Aplidin, a Marine Organism–Derived Compound with Potent Antimyeloma Activity In vitro and In vivo

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Abstract

Despite recent progress in its treatment, multiple myeloma (MM) remains incurable, thus necessitating identification of novel anti-MM agents. We report that the marine-derived cyclodepsipeptide Aplidin exhibits, at clinically achievable concentrations, potent in vitro activity against primary MM tumor cells and a broad spectrum of human MM cell lines, including cells resistant to conventional (e.g., dexamethasone, alkylating agents, and bortezomib) or novel anti-MM agents. Aplidin is active against MM cells in the presence of proliferative/antiapoptotic cytokines or bone marrow stromal cells and has additive or synergistic effects with some of the established anti-MM agents. Mechanistically, a short in vitro exposure to Aplidin induces MM cell death, which involves activation of p38 and c-jun NH2-terminal kinase signaling, Fas/CD95 translocation to lipid rafts, and caspase activation. The anti-MM effect of Aplidin is associated with suppression of a constellation of proliferative/antiapoptotic genes (e.g., MYC, MYBL2, BUB1, MCM2, MCM4, MCM5, and survivin) and up-regulation of several potential regulators of apoptosis (including c-JUN, TRAIL, CASP9, and Smac). Aplidin exhibited in vivo anti-MM activity in a mouse xenograft model. The profile of the anti-MM activity of Aplidin in our preclinical models provided the framework for its clinical testing in MM, which has already provided favorable preliminary results. [Cancer Res 2008; 68(13):5216–25]

Introduction

Although recent advances in the therapeutic management of multiple myeloma (MM) have improved its prognosis (1), no curative therapy currently exists for this plasma cell dyscrasia, which is the second most commonly diagnosed hematologic malignancy in the Western world (2). The identification of new classes of agents with anti-MM activity, especially in patients who relapse from or do not respond to available therapies, remains an urgent priority. Within this context, we evaluated a series of candidate antitumor agents derived from marine organisms. This effort was based on the rationale that several established or investigational anticancer drugs are derived from natural products (3). These compounds are produced by various plants and other organisms (mostly above sea level), and their antimitotic properties, which prompted the evaluation of their antitumor activity, may serve the teleologic purpose of protecting from predators or infections. Recently, increasing interest has been placed on oceanic organisms, primarily invertebrates (e.g., sponges, tunicates, bryozoans, and mollusks), as unique natural sources of a diverse array of natural products with potent antimitotic and/or antitumor properties, including compounds that have advanced to late-stage clinical trials (4, 5). An intriguing hypothesis about marine organism–derived compounds is that they may have evolved to be more potent than similar compounds from above-water organisms to compensate for the increased diffusion and thus rapidly decreasing protective concentration gradient of these compounds under water.

In this study, we characterize the anti-MM activity of one such marine-derived compound, Aplidin, which is a cyclic depsipeptide isolated from the Mediterranean tunicate Aplidium albicans (6). We show that Aplidin, at clinically relevant concentrations, has a potent activity against a broad panel of human MM cell lines and primary MM tumor cells, including cells resistant to conventional or novel anti-MM agents. Although cytokine- or cell adhesion–mediated interactions with the bone marrow microenvironment protect MM cells from conventional therapies (e.g., dexamethasone or cytotoxic chemotherapy; refs. 7, 8), Aplidin is able to overcome these protective effects in coculture models of MM cells with bone marrow stromal cells. Moreover, Aplidin has additive or synergistic effects with established anti-MM agents including bortezomib and immunomodulatory agents. Mechanistically, in vitro exposure of MM cells to clinically relevant doses of Aplidin for even a few hours is sufficient to induce their cell death. This involves activation of p38 signaling and is associated with suppression of a constellation of proliferative/antiapoptotic genes and up-regulation of several positive regulators of apoptosis. Aplidin also exhibited in vivo anti-MM activity in a mouse xenograft model. The profile of the anti-MM activity of Aplidin in preclinical in vitro and in vivo models,
coupled with its favorable safety profile in clinical trials for solid tumors, provided the framework for clinical studies of this agent in MM, where Aplidin has already shown encouraging preliminary results.

Materials and Methods

MM cell lines, primary MM tumor cells, stromal cells, normal hematopoietic cells, reagents, and antibodies. Detailed information on the MM cell lines, primary MM tumor cells, stromal cells, normal hematopoietic cells, reagents, and antibodies is included in Supplementary data.

Evaluations of cell viability, cell proliferation, cell cycle profiles, induction of apoptosis, and mitochondrial membrane potential. Previously described protocols were used to evaluate MM cell viability [with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric survival assay; refs. 9–13]; cell proliferation and cell cycle profiles (with the CD38-CD138/propidium iodide double-staining technique; ref. 14); induction of apoptosis (based on Annexin V-FITC/propidium iodide staining of myelomatous plasma cells; ref. 13); and changes in mitochondrial transmembrane potential (ΔΨm; using 3,3′-dihexyloxacarbocyanine iodide [DiOC6(3)] staining of cells; ref. 13). Detailed information on these protocols is included in Supplementary data.

Oligonucleotide microarray analyses, Western blots, c-jun NH2-terminal kinase in vitro kinase assays, and lipid raft isolation. Gene expression profiling, Western blot analyses, c-jun NH2-terminal kinase (JNK) in vitro kinase assays, and lipid raft isolation studies were done as in previous studies (12, 15–18) and are described in detail in Supplementary data.

Combinations of Aplidin with other anti-MM agents. The interaction between Aplidin and other anti-MM agents (dexamethasone, melphalan, doxorubicin, thalidomide, lenalidomide, and bortezomib) was analyzed with the CalcuSyn software (Biosoft). Data from cell viability assays (MTT) were expressed as fraction of cells affected by the dose in analyzed with the CalcuSyn software (Biosoft). Data from cell viability melphalan, doxorubicin, thalidomide, lenalidomide, and bortezomib) was
determined between Aplidin and other anti-MM agents (dexamethasone-sensitive MM1S and melphalan-sensitive U266 cells versus the effect of the drug on the MM1R and U266-LR7 isogenic sublines, which are resistant to dexamethasone and melphalan, respectively, we observed that Aplidin potently suppressed MM cell viability in a dose-dependent manner both in the drug-sensitive lines and in their drug-resistant sublines (Fig. 1B). Consistent with the studies on other MM cell lines, the IC50 values for these four cell lines were within the 1 to 10 nmol/L range (4.1, 4.7, 5, and 8.2 nmol/L for MM1S, MM1R, U266, and U266-LR7, respectively). Interestingly, this range of potency is similar to that observed for bortezomib (data not shown), a potent anti-MM agent used in the clinical practice (21, 22).

The effect of Aplidin on MM1S cells was time dependent: Inhibition of cell viability progressively increased from day 1 to day 3 of treatment with any given Aplidin dose (Fig. 1C). Interestingly, even a 24-hour exposure to Aplidin at concentrations as low as 5 nmol/L was sufficient to induce a >50% decrease in MM cell viability. To address the question of whether constant exposure to Aplidin was required for its anti-MM effect, we performed cell death commitment experiments: MM cells were exposed to a clinically relevant dose of Aplidin (40 nmol/L) for variable periods of time (ranging from 0 to 10 hours) and then were washed to remove the drug, followed by culture in drug-free media for 3 days. At that time, cell viability was assessed by MTT and expressed as percent cell viability compared with control cultures. These cell death commitment assays evaluated whether a short exposure of MM cells to Aplidin irreversibly commits them to undergo cell death. In all five MM cell lines tested (JJN-3, S6B45, NCI-H929, SKM1, and KMS-18), even a 1-hour exposure to Aplidin was sufficient to trigger, despite the subsequent washout of the drug, a >50% decrease in cell viability (Fig. 1D). In fact, in four of five lines tested (JJN-3, S6B45, SKM1, and KMS-18), a 1-hour exposure to Aplidin was sufficient to trigger a >70% decrease in MM cell viability. These results supported the notion that, whereas up to 24 hours may elapse before the full effect of Aplidin-induced cell death can be observed, a short exposure to the drug (1 hour) may be sufficient to irreversibly commit MM cells to their death.

Effect of Aplidin on MM cells cultured with exogenous interleukin 6, insulin-like growth factor I, and bone marrow stromal cells. We next evaluated the effect of Aplidin on MM cell proliferation induced by the stimulatory cytokines interleukin 6 (IL-6) and insulin-like growth factor I (IGF-I), as well as bone marrow stromal cells (Fig. 2). MM1S cells were treated with increasing doses of Aplidin in the presence or absence of IL-6 (1 nmol/L) or IGF-I (10 nmol/L). Neither of these growth factors

Results

In vitro activity of Aplidin against MM cell lines. We first evaluated, using MTT assays, the in vitro activity of Aplidin against a large panel of MM cell lines including cells resistant to various anti-MM agents. We observed that Aplidin exhibited potent in vitro anti-MM activity (Fig. 1): The majority (18 of 19) of MM cell lines treated with Aplidin at 10 nmol/L, a clinically achievable concentration (20), exhibited ≤30% viability compared with their respective controls (Fig. 1A; Supplementary Fig. S1; Supplementary Table S1). Aplidin-responsive cell lines included cells resistant to conventional anti-MM agents [e.g., melphalan (RPMI-8226/LR5), mitoxantrone (RPMI-8226/MR20), and doxorubicin (RPMI-8226/ DoxO)] as well as cells with low sensitivity to novel anti-MM agents [e.g., immunomodulatory thalidomide derivatives (S6B45, RPMI-8226/S); Fig. 1A]. Similarly, on comparison of the in vitro effect of Aplidin on dexamethasone-sensitive MM1S and melphalan-sensitive U266 cells versus the effect of the drug on the MM1R and U266-LR7 isogenic sublines, which are resistant to dexamethasone and melphalan, respectively, we observed that Aplidin potently suppressed MM cell viability in a dose-dependent manner both in the drug-sensitive lines and in their drug-resistant sublines (Fig. 1B). Consistent with the studies on other MM cell lines, the IC50 values for these four cell lines were within the 1 to 10 nmol/L range (4.1, 4.7, 5, and 8.2 nmol/L for MM1S, MM1R, U266, and U266-LR7, respectively). Interestingly, this range of potency is similar to that observed for bortezomib (data not shown), a potent anti-MM agent used in the clinical practice (21, 22).

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protected against Aplidin-induced growth inhibition (Fig. 2B and C). Binding of MM cells to patient bone marrow stromal cells triggered increased MM cell proliferation, which was attenuated by Aplidin (Fig. 2A). Even low doses of Aplidin (e.g., 10 nmol/L) display a potency similar to that of higher concentrations of bortezomib (e.g., 50 nmol/L). Importantly, Aplidin did not affect the viability of bone marrow stromal cells, as determined by MTT assays (data not shown).

**Efficacy of Aplidin against freshly isolated cells from MM patients.** The activity of Aplidin against MM plasma cells freshly isolated from 16 patients (8 newly diagnosed and 8 with relapsed/refractory disease) was next analyzed using a multiparametric cytometry protocol. This test allows for separate analysis of the induction of cell death in the myelomatous plasma cell population versus other bone marrow cells. Thirteen of 16 primary MM cell samples tested in vitro were sensitive to the drug. Two of the three nonresponsive MM primary cell samples were isolated from newly diagnosed patients and the third one was from a patient with refractory disease. Interestingly, of the 13 cases sensitive to Aplidin in vitro, 7 patients had shown clinical resistance to several treatments including alkylating agents and high-dose dexamethasone (all of them), autologous stem cell transplantation (5 cases), bortezomib (3 cases), and thalidomide-based regimens (2 cases) (Supplementary Table S2). Aplidin (100 nmol/L) induced cell death in >80% of MM plasma cells from 8 of 16 patients, and between 60% to 80% in 5 more patients. The average cytotoxicity of Aplidin (100 nmol/L) in sensitive patients was 83% (SD, 14.4; 95% confidence interval, 74.1–91.6). Even at a 10-fold lower dose of Aplidin (10 nmol/L), the average cytotoxic activity on MM plasma cells remained close to 60%. Supplementary Fig. S2A illustrates the effect of Aplidin on MM plasma cells, as well as on normal lymphoid and myeloid cells, from four representative patient cases. Interestingly, whereas Aplidin had a pronounced effect on the viability of MM cells, minimal toxicity was observed on normal lymphocytes and myeloid cells from the same patient samples (Supplementary Table S2; Supplementary Fig. S2A). Supplementary Fig. S2B depicts the results from the analysis of a representative primary MM patient sample, which exhibited a dose-dependent induction of cell death in MM plasma cells. Again, only minimal toxicity was observed on normal mononuclear and myeloid cells, even at high doses of Aplidin.

**Evaluation of combinations of Aplidin with other anti-MM agents.** Clinical experience in the therapeutic management of MM patients supports the concept that drug combinations can induce higher response rates compared with single-agent treatment (23–26). We therefore evaluated the effect of combinations of Aplidin with other anti-MM drugs on the viability of MM1S and U266-LR7 cell lines. Specifically, Aplidin was combined with conventional agents used in MM treatment (such as dexamethasone, doxorubicin, or melphalan), as well as with recently developed drugs (bortezomib, lenalidomide, or thalidomide). In these experiments, both nonconstant ratio (for suboptimal doses of drugs) and constant ratio experimental designs were used. The effects of single and combined treatments after 72 hours were...
evaluated by MTT assays, and data were analyzed with the Calcusyn program.

As shown in Fig. 3A, Aplidin increased the anti-MM effect of dexamethasone, melphalan, lenalidomide, thalidomide, and bortezomib. Analysis with the Calcusyn program indicated that Aplidin exhibited a synergistic effect when combined with lenalidomide or thalidomide (Fig. 3B); Aplidin combinations with dexamethasone or melphalan were additive; combinations of Aplidin and bortezomib presented an additive effect that was even moderately synergistic when low doses of both drugs were used. No synergism was observed in combinations with doxorubicin (data not shown). In experiments with U266-LR7 cells (resistant to melphalan), combinations with dexamethasone or melphalan were not amenable to evaluation with the Calcusyn program because the prerequisite of a dose-effect relationship for each of these drugs as single agents was not fulfilled. When Aplidin was combined with lenalidomide or thalidomide (Fig. 3C), a synergistic effect on U266-LR7 cells was again observed, although less pronounced than that against MM1S cells. The effect of Aplidin and bortezomib on U266-LR7 cells remained moderately synergistic at lower doses and additive at higher doses. Conversely, in U266-LR7 cells, the Aplidin plus doxorubicin combination remained antagonistic.

**Effects of Aplidin on the cell cycle and viability of MM cells.** Treatment with 10 nmol/L Aplidin induced a modest increase in the percentage of MM1S cells in the G2-M phase (Supplementary Fig. S3A). A similar increase in the percent of cells in G2-M was observed after treatment of MM1R cells with 1 nmol/L Aplidin: Percent of cells in G2-M was 19% in control cells versus 29% in 1 nmol/L Aplidin–treated cells (data not shown). Higher doses of Aplidin (100 nmol/L) triggered a clear sub-G0 accumulation (data not shown). The latter fact, together with the increased Annexin V staining obtained in patient plasma cells, indicates that Aplidin does not merely block the proliferation of MM cells but also actively triggers cell death. This hypothesis is also supported by a time-dependent increase in Annexin V staining of MM1S cells treated with Aplidin (Supplementary Fig. S3B). In addition, Aplidin induced DNA laddering in MM1S cells (data not shown).

To gain insights into the mechanism(s) of action of Aplidin on MM cells, we performed gene expression profiling, as well as biochemical and cell biological analyses, with emphasis on the pathways involved in the regulation of apoptosis. Gene expression profiling identified 805 transcripts that were significantly up-regulated or down-regulated by ≥2-fold with Aplidin treatment. The classification of these genes according to functional categories indicated that 9.8% were involved in apoptosis/responses to stress, 22.4% in the control of cell cycle/proliferation, 5.8% were kinases that participate in cell cycle and survival signaling, and 1% participated in adhesion (Supplementary Fig. S3C). These results suggest that genes involved in cell cycle and cell death regulation may participate in the mechanism of action of Aplidin. Indeed, microarray data showed that Aplidin down-regulated several genes required for cell proliferation and/or cell survival (MYBL2, BUB1, BUBR1, cyclin B1, cyclin E1, CENPF, MCM2, MCM4, MCM5, and survivin; Supplementary Fig. S3). In addition, Aplidin up-regulated the transcript levels of potential regulators of apoptosis, such as GADD45A, GADD45B, TRAIL, CASP9, CASP6, CIDE, and Smac (Supplementary Fig. S3D). Of interest, we also observed an increase in the expression of c-JUN and a decrease in the expression of MYC (Fig. 4; Supplementary Figs. S4–S8). Previous data from our group and others have shown that activation of the pathway and/or up-regulation of c-JUN expression is associated with the apoptosis induced by novel drugs such as bortezomib (11). Furthermore, extensive work in MM and other neoplasias has indicated that myc is an important oncogenic protein and a putative therapeutic target (as reviewed in ref. 27). A complete list of transcripts modulated by Aplidin treatment is included as a Supplementary table, and schematic representations of functional pathways involving Aplidin-modulated transcripts are depicted in Supplementary figures.

Drug-induced apoptotic cell death is frequently linked to the release from mitochondria to the cytosol of proteins, which then partner with other cytosol-resident proteins to activate caspases. Loss of mitochondrial membrane potential (Δψm) often reflects an increase in mitochondrial outer membrane permeability. Treatment of MM1S cells with Aplidin caused a decrease in Δψm,
indicative of an increase in mitochondrial outer membrane permeability (Fig. 4A). Bcl-2 family members act as important regulators of mitochondrial outer membrane permeability, and Western blot analyses indicated that Aplidin down-regulated the Bcl-2 family member Mcl-1 (Fig. 4B). No changes in Bcl-2 expression were induced by Aplidin, whereas a small increase in Bcl-X was noticed (Fig. 4B). Apoptosis triggered by Aplidin was further confirmed by cleavage of poly(ADP-ribose) polymerase.

Figure 3. Evaluation of combinations of Aplidin with other anti-MM agents. A, Aplidin (1.25 nmol/L) was combined with dexamethasone, bortezomb, melphalan, lenalidomide, or thalidomide; after 48 h, MTT assays were done on MM1S cells. B, calculated CIs of different double combinations for the MM1S cell line. A fraction affected-CI plot simulation is provided when a constant ratio design is used. CR, constant ratio; NCR, nonconstant ratio. C, CIs observed for the U266-LR7 cell line. CIs of <0.3, 0.3 to 0.7, 0.7 to 0.85, 0.85 to 0.90, 0.90 to 1.10, and >1.10 indicate strong synergism, synergism, moderate synergism, slight synergism, additive effect, and antagonism, respectively.
caspase-3, caspase-7, caspase-8, and caspase-9, with generation of active low Mr cleaved fragments (Fig. 4B). To investigate the importance of caspases in cell death triggered by Aplidin, we evaluated the ability of the pan-caspase inhibitor z-VAD-fmk, the caspase-8 inhibitor z-IETD-fmk, and the caspase-9 inhibitor z-LEHD-fmk to protect against Aplidin-induced apoptosis. As shown in Fig. 4C, preincubation with z-VAD-fmk attenuated Aplidin-induced cell death, whereas no effect was observed with caspase-8 and caspase-9 inhibitors, suggesting that only the blockade of both the intrinsic and extrinsic pathways can partially block Aplidin-induced apoptosis.

**Aplidin-induced translocation of Fas/CD95 death receptor into lipid rafts.** A novel mechanism regulating Fas/CD95 death receptor activation in apoptosis involves its translocation into lipid rafts (16). In this regard, Aplidin-induced apoptosis in leukemic cells involves translocation of Fas/CD95 in lipid rafts (28), and recruitment of death receptors in lipid rafts has recently been shown to be a crucial event in the anti-MM activity of the synthetic alkyl-lysophospholipid analogues edelfosine and perifosine (18). Therefore, we analyzed whether Aplidin induced translocation of Fas/CD95 death receptor in lipid rafts by isolating these membrane microdomains from untreated and drug-treated MM cells by taking advantage of their insolubility in Triton X-100 lysis buffer at 4°C, followed by discontinuous sucrose gradient centrifugation. GM1-containing lipid rafts, at the upper part (fractions 3–5) of the sucrose gradient (Fig. 5A), were identified using cholera toxin B subunit conjugated to horseradish peroxidase. We observed that Fas/CD95 was located in the soluble fractions (fractions 9–12) of the sucrose gradient and not in the detergent-insoluble lipid raft region in untreated MM144 cells, indicating that Fas/CD95 is excluded from the lipid rafts in untreated MM144 cells, in agreement with previous studies (18). Importantly, Aplidin treatment induced a partial recruitment of Fas/CD95 into the lipid raft region (fractions 3–5) of the sucrose gradient (Fig. 5A). Conversely, disruption of lipid rafts by methyl-β-cyclodextrin, which interferes with protein association with lipid rafts by cholesterol depletion (16), partially inhibited Aplidin-induced cell death in MM144 cells (Fig. 5B). These results suggest that Aplidin-induced cell death in MM cells involves, at least in part, Fas/CD95 translocation into lipid rafts.

**Figure 4.** Analysis of the pathways involved in Aplidin-induced apoptosis. A, MM1S cells were treated with Aplidin (100 nmol/L), and Δψm analyses done with [DiOC6(3)]/m emission by flow cytometry. B, MM1S cells were treated with Aplidin (100 nmol/L) for the indicated times, and expressions of poly(ADP-ribose) polymerase (PARP), caspase-3, caspase-7, caspase-8, caspase-9, Bcl-2, Bcl-X, Mcl-1, and myc proteins were analyzed by Western blotting of cell extracts with specific antibodies. C, effect of the caspase inhibitors z-VAD-fmk, z-IETD-fmk, and z-LEHD-fmk on Aplidin-induced cell death. MM1S cells were plated and pretreated, where indicated, with caspase inhibitors (50 μmol/L) for 60 min. Aplidin (100 nmol/L) was added to the corresponding samples and the experiment was continued for 6 and 12 h. Annexin V staining was carried out as described above.
The role of the JNK and p38 apoptotic pathways in the mechanism of action of Aplidin. To further explore the mechanism of action of Aplidin on MM cells, we analyzed the expression and activation status of JNK, p38, extracellular signal-regulated kinase (Erk)-1/2, and Erk5 in Aplidin-treated MM cells. Aplidin (100 nmol/L) induced activation of JNK, which was already detectable by 15 minutes of Aplidin treatment and reached a maximum at 1 to 3 hours (Fig. 5C). Activation of JNK was also observed in MM1R cells (data not shown). Aplidin also rapidly increased phosphorylation of p38. On the other hand, no significant effect on the Erk1/2 or Erk5 pathways was observed at times up to 6 hours of treatment. At later times, Aplidin decreased Erk1/2 and Erk5 expression. To further characterize the biological consequences of the activation of the JNK and p38 signaling pathways, cells were treated with or without inhibitors for p38 kinase (SB203580; 25 μmol/L) and JNK (SP600125; 25 μmol/L) before Aplidin treatment. Both the p38 inhibitor SB203580 and JNK inhibitor SP600125 partially rescued MM1S cells from Aplidin-induced cell death (Fig. 5D), implicating p38 and JNK activation in the mechanism of action of this drug. Comparison with bortezomib indicated differences in the sensitivity to mitogen-activated protein kinase (MAPK) inhibitors because the p38 inhibitor (SB203580) was more efficient in preventing the action of Aplidin (Fig. 5D). In fact, inhibition of the p38 route had a small effect on bortezomib-induced cell death (Fig. 5D), in agreement with the poor p38-stimulating activity of bortezomib (Fig. 5C).

Aplidin exhibits in vivo anti-MM activity in a xenograft-plasmocytoma murine model. In view of the potent in vitro activity of Aplidin against MM cell lines and freshly isolated primary MM patient cells, we next studied the in vivo anti-MM effect of Aplidin in a human plasmocytoma model in immunodeficient mice. To address the possibility that the side effect profile of Aplidin is strain dependent, we first conducted a pilot study to explore the potential toxicity of this drug in CB17-SCID mice. For this purpose, 20 mice were injected i.p. with different doses of Aplidin and were serially monitored for the development of any significant toxicity. Maximum tolerated dose for this strain was established at a total weekly dose of 700 μg/kg.

A separate cohort of 16 mice were treated with a total weekly dose of 700 μg/kg Aplidin at two different schedules of treatment: nine mice received Aplidin 100 μg/kg i.p. daily and seven were given Aplidin 140 μg/kg i.p. 5 days a week. Nine mice served as control cohort and received i.p. injections of the vehicle alone. The toxicity profile of Aplidin was quite favorable, evidenced by a body weight loss of only 6% to 8% in the treated groups as compared with the control group. Only one mouse in the 140 μg/kg cohort had to be euthanized (on day 26 after initiation of treatment) due to a significant loss in body weight (>20%). Aplidin treatment decreased tumor growth in both cohorts of treated mice, and this effect was greater in the group receiving 140 μg/kg of Aplidin 5 days a week (Fig. 6A). The differences in tumor volumes between the vehicle and the 140 μg/kg cohort were statistically significant from day 9 of treatment (P = 0.01) until the end of the experiment. With 100 μg/kg treatment, the tumor volume also decreased compared with the controls, but differences did not reach statistical significance (P = 0.08). Kaplan-Meier survival analysis revealed a significant prolongation of survival for the Aplidin-treated groups compared with the vehicle-treated controls (log-rank P = 0.02; Fig. 6B).

Figure 5. Effects of Aplidin treatment on Fas/CD95 translocation into lipid rafts and on JNK and p38 signaling. A, translocation of Fas/CD95 into lipid rafts in Aplidin-treated MM cells. Untreated MM144 cells (Control) and MM144 cells treated with 10 nmol/L Aplidin for 15 h (Aplidin) were lysed in 1% Triton X-100 and fractionated by centrifugation on a discontinuous sucrose density gradient. An equal volume of each collected fraction was subjected to SDS-PAGE before analysis of Fas/CD95 with a specific antibody. Location of GM1-containing lipid rafts (fractions 3–5) was determined using cholera toxin B subunit conjugated to horseradish peroxidase. Representative of three experiments. B, MM144 cells were untreated (Control) or pretreated with methyl–β–cyclodextrin (MCD) and then incubated with 10 nmol/L Aplidin (Aplidin) for 24 h and examined for the percentage of apoptotic cells by flow cytometry. Columns, mean of three independent determinations; bars, SE. C, MM15 cells were treated for the indicated times with 100 nmol/L Aplidin (left) or 25 nmol/L bortezomib (right) and lysed. One part of the cell extracts were used for the analyses of JNK activity using glutathione S-transferase-c-Jun. Another part of the extracts were used to detect p-Erk1/2, Erk1/2, p-p38, and poly(ADP-ribose) polymerase by Western blots. Erk5 was analyzed by Western blotting of anti-Erk5 immunoprecipitates. Shown are the results of an experiment that was repeated twice. D, MM15 cells were pretreated for 60 min with inhibitors for p38 kinase (SB203580) and JNK (SP600125) and subsequently treated with Aplidin for 6 and 12 h. Induction of cell death in each condition was assessed by Annexin V staining.
Antimyeloma Activity of Aplidin

The results of this preclinical study suggest a favorable profile of the in vitro and in vivo anti-MM activities of Aplidin. First, this compound exhibits potent in vitro activity against both MM cell lines and primary MM tumor cells, including cell lines resistant to established anti-MM treatments. Furthermore, Aplidin was able to induce cytotoxicity in myelomatous plasma cells obtained from patients with clinical resistance to most of the current treatments for the clinical management of MM, including combination chemotherapy regimens, autologous stem cell transplant, as well as novel agents such as bortezomib or thalidomide and their combinations. Interestingly, among the primary tumor samples tested with Aplidin, one of the most sensitive ones (patient sample no. 3) had shown clinical refractoriness to all of these treatments. These data suggest a different mechanism of action of Aplidin than other antimyeloma agents and suggest that it may overcome resistance generated against currently available therapies for MM. Moreover, our studies also show that the potency of Aplidin is high and comparable to that of bortezomib, a most potent anti-MM agent in vitro. Additional favorable aspects of Aplidin include the sparing of normal hematopoietic cells at concentrations that are still active against MM cells; the ability to overcome the protective effect of growth factors, such as IL-6 or IGF-I, on MM cells; and the additive (bortezomib and melphanal) or synergistic (dexamethasone, thalidomide, and lenalidomide) effects when Aplidin is combined with other anti-MM agents. Another positive feature of the anti-MM activity of Aplidin is that even a brief exposure of MM cells to this agent is capable of committing them to undergo cell death. Indeed, a 1-hour incubation with clinically relevant concentrations of Aplidin, based on pharmacokinetic studies from clinical trials in solid tumors (20), was capable of irreversibly initiating the process of MM cell death in vitro. Finally, this anti-MM activity of Aplidin in MM cells was confirmed in an in vivo setting, as evidenced by a delay in tumor growth and prolongation of survival in Aplidin-treated MM-bearing mice. Importantly, the toxicity profile of Aplidin in this model was favorable, as evidenced by the lack of major side effects.

Cell cycle studies indicated that low doses of Aplidin caused G2-M accumulation. In addition, molecular profiling studies indicated that Aplidin down-regulated several genes required for cell cycle progression. In addition to the cell cycle effects, Aplidin triggered increased Annexin V staining, decreased Δψm, and DNA laddering. It is important to emphasize the prompt increase in Annexin V staining, which is detectable early, after 3 hours of treatment. Due to this rapid proapoptotic action of Aplidin, we selected early times of exposure to Aplidin (4 hours) to identify genes involved in the triggering of cell death. These gene expression analyses identified several genes, including c-JUN and c-MYC, that might physically interact with Aplidin and function as exclusive mediator(s) of its antitumor effects. Our results offer new insights into the mechanism of action of Aplidin on tumor cells. Here we found that Aplidin induces apoptosis in MM cells, at least in part, through the translocation of the death receptor Fas/CD95 into lipid rafts. In addition, our data suggest that this is not the only mechanism of action of Aplidin. The difficulty in delineating a singular molecular target for Aplidin results, in part, from its complex molecular structure, which includes many potential biologically active moieties, and the pleiotropicity of its molecular sequelae. Both of these factors, which often apply as well for other similarly identified natural products, can confound our ability to pinpoint specific candidate molecule(s) that might physically interact with Aplidin and function as exclusive mediator(s) of its antitumor effects. Our studies, however, do provide insight into the pathways involved in the anti-MM activity of this compound. The rapid commitment of MM cells to undergo cell death on exposure to Aplidin and the early activation of JNK signaling are similar to the effects of the proteasome inhibitor bortezomib. However, whereas Aplidin also activates p38, bortezomib treatment is enhanced by p38 inhibition (29). Therefore, although equimolar concentrations of Aplidin and bortezomib have similar potency in rapidly killing MM cells in vitro, they have distinct differences in their molecular effects (11). This apparent dichotomy may be related to the ability of the p38 MAPK

Discussion

The results of this preclinical study suggest a favorable profile of the in vitro and in vivo anti-MM activities of Aplidin. First, this compound exhibits potent in vitro activity against both MM cell lines and primary MM tumor cells, including cell lines resistant to established anti-MM treatments. Furthermore, Aplidin was able to induce cytotoxicity in myelomatous plasma cells obtained from patients with clinical resistance to most of the current treatments for the clinical management of MM, including combination chemotherapy regimens, autologous stem cell transplant, as well as novel agents such as bortezomib or thalidomide and their combinations. Interestingly, among the primary tumor samples tested with Aplidin, one of the most sensitive ones (patient sample no. 3) had shown clinical refractoriness to all of these treatments. These data suggest a different mechanism of action of Aplidin than other antimyeloma agents and suggest that it may overcome resistance generated against currently available therapies for MM. Moreover, our studies also show that the potency of Aplidin is high and comparable to that of bortezomib, a most potent anti-MM agent in vitro. Additional favorable aspects of Aplidin include the sparing of normal hematopoietic cells at concentrations that are...
pathway to respond to different stimuli with diverse biological effects, which can also depend on the specific cellular context (30). For example, p38 can trigger apoptosis in some cells but inhibit its induction in other pathophysiologic settings (30). It has been proposed (as reviewed in ref. 31) that such stress-activated MAPK pathways may function as a double-edged sword: They may protect normal cells against environmental stress and malignant cells against forms of stress inherent to the neoplastic transformation (e.g., oncogenic “addiction,” drug treatments); conversely, they may also trigger apoptosis if the stress levels exceed a threshold (31), which in turn is influenced by the particular context in which the tumor cells are placed. We hypothesize that p38 activation, in the context of Aplidin treatment, is complemented by other molecular events (also triggered by the compound) that reinforce the proapoptotic role of p38. Such complementary molecular events include the suppression of mcy (an important regulator of proliferative/antiapoptotic responses for MM cells; ref. 27) or antiapoptotic molecules such as survivin (32, 33) and/or the increased expression of proapoptotic modulators such as TRAIL (9, 32, 34, 35) and Smac (36, 37).

This favorable preclinical profile of the anti-MM activity of Aplidin provided the rationale for the initiation of a clinical trial of this agent in patients with advanced MM (38). Preliminary results of this ongoing clinical study indicate that Aplidin has promising clinical anti-MM activity in heavily pretreated MM patients refractory to several conventional and novel anti-MM agents (e.g., bortezomib and/or lenalidomide; ref. 38). A high proportion of patients in this trial had received prior high-dose therapy and autologous stem cell transplant (60%), thalidomide (58%), and/or bortezomib (48%). Among 26 evaluable patients, 2 (8%) achieved partial response, 3 (12%) showed minor response, and 8 (31%) had stable disease. Due to the enhanced anti-MM activity observed with the in vitro combination of these agents, high-dose dexamethasone was added to Aplidin in patients not achieving response, and preliminary results of the clinical combination regimen are also very encouraging. Importantly, in the clinical trials Aplidin did not cause significant toxicities that are present in established anti-MM agents, such as neuropathy or myelosuppression (38). Furthermore, preliminary data indicate that its combination with dexamethasone is not associated with the thromboembolic complications observed with the combinations of glucocorticoids with thalidomide or lenalidomide (39, 40).

In conclusion, our preclinical data on the potent anti-MM activity of single-agent Aplidin and its potential in vitro synergistic/additive effect with other anti-MM agents, coupled with promising clinical data on the safety profile and objective clinical responses in heavily pretreated refractory patients, indicate that this novel compound (alone or in combination with other agents) may contribute to improve the outcome of MM patients.

Disclosure of Potential Conflicts of Interest


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