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Research Paper

Apoptolidin, a selective cytotoxic agent, is an inhibitor of F_0F_1-ATPase

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Abstract

Background: Apoptolidin is a macrolide originally identified on the basis of its ability to selectively kill E1A and E1A/E1B19K transformed rat glial cells while not killing untransformed glial cells. The goal of this study was to identify the molecular target of this newly discovered natural product.

Results: Our approach to uncovering the mechanism of action of apoptolidin utilized a combination of molecular and cell-based pharmacological assays as well as structural comparisons between apoptolidin and other macrocyclic polyketides with known mechanism of action. Cell killing induced by apoptolidin was independent of p53 status, inhibited by BCL-2, and dependent on the action of caspase-9. PARP was completely cleaved in the presence of 1 μM apoptolidin within 6 h in a mouse lymphoma cell line. Together these results suggested that apoptolidin might target a mitochondrial protein. Structural comparisons between apoptolidin and other macrolides revealed significant similarity between the apoptolidin aglycone and oligomycin, a known inhibitor of mitochondrial F_0F_1-ATP synthase. The relevance of this similarity was established by demonstrating that apoptolidin is a potent inhibitor of the F_0F_1-ATPase activity in intact yeast mitochondria as well as Triton X-100-solubilized ATPase preparations. The K_i for apoptolidin was 4–5 μM. The selectivity of apoptolidin in the NCI-60 cell line panel was found to correlate well with that of several known anti-fungal natural products that inhibit the eukaryotic mitochondrial F_0F_1-ATP synthase.

Significance: Although the anti-fungal activities of macrolide inhibitors of the mitochondrial F_0F_1-ATP synthase such as oligomycin, ossamycin and cytovaricin are well-documented, their unusual selectivity toward certain cell types is not widely appreciated. The recent discovery of apoptolidin, followed by the demonstration that it is an inhibitor of the mitochondrial F_0F_1-ATP synthase, highlights the potential relevance of these natural products as small molecules to modulate apoptotic pathways. The mechanistic basis for selective cytotoxicity of mitochondrial ATP synthase inhibitors is discussed.

Keywords: Apoptolidin; Apoptotic pathway; Macrolide; Mitochondrial F_0F_1-ATP synthase

1. Introduction

Drugs that can selectively sensitize cancer cells to apoptosis induction are crucial. Recently, Seto and coworkers uncovered a novel apoptosis inducer from a screen of compounds which can selectively sensitize E1A and E1A/E1B19K transformed cells to apoptosis with considerable potency [1,2] from *Nocardiopsis* sp. named apoptolidin [2] (Fig. 1). Our approach to uncovering the mechanism of action of this interesting polyketide utilized selected molecular and cell-based assays combined with structural comparison of the molecule with other polyketides of known mechanism. Using selected apoptolidin-sensitive cell lines, we examined the role of key apoptosis-related proteins such as p53, BCL-2, and caspases in apoptolidin-induced cell death. The results of these experiments suggested that apoptolidin acted upon a target associated with the mitochondria. In parallel, we also compared the structure of apoptolidin to that of other polyketides with a known mechanism of action. The closest structural homolog to apoptolidin uncovered from the literature was the mitochondrial F_0F_1-ATPase inhibitor oligomycin and the vacuolar V-ATPase inhibitor bafilomycin [3,4] (Fig. 1).

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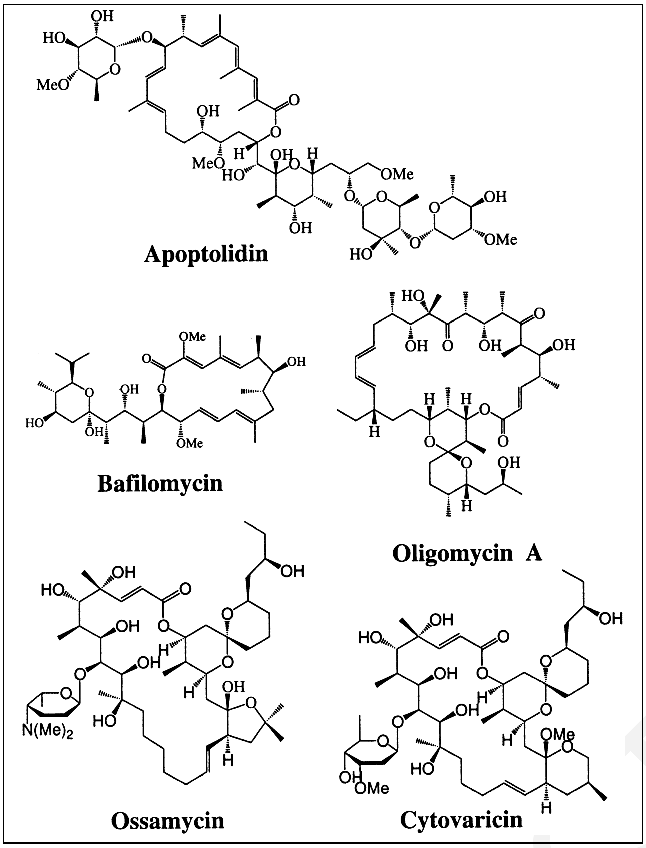

Fig. 1. Structures of apoptolidin, bafilomycin, oligomycin, cytovaricin, and ossamycin.

Since bafilomycin does not show significant selectivity in its cytotoxicity profile, we tested the ability of apoptolidin to inhibit the mitochondrial F_0F_1-ATPase. Our results confirmed that apoptolidin is indeed an inhibitor of the F_0F_1-ATPase. Further testing of apoptolidin against the NCI-60 cell line panel revealed that apoptolidin is a member of a family of known macrolide antibiotics.

2. Results

2.1. *Evaluation of the activity of apoptolidin against LYas lymphoma cells*

We have recently described that the apoptosis sensitivity of a mouse B cell lymphoma cell line (LYas) appears to be due to induction of genes that target the mitochondrial function [5,6]. Annexin V, which preferentially binds to phosphatidyl serine exposed on the surface of apoptotic and necrotic cells, and propidium iodide, which stains cells with permeabilized cytosolic membranes, were used to assay the cytotoxicity of apoptolidin. Fig. 2 shows the treatment of LYas cells with various concentrations of apoptolidin up to 6 h. At 3 h post-treatment with apoptolidin, Annexin V and propidium iodide positive cells began to appear in the culture. The minimum concentration of apoptolidin required for inducing apoptosis at the 3 h time-point was 200 nM, as judged by both stains. These results demonstrated the rapidity and potency with which cell death was induced in the LYas cell line by the natural product. Moreover, since LYas cells lack BCL-2 [6], this cell line could be used to study the effects of BCL-2 over-expression on the activity of the natural product (see below).

2.2. *Activity of apoptolidin in p53+/+ and p53−/− cell lines*

The *p53* gene product is an important factor in the induction of apoptosis in response to chemotherapeutic agents. Activation of p53 results in enhanced expression of the pro-apoptotic gene, *bax*. Conversely, null mutations in the p53 gene lead to increased resistance against a variety of chemotherapeutic agents (e.g. 5-fluorouracil) that induce alterations in nucleic acid structure and metabolism [7]. To determine whether the cytotoxic activity of apoptolidin was dependent or independent of p53 status, we used an isogenic pair of HCT116 p53−/− and p53+/+

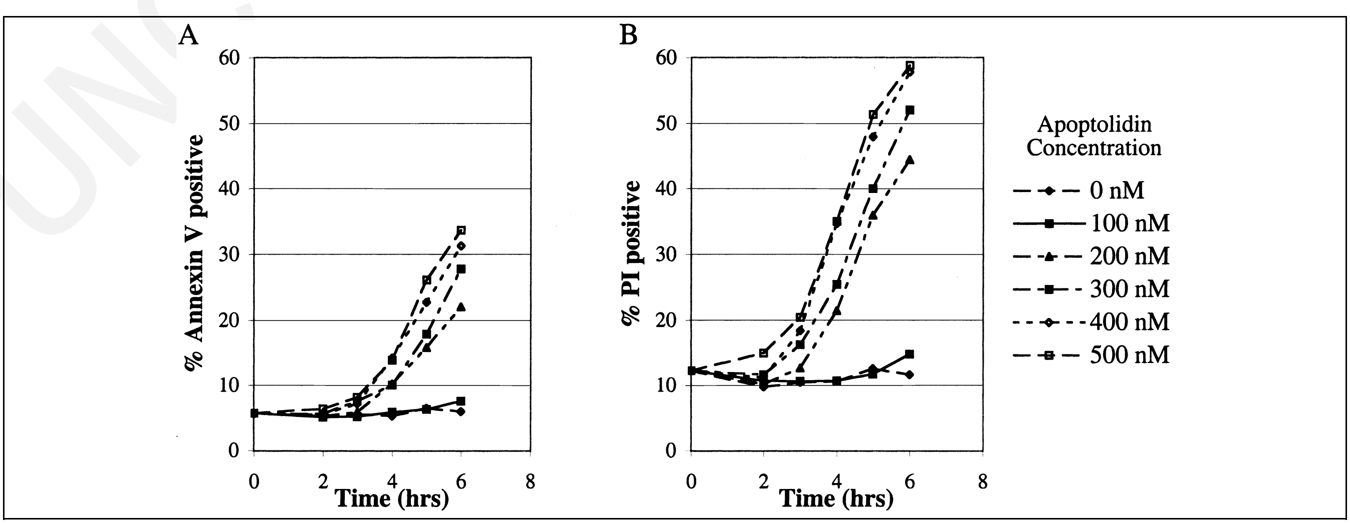

Fig. 2. Killing of LYas mouse lymphoma cells by apoptolidin as determined by FACS staining for Annexin V and propidium iodide.

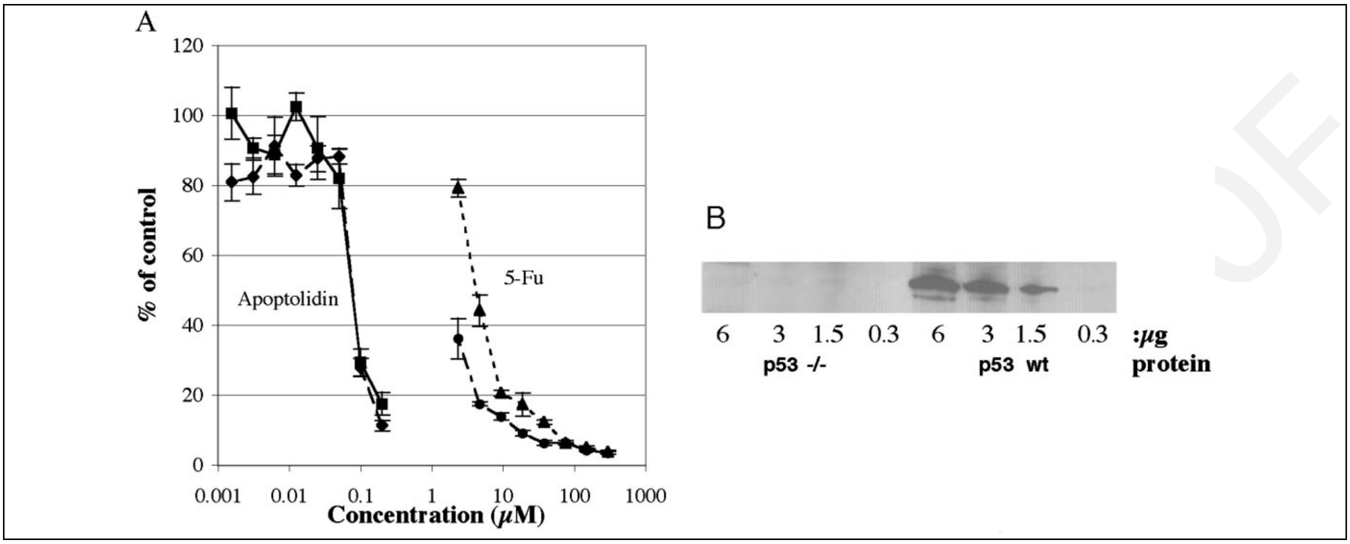

Fig. 3. Killing of HCT116 p53 wt or −/− cells with apoptolidin and 5-fluorouracil. A: Cell killing measured by MTT assay of cells treated for 7 days with drugs. Apoptolidin with p53 wt (◆) or p53 −/− (■) HCT116 cells. 5-Fluorouracil with p53 wt (●) or p53 −/− (▲). B: p53 Western blot of HCT116 p53 wt and −/− cells.

cell lines, developed by Vogelstein and coworkers [7,8]. The lack of change in the IC_{50} for the natural product in the two cell lines suggested that the cytotoxicity of apoptolidin is independent of p53 status (Fig. 3A). Control samples performed in parallel with 5-fluorouracil confirmed the p53 dependence of this drug (Fig. 3A), as shown previously [7]. Western blots also confirmed the lack of expression of the p53 protein in the knockout HCT116 cells as compared to the wild-type (wt) cells (Fig. 3B). These results demonstrate that, unlike most clinically relevant cytotoxic agents, the activity of apoptolidin was independent of the p53 status of a target cell. Therefore, either apoptolidin acted on a target downstream of p53, or its action involved a p53-independent apoptotic pathway.

2.3. Role of BCL-2 in apoptolidin-induced cell death

Mitochondrially associated proteins such as BCL-2 are key targets for apoptotic signals that are directed at the mitochondria. The p53-inducible protein Bax forms depolarizing pores in mitochondria and induces the opening of a permeability transition pore composed of adenine nucleotide transporter (ANT) and voltage-dependent anion channel [9–15]. In turn this perturbs the normal mitochondrial membrane potential ($\Delta\Psi_m$) and causes mitochondrial rupture and release of apoptogenic proteins including cytochrome *c* and AIF [16–18]. BCL-2 binds Bax and prevents its channel forming activity [9,13]. Additionally, BCL-2 inhibits mitochondrial release of pro-apoptotic proteins such as cytochrome *c* and AIF [19].

The importance of the BCL-2 protein was assessed by transfection of *bcl-2* into LYas cells as described in Section 5. LYas cells and LYas cells transfected with IRES GFP became Annexin V positive after 8 h treatment with 1 μM apoptolidin (Fig. 4A). In contrast, the BIG cell clone BIG1 was completely resistant to cell death induced by apoptolidin. To further verify that this phenotype of the BIG1 clone was not an artifact of the transfection procedure, four other BCL-2 expressing clones (BIG2–BIG5) were also shown to be resistant to apoptolidin (data not shown). The expression of *bcl-2* in the transfected cells was determined by Western blot analysis (Fig. 4B) and by BCL-2 specific FACS staining (data not shown). These results demonstrated that the activity of apoptolidin was inhibited by BCL-2. Taken together with the observation that the cytotoxicity was p53-independent, they also suggested that apoptolidin acted upon a mitochondrially associated target.

2.4. Role of caspases in apoptolidin-induced cell death

To obtain further evidence in support of the involvement of mitochondria in the mode of action of apoptolidin, we examined the effect of inhibiting various caspases on the activity of this natural product. In particular, caspase-9 was of interest, since it is activated upon the formation of the complex between mitochondrially released cytochrome *c*, Apaf-1, dATP, and caspase-9 [20–23]. Activated caspase-9 can activate caspase-3 [20]. Caspase-3-mediated cleavage of the inhibitor of caspase-activated deoxyribonuclease (ICAD) [24] leads to the activation of the caspase-activated deoxyribonuclease as well as cleavage of poly(ADP-ribose) polymerase (PARP), an important enzyme in DNA repair [25,26].

To determine whether the cytotoxicity of apoptolidin was caspase-dependent, caspase inhibitors with defined specificity were used [27]. In the presence of 140 μM z-LEHD.fmk (Enzyme Systems, Livermore, CA, USA), a caspase-9 specific peptide inhibitor, neither 1 μM etopo-

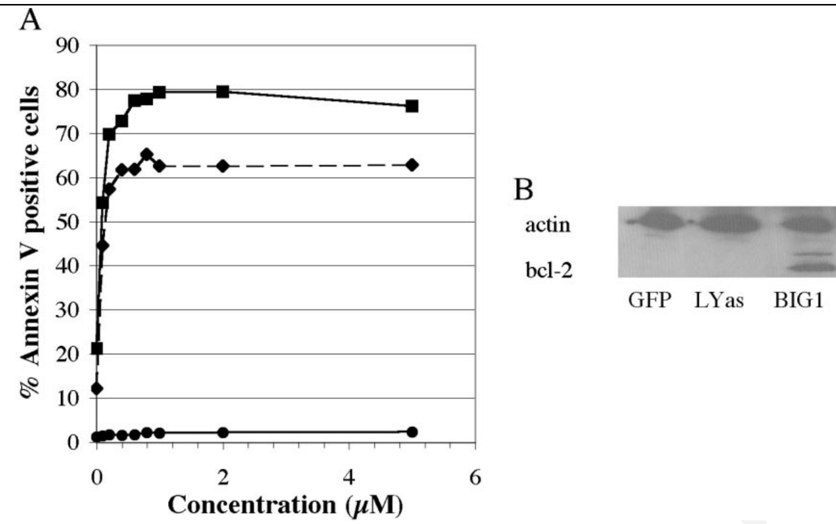

Fig. 4. Inhibition of killing by BCL-2. BIG transfected LYas cells were prepared as described in Section 5. A: Induction of Annexin V positive cells by apoptolidin with LYas/GFP (■), LYas (♦), and LYas/BIG1 (●) cells. B: Western blot for BCL-2 and actin for LYas/GFP, LYas, and LYas/BIG1 cells.

side nor 1 μM apoptolidin induced apoptosis in LYas cells after 6 h (Fig. 5A). Moreover, the pan-caspase inhibitor, z-VAD.fmk (Enzyme Systems, Livermore, CA, USA), which inhibits both caspase-3 and caspase-9, was also able to completely antagonize the activity of apoptolidin as well as etoposide on LYas cells (Fig. 5B). In both these experiments, etoposide was used as a control, since its activity is caspase-9-dependent [28]. To verify the activation of caspases by apoptolidin, PARP was shown to be completely cleaved in LYas cells after a 6 h treatment with 1 μM apoptolidin (Fig. 5C).

2.5. *Structural comparison between apoptolidin, oligomycin, and bafilomycin*

In parallel with the above biological studies, we also initiated studies on the chemistry of apoptolidin. Recently, we isolated a semi-synthetic derivative of apoptolidin that lacks the disaccharide moiety attached to C-27 but still retains some biological activity (manuscript in preparation). This prompted us to search the chemical database for macrolides with structural similarities to the apoptolidin aglycone. In particular, two natural products, oligomycin and bafilomycin (Fig. 1), drew our attention. The

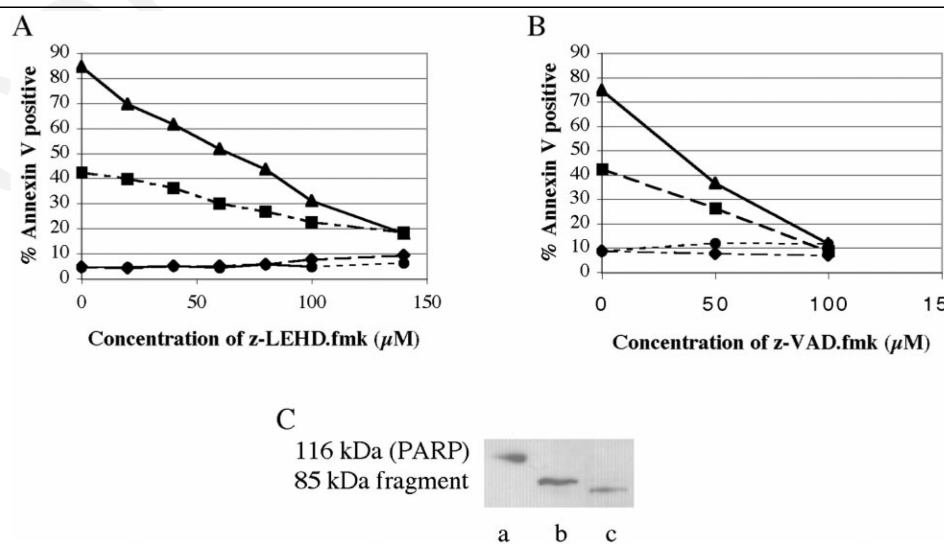

Fig. 5. Inhibition of apoptosis with the caspase-9 specific inhibitor z-LEHD.fmk (A), or pan-caspase specific inhibitor z-VAD.fmk (B) in LYas cells. Cells were treated for 6 h with z-LEHD.fmk and 1 μM etoposide (▲), 1 μM apoptolidin (■), no drugs (♦), DMSO control (●). C: Cleavage of PARP from 116 kDa size to activated 85 kDa fragment by 6 or 48 h treatment with 1 μM apoptolidin in LYas cells. 40 μg protein was applied in lanes a (untreated cells) and b (6 h apoptolidin), whereas 13 μg protein is applied in lane c (48 h apoptolidin).

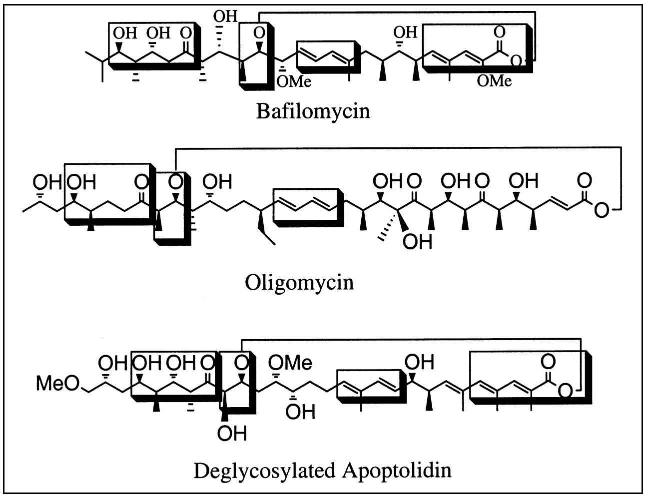

Fig. 6. Alignment of the polyketide backbones of apoptolidin, bafilomycin, and oligomycin A. Regions that are structurally related between apoptolidin and oligomycin or bafilomycin are boxed.

structures of their polyketide backbones are aligned with that of apoptolidin in Fig. 6.

Oligomycin is an inhibitor of the mitochondrial F_0F_1-ATPase [4], whereas bafilomycin selectively inhibits vacuolar ATPases [3,29]. Both molecules are known to be cytotoxic [29–32]. However, bafilomycin is relatively non-selective with a GI_{50} value of 10 nM against 80% of the cell lines in the NCI-60 panel (Fig. 7). In contrast, the activity of oligomycin shows significantly greater variability among different cell lines (Fig. 7). Approximately 35% of the cell lines are exquisitely sensitive to this natural product (GI_{50} = 10 nM); its potency against the remaining cell lines varies between 1 and 10 μM. Similar selectivity is also observed for ossamycin and cytovaricin (Figs. 1 and 7), two other structurally related macrolide inhibitors of the mitochondrial F_0F_1-ATPase. Compared to 37 000 other molecules tested in the NCI-60 screen, oligomycin, ossamycin, cytovaricin and apoptolidin are among the top 0.1% most cytoselective agents. In light of these as well as the above-mentioned results, we suspected that apoptolidin and oligomycin might share a similar mechanism of action.

2.6. *Identification of the molecular target of apoptolidin*

To test the hypothesis that apoptolidin might induce apoptosis in eukaryotic cells by inhibiting the same target as oligomycin, mitochondria were prepared from the lactate grown yeast strain DBY7286. ATP hydrolysis by the mitochondrial F_0F_1-ATPase was monitored in a coupled enzymatic system using pyruvate kinase and lactate dehydrogenase. In the presence of the electron transport inhibitor, antimycin, our yeast mitochondrial preparations were reproducibly found to have specific ATPase activity in the range of 1 μmol/min/mg protein, which is similar to the value reported by other workers in the field [4]. Moreover, the cytochrome *c* oxidase activity in our preparations was 0.4 μmol/min/mg, which also compared favorably with the literature [4]. Using these intact mitochondrial preparations, the K_i of apoptolidin was determined to be 5 μM (Fig. 8A). Control experiments showed that the K_i of oligomycin in the same assay was 1 μM. As expected, the vacuolar ATPase inhibitor bafilomycin had no inhibitory effect on ATPase activity up to 50 μM, which was the solubility limit of the compound under our assay conditions (data not shown). Control experiments, performed using Na^+/K^+ ATPase (Sigma) and the same coupled enzymatic assay system, confirmed that apoptolidin had no effect on either pyruvate kinase or lactate dehydrogenase (data not shown). Moreover, unlike ouabain, a specific inhibitor of Na^+/K^+ ATPase, apoptolidin was unable to inhibit this enzyme at concentrations as high as 90 μM.

The above results were consistent with the hypothesis that, like oligomycin, apoptolidin was an inhibitor of the eukaryotic F_0F_1-ATPase. However, since intact mitochondria were used, the possibility that apoptolidin inhibited the mitochondrial ATP–ADP translocator (ANT), which is required to shuttle ATP and ADP into and out of mitochondria, could not be overlooked. To eliminate this possibility, we directly assayed the activity of apoptolidin against Triton X-100-solubilized F_0F_1-ATPase. Extraction of yeast mitochondria with Triton X-100 was known to liberate active, oligomycin-sensitive F_0F_1-ATPase [33,34]. As shown in Fig. 8B, the K_i of apoptolidin against solubilized F_0F_1-ATPase (4 μM) compared well to its activity against intact mitochondria (5 μM). In a control experiment, oligomycin was confirmed to inhibit the activity of Triton X-100-solubilized ATPase with a K_i of 0.1 μM. Since ANT was not required for ATPase activity in this assay, our results confirmed that apoptolidin was a potent and selective inhibitor of the mitochondrial F_0F_1 ATPase.

3. Discussion

The isolation of new selective cytotoxic agents is an important goal in the treatment of cancer. Our studies on the mechanism of action of apoptolidin revealed the surprising discovery that F_0F_1-ATP synthase inhibitors have the potential to be selective agents, as illustrated by the ability of apoptolidin to kill rat glial transformed cells but not untransformed rat glial cells [2] and by the data shown in Fig. 7. The mechanism of action of apoptolidin was determined by a combination of targeted pharmacological assays and structural considerations. In particular, inhibition of cell killing by BCL-2 and a caspase-9 specific inhibitor suggested that cell death signal induced by apoptolidin involved a mitochondria-dependent pathway. Moreover, the total independence of apoptolidin activity on p53 hinted that apoptolidin acted downstream of p53, at or near the mitochondria. Finally, the fortuitous identification of structural similarities between the apoptolidin

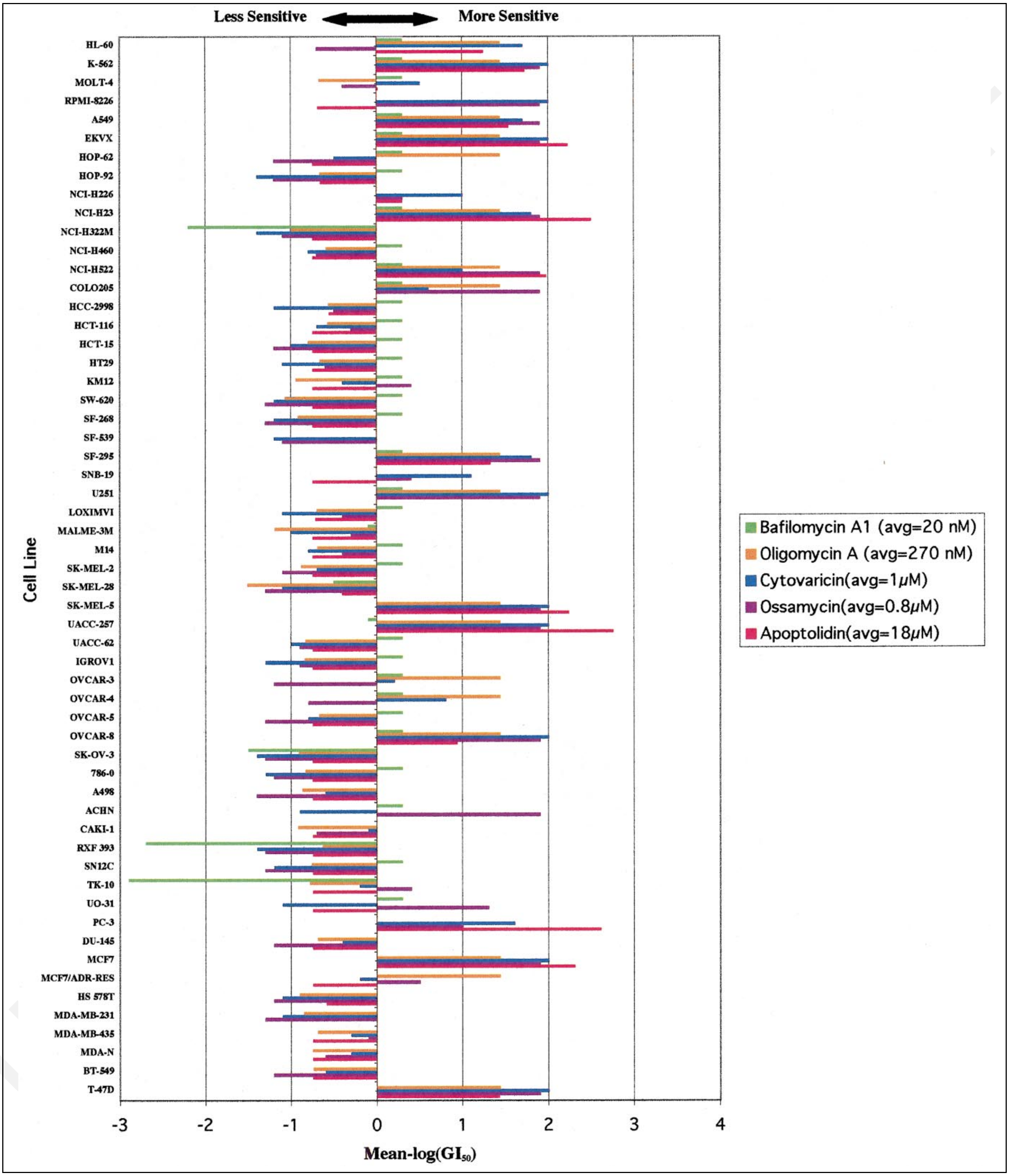

Fig. 7. Cytotoxic profiles of apoptolidin, oligomycin A, cytovaricin, ossamycin, and bafilomycin against the NCI-60 cell line panel. For more information regarding this assay, see http://dtp.nci.nih.gov/. Shown in the above bar graph is the activity (log scale) of each natural product against individual cell lines. The mean log(GI_{50}) values for bafilomycin, oligomycin A, apoptolidin, cytovaricin, and ossamycin are −7.7, −6.6, −4.7, −6, and −6.1, respectively.

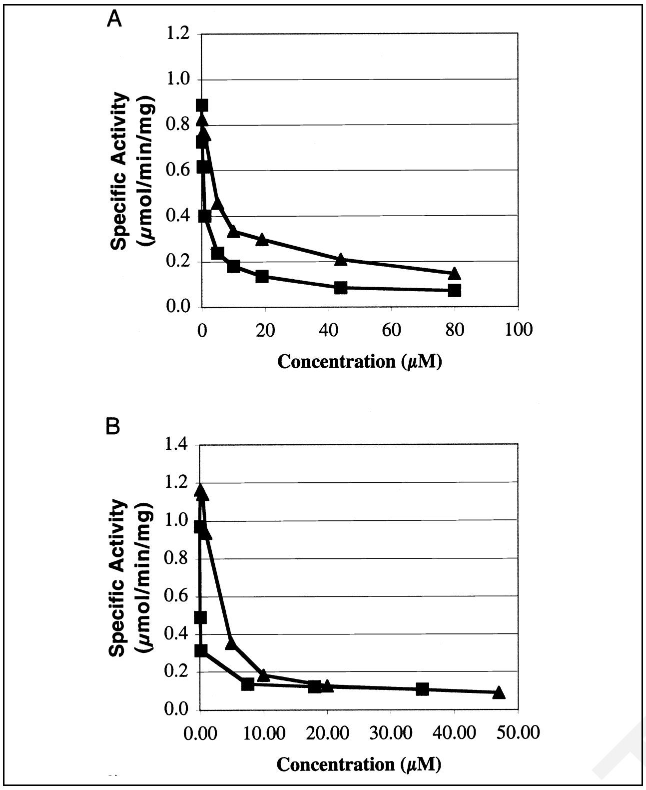

Fig. 8. Inhibition of yeast mitochondrial ATPase activity by apoptolidin (▲) and oligomycin (■): ATPase activity was measured by a coupled pyruvate kinase (PK), lactate dehydrogenase (LDH) enzyme assay system. NADH oxidation was monitored at 360 nm. A: Activity against intact mitochondria. B: Activity against 0.4% Triton X-100-solubilized mitochondria.

aglycone and oligomycin led us to establish that apoptolidin was an inhibitor of the mitochondrial ATP synthase.

Apoptolidin was originally identified on the basis of its ability to selectively kill E1A and E1A/E1B19K transformed rat glial cells while not killing untransformed glial cells or H-*ras*- and v-*src*-transformed cells. Many cancer cells maintain a high level of anaerobic carbon metabolism even in the presence of oxygen, a phenomenon that is historically known as the Warburg effect [38,39]. Recent results have led us to conclude that macrolide inhibitors of the mitochondrial F_0F_1-ATP synthase selectively kill aerobic, metabolically active tumor cells that do not exhibit the Warburg effect [40]. Furthermore we have shown that the master regulator of hypoxic and glycolytic gene expression, hypoxia-inducible factor 1α (HIF-1α), is expressed in Warburg type anaerobic cells but its protein expression is inhibited when these cells are switched to aerobic metabolism [40]. Interestingly, H-*ras*- and v-*src*-transformation has been shown to induce HIF-1α protein expression leading to a Warburg phenotype [41]. This could explain the resistance of cell lines transformed with these oncogenes to apoptolidin. Even though there are no available data of changes of HIF-1α protein expression with E1A transformation, we postulate that E1A is an aerobic transformation that probably does not induce HIF-1α protein expression and renders cells sensitive to apoptolidin.

It should be noted that, although the observed K_i for apoptolidin is 5-fold higher than that for oligomycin, this may be an underestimate of the potency of apoptolidin. In the course of our chemical studies on apoptolidin, we have observed a strong pH dependence in its stability (manuscript in preparation). Although the natural product is stable under acidic conditions, it rapidly degrades under alkaline conditions. Given that mitochondrial ATPase activity assays are typically performed at pH 8, the true K_i for apoptolidin may be lower than that reported in this study. Perhaps this could also account for the observation that the IC_{50} values for apoptolidin against LYas cells are substantially lower than the measured K_i against mitochondrial ATPase. Alternatively, this difference might be explained by a preference for mammalian ATPase over yeast ATPase, or by the possibility that apoptosis via this pathway is a dominant phenotype.

A recent report described the results of studies aimed at understanding the mechanistic basis for oligomycin-induced apoptosis [31]. DNA fragmentation in HL-60 cells induced by oligomycin was inhibited by serine protease inhibitors but not by caspase inhibitors including z-VAD.fmk. Furthermore, DNA fragmentation was not inhibited by ICAD in a cell free system. Our results show that apoptosis induced by apoptolidin in LYas cells is inhibited by z-VAD.fmk. Consistently, apoptolidin also induced cleavage of PARP which is known to be cleaved by activated caspase-3 [26]. The disparity in results could arise either due to slight differences in the interactions between these two natural products and their mitochondrial target, or as a result of the different assays employed to test for the induction of apoptosis.

With the discovery of the mechanism of action of apoptolidin, some intriguing questions remain to be answered. Paradoxically, ATP synthase inhibitors such as apoptolidin, oligomycin, cytovaricin, and ossamycin induce apoptosis, yet dATP is required for activation of caspases. A better understanding of the precise relationships between macrolide-mediated inhibition of the F_0F_1-ATPase and the well-known mitochondrial apoptosis pathway could provide new insights into the onset and progression of cancer.

4. Significance

Drugs that can selectively sensitize cancer cells to apoptosis induction are likely to play a vital role in cancer therapy. Although the anti-fungal activities of macrolide inhibitors of the mitochondrial F_0F_1-ATP synthase such as oligomycin, ossamycin and cytovaricin are well-documented, their unusual selectivity toward certain cell types is not widely appreciated. The demonstration that apoptolidin is an inhibitor of the mitochondrial F_0F_1-ATP synthase highlights the potential relevance of these natural products as small molecules to modulate apoptotic pathways. A better understanding of the mechanistic basis for this selective cytotoxicity might lead to increased interest in their potential utility as chemotherapeutic agents.

5. Materials and methods

5.1. Cells

LYas and LYar cells were grown in RPMI 1640 media and are sublines obtained from an apoptosis-sensitive B cell mouse lymphoma (TH-LY) [5]. HCT116 wt and p53 mutant cells were grown in McCoy's 5A media and were a kind gift of Dr. James Ford [8]. All cell culture media were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 50 U/ml streptomycin and cells were grown at 37°C, 5% CO_2 in air in a humidified incubator.

5.2. Drug additions

Apoptolidin was isolated from the producing organism as described previously [2]. Oligomycin A, bafilomycin, and cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Concentrated stock solutions of apoptolidin, oligomycin, and bafilomycin were prepared in phosphate-buffered saline (PBS) with less than 1% DMSO in the final drug dilution.

5.3. Preparation of bcl-2/IRES/GFP transfected LYas cells

LYas cells containing a *bcl-2*/GFP expression vector (BIG) and empty vector (GFP) were generated by adenovirus infection using previously described methods [35]. Briefly, helper-defective PHOENIX-Ampho packaging lines were transfected with GFP IRES expression vectors with or without human *bcl-2* inserted in the expression cassette. The resulting supernatants containing viral particles were used to infect LYas cells with the respective constructs. Cell cloning was performed by single cell FACS sorting of GFP positive cells into individual wells of a 96 well plate. Expression of BCL-2 was verified by internal staining for BCL-2 followed by FACS analysis as well as Western blot analysis.

5.4. Isolation of intact and Triton X-100-solubilized yeast mitochondria

Yeast mitochondria were isolated from a lactate grown *Saccharomyces cerevisiae* strain DBY7286 (*matA*, *ura*−/−) according to published procedures [36]. Briefly, 2 l shake flasks of yeast were grown up on semi-synthetic lactate medium at 30°C with vigorous shaking to an OD_{600} of 3. Cells were collected at $4000\times g$ and the wet weight of the pellet was determined. Cells were converted to spheroplasts by a 30 min incubation at 30°C with 2.5 mg Zymolyase 20T (ICN Biochemicals, St. Louis, MO, USA) per gram of packed cells in a volume of 2 ml per gram of packed cells in buffer A (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4). The Zymolyase 20T was washed out twice by centrifugation at $4000\times g$ and resuspension in buffer A. The spheroplasts were then resuspended in buffer B (0.6 M sorbitol, 20 mM K^+ MES, pH 6.0) with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and homogenized in a 40 ml glass Dounce homogenizer using 15 strokes with a tight-fitting pestle. The unbroken spheroplasts were collected by centrifugation at $1500\times g$ and rehomogenized with 15 strokes in buffer B plus PMSF. The nuclei and unbroken cells were separated by centrifugation at $1500\times g$ and the mitochondria were isolated from the supernatant by centrifugation at $12\,000\times g$ for 10 min. The mitochondrial pellet was then washed with buffer B and collected at $12\,000\times g$ for 10 min. Dark brown mitochondria were resuspended in buffer C (0.6 M sorbitol, 20 mM HEPES, pH 7.4). ATPase activity was measured within 6 h of preparing mitochondria. Protein concentrations were determined by the Lowry assay (Bio-Rad, Hercules, CA, USA).

To solubilize the F_0F_1-ATPase that is ordinarily attached to the inner membrane of mitochondria, 1 mg of purified yeast mitochondria (prepared as described above) were resuspended in 4 mM Tris/acetate, pH 7.4 [33]. This suspension was incubated with 0.75%, 1%, or 2% Triton X-100 for 20 min at 4°C. Solubilized mitochondria were then centrifuged at $100\,000\times g$ for 15 min at 4°C and the supernatant was tested for ATPase activity.

5.5. Assay for yeast mitochondrial ATPase activity

Mitochondrial ATPase activity was measured by standard methods [4]. Briefly, 20 μg of yeast mitochondrial protein (as measured by the Lowry method) was added to reaction buffer containing 50 mM Tris (pH 8.0), 1 mM ATP, 0.3 mM NADH, 3.3 mM $MgCl_2$, 2 μg/ml antimycin A, 1 mM phosphoenol pyruvate, 5 U/ml lactate dehydrogenase, and 2.5 U/ml pyruvate kinase at 28°C. Oxidation of NADH was followed at 360 nm over time. To establish the mitochondrial origin of the ATPase activity, published procedures were used to measure (mitochondrial) cytochrome *c* oxidase activity [37].

5.6. FACS assay for Annexin V and propidium iodide

Cells were treated with drugs for various times and then washed. Cells were stained with 5 μl/test Annexin V-FITC (Becton Dickinson, San Jose, CA, USA) for 15 min and washed three times. Next, the cells were stained with 1 μg/ml propidium iodide and washed two times. Cells were analyzed on the Facscan (Becton Dickinson) and the percentage of Annexin V and propidium iodide positive cells was quantified using FlowJo software for the Macintosh (Tree Star, Inc., San Carlos, CA, USA).

5.7. MTT assay

Drug dilutions were added to monolayer or suspension cells in 96 well plates in triplicate for varying times. MTT was then added to the wells at a final concentration of 0.5 mg/ml. Supernatant was removed after pelleting the reduced MTT crystals. The crystals were fully dissolved in 40 mM HCl in isopropanol. Plates were scanned on a microplate reader at 595 nm.

5.8. Cytotoxicity profiles in the NCI-60 cell line panel

The procedures for measuring GI_{50} values against a panel of selected human tumor cell lines are described on the web-site http://dtp.nci.nih.gov/. The activities of bafilomycin, ossamycin, and cytovaricin are documented on the same web-site. Oligomycin A and apoptolidin were submitted for similar analysis to the National Cancer Institute. The data are shown in Fig. 7.

5.9. Western blotting

For analysis of p53, BCL-2, and PARP expression levels, total cellular protein was isolated by lysing cells for 1 min at 98°C in a buffer of 2% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 6.8), 5% v/v glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, pH 6.8. Protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were subjected to 15% SDS–polyacrylamide gel electrophoresis and electroblotted to a nitrocellulose membrane. The membrane was blocked for 1 h in blocking buffer (PBS/Tween 20/10% milk) and then incubated for 4 h with 1:200 mouse anti-human p53 antibody (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1:400 hamster anti-human BCL-2 (6C8, BD Pharmingen, San Diego, CA, USA) or 1:3000 mouse anti-PARP (C2-10, BD Pharmingen). Membrane was then washed 2×15 min in blocking buffer followed by 2×15 min PBS/Tween washes. Membrane was then stained with 1:1000 sheep anti-mouse Ig (AP Biotech, Piscataway, NJ, USA) or 1:1000 anti-hamster IgG (Jackson Immunoresearch, West Grove, PA) directly conjugated to horseradish peroxidase for 1 h in blocking buffer and washed two times with blocking buffer and then two times with PBS/Tween. Bands were visualized using chemiluminescence with the ECL+ kit from AP biotech.

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