

Kahalalide F, a new marine-derived compound, induces oncosis in human prostate and breast cancer cells

Yajaira Suárez,^{1,2} Laura González,^{1,2}
Ana Cuadrado,^{1,2} Maite Berciano,³
Miguel Lafarga,³ and Alberto Muñoz²

¹Pharma Mar S.A., Madrid, Spain; ²Instituto de Investigaciones Biomédicas "Alberto Sols", Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Madrid, Spain; and ³Departamento de Anatomía y Biología Celular, Unidad de Biomedicina asociada al Instituto de Investigaciones Biomédicas (CSIC), Universidad de Cantabria, Santander, Spain

Abstract

Kahalalide F (KF) is a novel antitumor drug of marine origin under clinical investigation. KF showed a potent cytotoxic activity against a panel of human prostate and breast cancer cell lines, with IC₅₀ ranging from 0.07 μM (PC3) to 0.28 μM (DU145, LNCaP, SKBR-3, BT474, MCF7). Importantly, nontumor human cells (MCF10A, HUVEC, HMEC-1, IMR90) were 5–40 times less sensitive to the drug (IC₅₀ = 1.6–3.1 μM). KF cytotoxicity did not correlate with the expression level of the multidrug resistance MDR1 and of the tyrosine kinase HER2/NEU, and only slightly by the anti-apoptotic BCL-2 protein. KF action was triggered rapidly by short pulse treatments (15 min caused 50% maximum cytotoxicity). Neither a general caspase inhibitor (Z-VAD-fmk) nor transcription or translation inhibitors (actinomycin D, cycloheximide) blocked KF action. Flow cytometry analysis revealed that KF induced neither cell-cycle arrest nor apoptotic hypodiploid peak. Using mitochondrial (JC-1)- and lysosomal (LysoTracker Green, Acridine Orange)-specific fluorophores, we detected loss of mitochondrial membrane potential and of lysosomal integrity following KF treatment. Confocal laser and electron microscopy revealed that KF-treated cells underwent a series of profound alterations including severe cytoplasmic swelling and vacuolization, dilation and vesiculation of the endoplasmic reticulum, mitochondrial damage, and plasma membrane rupture. In contrast, the cell nucleus showed irregular clumping of chromatin into small, condensed masses, while chromatin disappeared from other nuclear domains, but the nuclear envelope was preserved and no DNA degradation was detected. Together, these data indicate

that KF induces cell death via oncosis preferentially in tumor cells. (Mol Cancer Ther. 2003;2:863–872)

Introduction

Kahalalide F (KF) (C₇₅H₁₂₄N₁₄O₁₆, M_r = 1476; Fig. 1A) is one of the families of natural depsipeptides isolated from the Hawaiian herbivorous marine mollusk *Elysia rufescens* (1, 2). Like other kahalalides, it is probably a secondary metabolite synthesized by the mollusk from peptides produced by a diet of the green algae *Bryopsis pennata*. KF has potent cytotoxic activity *in vitro* against cell lines from solid tumors including prostate, breast and colon carcinomas, neuroblastoma, chondrosarcoma, and osteosarcoma (3–6). In animal models *in vivo*, KF has also shown activity against human prostate cancer xenografts (4). Cytotoxicity against human tumor specimens has been seen with breast, colon, non-small cell lung, and ovarian carcinomas (7). Two phase I clinical trials in adult patients bearing advanced prostate cancer and pretreated solid tumors have identified the recommended dose for two different schedules (once a week and five consecutive days every 3 weeks) (8). Dose-limiting toxicity was elevation of aminotransferases in both trials. Recently, KF has entered into a phase II clinical trial.

The mechanism of KF action is mostly unknown. KF is a National Cancer Institute COMPARE-negative compound, which indicates that its cytotoxicity might be related to a unique mode of action. Human HeLa cervical cancer cells and monkey COS-1 fibroblasts treated with KF became dramatically swollen and developed large vacuoles that appeared to be consequence of changes in lysosomal membranes (9). However, the nature and characteristics of the cytotoxic effect of KF and mechanisms of cell death induction remain to be elucidated.

Clearly, the clinical use of KF will greatly benefit from the study of specificity and molecular determinants of its activity. Here we examine the action of KF in human prostate and breast cancer cells. KF rapidly induces a strong cytotoxic effect with IC₅₀ in the submicromolar range. We have examined the involvement of *BCL-2*, *MDR1*, and *HER2/NEU* genes in the mechanism of cytotoxicity of this compound. In addition, by flow cytometry and confocal and electron microscopy, we have characterized the cell death process induced by KF treatment. Together, our data characterize KF as a potent antitumor drug that induces predominantly necrotic cell death.

Materials and Methods

Cell Lines and Reagents

Human prostate cancer PC3, DU145, and LNCaP cells were grown in RPMI 1640 supplemented with 10% FCS, breast cancer SKBR-3, BT474, MCF7 and MDA-MB-231 cells, colon cancer LoVo cells, and IMR90 human diploid

Received 5/21/03; revised 6/16/03; accepted 6/19/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Grant support: Grant SAF01-2291 from Ministerio de Ciencia y Tecnología of Spain.

Requests for reprints: Alberto Muñoz, Instituto de Investigaciones Biomédicas "Alberto Sols", Arturo Duperier, 4, E-28029 Madrid, Spain. Phone: 34-91-585-4451; Fax: 34-91-585-4401. E-mail: amunoz@iib.uam.es

fibroblasts (supplied by Dr. M. Serrano, Centro Nacional de Biotecnología, Madrid, Spain) were grown in DMEM supplemented with 10% FCS and 1 mM glutamine (all from Life Technologies, Inc.-Invitrogen, Paisley, Scotland). MCF10A normal human mammary epithelial cells (supplied by Dr. D. Salomon, National Cancer Institute, Bethesda, MD) were cultured in a mixture (1:1) of DMEM:F12 media supplemented with 5% horse serum, 20 ng/ml human recombinant EGF (Upstate Biotechnology, Lake Placid, NY) and 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite (all from Life Technologies-Invitrogen), and 0.5 µg/ml hydrocortisone. Endothelial HMEC-1 (dermal microvascular) and HUVEC (umbilical cord) cells (supplied by Dr. B. Jiménez, Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, Spain) were grown respectively in DMEM supplemented with 10% FCS and M199 medium supplemented with 20% FCS, 10 ng/ml human recombinant EGF (Upstate Biotechnology), 20 mM HEPES, 10 µg/ml hydrocortisone, and 100 µg/ml heparin. MCF7/HER2 cells expressing an exogenous HER2/NEU gene (10) were supplied by Dr. A. Pandiella (Centro de Investigación del Cáncer, Salamanca, Spain); MDA-MB-231/BCL-2 cells expressing an exogenous BCL-2 gene (11) were supplied by Dr. A. López (Instituto López-Neyra de Biomedicina y Parasitología, Granada, Spain); and Lovo/DOX cells overexpressing the MDR1 gene were donated by Dr. Lola García-Grávalos (Pharma Mar S.A., Madrid, Spain), and were cultured as their parental cells.

KF is manufactured by Pharma Mar. Stock solutions were freshly prepared in dimethyl sulfoxide:ethanol (1:1) and diluted in the cell culture to final concentrations as indicated. Actinomycin D, cycloheximide, chloroquine, and valinomycin were from Sigma Chemical Co. (St. Louis, MI), and the Z-VAD-fmk general caspase inhibitor was from Calbiochem-Merck Biosciences (Darmstadt, Germany).

Antibodies

Primary antibodies used were: mouse monoclonal antibodies antihuman BCL-2 (DAKO, Copenhagen, Denmark), anti-MDR1 (Neo Markers, Fremont, CA), anti-HER2/NEU (Oncogene Research Products, Boston, MA) and anti-pan-Histone (clone H11-4, Roche Diagnostics, Indianapolis, IN); and rabbit polyclonal antibodies anti-calreticulin (Calbiochem-Merck) and anti-lamin B (Dr. S. Georgatos, University of Crete, Greece). As secondary antibodies, we used goat anti-mouse or anti-rabbit antibodies conjugated with FITC or Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PN).

Flow Cytometry Analysis

We used a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon ion laser. For cell-cycle analysis, asynchronous cells (500,000/ml) were cultured in the presence of different concentrations of KF. On incubation, the cells were harvested and washed in PBS, fixed in 70% cold ethanol for 30 min at -20°C, washed again in PBS, and incubated for 1 h in PBS containing 100 µg/ml RNase (Sigma) and 50 µg/ml propidium iodide (PI) (Sigma). Ten thousand events per

sample were acquired for data analysis using CELL-QUEST software by selective gating to exclude doublet cells. The results of the DNA content were modeled using ModFit (VERITY Software House, Inc., Topsham, ME). Lysosomal integrity was analyzed by the LysoTracker Green-uptake assay. The acidotropic dye LysoTracker Green (Molecular Probes, Eugene, OR), which accumulates in intact lysosomes, was diluted in culture media. Cells were cultured in the presence or absence of KF for the desired period of time and then medium containing 75 nM LysoTracker Green was added and cells were incubated for a further 30 min at 37°C. Cells were harvested, washed in PBS, and resuspended in 1 ml PBS. Green fluorescence of 10,000 events per sample was acquired for the data analysis using the FL1 channel. In some instances, cells were labeled for a further 15 min in the presence of 15 µg/ml PI to allow discrimination between live (PI non-permeant, no red fluorescence) and dead cells (PI permeant, high red fluorescence). For Acridine Orange (AO) uptake assays, cells were incubated as above but exposed to 5 µg/ml AO (Sigma) for 15 min at 37°C. AO is a lysototropic base and metachromatic fluorochrome exhibiting red fluorescence at high concentrations, when retained in its charged form in intact lysosomes. Red lysosomal fluorescence of 10,000 events per sample was determined by flow cytometry using FL3 channel. To measure mitochondrial membrane potential ($\Delta\Psi_m$), mitochondria were selectively probed with potential-sensitive JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) (Molecular Probes). JC-1 was stocked as 1 mg/ml in DMSO and freshly diluted with complete culture medium. After treatments, cells were harvested and incubated with medium containing JC-1 (10 µg/ml) for 10 min. Finally, cells were washed and resuspended in 1 ml PBS for flow cytometry analysis. JC-1 exhibits $\Delta\Psi_m$ -dependent accumulation in mitochondria, indicated by a shift in its fluorescence emission from green to red due to the formation of J-aggregates. Mitochondrial depolarization is so indicated by a decrease in red/green fluorescence ratio.

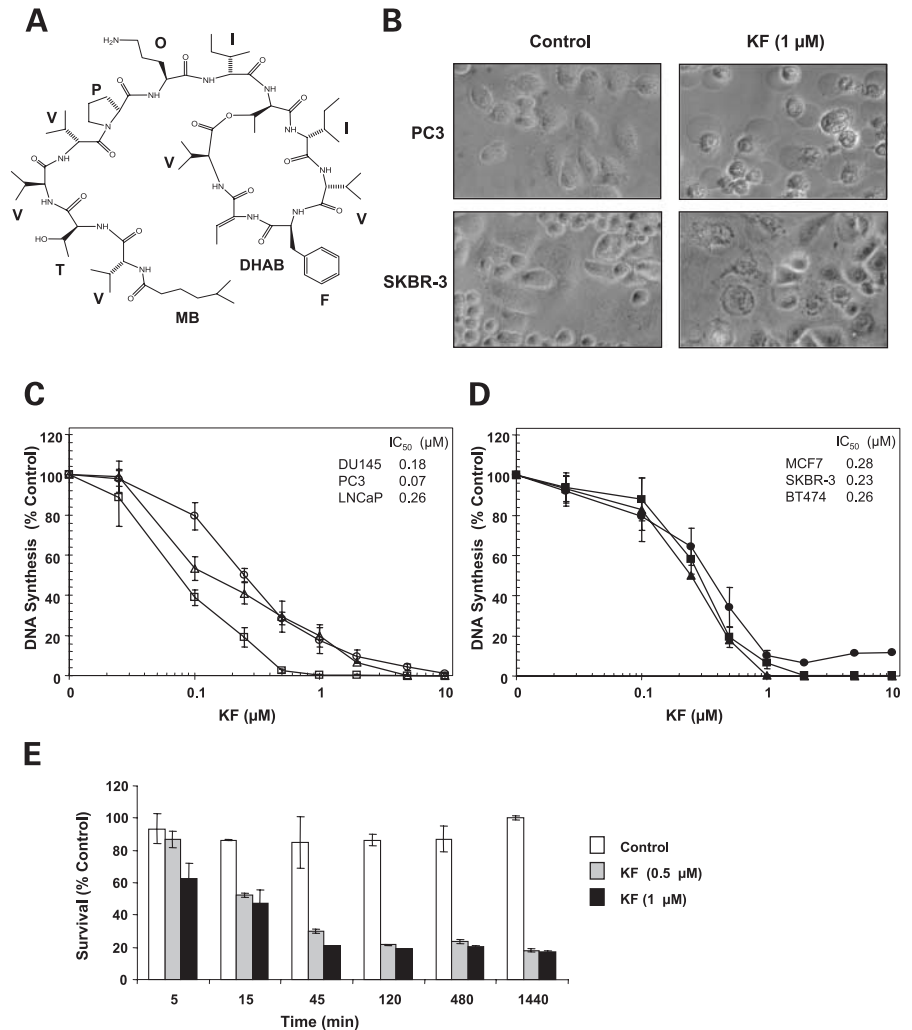
DNA Synthesis Measurements

Thirty thousand cells were seeded in 24-well dishes. After overnight incubation, cells were treated or not with KF in normal growth medium and pulsed with 1 µCi/ml [³H]thymidine (Amersham-Pharmacia Biotech, Piscataway, NJ) for 4 h at the indicated times posttreatment. At the end of the labeling period, the medium was removed and the cells were rinsed twice in PBS and fixed with chilled 10% trichloroacetic acid for 10 min. Trichloroacetic acid was then removed and the monolayers were washed in ethanol and air-dried at room temperature for 20 min. Thereafter, precipitated macromolecules were dissolved in 500 µl of 0.5 N NaOH-0.1% SDS and 450 µl of each sample were diluted in 5 ml of scintillation solution OptiPhase HighSafe (Wallac Scintillation Products). Radioactivity was measured on a 1209 RackBeta counter (LKB Wallac, Turku, Finland).

Crystal Violet Staining

To estimate cell mass, medium was removed and 24-well

Figure 1. Structure and effect of KF in human prostate and breast cancer cells. **A**, structure of KF containing a lateral chain formed by residues of threonine (T)-isoleucine (I)-ornithine (O)-proline (P)-valine (V)-valine-threonine-valine, and 5-methylbenzoic acid (MB) and a cyclized region containing phenylalanine (F)-valine-isoleucine-threonine-valine and dehydroaminobutyric acid (DHAB). **B**, phase-contrast micrographs of PC3 and SKBR-3 cells treated for 1 h with either vehicle (Control) or 1 μM KF. **C** and **D**, effect of KF on DNA synthesis in a panel of human prostate and breast cancer cell lines, respectively. Cells were cultured and incubated with KF at the indicated doses for 24 h and the level of DNA synthesis was estimated as described in "Materials and Methods" as percentage over untreated control cells and corresponds to the mean \pm SD of triplicates of three experiments. Prostate cancer cells: PC3 (\square), DU 145 (\triangle), and LNCaP (\circ). Breast cancer cells MCF-7 (\bullet), SKBR3 (\blacktriangle), and BT-474 (\blacksquare). IC_{50} values for each line are shown. **E**, effect of pulse treatments with KF on the PC3 cell viability. Cells were treated with KF (open bars, untreated cells; gray bars, 0.5 μM KF; black bars, 1 μM KF) for the indicated periods of time and then washed and incubated in a drug-free medium. Monolayers were stained following the crystal violet procedure 24 h after initial KF treatment and survival was estimated as percentage of staining over that of untreated cultures and corresponds to the mean \pm SD of triplicates of three experiments.



dishes were washed in PBS, fixed with 1% glutaraldehyde for 15 min, washed again in PBS twice, and stained with 200 μl 0.1% aqueous crystal violet for 20 min. Dishes were rinsed 4 times in tap water and allowed to dry. One hundred microliters 10% acetic acid were added and the content of each well was mixed before reading A_{595} .

DNA Gel Electrophoresis

After treatments, cells were harvested and washed in PBS. Cellular DNA was then extracted after overnight incubation at 37°C in a lysis buffer containing 25 mM EDTA, 100 mM NaCl, 0.5 mg/ml proteinase K, 0.5% SDS, and 10 mM Tris-HCl (pH 8.0). DNA was precipitated by 2 vol of 100% ethanol and 1/10 vol of 3 M sodium acetate. After centrifugation, samples were resuspended and incubated for 1 h at 37°C in TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.5) with 50 $\mu\text{g}/\text{ml}$ RNase. Supernatants were extracted with a 1:1 mixture of phenol:chloroform, precipitated as above and resuspended in TE buffer. Electrophoresis was carried out in 2% agarose gels in TAE buffer containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Gels were examined under UV light and photographed.

Immunostaining and Confocal Laser Microscopy Analysis

For indirect immunofluorescence, culture cells were washed twice in PBS, fixed with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 10 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature. Before immunostaining, fixed culture cell samples were sequentially incubated with 0.1 M glycine in PBS for 30 min, 1% BSA in PBS for 15 min, and 0.01% Tween 20 in PBS for 5 min. For immunolabeling, cells were rinsed in PBS containing 0.05% Tween 20 (PBS-Tw), incubated for 2 h at room temperature with primary antibodies diluted in PBS, washed in PBS-Tw, and incubated for 45 min with the appropriate secondary antibodies conjugated to FITC, or Texas Red (Jackson ImmunoResearch Laboratories). Some samples were counterstained with PI for the cytochemical demonstration of nucleic acids. Finally, the coverslips were mounted in VectaShield (Vector Laboratories, Peterborough, United Kingdom) and sealed with nail polish. Confocal microscopy was performed with a Bio-Rad

MRC-1024, using excitation wavelengths of 488 nm (for FITC) and 543 nm (for Texas Red). Each channel was recorded independently and pseudocolor images were generated and superimposed. Images were processed by the Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA).

Electron Microscopy Analysis

PC3 and SKBR-3 cells were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer for 15 min at room temperature. The cells were scraped, transferred to an Eppendorf tube, and centrifuged for 10 min. The pellets were washed with 0.1 M cacodylate buffer, postfixed in 2% osmium tetroxide, dehydrated in acetone, and embedded in araldite (Durcupan; Fluka, Switzerland). Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips EM-208 electron microscope operated at 60 kV.

Results

KF Showed Potent, Rapid Cytotoxic Activity against Human Prostate and Breast Cancer Cells

First, we studied the effect of KF on the proliferation of a panel of human prostate (PC3, DU145, LNCaP) and breast (SKBR-3, MCF7, BT474) cancer cell lines by estimating the level of DNA synthesis 24 h after treatment with different doses. Under the phase-contrast microscope, cells showed a dramatic morphological change soon after KF exposure, with intense swelling followed by cytoplasmic disintegration but no visible nuclear breakdown (Fig. 1B). In all cases, a dose-dependent inhibition of DNA synthesis was found, with IC_{50} around 0.2 μM (Fig. 1C) except for the androgen-independent PC3 line, which was more sensitive (IC_{50} = 0.07 μM). Wash-out experiments showed that short-pulse exposure to KF was sufficient to induce cytotoxicity, because 45 min treatment reduced cell survival as much as 24 h treatment, and 15 min treatment had a half-maximal effect (Fig. 1D).

Next, we examined whether KF cytotoxicity depends on the level of expression of three genes that are relevant for the action of many antitumor drugs: (a) *BCL-2*, an apoptosis inhibitor oncogene (12); (b) *MDR1*, encoding the P-glycoprotein responsible for extrusion of and resistance to multiple drugs, the overexpression of which is associated with poor clinical outcome (13, 14); and (c) *HER2/NEU* oncogene coding for a plasma membrane tyrosine kinase, the overexpression of which is linked to high malignancy and poor patient prognosis in breast cancer and other neoplasias (Ref. 15 and references therein). Only slight differences in KF sensitivity were found between MDA-MB-231 breast cancer cells overexpressing *BCL-2* protein (MDA-MB-231/*BCL-2*; IC_{50} = 0.74 μM) and the empty vector-transfected cells (IC_{50} = 0.39 μM) (Fig. 2A). Expression of an exogenous *MDR1* gene did not significantly alter the activity of KF against LoVo colon cancer cells (Fig. 2B). Likewise, KF action was also independent of the level of *HER2/NEU* expression, because no differences in drug sensitivity were found between parental and *HER2/NEU*-overexpressing MCF7

breast cancer cells (Fig. 2C). This result was confirmed by the finding that cell lines expressing either high (SKBR-3, BT-474), intermediate (LNCaP), or low (DU145, MCF7, MDA-MB-231) levels of *HER2/NEU* protein in Western blots (not shown) showed no significant differences in KF sensitivity (Fig. 1B). Moreover, PC3 cells showing highest sensitivity to KF express only moderate levels of *HER2/NEU* protein (not shown).

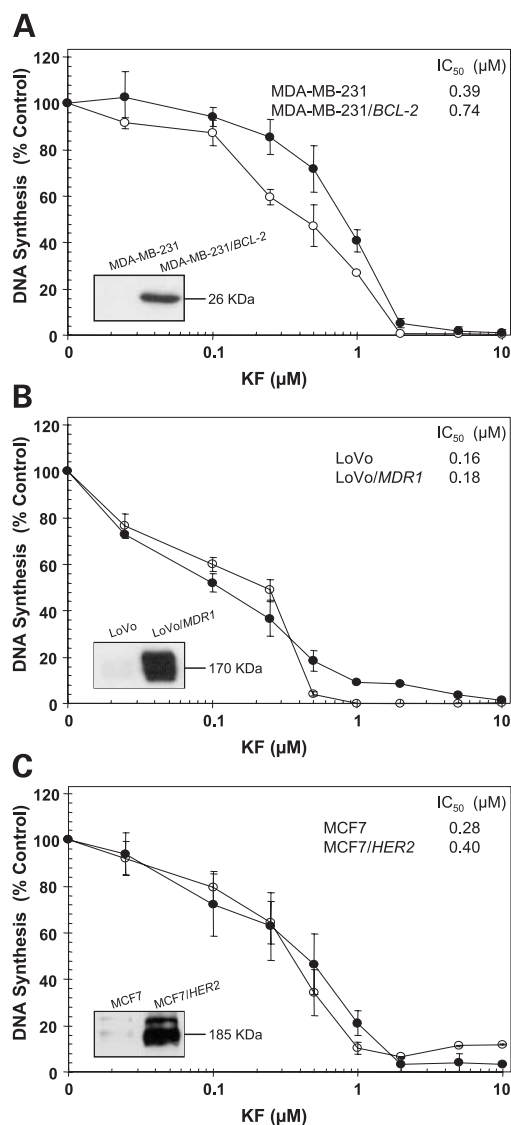


Figure 2. Effect of relevant cancer genes on KF cytotoxic action. Effect of *BCL-2* (A), *MDR-1* (B), and *HER2/NEU* (C) gene overexpression on KF cytotoxicity. Cells were incubated with KF at the indicated doses for 24 h and the level of DNA synthesis was estimated as described in "Materials and Methods." Values corresponding to percentage over untreated control cells are shown (mean \pm SD of triplicates obtained in two independent experiments). Solid circles, cells overexpressing the exogenous gene; empty circles, parental vector-transfected MDA-MB-231 (A), LoVo (B), and MCF7 (C) cells. IC_{50} values for each cell type are shown. Insets show the level of expression of the encoded *BCL-2*, *MDR1*, and *HER2/NEU* proteins analyzed by Western blotting using appropriate specific antibodies.

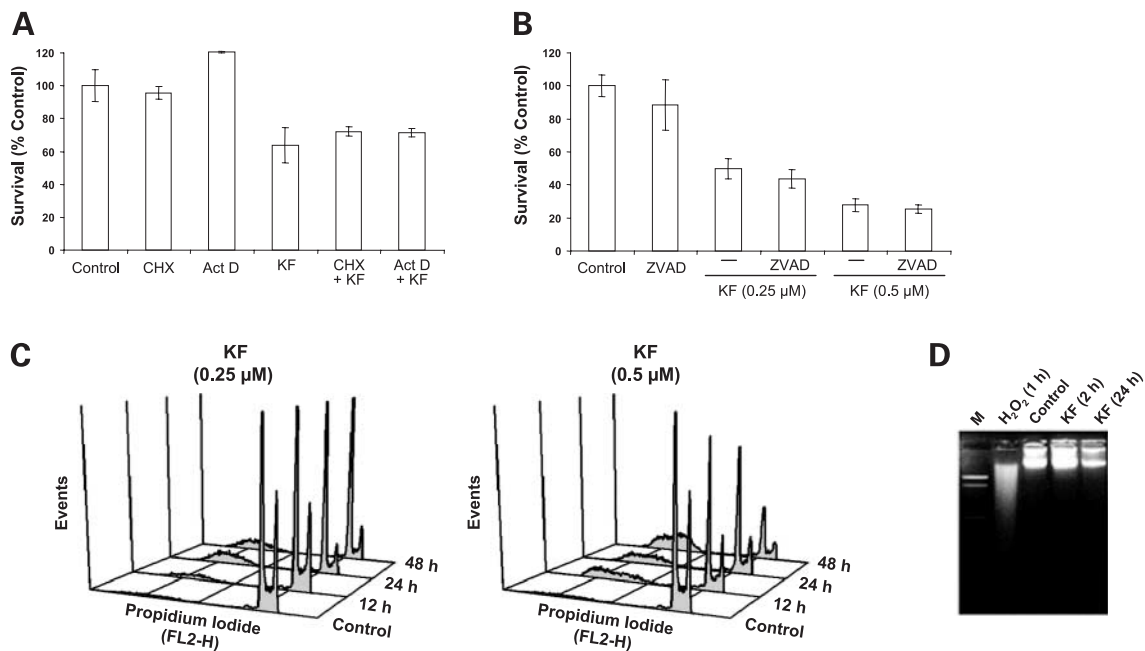


Figure 3. Characterization of KF-induced cell death. **A**, KF cytotoxicity is independent of gene expression. PC3 cells were pretreated or not for 2 h with 8 $\mu\text{g/ml}$ cycloheximide or 2 $\mu\text{g/ml}$ actinomycin D before treatment with the indicated doses of KF for additional 6 h. Cell viability was estimated by the crystal violet staining method. Values correspond to mean \pm SD of triplicates obtained in three independent experiments. **B**, KF cytotoxicity does not require caspase activity. PC3 cells were pretreated or not with 40 μM Z-VAD-fmk for 2 h before KF treatment for additional 22 h. Cell viability was estimated as in **A**. **C**, KF does not induce cell cycle arrest. PC3 cells were incubated in the absence or presence of 0.25 or 0.5 μM KF and at the indicated times were harvested and stained with PI as described in "Materials and Methods." Data were acquired using selective gating to exclude doublets. **D**, KF does cause DNA degradation. PC3 cells were treated with 0.5 μM KF for 2 or 24 h. DNA integrity was assayed after KF treatment by conventional DNA agarose gel electrophoresis as described in "Materials and Methods." Parallel cultures treated with 1% H_2O_2 for 1 h were used as control.

KF-induced Cytotoxicity Does Not Require Gene Expression or Caspase Activity and Does Not Cause Cell Cycle Arrest or DNA Degradation

To assess whether gene expression is required for KF action, we treated PC3 cells in the presence of inhibitors of transcription (actinomycin D) or translation (cycloheximide). At periods (6 h) causing no toxicity *per se*, neither of these two inhibitors affected the cytotoxic activity of KF (Fig. 3A). Likewise, pretreatment (24 h) with the general caspase inhibitor Z-VAD-fmk did not inhibit KF action, suggesting that caspases are not involved in its mechanism of action (Fig. 3B). In these same cells, the analysis by flow cytometry revealed a time-dependent increase in highly autofluorescent cell debris (as evidenced by forward and side-scatter properties) following KF exposure. In contrast, no hypodiploid peak (a hallmark of apoptosis) or arrest in any phase of the cell cycle was found. Furthermore, DNA analysis by gel electrophoresis showed that KF treatment did not induce the ladder-type of DNA degradation that is characteristic of apoptosis (Fig. 3D).

KF Causes Loss of Mitochondrial Membrane Potential and Alteration of Lysosomal Function

Mitochondrial depolarization is a common event on treatment of cells with antitumor drugs (16). Using the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria, we found that KF induced a rapid and transient loss of membrane potential ($\Delta\Psi_m$)

which is detected as soon as 2 h on KF addition by an increase in green and a decrease in red fluorescence emission (Fig. 4). At 24 h posttreatment, abundant non-specific highly auto-fluorescent cell debris was found, concomitantly with a decrease in the cell population undergoing mitochondrial depolarization, suggesting the induction by KF of $\Delta\Psi_m$ loss in a proportion of cells that are prone to die.

Putative effects of KF on lysosomes were studied using the fluorescent acidotropic probes LysoTracker and AO, which are retained in intact organelles. KF exposure led to a dose-dependent appearance of "pale cells" (deficient in intact lysosomes or with damaged lysosomes) that showed very low LysoTracker Green or AO fluorescence, respectively, indicating alteration of the lysosomal membrane permeability (Fig. 5A). Double labeling with LysoTracker Green and PI showed that the population of cells with reduced LysoTracker Green fluorescence incorporated a high-level PI, which corresponds to dying cells with heavily altered plasma membrane permeability (Fig. 5B). Emphasizing its effect on lysosomes, KF also enhanced the effect of the lysosomotropic agent chloroquine on the organelle integrity (Fig. 5C).

KF Induces a Rapid and Profound Alteration of Cell Architecture

First, we examined the alterations caused by KF in PC3 cells by immunostaining with specific antibodies followed by confocal laser microscopy analysis. KF drastically

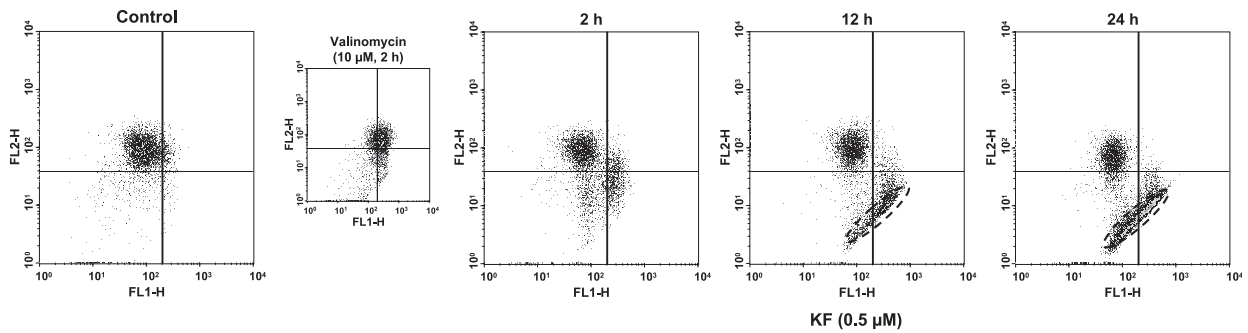


Figure 4. Effect of KF on the mitochondrial membrane potential ($\Delta\Psi_m$). Flow cytometry analysis of PC3 cells incubated with 0.5 μM KF for 2, 12, or 24 h and then stained with JC-1 as described in "Materials and Methods." Cells treated with 100 μM valinomycin for 2 h were used as control of depolarization. *Abscissas*, FL1-H values (green fluorescence, log scale); *ordinates*, FL2-H values (orange-red fluorescence, log scale). Dashed region indicates non-specific highly auto-fluorescent cell debris. Data correspond to a representative experiment out of three that gave similar results.

affected the ER, causing microvesiculation and disruption of the normal organization of the ER network observed in untreated cells using an anti-calreticulin antibody (Fig. 6, right panels). In contrast to what is usually observed during apoptosis, the staining with an anti-lamin B antibody showed that the integrity of nuclear envelope is preserved in KF-treated cells (Fig. 6, left panels). Likewise, the use of an anti-pan-Histone antibody revealed that KF induces chromatin condensation in small irregular aggregates distributed throughout the nucleus, but not the formation of peripheral, sharply delineated masses of condensed chromatin or apoptotic bodies, which are characteristic structural features of apoptosis (Fig. 6, middle panels).

To gain further insight into alterations caused by KF, we performed electron microscopy studies. In prostate PC3 and also in breast SKBR-3 cells, KF exposure leads to a rapid cytoplasmic swelling, with dramatic disruption of the normal architecture (Fig. 7). This includes extensive vesiculation of cytoplasmic organelles, dilation of the endoplasmic reticulum (ER) elements, cytoskeletal degradation, and rupture of the plasma membrane (Fig. 7, C, D, G, and H). In addition, mitochondria of PC3 cells were severely damaged, showing increased matrix density, some swelling of cristae, and wrapping by a single rough ER cistern, suggesting autophagy (Fig. 7B). In the case of the SKBR-3 cells, some mitochondria had increased electron

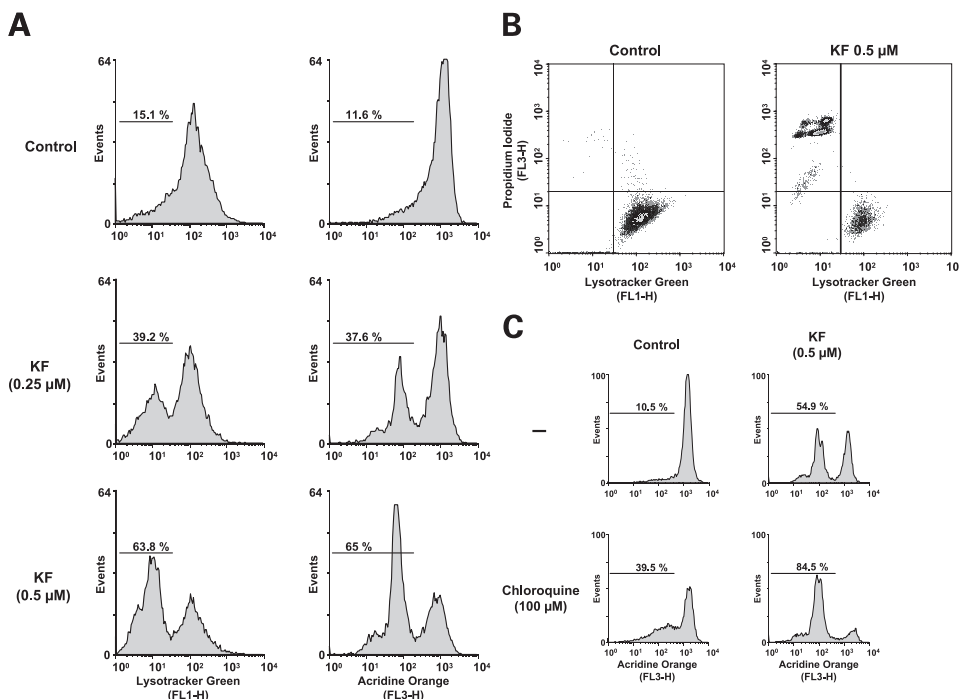


Figure 5. Effect of KF on lysosomal integrity. **A**, flow cytometry analysis of PC3 cells incubated with the indicated doses of KF for 4 h and then stained with LysoTracker Green or AO as described in "Materials and Methods." Intensity of lysosomal fluorescence and the percentages of cells with decreased green and red fluorescence ("pale cells") are shown. **B**, to examine lysosomal integrity and cell viability, PC3 cells were incubated with 0.5 μM KF for 4 h and stained with LysoTracker Green and PI as described in "Materials and Methods." *Abscissas*, FL1-H values (LysoTracker green fluorescence, log scale); *ordinates*, FL2-H values (PI orange-red fluorescence, log scale). Lower right quadrants, live cells with no damaged lysosomes (PI⁻/LysoTracker Green⁺); upper left quadrants, dead cells with damaged lysosomes (PI⁺/LysoTracker Green⁺). **C**, effect of combined treatment with KF and chloroquine on lysosome integrity. PC3 cells were incubated in the absence or presence of 100 μM chloroquine for 16 h and then treated or not with 0.5 μM KF for additional 4 h and then stained with AO. Intensity of lysosomal fluorescence and the percentages of cells with decreased red fluorescence ("pale cells") are shown.

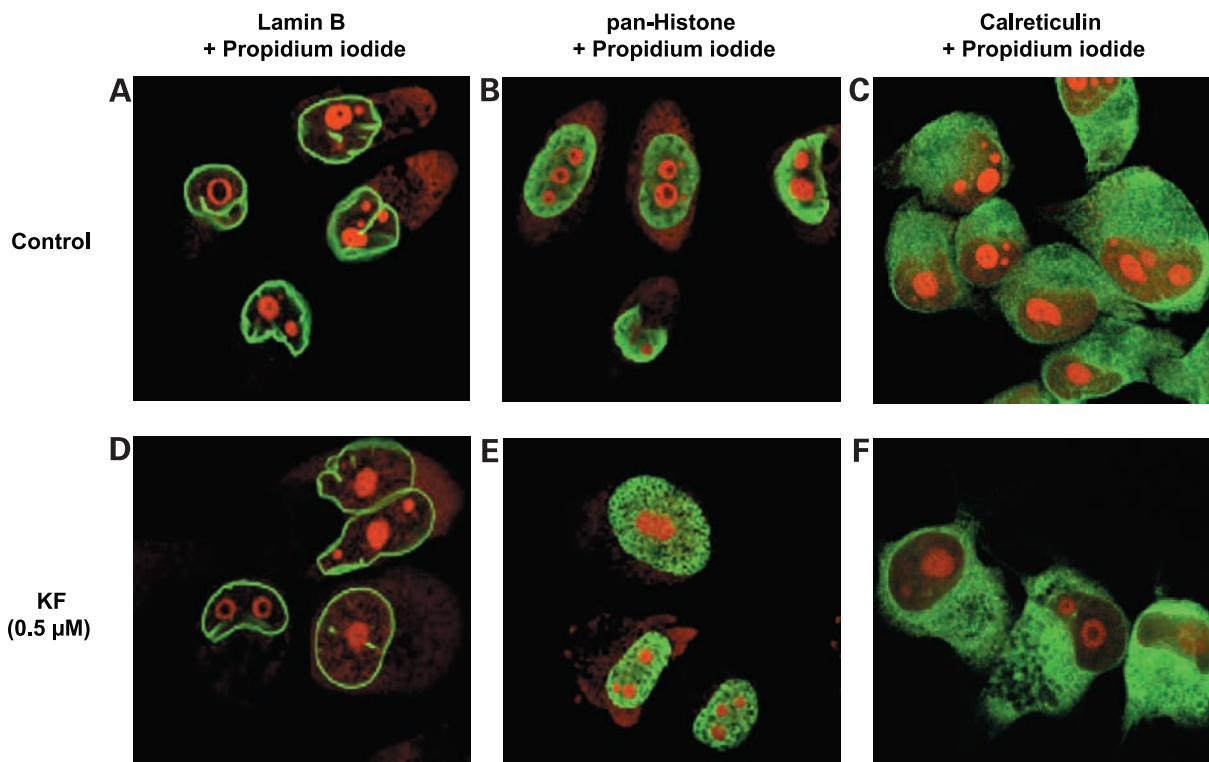


Figure 6. Confocal microscopy analysis of PC3 cells treated with KF. Confocal laser microscopy images representative of control (A–C) and KF-treated (D–F) PC3 cells. Double staining with an anti-lamin B antibody (green) and PI (red; A, D), anti-pan-Histone antibody (green) and PI (D, E), and anti-calreticulin antibody (green) and PI (C, F). Immunofluorescence labeling of lamin B, a marker of the nuclear dense lamina, demonstrates that the nuclear envelope is well preserved in PC3 cells exposed to KF, although these cells exhibit slight nuclear swelling and reduction of nuclear membrane invaginations in comparison with control cells (A and D), pan-Histone immunofluorescence illustrates the distribution of chromatin. The peripheral band of higher intensity observed in control cells (B), corresponds to the preferential distribution of heterochromatin in this nuclear region. KF treatment induces the condensation of chromatin in numerous small aggregates distributed throughout the nucleus (E). Calreticulin immunostaining, a marker of the ER, demonstrates a diffuse cytoplasmic labeling with a delicate network of higher staining intensity standing out (C). This pattern is dramatically altered in KF-treated PC3 cells due to the extensive vesiculation of the cytoplasm (F). Note the preservation of nucleoli, which appear counterstained with PI (red), in cells exposed to KF (D–F).

density and showed partial association with short cisterns of rough ER (Fig. 7F), but typical images of autophagy were not found. Cell nuclei were less affected in both PC3 and SKBR-3 cell lines. Main nuclear changes include irregular clumping of chromatin and the appearance of cleared nuclear domains free of chromatin (Fig. 7C). The nuclear envelope was preserved until the final stages of necrosis when cellular disintegration takes place (Fig. 7, C and F–H). Taken together, these results indicated that KF treatment causes a primary and preferential disruption of the cytoplasm architecture, while the cell nucleus is less affected at the early stages of drug exposure.

KF Has Reduced Toxicity on Normal Cells

To test whether KF has a preferential action on tumor cells *versus* normal cells that could provide a therapeutic window *in vivo*, we studied its inhibitory effect on DNA synthesis in four normal, non-tumor cell lines: IMR90, MCF10A, HMEC-1, and HUVEC (Fig. 8). As compared with the panel of tumor cell lines previously analyzed (Fig. 1), KF had a diminished inhibitory effect on normal cells. The comparison of the respective IC_{50} showed that normal cells were 5–40 times less sensitive to KF (Figs. 1 and 8).

Discussion

In this work we have examined the action of KF, a novel antitumor agent of marine origin under clinical investigation, in human prostate and breast cancer cells. KF showed potent cytotoxic activity against both types of tumor cells. This action is triggered very rapidly and does not require continuous presence of the drug. Soon after exposure to KF, cells initiate a death process including marked swelling and a series of profound morphological alterations that affect many cytoplasm organelles and the plasma membrane. These features are typical of the process named oncosis, a term describing the progression of cellular events leading to necrotic cell death (16–18). The cellular architecture is greatly affected as early as 1–3 h after KF treatment, and the integrity of crucial organelles such as mitochondria, ER, or lysosomes is severely compromised. In contrast, the nuclear structure is preserved, and no drastic alteration of chromatin or DNA degradation is detected. Under the electron microscope, a proportion of damaged mitochondria in PC3 cells appeared surrounded by rough endoplasmic cisterns, suggesting that KF may to some extent induce mitochondrial autophagy. The rapid

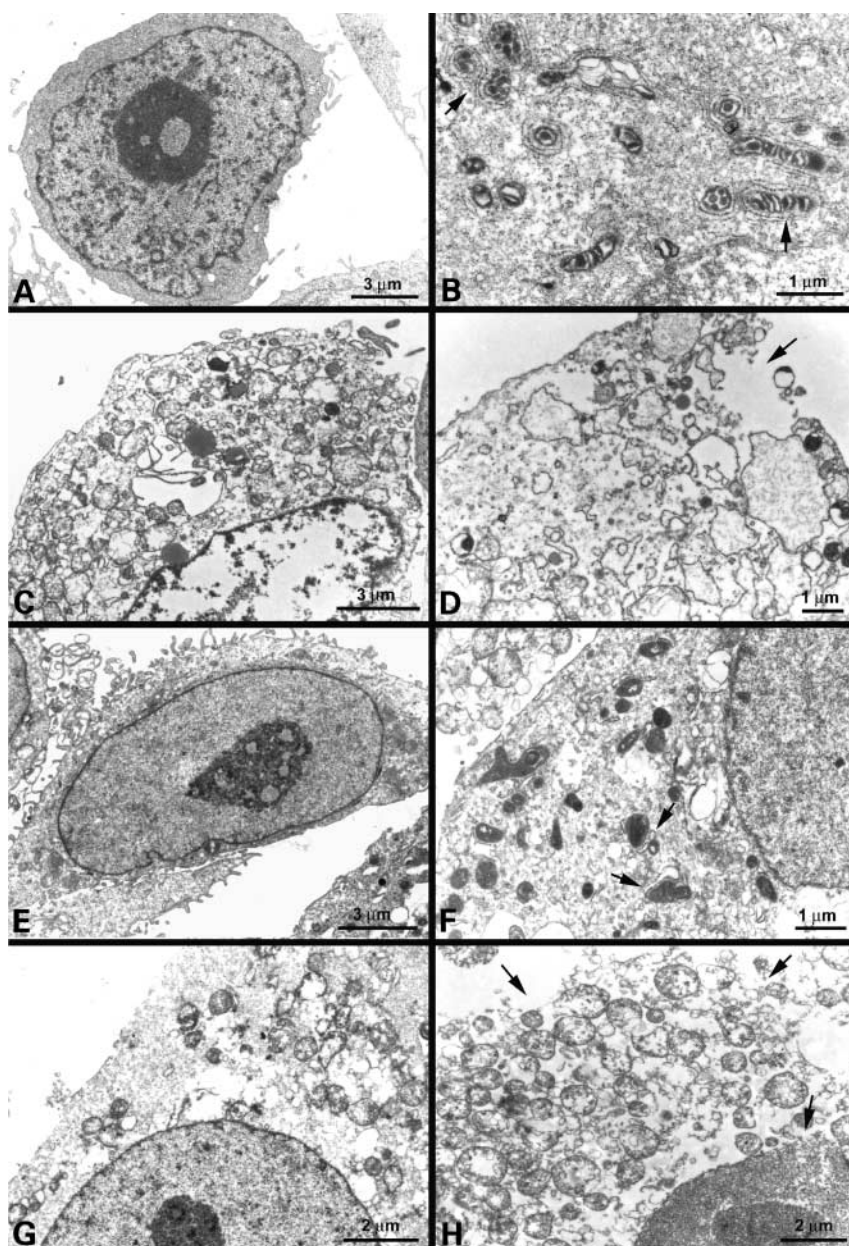


Figure 7. Electron microscopy analysis of PC3 and SKBR-3 cells treated with KF. Electron micrographs of untreated, control (**A**) and KF-treated PC3 (**B–D**) cells, and control (**E**) and KF-treated (**F–H**) SKBR-3 cells. **A**, untreated PC3 cells show a large cell nucleus with numerous small aggregates of condensed chromatin distributed throughout the nuclear interior and a prominent nucleolus. The cytoplasm appears as a perinuclear band. **B**, KF induces the formation of autophagic vacuoles (autophagosomes) in PC3 cells. All autophagosomes contain mitochondria with increased matrix density and appear enclosed by a cistern of rough ER (*arrows*). **C**, severe disruption of the nuclear and cytoplasmic organization by KF. Note the cellular swelling, the chromatin clumping, and the prominent vesiculation of the cytoplasmic membrane-bound organelles. **D**, advanced stage of necrotic cell death in PC3 cells treated with KF. Note the cytoplasmic swelling, the dilation of the ER elements, and the rupture of the plasma membrane (*arrows*). **E**, control untreated SKBR-3 cell with a disperse pattern of chromatin configuration and a prominent nucleolus. The cytoplasm exhibits abundant mitochondria. **F**, initial swelling and vesiculation of the cytoplasm in a SKBR-3 cell treated with KF. Mitochondria show increased electron density and some of them appear partially surrounded by short cisterns of rough ER (*arrows*). **G**, extensive cytoplasmic swelling and vesiculation in a SKBR-3 cell after 60 min of KF treatment. **H**, final stage of necrotic cell in a SKBR-3 cell treated with KF. The swollen cytoplasm contains remnants of vesiculated organelles. Note the rupture of both plasma membrane and nuclear envelope (*arrows*). This cytoplasmic degradation is accompanied by an overall chromatin condensation.

cytoplasmic swelling and the severe dysfunction and subsequent disintegration of endomembranes and plasma membrane lead to necrotic cell death. The alterations found suggest changes in the osmotic balance of the cell, possibly as a result of damage to cellular membranes, which we are currently investigating.

Our data indicate that KF has a novel mechanism of action among antitumor drugs, which is in line with the negative result of the NCI COMPARE program. Most anticancer drugs have been considered to induce apoptosis as a result of nuclear DNA damage, activation of membrane death receptors, or cytoskeletal alteration (Refs. 19–22 and references therein). Apoptosis is defined as an active, fixed-pathway process of cell death characterized by cell shrinking, cytoplasmic condensation, ladder DNA

degradation, and nuclear fragmentation resulting in the formation of apoptotic bodies. In contrast, oncosis was considered a passive death process, a consequence of the unspecific toxicity of life-incompatible physical conditions or chemical poisons (16). Today, however, apoptosis and oncosis are no longer believed to be mutually exclusive processes (18, 23) and, moreover, apoptosis is probably not the only, or even the main mechanism by which tumor cells are killed by anticancer drugs or radiotherapy (24). It is accepted that apoptosis and oncosis share certain mechanisms and alterations such as loss of mitochondria permeability and membrane potential, and that cell fate is mostly due to the intensity and duration of the death signal and the cell genetic and metabolic status (16). Likewise, caspase-independent apoptosis has been described, which

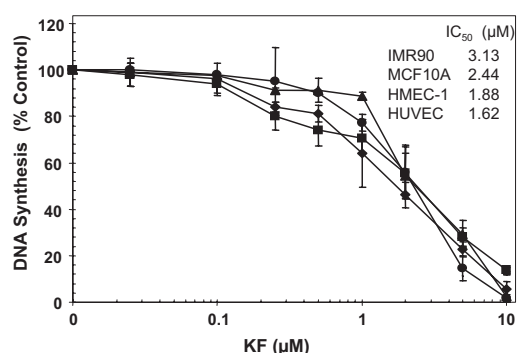


Figure 8. Effect of KF on normal cell proliferation. Cells were incubated with KF at the indicated doses for 24 h and the level of DNA synthesis was estimated as described in "Materials and Methods." Mean \pm SD obtained in triplicates of three independent experiments are shown. Cells: IMR90 (\blacktriangle), MCF10A (\bullet), HMEC-1 (\blacksquare), and HUVEC (\blacklozenge). IC₅₀ values are shown.

challenges the hypothesis that caspases activity is a definitive hallmark of the apoptotic process (25, 26). Our data indicate that KF action does not involve caspase activity but a rapid and severe destruction of cell organelles such as mitochondria and also ER and lysosomes. This is in line with the recent idea that the ER, Golgi apparatus, and lysosomes also play important roles in the integration and sensing of cellular damage in the initiation of cell death (27). We observed that KF induces lysosome damage, which agrees with a previous study in HeLa and COS-7 cells (9), and may contribute to necrotic cell death.

In addition to the morphological changes (cell swelling instead of shrinking), several biochemical results support the induction by KF of oncosis as opposed to apoptosis. First, the fact that no gene expression is required for KF to induce its effects. Second, that this family of proteases is not involved in KF action, as shown by the use of a general caspases inhibitor. Third, the absence of effects of KF on the cell cycle, because it does not induce the sub-G₀ hypodiploid peak that is characteristic of apoptosis or any phase-specific arrest. Fourth, the lack of DNA ladder degradation. And fifth, overexpression of the antiapoptotic *BCL-2* gene does not significantly affect KF action.

The finding that high levels of expression of *BCL-2*, *MDR1*, or *HER2/NEU* genes did not significantly reduce the cytotoxic activity of KF is relevant, given that overexpression of these genes is common in human carcinogenesis and involved in the pattern of primary and acquired resistance to anticancer treatment. These results also support a non-apoptotic mode of action (as discussed above for *BCL-2*), and imply differences in the mechanism of action, and probably of resistance, with most available antitumor drugs. The lack of effect of *MDR1* overexpression raises the possibility of combined treatments of KF with other drugs and of the use of KF as a second-line treatment in patients developing resistance to conventional drugs mediated by P-glycoprotein. Additionally, KF may be useful against tumors overexpressing *HER2/NEU* that are usually linked to high malignancy and bad prognosis.

The comparable activity of KF on the two series of cancer cell lines studied indicates that its action is mostly unrelated to p53 status (wild-type in MCF7 and LNCaP; mutated in PC3, DU145, MDA-MB-231, BT474, SKBR-3), and expression of androgen receptor (LNCaP are positive; PC3 and DU145 are negative) and estrogen receptor (MCF7 and BT474 are positive; MDA-MB-231 and SKBR-3 are negative).

Oncosis induction is sometimes thought to be a less specific mechanism than apoptosis, which may indicate potential side effects based on the features of necrotic cell death. Against this idea, the comparative study done in normal and tumor human cells shows that KF has a preferential cytotoxic activity on neoplastic *versus* normal cells. This result agrees with the promising toxicity data obtained in preclinical (28) and phase I clinical trials (8, 29).

Our results show that KF is a very potent cytotoxic drug that displays an unusual and interesting mode of action. We have characterized several key features of KF action in human prostate and breast cancer cells at the cellular and molecular level, which constitute the basis for additional in-depth studies and analysis of KF effects on other types of cancer cells. More studies are needed to fully characterize the antitumor action of KF including the identification of its primary target(s), the precise molecular mechanism of cytoplasmic organelle damage, or the basis for its preferential effect on tumor cells.

Acknowledgments

We thank those who appear in "Materials and Methods" for providing us with cells or antibodies, Robin Rycroft for his valuable assistance in the preparation of the English manuscript, and Teresa Martínez for her expert technical assistance.

References

- Hamann, M. T. and Scheuer, P. J. Kahalalide F: a bioactive depsipeptide from the sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J. Am. Chem. Soc.*, **115**: 5825–5826, 1993.
- Hamann, M. T., Otto, C. S., and Scheuer, P. J. Kahalalides: bioactive peptides from a marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. *J. Org. Chem.*, **61**: 6594–6660, 1996.
- Jimeno, J. M., Faircloth, G., Cameron, L., Meely, K., Vega, E., Gómez, A., Fernández Sousa-Faro, J. M., and Rinehart, K. Progress in the acquisition of new marine-derived anticancer compounds: development of ecteinascidin-743 (ET-743). *Drugs Future*, **21**: 1155–1165, 1996.
- Faircloth, G. T., Grant, W., Smith, B., Supko, J., Brown, A., Geldof, A., and Jimeno, J. Preclinical development of Kahalalide F, a new marine compound selected for clinical studies. *Proc. Am. Assoc. Cancer Res.*, **41**: 600, 2000.
- Faircloth, G. T., Smith, B., Grant, W., Jimeno, J., García-Grávalos, L., Scotto, K., and Shtil, A. Selective antitumor activity of Kahalalide F, a marine-derived cyclic depsipeptide. *Proc. Am. Assoc. Cancer Res.*, **42**: 213, 2001.
- Shao, L., Weissbach, L., Faircloth, G. T., Chabner, B. A., and Hornicek, F. J. *In vitro* anti-proliferative effect on sarcoma cells of ET-743 and other marine chemotherapeutics. *Proc. Am. Assoc. Cancer Res.*, **42**: 203, 2001.
- Medina, L. A., Gómez, L., Cerna, C., Faircloth, G., Yochmowitz, M., and Weitman, S. Investigation of the effects of Kahalalide F (PM92102) against human tumor specimens taken directly from patients. *Proc. Am. Assoc. Cancer Res.*, **42**: 213, 2001.
- Ciruelos, C., Trigo, T., Pardo, J., Paz-Ares, L., Estaun, N., Cuadra, C., Domínguez, M., Marín, A., Jimeno, J., and Izquierdo, M. A phase I

- clinical and pharmacokinetic (PK) study with Kahalalide F (KF) in patients (pts) with advanced solid tumors (AST) with a continuous weekly (W) 1-hour iv infusion schedule. 14th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, Frankfurt, Germany, November 19–22nd, 2002. Abstract 95. *Eur. J. Cancer*, **38** (Suppl. 7): S33, 2002.
9. García-Rocha, M., Bonay, P., and Avila, J. The antitumor compound Kahalalide F acts on cell lysosomes. *Cancer Lett.*, **99**: 43–50, 1996.
 10. Esparís-Ogando, A., Díaz-Rodríguez, E., Montero, J. C., Yuste, L., Crespo, P., and Pandiella, A. Erk5 participates in neuregulin signal transduction and is constitutively active in breast cancer cells over-expressing ErbB2. *Mol. Cell. Biol.*, **22**: 270–285, 2002.
 11. Ruiz-Ruiz, C., Muñoz-Pinedo, C., and López, A. Interferon- γ treatment elevates caspase-8 expression and sensitises human breast tumor cancer cells to a death receptor-induced mitochondria-operated apoptotic program. *Cancer Res.*, **60**: 5673–5680, 2000.
 12. Reed, J. C. Dysregulation of apoptosis in cancer. *J. Clin. Oncol.*, **17**: 2941–2953, 1999.
 13. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.*, **39**: 361–998, 1999.
 14. Tan, B., Piwnicka-Worms, D., and Ratner, L. Multidrug resistance transporters and modulation. *Curr. Opin. Oncol.*, **12**: 450–458, 2000.
 15. Lane, H. A., Beuvink, I., Motoyama, A. B., Daly, J. M., Neve, R. M., and Hynes, N. E. ErbB2 potentiates breast tumor proliferation through modulation of p27Kip1-Cdk2 complex formation: receptor overexpression does not determine growth dependency. *Mol. Cell. Biol.*, **20**: 3210–3223, 2000.
 16. Lemasters, J. J., Nieminen, A-L., Tian, Q., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A., and Herman, B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta*, **1366**: 177–196, 1998.
 17. Majno, G. and Joris, I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.*, **146**: 3–15, 1995.
 18. Van Cruchten, S. and Van den Broeck, W. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat. Histol. Embryol.*, **31**: 214–223, 2002.
 19. Thompson, C. B. Apoptosis in the pathogenesis and treatment of disease. *Science* (Washington DC), **267**: 1456–1462, 1985.
 20. Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**: 539–542, 1994.
 21. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. *Cell*, **108**: 153–164, 2002.
 22. Herr, I. and Debatin, K-M. Cellular stress response and apoptosis in cancer therapy. *Blood*, **98**: 2603–2614, 2001.
 23. Farber, E. Ideas in pathology: programmed cell death: necrosis *versus* apoptosis. *Mod. Pathol.*, **7**: 605–609, 1994.
 24. Finkel, E. Does cancer therapy trigger cell suicide? *Science* (Washington DC), **286**: 2256–2258, 1999.
 25. Candé, C., Cohen, I., Daugas, E., Ravagnan, L., Larochette, N., Zamzami, N., and Kroemer, G. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie*, **84**: 215–222, 2002.
 26. Lockshin, R. A. and Zakeri, Z. Caspase-independent cell deaths. *Curr. Opin. Cell Biol.*, **14**: 727–733, 2002.
 27. Ferri, K. F. and Kroemer, G. Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.*, **3**: E255–E263, 2001.
 28. Brown, A. P., Morrissey, R. L., Faircloth, G. T., and Levine, B. S. Preclinical toxicity studies of Kahalalide F, a new anticancer agent: single and multiple dosing regimens in the rat. *Cancer Chemother. Pharmacol.*, **50**: 333–340, 2002.
 29. Schellens, J. H., Rademaker, J. L., Horenblas, S., Meinhardt, W., Stokvis, E., De Reijke, T. M., Jimeno, J. M., López Lázaro, L., López Martín, J. A., and Beijnen, J. H. Phase I and pharmacokinetic study of Kahalalide F in patients with advanced androgen refractory prostate cancer. Proceedings of the 2001 AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, October 29th to November 2nd 2001. *Clin. Cancer Res.*, **7** (Suppl.): 42 (Abs. 207), 2001.