

Farnesylpyridinium, an analog of isoprenoid farnesol, induces apoptosis but suppresses apoptotic body formation in human promyelocytic leukemia cells

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Abstract 1-Farnesylpyridinium (FPy), an analog of isoprenoid farnesol, initially induced morphological changes similar to those of typical apoptosis in human leukemia HL-60 cells but FPy-treated cells were characterized by the absolute absence of final apoptotic events such as fragmentation into apoptotic bodies. FPy-induced cell death was considered to be apoptotic on the basis of the induction of DNA fragmentation and the protection against these events by the coaddition of a pan-caspase inhibitor. The increase in the cytoplasmic cytochrome *c* level supported the possibility that FPy-treated cells should have the ability to complete the entire apoptotic process ending in cell fragmentation and apoptotic body formation. At concentrations too low to induce apoptosis, FPy could suppress the induction of apoptotic body formation in HL-60 cells by typical inducers of apoptosis such as actinomycin D or anisomycin. FPy exhibited a cytochalasin-like effect on spatial arrangement of actin filament independent of its apoptosis-inducing activity. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Apoptosis; HL-60 cells; Farnesol; Apoptotic body; Actin

1. Introduction

Farnesyl pyrophosphate is an intermediate in the biosynthetic reaction of cholesterol and non-sterol isoprenoids, in which 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase produces mevalonate as a rate-limiting enzyme of the reaction [1]. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are also required for the post-translational modification of diverse cell proteins such as nuclear lamins,

G proteins and small GTP-binding proteins of the Ras superfamily [2]. The overall inhibition of isoprenoid biosynthesis can be attained by the addition of competitive inhibitors of HMG-CoA reductase such as lovastatin and these inhibitors have also been shown to induce apoptosis of various cell lines including human lung adenocarcinoma A549 [3–5]. Exposure of cells to farnesol (FOH) and geranylgeraniol results in apoptosis in a number of neoplastically transformed cells [6–9]. FOH was found to inhibit isoprenoid metabolism by accelerating degradation of HMG-CoA reductase rather than by acting as a competitive inhibitor of farnesyl:protein transferase [10,11].

Apoptosis is characterized by a series of morphological changes involving disappearance of cell surface microvilli, cell shrinkage, chromatin condensation and nuclear collapse [12,13]. Internucleosomal DNA fragmentation is another feature of apoptosis and is caused by a caspase-dependent activation of endonuclease [14]. At the final stage, apoptotic cells are fragmented into membrane-bound small particles, the so-called apoptotic bodies, possibly to enhance the phagocytic ingestion of dead cells and to enhance elimination of their macromolecular constituents [15–18]. The actin cytoskeleton appears to play an essential role in apoptotic morphological changes as deduced from the alteration of its spatial arrangement, resulting from filamentous actin disorganization and reorganization [15,16,19]. In apoptosis of HL-60 cells undergoing apoptosis, apoptotic body formation was suppressed by the coaddition of cytochalasin B, an agent which can inhibit actin polymerization [15].

FOH has previously been structurally modified to give tocotrienol [20–22], menaquinone-3 [23], farnesylthiosalicylic acid [24,25], farnesyl thioacetate [26] and farnesylamine [27–29], and most of these hybrid molecules have been shown to have greater *in vitro* tumor suppressive potency than FOH itself against various cell lines. This means that the cytotoxicity of FOH primarily depends on its isoprenoid hydrocarbon structure and can be elevated or modified by varying the structure of the attached group. We synthesized 1-farnesylpyridinium (FPy) as a novel analog of FOH (Fig. 1). FPy-treated HL-60 cells initially underwent morphological changes similar to those of typical apoptosis, but showed the complete absence of fragmentation into small particles or apoptotic body formation. We focussed our attention on the intracellular events involved in FPy-induced cell death accompanying this unusual morphological profile.

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Abbreviations: FPy, 1-farnesylpyridinium; FOH, farnesol; Cyt *c*, cytochrome *c*; Act D, actinomycin D; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PBS, phosphate-buffered saline; H₂CB, dihydrocytochalasin B; LPy, 1-laurylpyridinium; Z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; Z-Asp-CH₂-DCB, carbobenzoxy-L-aspart-1-yl-[(2,6-dichlorobenzoyl)oxy]methane

2. Materials and methods

2.1. Cell culture

The HL-60 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 µg/ml penicillin, and 100 µg/ml streptomycin. The cells were grown in a humidified incubator at 37°C under a 5% CO₂/95% air atmosphere and used for assays during the exponential phase of growth. Unless otherwise stated, confluent cells were then seeded in 1 ml of fresh medium in 48-well plates at an initial density of 10⁶ cells/ml and incubated with or without chemical treatment for the indicated times in each of the following experiments. Precultivation was normally done for 2 h in the fresh medium prior to the addition of each chemical.

2.2. Microscopic observations of morphology, nuclear fragmentation and actin microfilament

Cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS) and fixed with 1% glutaraldehyde in 100 µl of PBS for 1 h at room temperature. Fixed cells were washed with PBS, stained with 200 µM Hoechst 33258 in 20 µl of PBS, and observed and counted under phase-contrast fluorescence microscopes. Fixed cells were stained with rhodamine-phalloidine for visualization of actin microfilaments [19].

2.3. Assay of DNA fragmentation

Cells were harvested by centrifugation and incubated in 400 µl of lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM EDTA; 0.5% SDS; 1 mg/ml proteinase K) at 37°C overnight. After purification and enrichment of the DNA in each lysate, the finally obtained DNA samples were subjected to electrophoresis in 1% agarose gels [16].

2.4. Assay of mitochondrial membrane potential

Confluent cells were seeded in 1 ml of fresh medium at an initial density of 10⁶/ml, preincubated for 2 h and further incubated for 10 min after supplementation with 2 µM rhodamine 123. After incorporation of the fluorescent probe, cells were incubated for the indicated times with or without FPy, harvested by centrifugation and then suspended in 100 µl of PBS after they were washed once with PBS. The fluorescence intensity of each cell suspension was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm in a Cytofluor 2300 fluorescence spectrophotometer. The fluorescence intensity was used as an arbitrary unit representing the mitochondrial transmembrane potential.

2.5. Western blot analysis of cytochrome *c* (Cyt *c*)

Cells were seeded in 3 ml of fresh medium at an initial density of 10⁶/ml and incubated for the indicated times with or without addition of FPy. Cells were harvested by centrifugation and washed once with lysis buffer (30 mM Tris-HCl, pH 7.4; 5 mM KH₂PO₄; 40 mM KCl; 0.5 mM EDTA; 3 mM MgCl₂; 75 mM sucrose; 20 mM D-glucose). Cells were suspended in 300 µl of this buffer and lysed by the addition of 5 mg of digitonin and incubation at 37°C for 7 min. An aliquot (equivalent to 20 µg of protein) of the supernatant obtained after centrifugation of each cell lysate was dissolved in SDS-sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 10% mercaptoethanol; 20% glycerol; 0.002% bromophenol blue) and boiled for 5 min. The samples were subjected to SDS-PAGE on a 15% polyacrylamide gel followed by electrophoretic transfer of the proteins in the gel to a PVDF membrane (Amersham) for 4 h at 200 mA. The membrane was incubated with a primary antibody against Cyt *c* (1:1000 dilution) and then with horseradish peroxidase-linked secondary antibody (1:1000 dilution) against mouse IgG. The band of Cyt *c* was visualized by chemiluminescence using the ECL Western Blotting Detection kit (Amersham).

2.6. Synthesis of FPy

To prepare FPy, a mixture of farnesyl chloride (144 mg, 0.6 mmol) and dehydrated pyridine (2.5 ml) was heated at 80°C for 1 h. After cooling to room temperature, the resulting mixture was diluted with two parts of dichloromethane and applied onto a silica gel column. Elution with dichloromethane followed by 5% methanol in dichloromethane afforded FPy as a yellowish gummy liquid in 90% yield. Its chemical structure (Fig. 1) was elucidated based on the FAB(+)-MS *m/z* value of 284 (M⁺) which was detected using a JEOL JMS-700T

mass spectrometer, and the NMR spectrum determined using a JEOL JNM-LA400 NMR spectrometer in chloroform-*d*₁ at 40°C.

2.7. Other chemicals

FOH, farnesyl chloride, actinomycin D (Act D), anisomycin, Hoechst 33258, rhodamine 123 and dihydrocytochalasin B (H₂CB) were obtained from Sigma. Rhodamine-phalloidine was from Molecular Probes, Inc. 1-Laurylpyridinium (LPy) was a product of Wako Pure Chemical Co. (Japan). Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-fmk) and carbobenzoxy-L-aspart-1-yl-[(2,6-Dichlorobenzoyl)oxy]methane (Z-Asp-CH₂-DCB) were purchased from the Peptide Institute (Japan). The anti-Cyt *c* antibody was a product of Pharmingen.

3. Results and discussion

3.1. Evaluation of FPy-induced cell death

FOH-treated cells showed typical apoptotic morphological changes such as cell shrinkage and nuclear fragmentation, and were ultimately fragmented into apoptotic bodies, as shown in Fig. 2C,C'. FPy-induced cell death exhibited morphological changes similar to those in FOH-treated cells, but later exhibited an unusual morphological event, namely, failure in fragmentation into small particles or apoptotic bodies even at 6 h of incubation (Fig. 2B). The initially formed shrunken and spherical cell shape was maintained with no additional morphological changes for at least 12 h of incubation and, thereafter the whole cell structure was gradually lost without any sign of apoptotic body formation. As is the case for the main chain of the isoprenoid hydrocarbon of FPy, the linear chain of LPy consists of 12 carbon atoms with no methyl side chains (Fig. 1). LPy caused a different type of cytotoxicity, namely one accompanied by drastic cell lysis or plasma membrane collapse, reflecting an ability to induce necrotic cell death or a detergent-like property (Fig. 2D,D').

In spite of the failure of cell fragmentation, nuclear fragmentation was clearly observed with FPy-treated cells (Fig. 2B'), suggesting the possibility that FPy may selectively affect nuclear membrane assembly or interfere with some mechanism essential for maintenance of the nuclear structure.

3.2. FPy-induced intracellular events

As shown in Fig. 3, DNA ladders were observed in FPy-treated cells in a time-dependent and dose-dependent manner, along with the increase in the number of cells carrying fragmented nuclear particles. FPy-induced nuclear fragmentation may thus be an apoptotic event provoked along with endonuclease activation via a caspase family member. The caspase cascade functions to achieve a number of apoptotic events including actin depolymerization, poly(ADP-ribose) polymerase inactivation and endonuclease activation [14,30]. We examined whether or not the caspase cascade was involved in the progression of the unusual morphological changes in FPy-treated cells by using pan-caspase inhibitors such as Z-VAD-fmk and Z-Asp-CH₂-DCB [31–33]. FPy-induced nuclear collapse and DNA fragmentation were fully suppressed by the coaddition of each of these inhibitors at 100 µM (data not shown).

Several lines of evidence indicate that activation of caspase cascade can be achieved through mitochondrial Cyt *c* release [34,35]. Fig. 4 shows a clear time-dependent increase in the cytoplasmic Cyt *c* level in FPy-treated cells in parallel with time-dependent mitochondrial transmembrane depolarization. These FPy-induced intracellular events imply that FPy-treated

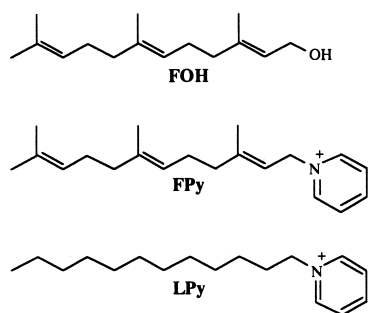


Fig. 1. Structures of FOH, FPy and LPy.

cells should have the ability to complete the entire apoptotic process ending in cell fragmentation and apoptotic body formation.

3.3. Protective effect of FPy against apoptotic body formation and actin organization

Cytochalasins are fungal metabolites that can prevent actin polymerization or cause actin depolymerization, thereby disrupting microfilament network interactions *in vivo* [36]. In accordance with previous reports [15,19], H₂CB did not provoke any apoptotic event by itself but could effectively prevent the formation of apoptotic bodies without causing any protective effect against other apoptotic events such as cell shrinkage and nuclear collapse induced with Act D (Fig. 5). H₂CB exhibited exactly the same type of protective effect against apoptotic body formation induced by anisomycin, an inhibitor of protein synthesis.

It was examined whether FPy can protect against apoptotic body formation in medium containing either Act D or anisomycin. FPy was found to induce apoptosis in $74 \pm 8\%$ of total cells at 50 μM by itself on the basis of the appearance of cells with fragmented nuclear particles (Fig. 5A). The ratio of apoptotic cells was further increased with the coaddition of Act D but their apoptotic bodies formation could be scarcely induced under the condition with 50 μM FPy (Fig. 5B). FPy significantly lost the ability to induce apoptosis at 12.5 μM by itself whereas the analog was still effective for preventing apoptotic body formation of Act D-treated cells at this concentration. FPy was more protective against apoptotic body formation of HL-60 cells when apoptosis was induced by anisomycin (Fig. 5C). These findings indicate that FPy can exhibit such a protective effect against apoptotic body formation independently of its apoptosis-inducing activity.

A fungal metabolite, jasplakinolide, is known to exhibit profound effects not only on the morphology of apoptotic cells but also on apoptosis induction in HL-60 cells [19]. Pronounced actin polymerization induced by the agent corresponds morphologically with prominent membrane blebbing, but with little apoptotic body formation. We therefore examined whether FPy can influence the actin polymerization status as is the case with H₂CB or jasplakinolide. As reported by Levee et al. [16], actin filaments were visible as a haze-like image brightly stained with the fluorescent probe in untreated cells at 6 h of incubation although individual microfilaments were not clearly resolved. When cells were treated with 1 μM Act D alone for 6 h, apoptotic bodies but not unfragmented cells were observed with brightly stained intracellular component, reflecting disorganization of actin microfilaments during the stage of apoptosis induction and its reorganization along with apoptotic body formation [16]. In accordance with that

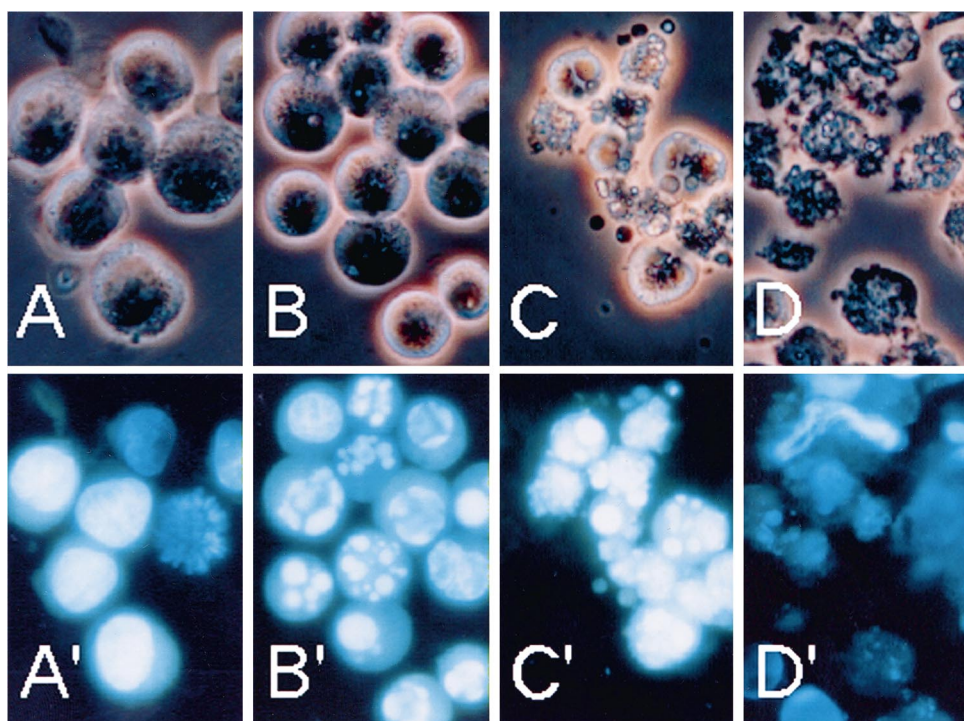


Fig. 2. Phase-contrast (A–D) and fluorescent microscopic observations (A'–D') of HL-60 cells incubated in medium alone (A,A'), medium with 50 μM FPy (B,B'), medium with 125 μM FOH (C,C') and medium with 12.5 μM LPy (D,D') for 6 h.

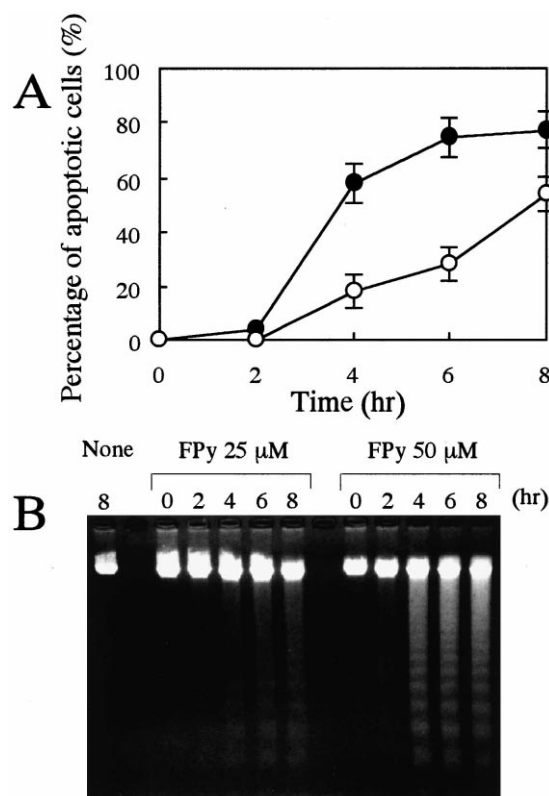


Fig. 3. Time course of changes in the number of cells carrying fragmented nuclear particles (A) and DNA ladder formation (B) during FPy treatment of HL-60 cells. Cells were incubated with 25 (\circ) or 50 (\bullet) μ M FPy for the indicated times. In (A), 200 cells were counted per each sample after Hoechst staining. Values are means \pm S.D. from triplicate assays.

fact, actin microfilaments were invisible in Act D-treated cells when they were protected against fragmentation into apoptotic bodies with the coaddition of 10 μ M H₂CB. Actin microfilaments were invisible when apoptotic cells were prevented

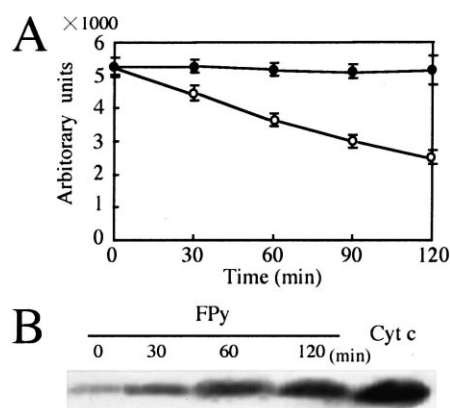


Fig. 4. Time course of changes in the mitochondrial membrane potential (A) and the cytoplasmic Cyt *c* level (B) during FPy treatment of HL-60 cells. In (A), cells were incubated in medium without (\bullet) or with (\circ) 50 μ M FPy for the indicated times. Values are means \pm S.D. from triplicate assays. In (B), cells were incubated in medium with 50 μ M FPy for the indicated times. Cyt *c* (500 ng) from bovine heart mitochondria (Sigma) was used as a standard in the Western blot analysis.

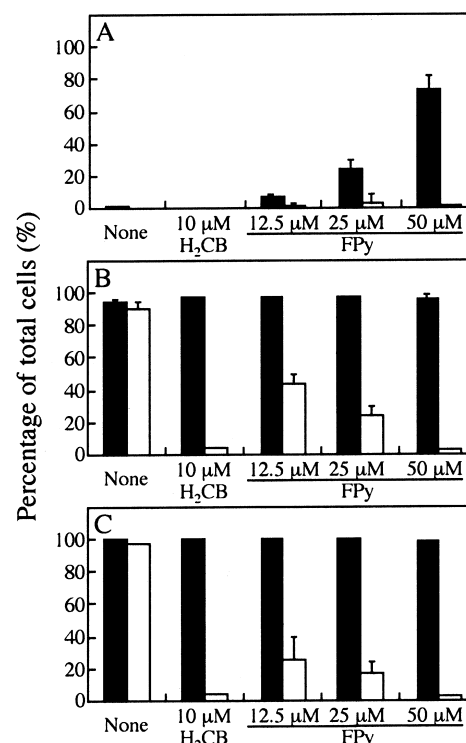


Fig. 5. Dose dependence of effects of FPy for induction of apoptosis (A) as well as for protection of apoptotic body formation induced by Act D (B) or anisomycin (C). In (A), cells were incubated in medium with H₂CB or FPy alone at the indicated concentrations for 6 h. Cells were incubated in medium with H₂CB or FPy at the indicated concentrations, and also supplemented with each of 1 μ M Act D for 6 h (B). In (C), 5 μ M anisomycin was used instead of Act D. Closed bars indicate the percentage of cells showing nuclear fragmentation and open bars indicate that of cells showing apoptotic body formation. In each sample of (A–C), 200 cells were counted and values are means \pm S.D. from triplicate assays.

from apoptotic body formation in medium with 50 μ M FPy alone as well as in medium with both 50 μ M FPy and 1 μ M Act D. These findings suggested that FPy showed an H₂CB-like activity such as preventing repolymerization of actin microfilaments that had been depolymerized due to its apoptosis-inducing activity.

In addition to the inhibition by cytochalasins, the formation of apoptotic bodies was prevented in HL-60 cells by the addition of benzamide or 3-aminobenzamide, an inhibitor of PARP [17], and in human myeloma RPMI 8226 cells by dexamethasone [18]. Dexamethasone was found to repress transglutaminase during the retinoic acid-induced apoptosis. Unlike the case with FPy and H₂CB, these agents could suppress nuclear condensation or fragmentation as well, although they were permissive for DNA fragmentation. Our results reveal that the structural modification of FOH generated an inhibitory activity against the mechanism essential for apoptotic body formation independent of its activity of apoptosis induction. The molecular target of FPy is currently under investigation with the aim of determining the reaction(s) most closely involved in spatial arrangement of actin filament.

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