Caspase-mediated apoptosis and caspase-independent cell death induced by irofulven in prostate cancer cells

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Abstract

Irofulven (hydroxymethylacylfulvene) is a novel antitumor drug, which acts by alkylating cellular macromolecular targets. The drug is a potent inducer of apoptosis in various types of tumor cells, whereas it is nonapoptotic in normal cells. This study defined molecular responses to irofulven involving mitochondrial dysfunction and leading to death of prostate tumor LNCaP-Pro5 cells. Irofulven caused early (2–5 hours) translocation of the proapoptotic Bax from cytosol to mitochondria followed by the dissipation of mitochondrial membrane potential and cytochrome c release at 4 to 12 hours. These effects preceded caspase activation and during the first 6 hours were not affected by caspase inhibitors. Processing of caspase-9 initiated the caspase cascade at ~6 hours and progressed over time. The activation of the caspase cascade provided a positive feedback loop that enhanced Bcl-2-independent translocation and cytochrome c release. General and specific caspase inhibitors abrogated irofulven-induced apoptotic DNA fragmentation with the following order of potency: pan-caspase ≥ caspase-9 > caspase-8/6 > caspase-2 > caspase-3/7 > caspase-1/4. Abrogation of caspase-mediated DNA fragmentation failed to salvage irofulven-treated cells from growth inhibition and loss of viability, demonstrating a substantial contribution of a caspase-independent cell death. Monobromobimane, an inhibitor of alternative caspase-independent apoptotic pathway that is mediated by mitochondrial permeability transition, antagonized both apoptosis, measured as phosphatidylserine externalization, and cytotoxicity of irofulven. Collectively, the results indicate that irofulven-induced signaling is integrated at the level of mitochondrial dysfunction. The induction of both caspase-dependent and caspase-independent death pathways is consistent with pleiotropic effects of irofulven, which include targeting of cellular DNA and proteins. [Mol Cancer Ther 2004;3(11):1385–96]

Introduction

Apoptosis induction is a desirable property for various anticancer drugs including those that target cellular DNA. Apoptosis induction in response to DNA-targeting drugs has been extensively studied and the role of DNA damage as a significant proapoptotic stimulus is well established (1). It is less understood what factors are responsible for the high variability of the timing and magnitude of apoptosis induction among different DNA-reactive drugs and in various cell models. We have postulated that such differences could reflect in part the interplay of DNA damage with drug effects on other cellular targets (2, 3). This idea takes into account the fact that various anticancer drugs classified as DNA-damaging alkylating agents tend to react mainly with cellular proteins, although their DNA adducts constitute only a small proportion of total cellular adducts (4, 5).

One of the novel anticancer drugs that targets both DNA and proteins is a semisynthetic sesquiterpenoid irofulven (hydroxymethylacylfulvene, MGI 114, or NSC 683863; Fig. 1; refs. 2, 3, 5, 6). Irofulven, which is currently under investigation in several phase I and II clinical trials (7, 8), shows in vivo antitumor activity against a broad spectrum of human tumor xenografts (9–13). In addition, the drug remains efficacious against various models of multidrug-resistant tumors (11, 14). Tumor shrinkage and complete curative effects were frequently observed (12, 13).

Consistent with its ability to cause tumor regression (12, 13), irofulven exhibits potent proapoptotic properties in various tumor cell systems (15–17), including models with apoptotic deficiencies, such as the lack of caspase-3 (18) and overexpression of antiapoptotic Bcl-2 (19). In contrast, several normal cell lines tested showed remarkable resistance to apoptosis induction by irofulven despite the formation of drug-macromolecule adducts at levels comparable with those in tumor cells (16, 20). For equivalent cellular drug uptake, irofulven was 3- to 4-fold less growth inhibitory to normal cells than to tumor cells (20). Moreover, drug-treated normal cells remained viable (nonapoptotic) and capable of resuming growth after a lag period (16, 20).
Biological activities of irofulven seem to be related to the alkylating properties of this drug (5, 21–23). Although the drug molecule has several potentially reactive electrophilic centers (21, 22), irofulven forms probably only monoaducts with cellular DNA (3, 5). DNA monoadducts are, in general, considered low lethality lesions (24). Accordingly, unlike irofulven, other DNA-reactive agents of clinical anticancer significance form either interstrand or intrastrand cross-links or double-strand breaks (24). In addition to DNA reactivity, however, irofulven readily alkylates sulphhydril groups (5, 21–23). In cellular systems, covalent binding of irofulven to protein sulphhydrils exceeds 3- to 10-fold drug binding to DNA (5, 23). This substantial protein binding, which notably involves targeting of key redox controlling proteins, could explain the ability of irofulven to distort protein redox homeostasis (2, 25).

Mitochondria are believed to play a central role in determining cell survival or death in response to diverse apoptotic stimuli including agents that cause either DNA damage or protein damage or distort protein redox status (1, 26–28). Responses triggered by DNA damage often involve a shift in the balance between proapoptotic and antiapoptotic members of the Bcl-2 family, which in turn increases the permeability of the outer mitochondrial membrane (26–29). The resulting release of cytochrome c and other apoptogenic components initiates caspase activation and triggers caspase-mediated apoptotic DNA fragmentation and eventually cell death (29). The Bax-mediated outer membrane permeabilization and the downstream caspase-mediated cell death tend to be inhibitable by Bcl-2 overexpression (19, 30).

In addition to the classic caspase-mediated apoptosis, mammalian cells can undergo caspase-independent apoptosis that is mediated by the dissipation of the inner mitochondrial membrane potential ΔΨm (inner membrane permeability transition, MPT) and the release of apoptosis-inducing factor (AIF; refs. 28, 31). The caspase-independent, AIF-mediated cell death pathway has been documented in various death models including exposure to some agents that affect protein redox status (28). Specifically, oxidative cross-linking of redox-sensing inner membrane proteins was found to directly cause Bcl-2-independent MPT and AIF release (28, 31–33). The triggering of caspase-independent mechanisms may circumvent the apoptotic resistance of those cancer cells that are insensitive to DNA damage induced by ionizing radiation and etoposide (34).

We hypothesized that the consequences of irofulven reactivity with its diverse cellular targets converge and integrate at the level of mitochondrial dysfunction. To better understand the nature of irofulven-induced apoptosis, we attempted to discern the primary apoptotic responses from the downstream effects and to identify factors/pathways that determine the fate of irofulven-treated cancer cells. Using prostate tumor cells LNCaP-Pro5, we show that irofulven-induced apoptosis involves early translocation of proapoptotic Bax from cytosol to mitochondria followed by mitochondrial dysfunction, cytochrome c release, caspase activation, and DNA fragmentation. Although caspase inhibition abrogates DNA fragmentation, it does not salvage irofulven-treated cells from growth inhibition and loss of viability. In contrast, a substantial reduction of irofulven-induced apoptosis and antagonized cytotoxicity is observed after blocking mitochondrial proteins involved in MPT. Thus, mitochondrial dysfunction seems to reflect a decisive point in the action of irofulven, resulting in cancer cell death via an interplay of caspase-dependent and caspase-independent routes.

Materials and Methods

Cell Culture and Drug Treatment

LNCaP-Pro5 cells (from Dr. C.A. Pettaway, MD Anderson Cancer Center, Houston, TX) were maintained in RPMI 1640 supplemented with 10% FCS, 2% MEM vitamin solution (Life Technologies, Grand Island, NY), 1% nonessential amino acids, sodium pyruvate (1 mmol/L), and L-glutamine (2 mmol/L) as described previously (16, 20). Cell numbers were determined by cell counting in a hemocytometer or in a model ZM Coulter counter (Beckman Coulter, Inc., Fullerton, CA). Cell viability (cell membrane integrity) was determined based on cell ability to exclude trypan blue. Stock solution of irofulven (MGI Pharma, Inc., Bloomington, MN) was prepared in DMSO and stored at −20°C protected from light. Unless stated otherwise, cells were treated for 4 hours with 10 μmol/L drug. At the end of drug treatment, cell monolayers were either harvested or washed twice with prewarmed medium and postincubated in fresh drug-free medium as indicated. When caspase inhibitors were used, they were added to cell cultures 1 hour prior to drug addition and were present during drug treatment as well as after incubation in irofulven-free medium. Inhibitors of caspase-1/4 [acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk)], caspase-2 [N-benzoyloxycarbonyl-Val-Ala-Ala-Asp(Ome)-fluoromethyl ketone (Z-VDVAD-fmk)], caspase-3/7 [acetyl-Asp-Glu-Val-Asp-chloromethyl ketone (Ac-DEVd-cmk)], caspase-9 [N-benzoyloxycarbonyl-Leu-Glu(Ome)-HisAsp(Ome)-fluoromethyl ketone (Z-LEHD-fmk)], and the broad-spectrum inhibitor, N-benzoyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethyl ketone (Z-VAD-fmk), were from Alexis Biochemicals (San Diego, CA). Caspase-8/6 inhibitor [N-benzoyloxycarbonyl-Ile-Glu(Ome)-Thr-Asp(Ome)-fluoromethyl ketone (Z-IETD-fmk)] was purchased from BIOMOL Research Labs (Plymouth Meeting, PA).
Cytotoxic Activity

Growth inhibitory activity was assayed as described previously (15) using the standard 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) assay. Exponentially growing cells were incubated with the drug for the indicated times and subjected to colorimetric reaction with MTT. The concentrations of mitochondrial proteins in the analyzed samples, which was also detected by Western blotting. The integrity of the isolated mitochondria preparations was assessed by the lack of release of cytochrome c oxidase detected with appropriate antibody in the cytosolic fractions.

Caspase Processing

The cleavage of pro-caspases from inactive to active forms was monitored by Western blotting, similar to the procedure described for Bcl-2-independent determinations. After treatment with drug as indicated, cells were harvested as described for Bax translocation determinations, washed with ice-cold PBS, and resuspended in HEPES (25 mmol/L, pH 7.4) containing 0.1% Triton X-100, 10% glycerol, DTT (5 mmol/L), and protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin). After 10 minutes of incubation on ice followed by three cycles of freezing-thawing (in liquid nitrogen and at 37°C), samples were centrifuged at 10,000 × g for 10 minutes at 4°C. Equal amounts of proteins were resolved by reducing SDS-PAGE. After protein transfer, nitrocellulose membranes were probed with the antibodies against human caspase-9 (mouse IgG, 1 μg/mL, Oncogene Research Products, Boston, MA), caspase-7 (mouse IgG, 1 μg/mL, PharMingen), caspase-8 (mouse IgG, 1 μg/mL, Oncogene), and caspase-3 (rabbit polyclonal, 1:1,000, PharMingen). Following incubation with respective horseradish peroxidase–conjugated secondary antibodies, caspase were visualized using enhanced chemiluminescence system and their signal quantitated as described for Bax.

DEVD Cleavage Activity

A spectrophotometric assay was used to measure the release of p-nitroanilide (pNA) from peptide conjugate Ac-DEVD-pNA (Alexis Biochemicals), a substrate for caspase-3 and caspase-7. Cell extracts obtained as described above for caspase processing were used as enzyme source for these determinations. Reactions were carried out in PBS (20 mmol/L, pH 7.2) containing NaCl (100 mmol/L), 10% sucrose, 0.1% Triton X-100, and β-mercaptoethanol (20 mmol/L). The reaction mixture containing Ac-DEVD-pNA (150 μmol/L) and cellular extract (40 μL) in the final volume of 100 μL was incubated at 37°C and absorbance at 405 nm was read after 0, 1, 2, and 3 hours of incubation. Parallel reactions containing activated caspase-3 (Alexis Biochemicals) were used as positive controls and DEVD proteolytic activity inhibitor (Ac-DEVD-cmk, 10 μmol/L) was used to assess nonspecific hydrolysis. The results are expressed as nanomoles of pNA per milligram of protein per hour with pNA as a standard.

Flow Cytometry Determinations of ΔΨm

ΔΨm was determined as described previously (19) using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), which emits red fluorescence when bound to mitochondrial membranes with high ΔΨm and green fluorescence in cells with depolarized mitochondrial membranes (37).

Briefly, irofulven-treated cells were harvested as described for Bax determinations and washed with PBS and with serum-free RPMI 1640 without phenol red and

Bax Translocation and Cytochrome c Release

At the end of treatments, attached cells harvested by gentle trypsinization were combined with detached cells and washed twice by centrifugation (5 minutes at 200 × g) with ice-cold PBS. The cytosolic and mitochondrial fractions were prepared according to Kluck et al. (36) with slight modification. Briefly, cell pellets were suspended (106 cells/200 μL) in isotonic buffer containing 10 mmol/L HEPES (pH 6.9), 200 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EGTA, 100 μmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, and incubated on ice for 15 minutes followed by homogenization in a Dounce homogenizer (20 strokes with type B pestle). After centrifugation at 1,000 × g for 10 minutes, supernatants were recentrifuged at 10,000 × g for 20 minutes at 4°C to pellet mitochondria. The postmitochondrial supernatants (cytosolic fractions) were sequentially passed through 0.2 and 0.1 μm filter to remove possible contaminating membrane fragments (36). The mitochondrial pellets were washed once with the isotonic buffer and solubilized in radiolmmunoprecipitation assay buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS (pH 7.2)] supplemented with protease inhibitors (100 μmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Protein concentrations in cytosol were determined by Bradford assay using bovine serum albumin as a standard, whereas concentrations in cytosol were determined by homogenization in a Dounce homogenizer (20 strokes with type B pestle). After centrifugation at 1,000 × g for 10 minutes, supernatants were recentrifuged at 10,000 × g for 20 minutes at 4°C to pellet mitochondria. The postmitochondrial supernatants (cytosolic fractions) were sequentially passed through 0.2 and 0.1 μm filter to remove possible contaminating membrane fragments (36). The mitochondrial pellets were washed once with the isotonic buffer and solubilized in radiolimmunoprecipitation assay buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS (pH 7.2)] supplemented with protease inhibitors (100 μmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Protein concentrations in cytosol were determined by Bradford assay using bovine serum albumin as a standard, whereas the concentrations of mitochondrial proteins in the presence of 0.6% SDS were determined spectrophotometrically at 280 nm (assuming that A280 = 0.21 corresponds to 10 μg/mL protein).

Both cytosolic and mitochondrial fractions containing equal amounts of protein were subjected to reducing SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific for Bax (rabbit anti-human, 1:1,000, PharMingen, San Diego, CA) or cytochrome c (mouse anti-human, 2 μg/mL, PharMingen) for 2 hours at room temperature. Following additional 1-hour incubation with horseradish peroxidase–conjugated secondary antibodies (goat anti-rabbit IgG 1:500, rabbit anti-mouse IgG 1:2,000), specific protein bands were visualized by chemiluminescence using enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Bands recorded on X-ray film were scanned using a Molecular Dynamics densitometer (Sunnyvale, CA) and their intensity was quantitated with IQ software (Molecular Dynamics). Bcl-2-independent and cytochrome c signals were normalized to the β-actin signal in the analyzed samples, which was also detected by...
glutamine. Finally, cells were resuspended in PBS. Aliquots of cell suspension were incubated for 25 minutes with JC-1 containing binding buffer (ApoAlert Mitochondrial Membrane Sensor, BD Biosciences Clontech, Palo Alto, CA) as per manufacturer’s protocol and analyzed immediately by flow cytometry on a Coulter EPICS ELITE flow cytometer. Typically, 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for green and red fluorescence, respectively. Events with high red and high green fluorescence were gated as measures of cells with high and low ∆Ψm, respectively.

**Annexin V Binding**

Phosphatidylserine externalization, a marker of early apoptotic events, was detected by binding of FITC-conjugated Annexin V, whereas counterstaining with propidium iodide (PI) allowed for the detection of cells with permeable cell membrane (19). Cells were treated with irofulven alone or in combination with inhibitors as indicated and harvested by trypsinization, washed with serum-free medium, and suspended in PBS at the density 1 × 10⁶ cells/mL. Aliquots of 1 × 10⁶ cells were suspended in binding buffer (500 μL, Annexin V-FITC staining kit, PharMingen). This cell suspension (100 μL) was stained with mouse anti-human Annexin V antibody (mlG type, 5 μL) and PI (500 μg/mL, 10 μL) for 15 minutes in the dark. The immunostaining was terminated by addition of binding buffer and cells were immediately analyzed by flow cytometry. The negative control included staining with mlGG only. Typically, 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for Annexin V and PI, respectively.

**Quantitative Apoptotic DNA Fragmentation**

The quantitative DNA fragmentation assay detects both early, high molecular weight DNA fragments and late residual oligonucleosomal-sized fragments (15, 16, 19, 20). Briefly, [C]-thymidine-prelabeled cells treated with drug as indicated were permeabilized in a hypotonic buffer followed by the extraction of fragmented DNA and collection of these fragments by centrifugation in respective supernatants, whereas nondegraded DNA remains in the nuclear pellet fraction. The results are expressed as the percentage of the total DNA released in the supernatants, corrected for the radioactivity released from untreated controls. Irofulven-generated double-stranded fragments were shown previously to peak at ~40 to 50 kbp after a 24-hour incubation of drug-treated cells (18, 38).

**Results**

This study centered on mitochondrial dysfunction and its consequences in the model of prostate cancer LNCaP-Pro5 cells in which irofulven has been shown previously to be a potent apoptosis inducer (16, 20). To identify early drug effects, we used a 4-hour exposure to irofulven (10 μmol/L) and a postincubation in drug-free medium, depending on a specific end point, for up to 12 to 16 hours elapsed time. These treatment conditions correspond to substantial but still incomplete growth inhibition (<90% inhibition; data not shown). To determine drug effects on cell growth and viability, longer times and a lower range of drug concentrations were used.

**Translocation of Proapoptotic Bax to Mitochondria**

The recruitment of the proapoptotic Bax protein to mitochondria is one of the early events that are essential for apoptosis triggering in various systems (29, 39). The effects of irofulven on the translocation of Bax from cytosol to mitochondria were examined by Western blotting using cytosolic and mitochondrial fractions from drug-treated cells (Fig. 2A). The quantification shows Bax signals in both fractions normalized to respective β-actin signals. Prior to drug treatment, Bax was detected predominately in the cytosolic fraction. A 2 to 4 hours of incubation with irofulven (10 μmol/L) resulted in ~2-fold increase in mitochondrial-associated Bax. Bax translocation progressed rapidly over time. At 8 hours elapsed time, there was an ~12-fold elevation of Bax signal in the mitochondrial fraction. Concurrently, the level of cytosolic Bax decreased markedly below the initial level (Fig. 2A). Whereas in some systems Bax translocation is promoted by the proteolytic cleavage of Bax molecule (40), Bax cleavage was not detected in irofulven-treated LNCaP-Pro5 cells.
To assess whether irofulven-induced Bax translocation is upstream from the caspase cascade, the levels of cytosolic Bax in irofulven-treated LNCaP-Pro5 cells were monitored in presence of the broad-spectrum caspase inhibitor Z-VAD-fmk and the initiator caspase-9 inhibitor Z-LEHD-fmk. The initial depletion of cytosolic Bax (up to 6 hours elapsed time) was not prevented by either inhibitor (data not shown). At 12 hours, however, Z-VAD-fmk significantly ($P < 0.02$) reduced the magnitude of irofulven-induced loss of Bax from the cytosolic fraction (Fig. 2B).

**Dissipation of $\Delta \Psi_m$**

The translocation of proapoptotic Bax translocation to mitochondria is expected to compromise mitochondrial membrane integrity, which may include the dissipation of $\Delta \Psi_m$ (41). Accordingly, flow cytometry determinations showed that irofulven affects $\Delta \Psi_m$ (Fig. 3). Whereas the majority of untreated LNCaP-Pro5 cells featured a high $\Delta \Psi_m$, the number of cells with low $\Delta \Psi_m$ after a 6-hour exposure to irofulven (10 $\mu$mol/L) slightly increased from $11.4 \pm 1.4\%$ to $20.2 \pm 2.7\%$ (Fig. 3A). The effect was further intensified at 12 hours, with $47 \pm 4.9\%$ cells showing low $\Delta \Psi_m$ (Fig. 3B).

The addition of Z-VAD-fmk prior to irofulven treatment had marginal effect on the fraction of cells with low

$\Delta \Psi_m$ at 6 hours elapsed time (Fig. 3A). At 12 hours, however, Z-VAD-fmk partially prevented the dissipation of $\Delta \Psi_m$ as illustrated by the decrease in the number of cells with low $\Delta \Psi_m$ from $47 \pm 4.9\%$ for irofulven alone to $30 \pm 0.5\%$ in presence of Z-VAD-fmk (Fig. 3B). These findings suggest that the activated caspases facilitate the collapse of $\Delta \Psi_m$.

**Cytochrome c Release**

Mitochondrial dysfunction promoted by Bax translocation usually leads to the leakage of cytochrome c from mitochondria (41). Accordingly, irofulven treatment of LNCaP-Pro5 cells increased the levels of cytoplasmic cytochrome c. The effect was detectable after 4 hours and progressed to reach a plateau at 6 to 12 hours (Fig. 4). Similar timing and progression of cytochrome c release was also detected in a related subline (LNCaP-LN3). The magnitude of this effect, however, was less pronounced than in LNCaP-Pro5 (data not shown), consistent with the lower apoptotic propensity of LNCaP-LN3 in response to irofulven (16).

The preincubation of cells with either the pan-caspase inhibitor or the caspase-9 inhibitor revealed that caspase dependency of cytochrome c release changes distinctly over time (Fig. 4). Both inhibitors failed to prevent irofulven-induced release of cytochrome c at 4 hours. At 6 hours elapsed time, however, cytochrome c release became partly caspase dependent as indicated by a significant inhibitory effect of both Z-VAD-fmk and Z-LEHD-fmk (78% and 60% inhibition, respectively). Partial inhibition by Z-VAD-fmk was also observed at 12 hours (40% inhibition; data not shown). Thus, irofulven-induced early release of
cytochrome c was not affected by the caspase inhibitors and preceded loss of ΔΨₘ. The temporal switch from caspase-independent to partially caspase-dependent release of cytochrome c parallels analogous switch for the dissipation of ΔΨₘ.

**Induction of Caspase Cascade**

Cytochrome c released to cytosol forms a multimeric complex with apoptotic protease-activating factor 1 and pro-caspase-9 leading to the activation of caspase-9 and downstream caspases (42). We thus examined the effects of irofulven on the processing of caspase-9 and of caspase-7 and caspase-3, which are directly activated by caspase-9 (42, 43). The temporal sequence of caspase processing/activation was assessed based on the conversion (cleavage) of the inactive pro-caspases to the enzymatically active truncated forms (Fig. 5A and B).

The activation of caspase-9 became apparent after 6 hours elapsed time (coinciding with the timing of profound cytochrome c release) and progressed at least up to 16 hours (Fig. 5A and B). Processing of caspase-7 shows a rapid progression and is essentially complete by 12 hours. The most delayed and least effective is the processing of the effector caspase-3, which is in line with the previously shown dispensability of that caspase for irofulven effects (18). As expected, caspase-9 inhibitor Z-LEHD-fmk and pan-caspase inhibitor Z-VAD-fmk abrogated downstream processing of caspase-7 and caspase-3 (Fig. 5C and D).

To further address the possible role of the executioner DEVD caspases 7/3, we examined the induction of DEVD-targeted proteolytic activity in the extracts from irofulven-treated cells using Ac-DEVD-pNA as the substrate. DEVD proteolytic activity increased from 7.6 ± 2.6 pmol pNA/μg protein/h in cell extracts from control cells to 54.4 ± 2.9 pmol pNA/μg protein/h in cells treated with irofulven (10 μmol/L) for 4 hours. With the consistent processing of caspase-3 and caspase-7, addition of a caspase-3/7 inhibitor (Ac-DEVD-CHO) at 10 μmol/L reduced DEVD proteolytic activity almost to the control level (12.1 ± 1.5 pmol pNA/μg protein/h).

**Caspase-Dependent DNA Fragmentation**

Activation of executioner caspases (such as caspase-3 and caspase-7) normally precedes the manifestation of apoptosis as massive DNA fragmentation (44). Consistent with the previous findings (16), DNA fragmentation induced by irofulven (5 μmol/L) progressed with time reaching the level of 43.6 ± 1.4% of total DNA after 12 hours (Fig. 6A) and 77.4 ± 5.2% after 24 hours. This irofulven-induced DNA fragmentation was profoundly suppressed (by 80.9 ± 4%) in the presence of the pan-caspase inhibitor Z-VAD-fmk (Fig. 6B). Nearly as potent were the inhibitors of caspase-9 and caspase-8/6 (Z-LEHD-fmk and Z-IEHD-fmk), producing 70.7 ± 1.3% and 77.2 ± 6.3% suppression, respectively. A more modest inhibition was produced by caspase-2 inhibitor Z-VDVAD-fmk (42.3 ± 6.0% inhibition) and by caspase-3 and caspase-7 inhibitor Ac-DEVD-fmk (32.9 ± 4.1% inhibition). Caspase-1 and caspase-4 inhibitor Ac-YVAD-cmk had marginal effect (11.2 ± 2.4% inhibition). These data further corroborate the initiator role of caspase-9 and show that irofulven-induced apoptotic DNA fragmentation results mostly from the caspase-mediated pathway.

**Inhibition of the Caspase Cascade Does Not Suppress the Antiproliferative Effects of Irofulven**

If the fate of irofulven-treated cells depended solely on the caspase-mediated route, the inhibition of the caspase cascade would be expected to rescue cells from death. To resolve this question, we compared the loss of cell viability (by trypan blue exclusion) and cell growth inhibition induced by irofulven (5 μmol/L) in the presence and absence of caspase inhibitors (Fig. 6C and D, respectively). Consistent with the data reported previously (20), cells treated with irofulven alone showed reduced viability at 8 hours from the beginning of drug insult, with strong...
progression through 12 and 24 hours (Fig. 6C). Z-VAD-fmk and other caspase inhibitors had marginal to slight effects on the irofulven-induced loss of cell viability. The effects of caspase inhibitors remained marginal also when related to the number of remaining cells (Fig. 6D). After a 24-hour exposure to irofulven (5 μmol/L) alone, the total cell number was reduced by 32 ± 5% compared with untreated control decreased observed of either Z-VAD-fmk or Z-LEHD-fmk (42% and 50%, respectively). Consequently, the estimated numbers of viable cells are only marginally higher in the presence of caspase inhibitors. Furthermore, Z-VAD-fmk had negligible effects on cell growth inhibition measured by the MTT assay (Fig. 6E; data not shown).

After 12, 24, and 48 hours, irofulven concentrations inhibiting the MTT signal by 50% (IC50) in the absence versus presence of Z-VAD-fmk amounted to 1.2 versus 1.9, 0.19 versus 0.23, and 0.10 versus 0.11 μmol/L, respectively. The inability of Z-VAD-fmk to rescue cells indicates a significant contribution of a caspase-independent component in irofulven effects on cell growth and viability.

**Monobromobimane Antagonizes Cell Growth Inhibition and Suppresses Apoptosis by Irofulven**

Irofulven-induced, caspase-independent cell death might reflect the known alternative route of apoptosis, which originates from MPT mediated by oxidative changes in critical redox-sensing mitochondrial membrane proteins (32, 34, 45). To test this possibility, we used monobromobimane, a protein reagent that prevents conformational changes in mitochondrial membrane proteins that are required to precipitate MPT (46). Thus, by blocking the early step in caspase-independent, AIF-mediated apoptosis, monobromobimane can serve as a mechanistic marker for this alternative route.

The ability of monobromobimane to interfere with irofulven-induced apoptosis was assessed using Annexin V binding assay. High Annexin V signal in this assay corresponds to phosphatidylserine externalization in cell membranes, which is a characteristic marker of both caspase-dependent and caspase-independent routes of apoptosis (47). The flow cytometry histograms for LNCaP-Pro5 cells incubated with irofulven (2.5 μmol/L) show large numbers of cells with high Annexin V signal and low PI signal (that are indicative of early apoptosis) as well as cells with high signal for both Annexin and PI (indicative of late apoptosis; Fig. 7A). Addition of monobromobimane 1 hour prior to irofulven attenuated the intensity of the Annexin signals. The quantification of flow cytometry data (Fig. 7B) showed that apoptotic cells are markedly reduced in the presence of monobromobimane.

**Figure 6.** Effects of caspase inhibitors on irofulven-induced apoptotic DNA fragmentation and cell growth. The following caspase inhibitors, when used as indicated below, were added at 25 μmol/L concentration 1 hour prior to the addition of irofulven: Z-VAD-fmk (the pan-caspase inhibitor), Z-LEHD-fmk (caspase-9), Z-IETD-fmk (caspase-8 and caspase-6), Z-VDVAD-fmk (caspase-2), Ac-DEVD-fmk (caspase-3 and caspase-7), and Ac-YVAD-cmk (caspase-1 and caspase-4). LNCaP-Pro5 cells were exposed to irofulven (5 μmol/L) for the indicated times in the presence or absence of caspase inhibitors followed by the determinations of respective apoptotic end points. A and C, untreated cells (○); irofulven alone (■); and irofulven in the presence of Z-VAD-fmk (▲), Z-LEHD-fmk (○), or Z-IETD-fmk (□). A, time course of apoptotic DNA fragmentation induced by irofulven (5 μmol/L) in the absence and presence of caspase inhibitors. Values of percentage of fragmented DNA were corrected for respective values in cultures without irofulven. B, inhibition of DNA fragmentation induced after 12-hour incubation with irofulven (5 μmol/L) by the indicated caspase inhibitors. C, time course of changes in cell viability. D, remaining cells after 24-hour exposure to irofulven (10 μmol/L) normalized to the number of cells in untreated cultures. TOTAL cells based on electronic cell counting represent both viable and nonviable cells. Values for viable cells are estimated by multiplying total cell number by respective relative viability values from C. Columns, mean of two experiments in duplicates; bars, SE. E, cytotoxicity of irofulven (HMAF) in the absence and presence of Z-VAD-fmk (25 μmol/L) measured using the MTT assay after 48-hour continuous exposure to the drug.
Because monobromobimane should mainly antagonize the MPT-mediated, caspase-independent route, monobromobimane was expected to be less antagonistic with regard to DNA fragmentation, which is largely mediated by the caspase cascade. Determinations of DNA fragmentation in the presence of monobromobimane corroborated this expectation. Compared with irofulven alone, monobromobimane exerted some protective effect, but its magnitude (~14% at 1 μmol/L monobromobimane; data not shown) seemed insufficient to account for the profound protection against apoptosis monitored by the Annexin V assay. Thus, the antagonistic effects of monobromobimane on irofulven-induced apoptosis are consistent with the inhibition of MPT/AIF pathway.

To determine whether monobromobimane antagonizes growth inhibition of irofulven-induced cells, cells were pretreated with monobromobimane for 1 hour prior to the drug addition for 48 hours. Following the recommendation for the median effect approach (35), these experiments were carried out using a constant ratio of irofulven to monobromobimane as opposed to fixed monobromobimane concentration in the Annexin assay. Cell growth was measured by the MTT assay and data were analyzed by plotting the combination index versus the fraction affected (Fig. 7C). In this plot, low to modest fraction affected values correspond to noncytotoxic to slightly cytotoxic concentrations of monobromobimane (data not shown). The interpretation of monobromobimane antagonism needs to be limited to this range, because combination index values at higher fraction affected values are likely to be influenced by nonspecific cytotoxicity of monobromobimane. The combination index values observed at fraction affected values up to ~0.35 are clearly in the range indicative of strong antagonistic or antagonistic interaction between irofulven and monobromobimane as per criteria of Chou and Talalay (35). This result further strengthens the possibility that the antiproliferative effects of irofulven originate in part from the MPT/AIF–mediated alternative apoptotic route.

Discussion

The consistently potent induction of apoptosis in various tumor systems is the hallmark of the novel anticancer drug, irofulven (15, 16, 18, 19). Previous investigations have identified DNA and proteins as major targets for alkylation by this drug in cancer cells (5). Various studies implicated both reactivities in apoptotic properties of irofulven (2, 5, 25). This report defines key molecular events that integrate irofulven-induced apoptotic signals and lead to cell death.

Mitochondrial dysfunction plays a central role in irofulven-induced apoptosis in prostate cancer LNCaP-Pro5 cells. An early step in this process is the translocation of proapoptotic Bax to mitochondria (Fig. 2) followed by the dissipation of ΔΨm (Fig. 3) and the leakage of cytochrome c (Fig. 4). The released cytochrome c apparently promotes the activation of caspases (Fig. 5) and gives rise to caspase-dependent DNA fragmentation.
Importantly, the apoptotic fate of irofulven-treated cells is determined not only by the caspase-mediated pathway but also via a coexisting caspase-independent route (Figs. 6 and 7). The nature and attributes of the irofulven-induced apoptotic cascade resulting in these two routes are discussed below in the context of the likely interplay between the drug-induced DNA damage signaling and the consequences of protein damage.

The observation that irofulven induces Bax translocation is consistent with the known effects of DNA damage signaling, although other apoptotic stimuli may also produce such effect (27, 29, 39). Especially relevant to the protein-targeting activities of irofulven are the reports that the oxidation of intracellular thiols facilitate the proapoptotic rearrangements of Bax/Bcl-2 balance (48, 49). Thus, irofulven-induced pro-oxidative changes in the protein redox status might additionally enhance Bax translocation resulting from DNA damage signaling.

Irofulven-induced Bax translocation seems to precede both cytochrome c release and loss of the inner membrane potential $\Delta \Psi_m$, which might reflect a causative role of Bax translocation. However, the initial $\Delta \Psi_m$ dissipation is still relatively small at the time of significant cytochrome c release. This pattern suggests that whereas the initial cytochrome c release depends largely on Bax translocation, $\Delta \Psi_m$ collapse might be in part Bax/Bcl-2 independent. Such interpretation is supported by our previous findings, which showed that forced overexpression of Bcl-2 had marginal effect on early irofulven-induced $\Delta \Psi_m$ collapse (19).

Bax/Bcl-2-independent $\Delta \Psi_m$ collapse resulting in the release of AIF is a well-established pathway in the action of agents that oxidize the critical thiol groups in mitochondrial membrane proteins (28, 50). In addition to globally distorting protein redox status (25, 51), irofulven directly reacts with a subset of mitochondrial proteins. Further studies are needed to clarify whether these effects indeed play a role in drug-induced inner membrane permeability transition. Consistent with such a role, however, is the observation that irofulven causes the release of AIF (in leukemic CEM cells). There is no doubt that irofulven-induced mitochondrial dysfunction is upstream from the caspase cascade, as Bax translocation, changes in $\Delta \Psi_m$, and cytochrome c release were consistently independent of caspase inhibitors at early times. However, the emergence of partial caspase dependency at later times strongly suggests that the activated caspasers provide a positive feedback loop, which further compromises mitochondrial integrity. Such interpretation is consistent with other reports that implicated caspase activation as a factor potentiating the collapse of $\Delta \Psi_m$ (52, 53).

The timing of caspase processing in response to irofulven and the effects of caspase inhibitors point to the activation of caspase-9 upstream in the cascade followed by the activation of caspase-7 and caspase-3. This order is consistent with the established role of caspase-9 as an initiator caspase (42, 43). To activate caspase-9, cytochrome c is thought to interact first with an adaptor molecule apoptotic protease-activating factor 1 and next with procaspase-9 (44). Such interaction can be efficient in LNCaP-Pro5 cells, which have clearly detectable levels of apoptotic protease-activating factor 1 that are unaffected by exposure to irofulven.2 Noteworthy, a recent study reported that the activation of caspase-9 is inhibited by the antiapoptotic redox-regulating protein thioredoxin (54). The ability of irofulven to interfere with the function of the thioredoxin system (25) may thus facilitate caspase-9 activation. This possibility indicates yet another level at which a crosstalk between irofulven effects on multiple cellular targets might self-potentiate apoptotic signaling.

Massive fragmentation of DNA in irofulven-treated LNCaP-Pro5 cells is clearly caspase dependent as evidenced by its near complete abrogation by the pancaspase inhibitor. General and specific caspase inhibitors abrogated irofulven-induced apoptotic DNA fragmentation with the following order of potency: pan-caspase $\geq$ caspase-9 $>$ caspase-8/6 $>$ caspase-2 $>$ caspase-3/7 $>$ caspase-1/4. The potent inhibitory effect of the caspase-9 inhibitor corroborates the role of this caspase as the main initiator of the caspase cascade in cells exposed to irofulven. It is less clear which caspases serve as executioners. Any significant role of caspase-1/4 can be ruled out, because the inhibitor of these caspases had marginal effect on irofulven-induced apoptotic DNA fragmentation. Although caspase-3 is commonly regarded as major executioner caspase, it was less efficiently processed in irofulven-treated LNCaP-Pro5 cells than other caspases. The modest inhibition of irofulven-induced DNA fragmentation in LNCaP-Pro5 cells observed in this study for the inhibitor of DEVD caspase-3 and caspase-7 is consistent with virtually no effect of this inhibitor in leukemic CEM cells (3). Moreover, previous studies with caspase-3-deficient MCF-7 cells showed that caspase-3 is dispensable for irofulven effects (18). The activation of caspase-7 accompanied apoptosis by irofulven in LNCaP-Pro5 cells (this study) and in pancreatic cancer cells (17) but was negligible in two breast cancer cell lines (18). Thus, although caspase-7 may contribute to the cascade in some models, it is unlikely to be universally critical for irofulven-induced apoptosis.

In contrast to weak effects of the caspase-3/7 inhibitor, a potent suppression of DNA fragmentation was caused by Z-IETD-fmk, the inhibitor of caspase-8/6. Irofulven was reported to induce caspase-8 processing in pancreatic cancer cells (17). In LNCaP-Pro5 cells, however, we could not detect any caspase-8 processing (by Western blotting) or any caspase-8 enzymatic activity in cell extracts from drug-treated cells (data not shown). These results suggest that the inhibition of DNA fragmentation by Z-IETD-fmk reflects the inactivation of caspase-6 rather than caspase-8. The possibility that caspase-6 could be an important executioner caspase in irofulven-induced apoptotic DNA fragmentation is consistent with analogous inference based on results in breast cancer cell lines (18).

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4 Liang and Woynarowska, unpublished data.
The findings of this report and data in other models (15, 16, 18, 19) show clearly that the death process initiated by irofulven-induced mitochondrial dysfunction has distinct attributes of apoptosis: early changes in cell membrane phospholipids, altered nuclear morphology, and DNA fragmentation, all of which precede the loss of cell membrane integrity (cell “viability”). It is important to underscore, however, that blocking the caspase cascade, which largely abrogates DNA fragmentation, minimally protects against the loss of membrane integrity and does not rescue irofulven-treated cells from growth inhibition. These observations provide unequivocal evidence that irofulven-induced apoptosis comprises not only caspase-dependent pathway but also caspase-independent death.

Although the classic route of apoptosis involves caspase-mediated phenomena, in a broader sense, apoptosis refers to systemic cell degradation “from inside” that precedes the loss of cell membrane integrity. The specific term “caspase-independent apoptosis” is increasingly used in the literature to denote a defined alternative apoptotic route (31, 32). This route involves membrane permeability transition that is initiated by changes in redox-sensing mitochondrial membrane proteins and proceeds in a Bcl-2-independent manner. MPT leads to the release of apoptogenic factors, notably AIF. AIF translocates to the nucleus, where it affects a long-range disruption of nuclear chromatin but not oligonucleosomal level DNA fragmentation. Like caspase-mediated apoptosis, AIF-mediated, caspase-independent apoptosis may also be induced by various stimuli. Nonetheless, the activation of caspase-independent apoptotic route, in addition to the caspase-mediated effects, is a characteristic feature of protein-reactive agents, such as diamide (31, 32, 55), but is not observed for DNA-damaging agents, such as etoposide, that do not react with proteins (34).

The pattern of irofulven effects is consistent with the contribution of the above-mentioned mechanistic attributes of caspase-independent apoptosis. First, preventing the reactivity of irofulven with the redox-sensing sulfhydryls in the mitochondrial membrane proteins (by preincubation with monobromobimane) partially antagonizes the apoptotic and antiproliferative effects of irofulven (Fig. 7). Secondly, as mentioned above, irofulven-induced membrane permeability transition seems to be partly Bcl-2 independent. Finally, irofulven-induced apoptosis is largely Bcl-2 independent, unlike apoptosis by etoposide that is almost completely abrogated by Bcl-2 overexpression (19). The idea that caspase-independent cell death induced by irofulven indeed reflects the AIF-mediated alternative apoptotic pathway (as suggested in the diagram in Fig. 8) is highly likely, but it still needs to be confirmed unequivocally. Further studies are needed, in particular, to explore drug effects on redox-sensitive mitochondrial proteins that might initiate AIF-mediated cell death.

Taken together, the reported findings reveal that the fate of irofulven-treated cells is most likely determined by a complex interplay among multiple signaling routes originated from drug effects on its diverse cellular targets (DNA and proteins). These signals converge and integrate at the mitochondrial level as the decisive point and diverge again downstream from mitochondria into two coexisting death routes (Fig. 8). The coinduction of multiple signaling pathways and apoptotic routes could play essential role in the ability of irofulven to preempt prosurvival attempts and facilitate the efficient elimination of cancer cells, including apoptosis-resistant models (2, 16, 18, 19). The irofulven paradigm of consistently potent apoptosis in cancer cells underscores the potential benefits of polytargeted anticancer approaches.

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