PM02734 (Elisidepsin) Induces Caspase-Independent Cell Death Associated with Features of Autophagy, Inhibition of the Akt/mTOR Signaling Pathway, and Activation of Death-Associated Protein Kinase

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Abstract

Purpose: PM02734 (elisidepsin) is a synthetic marine-derived cyclic peptide of the kahalalide family currently in phase II clinical development. The mechanisms of cell death induced by PM02734 remain unknown.

Experimental Design: Human non–small-cell lung cancer (NSCLC) cell lines H322 and A549 were used to evaluate PM02734-induced cytotoxicity, apoptosis, and autophagy, as well as effects on cell death–related signaling pathways.

Results: PM02734 at clinically achievable concentrations (0.5–1 μmol/L) was cytotoxic to H322 and A549 cells but did not cause nuclear fragmentation, PARP cleavage, or caspase activation, suggesting that classical apoptosis is not its main mechanism of cell death. In contrast, PM02734-induced cell death was associated with several characteristics of autophagy, including an increase in acidic vesicular organelle content, levels of GFP-LC3–positive puncta, elevation of the levels of Atg-5/12 and LC3-II, and an associated compromise of the autophagic flux resulting in increased number of autophagosomes and/or autolysosomes. Cotreatment with 3-methyladenine (3-MA) and downregulation of Atg5 gene expression by siRNA partially inhibited PM02734-induced cell death. PM02734 caused inhibition of Akt/mTOR signaling pathways and cotreatment with the Akt inhibitor wortmannin or with the mTOR inhibitor rapamycin led to a significant increase in PM02734-induced cell death. Furthermore, PM02734 caused the activation of death-associated protein kinase (DAPK) by dephosphorylation at Ser308, and downregulation of DAPK expression with siRNA caused also a partial but significant reduction of PM02734-induced cell death.

Conclusions: Our data indicate that PM02734 causes cell death by a complex mechanism that involves increased autophagosome content due for the most part to impairment of autophagic flux, inhibition of the Akt/mTOR pathway, and activation of DAPK. This unique mechanism of action justifies the continued development of this agent for the treatment of NSCLC.

Introduction

PM02734 (elisidepsin; Fig. 1A) is a synthetic marine-derived cyclic peptide belonging to the kahalalide family of compounds (1). We and others have previously shown that PM02734 is a potent cytotoxic agent both in vitro and in vivo in human non–small-cell lung cancer (NSCLC) cell lines (2). PM02734 is currently in phase II clinical development with preliminary evidence of antitumor activity. The cellular and molecular mechanisms of PM02734-induced cell death remain to be elucidated.

Type I programmed cell death (apoptosis) is a highly and genetically regulated cell suicide response to facilitate normal embryonic development and homeostasis of multicellular organisms. Apoptotic cell death is characterized by cell shrinkage, chromat condensation, nucleosomal DNA fragmentation, and formation of apoptotic bodies. Activation of the caspase family of cysteine proteases plays a crucial role in the regulation of apoptosis in the initial and execution stages (3). In addition, apoptosis is an important mechanism of drug-induced tumor cell killing and susceptibility to apoptosis in tumor cells is an important
IFN- mediated in response to various apoptotic stimuli such as with actin cytoskeleton (12). It plays a role as a positive calmodulin-regulated serine/threonine kinase associated shown to trigger cell death with autophagy (11), as mTOR (10). Interestingly, inhibition of mTOR signaling has been synthesis, cell-cycle progression, and cell proliferation way, downstream of PI3K/AKT, is implicated in protein pathway causes cell death associated with apoptosis and/or signaling pathway. Inhibition of the PI3K/AKT signaling nositide 3-kinase (PI3K)/AKT pathway acts as a cell survival or cell death pathway. The role of autophagy as a cell survival or cell death pathway is controversial, a number of studies have described cell death with autophagic features in response to various anti-tumor agents (6–8). The activation of the Class I phosphoinositide 3-kinase (PI3K)/AKT pathway is a cell survival signaling pathway. Inhibition of the PI3K/AKT signaling pathway causes cell death associated with apoptosis and/or autophagy (9). The activation of the mTOR signaling pathway, downstream of PI3K/AKT, is implicated in protein synthesis, cell-cycle progression, and cell proliferation (10). Interestingly, inhibition of mTOR signaling has been shown to trigger cell death with autophagy (11), as mTOR acts as a suppressor of the autophagic pathway.

Death-associated protein kinase (DAPK) is a calcium/calcmodulin-regulated serine/threonine kinase associated with actin cytoskeleton (12). It plays a role as a positive mediator in response to various apoptotic stimuli such as TNF-α (13), activation of Fas receptors, TNF-α, TGF-β (14), ceramide-, and p53-mediated apoptosis (15), as well as in the disruption of the extracellular matrix and resulting cell detachment. DAPK appears to function as a sensor that triggers early apoptotic events (16). In addition, DAPK acts as a tumor suppressor, as loss of DAPK expression is associated with hypermethylation is involved in tumor formation (17). Recent studies have shown that DAPK is a novel regulator of autophagic signaling in cell death mediated by estrogen receptor (ER) stress (18). DAPK belongs to a family of highly related death-associated kinases, which includes DRP-1 and ZIP kinases (19). DAPK contains a kinase domain, a calmodulin regulatory segment, 8 ankyrin repeats, a cytoskeleton binding region, and a death domain. The activation of DAPK depends on its dephosphorylation at serine 308 in the Ca<sup>2+</sup> calmodulin regulatory domain in response to cell death stimuli, resulting in its proapoptotic activation (20). In addition, activation of DAPK may have a direct role in the regulation of autophagic signaling pathways (21).

In this article, we show that PM02734 induces cell death associated with autophagic features including an increase in the number of acidic vacuole organelles, due, for the most part, to a remarkable compromise in the clearance of autophagosomes and/or autophagolysosomes. PM02734 also caused inhibition of AKT/mTOR signaling, and cotreatment with the AKT inhibitor wortmannin or with the mTOR inhibitor rapamycin enhanced PM02734-induced cytotoxicity associated with autophagic features. Furthermore, PM02734 induced the activation of DAPK and downregulation of DAPK gene expression by siRNA led to the attenuation of PM02734-induced cell death. PM02734 exhibited in vivo antitumor activity associated with the activation of the autophagic pathway in nude mice bearing human A549 NSCLC xenografts. Overall, these observations provide novel insight into the complex mechanism of PM02734-induced cell death that relays in part in its effects on the autophagic pathway at the level of steady-state number and clearance of autophagic compartments. Our findings should help building a rational basis for its therapeutic application either alone or in combination with other chemotherapeutic agents.

**Materials and Methods**

**Chemicals and antibodies**

PM02734 was manufactured by PharmaMar and dissolved in dimethyl sulfoxide (DMSO) as a stock concentration of 10 mmol/L and diluted to the indicated concentrations with culture medium. Polyclonal anti-caspase-8, caspase-9, caspase-3, anti-PARP antibody, anti-AKT, anti-phospho-AKT (Ser437) and anti-phospho-AKT (Thr308), anti-mTOR, and anti-phospho-mTOR (Ser2448), anti-S6 ribosomal protein, and anti-phospho-S6 ribosomal protein (Ser235/236), as well as anti-4E-BP1 antibody and anti-phospho-4E-BP1 (Thr37/46) antibodies were purchased from Cell Signaling Technology. Polyclonal anti-LC3 and anti-Arg-5 antibodies were obtained from Novus Biologicals. Polyclonal p62/SQSTM1 antibody and monoclonal anti-DAPK and anti-phospho-DAPK (Ser308) antibodies were purchased from Sigma-Aldrich. Other chemicals and reagents were obtained from Sigma-Aldrich, or from Biomol.

**Cell lines and cell culture**

Human NSCLC cell lines H322 and A549 were obtained from American Type Culture Collection. Cell lines were maintained in RPMI 1640 medium with 10% FBS and
maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2.

**Assay of cell viability and cell death**

Exponentially growing cells (1 × 10^5/mL) were plated in 96-well plates and allowed to attach overnight. Cells were exposed to various concentrations of PM02734 for the indicated times. Cell viability was assessed by the reduction of tetrazolium bromide (MTT) assay. For assay of cell death, cells were treated with PM02734 at different concentrations for the indicated times. After treatment, cell death was determined by trypan blue exclusion.

Figure 1. PM02734 induces caspase-independent cell death in H322 and A549 cells. A, chemical structure of PM02734. B, left, concentration-dependent cell death induced by PM02734. Cells were treated with various concentrations of PM02734 for 8 hours, and then cell death was assessed by trypan blue exclusion. B, right, time course of PM02734-induced cell death. H322 cells were treated with 0.5 μmol/L PM02734 and A549 cells were treated with 1 μmol/L PM02734 for the indicated times. After treatment, cell death was assessed by trypan blue exclusion. C, morphologic changes by PM02734 treatment in H322 and A549 cells. H322 cells were treated with 0.5 μmol/L PM02734 and A549 cells were treated with 1 μmol/L PM02734 for 8 hours and then stained with 4',6-diamidino-2-phenylindole (DAPI) solution. Morphologic and nuclear changes were assessed by fluorescence microscopy. Arrows indicate the condensed nuclei. D, left, effect of PM02734 on the activation of caspases. Cells were treated with 0.5 to 1 μmol/L PM02734 or with same volume of medium as negative control, or 1 μmol/L staurosporine as positive control. After treatment for 8 hours, the active form of cleaved caspase-8, caspase-9, and caspase-3, and cleavage of PARP protein, were determined by immunoblot analysis using the corresponding antibodies. β-Actin was used as a loading control. D, right, effect of Z-VAD-fmk, a pan-caspase inhibitor, on PM02734-induced cell death in A549 cells. Cells were treated with 1 μmol/L PM02734 or with 50 μmol/L Z-VAD-fmk alone, or cotreated with both agents, and cotreatment with 1 μmol/L staurosporine as a positive control. After a 24-hour treatment, cell death was determined by trypan blue exclusion. Data represent mean ± SD of three independent experiments. *, P < 0.05, compared with staurosporine alone.
blue exclusion as described by Scarlatti and colleagues (22).

Caspase activity assay

Cells were treated with 0.5 to 1 μmol/L PM02734 for 6 hours, and then cell extracts were prepared with extraction buffer. Ten microliter of cell extracts (10–30 μg of protein) were added into 100 μL of reaction mixture containing 12 μmol/L Ac-DEVD-pNA (Biomol) as the substrate for caspase-3 in a 96-well microplate. After incubation at room temperature for 120 minutes, the amount of p-nitroaniline–derived substrate cleavage by caspase-3 was determined in a microplate reader (Molecular Devices) at 405 nm.

Acridine orange staining for autophagy detection

Cells were treated with 0.5 to 1 μmol/L PM02734 for the indicated times. After treatment, cells were washed twice with PBS solution and then stained with 1 μg/mL acridine orange solution (Invitrogen) at 37°C for 15 minutes. The levels of acidic vesicular organelles (AVO) were assessed by counting the number of bright red fluorescent cells from a total of at least 200 cells with a Nikon fluorescence microscope.

Transfection with GFP-LC3 cDNA plasmid or siRNA

GFP-LC3 plasmid was a gift from Dr. Mizushima (Department of Bioregulation and Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Cells grown at about 70% confluence were transiently transfected with 1 μg/mL of GFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The localization of GFP-LC3 in transfected cells was assessed by fluorescence microscopy. For downregulation of Atg-5 and DAPK gene expression, Atg-5 siRNA and DAPK siRNA were purchased from Dharmacon. Transfections of siRNA were carried out with Lipofectamine 2000 according to the manufacturer’s instructions.

Transmission electron microscopy

A549 cells were treated with 1 μmol/L PM02734 or with the same volume of medium containing 0.1% DMSO as a control at 37°C for 3 hours. After treatment, cells were washed twice with PBS, and then fixed with 0.5 mL of ice-cold glutaraldehyde (2.5% in 0.1 mol/L cacodylate buffer, pH 7.4) at 4°C overnight. After washing, cells were fixed in 1% OsO4 and embedded in polybed resin. The ultrathin sections were doubly stained with uranyl acetate and lead citrate and analyzed by transmission electron microscopy (JEOL).

Immunoblot analysis and LC3-II flux measurements

Cells were scraped from culture dishes, and cell lysates were prepared with lysis buffer. Immunoblot analysis was conducted as previously described (23). Autophagic flux was calculated from the densitometric analysis of the immunoblots for LC3, as the ratio of LC3-II levels in cells in the presence of bafilomycin over untreated cells (LC3 flux), or the amount of LC3-II in bafilomycin-treated cells minus that in untreated cells (LC3 net flux; ref. 24).

Assay of DAPK activity

Endogenous DAPK was immunoprecipitated from PM02734-treated A549 cells at the indicated times and subjected to an in vitro kinase assay using myosin light chain kinase as a substrate, as described by Jin and colleagues (25). DAPK activity was assessed by measurement of phosphorylated MLC by immunoblot analysis using anti-phospho-MLC antibody.

Antitumor activity in vivo

Exponentially growing A549 cells (2 × 10⁶ cells) were subcutaneously injected into the flank of nude mice. Seven days after inoculation mice bearing tumors (volume around 100 mm³) were divided into 2 groups of 3 animals and treated with 0.1 mg/kg PM02734 or the same volume of vehicle as control by tail vein 3 times per week for 2 weeks. Body weight and tumor size were monitored at the indicated time points. All animal experiments were conducted in accordance with Institutional Protocol for Animal Experiments of the Albert Einstein College of Medicine. After 2 weeks, mice were sacrificed, and tumor tissues from control and PM02734-treated mice were harvested for assay of apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining or for assay of autophagy as measurement of the levels of LC3-II by either immunohistochemical staining or by immunoblotting using a polyclonal anti-LC3 antibody (NB600-1384; Novus Biologicals).

Statistical analysis

All data are presented as mean ± SD. Differences between groups were analyzed for statistical significance using a 2-tailed Student’s t test. P < 0.05 was used as the significance level.

Results

PM02734-induced cell death is not associated with activation of the classic apoptotic pathway in H322 and A549 cells

We have previously studied the cytotoxic effect of PM02734 on a panel of human NSCLC cell lines and observed that H322 and A549 cell lines are highly sensitive to PM02734 with IC₅₀ values of 0.5 to 1 μmol/L (2). Initially, we treated H322 and A549 cells with varying concentrations of PM02734 for 8 hours or with 0.5 μmol/L PM02734 (H322 cells) or 1 μmol/L PM02734 (A549 cells) for the indicated times to evaluate drug-induced cell death as measured by trypan blue exclusion. As shown in Figure 1B, PM02734 induces cell death in a concentration- and time-dependent manner, about 12% to 17% cell death can be detected at 4 hours after drug exposure and 37% to 40% at 24 hours. By morphologic assessment, PM02734 induced disruption of membrane...
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integrity and nuclear condensation but did not cause chromatin fragmentation (Fig. 1C), suggesting that cell death induced by PM02734 does not have the characteristics of classical apoptosis. To further verify drug-induced membrane disruption, we treated A549 cells with varying concentrations of PM02734 for 6 hours and then stained them with fluorescein isothiocyanate (FITC)-labeled Annexin V. PM02734 treatment caused FITC-labeled Annexin V–positive staining cells with exposure of phosphatidylserine outside the plasma membrane occurring at 1 μmol/L and increasing at 10 μmol/L (data not shown). We next explored whether PM02734-induced cell death was associated with caspase activation. Immunoblot analysis revealed that PM02734 treatment for 8 hours did not markedly induce the formation of the active forms of cleaved caspase-8, caspase-9, and caspase-3, whereas the active forms of caspase-8 (43 and 41 kDa), caspase-9 (39 and 37 kDa), and caspase-3 (28, 19, and 17) were clearly observed in the tested cell lines following treatment with 1 μmol/L staurosporine. Consistently, the cleavage of PARP, which is a substrate of caspase-3 and a well-known apoptotic hallmark, was observed only in cells treated with staurosporine but not in PM02734-treated cells (Fig. 1D, left). Quantitative analysis of caspase-3 activity by colorimetric assay also showed that PM02734 treatment did not alter caspase-3 activity in the tested cell lines, whereas staurosporine treatment led to an increase in caspase-3 activity (data not shown). Moreover, cotreatment with 50 μmol/L Z-VAD-fmk, a pan caspase inhibitor, failed to inhibit PM02734-induced A549 cell death but effectively reduced staurosporine-induced A549 cell death (Fig. 1D, right). These data suggest that PM02734-induced cell death is not associated with the caspase-dependent apoptotic signaling cascade.

PM02734-induced cell death is associated with the activation of the autophagic signaling pathway in H322 and A549 cells

Because PM02734-induced cell death appeared to lack the characteristics of classical apoptotic type 1 cell death, we sought to determine whether it was associated with changes in the autophagic pathway previously described as characteristics of type 1 cell death. We first determined the effect of PM02734 on the levels of AVOs by staining tested cells with acidic vesicle solution as described by Paglin and colleagues (26). Figure 2A, left, shows acidic vesicle organelles in both tested cell lines following treatment with PM02734 for 8 hours, the cytoplasm fluorescence changing from bright green to bright red. Quantitative analysis showed about 60% to 66% cells with an expansion of their AVOs as compared with 15% to 23% in control cells (P < 0.01; Fig. 2A, right). These results support that treatment with PM02734 results in enlargement of the endosomal/lysosomal system in cancer cells. To determine the possible autophagic nature of the PM02734-induced compartments, next, we determined the effect of the drug on the intracellular localization of microtubule-associated protein 1 light chain 3 (LC3), a specific marker of autophagosomes (27), in the tested cell lines. Initially, we transiently transfected cells with GFP-LC3 plasmid for 36 hours and then treated them with PM02734, or with the same volume of medium containing 0.1% DMSO as a control, for an additional 8 hours. The cellular localization of GFP-LC3 protein was assessed by fluorescence microscopy. As shown in Figure 2B, left, representative fluorescence micrographs show a punctate pattern of GFP-LC3 in PM02734-treated cells in contrast to the diffuse pattern in control cells. Quantitative analysis revealed a significant increase in cells with GFP-LC3 punctate pattern in PM02734-treated cells as compared with control cells (P < 0.001), that is, ~38% versus ~20% in H322 cells and ~36% versus ~17% in A549 cells, respectively (right). To further verify an increase in autophagic markers induced by PM02734, we assessed the effect of PM02734 on the levels of LC3 (Fig. 2B, right). Figure 2C, left, the immunoblot analysis revealed that PM02734 treatment led to a time-dependent increase in the levels of conjugated Atg-5 and LC3-II proteins in both tested cell lines. Quantitative analysis indicated that PM02734 treatment led to an increase in Atg-5/G-12 and LC3-II levels as early as 4 hours after drug exposure and gradually increasing thereafter (Fig. 2C, right). To determine whether PM02734-induced accumulation of LC3-II is caused by enhanced formation of autophagic vacuoles or by blockade of autophagic vacuole clearance, we treated cells with PM02734 in the absence or presence of the inhibitor of lysosomal proteolysis bafilomycin A1 (BFA; ref. 24). As shown in Figure 2D (top), LC3-II levels were increased in PM02734-treated cells compared with that in control cells both in the presence and in the absence of bafilomycin. However, measurement of the increase in LC3-II after addition of bafilomycin (which reflects the amount of autophagosomes degraded in lysosomes) revealed that differences between PM02734-treated cells and untreated cells were no longer evident (Fig. 2D). These results support that the increase in the amount of autophagosomes was in large part resulting from their compromised clearance by lysosomes. Reduced autophagosome clearance was also supported when following the degradation of p62/SQSTM1, a polyubiquitin-binding protein that is degraded by autophagy (29, 30). Although levels of p62 were markedly reduced in H322 and A549 cells treated with PM02734, the difference was not due to increased degradation as the lower p62 content in treated cells was still evident upon bafilomycin blockade of lysosomal degradation (Fig. 2D); however, the fact that even after treatment of PM02734, we found a slight but consistent increase in p62 levels in bafilomycin-treated cells supports that some level of autophagosomes/lysosome fusion still occurs in these cells. These data suggest that PM02734 may lead to increase content of cellular autophagosomes (increased Atg-5/12 levels) by preventing their clearance (reduced autophagic flux; Fig. 2D, bottom). Finally, we confirmed...
the increased content of autophagic vacuoles by transmission electron microscopy in A549 cells following treatment with 1 μmol/L PM02734, or with the same volume of medium containing 0.1% DMSO as a control, for 8 hours. As shown in Figure 2E, treatment with PM02734 caused the accumulation of autophagic vacuoles, which exhibited autophagosome and/or autolysosomal characteristics (double and multilayer membranes containing identifiable mitochondrial and cytoplasmic components), whereas only a few vacuoles were observed in control cells. Overall, these results suggest that PM02734-induced cell death may result, at least in part, from the abnormal accumulation of autophagic compartments, which has been shown to be toxic for cells in different cellular settings (31).
Effects of 3-MA and downregulation of Atg-5 expression on PM02734-induced autophagic cell death in H322 and A549 cells

To confirm the contribution of the increase in the autophagic compartment to PM02734-induced cell death, we analyzed the effect of 3-methyladenine (3-MA), a well-known inhibitor of autophagosome formation (32). First, we cotreated H322 and A549 cells with 2 mmol/L 3-MA and 0.5 to 1 μmol/L PM02734 for 8 hours, and then harvested cell pellets to determine the amount of the autophagic marker LC3-II. As expected, PM02734 treatment resulted in increased levels of LC3-II in H322 and A549 cells. 3-MA treatment led to a significant decrease in PM02734-induced LC3-II expression in the 2 cell lines (Fig. 3A and B, top and middle). In addition, we tested whether cotreatment with 3-MA could affect PM02734-induced cell death. As shown in Figure 3A and B, bottom, cotreatment with 2 mmol/L 3-MA resulted in a significant but partial inhibition of PM02734-induced cell death, as measured by MTT assay, in H322 and A549 cell lines. Given...
that Atg-5 protein is essential for induction of autophagy (33), we next determined whether the downregulation of Atg-5 gene expression by transfection with Atg-5 siRNA could affect PM02734-induced cell death. As shown in Figure 3C and D, downregulation of Atg-5 gene expression produced a significant although partial attenuation of PM02734-induced cell death as measured by MTT assay in H322 and A549 cells. The fact that both chemical and genetic blockade of autophagosome formation reduced cell death confirmed that the observed accumulation of autophagosomes in cells treated with this drug contributes at least, in part, to its cellular toxicity.

Role of Akt/mTOR signaling pathways in PM02734-induced cell death in H322 and A549 cells

Activation of the Akt signaling pathway has been shown to be involved in cell survival through phosphorylation at Thr308 by PDK1 or at Ser473 by mTOR kinase (34). mTOR, one of downstream targets of Akt, plays a central role in the regulation of protein synthesis by phosphorylation of p70 S6 kinase and 4E-BP1 (35). Recent investigations have showed that the inhibition of this signaling pathway is linked to the triggering of autophagy (36). Thus, we sought to test whether PM02734 could induce autophagy by inhibition of the Akt/mTOR signaling pathways. As shown in Figure 4A, PM02734 treatment caused a marked reduction in Akt phosphorylation at Ser473 and Thr308, along with the inhibition of phosphorylation of mTOR at Ser2448, as early as 4 hours after exposure and increasing thereafter. In parallel, PM02734 inhibited the phosphorylation of S6 ribosomal protein and 4E-BP1, which are downstream targets in response to Akt/mTOR signaling. Of interest, 4E-BP1 appeared to be more susceptible to phosphorylation during the 4- to 24-hour period after drug exposure (Fig. 4B, right). To further confirm that PM02734-induced DAPK dephosphorylation was already observed at 2 hours of drug exposure. To further explore the role of Akt/mTOR signaling in the regulation of PM02734-induced cell death, we cotreated H322 and A549 cells with PM02734 and the Akt inhibitor wortmannin or the mTOR inhibitor rapamycin for 24 hours. Following treatment, cell viability was determined by MTT assay, and the LC3-II levels were assayed by immunoblotting. As shown in Figure 4B, either cotreatment with wortmannin or with rapamycin resulted in a significant enhancement of cell death as compared with PM02734 alone (P < 0.05 and 0.01). Although wortmannin has been shown to inhibit PI3K class III and compromise autophagosome formation, in a similar manner to previously shown for 3MA, this did not seem to be the case in these cells in the conditions of our assay. In fact, the immunoblot analysis revealed an increased amount of LC3-II in these cells when treated with wortmannin almost comparable to the one induced by rapamycin. Addition of PM02734 further increased LC3-II levels in cells treated with either wortmannin or rapamycin, further supporting that the effect of PM02734 was not only at the level of autophagosome formation but also on autophagosome clearance (Fig. 4C).

To further test whether cotreatment with Akt and mTOR inhibitors could affect PM02734-induced autophagic flux, we also determined the intracellular levels of p62 protein. As shown in Figure 4D, PM02734 markedly suppressed p62 expression as compared with that in control cells. Treatment with Akt inhibitor wortmannin alone led to a remarkable accumulation of p62 protein; however, treatment with mTOR inhibitor rapamycin caused an effective decrease in p62 protein. Of interest, cotreatment with PM02734 resulted in a significant suppression of p62 levels as compared with wortmannin or rapamycin alone (P < 0.01). These results suggest that the inactivation of Akt/mTOR pathway by PM02734 may be involved in the regulation of drug-induced autophagy and cell death.

Role of activation of DAPK in PM02734-induced cell death in A549 cells

Recent studies have shown that the activation of DAPK is associated with the triggering of types I and II cell death (5, 19, 37). Thus, we wanted to test whether PM02734-induced cell death could be associated with the activation of DAPK in A549 cells. Initially, we tested the effect of PM02734 on DAPK expression and found that PM02734 treatment caused the degradation of full-size DAPK (160 kDa) accompanied by increased levels of its corresponding cleaved fragments (100 and 60 kDa) in a concentration- and time-dependent manner (Fig. 5A). Although the significance of DAPK degradation remains largely unknown, several studies have shown that the generation of proteolytic DAPK fragments (100 or 60 kDa) is involved in the initiation of the apoptotic and autophagic cascades (20, 38). Furthermore, recent investigations have shown that the activation of DAPK depends on its dephosphorylation at Ser308 (20). We therefore determined the effect of PM02734 on DAPK phosphorylation at Ser308 by immunoblot analysis using the corresponding antibody. Of interest, PM02734 treatment strongly induced the dephosphorylation of DAPK at Ser308 in a concentration- and time-dependent manner. As shown in Figure 5B, left, 1 μmol/L PM02734 led to about 80% of DAPK dephosphorylation at Ser308 in A549 cells. Time course studies showed about 65% DAPK dephosphorylation as early as 1 hour after PM02734 exposure and about 80% DAPK dephosphorylation during the 4- to 24-hour period after drug exposure (Fig. 5B, right). To further confirm that PM02734-induced DAPK dephosphorylation was correlated with the enhancement of DAPK activity, we used MLC as a substrate to determine the effect of PM02734 on DAPK activity in vitro using immunoprecipitated endogenous DAPK as described in Materials and Methods. As shown in Figure 5C, DAPK activity was increased by about 150% as early as 1 hour of drug exposure and reached its maximum (280% to 250%) after 2 to 4 hours of exposure, declining to basal levels after 24-hour exposure. Moreover, we determined the effect of downregulation of DAPK expression on PM02734-induced cell death. As shown in Figure 5D, downregulation of DAPK expression by siRNA resulted in a pronounced and significant attenuation of PM02734-induced cell growth inhibition as measured by MTT assay. In addition, down-regulation of DAPK by siRNA transfection also resulted in a

Figure 3C and D, downregulation of Atg-5 gene expression could affect PM02734-induced cell death. As shown in
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Figure 4. Role of inhibition of Akt/mTOR-related signaling pathways in PM02734-induced cell death in H322 and A549 cells. A, effect of PM02734 on the phosphorylation of Akt/mTOR signaling. H322 cells were treated with 0.5 μmol/L PM02734 and A549 cells were treated with 1 μmol/L PM02734 for the indicated times. After treatment, cells were harvested and cell extracts were prepared for immunoblot analysis. The levels of total Akt and mTOR, and phospho-Akt, and phospho-mTOR, total S6 ribosomal protein (RP) and 4E-BP1, as well as phospho-S6 RP and phospho-4E-BP1 were detected by immunoblot analysis using the corresponding antibodies. β-Actin was used as a loading control. B, effects of cotreatment with wortmannin and rapamycin on PM02734-induced cell death. Cells were treated with 0.5 to 1 μmol/L PM02734 or 10 μmol/L wortmannin (wort), an Akt inhibitor, or 1 μmol/L rapamycin (rap), an mTOR inhibitor alone or in combination with PM02734 for 24 hours. After treatment, cell viability was determined by MTT assay. Each column represents the mean ± SD of 3 independent experiments. *, P < 0.05; **, P < 0.01, compared with PM02734 alone. C, effects of cotreatment with wortmannin and rapamycin on PM02734-induced increase of LC3 protein levels. Cells were treated with PM02734, wortmannin, or rapamycin alone, or with combinations of PM02734 and either other agent as described above. After treatment, cells were harvested and cell extracts were prepared for immunoblot analysis. The levels of LC3-II were detected by immunoblot using a polyclonal anti-LC3 antibody. β-Actin was used as a loading control (top). After normalization to β-Actin, the relative levels of LC3-II were analyzed by comparison with control which was given the value of 1. Each column represents the mean ± SD of 3 independent experiments. *, P < 0.05 and **, P < 0.01 (bottom). D, effects of PM02734, wortmannin, rapamycin alone, or combined treatment on intracellular p62 levels. After treatment as described above, cells were harvested and cell lysates were prepared for detection of p62 protein by immunoblot using a polyclonal anti-p62 antibody. β-Actin was used as a loading control (top). After normalization with β-Actin, the relative levels of p62 were compared with control which was given the value of 1. Each column represents the mean ± SD of 3 independent experiments. **, P < 0.01 (bottom).

PM02734 inhibition of human NSCLC A549 xenografts is associated with autophagosome accumulation

To investigate the antitumor efficacy of PM02734 in vivo, we conducted a dose escalation toxicity trial in mice and found that an intravenous dose of 0.1 mg/kg was well tolerated. We then established a xenograft model of human A549 NSCLC cells in nude mice. Exponentially growing A549 cells (2 × 10⁵ cells) were subcutaneously inoculated in the flank of nude mice. Seven days after inoculation, when tumor volume is about 100 mm³, mice were randomly assigned to control (vehicle) and PM02734 treatment groups. Mice bearing established A549 tumors were given intravenous injections of 0.1 mg/kg PM02734 or the same volume of vehicle 3 times per week for 2 weeks. As shown in Figure 6A, top, PM02734 caused about 50%
The expression of DAPK was monitored by immunoblot using anti-DAPK antibody, 

hours. After transfection, cells were treated with various concentrations of PM02734 for 24 hours, and cell viability was determined by MTT assay. 

Figure 5. Role of the activation of DAPK in PM02734-induced cell death in A549 cells. A, PM02734 induces the degradation of DAPK in a concentration- and time-dependent manner. A549 cells were treated with various concentrations of PM02734 for 8 hours or with 1 μmol/L PM02734 for the indicated times. Following treatment, cells were harvested and cell extracts were prepared for immunoblot analysis. The full size of DAPK and its cleaved fragments were detected by immunoblot with a monoclonal anti-DAPK antibody (left). β-Actin was used as a loading control. The relative levels of full-size and cleaved DAPK were analyzed in comparison with control or exposure time 0 (right). Each column represents the mean ± SD of 3 independent experiments. *, P < 0.05; **, P < 0.01, compared with control or with time 0. B, PM02734 induces DAPK dephosphorylation at Ser308 in a concentration- and time-dependent manner. Cells were treated with PM02734 as described above. After treatment, cell extracts were prepared for assay of DAPK phosphorylation at Ser308 by immunoblot using a monoclonal anti-phospho-DAPK antibody. β-Actin was used as a loading control. The relative level of phospho-DAPK at Ser308 was analyzed in comparison with control or with exposure time 0. Each column represents the mean ± SD of 3 independent experiments. ***, P < 0.001, compared with control or with time 0. C, effect of PM02734 on DAPK activity. A549 cells were treated with 1 μmol/L PM02734 for the indicated times. Following treatment, endogenous DAPK was immunoprecipitated, and DAPK activity was assessed by measurement of MLC phosphorylation as described in Materials and Methods. Each point represents the mean of 2 independent experiments. **, P < 0.01, compared with control or with time 0. D, effect of downregulation of DAPK gene expression by siRNA on PM02734-induced cell viability. A549 cells were transiently transfected with mock or DAPK siRNA or nontransfection for 24 hours. After transfection, cells were treated with various concentrations of PM02734 for 24 hours, and cell viability was determined by MTT assay. The expression of DAPK was monitored by immunoblot using anti-DAPK antibody, β-actin was used as a loading control (right). Each point represents the mean ± SD of 3 independent experiments. **, P < 0.01, compared with nontransfection. E, effect of downregulation of DAPK on PM02734-induced LC3-II expression. siRNA-transfected or controlled A549 cells were treated with 1 μmol/L PM02734 or with the same volume of medium as control for 8 hours. After treatment, cell extracts were prepared for assay of LC3 expression by immunoblot using a polyclonal anti-LC3 antibody. β-Actin was used as a loading control. The relative LC3-II levels were analyzed in comparison with control which was assigned a value of 1. Each column represents mean ± SD of 3 independent experiments. *, P < 0.05, compared with control cells.
tumor growth inhibition ($P < 0.05$) as compared with control animals. No body weight loss was observed in drug-treated mice compared with control mice (Fig. 6A, bottom). To investigate the mechanism of PM02734-induced tumor growth inhibition in vivo, mice were sacrificed and tumor tissues collected 1 day after the last PM02734 dose. Apoptosis was assessed by TUNEL staining and autophagy by LC3 expression by immunohistochemical staining and immunoblot analysis. Figure 6B shows no differences in the fraction of TUNEL-positive staining cells between PM02734-treated tumors and control tumors. In contrast, the expression of total cytoplasmic LC3 detected by immunohistochemical staining was markedly elevated in PM02734 treated tumors compared with control tumors (Fig. 6C). Consistently, the immunoblot analysis revealed that LC3-II levels were higher in PM02734-treated tumors than in control tumors, whereas no differences were observed between PM02734 treatment and control tumors in Bcl-2 and Bax levels (Fig. 6D). All these data suggest that autophagosome accumulation is associated with PM02734-induced tumor growth inhibition in vivo.

**Discussion**

Our results indicate that PM02734-induced cell death is associated with features of autophagy including an increase in cellular content of AVOs and compromised autophagic clearance. The role of autophagy in cancer therapeutics is still controversial. Autophagy has been found to play a role as a cell survival mechanism by which cells can damage cytoplasmic proteins and organelles through lysosomal degradation and survive metabolic stress (39). On the other hand, autophagy has also been found to contribute to type II programmed cell death in response to hypoxia, chemotherapeutic agents, virus infection, and toxins (40). Our results suggest that the observed abnormal increase of the autophagic compartment can only partially explain...
the cytotoxic effect of PM02734, as cotreatment with 3-MA, a well-known autophagy inhibitor, or Agt-5 siRNA, an essential component for autophagosome formation, had only a partial protective effect on PM02734-induced cell death in H322 and A549 cells. It is possible that the PM02734-induced increase in autophagosome content could be, in part, related to induction of autophagy through its inhibitory effect on the Akt/mTOR signaling pathway and activation of DAPK. The accumulation of autophagic vacuoles induced by PM02734 was confirmed in vitro in A549 xenografts. This study is the first to show that PM02734-induced antitumor effect is, in part, mediated by an autophagic mechanism.

Apoptosis is an active form of cell death characterized by cell shrinking, cytoplasmic condensation, DNA ladder degradation, and nuclear fragmentation resulting in the formation of apoptotic bodies. In contrast, type II programmed cell death is characterized by the presence of visible autophagic cytoplasmic vacuoles, mitochondrial dilation, enlargement of the endoplasmic reticulum and the Golgi apparatus, and nuclear condensation without DNA laddering (41). Apoptosis and cell death with autophagy are not mutually exclusive and can coincide in vivo in certain tissues and in cell culture (42). In addition, apoptosis and autophagy may share certain mechanisms and alterations such as loss of mitochondrial permeability and membrane potential (43). The fact that PM02734-induced cell death could only be attenuated partially with autophagy inhibitors suggests that other mechanisms of cell death are involved in PM02734-induced caspase-independent cell death. In a previous work, we and others have shown that kahalalide F, a parent compound of PM02734, induces necrotic cell death (type III programmed cell death) linked to damage of the endoplasmic reticulum, Golgi apparatus, and the activation of lysosomes, as well as loss of the mitochondrial membrane potential and release of lactate dehydrogenase (LdH) from cytoplasm (44). Preliminary studies also show that PM02734 leads to a rapid depletion of intracellular ATP and loss of mitochondrial membrane potential in H322 and A549 cells (data not shown). It has been suggested that activation of necrotic signaling pathways may also be involved in PM02734-induced cell death.

An emerging body of evidence indicates that the Akt/mTOR pathway plays a crucial role in the regulation of both apoptosis and autophagy (36). We present here data indicating that PM02734 markedly inhibits Akt phosphorylation at Ser473 and at Thr308, as well as mTOR phosphorylation at Ser2448. In addition, we observed that downregulation of mTOR protein by PM02734 may explain at least in part mTOR dephosphorylation. Time course studies indicated that the inhibition of the Akt/mTOR signaling pathway occurred as early as 4 hours after PM02734 exposure, suggesting that the inhibition of Akt/mTOR is an early event in the drug-induced cell death process. As expected, phosphorylation of S6 ribosomal protein and 4E-BP-1, downstream targets of Akt/mTOR pathway, were inhibited by PM02734. However, we found that PM02734 caused degradation and dephosphorylation of 4E-BP-1, a translation factor for protein synthesis and a downstream element of the Akt/mTOR axis 2 hours after PM02734 exposure, suggesting that inactivation of the S6 ribosomal protein/4E-BP1 pathway preceded Akt/mTOR inhibition. Although the pharmacologic significance of PM02734-induced downregulation and inactivation of Akt/mTOR downstream events is unknown, these findings suggest that the effect of PM02734 on this pathway could be 2-fold; in one hand, it may induce autophagosome formation, which associated with the observed compromise in clearance would lead to cellular clogging, and in addition, it may directly or indirectly affect protein biosynthesis, which could further lead to suppression of cell proliferation and cell growth. Wortmannin has been shown to be an autophagy inhibitor, via inhibition of class III PI3K (45); however, its effect over class I PI3K would result in mTOR inhibition. In this work, we found that wortmannin markedly induces accumulation of the intracellular levels of LC3 in H322 and A549 cells, indicating that this compound may have a preferential effect on class I PI3K in these cells (45), as it did not seem to impact the conversion of LC3-I into LC3-II, or could be interfering with autophagosome clearance by lysosomes. Recent investigations have shown that autophagy inhibition at an early stage could trigger cell survival signaling pathways, preventing death due to stress; whereas, inhibition of apoptosis at a late stage could cause accelerated cell death under autophagy-inducing conditions via the activation of apoptotic signaling (46). Further studies are required to determine whether a combined effect of PM02734 activating early autophagy steps and compromising late autophagic stages may be behind its marked negative effect on cell viability. Interestingly, we here present data that cotreatment with 3-MA, which is an early autophagy inhibitor (45), led to the attenuation of PM02734-induced cell death, whereas cotreatment with wortmannin, which may inhibit autophagy at a late stage as discussed above, caused a significant enhancement of PM02734-induced cytotoxicity in 2 tested cell lines. Consistent with our findings, Shingu and colleagues recently reported that inhibition of autophagy by 3-MA attenuated imatinib-induced cell death, but inhibition of autophagy at a late stage with bafilomycin A1 enhanced drug-induced cytotoxicity in human malignant glioma cells (47). In addition, we also observed that cotreatment with the mTOR inhibitor rapamycin and PM02734 resulted in an additive effect on cell death associated with an increase in autophagosome content as shown by increase in LC3-II, suggesting that the inhibition of mTOR signaling may play a role in PM02734-induced autophagy. Overall, the role of the PI3K/Akt/mTOR axis in modulating PM02734-induced cell death appears highly complex and deserves further investigation. DAPK, Ca \(^{2+}\)/calmodulin-regulated serine/threonine kinases, has a wide range of functions in the regulation of cell growth and cell death. It functions as a tumor suppressor, is involved in p53-mediated checkpoint control resulting in inhibition of oncogene-induced cell...
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transformation, and is also an antitumor metastasis factor (12). Recent studies have shown that DAPK acts as a positive mediator, regulating different types of cell death signaling pathways including apoptosis and autophagy (18, 21). Of note, data presented in this work indicate that PM02734 induces the cleavage of full-size DAPK (160 kDa) into 2 small fragments (100 and 60 kDa) in a dose- and time-dependent manner. The biological role of such cleavage is unclear, although Shamloo and colleagues (38) have reported that the cleavage of full-size DAPK resulting in 100 and 60 kDa fragments was correlated with the induction of cell death in damaged rat brain by ischemic injury, suggesting that these cleaved fragments may represent the activated form of DAPK. Moreover, a number of investigations have shown that the activation of DAPK depends on its dephosphorylation at Ser308 (21). Consistently, we also found that PM02734 at a low concentration (1 μmol/L) induces DAPK dephosphorylation at Ser308 as early as 1 hour after exposure, and an increase in DAPK dephosphorylation over time. Accordingly, DAPK activity was induced after PM02734 exposure for 1 hour, peaked at 4 to 8 hours and declined thereafter, suggesting that the activation of DAPK may be an initiator of cell death. Of interest, downregulation of DAPK expression by siRNA caused a significant attenuation of PM02734-induced cytotoxicity and also blocked drug-induced LC3-II accumulation, suggesting that the activation of DAPK may contribute to PM02734-induced cell death with autophagic features. Recently, Harrison and colleagues reported that DAPK can interact with microtubule-associated protein 1B, which was proven to interact with autophagosomal protein LC3 (LC3), resulting in the stimulation of DAPK-dependent membrane blebbing and autophagy (48). In addition, Zalevkar and colleagues showed that DAPK phosphatases beclin 1, an essential protein for autophagy in breast cancer 119, resulting in the dissociation of beclin 1 from Bcl-XL and induction of autophagy (49). In addition, we here observed that downregulation of DAPK by siRNA causes greater extent of blockade of PM02734-induced cell death than direct inhibition of autophagy by 3-MA treatment or by Atg-5 downregulation, suggesting that other lethal caspase-independent pathways induced by DAPK such as activation of programmed necrosis may be involved in PM02734-induced cell death. Thus, the cellular and molecular mechanism by which PM02734 induces DAPK activation and cell death associated with autophagic features deserves to be studied further. Consistent with the in vitro results, we found that PM02734 has antitumor activity in vivo and that the extent of LC3-II, an autophagic marker, was elevated in PM02734-treated xenograft tumors, as measured by immunohistochemical staining and immunoblot analysis. However, TUNEL assay and the levels of Bcl-2 and Bax did not show evidence of apoptosis after PM02734 treatment.

In summary, we have shown that PM02734 induces cell death in human NSCLC cell lines both in vitro and in vivo by a complex mechanism that appears to involve accumulation of autophagosomes, mainly by compromise of autophagosome clearance along with inhibition of the Akt/mTOR signaling pathway and activation of DAPK. Consistent with in vitro results, PM02734 inhibits tumor growth accompanied by marked accumulation of autophagosomes in vivo in NCI-H449 xenografts. These observations represent a potentially important step toward understanding the mechanism of lethality of this structurally unique agent and may be of help in rationally integrating PM02734 with other agents for the treatment of NSCLC tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Retraction: PM02734 (Elisidepsin) Induces Caspase-Independent Cell Death Associated with Features of Autophagy, Inhibition of the Akt/mTOR Signaling Pathway, and Activation of Death-Associated Protein Kinase

The authors wish to retract the article titled "PM02734 (Elisidepsin) Induces Caspase-Independent Cell Death Associated with Features of Autophagy, Inhibition of the Akt/mTOR Signaling Pathway, and Activation of Death-Associated Protein Kinase," which was published in the August 15, 2011, issue of Clinical Cancer Research.  

After an unidentified concerned reader made allegations of image mishandling in several figures, the authors requested that their medical school conduct an investigation as per its established protocol. The investigators concluded that there was enhancement of bands in some of the questioned figures and that the changes were subtle and seen only on special analysis. One of the coauthors, who since has returned to his home country, accepted full responsibility for the changes. He never mentioned the enhancements to any of the authors before manuscript submission. In no case would the conclusions from the experiments have been different if no alterations had been made. Independent repetition of key experiments by another senior investigator showed reproducibility of the major findings. In view of the reproducibility of the original data by a third party, the authors continue to stand by the conclusions of the article. However, because the submission did not meet the standards for manuscript submission to Clinical Cancer Research as described in the Instructions to Authors, the authors voluntarily retract this article. The authors apologize to the readers for the inconvenience that this oversight may have caused.

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