-wt, pSGT-Abl-D565A, and pcDNA3-Abl-TM (pcDNA3-Abl-D565A-D644A-D958A) were constructed as previously described (26, 27). All mutations were confirmed by sequencing. For c-Jun– and c-Abl–specific knockdown experiments, MM.1S cells were transiently transfected with indicated amounts of small interfering RNA (siRNA) SMARTpool for c-Jun, c-Abl, or nonspecific control duplexes (pool of four; Upstate Cell Signaling Solutions/Dharmacon RNA Technologies, Lafayette, CO) using the Cell Line Nucleofector Kit V Solution (Amaxa Biosystems, Cologne, Germany).

Annexin/propidium iodide stain. MM.1S cells were treated with either vehicle or adaphostin for 24 and 48 h, washed with PBS, and evaluated for dual Annexin V and propidium iodide staining by fluorescence-activated cell sorting analysis.

Isobologram analysis. The interaction between pazopanib and IMiDs (Lenalidomide and Actimid), bortezomib, or melphalan was analyzed using the CalcuSyn software program (Biosoft, Ferguson, MO), which is based on the Chou-Talalay method. When combination index (CI) = 1, this equation represents the conservation isobologram and indicates additive effects. CI < 1 indicates synergism; CI > 1 indicates antagonism.

Results

Adaphostin inhibits growth of MM cells. The therapeutic potential of adaphostin has been shown in AML, CML, and CLL (3, 4, 6, 7). To ascertain whether the antineoplastic activities of adaphostin extend to MM, we first evaluated its effect on proliferation in a panel of MM cell lines. Adaphostin treatment induced dose-dependent growth inhibition in dexamethasone-sensitive MM.1S, dexamethasone-resistant MM.1R and RPMI 8226, and doxorubicin-sensitive RPMI (Dox40), OPM1, and OPM2 MM cell lines (Fig. 1*A*). In contrast, treatment with adaphostin induced only minor changes in the growth of peripheral blood mononuclear cells (5).

Bone marrow stromal cells (BMSC) enhance growth, survival, and drug resistance of MM cells mediated via direct MM cell-stromal cell contact as well as via growth factors, cytokines, and chemokines (e.g., vascular endothelial growth factor and IL-6). Our results show that adaphostin treatment also inhibits proliferation of MM cells adherent to BMSCs in a dose-dependent manner (Fig. 1*B*).

Adaphostin induces c-Jun up-regulation and c-Abl cleavage. Adaphostin is significantly less effective than imatinib mesylate at inhibiting Bcr-Abl activity *in vitro* (5). As noted above, multiple biochemical activities seem to underlie the antineoplastic activities of adaphostin. In an attempt to delineate adaphostin-responsive pathways in MM, serial expression profiles were obtained in MM.1S cells after treatment for 2, 6, and 12 h. Among genes that showed $\geq$2-fold changes in expression level after 6 and 12 h of treatment, 192 genes were up-regulated and only 27 were down-regulated (Supplementary Table S1). Analysis with the Ingenuity software (28) showed seven pathways with a score of >1 (range, 13–23; see Materials and Methods; Fig. 1*C*, and data not shown); importantly, four of these cascades converged on the transcription factor c-Jun,

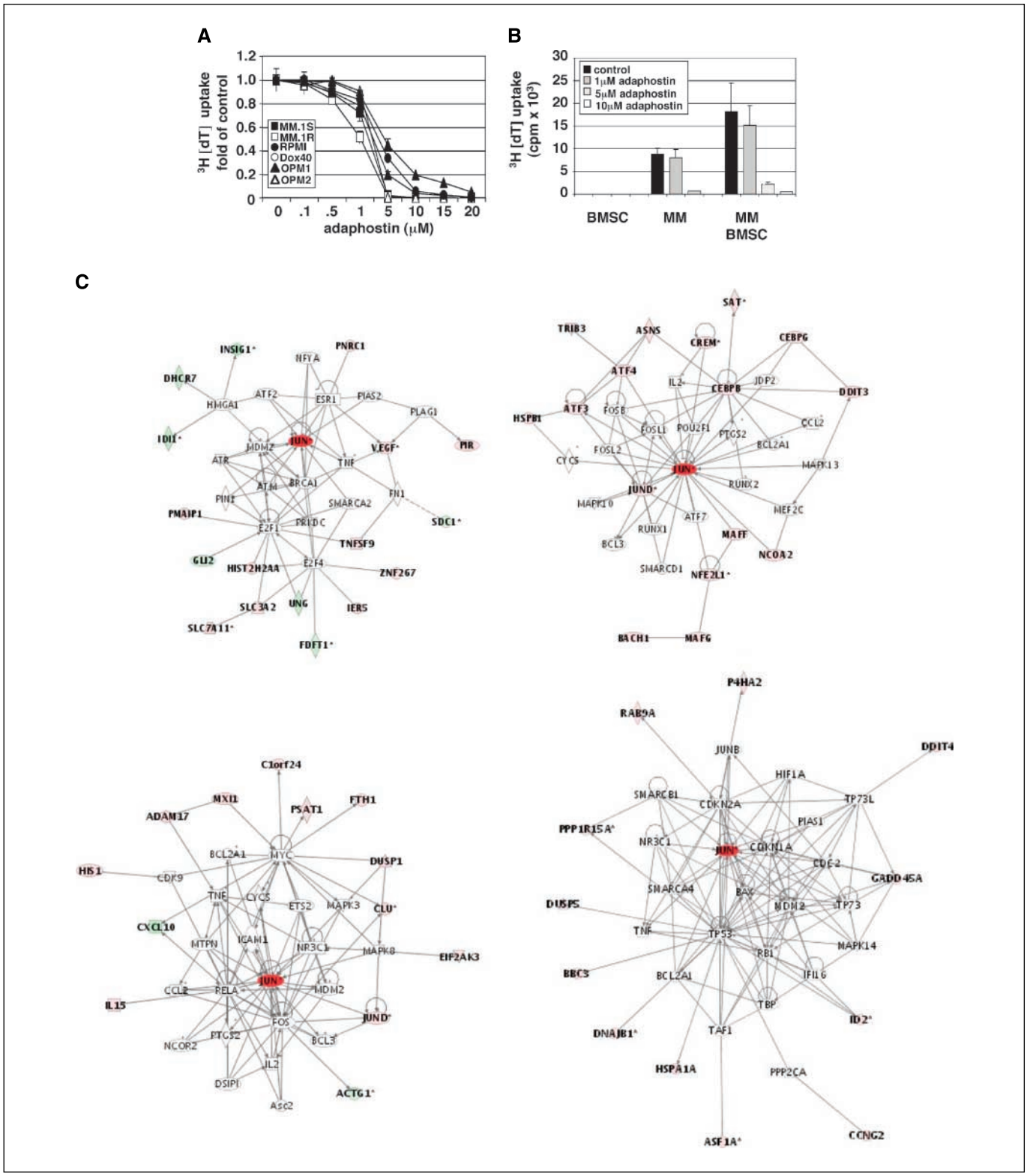

Figure 1. Adaphostin inhibits growth of MM cells, as well as BMSC-triggered MM cell growth, and induces c-Jun up-regulation. *A,* dose-related effect of adaphostin on proliferation of indicated MM cell lines. [^{3}H]dT uptake was measured during the last 10 to 12 h of 24-h and 48-h cultures. *Points,* mean [^{3}H]dT uptake of triplicate or quadruplicate cultures; *bars,* SD. *B,* adaphostin inhibits proliferation of MM cells adherent to BMSCs. MM cells were cultured with or without BMSCs. Adaphostin was added at the indicated concentrations and proliferation was measured by [^{3}H]dT uptake. *Columns,* mean of experiments done in triplicate; *bars,* SD. *C,* adaphostin-triggered up-regulation of c-Jun gene expression. Alterations in gene expression of MM cells treated with adaphostin versus control cells were delineated by oligonucleotide microarray analysis (human U1333A 2.0 Affymetrix GeneChip), followed by data analysis using the Ingenuity Pathway Analysis software. The four pathways identified by the Ingenuity Pathway Analysis software that included JUN are indicated. *Boldface,* genes showing overexpression (*red/pink*) or down-regulation (*green*) after treatment with adaphostin. *Asterisks,* genes that are present in more than one altered network (for additional information, see also http://www.ingenuity.com).

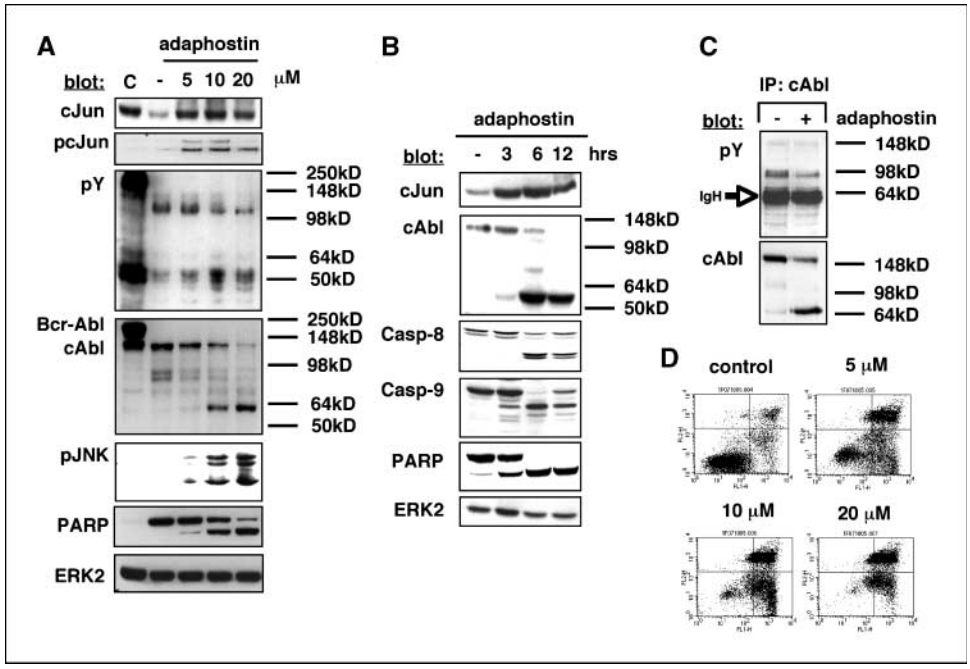

Figure 2. Adaphostin-induced protein profiling. Dose- and time-dependent protein profiling of MM cells exposed to adaphostin. MM.1S cells were exposed to adaphostin at indicated doses (*A*) or indicated time intervals (*B*), followed by immunoblot analysis of the lysates with the indicated antibodies. *C,* adaphostin-induced c-Abl cleavage. MM.1S cells were treated with adaphostin and c-Abl immunoprecipitates (*IP*) from whole-cell lysates were analyzed by Western blotting with c-Abl monoclonal antibody. *A* to *C,* molecular mass markers are given in kilodaltons. *D,* adaphostin-induced MM cell apoptosis. MM.1S cells were treated with indicated concentrations of adaphostin. Representative dot plots stained with Annexin (*X axis*) and propidium iodide (*Y axis*), followed by flow cytometric analysis.

suggesting its central role in response of MM cells to adaphostin (Fig. 1*C* and Supplementary Fig. S1). Indeed, c-Jun exhibited an early 13-fold increase at 2 h and its expression level increased (>100-fold increase) thereafter. Immunoblot assays confirmed that c-Jun protein up-regulation was also triggered by adaphostin in a dose- and time-dependent manner (Fig. 2*A* and *B*). JunD and CDKN1C exhibited 4- and 16-fold increased expression, respectively, whereas JunB, Fos, and other Fos family members were not altered (Fig. 1 and data not shown).

Because adaphostin was developed as an alternative to imatinib mesylate, we next evaluated its effects on c-Abl in MM cells. Consistent with previous reports, c-Abl was strongly expressed in MM.1R, RPMI, RPMI-Dox40, OPM1, and OPM2 MM cells (Supplementary Fig. S2; see refs. 20, 21). Adaphostin triggered cleavage of c-Abl in MM cells, evidenced by immunoblot analysis of both whole-cell lysates (Fig. 2*A* and *B*) and immunoprecipitates (Fig. 2*C*). Moreover, adaphostin induced marked changes in the tyrosine phosphorylation of several proteins including JNK, with only minor

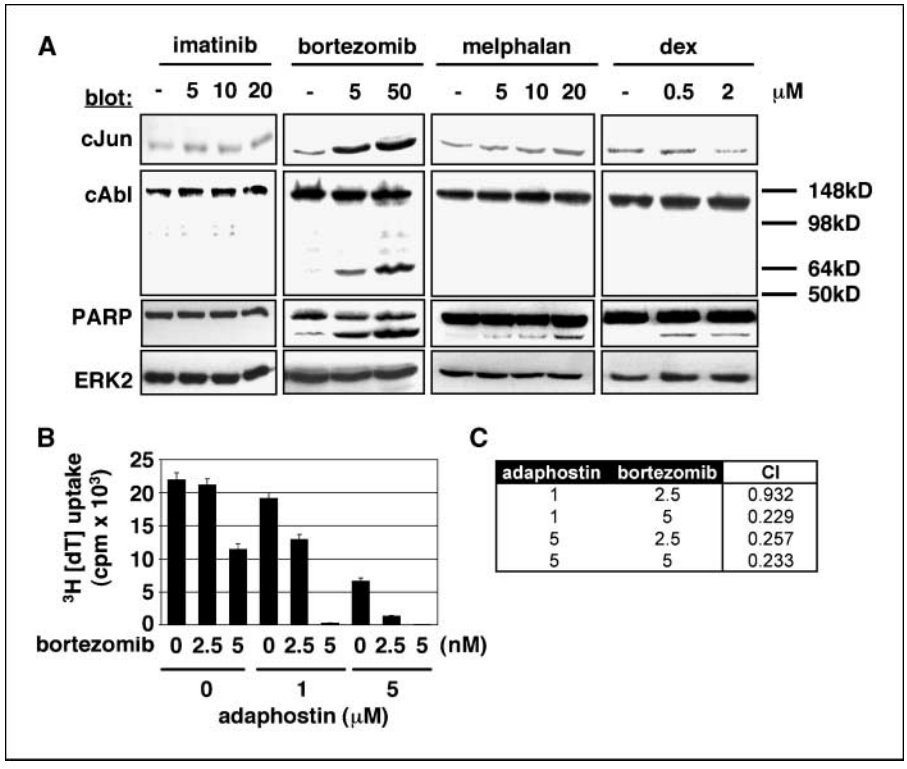

Figure 3. Bortezomib, but not imatinib mesylate, melphalan, or dexamethasone, triggers c-Abl cleavage. *A,* bortezomib, but not imatinib mesylate, melphalan, or dexamethasone (*dex*), triggers c-Jun up-regulation and c-Abl cleavage. MM.1S cells were treated with indicated doses of imatinib mesylate, bortezomib, melphalan, or dexamethasone, followed by immunoblot analysis of the lysates with the indicated antibodies. *B* and *C,* synergistic inhibition of BMSC-triggered MM cell growth by low-dose adaphostin and bortezomib. MM cells were cultured with or without BMSCs in the presence or absence of adaphostin and bortezomib, and proliferation was then measured by [^{3}H]dT uptake. *B, columns,* mean of experiments done in triplicate; *bars,* SD. *C,* isobologram analysis. CI < 1 indicates synergism.

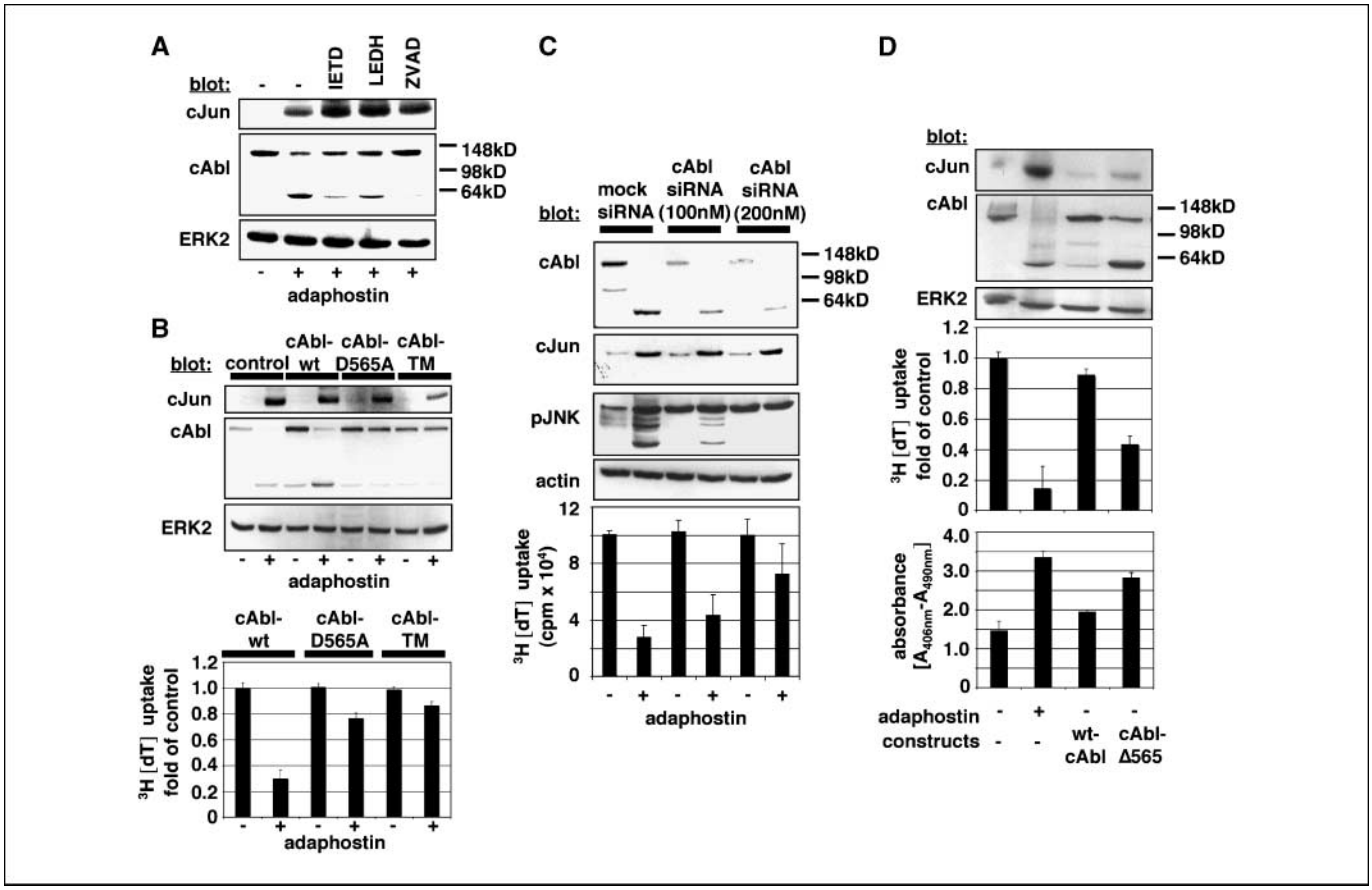

Figure 4. Caspase-dependent cleavage of c-Abl in MM cells induces inhibition of MM cell growth and induction of apoptosis. *A,* caspase-dependent cleavage of c-Abl. MM.1S cells were preincubated with caspase-8, caspase-9, or pan-caspase inhibitors, followed by treatment with adaphostin. Immunoblot analysis was done with the indicated antibodies. *B,* c-Abl cleavage induces inhibition of MM cell growth. MM.1S cells transfected with wild-type c-Abl (c-Abl-wt), c-Abl mutated at D565 (c-Abl-D565A), or c-Abl mutated in all caspase cleavage sites (TM-Abl) were treated with adaphostin, followed by either immunoblotting with the indicated antibodies (*top*) or assay for [^{3}H]dT uptake (*bottom*). *C,* depletion of c-Abl protects against adaphostin-induced inhibition of MM cell growth. MM.1S cells were transiently transfected with mock (200 nmol/L) and c-Abl siRNA (indicated concentrations) and treated with adaphostin, followed by immunoblotting with the indicated antibodies (*top*) or assay for [^{3}H]dT uptake (*bottom*). *D,* c-Abl fragment (cAbl-Δ565) markedly down-regulates MM cell proliferation and induces MM apoptosis. MM.1S cells were transiently transfected with wt-cAbl or cAbl-Δ565 followed by immunoblotting with the indicated antibodies (*top*) or assay for [^{3}H]dT uptake (*middle*) or DNA fragmentation (*bottom*).

or no changes in c-Abl (145 kDa) phosphorylation (Fig. 2*A* and *C*), as well as cleavage of caspase-8, caspase-9, and PARP (Fig. 2*B*). MM cell apoptosis was confirmed both by Annexin V/propidium iodide staining (Fig. 2*D*) and DNA fragmentation assays in both MM.1S and primary patient MM cells (data not shown). Similar results were shown in MM.1R, OPM1, OPM2, RPMI, and RPMI-Dox40 (Dox40) MM cell lines (Supplementary Fig. S2). Taken together, these data show that adaphostin-induced inhibition of MM cell growth and survival is associated with c-Jun up-regulation and cleavage of c-Abl.

Bortezomib, but not imatinib mesylate, melphalan, and dexamethasone, triggers c-Abl cleavage. To assess the specificity of these adaphostin-induced molecular effects in MM, we next tested melphalan and dexamethasone conventional MM chemotherapeutics, as well as the novel proteasome inhibitor bortezomib, for their ability to trigger up-regulation of c-Jun and cleavage of c-Abl. Unlike adaphostin, imatinib mesylate, melphalan, and dexamethasone did not induce c-Jun up-regulation or c-Abl cleavage. Importantly, bortezomib, similar to adaphostin, induced both c-Jun up-regulation and c-Abl cleavage (Fig. 3*A*). This observation is consistent with reports that the proteasome inhibitor MG-132 triggers c-Abl cleavage and cell death in both suspension and adherent solid tumor cells (29). The effect of adaphostin combined with bortezomib, melphalan, or dexamethasone in BMSC-MM cell cocultures was next evaluated. Importantly, isobologram analysis showed that adaphostin and low-dose bortezomib induces marked synergistic effects on MM cell growth (Fig. 3*B* and *C*). In contrast, no additional cytotoxicity was observed with adaphostin combined with melphalan or dexamethasone (data not shown).

Caspase-dependent cleavage of c-Abl and its functional effect in MM cells. The antiproliferative and proapoptotic functions of c-Abl have previously been described in human lymphoid cells (17, 18, 27) via caspase-dependent cleavage at D565, D644, and D958 (27, 30). Moreover, the 60-kDa fragment generated by cleavage at the c-Abl residue D565 is the only cleavage product detectable after longer periods of caspase activation (30). We next sought to determine whether caspases mediate adaphostin-triggered cleavage of c-Abl in MM cells. We therefore incubated MM cells with caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEDH-fmk), as well as pan-caspase inhibitor (z-VAD-fmk), before exposure to adaphostin and then monitored for c-Jun expression and c-Abl cleavage. Whereas c-Jun up-regulation was unaffected, c-Abl cleavage was partially reduced by inhibition of caspase-8

and caspase-9 and nearly completely abrogated by pan-caspase inhibition (Fig. 4*A*). Taken together, these data show that adaphostin activates caspases, which in turn cleave c-Abl predominantly at D565, thereby generating a 60-kDa fragment in MM cells.

c-Abl cleavage by adaphostin inhibits MM cell growth. To correlate adaphostin-triggered c-Abl cleavage with alterations in MM cell growth and survival, we next transiently transfected MM cells with wild-type c-Abl or with c-Abl mutants, which harbor Asp-Ala substitutions at one (D565) or all three caspase cleavage sites (TM; refs. 27). Transfection efficacy was validated by immunoblot analysis. Adaphostin-triggered c-Abl cleavage is abrogated in MM cells transfected with either c-Abl-D565A or c-Abl-TM, but not with wild-type c-Abl. In contrast, adaphostin-induced up-regulation of c-Jun expression was not affected (Fig. 4*B, top*). Moreover, the sensitivity to adaphostin was significantly reduced in MM cells transfected with c-Abl-D565A and c-Abl-TM compared with wild-type c-Abl (Fig. 4*B, bottom*).

To further show the pivotal role of c-Abl cleavage in inhibiting MM cell growth, MM.1S cells were transiently transfected with c-Abl siRNA before treatment with adaphostin. As shown in Fig. 4*C*, protein expression of both uncleaved and cleaved c-Abl was markedly down-regulated in a dose-dependent manner compared with cells transfected with mock siRNA. c-Abl–specific down-regulation was confirmed by immunoblotting with antibodies against actin (Fig. 4*C*) and ERK2 (data not shown). Adaphostin-induced up-regulation of c-Jun was unchanged, providing further evidence of its role upstream of c-Abl. Importantly, adaphostin-induced JNK phosphorylation in MM cells was inhibited upon c-Abl depletion, showing a pivotal role for the c-Abl fragment mediating JNK activation. These data are consistent with previous studies in other cell models, in which c-Abl cleavage leads to JNK activation (31, 32), as well as with our own studies showing marked JNK activation in MM cells triggered by bortezomib (33, 34). Conversely, depletion of c-Abl by specific siRNA protected against adaphostin-induced inhibition of MM cell proliferation (Fig. 4*C*). Consistent with these data, transient transfection with c-Abl fragment cAbl-Δ565, but not with wt-cAbl, significantly down-regulated thymidine uptake (Fig. 4*D, middle*) and increased DNA fragmentation (Fig. 4*D, bottom*), as in adaphostin-treated MM cells (Fig. 4*D*). Taken together, these data strongly indicate that cleavage of c-Abl contributes to the cytotoxicity of adaphostin in MM cells.

Depletion of c-Jun inhibits adaphostin-induced c-Abl cleavage and decreases MM cell growth. The requirement for c-Jun up-regulation in caspase-mediated cleavage of c-Abl was next investigated. MM cells transiently transfected with c-Jun siRNA were treated with adaphostin and examined for c-Abl cleavage, as well as associated effects on MM cell growth. As shown in Fig. 5*A*, both adaphostin-induced up-regulation of c-Jun expression and c-Abl cleavage were markedly inhibited upon c-Jun depletion. Specific down-regulation of c-Jun was validated by immunoblotting with antibodies against actin (Fig. 5*A*) and ERK2 (data not shown). Importantly, the sensitivity of MM cells to adaphostin was markedly decreased, as evidenced by [^{3}H]dT uptake (Fig. 5*A*). Consistent with these data, transient transfection with wt-cJun, but not with the empty vector (pCDNA3), markedly down-regulated MM cell proliferation (Fig. 5*B, top*) and increased DNA fragmentation (Fig. 5*B, bottom*), as in adaphostin-treated MM cells. Similar results were obtained using Annexin V staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay (data not shown). Taken together, these data indicate that up-regulation of c-Jun expression initiates caspase activation and subsequent c-Abl

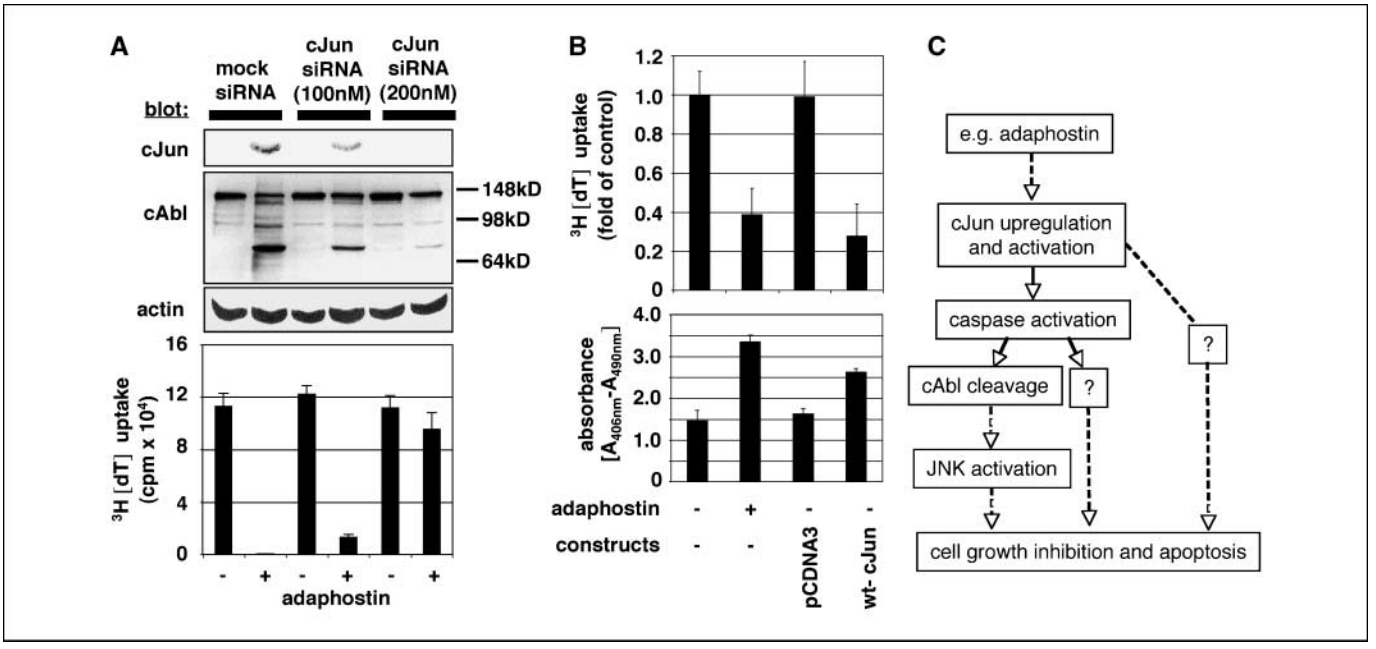

Figure 5. c-Jun–induced c-Abl cleavage triggers inhibition of MM cell growth and induction of apoptosis. *A,* depletion of c-Jun abrogates adaphostin-induced c-Abl cleavage and decreased MM cell growth. MM.1S cells were transiently transfected with mock (200 nmol/L) and c-Jun siRNA (indicated concentrations) and treated with adaphostin, followed by either immunoblotting with the indicated antibodies (*top*) or assay for [^{3}H]dT uptake (*bottom*). *B,* transient transfection with wt-cJun but not empty vector (pcDNA3) markedly down-regulates MM cell proliferation (*top*) as well as increases DNA fragmentation (*bottom*). *C,* model of adaphostin-induced effects in MM. Adaphostin-triggered c-Jun up-regulation activates caspases in MM cells and generates a 60-kDa c-Abl product, which in turn induces cell growth arrest and/or apoptotic cell death.

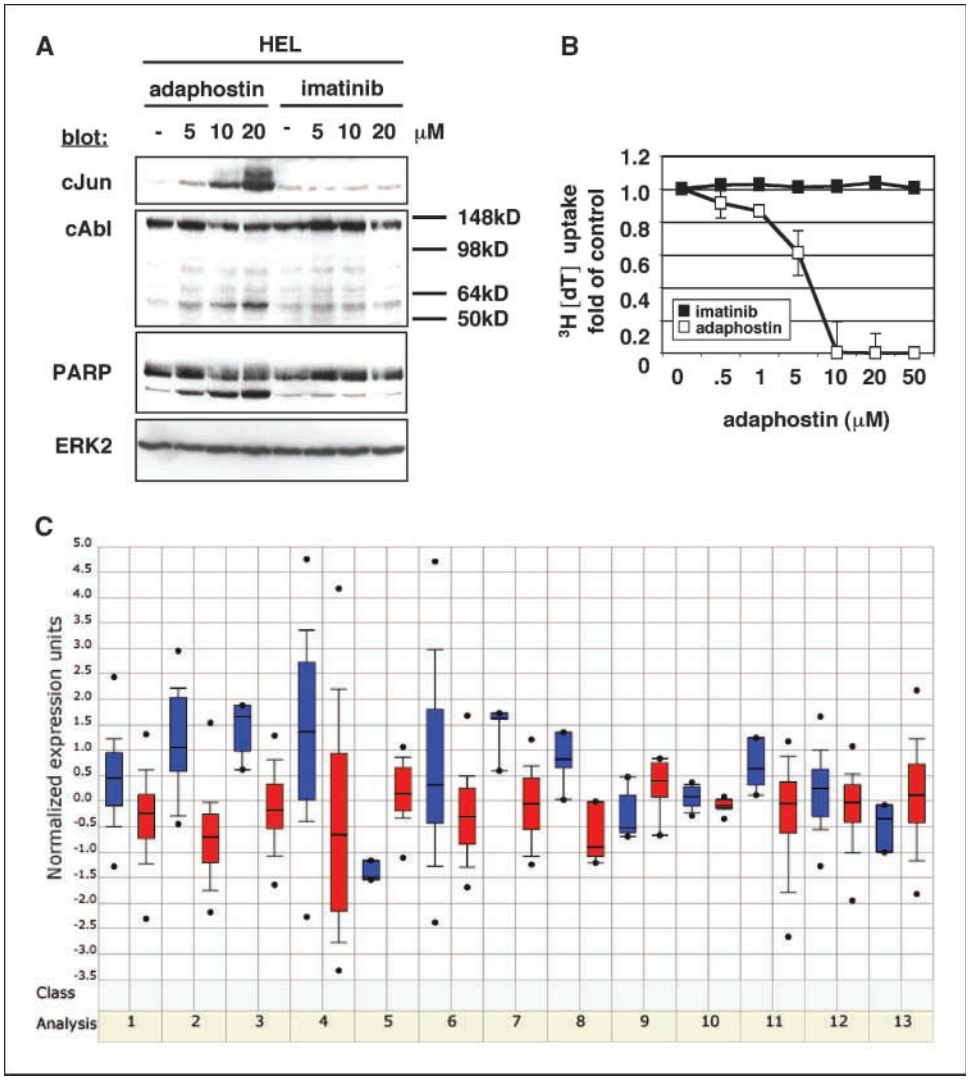

Figure 6. Role of c-Jun up-regulation in inhibiting tumor cell growth and apoptosis. *A* and *B,* adaphostin-induced c-Jun up-regulation and c-Abl cleavage in Bcr-Abl–negative erythroleukemia cells (Jak2V617F mutation) are associated with growth inhibition. HEL cells were treated with adaphostin or imatinib mesylate, followed by either immunoblot analysis of the lysates with the indicated antibodies (*A*) or assay for ^{3}H[dT] uptake (*B*). *C,* Oncomine analysis of multiple tumors. Only data sets showing a significant dysregulation of c-Jun expression when compared with expression values in normal samples are shown. *Blue,* expression level of c-Jun across normal samples; *red,* expression level of c-Jun across tumor samples. Data sets showing significant down-regulation of c-Jun in tumor cells when compared with normals include 1 liver (40), 2 seminoma (41), 3 breast carcinoma (42), 4 breast carcinoma (43), 6 diffuse large B-cell lymphoma (44), 7 breast ductal carcinoma (45), 8 breast lobular carcinoma (45), 10 prostate carcinoma (46), 11 squamous lung cancer carcinoma (47), and 12 prostate cancer (48). In contrast, data sets of 5 glioblastoma multiforme (49), 9 renal clear cell carcinoma (50), and 13 lung adenocarcinoma (47) show c-Jun up-regulation.

cleavage, MM growth inhibition, and apoptosis (Fig. 5*C*). Additional mechanisms that may contribute to c-Jun–induced tumor cell death are under active investigation, including caspase-triggered cleavage of proteins other than c-Abl and differential regulation of death-preventing versus death-enhancing members of the Bcl-2 gene family (13).

Adaphostin induces similar effects in imatinib mesylate–resistant erythroleukemia cells. We next examined whether similar effects were observed in settings other than myeloma. Remarkably, this mechanism also contributes to adaphostin-triggered growth inhibition and apoptosis in the CML cell line K562 (Bcr-Abl expressing) and the erythroleukemia cell line HEL (Jak2V617F expressing; Fig. 6*A*) because treatment of both cell lines with adaphostin triggered up-regulation of c-Jun and c-Abl, as well as PARP cleavage. These effects were somewhat diminished in imatinib-sensitive, Bcr-Abl–positive K562 cells, in which one c-Abl allele is lost to the Bcr-Abl fusion (data not shown). Importantly, adaphostin, but not imatinib mesylate, induced dose-dependent inhibition of HEL cell growth (Fig. 6*B*). Given our results suggesting that c-Jun up-regulation induces cell growth inhibition and apoptosis in MM cells, we did Oncomine analysis (35) of 13 tumor data sets representing a broad spectrum of tumor types. Nine of these tumors, including diffuse large B-cell lymphoma, show significantly lower levels of c-Jun expression in tumor cells versus normal cellular counterparts (Fig. 6*C*).

Discussion

The present study shows a role for c-Jun–induced caspase activation and associated c-Abl cleavage in the inhibition of MM cell growth and induction of apoptosis. Functional sequelae of c-Jun up-regulation were investigated using adaphostin, a compound originally investigated as an alternative to the 2-phenylaminopyrimidine derivative imatinib mesylate. These studies identify a novel mechanism whereby c-Jun modulates MM cell growth and also provide a preclinical rationale for use of adaphostin, alone and in combination, to augment MM cell cytotoxicity and improve patient outcome.

c-Jun not only induces cell growth and oncogenic transformation (11, 12) but may also mediate cell death either by acting as a transcriptional regulator or by triggering caspase-mediated cleavage of proteins. Potential explanations for its opposing effects include cell type specificity, the availability of external and internal survival factors, and specific spectra of binding partners. In addition to the

above data, our recent studies show a trend toward a shorter overall survival and event-free survival in 11 of 67 (17%) MM patients with low levels of c-Jun compared with patients with normal or high levels of c-Jun (36). Taken together, these data suggest that low c-Jun expression may correlate with poor survival, which requires validation in larger studies. They also suggest that adaphostin and bortezomib, by increasing c-Jun expression and inducing apoptosis, may be particularly useful in this subset of patients.

c-Abl has been associated with DNA replication, recombination, repair, G_1 growth arrest, and apoptosis (19); as for c-Jun, phosphorylated protein-protein interactions are implicated in the regulation and function of c-Abl (18). c-Abl activity is reported to play only a minor role in MM pathogenesis because tyrosine phosphorylation of c-Abl in MM cells is low and unaffected by treatment with imatinib mesylate (21). Moreover, a phase II trial of imatinib mesylate in patients with refractory/relapsed MM showed little activity (23). However, our studies show that cleavage of c-Abl inhibits MM cell growth and induces apoptosis of MM cells both alone and in the bone marrow microenvironment. These effects are predominantly mediated via up-regulation of c-Jun, followed by cleavage of c-Abl. Importantly, after caspase-triggered cleavage, c-Abl products are regulated by ubiquitination and the proteasome (29). The marked synergistic effects of adaphostin and bortezomib observed in our study may, at least in part, be due to the inhibition of degradation of ubiquitinated proteins and the stabilization of cleaved c-Abl. Consistent with our data, the combination of adaphostin with the investigational proteasome inhibitor MG-132, as well as with bortezomib, is strongly synergistic in a variety of leukemia cell lines (8). Importantly, c-Abl also neutralizes the inhibitory effect of murine double minute-2 on p53 (37), whereas bortezomib induces up-regulation of p53 by inhibiting the ubiquitin-proteasome pathway (33). Therefore, complex formation of c-Abl with p53 (17) may be another mechanism to induce synergistic effects on G_1 arrest in MM cells.

Our data both highlight significant differences in the mode of action of adaphostin versus imatinib mesylate and provide the framework for combined therapy to induce synergistic cytotoxicity in multiple human leukemia cell lines (38). Specifically, we suggest that c-Abl cleavage may contribute to adaphostin-triggered effects in CML cells, consistent with previous studies reporting a decrease of p210 Bcr-Abl after adaphostin treatment. Our data strongly suggest that decreased p210 Bcr-Abl expression in these studies is associated with an increase of c-Abl cleavage products. Moreover, reactive oxygen species generation is one of the initial events mediating adaphostin-induced death in CML cells, and adaphostin-induced oxidative injury of leukemic cells activates caspases, thereby amplifying this cell death process (7, 8). Adaphostin induces significantly greater CML cell apoptosis with less inhibition of Bcr-Abl phosphorylation than imatinib mesylate, consistent with this view (5).

Ongoing studies are delineating the downstream molecular mechanisms mediating the antiproliferative and proapoptotic functions of c-Abl in MM. It is possible that c-Abl–induced JNK phosphorylation triggers the translocation of JNK to mitochondria, initiates the release of cytochrome *c* and Smac/DIABLO, and thereby triggers mitochondrial death signaling cascades (39). We are also testing whether protein interaction of cleaved c-Abl with retinoblastoma 1, ataxia telangiectasia mutated, p73, breast cancer 1, RNA polymerase II, Rad51, protein kinase C, DNA-dependent protein kinase, or p53 (17–19) contributes to adaphostin- and/or bortezomib-induced inhibition of MM cell growth and apoptosis.

Besides specific protein-protein interactions, the cellular localization of both full-length c-Abl and caspase-induced c-Abl fragments may play a crucial role in defining specific functions of c-Abl. Specifically, the first caspase-induced cleavage of c-Abl at Asp^{958} generates 120- and 22-kDa fragments; the second cleavage of the 120-kDa fragment at Asp^{565} generates a 60-kDa fragment. The 120-kDa fragment loses the nuclear exclusion signal and the actin-binding domain, whereas the 60-kDa c-Abl fragment retains the SH3, SH2, and kinase domains and loses both the nuclear localization and nuclear export signals, as well as the DNA- and actin-binding regions (27, 30). Our data indicate that the major 60-kDa c-Abl fragment is responsible for the antiproliferative and proapoptotic effects in MM cells; our ongoing studies are investigating the functional relevance of its subcellular localization in MM.

The present report therefore shows for the first time that c-Jun up-regulation (triggered by bortezomib or adaphostin) activates caspases in MM cells, which generate a 60-kDa c-Abl product, which in turn induces cell growth arrest and/or apoptotic cell death. It delineates a novel mechanism of bortezomib- and adaphostin-induced MM cell growth inhibition and apoptosis. Importantly, our data suggest that this mechanism may be operative not only in MM but also in other malignancies including CML and solid tumors, thereby providing the preclinical rationale for use of adaphostin, both alone and in combination, to improve patient outcome.

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