Flex-Hets differentially induce apoptosis in cancer over normal cells by directly targeting mitochondria

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Abstract

Flex-Het drugs induce apoptosis in multiple types of cancer cells, with little effect on normal cells. This apoptosis occurs through the intrinsic mitochondrial pathway accompanied by generation of reactive oxygen species (ROS). The objective of this study was to determine if direct or indirect targeting of mitochondria is responsible for the differential sensitivities of cancer and normal cells to Flex-Hets. Mitochondrial effects and apoptosis were measured using JC-1 and Annexin V-FITC dyes with flow cytometry. Bcl-2, Bcl-x_L, and Bax were measured by Western blot. Flex-Hets induced mitochondrial swelling and apoptosis in ovarian cancer cell lines but had minimal to no effects in a variety of normal cell cultures, including human ovarian surface epithelium. Effects on inner mitochondrial membrane (IMM) potential were variable and did not occur in normal cells. Two different antioxidants, administered at concentrations shown to quench intracellular and mitochondrial ROS, did not alter Flex-Het–induced mitochondrial swelling, loss of IMM potential, or apoptosis. Inhibition of protein synthesis with cycloheximide also did not prevent Flex-Het mitochondrial or apoptosis effects. Bcl-2 and Bcl-x_L levels were decreased in an ovarian cancer cell line but increased in a normal culture, whereas Bax expression was unaffected by Flex-Hets treatment. In conclusion, ROS seems to be a consequence rather than a cause of mitochondrial swelling. The differential induction of apoptosis in cancer versus normal cells by Flex-Hets involves direct targeting of mitochondria associated with alterations in the balance of Bcl-2 proteins. This mechanism does not require IMM potential, ROS generation, or protein synthesis. [Mol Cancer Ther 2007;6(6):1814–22]

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Introduction

To kill cancer cells without harming healthy cells is the ultimate objective of cancer therapy. This objective seems to be met by a novel class of synthetic compounds called flexible heteroarotinoids (Flex-Hets). *In vitro* studies showed that the Flex-Hets, called SHetA2, SHetA3, and SHetA4, induced differentiation and apoptosis in cancer cell lines and primary cultures of cancer cells (1, 2). The Flex-Het compound called SHetA2 was chosen as the lead Flex-Het because it induced the highest levels of apoptosis in multiple cancer types. Micromolar concentrations of SHetA2 were effective against all of the cell lines in the National Cancer Institute's Human Tumor Cell Line Panel in addition to cervical and head and neck cancer cell lines (3, 4). Although this broad spectrum of activity may lead one to speculate that Flex-Hets will be toxic to all cell types, comparison of effects on normal versus cancer cells reveals a 10-fold reduced activity of Flex-Hets on normal endometrial versus ovarian cancer cells. *In vivo* studies showed that Flex-Hets inhibit tumor growth without evidence of toxicity, skin irritation, or teratogenicity (3, 5).

Flex-Hets were developed from a class of retinoid drugs, heteroarotinoids, by replacing the conventional two-atom linker with a more flexible urea or thiourea linker (2). Although the retinoids exert their activities through nuclear retinoic acid receptors, the Flex-Hets do not require the retinoid receptors for their action, nor are their activities prevented by retinoid receptor antagonists (2, 4). This may explain the lack of conventional retinoid toxicities, such as skin irritation or teratogenicity, observed when Flex-Hets were tested in animal models designed to specifically test for these toxicities (3, 5). The only retinoid activity retained by Flex-Hets is their ability to induce differentiation or reverse the cancerous phenotype (1). The major difference between Flex-Hets and retinoids is the ability of Flex-Hets to induce potent apoptotic activity in cancer cells. The mechanism of this activity has been shown to occur through the intrinsic mitochondrial pathway associated with loss of mitochondrial membrane integrity, generation of reactive oxygen species (ROS), release of cytochrome *c* from mitochondria, and activation of caspase-3 in head and neck cancer cell lines (4). Generation of ROS was confirmed in ovarian cancer cell lines (2).

Targeting the mitochondria is a logical explanation for the differential effects of Flex-Hets on normal versus cancer cells. Because cancer cells have increased metabolism and

mitochondrial mutations (reviewed in ref. 6), their mitochondria may be more unstable and therefore more sensitive to Flex-Het perturbations. It is important to study the Flex-Het mitochondrial effects in normal cells because alterations of normal mitochondrial activity could induce significant side effects when used as pharmaceuticals. Another concern regarding toxicity is the potential that the mitochondrial effects are indirectly caused by generation of ROS. ROS are generated naturally by the mitochondrial electron transport chain and $\sim 0.1\%$ leak out of the mitochondria to form superoxide, O_2^-. The levels of ROS are carefully controlled in the cell by detoxifying enzymes, such as the superoxide dismutases, glutathione peroxidase, and catalase, to avoid ROS-induced damage of cellular molecules (reviewed in ref. 7). Therefore, drugs that nonspecifically generate ROS to levels that surpass what these enzymes can control could cause severe cellular damage and potential carcinogenic changes.

An alternative mechanism to ROS generation that could be responsible for Flex-Het induction of the intrinsic apoptosis pathway is through disregulation of the balance between the Bcl-2 family of mitochondrial proteins. The Bcl-2 family members are classified based on their number of Bcl-2 homology domains (BH1, BH2, BH3, and BH4) and their regulation of apoptosis (8). The proapoptotic Bax and Bak family members are multidomain proteins that form mitochondrial pores leading to membrane destabilization, release of cytochrome c, and induction of apoptosis. The antiapoptotic Bcl-2 and Bcl-x_L family members are multidomain proteins that interrupt this pore formation, whereas the BH3-only proteins either suppress the antiapoptotic proteins or activate the proapoptotic proteins. Thus, the balance of these proteins in the mitochondrial membrane is an important regulatory mechanism of cell fate.

The hypothesis of this study is that Flex-Hets induce differential apoptosis in cancer cells over normal cells by directly targeting mitochondria independent of protein synthesis or ROS generation. The objectives were to compare the effects of three Flex-Hets (SHetA2, SHetA3, and SHetA4; see Fig. 1 for structures) on mitochondria, Bcl-2 proteins, and apoptosis in ovarian cancer cell lines in comparison with primary and immortalized cultures of normal human cells and to determine if the effects require generation of ROS or synthesis of RNA or protein.

Materials and Methods

Cell Lines and Primary Cultures

The ovarian cancer cell lines A2780 (obtained from Dr. Michael Birrer, National Cancer Institute, Bethesda, MD) and OVCAR-3 (obtained from the American Type Tissue Culture Collection) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), antibiotic/antimycotic, 1 mmol/L sodium pyruvate, and 1 mmol/L HEPES buffer. Antimycotic was omitted from the medium for OVCAR-3. The SK-OV-3 ovarian cancer cell line (American Tissue Culture Collection) was cultured in McCoy's 5a medium with 1.5 mmol/L L-glutamine, 10%
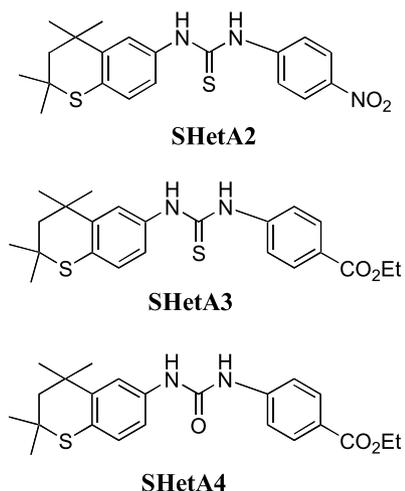

Figure 1. Chemical structures of Flex-Hets.

FBS, and antimycotic/antibiotic. The Caov-3 ovarian cancer cell line (American Type Culture Collection) was cultured in DMEM with 4 mmol/L L-glutamine, 10% FBS, and antibiotic/antimycotic.

Primary cultures of human ovarian surface epithelium (HOSE) and menstruated endometrial cells were obtained from patients and volunteers under Institutional Review Board–approved protocols. HOSE cells were scraped from the ovary using a CytoSoft Cytology Brush (Cardinal Health, Inc.) and immediately transferred into centrifuge tubes containing 15 mL of sterile cell suspensions and centrifuged at $600 \times g$ for 10 min, and the resulting pellet was resuspended in 1 mL of sterile medium and transferred to a six-well tissue culture plate. HOSE and immortalized HOSE (IOSE80, obtained from Dr. Michael Birrer) were cultured in Medium 199/MCDB105 (1:1; Sigma) supplemented with 15% FBS and antibiotic/antimycotic (Invitrogen Corp.). Primary endometrial cultures were collected as previously described (9) and maintained in MEM containing 1 mmol/L sodium pyruvate, 10% FBS, and antibiotic/antimycotic. Primary cultures of gingival fibroblast cells (PGF12) were obtained during the tooth extraction from healthy 27-year-old females under an Institutional Review Board–approved protocol and provided by Barbara Safiejko-Mroczka (University of Oklahoma, Norman, OK). PGF12 cells were cultured in DMEM containing 1 mmol/L sodium pyruvate, L-glutamine, D-glucose, 10% FBS, and antibiotic/antimycotic.

Cell Plating and Drug Treatments

The Flex-Hets were synthesized by K. Darrell Berlin, Ph.D., as previously described (2); dissolved in DMSO at a 0.01 mol/L, stored in 50-μL aliquots at $-20°C$; and manipulated under subdued lighting to protect from photo-oxidation. For the cytotoxicity and proliferation assays, cells were inoculated into 96-well microtiter plates at densities of 1,000 per well. For the other assays, cells were plated in six-well tissue culture dishes at a cell density

of 1×10^5 per well for apoptosis analysis and dihydroethidium staining or at 1×10^6 per well for mitochondria analysis or 2×10^5 per well for MitoSox staining. For Western blot analysis, cells were plated in 10-cm plates at concentrations that would achieve 90% confluency before drug treatment the next day. After allowing cells to adhere to the dishes overnight, parallel cultures were treated with varying concentrations of Flex-Hets, 30 nmol/L actinomycin D, 1 μmol/L cycloheximide, and/or the same volume of DMSO solvent for various times before processing for analysis. For the antioxidant studies, cultures were pretreated with butylated hydroxyanisol (BHA; 50 μmol/L) or MnTBAP (100 μmol/L) for 16 h before SHetA2 or DMSO treatments.

Growth Assays

The CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega) was used to measure metabolically active cells remaining after 72 h of incubation with or without a range of Flex-Het concentrations. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), a novel tetrazolium compound, was added to each well and incubated for 4 h followed by addition of a solubilization/stop solution. After an overnight incubation, the absorbance at 540 nm (A_{540}) of each well was determined using a Dynex MRX Revelation microtiter plate reader. Treatments were done in duplicate, and the growth indices were derived by dividing the average A_{540} of each treatment by the average A_{540} of control cultures.

The CyQUANT NF assay (Invitrogen) was used to measure cell number present after 72 h of incubation with or without a range of Flex-Het concentrations. This assay is based on measurement of cellular DNA content via fluorescent dye binding. After treatment, the medium was removed, and the cells were incubated with CyQuant dye for 45 min at 37°C. The fluorescence intensity of each sample was measured using a fluorescence microplate reader with excitation at 485 nm and emission detection at 530 nm. Treatments were done in triplicate, and the growth indices were derived by dividing the average A_{530} of each treatment by the average A_{530} of control cultures.

MitoSOX Staining

Cultures grown and treated on collagen type I–coated plates were gently washed thrice with warm PBS and then covered with 1 mL of 5 μmol/L MitoSOX reagent working solution. The cells were incubated for 10 min at 37°C and protected from light. The cultures were then gently washed thrice with warm PBS and counterstained with 5 μmol/L Hoescht 33342 solution. Photomicrographs of the MitoSox and Hoechst stains were acquired at ×40 using identical imaging variables of a Nikon TE2000-U Microscope for each treatment. The MitoSox and Hoechst images were merged using Lucia Software.

Flow Cytometry

A FACSCalibur (Becton Dickinson) automated bench-top flow cytometer was used for all flow cytometry experiments. Flow cytometry data were quantified using Summit for MoFlo Acquisition and Sort Control Software (Cytomation, Inc). The details for each type of experiment are described separately below.

Dihydroethidium

Generation of intracellular O$_2$ by SHetA2 was assessed by oxidation of dihydroethidium (Molecular Probes) to ethidium. Cell cultures were trypsinized and resuspended in
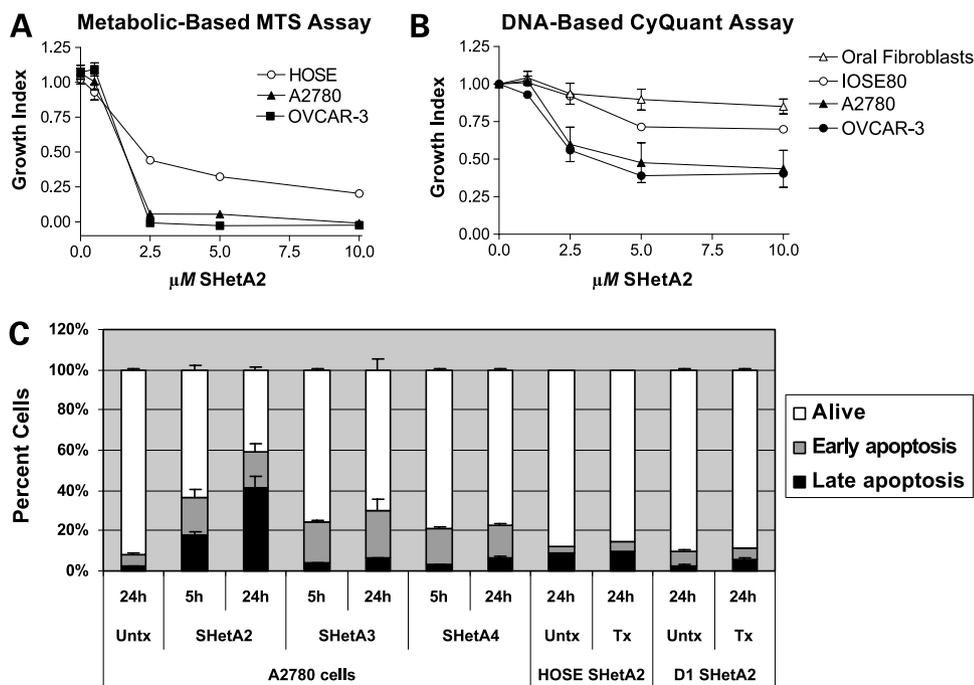

Figure 2. Differential effects of SHetA2 on growth index and apoptosis in ovarian cancer versus normal HOSE cells. **A,** cytotoxicity assays of A2780 and OVCAR-3 ovarian cancer cell lines and HOSE primary cultures treated with SHetA2 for 72 h. Results represent the average of two independent experiments each done in duplicate. **B,** proliferation assay of A2780 and OVCAR-3 ovarian cancer cell lines, IOSE80 cells, and oral fibroblasts treated with SHetA2 for 72 h. **C,** early and late apoptosis/necrosis was measured over 24 h in Flex-Het–treated A2780, HOSE, and normal endometrial (D1) cells by staining with Annexin V-FITC and propidium iodide followed by dual-laser flow cytometry. Representative of four independent experiments. Tx, treated; Untx, untreated.

Figure 3. Flex-Het effects on mitochondrial swelling and membrane (*Mb*) integrity in ovarian cancer cell lines. A2780 or OVCAR-3 cultures were treated with 10 μmol/L SHetA2 for the indicated times, stained with JC-1 dye, and analyzed by flow cytometry. **A,** representative histograms of two independent experiments done in duplicate. *Light curve,* untreated cultures; *dark curve,* cultures treated with SHetA2. An increase in green fluorescent (*Fl*) intensity (rightward shift) represents mitochondrial swelling, whereas a decrease (leftward shift) in red fluorescence indicates loss of mitochondrial membrane potential. **B** and **C,** time course of SHetA2 action in A2780 (**B**) and OVCAR-3 (**C**). The amounts of shift in green fluorescence intensity (■) and red fluorescence intensity (○) were calculated by subtracting the mean fluorescence intensity of treated cultures from the mean fluorescence intensity of the untreated control cultures.
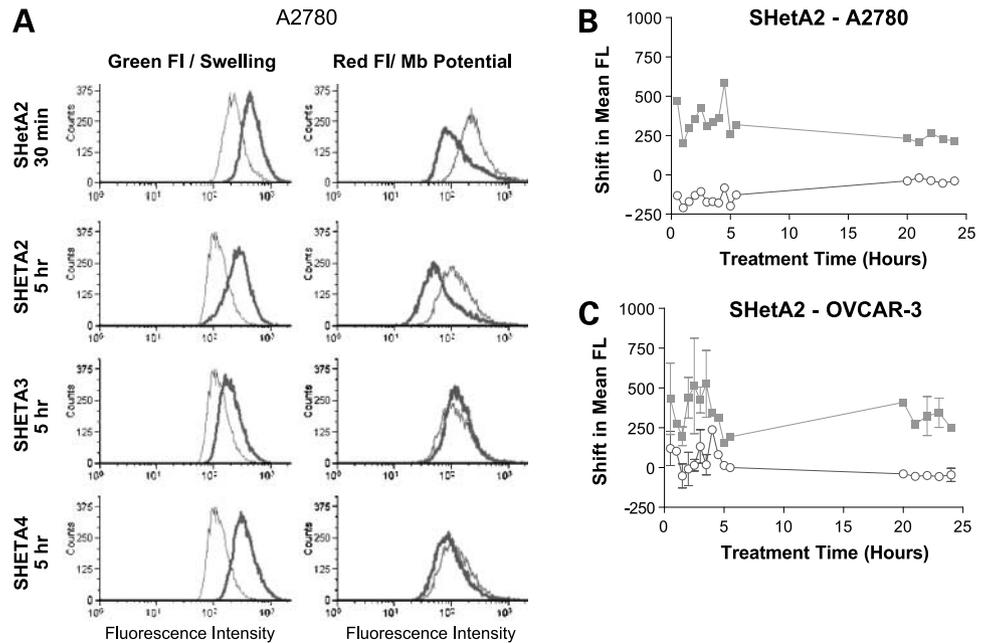
1 mL of PBS. Cell suspensions were incubated for 30 min with 10 μmol/L dihydroethidium, and the fluorescence of ethidium inside the cells was measured by flow cytometry using an excitation wavelength of 488 nm and an observation wavelength of 530 nm.

Mitochondrial Effects

Mitochondrial transmembrane potential ($\Delta\psi_m$) was assessed by measurement with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolylcarbocyanine iodide (JC-1; Molecular Probes; T3168). Cell cultures were trypsinized, resuspended in 1 mL of PBS, and incubated with 10 μg/mL JC-1 dye for 15 min. Both red and green fluorescence emissions were analyzed by flow cytometry using an excitation wavelength of 488 nm and observation wavelengths of 530 nm for green fluorescence and 585 nm for red fluorescence.

Apoptosis Assay

The Vybrant Apoptosis Assay kit #3 (Molecular Probes) was used to measure apoptosis and necrosis. Tissue culture medium in each well was collected to harvest cells that had already lifted off the tissue culture plate. These were combined with adherent cells that were harvested by trypsinization. The cells were pelleted by centrifugation and resuspended in 100 μL of 1× annexin-binding buffer and then incubated with 5 μL of Annexin V conjugated to fluorescein (Annexin V-FITC) and 1 μL of 100 μg/mL propidium iodide for 15 min at room temperature. The solution was then mixed gently with an additional 400 μL of 1× annexin-binding buffer, and the samples were evaluated with flow cytometry at an excitation wavelength of 488 nm and observation wavelengths of 530 and 575 nm.

Western Blot Analysis

Cultures were harvested with a cell scraper, washed once with PBS, and pelleted. The CytoBuster protein extraction regent (Novagen) and protease inhibitor cocktail (Sigma) were used to extract proteins, and concentrations were determined using Pierce BCA protein assay kit. Proteins (100 μg) were separated by 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes, which were then blocked with 5% nonfat milk in 0.1% Tween 20-TBS for 2 h at room temperature. The membranes were immunoblotted with one of the following primary antibodies: mouse monoclonal Bcl-2 (100), Bcl-x$_L$ (H5), Bax (2D2; Santa Cruz Biotechnology). After further washing, the blots were incubated with the appropriate horseradish peroxidase–conjugated secondary antibody. Antibody binding was detected with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Membranes were stripped and re-probed with mouse monoclonal β-actin (C4; Santa Cruz Biotechnology) as a protein loading control.

Results

Differential Flex-Het Effects on Growth and Apoptosis in Cancer versus Normal Ovarian Cells

Although differential sensitivities of ovarian cancer lines versus normal endometrial cells to Flex-Hets were previously shown (2), these effects could be due to the fact that the cancer cells were derived from a different organ site than the normal cells. In this study, the differential effects of the most potent Flex-Het (SHetA2) on two ovarian cancer cell lines (OVCAR-3 and A2780) and normal HOSE cells were compared using a standard cytotoxicity assay. There were no significant growth effects at concentrations below 1 μmol/L SHetA2. At concentrations of ≥2.5 μmol/L SHetA2, the growth indices of HOSE ranged from 0.4 to 0.2, whereas the growth indices of the cancer cell lines were ≤0.05 (Fig. 2A), showing a greater sensitivity of the cancer cells to SHetA2 in comparison with the normal cells.

These results could be biased by the type of growth assay used, however, because the MTS cytotoxicity assay measures mitochondrial dehydrogenase enzyme activity as a representation of metabolically active cells, and the hypothesis being tested involves mitochondria. Therefore, a second assay based on DNA content (CyQuant assay) that is a more direct measure of cell number was also used to compare the same two ovarian cancer cell lines with additional cultures of non-cancerous cells (Fig. 2B). The decrease in survival of the treated ovarian cancer cell lines was not as dramatic in the DNA-based assay in comparison with the MTS assay, indicating that a proportion of the cells remaining in the treated cultures were metabolically inactive. The non-cancerous cells included an immortalized HOSE line (IOSE80) obtained from a separate individual as the primary HOSE culture and included a primary culture of human oral fibroblasts. Differential sensitivities were apparent between the cancer and non-cancer cell lines, with the DNA-based assay confirming that the results are not dependent upon the mitochondria or individual cultures used.

Apoptosis and necrosis were evaluated in A2780 and HOSE cells using Annexin V-FITC, which detects translocation of phosphatidylinositol from the inner to outer cell membrane during early apoptosis and propidium iodide, which can enter the cell in late apoptosis or necrosis (Fig. 2B). Apoptosis was observed in A2780 ovarian cancer cells within 5 h of treatment with SHetA2, SHetA3, or SHetA4. Apoptosis could still be detected at 24 h, after which most of the cells were gone. SHetA2 did not induce apoptosis or necrosis in HOSE cells or normal endometrial cells (D1) even after treatment for 24 h.

Differential Effects of Flex-Hets on Mitochondria in Cancer versus Normal Cells

Previous studies showed that SHetA2 induced apoptosis through the intrinsic mitochondrial pathway in head and neck cancer cell lines (4). In this study, the time courses of Flex-Het effects on mitochondria in ovarian cancer cell lines and HOSE were studied using the JC-1 dye and flow cytometry. JC-1 is a cationic dye that accumulates in mitochondria. Monomers of JC-1 dye fluoresce in the green range (525 nm), which is used as a measure of mitochondrial density in cells. JC-1 accumulation in the mitochondria is dependent on the mitochondrial membrane potential. Under normal conditions, JC-1 accumulation in the mitochondria leads to clumping of the dye into J-aggregates that fluoresce in the red range (590 nm). Loss of mitochondrial membrane integrity or potential leads to loss of aggregates and decreased red fluorescence. Within 30 min of treatment, 10 μmol/L SHetA2 induced mitochondrial swelling indicated by an increase (rightward shift) in green fluorescence (Fig. 3A), which was sustained over the 24-h evaluation period in both A2780 and OVCAR-3 cell lines (Fig. 3B and C, respectively). A loss of mitochondrial membrane integrity was noted within 30 min of SHetA2 treatment in the A2780 cell line but not the OVCAR-3 cell line (Fig. 3B versus C). To determine the generality of these mitochondrial effects, the SHetA3 and

SHetA4 Flex-Hets that differ from SHetA2 by single structural alterations were evaluated on both cell lines and found to induce swelling but not loss of mitochondrial membrane integrity (Fig. 3A for A2780; data not shown for OVCAR-3).

To determine if the differential effects of SHetA2 on growth and apoptosis in cancer versus normal cells correlated with mitochondrial effects, normal endometrial cells (Fig. 4A) and HOSE cells (Fig. 4B) were evaluated with the JC-1 dye. Mitochondrial swelling was only slight in normal endometrial cells and did not occur at all in HOSE cells treated with SHetA2 over a 24-h treatment period. Neither cell type exhibited loss of mitochondrial membrane integrity over this 24-h period (Fig. 4A and B).

Role of ROS in the Flex-Het Mechanism

Our previous studies showed that SHetA2 induces ROS in ovarian and in head and neck cancer cell lines (2, 4). To test the hypothesis that SHetA2 exerts mitochondrial effects through generation of intracellular ROS, the ability
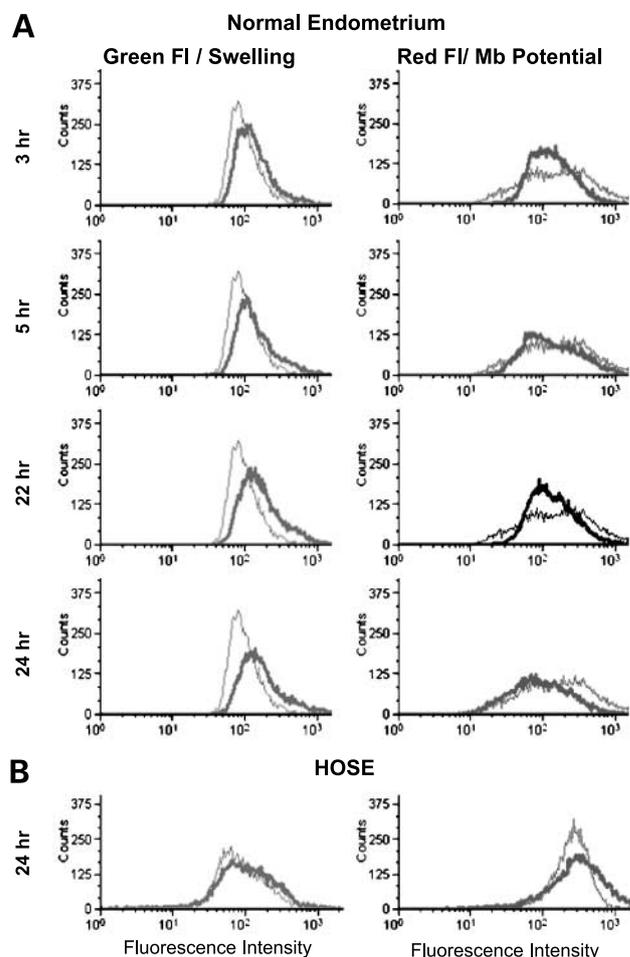

Figure 4. Minimal mitochondrial effects of SHetA2 on normal endometrial and HOSE cells. Primary cultures were treated with 10 μmol/L SHetA2 for the indicated times, stained with JC-1 dye, and analyzed by flow cytometry. *Light curve,* untreated cultures; *dark curve,* cultures treated with SHetA2.
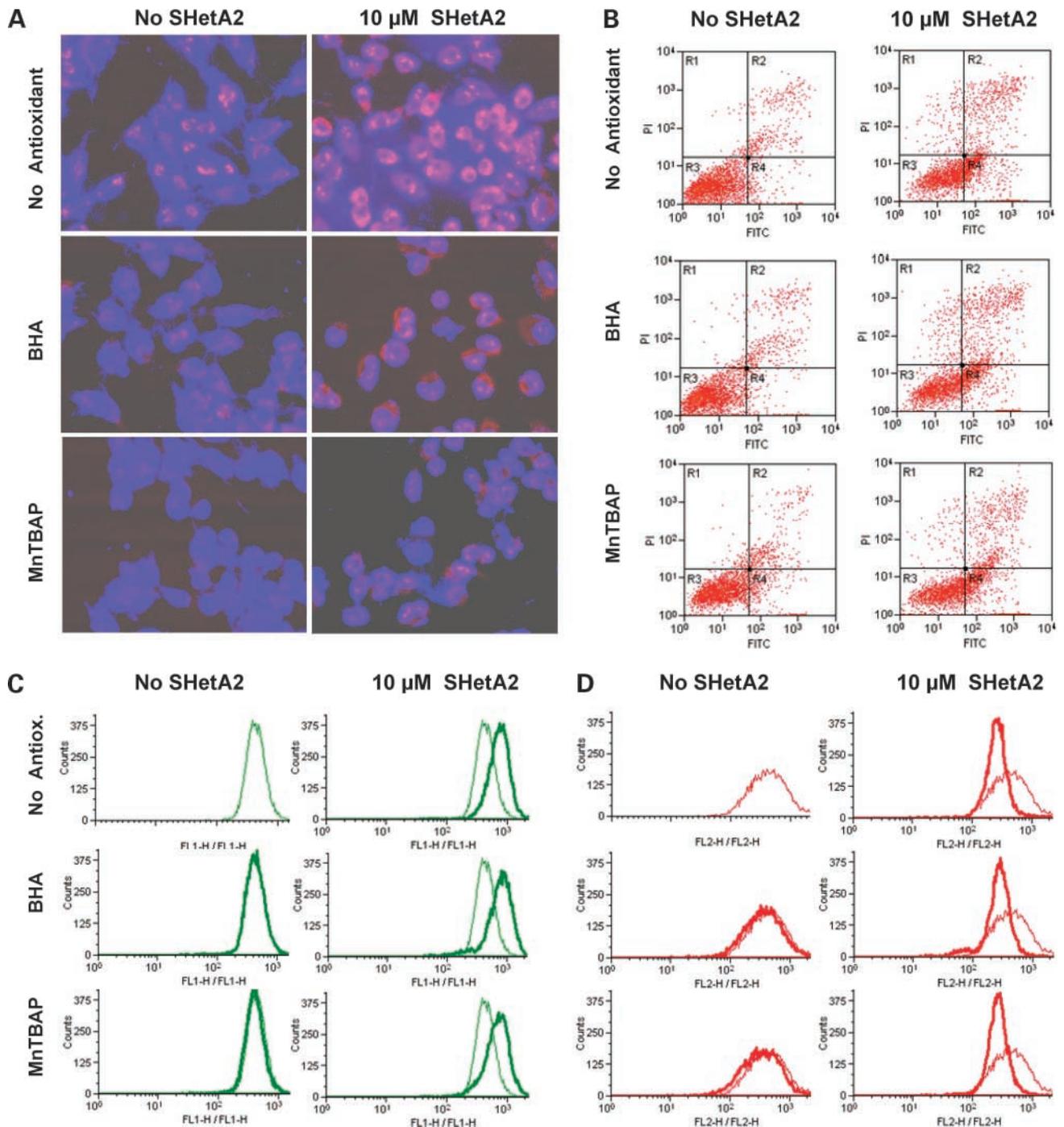
Figure 5. Antioxidants inhibit SHetA2-generated ROS levels but not apoptosis or mitochondrial effects. Cultures were pre-treated with BHA (50 μmol/L) or MnTBAP (100 μmol/L) for 16 h before treatment with SHetA2 or solvent only. **A,** photomicrographs (×40) of cells stained with MitoSOX (*red*) and Hoechst (*blue*) dyes showed that both BHA (50 μmol/L) and MnTBAP (100 μmol/L) can effectively inhibit SHetA2-induced intracellular ROS levels. **B,** dual flow cytometric analysis of Annexin V-FITC and propidium iodide (*PI*) staining. Living cell populations are clustered in the R3 quadrant; cells in early apoptosis are in the R4 quadrant; late apoptotic/necrotic cells are in the R2 quadrant. JC-1 dye and flow cytometry were used to measure mitochondrial swelling (**C**) and (**D**) membrane potential. *Light curve,* cultures grown in the absence of SHetA2; *dark curve,* cultures treated with SHetA2.

of SHetA2 to induce apoptosis in the presence of the antioxidants BHA or MnTBAP was measured. MnTBAP is a metalloporphyrin catalytic antioxidants that mimics Mn superoxide dismutase and scavenges a wide range of ROS, including superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxyl radicals (10). BHA is a synthetic phenol that can scavenge ROS by donating a hydrogen atom to oxygen radicals (11). Dihydroethidium dye was used to

confirm the quenching of SHetA2-generated ROS at the cellular level. When exposed to superoxide radicals, the predominant ROS in mitochondria in living cells, dihydroethidium is oxidized to ethidium, which binds DNA in the nucleus and produces a strong red fluorescence. It is possible, however, that this dye does not penetrate the mitochondria sufficiently to identify small inner-mitochondrial ROS levels that may be involved. Therefore, an additional dye called MitoSOX that is selectively targeted to the mitochondria was used. Once in the mitochondria, MitoSOX is oxidized by superoxide and exhibits bright red fluorescence upon binding to mitochondrial nucleic acids. Parallel cultures treated with various combinations of the antioxidants and SHetA2 were evaluated with dihydroethidium, MitoSOX, JC-1, and Annexin V-FITC/propidium iodide dyes. Dihydroethidium confirmed that the antioxidants inhibited cellular superoxide generation by Flex-Hets at the concentrations used in this experiment (data not shown). MitoSOX staining showed that the antioxidants suppressed mitochondrial ROS generation (Fig. 5A), at concentrations that did not prevent apoptosis (Fig. 5B), mitochondrial swelling (Fig. 5C), or loss of mitochondrial membrane integrity (Fig. 5D), induced by SHetA2 in A2780 ovarian cancer cells. The inability of BHA or MnTBAP antioxidants to prevent SHetA2 mitochondrial effects was also observed in two additional ovarian cancer cell lines (SK-OV-3 and Caov-3; Fig. 6), further supporting the conclusion that ROS generation is not the mechanism by which SHetA2 induces mitochondrial swelling.

Role of RNA and Protein Synthesis in the Flex-Het Mechanism

To evaluate whether SHetA2 directly targets the mitochondria or exerts effects through the regulation of gene expression, the role of RNA and protein synthesis in the SHetA2 mitochondria effects were tested in the presence of actinomycin D and cycloheximide, to inhibit RNA and protein synthesis, respectively (Fig. 7). Actinomycin D alone induced mitochondrial swelling (compare light curve in top and second rows); thus, the ability to modulate the induced swelling of SHetA2 could not be evaluated. Cycloheximide did not affect mitochondrial swelling as a single agent, nor did it prevent mitochondrial swelling induced by SHetA2, indicating that protein synthesis is not required for SHetA2 induced mitochondrial swelling. The involvement of protein synthesis in SHetA2 regulation of growth and apoptosis was tested at concentrations of 1, 20, and 40 μmol/L cycloheximide, but none of these treatments altered the level of growth inhibition or apoptosis induced by SHetA2 (data not shown).

SHetA2 Differentially Alters Levels of Bcl-2 and Bcl-x_L

The Bcl-2 family of proteins are potential molecular targets for Flex-Hets because they reside in the mitochondrial membrane and regulate permeabilization leading to release of cytochrome *c* and induction of apoptosis. The effects of SHetA2 on expression of the antiapoptotic Bcl-2 and Bcl-x_L and the proapoptotic Bax proteins were evaluated by Western blot (Fig. 8). Proteins were extracted from the A2780 ovarian cancer cell line and the primary endometrial cultures treated with SHetA2 or vehicle control over a series of time points. SHetA2 caused decreased Bcl-2 and Bcl-x_L expression in the cancer cell line in contrast to the increased expression of these proteins in the primary non-cancerous culture. The amount of the decreased expression levels in cancer cells and increased expression levels in non-cancer cells increased with time and was maximal at 16 h. The proapoptotic Bax protein was unaffected in either cell type.
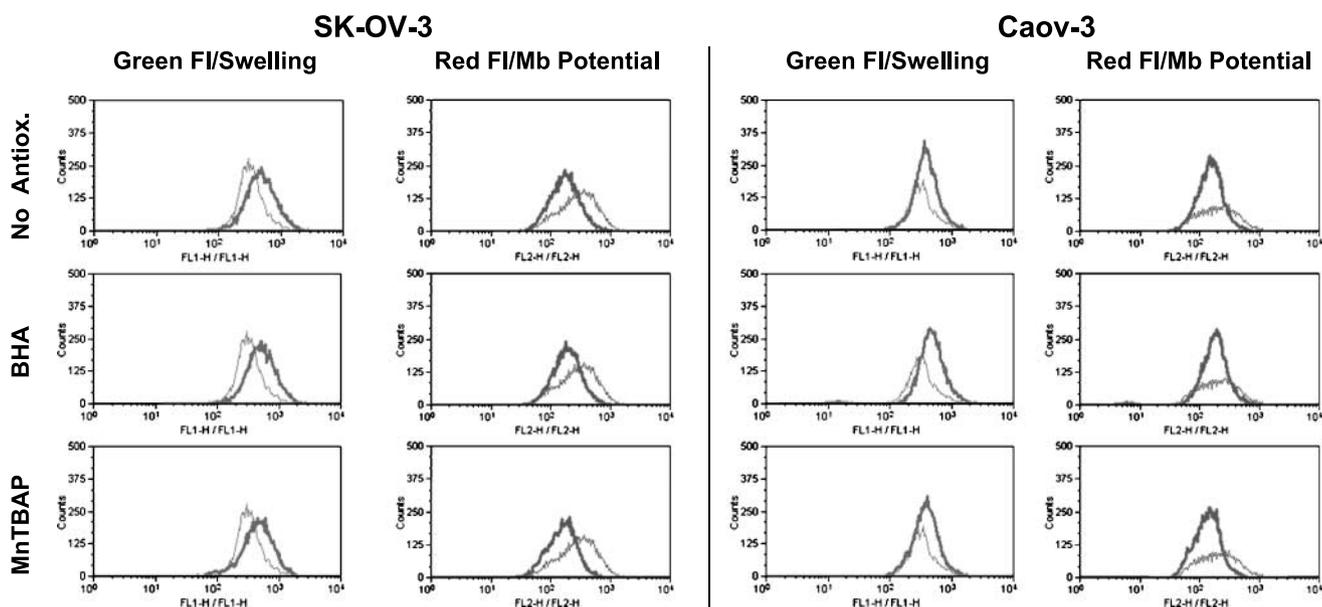

Figure 6. Studies with additional ovarian cancer cell lines confirm that ROS generation is not required for mitochondrial effects. SK-OV-3 and Caov-3 ovarian cancer cell lines were treated as described in Fig. 5. JC-1 dye and flow cytometry were used to measure mitochondrial swelling and membrane potential. *Light curve,* cultures grown in the absence of SHetA2; *dark curve,* cultures treated with SHetA2.
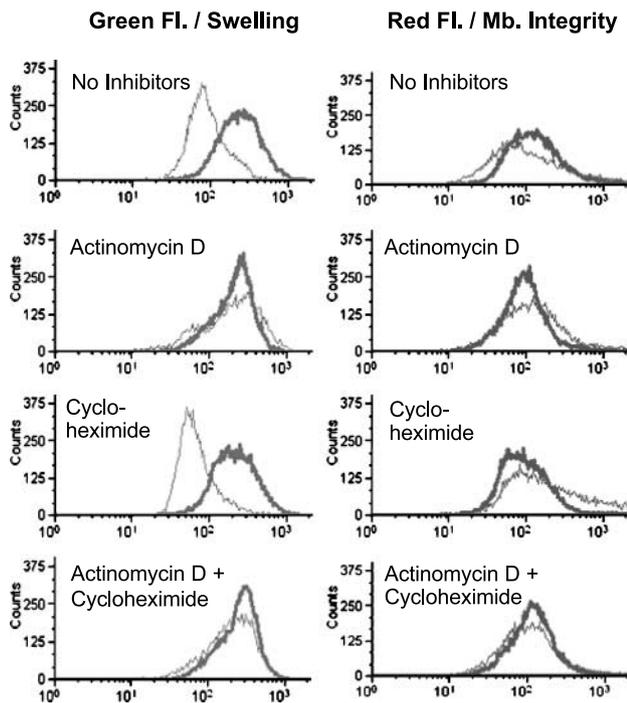
Green Fl. / Swelling **Red Fl. / Mb. Integrity**

Figure 7. Protein synthesis is not required for SHetA2 action. OVCAR-3 cultures were treated with 10 μmol/L SHetA2 for 12 h in the presence or absence of cycloheximide and/or actinomycin D and then stained with JC-1 and analyzed by flow cytometry. *Light curve,* untreated cultures; *dark curve,* cultures treated with SHetA2.

Discussion

The results of this study indicate that direct targeting of mitochondria by Flex-Hets is responsible for the differential effects of these drugs in cancer versus normal cells. In cancer cells, the effects of Flex-Hets on growth and apoptosis are associated with rapid swelling of mitochondria. In normal cells, the increased resistance to Flex-Hets is associated with minimal to no mitochondrial swelling. The opposite effects of SHetA2 levels of antiapoptotic Bcl-x_L and Bcl-2 proteins in cancer versus non-cancer cells provides a potential molecular mechanism for the differential effects of Flex-Hets on mitochondria and apoptosis. The differential effects of the lead Flex-Het, SHetA2 on growth and apoptosis were observed in ovarian cancer cell

lines in comparison with ovarian surface epithelial cells (HOSE and IOSE80) derived from separate donors and the same organ site as the cancer cell lines. This shows that the previous observation of differential effects in ovarian cancer cell lines in comparison with normal endometrial cells is a general phenomenon and not due to the organ site from which the cells were derived. SHetA2 sensitivity of all of the cell lines in the National Cancer Institute human tumor cell line panel (3) and increased resistance of four different types of non-cancerous cultures (HOSE, IOSE80, normal endometrial, and gingival fibroblast cultures) in this study suggest that the greater sensitivity of cancer cells is due to a property inherent to the cancer state and not a coincidence of the cell lines or cultures tested.

To determine if ROS caused or were a consequence of the mitochondrial effects, ROS generated by SHetA2 were quenched with two different antioxidants: BHA and MnTBAP. Dihydroethidium dye was used to confirm quenching of cellular ROS, and MitoSox dye was used to confirm quenching of ROS inside the mitochondria. Antioxidant conditions that suppressed increased cellular or mitochondrial ROS generation did not attenuate mitochondrial effects or apoptosis by SHetA2, indicating that ROS generation is a consequence and not a cause of Flex-Het action on mitochondria. The inability of BHA and MnTBAP to prevent mitochondrial swelling or loss of membrane potential was observed in three different ovarian cancer cell lines.

The rapid induction of mitochondrial swelling within 30 min of Flex-Het treatment suggest that the mitochondria is the direct target of Flex-Hets; however, it is possible that rapid regulation of gene and protein expression within 30 min could contribute to the Flex-Het mechanism of mitochondrial swelling. In this study, the inability of cycloheximide to prevent mitochondrial swelling ruled out a role for regulation of protein synthesis in the mechanism.

The molecular mechanism of Flex-Het effects on mitochondria in cancer cells includes upsetting the balance of antiapoptotic to proapoptotic Bcl-2 proteins by decreasing the levels of antiapoptotic Bcl-x_L and Bcl-2, without affecting the proapoptotic Bax levels. The role of these proteins in regulating outer mitochondrial membrane permeabilization and the inconsistent effects of Flex-Hets on inner mitochondrial membrane (IMM) potential suggest that the mechanism of Flex-Hets targets the outer mitochondrial membrane and not the IMM. For instance,
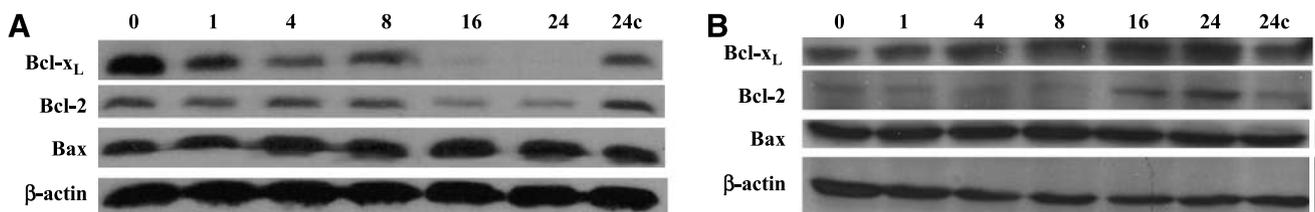

Figure 8. Western blotting of antiapoptotic and proapoptotic proteins of the Bcl-2 family in A2780 (**A**) and D1 cells (**B**). A2780 or D1 cultures were treated with 10 μmol/L SHetA2 for the indicated times. CytoBuster protein extraction reagent and protease inhibitor were used to extract protein. The result showed that the ratio of antiapoptotic (Bcl-x_L and Bcl-2) and proapoptotic (Bax) proteins of the Bcl-2 family was significantly decreased in SHetA2 treated A2780 ovarian cancer cells (**A**) but not in normal endometrial cells (**B**).

A2780, SK-OV-3, and Caov-3 consistently exhibited loss of membrane potential in response to SHetA2, whereas OVCAR-3, which was equally sensitive to the drug, did not. Outer mitochondrial membrane permeabilization induced by the Bax or Bak proteins seems to be a separate, but integrated, mechanism from IMM permeabilization (12). Therefore, outer mitochondrial membrane permeabilization induced by Flex-Hets may lead to IMM permeabilization, depending on the state of the mitochondria.

Pharmaceuticals that modulate mitochondrial activity can cause severe side effects (13). Arsenic trioxide, a Food and Drug Administration–approved drug for the treatment of acute promyelocytic leukemia (14), induces apoptosis by disruption of the IMM potential (15). The main side effects of this drug include prolongation of the cardiac QT interval, torsade de pointes, congestive heart failure, hypokalemia, hypomagnesemia, and leukocytosis (16). Lonidamine, a derivative of indazole-3-carboxylic acid, also induces apoptosis through IMM potential (17). The most frequent toxicities of this drug are gastrointestinal and hematologic side effects (18). In this study, the greater resistance of normal ovarian, endometrial, and oral fibroblast cells to SHetA2 indicates that the potential for these side effects is reduced for Flex-Hets. To date, however, no toxicities have been noted for SHetA2 in animal models (3, 5). The efficacy, toxicity, pharmacokinetics, and formulation of SHetA2 were evaluated in the National Cancer Institute's Rapid Access to Intervention Development (RAID) program (Application 196, Compound NSC 726189) and now in the Rapid Access to Preventive Intervention Development (RAPID) program. The RAID pharmacokinetic studies showed that micromolar concentrations of SHetA2 can be achieved in mice (19), indicating that concentrations sufficient to differentially induce apoptosis in cancer cells over normal cells can be targeted in clinical trials.

The additional activity of differentiation induced by Flex-Hets can be observed at concentrations below 3 μmol/L. At higher concentrations, the cells are killed by apoptosis. These concentrations, however, are relevant to short-term (2–3 days) monolayer culture assays. When the cells are grown within a three-dimensional extracellular matrix in organotypic cultures, and the drugs are administered at 1 μmol/L for 4 days to 2 weeks, both differentiation and apoptosis can be observed within the same cultures (1). What determines if a cell will undergo differentiation or apoptosis in response to 1 μmol/L Flex-Hets has yet to be determined. Microarray analysis identified specific patterns of gene expression that occur over time in cancer cells undergoing differentiation and apoptosis. Validation of these identified genes is being pursued in an effort to identify the molecular mechanism of Flex-Het differentiation and apoptosis and have already led to the discovery that Flex-Hets inhibit angiogenesis.[5]

In conclusion, the results of this study indicate that the mechanism of differential apoptosis activity by Flex-Hets on cancer versus normal cells involves direct targeting of the mitochondria, independent of ROS generation and protein synthesis. The Bcl-2 and Bcl-x_L proteins seem to be associated with the mechanism of the differential effects on cancer versus normal cells. The differential effects on mitochondria in cancer versus normal cells reduce the potential for induction of severe side effects.

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