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Apoptosis induction by the dual-action DNA- and protein-reactive antitumor drug irofulven is largely Bcl-2-independent☆

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Abstract

The overexpression of Bcl-2 is implicated in the resistance of cancer cells to apoptosis. This study explored the potential of irofulven (hydroxymethylacylfulvene, HMAF, MGI 114, NSC 683863), a novel DNA- and protein-reactive anticancer drug, to overcome the anti-apoptotic properties of Bcl-2 in HeLa cells with controlled Bcl-2 overexpression. Irofulven treatment resulted in rapid (12 hr) dissipation of the mitochondrial membrane potential, phosphatidylserine externalization, and apoptotic DNA fragmentation, with progressive changes after 24 hr. Bcl-2 overexpression caused marginal or partial inhibition of these effects after treatment times ranging from 12 to 48 hr. Both Bcl-2-dependent and -independent responses to irofulven were abrogated by a broad-spectrum caspase inhibitor. Despite the somewhat decreased apoptotic indices, cell growth inhibition by irofulven was unaffected by Bcl-2 status. In comparison, Bcl-2 overexpression drastically reduced apoptotic DNA fragmentation by etoposide, acting via topoisomerase II-mediated DNA damage, but had no effect on apoptotic DNA fragmentation by helenalin A, which reacts with proteins but not DNA. Irofulven retains its pro-apoptotic and growth inhibitory potential in cell lines that have naturally high Bcl-2 expression. Collectively, the results implicate multiple mechanisms of apoptosis induction by irofulven, which may differ in time course and Bcl-2 dependence. It is possible that the sustained ability of irofulven to induce profound apoptosis and to block cell growth despite Bcl-2 overexpression may be related to its dual reactivity with both DNA and proteins.

Keywords: Irofulven; Helenalin A; Bcl-2 overexpression; Apoptosis; Mitochondria; Protein damage

1. Introduction

The goal of cancer chemotherapy is to eradicate cancer cells, preferably through coordinated cell death, as in the apoptotic process. Apoptosis is thought, in general, to be initiated via two major routes involving either a death receptor pathway or a signaling pathway leading to mitochondrial dysfunction [1,2]. Blocks to either route are implicated in the resistance of cancer cells to drug-induced cell death [3]. There is substantial cross-talk between both pathways, and mitochondrial dysfunction is viewed as a general point of no return in the execution of apoptosis [4,5].

Whether or not an apoptotic stimulus leads to mitochondrial dysfunction can be critically dependent upon the interplay of the Bcl-2 family of proteins. Tipping the delicate balance between the pro- and anti-apoptotic members of the Bcl-2 family can lead to either increased cytochrome *c* release and mitochondrial dysfunction or attenuation of apoptosis signaling and continued unimpeded mitochondrial function [6,7]. Among the several known mammalian anti-apoptotic members of the Bcl-2

☆ Preliminary accounts from these studies have been presented at the 91st Annual Meeting of the American Association for Cancer Research, April 1–5, 2000, San Francisco, Abstract 1799, and at the 11th NCI-EORTC Symposium on New Drugs in Cancer Therapy, Amsterdam, November 7–10, 2000, Abstract 303.

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Abbreviations: JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; GI_{50}, drug concentration inhibiting relative cell growth by 50%; $\Delta\Psi_m$, mitochondrial membrane potential; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PTPC, permeability transition pore complex; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone.

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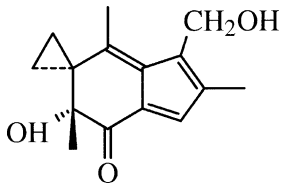

Fig. 1. Structure of irofulven.

family, apoptosis inhibition by Bcl-2 itself has been the most extensively documented [5,6,8–13]. Bcl-2 overexpression inhibits apoptosis induced by diverse anticancer drugs [11,14–18]. The chemoresistance of some leukemias, lymphomas, and colon, prostate, and lung cancers can be traced to overexpression of Bcl-2, or to increased ratios of anti-/pro-apoptotic Bcl-2 family members [7,11,14,18–26]. Clinically, increased Bcl-2/Bax ratios are often observed in tumors of patients found to be unresponsive to various chemotherapeutic treatments [22,27–30].

Irofulven (HMAF, hydroxymethylacylfulvene, MGI 114, NSC 683863, Fig. 1) is a novel pro-apoptotic antitumor drug currently undergoing a Phase III trial for gemcitabine-refractory pancreatic cancer, and several Phase II clinical trials [31]. A broad spectrum of antitumor activity [32–36] and a potent induction of apoptosis are the most prominent features of the action of irofulven [37–40]. Moreover, irofulven can differentiate between tumor and normal cells, essentially being reversibly cytostatic and thus non-apoptosis-inducing in various types of normal cells [39,40]. The reactivity of irofulven with cellular macromolecules, including alkylation of both DNA and proteins [37,38,41], suggests that the drug may promote apoptosis in tumor cells via multiple mechanisms.

DNA damage is generally recognized as an important apoptotic stimulus that initiates death signaling. Thus, irofulven–DNA adducts are highly likely to initiate apoptotic responses in drug-treated cells. The DNA-damage initiated pathway, however, is known to be subject to inhibition by Bcl-2 overexpression [42–45]. On the other hand, functional protein damage by agents that react only with proteins seems to promote apoptosis by mechanisms that have limited dependence on Bcl-2 [46–55]. We proposed that protein adducts (functional protein damage) may enhance apoptosis by dual-acting agents such as irofulven [56]. Hence, it seemed likely that irofulven-induced protein damage and a resulting pro-oxidative distortion of redox homeostasis might overcome the anti-apoptotic effects of Bcl-2.

In this study, we explored the effects of forced Bcl-2 overexpression on the ability of irofulven to induce apoptosis and inhibit cell growth. The results demonstrate that irofulven remains profoundly pro-apoptotic under the conditions of overexpression of the anti-apoptotic protein Bcl-2. The pattern of irofulven's effects and the comparison with the protein-reactive drug helenalin and the DNA-damaging drug etoposide implicate the dual DNA- and protein-reactivity of irofulven in its apoptotic properties.

2. Materials and methods

2.1. Drugs

Stock solutions of irofulven (from MGI Pharma), helenalin A (Calbiochem), and etoposide (Sigma) were prepared in DMSO and stored protected from light at -20°.

2.2. Cell culture and drug cytotoxicity

BH2 cells, a subline of HeLa S3 cells stably transfected with a tetracycline-controlled Bcl-2 overexpressing system, were developed and made available to the research community by Yin and Schimke [57] and distributed free of charge by Clontech Laboratories Inc. BH2 cells were cultured in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum either in medium containing 2 μg/mL of tetracycline (Bcl-2 OFF) or without the addition of tetracycline (Bcl-2 ON) as described by Yin and Schimke [57]. Tetracycline, when used, was continuously present in culture starting at least 3 days before an experiment to ensure the complete inhibition of Bcl-2 expression. The presence of tetracycline did not affect the growth properties of untreated cells adversely.

Drug cytotoxicity was measured by a standard colorimetric MTT assay as described previously [38,58]. Briefly, exponentially growing cells, either with or without tetracycline, were plated at 1.5×10^3 cells/0.2 mL in a 96-well plate and incubated with a series of irofulven concentrations for 72 hr followed by the addition of MTT for the colorimetric reaction. The results were expressed as GI_{50} values (drug concentrations inhibiting relative cell growth, RG, by 50%), using the equation: $RG = (T_r - T_0)/(Con - T_0)$, where T_r and Con are the MTT absorbance signals for drug-treated and control cells, respectively, and T_0 is the initial (time zero) absorbance.

2.3. Detection of Bcl-2 by Western blot analysis

Total protein extracts were obtained as described previously [58]. Protein concentration in the lysates was determined using the Pierce bicinchoninic acid (BCA) assay with BSA as the standard (Pierce). Protein samples (50 μg) were subjected to reducing SDS–PAGE electrophoresed on a 12% SDS–polyacrylamide gel [58] and then electro-transferred to nitrocellulose membrane (Bio-Rad Laboratories) [59]. Membranes were blocked for non-specific staining with 10% non-fat dry milk for 1 hr at room temperature [59] and next probed with antibody specific for Bcl-2 (1:2000, mouse anti-human Bcl-2 specific antibody, clone YTH-8C8, R&D Biosystems) and then with corresponding horseradish peroxidase-conjugated secondary antibody (1:500 goat anti-mouse IgG). Specific protein bands were visualized using a chemiluminescence system, ECL (Amersham Biosciences).

2.4. Flow cytometric determinations of $\Delta\Psi_m$

$\Delta\Psi_m$ was determined using the fluorescent lipophilic cationic probe JC-1. Irofulven-treated cells were harvested either by a mild trypsinization or by scraping and were combined with any cells already detached to the medium during the treatment. Cells were washed twice by centrifugation for 5 min at 200 *g* at room temperature in serum-free RPMI 1640 medium without phenol red and glutamine and were resuspended in PBS. Aliquots (1 mL) containing 1×10^6 cells were supplemented with 1 mL of binding buffer with JC-1 (ApoAlert™ Mitochondrial Membrane Sensor, BD Biosciences Clontech) as per the protocol of the manufacturer. Cell suspensions were incubated for 25 min at 37° and then analyzed immediately by flow cytometry on a Coulter EPICS ELITE flow cytometer (Beckman Coulter). 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 indicated in sample histograms (see Fig. 2B) as measures of cells with high and low mitochondrial membrane potential, respectively.

2.5. Flow cytometric determinations of phosphatidylserine externalization (annexin V binding)

Cells were treated with irofulven as indicated and harvested as described for $\Delta\Psi_m$ determinations. Harvested cells were washed with serum-free RPMI 1640 medium without phenol red and glutamine, counted, and suspended in PBS at a density 1×10^6 cells/mL. After an additional wash with PBS, aliquots of 1×10^6 cells were suspended in 500 μL of binding buffer (Annexin V-FITC Staining Kit, BD Biosciences Pharmingen). One hundred microliters of this cell suspension was stained with 5 μL of annexin V conjugated to fluorescein isothiocyanate (from Annexin V-FITC Staining Kit) and 10 μL of PI (500 μg/mL) for 15 min in the dark followed by the addition of 400 μL of binding buffer. Cells were placed on ice and immediately analyzed by flow cytometry. Typically, 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for annexin and PI, respectively.

2.6. Quantitative apoptotic DNA fragmentation

The quantitative fragmentation assay, described in detail previously [37,39,40], detects both early, high-molecular-weight DNA fragments and late residual oligonucleosomal-size fragments. In this assay, drug-treated [^{14}C]thymidine-prelabeled cells were permeabilized in a hypotonic buffer followed by the extraction of fragmented DNA. Under these conditions, undegraded DNA remains in the nuclear pellet fraction while chromatin fragments are in the supernatants. The results are expressed as a percentage of the total DNA released in the supernatants, corrected for the radioactivity released from untreated controls (typically 13 and 16% for Bcl-2 ON and OFF, respectively). Z-VAD-fmk (200 μM) was added 2 hr prior to the addition of irofulven in samples treated with this pan-caspase inhibitor.

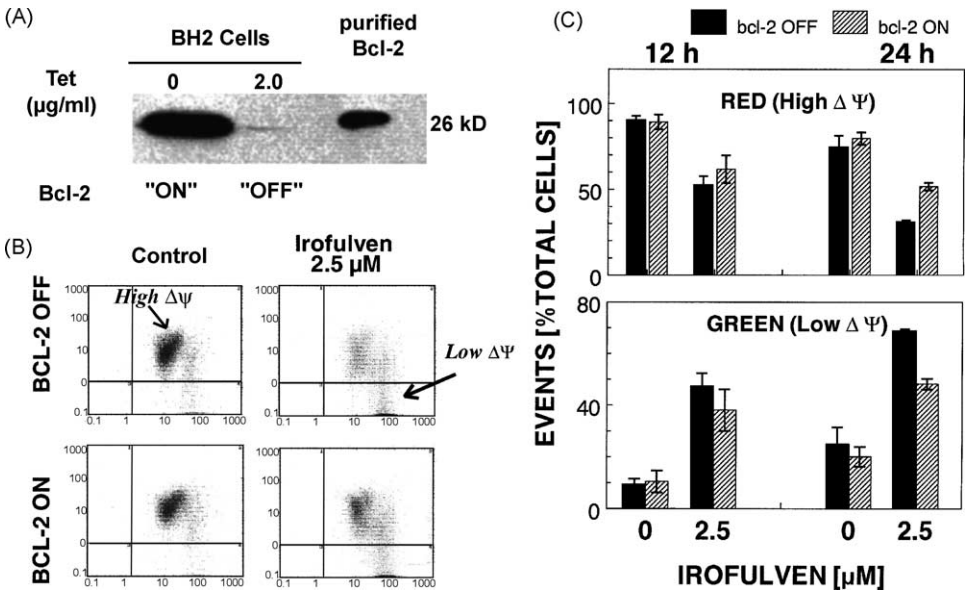

Fig. 2. Irofulven-induced mitochondrial permeability transition in BH2 cells under Bcl-2 ON and OFF conditions. (A) Western blot analysis of Bcl-2 protein from BH2 cells grown in the presence or absence of 2 μg/mL of tetracycline, referred to as "Bcl-2 ON" and "Bcl-2 OFF," respectively. A 26-kDa mark indicates the position of the Bcl-2 band. (B and C) Changes in $\Delta\Psi_m$ were followed by flow cytometric analysis using ApoAlert Mitochondrial Membrane Sensor. High red and green fluorescence correspond to cells with high and low $\Delta\Psi_m$, respectively. Panel B: Representative examples of flow cytometric histograms after 12 hr of drug treatment. Panel C: Quantitation of changes in red and green fluorescence for 12- and 24-hr drug treatment times averaged (±SEM) from two independent experiments carried out in duplicate.

3. Results

Apoptotic responses to irofulven treatment were followed in BH2 cells, a subline of HeLa S3 cells developed by Schimke and co-workers [57,60] with forced overexpression of Bcl-2 in the absence of tetracycline. Under these conditions, herein referred to as "Bcl-2 ON," BH2 cells were confirmed to have abundant levels of *bcl-2* message (data not shown) and Bcl-2 protein (Fig. 2A). In contrast, BH2 cells grown in the presence of 2 μg/mL of tetracycline had marginal levels of Bcl-2 protein (condition referred to as "Bcl-2 OFF," Fig. 2A).

3.1. Collapse of $\Delta\Psi_m$ in both Bcl-2 OFF and Bcl-2 ON cells

Since mitochondrial effects can be rate limiting in apoptosis [4], prevention of mitochondrial dysfunction by excess Bcl-2 can have a decisive effect on the fate of a cell. Thus, the effects of irofulven on $\Delta\Psi_m$ were examined under the conditions of Bcl-2 ON and OFF using flow cytometric determinations with a fluorescent sensor of $\Delta\Psi_m$, JC-1. In cells with normal mitochondria (high $\Delta\Psi_m$), JC-1 molecules bound as multimers in mitochondrial membranes, emitting red fluorescence. In cells with depolarized mitochondria, the dye is mainly present in the cytoplasm as a monomer, emitting green fluorescence [61,62].

In the control cells with no drug treatment, the majority of cells had a high $\Delta\Psi_m$ regardless of their Bcl-2 status, as indicated by a high red JC-1 fluorescent signal (Fig. 2B). After treatment with irofulven, however, the fraction of cells with red fluorescence decreased. Concurrently, the fraction of cells with green fluorescence (dissipated $\Delta\Psi_m$) became substantial. This collapse of $\Delta\Psi_m$ could be detected after a 12-hr drug treatment. The effect was somewhat more pronounced in cells with Bcl-2 OFF than with Bcl-2 ON, but the difference was not significant. An extensive collapse of $\Delta\Psi_m$ with both Bcl-2 OFF and ON was also seen at 24 hr. At this time, however, the down-regulating influence of Bcl-2 ON was slightly more significant ($P = 0.06$) than after 12 hr. These results demonstrated clearly that irofulven causes mitochondrial dysfunction in the absence as well as in the presence of Bcl-2 overexpression. At the most, Bcl-2 seemed to slow down the progression of the initial $\Delta\Psi_m$ collapse.

3.2. Possible independence of the earliest apoptotic events of the Bcl-2 status

To discern between possible differences in Bcl-2-dependence or the kinetics of apoptosis, we analyzed the effects of irofulven on apoptosis at various stages. Changes in plasma membrane phospholipids, such as externalization of phosphatidylserine residues in the outer plasma membrane, are a characteristic marker of early apoptotic events [63,64]. Phosphatidylserine externalization can be conveniently detected by fluoresceinated annexin V binding. Counterstaining with PI, which detects cells with compromised cell membrane integrity, allows one to distinguish among necrotic, early-apoptotic, and late-apoptotic cells [65,66] (Fig. 3A).

Representative flow cytometric histograms (Fig. 3A) illustrated profound shifts in annexin V and PI signals in BH2 cells following irofulven treatment for 12 hr. The appearance of cells with a high annexin signal and a low PI signal is characteristic of early apoptosis. The progression of apoptosis results in cells with a high annexin signal and a high PI signal, characteristic of late apoptosis (secondary necrosis).

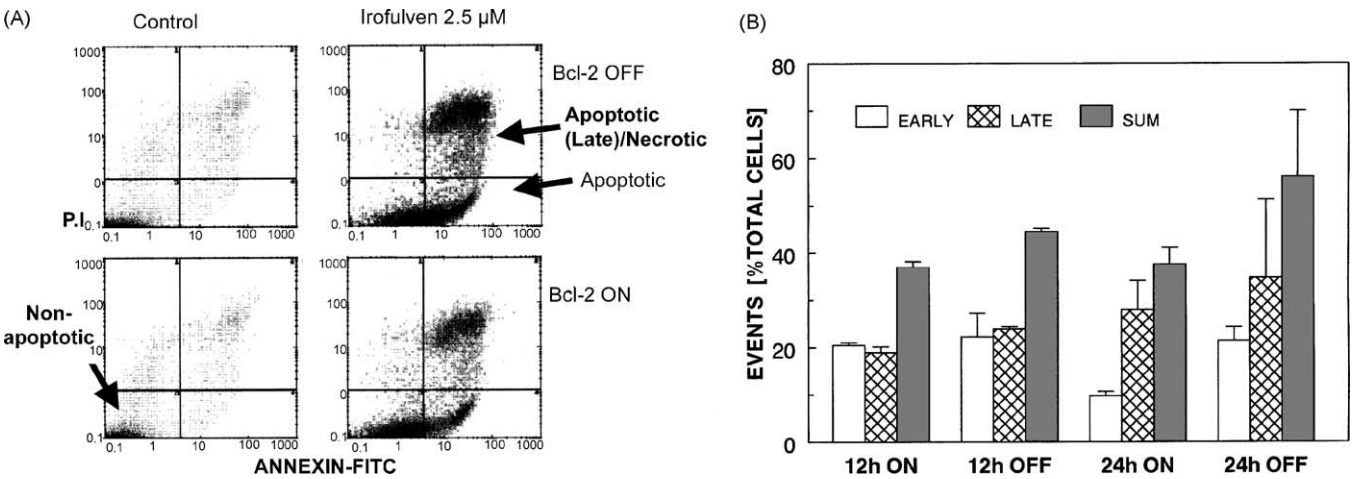

Fig. 3. Early and late apoptosis induced by 2.5 μM irofulven in BH2 cells under Bcl-2 ON and OFF conditions: flow cytometric analysis of phosphatidylserine externalization (annexin staining) with PI counter-staining. (A) Representative examples of flow cytometric histograms after 12 hr of drug treatment. The appearance of cells with a high annexin signal and a low PI signal is characteristic of early apoptosis. Progression of apoptosis results in cells with a high annexin signal and a high PI signal (secondary necrosis). (B) Quantitation of early, late, and total apoptosis after 12- and 24-hr treatments with 2.5 μM irofulven. The values shown are means (±SEM) from two independent experiments carried out in duplicate and are corrected for respective values in control cells.

The quantitation of these effects (Fig. 3B) shows that a relatively brief 12-hr incubation with irofulven-induced profound apoptosis regardless of Bcl-2 status. Bcl-2 overexpression after a 12-hr irofulven treatment only marginally reduced the proportion of cells in the combined early and late apoptotic compartments, compared to Bcl-2 OFF conditions. Thus, it appears that the earliest apoptotic events induced by irofulven and detected by annexin binding can be independent of Bcl-2 status. The differences in the response to irofulven between Bcl-2 ON and Bcl-2 OFF cultures increased after 24 hr with the drug. In particular, in the Bcl-2 ON cultures (Fig. 3B), the early apoptosis compartment was diminished markedly after 24 hr compared to both 12 hr for Bcl-2 ON and 24 hr for Bcl-2 OFF. The differences between the late apoptosis compartments were not significant, which is consistent with the belief that the cells in late apoptosis, after 24 hr, represent primarily cells that were in the early apoptosis phase after 12 hr of drug treatment.

3.3. Partial dependence of apoptotic DNA fragmentation on Bcl-2 status

DNA breakage, an event downstream from the caspase cascade, is another hallmark of apoptosis. With other cell lines, irofulven tended to produce massive high-molecular-weight DNA fragmentation (peaking at ~40–50 kbp after a 24-hr incubation), although no oligonucleosomal-size fragments (“DNA ladder”) were observed [37]. To assess whether Bcl-2 overexpression affects DNA fragmentation in BH2 cells, we used a quantitative DNA fragmentation assay [37,39,40] which measures DNA fragments that can be eluted from the nuclei of drug-treated cells, and correctly quantitates not only oligonucleosomal fragments but also large fragments. In this assay, the background levels of apoptosis were themselves suppressed by Bcl-2 overexpression. Control levels of DNA fragmentation were typically 13.2 ± 0.7 and $16.3 \pm 0.6\%$ (SEM, 24 hr, $N = 34$–46) for Bcl-2 OFF and Bcl-2 ON, respectively.

Under Bcl-2 OFF conditions, irofulven induced high levels of apoptotic DNA fragmentation in BH2 cells (Fig. 4). Fragmented DNA was detected at drug concentrations as low as 0.5 μM after treatment times of 12 hr or more (Fig. 4A). Under Bcl-2 ON conditions, DNA fragmentation was reduced significantly after 12 and 24 hr of drug treatment. However, the Bcl-2-independent component of DNA fragmentation remained profound ($21.8 \pm 2.6\%$ (SEM) at 2.5 μM, 12 hr, $N = 5$). Moreover, the partial dependence of irofulven-induced DNA fragmentation on Bcl-2 status became insignificant after a prolonged (48-hr) incubation (Fig. 4C). Given that the Bcl-2 ON conditions in the BH2 system result in supra-physiologically high Bcl-2 protein levels, DNA fragmentation data provided further evidence that irofulven-induced apoptosis has at least two components, one of which is profoundly insensitive to Bcl-2 inhibition. Both components seem to be mediated by the

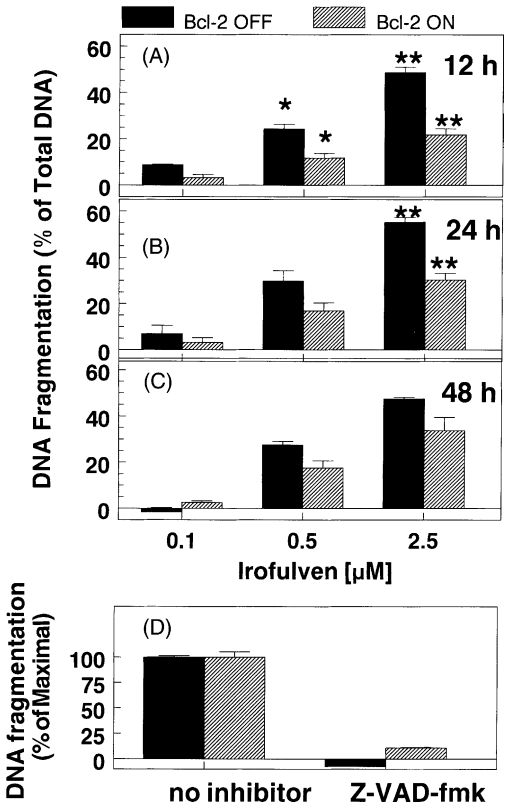

Fig. 4. Apoptotic DNA fragmentation in BH2 cells incubated with irofulven with forced overexpression of Bcl-2 OFF or ON. (A–C) DNA fragmentation after the indicated times of drug treatment. The values of the percentage of fragmented DNA are means (±SEM) from 3–12 independent experiments carried out in triplicate and are corrected for respective values in control cells. Significant differences in sample means at individual times and drug concentrations are indicated by (∗) and (∗∗) for $P < 0.05$ and 0.005, respectively. (D) Inhibition of irofulven-induced DNA fragmentation by the pan-caspase inhibitor Z-VAD-fmk (200 μM) assayed after 24 hr of treatment with 2.5 μM drug. Data are expressed as the percentage of DNA fragmentation in the absence of caspase inhibitor (mean values ± range from duplicate cultures).

caspase cascade, since a broad-spectrum caspase inhibitor Z-VAD-fmk completely abrogated irofulven-induced DNA fragmentation, regardless of Bcl-2 status (Fig. 4D).

Additional DNA fragmentation experiments compared the effects of Bcl-2 overexpression on apoptotic responses to irofulven and to the model single-action drugs etoposide, which essentially produces only DNA lesions (mediated by topoisomerase II [42]), and helenalin A, a sesquiterpenoid like irofulven, which binds to proteins but not to DNA [67]. The results demonstrated profound differences in the effects of Bcl-2 on apoptosis by these three drugs (Fig. 5). Whereas etoposide produced rather high levels of DNA fragmentation with Bcl-2 OFF, Bcl-2 ON conditions severely attenuated these effects (Fig. 5A). This Bcl-2 dependency is consistent with previous findings for etoposide [42,68]. In contrast, helenalin A produced less DNA fragmentation, but the effects of this drug were essentially independent of the Bcl-2 status (Fig. 5A). Bcl-2-independent DNA fragmentation was also produced by diamide, another protein-reactive agent (data not shown). Irofulven responses resembled

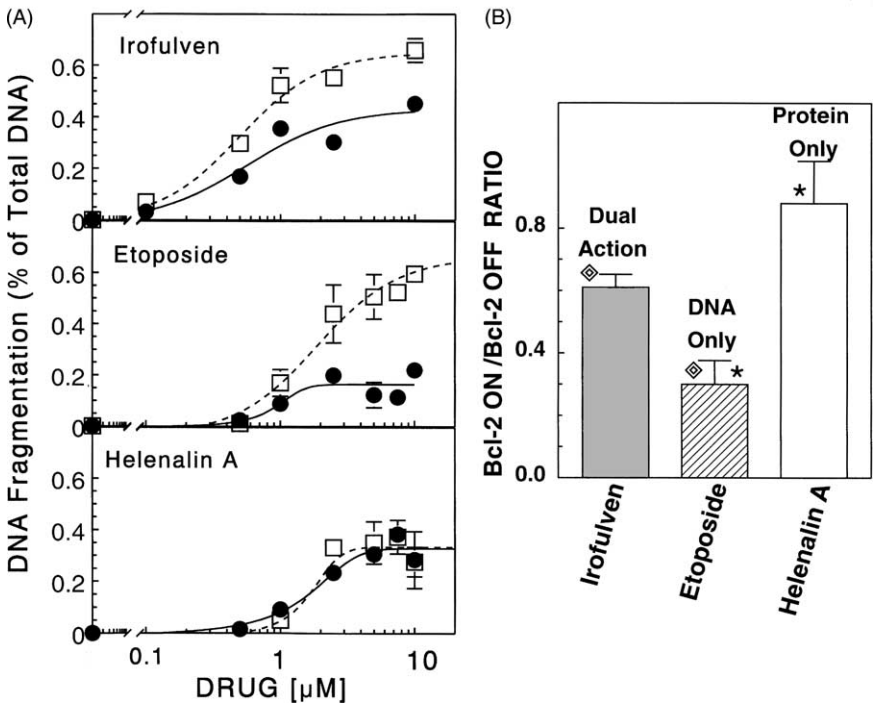

Fig. 5. Comparison of apoptotic DNA fragmentation in BH2 cells with forced overexpression of Bcl-2 OFF or ON in response to irofulven and model single-action drugs, etoposide (essentially producing only DNA lesions) and helenalin (protein damage only). (A) DNA fragmentation after 24 hr of drug treatment with Bcl-2 ON (solid symbols and lines) and OFF (open symbols and dashed lines). The values of percentage of fragmented DNA are means (±SEM) from 2 to 15 independent experiments carried out in triplicate and are corrected for respective values in control cells. Some of the points for irofulven are re-plotted from Fig. 3B. (B) The ratios of apoptotic DNA fragmentation under Bcl-2 ON and OFF calculated from the data in panel A as the average ratio (±SEM) using drug concentrations that produced DNA fragmentation ranging from 20 to 55%. Significant differences in means between irofulven and etoposide and helenalin and etoposide are indicated by diamonds and asterisks, respectively (for both, $P < 0.05$, *t*-test).

etoposide in terms of the magnitude of DNA fragmentation with Bcl-2 OFF, but were significantly less attenuated by Bcl-2 ON (Fig. 5A and B).

3.4. Irofulven cytotoxicity in the presence of high levels of Bcl-2

To address the question of whether the apoptotic effect of reduced DNA fragmentation can be relevant to the overall antiproliferative effects of irofulven, we compared drug cytotoxicity in Bcl-2 ON and OFF cultures of BH2 cells. The results demonstrated that drug-induced cell growth inhibition after 72 hr of continuous exposure was virtually identical regardless of Bcl-2 status, with GI_{50} values of 0.16 and 0.15 μM for Bcl-2 ON and OFF, respectively (Fig. 6). Like irofulven, helenalin A also showed similar cytotoxicity with Bcl-2 ON and OFF with the GI_{50} values of 0.52 and 0.38 μM, respectively, while etoposide produced somewhat more disparate GI_{50} values of 0.87 and 0.56 μM, respectively.

To extend the findings with irofulven, we used NCI databases to plot Bcl-2 RNA levels (http://dtp.nci.nih.gov/mtargets/mt_index.html) versus irofulven cytotoxicity in the NCI panel of various cancer cell lines (http://dtpsearch.ncifcrf.gov/htbin/nsc_search.exe). No obvious correlation existed between the GI_{50} values and the Bcl-2 RNA levels (Fig. 7A). Furthermore, a number of cell lines were identified that are highly susceptible to irofulven, with GI_{50} values below 0.5 μM despite clearly elevated Bcl-2 message. Like growth inhibition, the ability of irofulven to induce apoptosis in several cell lines was also not correlated

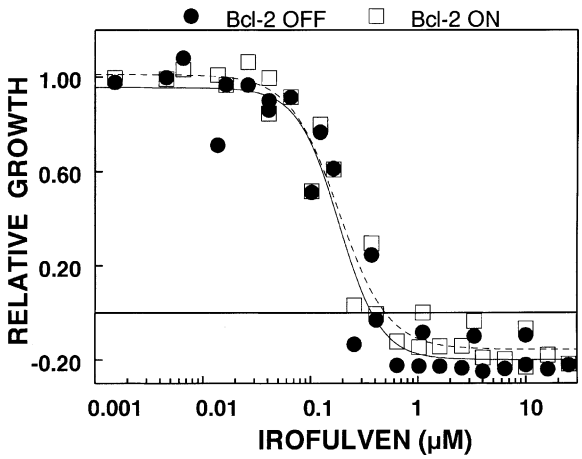

Fig. 6. Cytotoxicity of irofulven in BH2 cells incubated with irofulven with forced overexpression of Bcl-2 OFF or ON. BH2 cells grown in the presence (□) or absence (●) of 2 μg/mL of tetracycline (Bcl-2 OFF and ON, respectively) were incubated with irofulven for 72 hr before measuring cell growth by the MTT assay. Average values from three separate experiments are plotted, reflecting GI_{50} values of 0.15 and 0.16 μM for Bcl-2 OFF and ON, respectively. Similar results were obtained by cell counts, with GI_{50} values of 0.19 and 0.20 μM drug for Bcl-2 ON and OFF, respectively (profiles not shown).

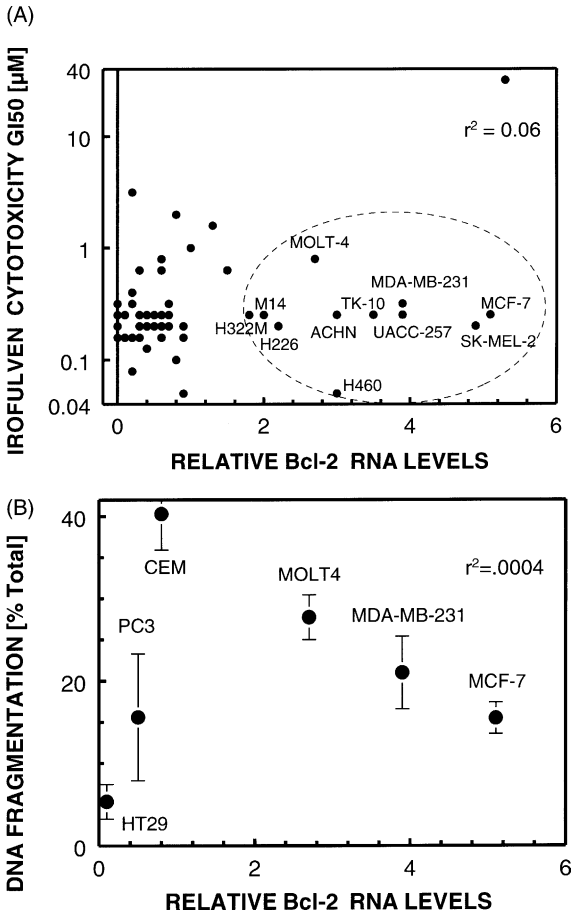

Fig. 7. Lack of correlation between irofulven cytotoxicity and apoptosis induction in cancer cells and Bcl-2 levels. (A) Cytotoxicity of irofulven (GI_{50} values from the MTT assay) in an NCI panel of various cancer cell lines plotted against Bcl-2 RNA levels (by dot-blot hybridization expressed relative to levels in the BT549 cell line) using data from NCI Developmental Therapeutics Program databases on Drug Cancer Screening (http://dtp.nci.nih.gov/docs/chemname_search.html) and Molecular Targets (http://dtp.nci.nih.gov/mtargets/mt_index.html), respectively. Several cell lines that are highly responsive to irofulven despite their reported high Bcl-2 levels are encircled with a dotted line. The lack of correlation is indicated by $r^2 = 0.06$. (B) Irofulven-induced apoptotic DNA fragmentation (24-hr incubation, 5 μM drug) in several cell lines plotted against Bcl-2 RNA levels. Bcl-2 data are from the Molecular Targets database (see panel A). Fragmentation data for MOLT4 cells are means (±SEM) from triplicate determinations. Fragmentation data for CEM, HT29, and PC-3 cells are from [40]. For MCF-7 and MDA-MB-231, the plotted values for 5 μM drug are interpolated from the previously published [58] data for 2 and 10 μM drug.

with the levels of Bcl-2 expression (Fig. 7B). For example, irofulven produced markedly more potent apoptotic responses in breast cancer MCF-7 and MDA-MB-231 cells, which exhibit high levels of Bcl-2 expression, than in Bcl-2-poor HT29 cells (Fig. 7B).

4. Discussion

Apoptosis induction by the promising anti-cancer drug irofulven is likely to reflect a combination of drug effects on multiple cellular targets [56]. Irofulven alkylates cellular DNA, which should induce damage signaling, a well-known initiator of the death pathway. Independently, irofulven binds covalently and irreversibly to cellular proteins, which include key regulators of apoptosis execution [38,40]. By affecting multiple intracellular targets that may potentially have different effects on the apoptotic process, irofulven may overcome various specific cellular anti-apoptotic defenses. Indeed, irofulven has been shown recently to bypass apoptosis resistance due to caspase-3 deficiency [58]. This study demonstrates that overexpression of the anti-apoptotic protein Bcl-2 is also unable to abrogate irofulven-induced cell death.

Irofulven remains strongly pro-apoptotic under supra-physiological levels of Bcl-2, as documented by several endpoints. Bcl-2 overexpression seems largely inconsequential to the potent early depolarization of the mitochondrial membrane after drug treatment (Fig. 2). Apoptotic phosphatidylserine externalization was essentially unaffected at 12 hr of treatment and only marginally reduced upon Bcl-2 overexpression at 24 hr (Fig. 3). Furthermore, the massive irofulven-induced apoptotic DNA fragmentation, while attenuated, was still profound in Bcl-2 overexpressing cells (Fig. 4).

These results are consistent with the notion that the earliest effects in the apoptosis induced by irofulven, including mitochondrial dysfunction, are Bcl-2-independent. Bcl-2, in general, blocks changes in the PTPC, preventing mitochondrial membrane depolarization and a subsequent release of cytochrome *c* and other pro-apoptotic factors [27,69–71]. However, overexpressed levels of Bcl-2 failed to prevent the mitochondrial permeability transition (MPT) induced by irofulven at 12 hr (Fig. 2). One explanation for this failure could be a distorted interplay among Bcl-2 family members within the mitochondrial membrane [6,10,11]. The Bcl-2-independent effects of some pro-apoptotic anticancer drugs were suggested to originate from the cleavage of pro-apoptotic Bax to a form that no longer interacts with Bcl-2 but still promotes permeability transition [72]. In the case of irofulven, however, this possibility seems unlikely. Irofulven does cause Bax translocation to mitochondria, which is followed by cytochrome *c* release, but without any detectable Bax cleavage [56,73]. Alternatively, a caspase-3-dependent cleavage of Bcl-2 can promote mitochondrial release of cytochrome *c* [74–76]. However, no indications of Bcl-2 cleavage accompanied irofulven-induced apoptosis in leukemic CEM (Herzig *et al.*, unpublished data). In addition, irofulven remains pro-apoptotic in caspase-3-deficient cells [58]. Further studies of the complex interrelationships among $\Delta\Psi_m$ dissipation, mitochondrial pore opening [5,6,12,13], and other members of the Bcl-2 family [77] are needed to uncover the molecular events underlying the Bcl-2-independent component in irofulven-induced mitochondrial dysfunction.

One specific possibility worth consideration is direct interference with mitochondrial membrane proteins. Purely protein-reactive agents, such 1,1′-azobis(*N*,*N*-dimethylfor-

mamide) (diamide) and helenalin, are also known to induce apoptosis in a Bcl-2-independent manner [48,51,52]. Although diamide and related protein cross-linking agents bind to numerous cellular proteins, the covalent modification of a PTPC protein, adenine nucleotide transporter (ANT), was proposed to lead to Bcl-2-independent mitochondrial membrane permeability transition [52]. It is tempting to speculate that, analogous to purely protein-reactive agents, irofulven reactivity with sulfhydryl-containing proteins may play a broader role in overcoming the anti-apoptotic action of Bcl-2. An intriguing analogy is offered by the recent findings with helenalin [55], a sesquiterpene lactone that belongs to the same broader class of compounds as irofulven. Like irofulven, helenalin is capable of reacting with protein sulfhydryls [78] but, unlike irofulven, helenalin probably does not react with DNA [67]. The protein reactivity is apparently sufficient for potent caspase-dependent but Bcl-2-independent apoptosis by helenalin ([55] and Fig. 5), similar to apoptosis by irofulven. The broad reactivity with multiple cellular proteins of both helenalin [55,67,78] and irofulven ([38] and Herzig *et al.*, data not shown) may help to derail the known function of Bcl-2 as an antioxidant [7,47,79–81]. Similar to the protein-reactive diamide, unrepaired irofulven–protein adducts are associated with a pro-oxidative distortion of the redox homeostasis [56,59]. Pro-oxidative changes, in general, are known to facilitate apoptosis execution [49]. In particular, a key role in the repair of protein damage and in redox-mediated anti-apoptotic responses to various anticancer agents is played by the thioredoxin system [82–84]. In that context, the demonstrated ability of irofulven to bind to thioredoxin and thioredoxin reductase ([38,56] and Herzig *et al.*, unpublished data) might contribute to counteracting the antioxidative effects of Bcl-2 at the cellular level.

Although excessive Bcl-2 cannot halt irofulven-induced apoptosis, it may slow its progression somewhat. This component of irofulven-induced apoptosis that is slowed by Bcl-2 might reflect a canonical Bcl-2-mediated inhibition of cellular responses to drug–DNA adducts. For a variety of drugs, including etoposide, whose mechanism implicates DNA damage as the apoptotic stimulus, Bcl-2 overexpression conferred a significant resistance to drug-induced apoptosis ([74,85,86] and Fig. 5). In addition to drug-specific responses caused by the primary DNA lesions, mitotic catastrophe in cells with unrepaired DNA damage could give rise to ‘‘generic,’’ secondary apoptotic responses that can be expected to be inhibitable by the canonical Bcl-2 mechanism. This scenario is consistent with the anti-apoptotic effect of Bcl-2 being most evident at 24 hr, a time frame that seems sufficient for some affected cells to have attempted mitosis. Finally, the Bcl-2-inhibitable component of irofulven apoptosis may also be related to events downstream from mitochondrial dysfunction. Downstream effects, including the caspase cascade, are known to affect the kinetics of cell death by providing positive feedback, secondary routes that enhance the pro-apoptotic mitochondrial changes [75]. Distinct from the direct mitochondrial effects, Bcl-2 can down-regulate events associated with this positive feedback loop, for example, by sequestering pro-caspases [69]. Whereas the mitochondrial events seem central for irofulven-induced apoptosis, a contribution of positive feedback loops, particularly at later times, has been implicated in the irofulven-induced apoptosis in LNCaP-Pro5 cells [56,73]. Collectively, these data suggest that irofulven-induced apoptosis may have different underlying molecular characteristics at various times from the beginning of the pro-apoptotic insult.

A comparison of Bcl-2 dependency of irofulven-induced apoptotic DNA fragmentation with the effects of etoposide, which produces mainly DNA lesions, and those of helenalin, which causes protein damage only, demonstrates clearly that irofulven responses share the traits expressed by both single-action agents. The high magnitude of apoptotic fragmentation in the absence of Bcl-2, observed for both irofulven and etoposide, may reflect the ability of both drugs to damage DNA. On the other hand, the significantly lower attenuation of irofulven-induced DNA fragmentation by overexpressed Bcl-2 is consistent with the contribution of protein damage, as the effects of helenalin were essentially Bcl-2-independent.

Regardless of the specific molecular details, the Bcl-2-independent effects of irofulven are likely to determine the fate of the cell. Bcl-2 overexpression had no effect on cell growth inhibition by irofulven in the BH2 system (Fig. 6). Moreover, irofulven remains highly cytotoxic and pro-apoptotic in several cancer cell lines with naturally elevated levels of *bcl-2* message (Fig. 7). These data again bring up the analogy to the pure protein damage induced by diamide, for which the resulting cell death is not prevented by Bcl-2 [52].

Taken together, the reported findings strongly suggest that irofulven is likely to maintain its apoptotic potential under elevated Bcl-2 levels in naturally occurring tumors. Such potent pro-apoptotic properties are generally highly desirable since they reduce the chances for selecting more aggressive and/or drug-resistant tumor phenotypes, which are often responsible for the failure of classical therapies. Killing Bcl-2-overexpressing cells could be particularly important, since high Bcl-2 levels were suggested to increase the metastatic potential of a cancer cell [87]. Thus, irofulven may be useful as a treatment modality in the case of pharmacologically inauspicious apoptosis-resistant tumors with elevated Bcl-2. The potential utility of dual-acting anticancer agents, such as irofulven, warrants further mechanistic studies to better understand how the combination of drug-induced DNA and protein damage circumvents anti-apoptotic cell defenses, such as Bcl-2.

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