Ectopic expression of Bcl-2 or Bcl-xL suppresses p-fluorophenylalanine-induced apoptosis through blocking mitochondria-dependent caspase cascade in human Jurkat T cells

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Abstract

p-Fluorophenylalanine (FPA), a phenylalanine analog, is able to induce apoptotic cell death of human acute leukemia Jurkat T cells. To better understand the mechanism by which FPA induces apoptotic cell death, the effect of ectopic expression of antiapoptotic proteins, Bcl-2 and Bcl-xL, on FPA-induced apoptosis was investigated by employing Jurkat T cells transfected with Bcl-2 gene (JT/Bcl-2) or Bcl-xL gene (J/Bcl-xL) and Jurkat T cells transfected with vector (JT/Neo or J/Neo). When Jurkat T cells, JT/Neo or J/Neo, were exposed to FPA at concentrations ranging from 0.63 to 5.0 mM, the cell viability determined by MTT assay declined in a dose-dependent manner. In addition, apoptotic DNA fragmentation along with several apoptotic events such as caspase-8 activation, Bid cleavage, mitochondrial cytochrome c release, caspase-9 activation, caspase-3 activation, and degradation of PARP was induced. However, the FPA-induced cytotoxic effect, activation of caspase-8, and cleavage of Bid were significantly abrogated by ectopic expression of Bcl-2 or Bcl-xL. At the same time, there was marked reduction in the level of cytochrome c release from mitochondria, caspase-9 activation, caspase-3 activation, and degradation of PARP. These results indicate that caspase-8 activation, Bid cleavage, and mitochondrial cytochrome c release with subsequent activation of the caspase cascade are negatively regulated by Bcl-2 or Bcl-xL, and are thus required for FPA-induced apoptosis in Jurkat T cells.

Key words – p-fluorophenylalanine, apoptosis, cytochrome c-dependent caspase cascade, Bcl-2, Bcl-xL.

Introduction

p-Fluorophenylalanine (FPA) is a phenylalanine analog, which has enough structural similarity to compete with phenylalanine for phenylalanyl tRNA activation and for its incorporation into cellular proteins. Previously it has been shown that amino acid analogs possess toxicity against microorganisms including bacteria [4,7], yeasts [18,24] and viruses [14], and animal [20] and plant cells [15]. Based on their cytotoxicity, several studies have proposed the possible application of amino acid analogs to pharmacological treatment of malignant tumors [3,5,8,19,23]. However, the molecular and cellular basis underlying these inhibitory effects of amino acid analogs toward tumor cells still remains largely unknown.

Recently, we have shown that the cytotoxicity of L-canavanine, an L-arginine analog, toward human acute leukemia Jurkat T cells is attributable to the induced apoptosis [10]. The L-canavanine-induced apoptosis appeared to accompany mitochondrial cytochrome c-
independent activation of caspase-3, which could be interrupted by ectopic expression of Bcl-2 or Bcl-xL, suggesting that L-canavanine may induce apoptotic cell death of Jurkat T cells by triggering a conserved caspase cascade, leading to caspase-3 activation without involving mitochondrial cytochrome c release. More recently, we have found that thialysine, a lysine analog, can induce apoptotic cell death in Jurkat T cells via caspase-8 activation, Bid cleavage and mitochondrial cytochrome c release with resultant activation of caspase-9 and -3 (unpublished results). Since these results suggest that there are at least two different apoptotic pathways leading to caspase-3 activation in amino acid analog-induced apoptosis: a mitochondrial cytochrome c-independent pathway for L-canavanine and a mitochondrial cytochrome c-dependent pathway for thialysine, we have decided to examine if there is another apoptotic signaling pathway converging to caspase-3 activation in amino acid analog-induced apoptosis.

In the present study, to understand the mechanism underlying the apoptotic effects induced by amino acid analogs, we investigated the apoptotic activity of \( \beta \)-fluorophenylalanine (FPA), a phenylalanine analog, against Jurkat T cells, focusing on its death signaling pathway converging to caspase-3 activation. In addition, we investigated how these signaling events are modulated by antiapoptotic proteins Bcl-2 and Bcl-xL. The results show that FPA is able to induce apoptotic cell death in Jurkat T cells via caspase-8 activation, Bid cleavage, and mitochondria-dependent death signaling pathway, which appears to be similar with the death pathway for thialysine. These FPA-induced apoptotic changes were abrogated by ectopic expression of Bcl-2 or Bcl-xL, indicating that Bcl-2 and Bcl-xL are critical negative regulators of FPA-induced apoptotic cell death.

**Materials and Methods**

**Reagents, antibodies, and cells**

\( \beta \)-Fluorophenylalanine (FPA) was purchased from Sigma Chemical (St. Louis, MO, USA). The ECL Western blotting kit was from Amersham (Arlington Heights, IL, USA). Anti-cytochrome c antibody was purchased from Pharmingen (San Diego, CA, USA), and anti-PARP, anti-Bcl-2 and anti-Bcl-xL were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-caspase 3 and anti-FasL antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Human acute leukemia Jurkat T cell clones JT/Neo, J7/1Bcl-2, J7/Neo, and J7/Bcl-xL were supplied by Dr. Dennis Taub (Gerontology Research Center, NIA/NIH, Baltimore, MD, USA). Jurkat T cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 10% FBS (UBI, Lake placid, NY, USA), 20 mM HEPES (pH 7.0), 5\( \times \)10\(^{-3}\) M 2-ME, and 100 \( \mu \)g/ml gentamycin.

**Cytotoxicity assay**

The cytotoxic effect of FPA on Jurkat T cells was analyzed by MTT assay reflecting the cell viability. For MTT assay, Jurkat T cells (5\( \times \)10\(^4\)) were added to a serial dilution of FPA in 96-well plates. At 20 h after incubation, 50 \( \mu \)l of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. After centrifugation, the supernatant was removed from each well and then 150 \( \mu \)l of DMSO was added to dissolve the colored formazan crystal produced from MTT. OD values of the solutions were measured at 540 nm by plate reader.

**DNA fragmentation analysis**

Apoptotic DNA fragmentation induced in Jurkat T cells following FPA treatment was determined as previously described [9]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (0.5% Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, and pH 7.4) for 20 min on ice. After centrifugation for 15 min at 14,000 rpm, the supernatant is collected and treated for 2 h at 50°C with proteinase K and subsequently with RNase for 4 h at 37°C. After extraction
with an equal volume of buffer-saturated phenol, the DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 0.5 M NaCl and visualized following electrophoresis on a 1.2% agarose gel.

Flow cytometric analysis

Cell cycle progression of Jurkat T cells following FPA treatment was analyzed by Flow cytometry as described elsewhere [11]. Approximately $1 \times 10^6$ cells were suspended in 100 $\mu$l of PBS, and 200 $\mu$l of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended with 12.5 $\mu$g of RNase in 250 $\mu$l of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37°C for 30 min before staining the cellular DNA with 250 $\mu$l of propidium iodide (50 $\mu$g/ml) for 30 min at room temperature. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content, based on increased red fluorescence.

Preparation of cell lysate and Western blot analysis

Cellular lysates were prepared by suspending $5 \times 10^6$ Jurkat T cells in 200 $\mu$l of lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM MgCl$_2$, 0.1% Triton X-100, 25 mM MOPS, 2.5 $\mu$g/ml proteinase inhibitor E-64, and pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. An equivalent amount of protein lysate (20–30 $\mu$g) was denatured with SDS sample buffer, and subjected to electrophoresis on 4–12% SDS gradient polyacrylamide gel with MOPS buffer. For detection of caspase-3 activation and mitochondrial cytochrome c release, protein lysates were electrophoresed on 10% SDS gradient polyacrylamide gel with MES buffer. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Detection of each protein was carried out with the ECL Western blotting kit (Amersham, Arlington, Heights, IL, USA) detection solution according to the manufacturer’s instructions.

Detection of Bid and mitochondrial cytochrome c in cytosolic protein extracts

To assess Bid cleavage and mitochondrial cytochrome c release in Jurkat T cells following FPA treatment, cytosolic protein extracts were obtained. Briefly, approximately $5 \times 10^6$ cells treated with FPA were washed with cold PBS three times and then suspended in 0.5 ml of lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF, 2.5 $\mu$g/ml E-64, 20 mM HEPES, and pH 7.2). The cells were allowed to swell on ice for 30 min and were homogenized with a Dounce homogenizer with 50 strokes. The homogenates were centrifuged at 3,500 rpm for 10 min at 4°C, and the supernatants were centrifuged at 13,700 rpm for 15 min at 4°C. The supernatants were harvested as cytosolic extracts free of mitochondria, and analyzed for the alteration in the level of cytosolic Bid or mitochondrial cytochrome c release by Western blotting.

Results and Discussion

Cytotoxic effect of FPA on Jurkat T cells was attenuated by ectopic expression of Bcl-2 or Bcl-xL

The cytotoxicity of FPA on Jurkat T cells were analyzed by MTT assay. When Jurkat T cells (JT/Neo and J/Neo) were treated with FPA at various concentrations ranging from 0.63 to 5.0 mM for 24 h, cell viability appeared to decline significantly in a dose-dependent manner (Fig. 1A and B). After treatment with 0.63 mM FPA, the viability sustained at the level of 75–80%. However, cell viability declined in a dose-dependent manner, and remained at a minimal level (38–42%) in the presence of 5.0 mM FPA. Sequentially, we examined whether the cytotoxic effect of FPA is modulated by ectopic expression of Bcl-2 or Bcl-xL. As shown in Fig. 1A and B, the cytotoxic effect of FPA was significantly attenuated by the presence of Bcl-2 or Bcl-xL, indicating...
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Fig. 1. Inhibitory effect of Bcl-2 (A) or Bcl-xL (B) on cytotoxicity induced by p-fluorophenylalanine (FPA). Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) or Bcl-xL (J/Bcl-xL) and individual control (JT/Neo and J/Neo) cells were incubated at density of 5\times10^5/well with various concentrations of FPA in 96-well plate. After incubation for 20 hr, MTT was added for additional 4 h. The cells were processed to assess the colored formazan crystal produced from MTT as an index of cell viability.

that the FPA-induced apoptotic pathway involves at least a conserved death signaling event which can be blocked by the antiapoptotic role of Bcl-2 and Bcl-xL.

FPA-induced apoptotic DNA fragmentation in Jurkat T cells was blocked by ectopic expression of Bcl-2 or Bcl-xL.

Since cytotoxic effect of FPA appeared to significantly decline by ectopic overexpression of Bcl-2 or Bcl-xL, we have decided to investigate if the reduction in the cytotoxicity of FPA by Bcl-2 or Bcl-xL results from their protective roles against FPA-induced apoptotic DNA fragmentation. As shown in Fig. 2A and C, FPA-induced apoptotic DNA fragmentation in Jurkat T cells (JT/Neo and J/Neo) was easily detectable with a maximum level at concentrations ranging from 1.25 to 5.0 mM FPA. However, FPA-induced apoptotic DNA fragmentation was completely abrogated by ectopic expression of Bcl-2 or Bcl-xL. These results confirm that the protective effect of Bcl-2 and Bcl-xL on cytotoxicity of FPA toward Jurkat

Fig. 2. Inhibition of FPA-induced apoptotic DNA fragmentation by Bcl-2 (A and B) or Bcl-xL (C and D). Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) or Bcl-xL (J/Bcl-xL) and individual control cells (JT/Neo and J/Neo) were incubated at a density of 5\times10^5/well with various concentrations of FPA for 24 h. The cells were collected and were processed to analyze apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis.
T cells is due to their blocking of apoptotic DNA fragmentation. On the other hand, although the ectopic expression of Bcl-2 or Bcl-xL completely prevents FPA-induced apoptotic DNA fragmentation, the cell viability was not completely recovered under the same conditions. These results indicate that FPA not only induces the apoptotic DNA fragmentation, but also affects the cell cycle progression to reduce cell proliferation.

Jurkat T cells overexpressing Bcl-2 or Bcl-xL failed to undergo apoptosis as well as cell cycle progression in the presence of FPA.

DNA fragmentation analysis showed that ectopic expression of Bcl-2 or Bcl-xL completely prevents FPA-induced apoptosis. To confirm the effect of Bcl-2 or Bcl-xL on FPA-induced apoptosis and to investigate whether there is an apoptotic change in the cell cycle distribution of Jurkat T cells following FPA treatment, Jurkat T cells overexpressing Bcl-2 or Bcl-xL and their control cells were analyzed by Flow cytometry after treatment at various concentrations of FPA for 24 h. When either JT/Neo or J/Neo cells were treated with FPA at concentrations ranging from 0.63 to 5.0 mM for 24 h, the sub-G1 peak representing apoptotic cells was easily detectable and increased dose-dependently, in accordance with the results from apoptotic DNA fragmentation analysis (Fig. 3A and 4A). In addition, although the S phase cells were not changed significantly, the cells in the G1 and G2/M appeared to decline when there was a marked increase in the number of sub-G1 cells after FPA treatment. Under the same conditions, Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) or Bcl-xL (J/BclxL) showed no detectable increase in the level of sub-G1 peak, confirming the protective effect of Bcl-2 and Bcl-xL on FPA-induced apoptotic DNA fragmentation (Fig. 3B and 4B). However, the S phase cells for both JT/Bcl-2 and J/Bcl-xL appeared to decline in the presence of FPA dose-dependently, whereas the accumulation of the cells in the G1 and G2/M phase was enhanced. As shown in Fig. 5A and B, after incubation for 24 h, the initial cell number (4×10^5/ml) for JT/Bcl-2 or J/Bcl-xL increased to 1×10^6/ml in the absence of FPA, suggesting that the cells were able to complete one round of the cell cycle within 24 h. On the contrary, in the presence of 0.63~5.0 mM FPA, the cell growth appeared to decline in a dose-dependent manner. The cell number after incubation for 24 h at a concentration of 5.0 mM was equal to the initial cell number, indicating that the cells failed to proceed the cell cycle in the presence of 5.0 mM FPA. These results demonstrate that the ectopic expression of Bcl-2 or Bcl-xL can protect Jurkat T cells from FPA-induced apoptosis but not from FPA-induced arrest of the cell cycle progression. In addition, these
Fig. 4. Effect of Bcl-xL on apoptotic change in the cell cycle distribution in Jurkat T cells after treatment of FPA. Both Jurkat T cells overexpressing Bcl-xL (J/J/Bcl-xL) (B) and control cells (J/J/Neo) (A) were incubated at a density of 5×10⁶/well with various concentrations of FPA for 24 h, and the cells were harvested. The analysis of cell cycle distribution was performed on an equal number of cells (2×10⁶) by flow cytometry.

Fig. 5. Effect of FPA on cell proliferation of Jurkat T cells overexpressing Bcl-2 (J/J/Neo) or Bcl-xL (J/J/Bcl-xL) and individual control cells (J/J/Neo and J/J/Neo). Jurkat T cells overexpressing Bcl-2 (J/J/Bcl-2) or Bcl-xL (J/J/Bcl-xL) and individual control cells were incubated at density of 4×10⁶/ml with various concentrations. After incubation for 24 h, the cells were stained with trypsin blue and the viable cell number was counted with hemocytometer.

Results indicate that FPA blocks the G1/S and G2/M transition of the cell cycle progression, and suggest that the majority of Jurkat T cells (J/J/Neo and J/J/Neo) that fail to complete these two transition points in the presence of FPA may undergo apoptotic cell death.

FPA-induced apoptotic events including caspase-8 activation, Bid cleavage, and mitochondria-dependent caspase cascade were blocked by ectopic expression of Bcl-2 or Bcl-xL.

When we performed Western blot analysis to investigate the death signaling pathway responsible for FPA-induced apoptosis of Jurkat T cells (J/J/Neo and J/J/Neo), a decline in the level of pro-caspase-8 (55 kDa) and cytosolic level of Bid, which reflects activation of caspase-8 and resultant cleavage of Bid to truncated Bid (tBid), was easily detectable (Fig. 6 and 7). At the same time, mitochondrial cytochrome c release into cytosol, cytochrome c-dependent activation of caspase-9, caspase-3 activation, and cleavage of poly (ADP-ribose) polymerase (PARP) were detectable. This cleavage of PARP by active caspase-3 is proposed as a marker of apoptosis in many experimental models [13]. As shown in Fig. 6 and 7, the...
Fig. 6. Western blot analysis of overexpression of Bcl-2 (A) and its inhibitory effect on caspase-8 activation (B), Bid cleavage (C), mitochondrial cytochrome c release (D), caspase-9 activation (E), caspase-3 activation (F), cleavage of PARP (G), and FasL (H) induced by FPA. Both Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) and control cells (JT/Neo) were incubated at a density of 5×10⁶/well with indicated concentrations of FPA and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. The membrane was probed with each primary antibody and then with a horse-radish peroxidase conjugated secondary antibody. Detection of each protein was performed using the ECL Western blotting detection system.

appearance of active caspase-9 (35 kDa) and active caspase-3 (19 kDa) in the presence of FPA was proportional to the decrease in the level of the inactive pro-caspase-9 (46 kDa) and -3 (32 kDa). These results suggest that FPA treatment in Jurkat T cells (JT/Neo and J/Neo) induce caspase-8 activation and cleavage of Bid, which can cause cytochrome c release from mitochondria and subsequent activation of cytochrome c-dependent caspase cascade, leading to PARP cleavage and apoptotic DNA fragmentation. However, it remains unclear whether FPA-mediated caspase-8 activation with resultant cleavage of Bid, and mitochondrial cytochrome c release are prerequisite for FPA-induced apoptotic cell death.

Recently, it has been reported that antiapoptotic regulatory protein Bcl-2 and its homolog Bcl-xL can protect cells from apoptosis induced by diverse signals.
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such as Fas ligation, ionizing radiation, hypoxia, or chemotherapeutic agents [1,6,16,17,21]. The antiapoptotic role of Bcl-2 and Bcl-xL is initially known to center around their prevention of cytochrome c release from mitochondria [12,22]. Although the mechanism by which Bcl-2 or Bcl-xL can block caspase-8 activation remains to be elucidated, it has been suggested that Bcl-2 or Bcl-xL directly prevents caspase-8 activation as well as Bid cleavage by caspase-8, resulting in suppression of the mitochondria-dependent apoptotic pathway [2]. We decided to take advantage of this antiapoptotic role of Bcl-2 and Bcl-xL to examine whether FPA-induced caspase-8 activation, Bid cleavage, and mitochondrial cytochrome c release comprise a conserved death signaling pathway that is sensitive to antiapoptotic action of Bcl-2 or Bcl-xL, and that caspase-8 activation and Bid cleavage as well as mitochondrial cytochrome c release are crucial steps for FPA-induced apoptosis. In this regard, we investigated the effect of ectopic overexpression of Bcl-2 or Bcl-xL on FPA-induced caspase-8 activation and Bid cleavage, and subsequent mitochondria-mediated apoptotic events including cytochrome c release, caspase-9 activation, caspase-3 activation, and PARP cleavage by employing Jurkat T cells transfected with Bcl-2 gene (JT/Bcl-2) (Fig. 6) or Bcl-xL gene (J/Bcl-xL) (Fig. 7) and individual control cells. As shown in Fig. 6A and 7A, the ectopic expression of Bcl-2 or Bcl-xL in Jurkat T cells was confirmed by Western blot analysis. When the effect of ectopic expression of Bcl-2 or Bcl-xL on FPA-mediated caspase-8 activation and Bid cleavage was investigated by Western blot analysis, both caspase-8 activation and Bid cleavage inducible in the presence of 0.63~5.0 mM FPA appeared to be completely abrogated by ectopic expression of Bcl-2 or Bcl-xL. Under these conditions, FPA-mediated mitochondrial cytochrome c release, caspase-9 activation, caspase-3 activation, and degradation of PARP declined to an undetectable or barely detectable level. These results indicate that caspase-8 activation, Bid cleavage, and mitochondrial cytochrome c release with subsequent activation of the caspase cascade are negatively regulated by Bcl-2 or Bcl-xL, and are thus required for FPA-induced apoptotic DNA fragmentation. These results demonstrate that FPA sequentially causes caspase-8 activation, Bid cleavage, and mitochondrial cytochrome c release leading to sequential activation of caspase-9, and -3 in Jurkat T cells. Previously we have shown that the L-arginine analog, L-canavanine induces apoptotic cell death of Jurkat T cells via mitochondrial cytochrome c-independent caspase-3 activation [10] and the lysine analog, thialysine induces apoptotic cell death of Jurkat T cell via mitochondrial cytochrome c-dependent caspase-3 activation (unpublished data). These previous results together with the present results suggest that caspase-3 activation is commonly involved in amino acid analog-mediated apoptosis. However, it is likely that there are at least two different apoptotic pathways leading to caspase-3 activation in amino acid analog-induced apoptosis: a mitochondrial cytochrome c-independent pathway for L-canavanine and a mitochondrial cytochrome c-dependent pathway for thialysine and p-fluorophenylalanine.

Taken together these results demonstrate that the cytotoxicity of FPA toward Jurkat T cells is attributable to apoptotic cell death mediated by a mitochondria-dependent death signaling pathway that includes caspase-8 activation, Bid cleavage, mitochondrial cytochrome c release, caspase-9 activation, and caspase-3 activation. The results also indicate that FPA-induced commitment of apoptosis is negatively regulated by ectopic expression of Bcl-xL through its protective roles against caspase-8 activation, Bid cleavage, and mitochondrial cytochrome c release with resultant activation of the caspase cascade. The mechanism by which FPA induces caspase-8 activation remains to be elucidated, and will be investigated in our future studies.
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References

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초록: Jurkat T 세포에 있어서 p-fluorophenylalanine에 의해 유도되는 세포자살의 Bcl-2 및 Bcl-xL에 의한 저해 기전

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Phenylalanine의 구조유사체인 pfluorophenylalanine (FPA)은 인체 급성백혈병세포주인 Jurkat T 세포의 세포자살을 유도한다. FPA에 의한 세포자살에 미치는 Bcl-2 또는 Bcl-xL의 영향을 조사하기 위해, Bcl-2 또는 Bcl-xL을 stable transfection하거나 empty vectors만을 transfection한 Jurkat 세포를 이용하여 FPA의 세포독성과 FPA에 의한 세포내 세포주살 신호전달경로를 비교 분석하였다. Jurkat T 세포에 0.63~5.0 mM의 FPA를 처리하였을 때 세포의 생존도는 농도에 비례하여 감소하였다. 또한 세포자살관련 DNA fragmentation, caspase-8 activation, Bid cleavage, mitochondria로 부터의 cytochrome c 방출, caspase-9 및 -3 activation, PARP degradation 등이 유도되었다. 한편, FPA에 의해 유도되는 이러한 일련의 생화학적 현상들은 Bcl-2 또는 Bcl-xL의 overexpression에 의해 현저히 저해되었다. 이상의 결과들은 caspase-8 activation, Bid cleavage, mitochondrial cytochrome c 방출에 의해 환경화되는 caspase cascade 등의 현상이, Bcl-2 또는 Bcl-xL에 의해 역제될을 나타내며 FPA에 의해 유도되는 세포자살에 필요한 과정임을 시사한다.