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The mechanism of action of Kahalalide F: Variable cell permeability in human hepatoma cell lines

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Abstract

Kahalalide F (KF) is a small natural peptide that showed activity *in vitro* and *in vivo*. The dose-limiting toxicity in clinical trials was transaminitis. We investigated the cytotoxicity of KF in cell lines from breast, ovary, prostate and colon cancers, but focused on hepatoma cell lines, performing mechanistic studies in HepG2 (IC50 = 0.3 μM) and PLC/PRF/5C (IC50 = 5 μM). Following KF exposure, HepG2 cells demonstrated profound ATP depletion, associated with cell swelling and cell blebbing, and increased permeability to propidium iodide (PI), annexin V (AV) and release of lactate dehydrogenase (LDH). PLC/PRF/5C cells retained their cell structure, but were permeable to PI and, following exposure to high concentrations of KF, to AV. The pattern of cell permeability is similar to maitotoxin, another small cytotoxic peptide, but the differential effects on the cell membrane induced by KF in HepG2 and PLC/PRF/5C suggest specific interactions with membranes or proteins. This could lead to better drug design aimed at exploiting the potential for cell selectivity.
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1. Introduction

Natural products and their derivatives have traditionally been a common source of drugs. Among them, cytotoxic peptides are synthesised by a large number of organisms from plants to animals. They can interact with lipid bilayers to alter the membrane structure leading to cell death (for review [1]). Their potentially new mechanisms of action may therefore be a novel approach to cancer chemotherapy. Although combinatorial chemistry is extensively used for drug discovery, the diversity of natural products may be more advantageous. ''Nature-optimised'' peptides may in fact be a better starting point for drug design than synthetic molecules [2]. The identification of the pharmacological properties of small cytotoxic peptides can therefore be used to optimise their structures and to improve their clinical utility.

Kahalalide F was isolated from a Hawaiian herbivorous marine mollusk, *Elysia rufescens*. It has shown potent cytotoxic activity *in vitro* in a number of cancer cell lines [3,4]. In preclinical studies, KF also showed antitumour activity against androgen-resistant prostate cancer xenografts [5]. In phase I studies using either weekly 1 h-infusion [6] or a daily × 5 regimen [7], elevation of liver transaminases was the dose-limiting toxicity, but stable disease in hepatocellular carcinomas and a decrease in Prostate Specific Antigen (PSA) in one patient with androgen-resistant prostate cancers was also reported.

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In vitro studies in prostate and breast cancer cells revealed that KF induced oncosis [4]. Apoptosis and oncosis are two forms of cell death that were thought to be distinct but now appear to often coexist (for review [8]). Oncosis leads to necrosis with karyolysis and cell swelling while apoptosis leads to necrosis with karyorhexis and cell shrinkage [9]. ATP depletion plays a central role in this process; apoptosis requires high levels of ATP to proceed while profound ATP depletion will direct cells towards oncosis. Since the mitochondrion is the main producer of ATP, the balance between lack of ATP production by the mitochondria and ATP production through glycolysis determines whether apoptosis or oncosis will prevail [8,10]. Moreover, during the development of oncosis, a pattern of membrane alterations occur, with permeation of small molecules such as propidium iodide (PI) or ethidium bromide evolving to progressively larger molecules such as high molecular weight dextrans [11]. Finally, oncosis can be induced by a variety of compounds; natural toxins such as maitotoxin [12], chemicals such as dibenzocarbazole [13] and even DNA-damaging agents such as etoposide and nitrogen mustard [14].

So far, the induction of oncosis by KF has been studied in cell lines that were highly sensitive to the drug. In order to better understand the determining events of KF-induced cytotoxicity, we were particularly interested in identifying differential features between cell lines resistant and sensitive to KF. As the liver appears to be a target organ for this compound, both in terms of efficacy and toxicity, we evaluated the cytotoxicity of KF on a panel of hepatoma cell lines and compared it with other cell lines from breast, ovary, prostate and colon cancers. We further investigated two hepatoma cell lines HepG2 and PLC/PRF/5C, sensitive and resistant to KF respectively to establish the key elements of KF-induced cell death.

2. Materials and methods

2.1. Tissue culture

Cells were routinely cultured in either DMEM or RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The PEO1 cell line was derived from the ascites of a patient with a poorly differentiated serous adenocarcinoma at the Edinburgh Medical Oncology Unit [15]. The OVCAR5 cell line was kindly donated by Dr. Hamilton (Fox Chase Institute, PA) and the HCT116 cell lines by Dr. Vogelstein. The 41M, A2780, HCT15, COLO205, SW620, PLC/PRF/5C, Sk-Hep-1, Hep3B and Huh-7D12 cell lines were obtained from the European Tissue Culture Collection (Porton Down, UK). SKOV-3, HT29, Sk-Br3 and HepG2 cell lines were obtained from the American Type Culture Collections (Mannassas, VA).

2.2. Growth inhibition using sulforhodamine B assay

Cells were seeded at 1500–6000 cells per well in 96-well plates, in a volume of 150 μl. The following day, cells were exposed to KF (0.25 nM–10 μM) for 1 or 96 h. After drug exposure, cells were washed with PBS and replaced in drug-free medium. After 72 h, cells were fixed with trichloracetic acid and stained with sulforhodamine B (SRB). Optical densities were measured at 540 nm with a Biohit BP-800 (Bio-Hit, Helsinki, Finland). Growth inhibition curves were plotted as percentage of control cells and IC50 values were determined by Graphpad Prism 3 Software (Graphpad Software, San Diego, CA).

2.3. Quantitation of intracellular ATP

HepG2 and PLC/PRF/5C cells were seeded into luminometer grade 96-well plates at a density of 13,000 and 7000 cells/well, respectively. After 48 h, cells were treated with KF (0.1–5 μM for HepG2, 1–50 μM for PLC/PRF/5C). Cell media was then removed and cells lysed with 50 μl of solution A (45 μl L-4 buffer (25 mM HEPES, 5 mM $MgCl_2$, 140 mM NaCl) + 5 μl ATP somatic cell releasing reagent (Sigma, Gillingham, UK)) for 5 min. ATP was determined by addition of 50 μl of solution B (0.2 mM D-luciferin (Molecular Probes, Paisley UK) + 5 μg/ml luciferase (Promega, Southampton, UK) in L-4 buffer) into each well. Immediate readings were taken using Berthold Microlumat plus LB 96V. Results are expressed as percentage of ATP levels in control wells.

2.4. Detection of membrane permeability to propidium iodide and annexin V

Annexin V/PI staining was performed using TACS Annexin V-FITC apoptosis detection kit (R&D Systems, Abington, UK) according to Lecoeur [16]. Log-phase cells were exposed to KF (0.125–10 μM) for 1 h. At the end of the incubation period, cells were washed with PBS, and resuspended in 1× annexin buffer containing 75 ng/sample of Annexin V-FITC and 50 ng/sample of PI. Cells were analysed using Becton-Dickson FACScalibur and Cell Quest software. FITC and PI emissions were detected in the FL-1 (band pass 530 nm, band width 30 nm) and FL-2 (band pass 585 nm, band width 42 nm) channels, respectively. Cells were first analysed for the forward scatter *vs.* side scatter distributions. Cell debris characterised by low FSC/SSC were excluded from the analysis. The viable cells were then analysed on the FL-1 *vs.* FL-2 plot. Cells stained with PI or annexin V only were used to set up the compensation value between the two channels. Untreated cells (low PI and AV) stained with both PI and AV were then processed to define cut-off values of positivity on both

channels and define the 4 quadrants of interest: PI−/AV−, PI+/AV−, PI−/AV+. The samples (10,000 cells) were then analysed using the same settings to measure the distribution of the cells in the different quadrants: impermeable to both PI and AV (PI−/AV−), permeable to PI but not AV (PI+/AV−), permeable to AV but not PI (PI−/AV+), and permeable to both PI and AV (PI+/AV+).

2.5. LDH release assay

Log-phase cells were seeded in 96-well plates as previously described for the SRB assay. Twenty four hours later, cells were treated with KF for 1 h. LDH release in culture medium was measured using Tox 7 kit (Sigma, Gillingham, UK) according to manufacturer's instructions. Results are expressed as percentage of LDH release in control cells and are mean ± SD of at least three experiments performed in triplicates.

2.6. KF uptake in hepatoma cells

Exponentially growing HepG2 and PLC/PRF/5C cells were exposed to KF (0.1–10 μM) for 1 h. At the end of drug exposure, cells were washed with PBS and centrifuged at 4 °C for 5 min. Cell pellets were resuspended in PBS and cellular proteins precipitated by addition of acetonitrile. After centrifugation at 13,000*g* for 10 min, the supernatant was analysed by HPLC. Chromatographic analyses were performed on a 2690 (Alliance) Separation Module equipped with a 996 Photodiode Array Detector, all from Waters (Milford, MA). Instrument control and data acquisition were performed using Chromeleon software (Version 6.5) (Dionex Corporation, Sunnyvale, CA). Partial loop injections of 20 μl were made on a Phenomenex Luna C18 column (150 × 4.6 mm, 5 μm). The column was used at 30 °C and the mobile phase consisted of a mixture of 45% (v/v) H_2O, 55% (v/v) MeCN and 0.1% (v/v) TFA. The run time was 10 min and KF was detected at 200 nm using UV detection with a retention time of 5.2 min. Experiments were performed three times and results presented are mean ± SD.

3. Results

3.1. Cytotoxicity of KF on a panel of human cancer cell lines

The cytotoxic effect of KF was determined in a large panel of 20 cancer cell lines derived from different tumour types (ovary, breast, prostate, colon and liver) (Fig. 1). The range of IC50 values was narrow, ranging from 0.3 to 5.3 μM after 1 h exposure (median = 0.9 μM) and from 0.2 to 4 μM using a continuous exposure (median = 0.75 μM). HepG2, and PC3 were the most sensitive to KF (IC50 of 0.3 μM) while PLC/PRF/5C, HCT-116, SW620 and PE01 displayed IC50 values from 3.6–5.3 μM. The effect of KF was not time-dependent since the ratio of IC50 values observed following 1 h exposure and continuous exposure was not significantly different in most cases. Among the hepatoma cell lines, HepG2 was one of the most sensitive and PLC/PRF/5C the most resistant.

HepG2 is derived from a well differentiated hepatocellular carcinoma expressing a number of hepatocyte-specific proteins such as albumin, α1-antitrypsin, transferrin but also alpha-foetaprotein (AFP) [17]. PLC/PRF/5C (Alexander cell line) is a hepatocellular carcinoma expressing hepatitis B surface antigen as well as AFP [18].

The kinetics of cellular changes induced by KF were monitored during the course of 1 h exposure to the drug. Pictures of control cells and cells exposed to KF for 1 h (0.5 μM for HepG2 and 5 μM for PLC/PRF/5C) are presented in Fig. 2. Both cell lines demonstrated rapid cell blebbing followed by cell swelling. Although the pattern was similar in both cell lines, HepG2 cells rounded up and detached from the plastic after 1 h while the swelling in PLC/PRF/5C cells appeared more controlled and adherence to the support was maintained.

3.2. KF induces a different ATP depletion in HepG2 and PLC/PRF/5C

Concentration- and time-dependent depletion of ATP was determined in both HepG2 (Fig. 3(a)) and PLC/PRF/5C cells (Fig. 3(b)). Both cell lines showed a concentration-dependent ATP depletion after KF exposure. However, the depletion occurred at lower concentrations of KF in HepG2 than in PLC/PRF/5C cells. Fifty percent depletion was achieved using 0.5–0.7 μM KF in HepG2 cells but required 4–6 μM KF in PLC/PRF/5C, these concentrations being consistent with the IC50 values obtained with the cytotoxicity assay.

A time-course of ATP depletion (Fig. 3(a) and (b)) showed that ATP levels dropped rapidly and reached a steady state after 15 min in HepG2 and 30 min in PLC/PRF/5C.

3.3. KF induces different membrane alterations in HepG2 and PLC/PRF/5C cells

Permeability to both propidium iodide (PI) and annexin V (AV) was assessed in HepG2 and PLC/PRF/5C cells using flow cytometry according to the method of Lecoeur and colleagues [16]. Propidium iodide is a small molecule (614 Da) usually excluded from cells when the cell membrane is intact. Annexin V (∼35 KDa) binds to phosphatidyl-serine (PS) and has been used commonly to detect the early phases of

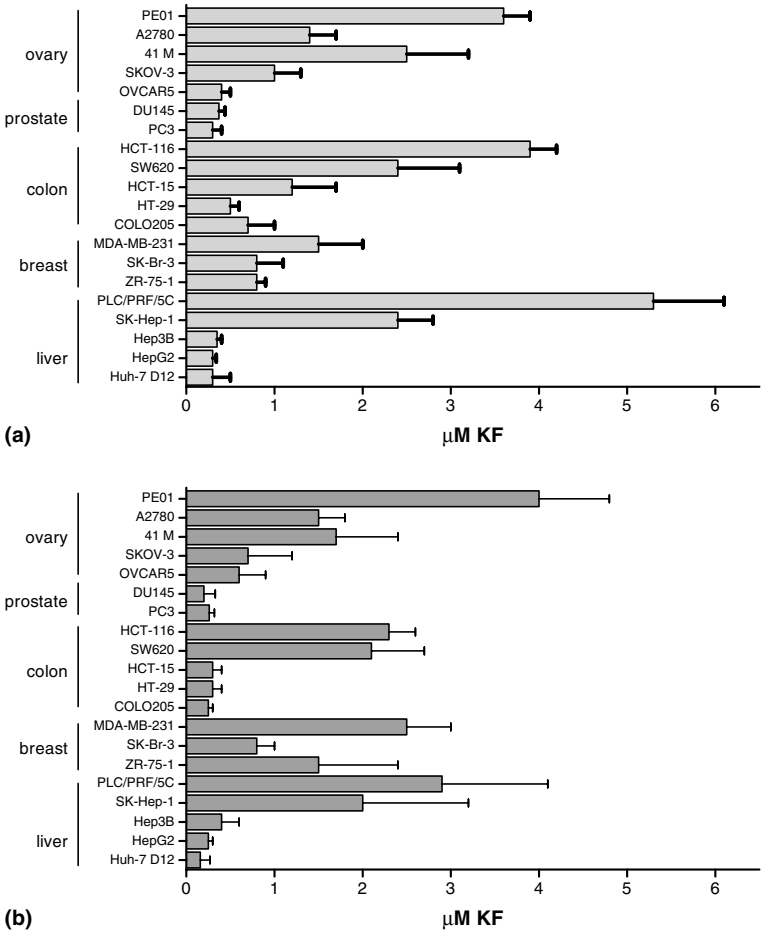

Fig. 1. Concentrations of KF inhibiting 50% of cell growth on a panel of cell lines from ovary, prostate, colon, breast and liver as determined by the SRB assay. KF was exposed to cells for: (a) 1 h or (b) continuously (96 h). Results are mean ± SEM of at least three independent experiments.

apoptosis when PS is exposed on the outer membrane of the cells. Oncotic cells also show positivity to AV due to either the presence of PS on the outer membrane of the cells or to large pores allowing AV to access PS located in the inner cell membrane. Cells presenting both PI and AV staining (PI+/AV+) must therefore display extensive membrane damage or an oncotic phenotype while cells permeable to PI have acquired only limited membrane damage.

A typical dot-plot presenting the distribution of the cells according to their positivity to PI and AV staining is presented in Fig. 4. Untreated HepG2 cells clustered in the lower left quadrant of the dot-plot and show the exclusion PI and low AV staining (Fig. 4(a)). After exposure to KF, the fraction of cells in the upper left quadrant (PI+/AV+) increased and suggests membrane damage and oncosis (Fig. 4(b)). The percentage of cells present in each quadrant after 1-h exposure to increasing concentrations of KF is summarised in Table 1. In both cell lines, the PI−/AV+ fraction (apoptotic fraction) remained low over the range of KF concentrations tested (1–3% for HepG2 and 3–6% for PLC/PRF/5C cells). In HepG2 cells, the PI+/AV+ fraction increased with KF concentrations from 2% to 40% between 0.125 and 0.5 μM associated with a limited increase of PI-only positive cells (1–11%). However, in PLC/PRF/5C cells, although the PI+/AV+ fraction increased from 3% to 24%, a parallel increase of the PI+/AV− fraction from 2% to 26% was also observed for KF concentrations higher than in HepG2 cells (0 and 8 μM). The PI+/AV− fraction can correspond to either bare nuclei or to cells that are permeable to small molecules such as PI but impermeable to high molecular weight molecules such as AV. Since the cellular structure was maintained in PLC/PRF/5C cells over the course of the exposure to the drug (Fig. 2), this fraction is unlikely to correspond to bare nuclei and probably represents cells with limited cell membrane damage.

3.4. KF induces LDH release from HepG2 cells

In order to ascertain whether the permeability of PLC/PRF/5C cells was affected by KF, we measured the release of lactate dehydrogenase (LDH) into the

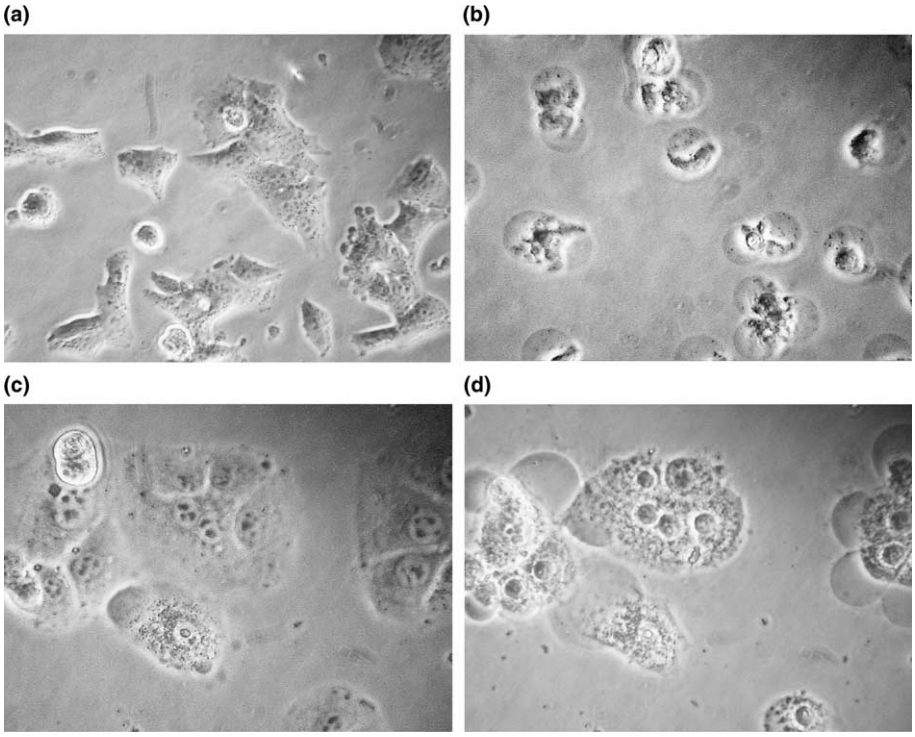

Fig. 2. Images of cells treated with KF: (a) untreated HepG2 cells; (b) HepG2 + 0.5 μM KF for 1 h; (c) untreated PLC/PRF/5C cells; (d) PLC/PRF/5C cells + 5 μM KF for 1 h.

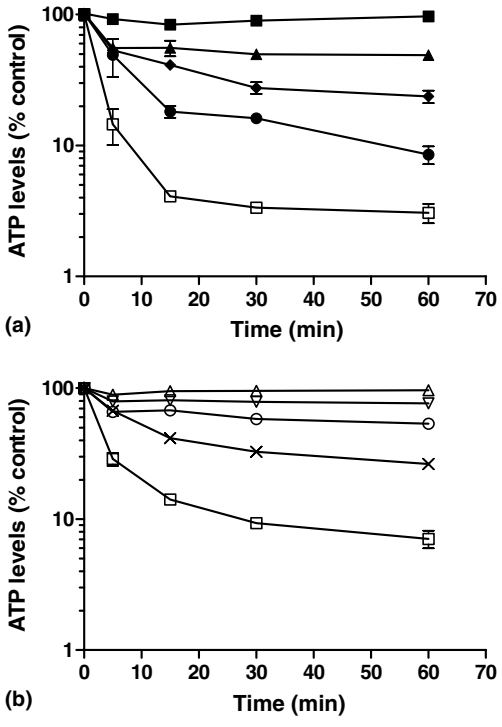

Fig. 3. KF-induced depletion of ATP in HepG2 and PLC/PRF/5C cells. Results are expressed as percentage of ATP levels in untreated cells: (a) time-course (0–60 min) of ATP levels in HepG2 cells exposed to 0.1 (■), 0.5 (▲), 1 (◆), 2.5 (●) and 5 (□) μM of KF; (b) time-course (0–60 min) of ATP levels in PLC/PRF/5C cells exposed to 1 (△), 2.5 (▽), 5 (○), 10 (×) and 25 (□) μM of KF. Results are mean ± SEM of three experiments performed in triplicates.

tissue culture medium (Fig. 5). LDH is a high molecular weight protein (140 KDa) that is abundant in cells and is released when the membrane structure is altered. HepG2 cells showed a significant increase in levels of released LDH at concentrations of KF greater than 0.25 μM up to 230% ± 10 of control cells at 2 μM of KF for 1 h. Conversely, LDH release was limited in PLC/PRF/5C cells, up to 138% ± 11 at 10 μM KF for 1 h. PLC/PRF/5C cells are therefore permeable to small molecules like PI but the damage to the cell membrane is not sufficient to enable LDH to be released from the cells.

3.5. KF uptake in HepG2 and PLC/PRF/5C cells

Earlier studies have suggested that KF targets lysosomes. In order to determine whether the differences in membrane damage observed between HepG2 and PLC/PRF/5C cells were linked to a difference in KF uptake between the two cell lines, we determined the uptake of KF after 1-h exposure to the drug (Fig. 6). KF was extracted from cells using acetonitrile precipitation. No subcellular fractionation was performed before extraction and therefore the results presented correspond to KF accumulation in cell membranes and cytosolic fractions. KF was detectable in HepG2 cells after exposure to 0.5 μM of drug while it remained undetectable in PLC/PRF/5C cells at 0.5 and 1 μM. KF cellular concentrations increased in HepG2 from 0.5 to 5 μM

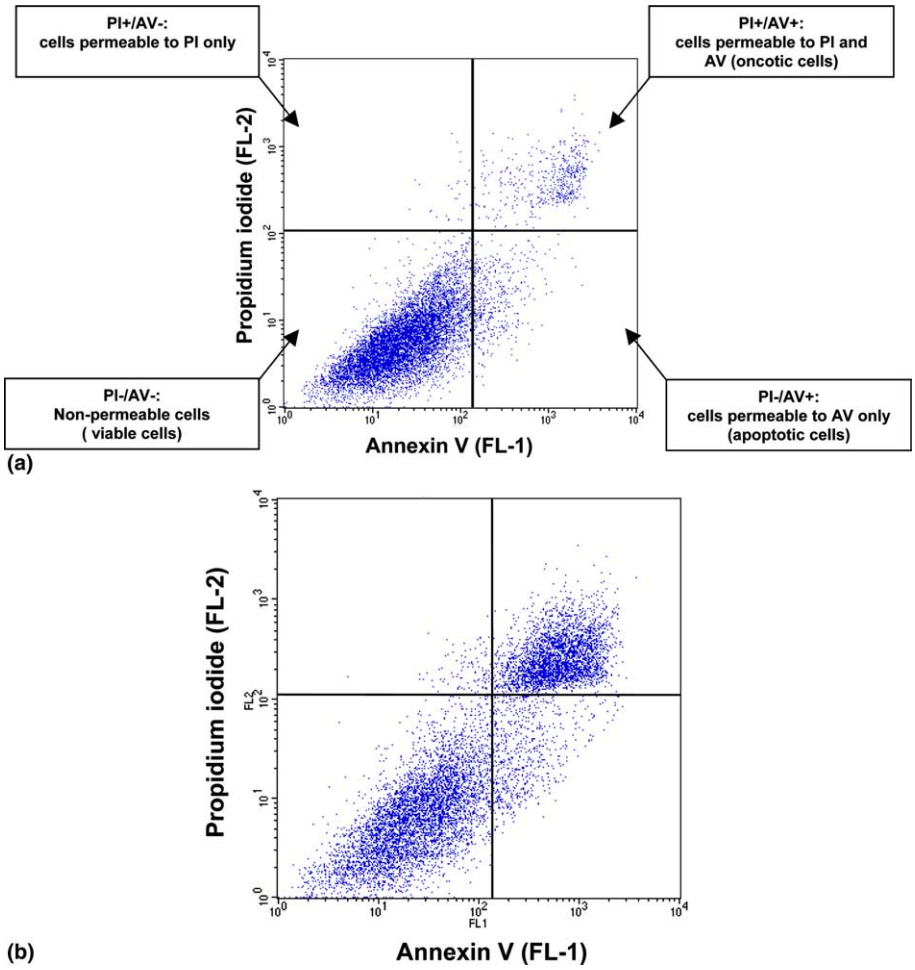

Fig. 4. Analysis of KF-induced permeability changes in HepG2 cells by flow cytometry. Cells: (a) untreated and (b) treated with 0.25 μM KF for 1 h were analysed by flow cytometry after staining with PI and Annexin V-FITC (AV). The cells distribute in four quadrants: PI−/AV− (viable cells), PI+/AV− (cells stained with PI only), PI+/AV+ (cells stained with both PI and AV: oncotic cells appear in this quadrant), and PI−/AV+ (cells stained with AV only: apoptotic cells appear in this quadrant). Results are expressed as percentages of the cell population present in each quadrant.

Table 1
Permeability of HepG2 and PLC/PRF/5C cells to propidium iodide (PI) and annexin V (AV)

HepG2				
KF concentration (μM)	**0**	**0.125**	**0.25**	**0.5**
Cells not staining PI or AV	94 ± 3	94 ± 2	84 ± 6	42 ± 23
Cells staining for AV	1 ± 1	1 ± 0	2 ± 1	3 ± 2
Cells staining for PI	1 ± 1	1 ± 1	3 ± 4	11 ± 9
Cells staining for PI and AV	2 ± 1	3 ± 1	9 ± 6	40 ± 20
PLC/PRF/5C				
KF concentration (μM)	**0**	**4**	**8**	**10**
Cells not staining PI or AV	89 ± 6	73 ± 7	47 ± 10	39 ± 8
Cells staining for AV	3 ± 3	3 ± 3	3 ± 3	6 ± 4
Cells staining for PI	2 ± 2	10 ± 2	23 ± 9	26 ± 5
Cells staining for PI and AV	3 ± 2	9 ± 5	17 ± 12	24 ± 7

Cells were exposed to various concentrations of KF for 1 h and analysed by flow cytometry for cellular staining to PI and AV. Results are presented as percentages of the overall population and are mean ± SD of three experiments performed in duplicates.

and a similar uptake was observed in HepG2 at 5 μM KF and PLC/PRF/5C cells exposed to 10 μM KF (130 ± 37 and 137 ± 46 pmol/10^6 cells, respectively). Considering the structural changes observed in HepG2 cells exposed to 0.5 μM KF (Fig. 2), KF uptake was not considered for concentrations greater than 5 μM.

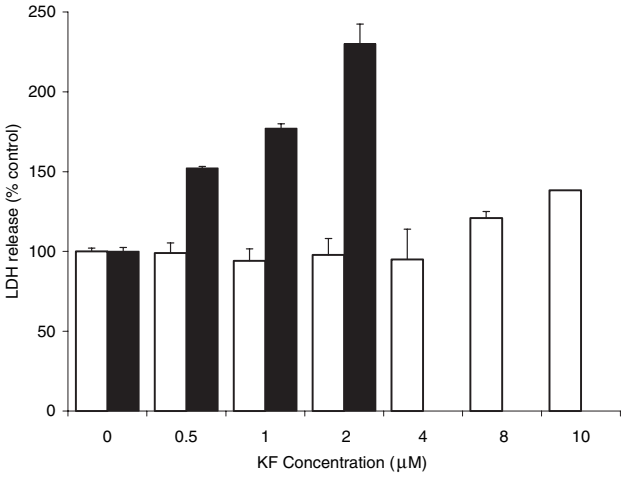

Fig. 5. LDH release by HepG2 (■) and PLC/PRF/5C (□) after exposure to KF for 1 h. Results are expressed as percentage of LDH released in the cell culture medium in control cells and are means ± SD of at least three independent experiments performed in triplicates.

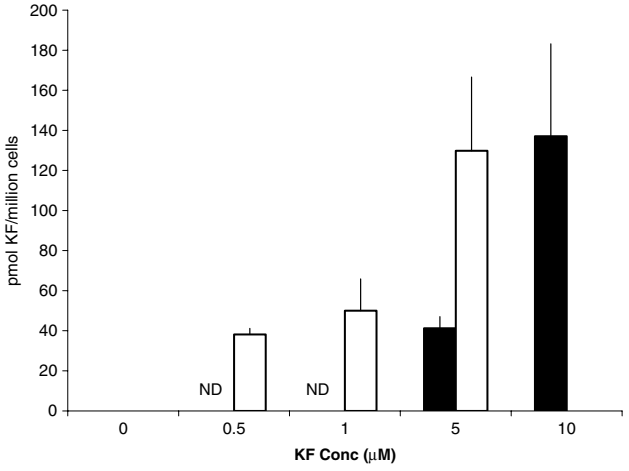

Fig. 6. Uptake of KF in HepG2 (□) and PLC/PRF/5C (■) after being exposed to KF for 1 h. Cellular concentrations of KF were determined by HPLC and expressed as pmol KF/10^6 cells. Results are mean ± SD of two independent experiments performed in triplicates.

4. Discussion

Kahalalide F (KF) is a cytotoxic peptide extracted from the mollusk, *E. rufescens*. KF has cytotoxicity *in vitro* and antitumour activity *in vivo* in a number of tumour models [4,5]. It has also been tested in the clinic in phase I in patients with refractory prostate cancers [7] and the dose-limiting toxicity was transaminitis.

In this study, we have shown that KF induces a cytotoxic effect in a number of cell lines from breast, prostate, ovary, liver and colon over a relatively narrow range of IC50 values. KF is particularly active in prostate (confirming previous *in vitro* studies [4]) and hepatoma cell lines. We focused the main part of our study on two hepatoma cell lines, KF-sensitive HepG2 (mean IC50 = 0.3 μM) and KF-resistant PLC/PRF/5C (mean IC50 = 5.3 μM) in order to identify specific features of drug resistance or sensitivity. KF-induced extensive cell swelling and blebbing in both cell lines. However, while HepG2 cells rounded up and detached from the tissue culture substrate after 1-h exposure to the drug, PLC/PRF/5C maintained a relative cell structure and the adherence to plastic despite an extensive increase in cell volume. These cellular changes were associated with ATP depletion in a concentration-dependent fashion in both HepG2 and PLC/PRF/5C cells but the depletion was slower in PLC/PRF/5C cells. These results are consistent with observations reported in COS-7, Hela, prostate and breast cancer cells exposed to KF [3,4]. Hepatocytes maintained in hypoxic conditions [11,19] and Jurkat cells in presence of dideoxyforskolin [20] also display similar cell swelling associated with ATP depletion consistent with oncotic cell death.

Important membrane alterations were observed after exposure of HepG2 cells to KF and included the rapid permeablity to cationic PI and large molecules such as annexin V and LDH, suggesting large pore formation. Conversely, the permeability of PLC/PRF/5C cells was limited to PI without permeation to AV or release of LDH unless high concentrations of KF were used (>10 μM). Also, lower cellular concentrations of KF were measured in PLC/PRF/5C and may suggest that the size of the pores formed in these cells was too small to accommodate the 1476 Da KF molecule. In hepatocytes subject to hypoxia, a similar pattern of membrane permeation develops; the opening of glycine-sensitive chloride channels induces the permeation to anions such as calcein (623 Da) followed by permeation to cationic PI (414 Da) and fluorescein dextrans (40 KDa) [11]. MDCK cells subject to profound ATP depletion also present membrane defects evolving from glycine-sensitive aqueous pores only permeable to cationic PI to larger pores permeable to high molecular weight dextrans [21]. Maitotoxin is a natural cytotoxic peptide, which also induces membrane permeation with an increase in permeability from ethidium bromide (314 Da) to LDH (140 KDa), which is linked to the opening of calcium channels [12,22]. The pattern of permeation observed in this study in HepG2 cells and the timeframe of the events is similar to that reported with maitotoxin [12]. However, the impact of KF on calcium channels has still to be established. Moreover, glycine did not appear to prevent KF cytotoxicity (data not shown) while it blocks cell lysis induced by maitotoxin in bovine aortic endothelial cells [23].

The similarity in the pattern of permeabilisation between maitotoxin and KF suggests that KF may act like other natural cytotoxic peptides. Although KF can interact with the lipid bilayer, it is unlikely to form pores by itself since at least 20 amino acids are required to span the lipid bilayer. However, we cannot exclude the possibility that KF forms multimers. The composition

of phospholipid bilayer greatly influences the formation of channels by well-known channel-forming cytotoxic peptides and may therefore explain the difference between HepG2 and PLC/PRF/5C cells [1]. KF may also impact on ATP formation or interact with ion-channels. More studies are required to ascertain whether KF has a similar mechanism of action as maitotoxin.

The differential pattern of cell permeability observed between HepG2 and PLC/PRF/5C cells suggests that specific proteins or membrane interactions are involved. Therefore, identifying these cellular factors may guide the targeted use of KF and the development of analogues demonstrating enhanced selectivity.

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