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Bryostatin analogue-induced apoptosis in mantle cell lymphoma cell lines

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Abstract

The anti-cancer effects of bryostatin-1, a potent diacylglycerol analogue, have traditionally been attributed to its action on protein kinase C. However, we previously documented apoptosis in a B non-Hodgkin lymphoma cell line involving diacylglycerol analogue stimulation of Ras guanyl-releasing protein, a Ras activator, and Bim, a proapoptotic Bcl-2 family protein. To further explore the role of Bim, we examined several Bim-deficient B non-Hodgkin lymphoma cells for their responses to pico, a synthetic bryostatin-1-like compound. The Bim⁻ mantle cell lymphoma cell lines Jeko-1, Mino, Sp53, UPN1, and Z138 and the Bim⁺ cell line Rec-1, as well as the Burkitt lymphoma cells lines BL2 (Bim⁻) and Daudi (Bim⁺), were examined for their response to pico using assays for proliferation and apoptosis as well as biochemical methods for Ras guanyl-releasing proteins and Bcl-2 family members. With the exception of UPN1, mantle cell lymphoma cell lines underwent pico-induced apoptosis, as did BL2. In some cases, hallmarks of apoptosis were substantially diminished in the presence of mitogen-activated protein kinase kinase inhibitors. Pico treatment generally led to increased expression of proapoptotic Bik, although the absolute levels of Bik varied considerably between cell lines. A pico-resistant variant of Z138 exhibited decreased Bik induction compared to parental Z138 cells. Pico also generally decreased expression of anti-apoptotic Bcl-XL and Mcl1. Although, these changes in Bcl-2 family members seem unlikely to fully account for the differential behavior of the cell lines, our demonstration of a potent apoptotic process in most cell lines derived from mantle cell lymphoma encourages a re-examination of diacylglycerol analogues in the treatment of this subset of B non-Hodgkin lymphoma cases.

Bryostatin-1 is a potent diacylglycerol (DAG) analogue that exhibits a range of promising therapeutic activities. Unlike DAG analogues such as phorbol 12-myristate, 13-acetate,

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

bryostatin-1 is not a tumor promoter [1]. Based on promising preclinical data, bryostatin-1 has been evaluated as both a single agent and an adjuvant to conventional anticancer therapies, including those aimed at leukemia and lymphoma [2]. Although bryostatin-1 is well tolerated, its efficacy has been documented on a sporadic basis only.

Traditionally, the activities of both natural DAG and DAG analogues have been interpreted in terms of their binding to, and activation of, protein kinase C (PKC) family members. Conventional and novel species of PKC possess C1 domains that bind DAG and DAG analogues, contributing to membrane recruitment and activation of these enzymes. Because prolonged activation of PKCs can lead to proteolysis of these enzymes, DAG analogue effects have also been interpreted in terms of PKC downregulation [2].

Besides PKCs, several other classes of proteins with DAG and DAG-analogue-binding C1 domains have been described [3,4]. We have shown that some members of the Ras guanyl-releasing protein (RasGRP) family of Ras activators bind DAG and DAG analogues such as phorbol esters and bryostatin-1 with low nanomolar affinity [5,6]. RasGRPs are functionally analogous to Sos proteins, but they are preferentially expressed in lymphocytes, where they link immune receptors and phospholipase C-generated DAG to Ras signaling. Recruitment of RasGRPs to cellular membranes by DAG and DAG analogues brings them into proximity with their substrate, membrane-bound Ras. DAG and DAG analogues additionally activate RasGRPs by PKC-mediated regulatory phosphorylation [7–9]. The interaction of activated RasGRPs with Ras at the cell membrane results in conversion of inactive Ras-GDP to active Ras-GTP, triggering signaling through the Raf-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) kinase cascade [10]. Activation of this pathway is a key process during immune receptor signaling and contributes to both immune cell development and effector function [10].

We have shown that bryostatin-1 and a synthetically accessible and tunable bryostatin-like compound called pico (or picolog) are indistinguishable in several RasGRP-based assays, including those based on primary human lymphocytes [11]. Additionally, we recently reported that exposure to either bryostatin-1 or pico induces extensive apoptosis in the cell line Toledo, which was derived from a germinal type B non-Hodgkin lymphoma (B-NHL) [12]. RasGRPs are almost certainly drug targets in this system because apoptosis was blocked effectively in the presence of MEK inhibitors or PKC inhibitors that block regulatory phosphorylation of RasGRPs.

We also presented evidence that apoptosis arose from proapoptotic phosphorylation of the pro-death Bcl-2 family member Bim. Bim is a key regulator of lymphocyte apoptosis [13]. Bim has properties consistent with a tumor suppressor; Bim coding sequences are frequently mutated or epigenetically silenced in several types of B-NHL, including mantle cell lymphoma (MCL) and Burkitt lymphoma (BL) [14,15]. In light of our proposal that Bim played a key role in DAG analogue-induced apoptosis in Toledo B-NHL cells, we were interested in examining the response of cells lacking Bim to the bryostatin-1 analogue pico. Unexpectedly, pico induced apoptosis in most MCL cell lines, regardless of Bim status. The Bim-deficient BL cell line BL2 was also sensitive. Our results suggest that bryostatin-1 and related compounds might have efficacy in some cases of MCL and BL.

Materials and methods

Reagents and antibodies

Pico was synthesized as described previously [16], dissolved in dimethyl sulfoxide (DMSO) at 1.0 mM and stored at -20°C . Except for the dose-response studies, pico was used at 100 nM. The MEK inhibitor U0126 was purchased from Cell Signaling (Danvers, MA, USA) and was used at 10 μM . The MEK inhibitor PD184352 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at 2 μM . Tetramethylrhodamine ethyl ester (TMRE) was purchased from Molecular Probes (Carlsbad, CA, USA). Doxycycline hyclate and Thiazolyl blue tetrazolium bromide were purchased from Sigma (St Louis, MO, USA).

The following antibodies were used to detect proteins by immunoblotting: phospho-ERK (#9101), Bik (#4592), Bim (#2819), Bcl-XL (#2762), and Mcl1 (#4572) were from Cell Signaling. Bcl-2 antibodies were purchased from BD Biosciences (#551107 and #551051, clone 6C8) and from Cell Signaling (#4223; clone D55G8). Antibodies to detect actin were purchased from Sigma (#2066) and total ERK antibody was from Santa Cruz Biotechnology (sc94). Anti-Ras antibody (#05-516 clone 10) was from Millipore (Billerica, MA, USA). Anti-RasGRP antibodies have been described previously [8,17].

Cell lines, variant cell isolation, and gene transfer

The MCL cell lines Jeko-1, Mino, Sp53, UPN1, and Z138 were provided by Jose Martinez-Climent (University of Navarra, Pamplona, Spain), as was the BL cell line BL2. Rec-1 and Daudi were provided by Drs Raymond Lai and Andrew Shaw, respectively (Cross Cancer Institute, Edmonton, Alberta, Canada). After prolonged exposure of Z138 cells to 100 nM pico, the pico-resistant variant Z-CV4 was obtained by limiting dilution.

The cell lines used here were selected on a nonrandom basis. The Bim⁻ cell lines were selected to examine the role of Bim in DAG analogue-induced apoptosis. Rec-1 was subsequently included in the analysis to increase the size of the MCL collection and was found to be Bim⁺. Daudi was found to be pico-resistant and was selected here as a BL control for BL2. However, Daudi is derived from an endemic BL and is Epstein-Barr virus-positive, while BL2 is derived from a sporadic BL and is virus-negative. Besides confirming the presence or absence of Bim, the cell lines were typed using a cyclin D1 antibody by immunoblotting to confirm that, compared to the BL cell lines, all MCL cell lines overexpressed cyclin D1 (data not shown).

To express Bik and Bcl-2 complementary DNAs, we used a doxycycline-inducible retrovirus system (Lenti-X Lentiviral Expression Systems; Clontech Laboratories, Inc., Mountain View, CA, USA). Briefly, lymphoma cells were first transduced with the LVX-Tet-On advanced lentivirus (Neo+) followed by selection in G418 (1.0 mg/mL). Resistant cells were then transduced with the LVX-Tight-Puro vector or sequence-verified derivatives expressing wild-type human Bik or Bcl-2 complementary DNAs, followed by selection in puromycin (2.5 $\mu\text{g}/\text{mL}$). Complementary DNA expression was induced by addition of doxycycline (2 $\mu\text{g}/\text{mL}$).

Biochemical assays

Detection of RasGRP1, RasGRP3, and Bcl-2 family proteins by immunoblotting and Ras signaling assays were carried out as described previously [8,12,17,18]. To study co-precipitation of Bik with Bcl-2, we used an anti-Bcl-2 monoclonal antibody (clone 6C8) to precipitate Bcl-2 from lysates made in CHAPS detergent, as we described previously [12]. ImageQuant TL2005 was used to quantify band intensity.

Proliferation and apoptosis assays

To document the effect of pico on proliferation, cultures were incubated in medium with 100 nM pico or DMSO (control). Cells were enumerated daily with an electronic cell counter (Coulter Model Zf). To determine the fraction of cells that had lost mitochondrial outer membrane integrity, TMRE staining was performed as described previously [12]. Likewise, the terminal deoxynucleotidyl transferase dUTP nick end labeling assay (In Situ Cell Death Detection Kit; Roche, Indianapolis, IN, USA) was used to detect terminally apoptotic cells. The CaspACE kit (Promega, Madison, WI, USA), which is based on binding of a fluorescent ZVAD caspase inhibitor, was used according to the supplier's instructions to detect global caspase activation. These three apoptosis assays were monitored by flow cytometry using FACSCalibur with an argon laser and CellQuestPro software (BD Biosciences, San Jose, CA, USA). To perform dose-response experiments, we used the MTT assay, which measures the accumulation of viable cells. Cells were seeded in 96-well plates in medium lacking pico or containing pico at various concentrations, followed by incubation for 3 to 7 days, depending on the proliferation rate of the cell line in medium lacking pico. After incubation, the thiazolyl blue tetrazolium bromide was added at a final concentration of 0.5 µg/mL. After 3 hours incubation at 37°C, the formazan precipitate was solubilized by adding isopropanol acidified by addition of HCl (0.04 M). The absorbance at 560 nm was quantified using a Synergy HT microplate reader (BioTek Instruments, Inc, Winooski, VT, USA).

Statistical analysis

Results are expressed as mean $\pm$ standard error of mean. Comparisons between two groups were performed using the two-tailed Student's *t* test for unpaired samples, with equal variance.

Results

Pico stimulates proapoptotic pathways in most MCL and some BL cell lines

To examine the effect of DAG analogue treatment on MCL cell lines, cells were incubated with DMSO or with 100 nM pico for up to 10 days and daily observations were made by microscopy. To determine whether observed effects of pico were dependent on signaling through MEK, each type of culture was incubated either without or with the MEK inhibitor U0126. The MCL cell lines Jeko-1, Mino, Rec-1, SP53, and Z138 assumed a granular appearance after 3 to 5 days of pico treatment. The effect of pico treatment on cell morphology was partially inhibited by the presence of U0126, although this inhibitor alone had no effect on cell morphology (data not shown). Although the morphology of cells

resembled that reported previously for Toledo cells, the killing process is much slower. Apoptosis is complete in Toledo cells by 48 hours [12], at which time no signs of death are evident in the cell lines studied here. At the level of cell morphology, MEK inhibition afforded only partial protection from the effects of pico in the studies reported here, whereas it provided complete protection in Toledo cells. The MCL cell line UPN1 showed no signs of pico-induced cell death (Fig. 1A). Cell-counting experiments confirmed that proliferation of sensitive MCL cell lines was substantially inhibited by pico, while the effect on UPN1 was more muted (Fig. 1B). The Bim⁻ cell line BL2 was also sensitive to pico, while Daudi was resistant.

After 7 days treatment of pico-sensitive cells, nuclear DNA fragmentation in pico-sensitive cell lines was evident as demonstrated using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay in conjunction with flow cytometry (Fig. 2A). In some cases, for example Daudi and BL2, significant apoptosis was also observed in the DMSO-treated cultures, but this likely arose from culture overgrowth. After 3 days, pico treatment, sensitive cells exhibited global activation of caspases (Fig. 2B). In several cases, caspase activation was substantially diminished by the MEK inhibitor U0126 (Fig. 2B; Table 1). The apoptotic response apparently involves the mitochondrial pathway because pico treatment of sensitive cell lines for 3 days resulted in loss of TMRE staining, a marker of mitochondrial integrity (Table 1). Because there is some concern that U0126 is not completely specific for MEK, we compared two different MEK inhibitors, U0126 and PD184352, to confirm the involvement of the canonical Ras signaling pathway in Jeko-1 cells (Fig. 2C). In contrast, pico-induced apoptosis was relatively insensitive to MEK inhibition in SP53, suggesting the existence of MEK-independent signaling pathways (Table 1). Pico-treated UPN1 cells exhibited only a modest loss of TMRE staining after pico treatment (Table 1).

Using the MTT assay, we showed that pico concentrations in the low nanomolar range effectively inhibited accumulation of viable cells in many of the sensitive cell lines (Supplementary Figure E1; online only, available at www.exphem.org). In contrast, Daudi and UPN1 were only marginally sensitive to 100 nM pico in this assay (Supplementary Figure E1; online only, available at www.exphem.org).

Expression of functional RasGRPs

To confirm the involvement of RasGRPs in pico-induced apoptosis, we examined each of our cell lines for RasGRP expression and used Ras signaling assays. Our previous studies indicated that RasGRP1 was expressed in T cells, while RasGRP1 and RasGRP3 were co-expressed in B cells [8,18]. Using immunoblotting, we tested each cell line for both proteins. Our BL cell lines express both RasGRP1 and RasGRP3 as expected of B cells. Among the MCL cell lines, only Z138 exhibited a typical B-cell pattern of RasGRP1/3 expression. Other MCL cell lines expressed either relatively low levels of RasGRP1, RasGRP3, or both (Fig. 3A). These and other biochemical results are summarized in Table 2. Pico treatment for 10 minutes caused most cell lines to increase levels of Ras-GTP (Fig. 3B). In Mino and Z138, high basal Ras-GTP in untreated cells largely obscured this effect, although in other experiments with Z138, at least, a modest pico-induced increase was apparent (Table 2). The results in Figure 3 indicate that UPN1, the pico-resistant MCL cell line, only marginally

activated Ras in response to pico treatment. To examine signaling in a longer time frame, we compared Jeko-1 and UPN1 in a pico treatment time-course experiment in terms of their phospho-ERK levels. UPN1 had relatively muted pico-induced RasGRP-ERK signaling (Fig. 3C).

Expression of Bcl-2 family members

Because Bcl-2 family proteins are key regulators of the mitochondrial apoptosis pathway, we examined the expression of the most commonly expressed members using immunoblotting (summarized in Table 2). Most of the cell lines used in this study were selected on the basis of their Bim-negative status and this finding was confirmed (Supplementary Figure E2; online only, available at www.exphem.org). However, the MCL cell line Rec-1 and the BL cell line Daudi express readily detectable BimEL, the most abundant splice form of the protein (Supplementary Figure E2; online only, available at www.exphem.org). As seen in many cell types [19], ERK activation leads to BimEL phosphorylation and ubiquitination, as evidenced by reduced electrophoretic mobility.

Bcl-2 was undetectable in Daudi and expressed at a very low level in UPN1 (Supplementary Figure E2; online only, available at www.exphem.org). Similar results were obtained with three different anti-Bcl-2 antibodies. Because of the evidence that Bcl-2 can be converted to a proapoptotic protein upon physical association with the protein Nur77 [20], we hypothesized that the deficiency of Bcl-2 might contribute to the pico resistance of Daudi and UPN1 cells. However, engineered expression of Bcl-2 in UPN1 at levels comparable to those seen in our sensitive cell lines using the pLVX-Tight-Puro vector did not convert UPN1 cells to a pico-sensitive phenotype (Supplementary Figure E3; online only, available at www.exphem.org). Pico treatment did not reproducibly influence endogenous Bcl-2 expression levels.

The BH3-only protein Bik was readily detectable in BL2, Daudi, Mino, and Z138 (Fig. 4A). Furthermore, the levels increased after 3 days of pico treatment. The basal levels of Bik were much lower in Jeko-1 and SP53, although still somewhat increased after pico treatment. In many such assays, Bik recovered from pico-treated cells migrated as a doublet or as a bottom-heavy band, suggesting that the protein was subject to post-translational modification, such as proteolytic cleavage. Bik was not convincingly detectable in Rec-1 or UPN1, regardless of pico treatment. However, wild-type Bik coding sequences were recovered from RNA isolated from UPN1 cells.

BH3-only proteins are thought to work at least in part by binding anti-apoptotic proteins such as Bcl-2, thereby titrating these latter proteins and reducing their ability to sequester proapoptotic proteins such as Bak and Bax [21,22]. Accordingly, we examined the abundance of Bik complexed with Bcl-2 using a co-immunoprecipitation assay. In both Jeko-1 (low Bik-expressing) and BL2 cells (relatively high Bik expressing), pico treatment increased the amount of co-precipitated Bik, arguing that the pico-induced Bik expression levels might be physiologically relevant to induced apoptosis in these cell lines (Fig. 4B). Increased Bcl-2 expression in BL2 cells may have also contributed to this effect in this experiment. Pico treatment for 3 days caused decreases in the levels of Bcl-XL and Mcl1 in most cell lines (Fig. 4A). However, as in the case of Bik, there was considerable variability

in the basal expression levels between these cell lines. Notably, perhaps, quantitative changes in Bcl-XL in pico-treated UPN1 were, on average, relatively minor, while changes in Mcl1 were not significant. The expression of other Bcl-2 family proteins did not vary between cell lines, or in response to pico, in ways that offered hints about pico-induced apoptosis (data not shown).

Z-CV4, a spontaneous pico-resistant variant of Z138 expresses less pico-induced Bik

Most pico-sensitive cell lines undergo 100% cell death after prolonged exposure to pico. After prolonged treatment of Z138 cells with pico, however, we observed eventual outgrowth of pico-resistant cells. The clonal variant Z-CV4 grows in pico-containing medium and is less sensitive to pico-induced loss of TMRE staining (Fig. 5A). In principle, pico resistance might arise from a failure of the drug to stimulate RasGRP-ERK signaling. This is not the case in Z-CV4, however, as shown by the analysis of phospho-ERK levels in a pico time-course experiment (Fig. 5B). ERK was activated similarly in Z138 and its derivative Z-CV4 from 1 to 6 hours, after which the phospho-ERK levels dropped to slightly below basal levels. The same experiment demonstrated that Bik accumulates dramatically between 24 and 72 hours in Z138, but much less so in Z-CV4 (Fig. 5B, C). To demonstrate the robust nature of this result, we quantified Bik levels induced by 3 days of pico treatment in three independent experiments. Although basal Bik levels are only modestly lower in Z-CV4, pico-induced levels are significantly lower. Z138 and Z-CV4 express similar levels of Bcl-XL and Mcl1 and downregulate these proteins similarly in response to pico (data not shown).

To confirm the role of Bik in pico-induced apoptosis, the pico-resistant Z-CV4 clone was engineered to express Bik under doxycycline regulation. After inducing protein expression with doxycycline for 24 hours, cells were treated with 100 nM pico for 3 days and the integrity of the outer mitochondria membrane was assessed by TMRE staining (Fig. 5D). Cells overexpressing Bik after doxycycline induction underwent more TMRE loss than both the un-induced and parental (empty vector) cells. The loss of outer mitochondrial membrane integrity was even more pronounced after induction of Bik by doxycycline followed by treatment with pico (Fig. 5E). Immunoblotting confirmed that these effects on apoptosis were achieved with only modest levels of Bik overexpression. Collectively, these results implicate the pico-induced increase in Bik in pico-induced apoptosis in Z138, although decreases in Mcl1 and Bcl-XL might also play a role.

Discussion

We show here that, in response to pico treatment, five of six MCL cell lines undergo apoptosis by a protracted mechanism, while a single cell line, UPN1, is insensitive to pico-induced apoptosis. The Bim-negative BL cell line BL2 also undergoes pico-induced apoptosis. Pico-induced apoptosis is at least partially dependent on MEK activity, implicating Ras-ERK signaling. MEK-independent apoptosis might arise from RasGRP-dependent activation of noncanonical Ras signaling pathways or from signaling through unrelated pathways governed by C1 domain proteins. The MCL cell lines express RasGRP1, RasGRP3, or both. In most cases, we confirmed that pico could increase Ras-GTP after pico

treatment. UPN1 exhibited impaired Ras activation compared to the other cell lines and in a time-course experiment showed diminished ERK activation compared to Jeko-1. However, the relationship between apoptosis and ERK activation is not straightforward. At later time points, pico-sensitive Z138 usually exhibited phospho-ERK levels below that of untreated cells, a result suggesting negative feedback regulation. Furthermore, UPN1 cells consistently showed ERK activation after 3 days pico treatment.

The apoptotic response of our MCL cell lines differs from the more rapid killing previously described for the germinal center cell line Toledo, in which we implicated Bim [12], although both mechanisms involve canonical Ras-ERK signaling and entail the mitochondrial pathway. Interestingly, recent studies with mouse primary immature B cells have illuminated a RasGRP1-dependent proapoptotic pathway [23]. In this case, PKC- δ and calcium appear to activate RasGRP1, which possesses calcium-binding EF hands [24]. Earlier studies implicated RasGRP1 in positive regulation of apoptosis in an immature B-cell line by BCR stimulation [25]. Strangely, this mechanism was independent of MEK, but dependent on signaling through another pathway that regulates the nuclear factor- κ B transcription factor. Thus, RasGRPs in B cells can promote apoptosis by a variety of mechanisms.

MCL cell line apoptosis does not require Bim, but nor does it require the absence of Bim, since Rec-1 demonstrated pico sensitivity and yet is Bim-positive. Pico-induced changes in other Bcl-2 family members, such as increased Bik and decreased Bcl-XL and Mcl1, likely contributed to the induction of apoptosis. However, these changes in protein levels appear unable to account for the properties of all of our cells. For example, Bik levels were relatively low in some sensitive cells compared to others. Nonetheless, the analysis of a pico-resistant clonal variant derived from Z138 suggests that this proapoptotic protein can participate in pico-induced apoptosis in some cases. In Jeko-1 and BL2, at least, this excess Bik appears to form physiologically active complexes with Bcl-2. Previous studies of apoptosis induced by interleukin-21 treatment of MCL cell lines also implicated Bik [26]. Somatic mutations affecting Bik coding sequences have been documented in peripheral lymphoma [27], arguing that Bik plays an important role in culling B cells under particular circumstances or at certain stages of development. The decreased expression of the anti-apoptotic proteins Bcl-XL and Mcl1 after 3 days of pico treatment also likely contributed to pico-induced apoptosis. The resistance of the exceptional MCL cell line, UPN1, might arise from defective RasGRP-ERK signaling during the first 24 hours after pico treatment, the absence of Bik induction and ineffective Bcl-XL and Mcl1 downregulation. Other factors likely contribute to the relative pico resistance of UPN1, however. Additionally, the documented changes in Bik and Bcl-XL do not necessarily lead to apoptosis of malignant B cells, because our sole pico-resistant BL cell line, Daudi, also shows these induced changes.

Despite these uncertainties, the present studies further support the idea that RasGRPs could constitute viable drug targets that can be regulated by medicinal DAG analogues, such as bryostatin-1 and related compounds such as pico. Medicinal DAG analogues alone or in combination with other therapeutic agents might represent a potential strategy for treating MCL, a disease that has a dismal prognosis and no standard therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Pico treatment induces morphology changes and inhibits cell proliferation in some MCL and BL cell lines. (A) Cell lines were incubated for 3 days with DMSO or pico and cell density and morphology were recorded by phase-contrast photomicroscopy. Results shown for Jeko-1 are representative of other pico-sensitive cell lines. (B) Cells were incubated in the presence of DMSO (control) or 100 nM pico and enumerated at days 2, 4, and 7. In the left Figure, the proliferation of each cell line in pico is shown as a percent of that observed with

DMSO. The Figures on the right show representative proliferation curves for Jeko-1 (sensitive) and UPN1 (resistant).

Figure 2.

Pico-induced death occurs by a mitochondrial apoptotic mechanism. **(A)** In Situ Cell Death Detection kit (terminal deoxynucleotidyl transferase dUTP nick end labeling) was used to detect DNA strand breaks in apoptotic cells after 7 days of pico treatment. **(B)** CaspACE-FITC-VAD-FMK in situ marker was used to detect activated caspases as an in situ marker of apoptosis in cells after 3 days of pico treatment. U0126 was included in some assays to demonstrate MEK-dependence of pico-induced caspase activation. **(C)** TMRE staining was performed to demonstrate loss of outer mitochondrial membrane integrity in the pico-

sensitive cell line Jeko-1 after 3 days pico treatment. The inhibitors U0126 and PD184352 were included to demonstrate the dependence of the effect on MEK.

Figure 3.

RasGRP expression and signaling in MCL and BL cell lines. (A) Expression of RasGRP1 and RasGRP3 in MCL and BL cell lines was documented by immunoblotting. (B) Ras-GTP levels without and with pico treatment (10 minutes, 100 nM). Ras-GTP in lysates was recovered on the basis of binding to recombinant Ras binding domain of Raf and compared to total Ras levels using immunoblotting. (C) Jeko-1 and UPN1 were treated with 100 nM pico and cells were harvested at the indicated time points for analysis of phospho-ERK and total ERK using immunoblotting.

Figure 4.

(A) Upregulation of functional Bik and downregulation of Bcl-XL and Mcl1 after 3 days incubation without or with pico. Protein lysates (300 µg/lane) were blotted with antibodies to detect Bik, Bcl-XL, Mcl1, phospho-ERK, and ERK. Band intensities for each of Bik, Bcl-XL, and Mcl1 were normalized to results obtained with ERK in the same experiment. The mean of three such values derived from independent biological replicates was normalized to that obtained with untreated samples (set at 1.0) and expressed as a histogram showing the standard error of mean. (B) Jeko-1 and BL2 were incubated with pico or DMSO for 3 days

and cells were lysed in CHAPS. Bcl-2 was immunoprecipitated and co-precipitated Bik was detected by immunoblotting. PNS = post-nuclear supernatant assayed directly.

E **Figure 5.**

Reduced apoptosis and reduced Bik upregulation in Z-CV4, a pico-resistant variant of Z138.

(A) TMRE staining was assessed after 3 days incubation in DMSO or 100 nM pico. (B) Z138 and Z-CV4 were treated for the indicated times with pico and were analyzed for their levels of Bik, phospho-ERK, and actin (loading control). (C) For Z138 and Z-CV4 the levels of Bik (normalized to actin) were determined after 3 days incubation with DMSO or pico treatment. The results of three separate experiments were analyzed. (D) Engineered expression of Bik in Z-CV4 increased the level of TMRE-negative cells, especially in the

presence of pico. (E) The results of three independent TMRE staining experiments were analyzed (top). Engineered, doxycycline-induced expression of Bik of Z-CV4 was confirmed by immunoblotting (bottom). ERK was used as a loading control.

Table 1

Effects of pico and the MEK inhibitor U0126 on apoptosis as assessed by CaspACE and TMRE

% Apoptotic signal*	CaspACE				TMRE			
	DMSO	Pico (100 nM)	U0126 (10 μ M)	Pico + U0126	DMSO	Pico (100 nM)	U0126 (10 μ M)	Pico + U0126
BL2	3.9	76	8	20	0.5	75	1	65
Daudi	4.2	12.9	10	12.4	1.5	1.5	6	4
Jeko-1	3.4	41.4	5.7	15	2	31	4	6
Mino	ND	ND	ND	ND	68.9	98.1	65.3	73.7
Rec-1 [†]	9.7	38.9	17	25.9	–	–	–	–
SP53	18.1	53.5	31.2	56.9	41.7	99.5	29.8	90.8
UPNI	3.5	6	6.9	7.5	10	20	15	17
Z138	7	20	6	3	6	36	5.5	27

CaspACE = FITC-VAD-FMK in situ marker; ND = not done.

* Percent of CaspACE-positive cells or percent of TMRE-negative cells (average of two independent experiments).

[†] Rec-1 cell line consistently failed to stain with TMRE.

Table 2

Summarized protein expression results for the cell lines included in the study

Cell line	RasGRP-1		RasGRP-3		RasGTP*		P-ERK*		Bim	Bcl-2		Bik [†]		Bcl-XL [‡]		MCL-1 [‡]	
	+	++	+	++	DMSO	Pico	DMSO	Pico		DMSO	Pico	DMSO	Pico	DMSO	Pico	DMSO	Pico
BL2	+	++	+	++	+	++	-	+	-	++	++	+	++	++	++	+	+
Daudi	+	++	+	++	+	++	-	+	++	-	++	+	++	+	++	+	+
Jeko-1	+	++	+	++	+	++	-	+	-	++	++	+	++	+	++	+	+
Mino	-	+	+	++	+	++	+	+	-	++	++	+	++	+	++	-	-
Rec-1	++	+	-	+	+	++	-	+	++	++	++	-	++	+	++	+	+
SP53	-	+	+	++	+	++	+	++	-	++	++	+	++	+	++	-	-
UPN1	+	++	-	+	+	++	-	+	-	+	++	-	++	+	++	+	+
Z138	+	++	+	++	+	++	+	++	-	++	++	+	++	+	++	+	+

- = not detectable; + = relatively weak band; ++ = relatively strong band, compared to untreated (Bik, Ras-GTP, and P-ERK), pico-treated (Bcl-XL, Mcl1), or other cell lines (RasGRP1, RasGRP3, Bcl-2, Bim).

* DMSO compared to 10 minutes of 100 nM pico treatment.

[†] DMSO compared to 3 days of 100 nM pico treatment.

[‡] Low expressors.