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Antitumor activity of bovine lactoferrin and its derived peptides against HepG2 liver cancer cells and Jurkat leukemia cells

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Abstract Liver cancer and leukemia are the fourth and first causes, respectively, of cancer death in children and adults worldwide. Moreover, cancer treatments, although beneficial, remain expensive, invasive, toxic, and affect the patient's quality of life. Therefore, new anticancer agents are needed to improve existing agents. Because bovine lactoferrin (bLF) and its derived peptides have antitumor properties, we investigated the anticancer effect of bLF and LF peptides (LFcin17-30, LFampin265-284 and LFchimera) on liver cancer HepG2 cells and leukemia Jurkat cells. HepG2 and Jurkat cells were incubated with bLF and LF peptides. Cell proliferation was quantified by an MTT assay, and cell morphology and damage were visualized by light microscopy or by phalloidin-TRITC/DAPI staining. The discrimination between apoptosis/necrosis was performed by staining with Annexin V-Alexa Fluor 488 and propidium iodide, and the expression of genes related to apoptosis was analyzed in Jurkat cells. Finally, the synergistic interaction of bLF and LF peptides with cisplatin or etoposide was assessed by an MTT assay and the combination index. The present study demonstrated that bLF and LF peptides inhibited the viability of HepG2 and Jurkat cells, inducing damage to the cell monolayer of HepG2 cells and morphological changes in both cell lines. bLF, LFcin17-30,

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and LFampin265-284 triggered apoptosis in both cell lines, whereas LFchimera induced necrosis. These results suggested that bLF and LF peptides activate apoptosis by increasing the expression of genes of the intrinsic pathway. Additionally, bLF and LF peptides synergistically interacted with cisplatin and etoposide. In conclusion, bLF and LF peptides display anticancer activity against liver cancer and leukemia cells, representing an alternative or improvement in cancer treatment.

Keywords Bovine lactoferrin · Lactoferrin peptides · Antitumor · Liver cancer · Leukemia

Introduction

Cancer is defined as malignant growth due to uncontrolled cell division (Roy and Saikia 2016). Cancer is classified in solid tumors and in hematologic or blood cancers. Liver cancer is a solid tumor and is the sixth most common type of cancer and the third cause of death, with approximately 830, 180 deaths reported worldwide in 2020, and hepatocellular carcinoma (HCC) represents the 85–90% of primary liver cancers (Cancer, I.A.f.R.o. 2020). On the other hand, Leukemia is a type of liquid or blood cancer characterized for an increased number of leucocytes in the blood and/or the bone marrow. Leukemia may be present at all ages; nevertheless, the acute lymphoblastic leukemia (ALL) is more common in early childhood and rare in adults (Juliusson et al. 2016; Seth and Singh 2015), and 311, 594 persons died due to leukemia in 2020 (Cancer, I.A.f.R.o. 2020).

Despite advances in cancer treatments for solid tumors (HCC) and blood cancers (leukemia), the toxicity remains high, and the secondary effects reduce the quality of life and may even cause death. Conventional chemotherapy is ineffective for patients with HCC (Bruix et al. 2016). On the other hand, in children with ALL severe adverse effects are presented, including infections (Kato and Manabe 2018). Thus, the endpoint of treatments is to increase survival and avoid complications to improve patients' quality of life.

Lactoferrin (LF) is a 703 amino acid glycoprotein that is an effector of the immune system of mammals, and it is secreted by glandular epithelial cells and found mainly in milk and in the secondary granules of neutrophils (Baker and Baker 2005a). The native LF and their LF-peptides exhibit antimicrobial properties and modulates the immune system in vitro and in vivo (Vogel 2012; Baker and Baker 2005b; Vorland 1999). Interestingly, previous studies have shown than LF has anticancer activity reducing cell growth in vitro and in vivo. It has been reported against several types of carcinoma, breast cancer (Pereira et al. 2016; Gibbons et al. 2015), colon cancer (Li et al. 2017; Sugihara et al. 2017).

LF is a protein that has several biological functions, but the mechanism of action related to its antitumor activity is not completely understood. LF inhibits cell viability and the growth of tumors (Li et al. 2017; Wolf et al. 2003; Wolf et al. 2007; Wei et al. 2015). Several studies have shown that LF alters gene expression related to apoptosis, but the intrinsic or extrinsic pathway has not been established and may vary according to cell line (Gibbons et al. 2015; Fujita et al. 2004; Chea et al. 2018; Luzi et al. 2017). In addition, studies have shown that LF alters genes related to cell cycle arrest (Chea et al. 2018; Xiao et al. 2004). Moreover, the activity of bLF and LF peptides in combination with chemotherapeutics has been demonstrated, showing potential for reducing the cytotoxicity of drugs, consequently avoiding side effects in patients (Ramirez-Sanchez 2020).

Some synthetic LF peptides have been demonstrated to be effective in vivo and in vitro against several types of cancer; however, the mechanism of action has not been fully explored (Massodi et al. 2009; Eliassen et al. 2002; Lu et al. 2016; Onishi et al. 2008; Meng et al. 2017). Due to the therapeutic potential of LF and LF peptides to prevent or treat cancer, it is necessary to completely understand the molecular mechanisms, to evaluate their properties against different types of cancer and to investigate their use in combination with chemotherapeutic drugs. We previously demonstrated that bLF and LF peptides affect the cell viability of endometrial and cervical cancer cell lines, and we explored the mechanism of action (Ramirez-Sanchez 2020). However, there only a few reports of the effects of bLF and LF peptides on liver cancer and leukemia (Wei et al. 2015). Therefore, the present study investigated the anticancer activity of bLF, LF peptides (lactoferricin17-30 and lactoferrampin265-284) and a lactoferrin chimera against liver cancer HepG2 cells

(hepatocellular carcinoma) and leukemia Jurkat cells (acute lymphoblastic leukemia) in vitro.

Material and methods

Lactoferrin and LF peptides

Bovine lactoferrin was obtained from Abial (Cantabria, Spain). To confirm the purity of bLF (> 98%), SDS−PAGE gels were stained with silver nitrate. bLF concentration was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm and 1% solution) (Valenti et al. 1999). The bLF iron saturation was measured by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation) and detected at approximately 20%. The lipopolysaccharide contamination of bLF, which was estimated by a Limulus Amebocyte assay (LAL Pyrochrome kit, Thermo Fisher Scientific, Waltham, MA, USA), was equal to 0.7 ± 0.06 ng/mg of bLF (Cutone et al. 2014). The LFcin17-30, LFampin265-284 and LFchimera synthetic peptides were obtained by solid phase peptide synthesis using Fmoc chemistry as described previously (Bolscher et al. 2009).

Cell culture

The HepG2 hepatocellular cancer cell line (ATCC HB-8065™) and the Jurkat leukemia cell line (ATCC® TIB-152™) were purchased from ATCC®. HepG2 and Jurkat cells were maintained in DMEM and RPMI-1640, respectively, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were cultured at 37 °C and 5% CO_2.

Cell viability measurement by MTT assay

HepG2 (2×10^4) and Jurkat (1×10^6) cells were seeded into a 96-well plate and incubated at 37 °C and 5% CO_2 for 24 h. Cells were then treated with bLF and LF peptides at concentrations of 1, 10 and 20 µM, and treatment with medium alone was used as a control. HepG2 cells were treated for 6, 12 and 24 h, and Jurkat cells were treated for 4, 6 and 8 h. The treatment times used for HepG2 were selected based on previous experiments performed in adherent cells (HeLa, SiHa, KLE and HEC-1A) (Ramirez-Sanchez 2020), while the treatment times used for Jurkat cells were based on previous reports in which the activity of bLF and LF peptides against Jurkat cells and other cancer cells were reported and considering the high growth rate of this suspension cell line (Mader et al. 2005; Guerra et al. 2019; Igder et al. 2013). Cisplatin was used as a reference at concentrations of 10, 20 and 40 µM in HepG2 cells, and etoposide was used as a reference at concentrations of 10, 25, 50 and 100 µM in Jurkat cells. After the treatments, cells were washed twice, and 90 µL of PBS was added. Then, 10 µL of MTT solution (5 mg/mL, Sigma) was added to each well followed by incubation for 4 h at 37 °C and 5% CO_2. Then, 100 µL of 0.1 N HCl/isopropanol (Sigma) was added to each well to dissolve the formazan crystals. The amount of reduced MTT was measured by spectrophotometry using a microplate reader at 620 nm. The reduction in cell viability was calculated using the following equation: $(A_{(experimental)}/A_{(control)}) \times 100\%$; where A is the absorbance at 620 nm.

Giemsa staining for cell monolayers

HepG2 cells were seeded in an 8-well chamber slide (Nunc ™ Lab-Tek ™ II Chamber Slide ™ System, Cat no. 154941, Thermo Fisher Scientific) at a density of 2×10^5 cells per well to obtain > 90% confluence. After 24 h of incubation at 37 °C and 5% CO_2, cells were treated with bLF (1, 10 and 20 µM), LF peptides (1, 10 and 20 µM), cisplatin (40 µM) or medium alone for 6, 12 and 24 h. Cells were washed and fixed with 70% methanol for 15 min at RT. Cells were then washed with PBS and stained with Giemsa for 30 min at room temperature (RT). After disassembling chamber slides and mounting the coverslip in Gelvatol mounting medium, images were acquired by an LMD7 microscope (Leica) using a 40× objective.

Fluorescence microscopy

HepG2 cells were seeded as described previously in an 8-well chamber slide and incubated with bLF (20 µM), LF peptides (20 µM), cisplatin (40 µM) or medium alone for 12 and 24 h. Jurkat cells were seeded in a 24-well plate at a density of 1×10^6 cells per well and treated with bLF (20 µM), LF peptides (20 µM) or etoposide (100 µM) for 4 and 8 h. HepG2

and Jurkat cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 20 min at 4 °C in the dark. Cells were permeabilized with 0.2% Triton X-100 for 5 min and stained with DAPI and phalloidin-TRITC (Cat no. P1951; Sigma−Aldrich) for 20 min. The samples were mounted in Gelvatol mounting medium, and images were acquired by an LMD7 fluorescence microscope (Leica) using a 100× oil immersion objective and the specific filter settings for DAPI (385–400 nm) and TRITC (475–490 nm).

Annexin V/PI assay

HepG2 cells were seeded in an 8-well chamber slide as described previously and treated with bLF (20 μM), LF peptides (20 μM), cisplatin (40 μM) or medium alone for 12 and 24 h. Jurkat cells were seeded in a 96-well plate at a density of 1×10^6 cells per well and treated with bLF (1, 10 and 20 μM), LF peptides (1, 10 and 20 μM) or etoposide (100 μM) for 4 and 8 h. Apoptotic and necrotic cells were detected using the Alexa Fluor® 488 Annexin V/dead Cell Apoptosis kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. After treatments, cells were cultured with 1×Binding Buffer and incubated with Alexa Fluor® 488 Annexin V and propidium iodide (PI) for 15 min in the dark. Cells were fixed with 4% PFA for 20 min at 4 °C. Finally, cells were mounted in Gelvatol mounting medium, and images were obtained with an LMD7 Leica Microscope using a 100×oil immersion objective and the specific filter settings for Alexa Fluor® 488 (FITC filter) and TRITC. Apoptosis or necrosis was classified by differences in fluorescence according to the Alexa Fluor® 488 Annexin V/dead Cell Apoptosis kit as follows: viable cells any color; early apoptosis, green; late apoptosis, green and red; and necrosis, red.

Primer design

The primers were designed using Oligo 7 Primer Analysis Software (Molecular Biology Insights, CO, USA) and verified in *Basic Local Alignment Search Tool* (BLAST) from *National Center for Biotechnology Information* (NCBI). The synthesis of primers was performed by *Integrated DNA Technologies* (Coralville, IA, USA). A total of 12 genes were amplified. The following eight proapoptotic genes were amplified: Fas receptor or CD95 (FAS), cytochrome C (CYCS), Bcl2-associated X protein (BAX), caspase-8 (CASP8), Fas-associated protein with death domain (FADD), BH3-like motif containing, cell death inducer (BLID), caspase-9 (CASP9) and caspase-3 (CASP3). The following three antiapoptotic genes were amplified: B-cell lymphoma 2) (BCL2), Bcl-2-like 1 (BCL2L1) and CASP8 and FADD-like apoptosis regulator (CFLAR or c-FLIP). The β-actin (ACTB) housekeeping gene was also amplified (Supplementary Table 1).

Expression analysis

Jurkat cells at a density of 1×10^6 cells per well were treated with bLF and LF peptides (1, 10 and 20 μM) for 2 and 4 h at 37 °C and 5% CO_2. As controls, 5 μg/mL anti-Fas mAb and untreated cells were used. Total RNA was extracted using the MagNA Pure LC RNA Isolation Kit—High Performance (Roche Applied Science, Germany, Cat no. 03542394001) according to the manufacturer's instructions and quantified by spectrophotometry using a NanoDrop™ 1000 (Thermo Fisher Scientific, USA). Amplification of cDNA and qPCR were performed in one step using QuantiFast® SYBR® Green qRT−PCR (Qiagen, CA, USA) and the CFX96 Real Time System coupled to a C1000 Thermal Cycler (Bio-Rad, CA, USA). Using Relative Expression Software Tool (REST) software (Qiagen, CA, USA), the comparative CT method based on the $2^{\Delta\Delta CT}$ formula was utilized to analyze gene expression.

Synergistic effect with chemotherapeutics

The effective concentration at 50% (EC_{50}) of bLF, LF peptides, cisplatin and etoposide in HepG2 or Jurkat cells was determined in previous experiments using MTT assays. Combinations of bLF and LF peptides with cisplatin or etoposide (HepG2 or Jurkat cells, respectively) were tested at constant ratios of 0.25. 0.5, 1, 1.25 and 1.5 of EC_{50} in combination. Untreated cells with only medium were used as a positive control of viability. MTT assays were performed as described above, and the effect at 50% of each combination was identified. The results were analyzed with the combination index (CI) according to the Chou-Talalay method (Chou 2010) as follows:

CI < 1 indicates synergy, CI = 1 indicates an additive effect and CI > 1 indicates antagonism.

Statistical analysis

All experiments were performed in duplicate. Data are expressed as the mean ± standard error of the mean (SEM). The statistical significance of differences between means was determined by one-way analysis of variance (ANOVA) followed by the Dunnett test for post hoc multiple comparisons using SigmaPlot software version 12.0 (CA, USA). $p < 0.05$ was considered statistically significant.

Results

bLF and LF peptides decrease the viability of hepatocellular and leukemia cell lines

To determine the effect of bLF and LF peptides on the viability of HepG2 and Jurkat cells, MTT assays were performed (Fig. 1A, B). The activity of bLF and LF peptides in HepG2 cells was lower than expected after short periods of incubation (Fig. 1A). Compared to the untreated control, bLF (1 μM) and LFchimera (20 μM) reduced cell viability by 36 and 51%, respectively. However, the activity of 1 μM bLF may be an experimental variation in comparison with its higher concentrations. LFcin17-30 and LFampin265-284 did not exert any inhibitory activity after 6 h of incubation. Cisplatin exhibited similar activity to bLF and LFchimera with 36% cell viability at the higher concentration (40 μM) (Fig. 1A). However, after 12 h, bFL decreased the cell viability by nearly 40% at higher concentrations compared to untreated cells, and similar results were found with LFchimera. The highest effect of bLF was observed at 24 h, and 10 μM and 20 μM bLF decreased the viability of HepG2 cells by 21% and 29%, respectively, compared to untreated cells ($p < 0.05$). Similar to that at 12 h, LFchimera maintained its activity after 24 h. LFcin17-30 and LFampin265-284 displayed effects after 12 h with approximately 50% cell viability, but

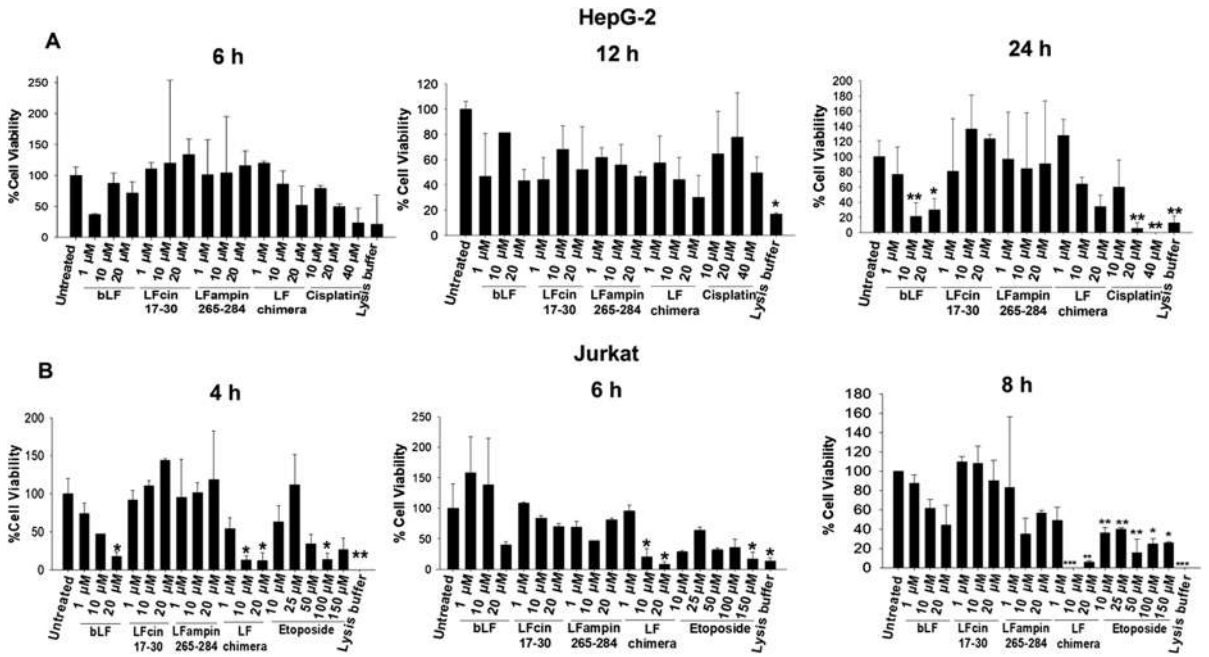

Fig. 1 Effect of bLF and LF-peptides in cell viability of hepatocellular cancer and leukemia cell lines. HepG-2 (**A**) and Jurkat (**B**) cancer cells were cultured with 1, 10 and 20 μM bLF and LF-peptides: LFcin17-30, LFampin265-284 and LFchimera for 6, 12 and 24 h in HepG-2 and for 4, 6 and 8 h in Jurkat cells. Cisplatin was used as a control in HepG-2 at concentrations of 10, 20 and 40 μM. Etoposide was used in Jurkat cells at concentrations of 10, 25, 50, 100 and 150 μM. Cell viability was then determined by MTT assay. *Columns,* percentage of viable cells; *bars*, standard deviation (SD); $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$

after 24 h, the inhibitory effect decreased and was not significant different compared to untreated cells (Fig. 1A). Finally, cisplatin decreased cell viability to approximately 0% after 24 h compared to untreated cells ($p < 0.05$). The percentages of HepG2 cell viability are shown in Supplementary Table 2. In HepG2 cells, bLF exhibited a time-dependent effect, and LFchimera presented a concentration-dependent effect. Moreover, cisplatin presented a time- and concentration-dependent effect in HepG2 cells. The remaining treatments were not time- or concentration dependent.

In Jurkat cells (Fig. 1B), LFchimera exerted a significant effect at 10 and 20 µM, resulting in approximately 6% cell viability compared to untreated cells at 8 h. In addition, a cell viability lower than 20% at all incubation times was obtained after treatment with 10 and 20 µM LFchimera (Fig. 1B). Similarly, bLF had an inhibitory activity against Jurkat cells with the highest inhibitory effect at 20 µM after 4 h, resulting in 17.5% cell viability ($p < 0.05$). Although the effect of bLF was reduced at 6 and 8 h with an approximate cell viability of 40% (with a lack of statistical significance), bLF continued to exert inhibitory activity even when used at the highest concentration (Fig. 1B). The highest inhibitory activity of LFcin17-30 and LFampin265-284 occurred at 6 h, but apparently it was not statistically significant compared to control cells, which may have been due to data fluctuation. Etoposide induced a higher effect at 100 µM after 4 h with 13.5% cell viability ($p < 0.05$), and its effect was similar to that of bLF, decreasing viability over time (Fig. 1B). In general, etoposide concentrations of 50, 100 and 150 µM exerted inhibitory activity (less than 40% cell viability) at 4, 6 and 8 h, and most data were significant ($p < 0.05$) (Fig. 1B). The percentages of viable Jurkat cells are shown in Supplementary Table 3. In Jurkat cell, only bLF and LFchimera exhibited a concentration-dependent effect.

bLF and LFchimera exerted significant inhibitory activity against both cell lines (HepG2 and Jurkat cells). Regardless of the lack of statistical significance of some of these data, especially for LF peptides (LFampin265-284), which decreased cell viability of Jurkat cells, the inhibitory activity was demonstrated in the complementary experiments. Thus, these results supported the anticancer potential of bLF and LF peptides and suggested that the lack of significance in the results of MTT assays may be due to data fluctuation.

bLF and LF peptides damage the cell monolayer of HepG2 cells

The damage caused by bLF and LF peptides on hepatocellular cancer HepG2 cells was visualized by light microscopy on 80–90% confluent cell monolayers stained with Giemsa. The objective of this experiment was to visualize the morphological changes caused by the treatments in the monolayer formed by HepG2 cells, which is not possible for Jurkat cells because they are suspension cells. Compared to the untreated control, LFchimera and bLF disrupted the monolayer integrity and caused cell detachment. However, both treatments may have different mechanisms as indicated by different cell morphology changes. Both bLF and LFchimera showed great loss of cells (Fig. 2A, B, *asterisks*), but the synthetic LFchimera peptide severely damaged and perhaps lysed the cells (Fig. 2A, B, *arrowheads*). Similar to bLF, LFcin17-30 and LFampin265-284 also caused damage to the cell monolayer (Fig. 2, *simple arrows*) and a tendency toward cell clustering (Fig. 2, *arrowheads*). Cisplatin caused the cells to be detached (Fig. 2A, B, *asterisks*).

The highest inhibitory effect of bLF and LFchimera was found after 24 h at a concentration of 20 µM. The effect of LFcin17-30 and LFampin265-284 on cells was reduced from 12 to 24 h. Cisplatin maintained its effect with no cells remaining on the monolayer (Fig. 2). Moreover, bLF and LFchimera presented a time-dependent effect in these cells.

bLF and LF peptides induce cellular morphological changes in hepatocellular cancer and leukemia cell lines

Because the treatments damaged the cell monolayer, the bLF- and LF peptide-induced morphological changes in HepG2 and Jurkat cells were analyzed. The actin cytoskeleton was stained with phalloidin-TRITC, and the nucleus was stained with DAPI. HepG2 cells (Fig. 3) were treated for 12 and 24 h, but only the most representative period of incubation is shown (12 h). At 12 h, higher inhibitory activity and a clearer effect on cell morphology were observed. In HepG2 cells, bLF induced cell rounding and shrinkage as well as DNA fragmentation compared to

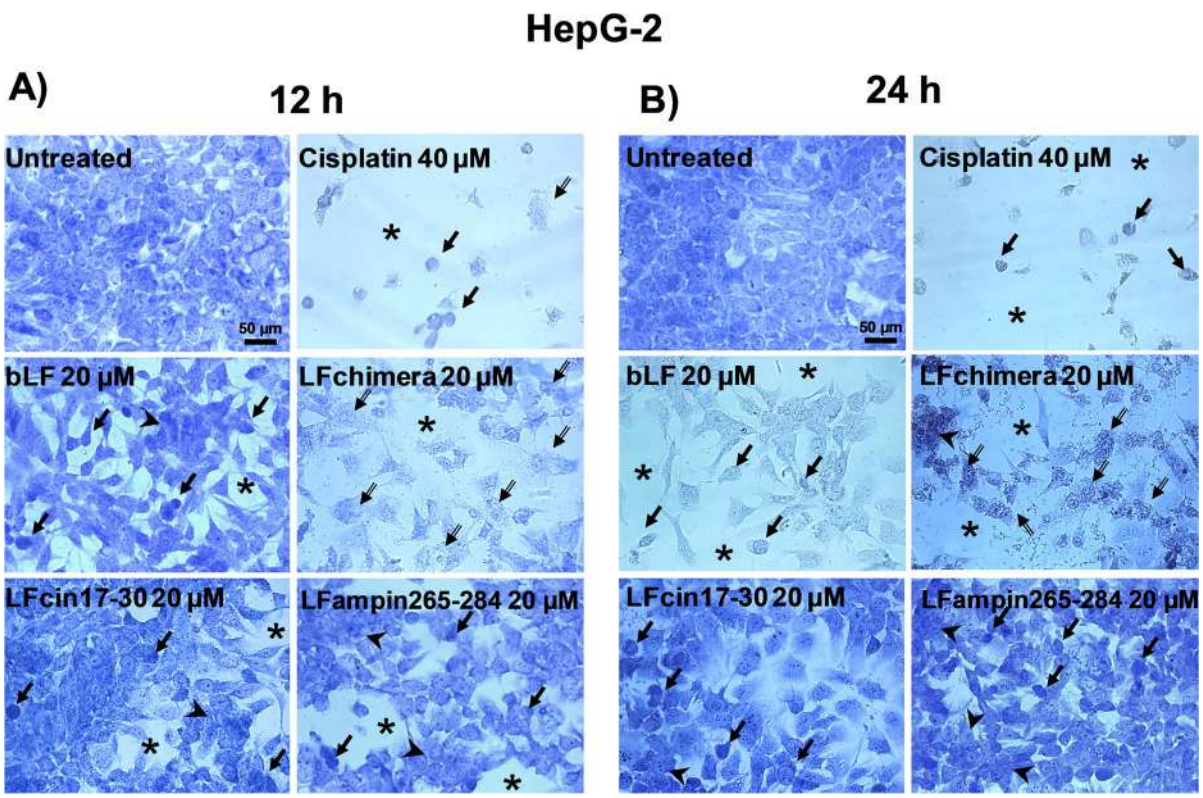

Fig. 2 Damage caused by bLF and LF-peptides on hepatocellular cancer cells monolayers. The cells were cultured with 20 µM of bLF and LF-peptides for 12 h (**A**) and 24 h (**B**). The cells were stained with Giemsa and visualized by light microscopy and the 40 X objective. *Simple arrows,* cell rounding and shrinkage; *double arrows*, cell swelling, lysis, cell debris; *arrowheads*, cell clustering; *asterisks*, cell detachment

untreated cells. LFcin17-30 and LFampin265-284 also showed alterations in the cytoskeleton and DNA, but these effects were mild compared to bLF (Fig. 3, *simple arrows*). Furthermore, LFchimera had different effects than bLF, LFcin17-30, LFampin265-284 and the chemotherapeutic drugs because it caused degradation of the actin cytoskeleton and DNA (Fig. 3, *arrowheads*). The most drastic effect in cells was exerted by cisplatin because the cells presented cytoplasm reduction, DNA degradation and cytoskeleton degradation (Fig. 3, *simple arrows*).

Jurkat cells were incubated with the different treatments at 4 and 8 h (Fig. 4), but only the most representative period is shown (8 h). Compared to untreated cells, bLF caused DNA fragmentation, cell shrinkage and cytoplasm reduction, while LFcin17-30 and LFampin265-284 showed alterations in DNA and nuclei morphology (Fig. 4, simple arrows). Etoposide induced similar damage to that caused by bLF. In contrast, LFchimera caused a loss of cell structure, DNA fragmentation and DNA degradation. In both cell lines, the treatments followed the same mechanism, inducing similar changes in cell morphology. For instance, bLF, LFcin17-30 and LFampin265-284 caused typical signs of apoptosis, while LFchimera induced cell lysis, an indicator of necrosis. Thus, we next investigated the type of cell death in more detail.

bLF and LF peptides induce different types of cell death in hepatocellular and leukemia cell lines

Because bLF and LF peptides induced morphological changes in HepG2 and Jurkat cells, we next investigated the cell death mechanisms using Annexin V and propidium iodide staining. In HepG2 cells, bLF induced apoptosis (Fig. 5A) as indicated by a well-defined pattern of Annexin V binding to the apoptotic plasma membrane of cells,

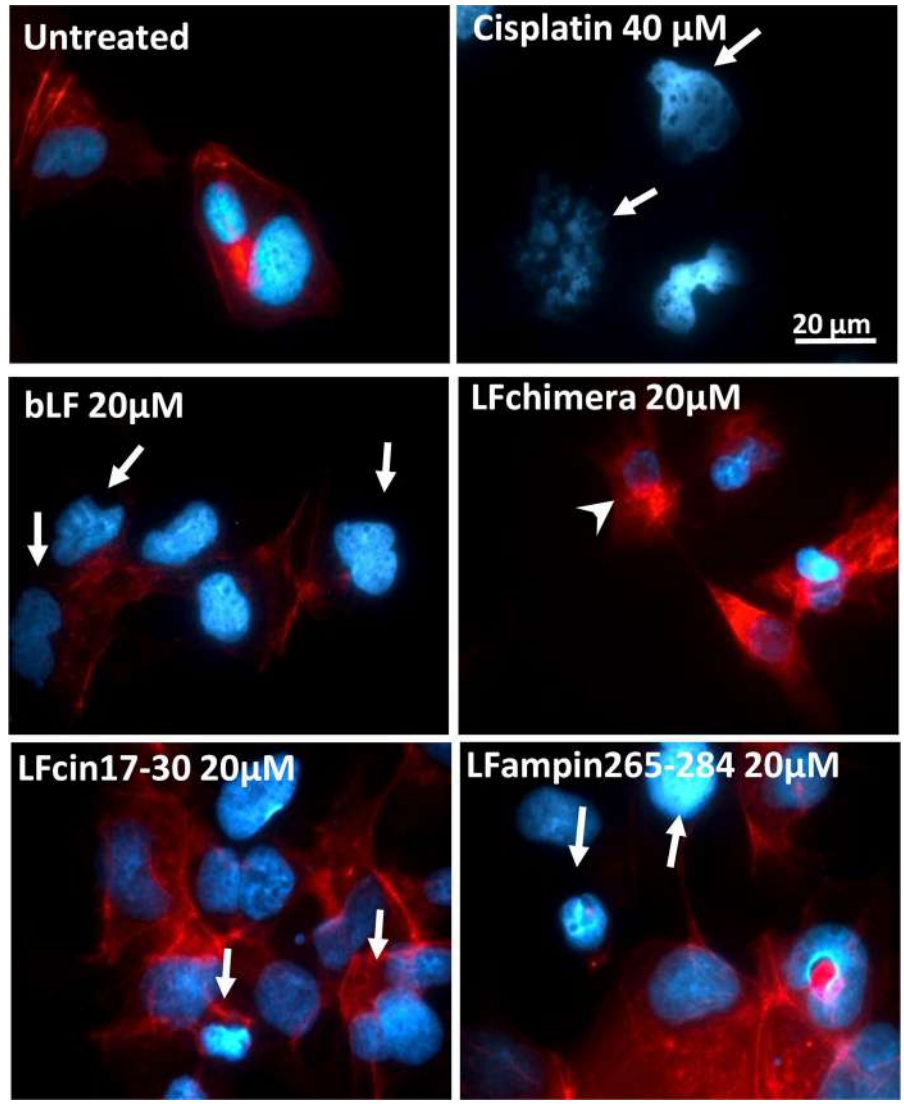

Fig. 3 Morphological changes induced by bLF and LF-peptides on hepatocellular cancer cells. bLF and LF-peptides 20 µM were incubated with HepG-2 cells for 12 h. After, the cells were fixed and stained with DAPI and phalloidin-TRITC. The images were captured with a fluorescence microscope using an immersion oil 100× objective. *Simple arrows,* DNA damage, fragmentation, or condensation; *arrowheads* actin cytoskeleton disruption and DNA degradation

and some cells were stained with propidium iodide, suggesting late stages of apoptosis. Conversely, LFchimera induced necrosis as indicated by a diffuse pattern of Annexin V and propidium iodide staining, indicating Annexin V binding to dispersed components of cell plasma membranes, demonstrating membrane disruption and cell lysis (Fig. 5A). Moreover, LFcin17-30 and LFampin265-284 induced both apoptosis and necrosis (Fig. 5A). In these cases, treated cells showed multiple staining patterns as follows: only Annexin V (early apoptosis); Annexin V and propidium iodide (late apoptosis or necrosis); and only propidium iodide staining (primary necrosis) (Fig. 5A).

In Jurkat cells, bLF induced apoptosis (Fig. 5B) as indicated by early and late apoptosis events. The cells were stained with only Annexin V or both Annexin V and propidium iodide with a well-defined and organized pattern.

In contrast, LFchimera induced necrosis in Jurkat cells as indicated by a diffuse pattern of Annexin V and propidium iodide staining, demonstrating membrane disruption and random degradation of DNA (Fig. 5B). The LFcin17-30 and LFampin265-284 peptides induced early and late apoptosis in Jurkat cells similar to etoposide and anti-Fas (Fig. 5B).

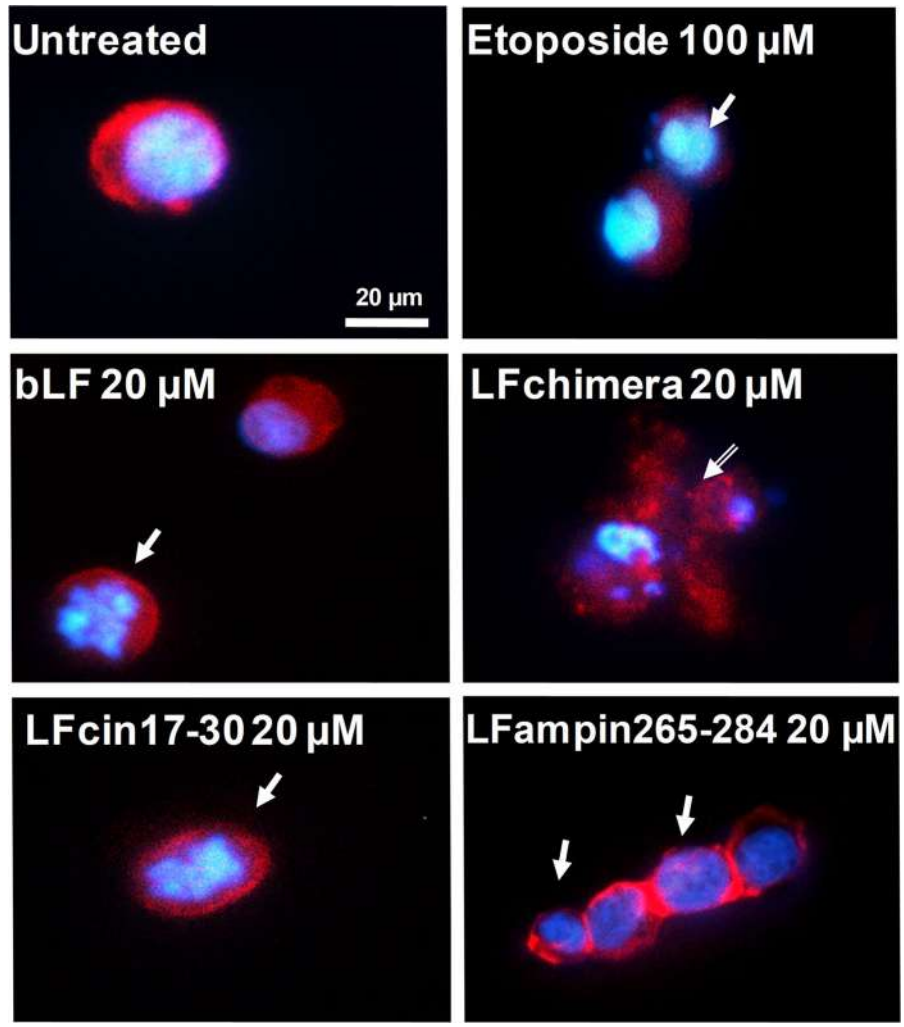

Fig. 4 Morphological changes induced by bLF and LF-peptides on leukemia cells. bLF and LF-peptides 20 µM were incubated with Jurkat cells for 8 h. After, the cells were fixed and stained with DAPI and phalloidin-TRITC. The images were captured with a fluorescence microscope using an immersion oil 100× objective. *Simple arrows,* DNA damage, fragmentation, or condensation; *arrowhead* actin cytoskeleton disruption and DNA degradation

bLF and LF peptides influence apoptotic gene expression in leukemia cell lines

We next investigated the pathways and genes involved in the mechanism of action of bLF and LF peptides. The expression of proapoptotic and antiapoptotic genes was analyzed in Jurkat cells after 2 and 4 h of treatment by qRT−PCR. The expression analysis was performed in Jurkat cells because a greater inhibitory effect was observed in these cells. With the different treatments, a higher incidence of apoptotic events occurred in Jurkat cells compared to HepG2 cells. Additionally, the concentrations of bLF and LF peptides used in this assay were lower than the concentrations that demonstrated notable cell damage, detachment or decrease in cell viability in the previous experiments to avoid error in results due to loss of cells and RNA that is used to measure the relative expression of apoptotic genes.

Treatment with bLF and LF peptides for 2 h did not cause significant differences compared to the anti-Fas positive control. However, treatment with bLF and LF peptides for 4 h initiated the apoptotic process (Figs. 6 and 7). All expression levels were normalized to the untreated control, which was set as 1.0. After 4 h of treatment, the mRNA expression levels of cytochrome C (*CYCS*) and *BLID* were significantly increased, and the mRNA expression levels of caspase-9 were slightly increased; these genes represent the intrinsic or mitochondrial pathway of apoptosis (Fig. 6). The *CYCS* gene was altered as follows: increased by 1.208-and 1.41-fold after

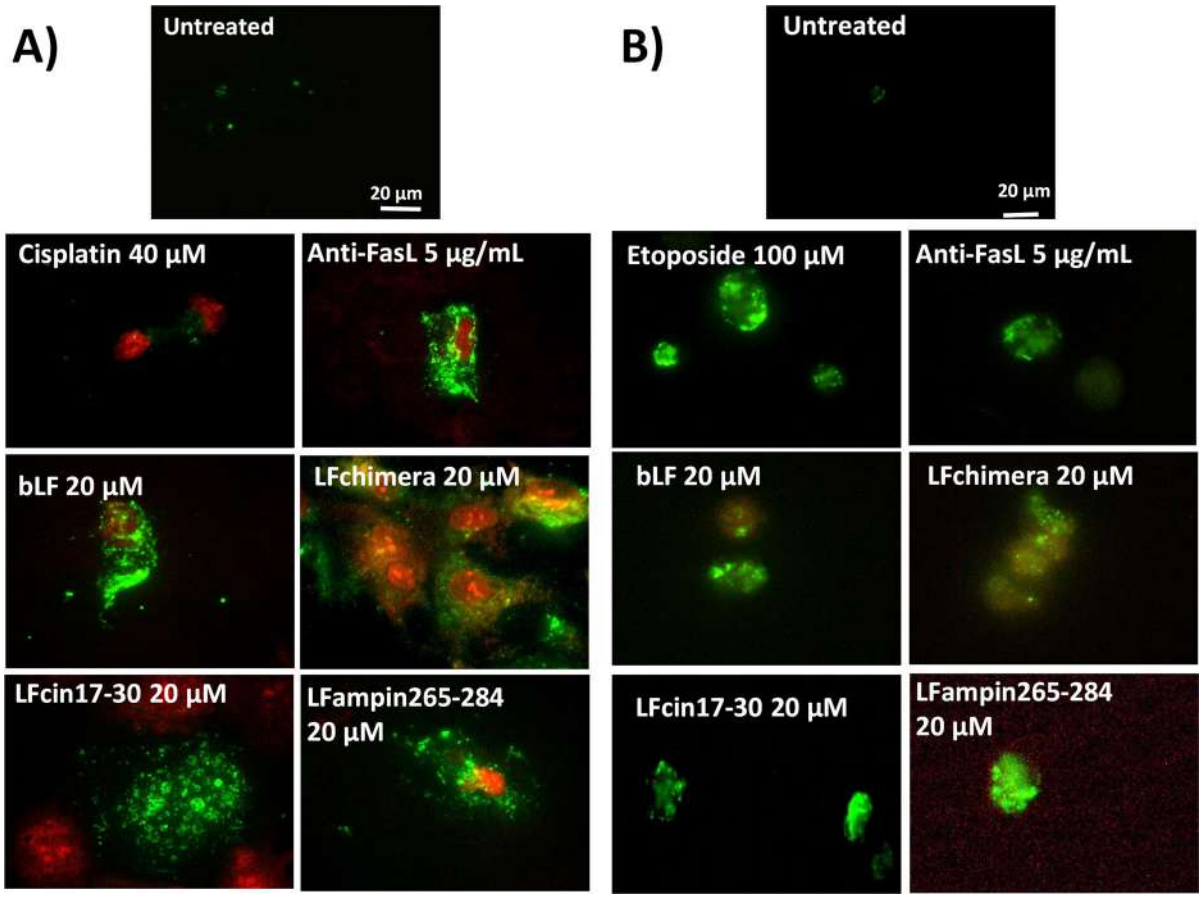

Fig. 5 Induction of cell death by different mechanisms by bLF and LF-peptides in hepatocellular cancer (**A**) and leukemia cells (**B**). The annexin V and propidium iodide staining was carried out after 12 h of incubation of bLF and LF-peptides 20 µM in HepG-2 cells and 4 h in Jurkat cells. Cisplatin 40 µM were gemcitabine 50 µM were tested in HepG-2. Etoposide 100 µM was used in Jurkat cells. In both cell lines, the apoptotic control anti-Fas (5 µg/mL) and untreated cells were used as controls. Then, cells were stained with annexin v-Alexa fluor 488 and propidium iodide. The images were visualized and captured with a fluorescence microscope using the immersion oil 100× objective. *Annexin V+/PI −*, early apoptosis; *annexin V+/PI+*, late apoptosis or necrosis; *annexin V −/PI+*, necrosis; *annexin V −/PI −*, viable cells

treatment with 1 and 20 µM LFcin17-30, respectively; increased by 1.319-fold after treatment with 20 µM LFampin265-284; and increased by 1.468- and 1.78-fold after treatment with 1 and 10 µM LFchimera, respectively. The *BLID* gene altered as follows: increased by 1.294-fold after treatment with 10 µM bLF; increased by 1.448, 1.367 and 1.343-fold after treatment with 1, 10 and 20 µM LFcin17-30, respectively; and increased by 1.301-fold after treatment with 20 µM LFampin265-284. The *CASP9* gene was increased by 1.098- and 1.023-fold after treatment with 20 µM LFampin265-284 and 20 µM LFcin17-30, respectively.

The *FAS* gene, which is related to the extrinsic pathway, was increased by 1.203-fold after treatment with 20 µM LFampin265-284. The remaining proapoptotic genes were similar to the untreated control or slightly reduced. As expected, almost all the genes were increased by 1.18-to 2.274-fold after anti-Fas treatment (Fig. 6).

The *BCL2L1* and *CFLAR* antiapoptotic genes were decreased by most of the treatments (Fig. 7). *CFLAR* was decreased by 0.746- and 0.638-fold after treatment with 1 and 10 µM LFchimera, respectively. The expression of *BCL-2* was altered by bLF (10 µM), LFcin17-30 (20 µM), LFampin265-284 (20 µM) and

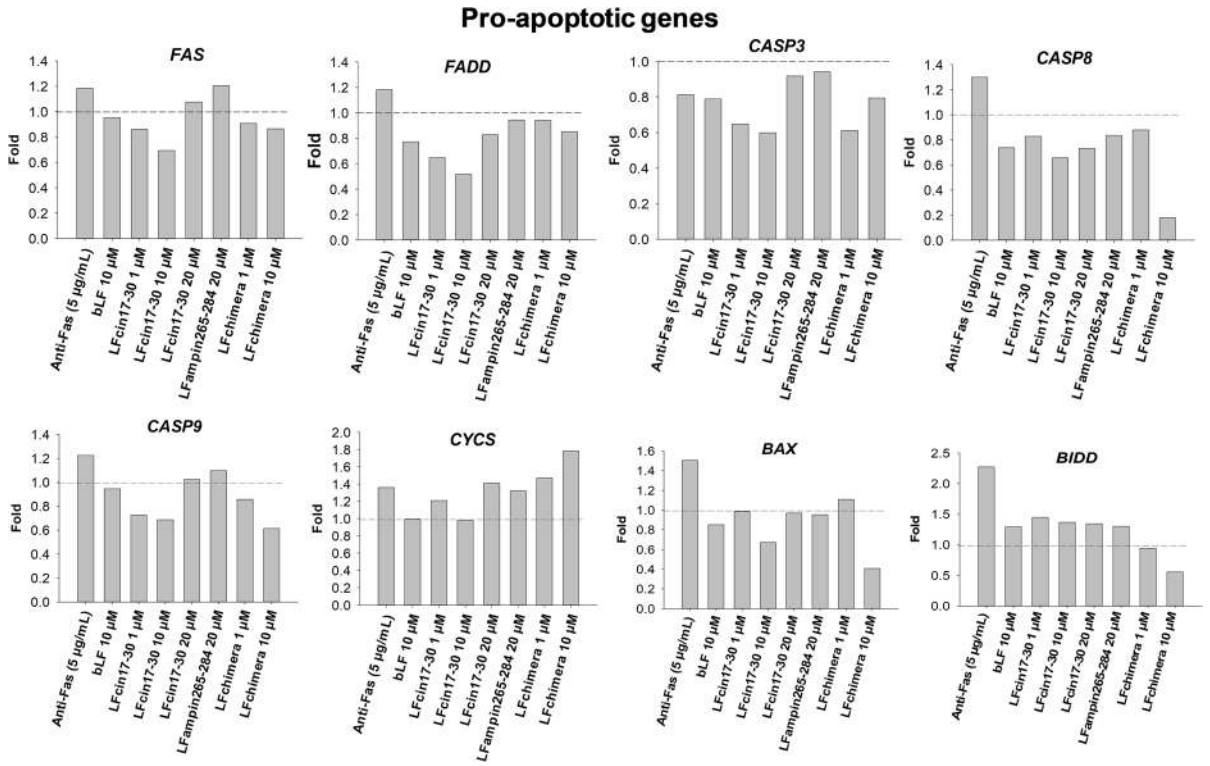

Fig. 6 Alteration of pro-apoptotic genes by bLF and LF-peptides in leukemia cells (Jurkat). bLF 10 uM, LFcin17-30 1, 10 and 20 µM, LFampin265-284 20 µM and LFchimera 1 and 10 µM were incubated with Jurkat cells for 4 h. As a positive control of apoptosis, anti-Fas was used. Expression analysis was carried out by RT-qPCR. The untreated cells are used as reference of 1.0 fold

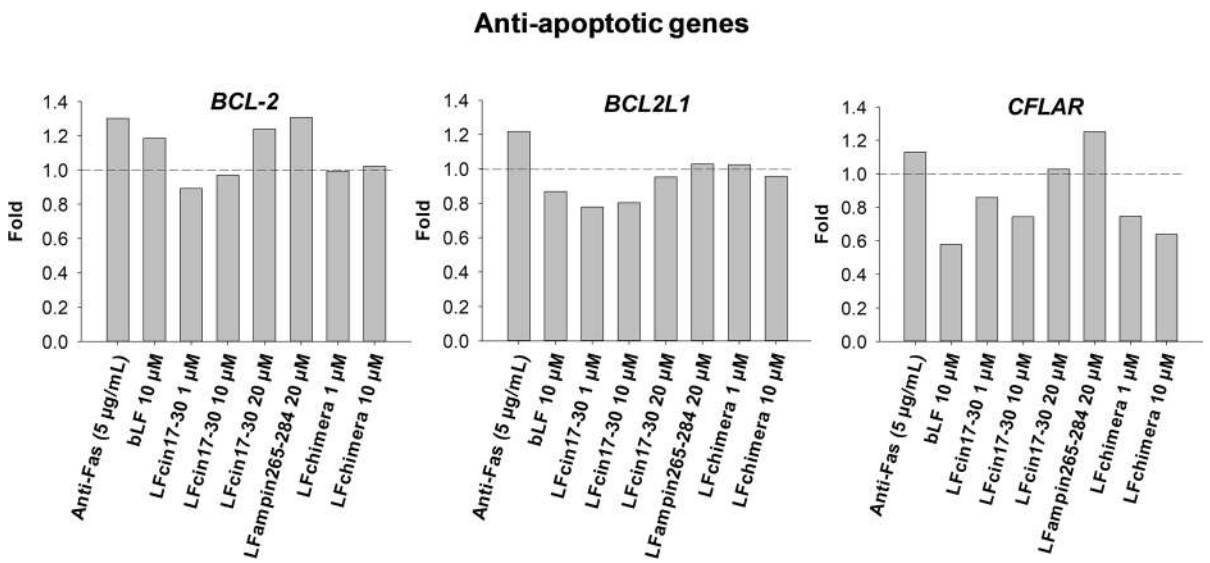

Fig. 7 Alteration of anti-apoptotic genes by bLF and LF-peptides in leukemia cells (Jurkat). bLF and LF-peptides (1, 10 and/or 20 µM) were incubated with Jurkat cells for 4 h. As a positive control of apoptosis, anti-Fas was used. Expression analysis was carried out by RT-qPCR. The untreated cells are used as reference of 1.0 fold

anti-Fas (Fig. 7). The gene expression data of Jurkat cells treated with bLF and LF peptides are shown in Supplementary Table 4.

Effect of bLF and LFchimera in combination with cisplatin or etoposide in HepG2 and Jurkat cells

We next investigated the effect of bLF and LF peptides in combination with commonly used chemotherapeutic drugs (cisplatin or etoposide) against hepatocellular cancer and leukemia cell lines (Figs. 8 and 9). Almost all of the combinations of bLF and LF peptides affected the viability of HepG2 cells, except 0.25 and 0.5 µM bLF combined with 10 and 20 µM cisplatin, respectively, as well as LFampin265-284 (2.5 µM) combined with 10 µM cisplatin and LFchimera (5 µM) combined with 10 µM cisplatin. The remaining combinations decreased the viability of HepG2 cells by approximately 25% to 0% ($p < 0.05$) (Fig. 8). Regarding Jurkat cells, the combination of 2.5, 10, 12.5 and 25 µM bLF with the same concentration of etoposide decreased the viability of Jurkat cells to 0 to near 30% after 4 h of incubation compared to untreated cells ($p < 0.05$). Additionally, the combination of 1.25 and 1.5 µM LFchimera with 12.5 and 15 µM etoposide decreased the viability of these cells to 30 to 40% ($p < 0.05$), but the other LFchimera combinations did not show significant activity (Fig. 9). Regarding LFcin17-30 and LFampin265-284 in combination with etoposide, the highest activity was found after 6 h of incubation with Jurkat cells. The combinations of 25 and 30 µM LFcin17-30 with 31.5 and 37.5 µM etoposide, respectively, decreased the viability of cells to less than 7% compared to untreated Jurkat cells ($p: < 0.05$). Additionally, 10, 12.5, and 15 µM LFampin265-284 in combination with 25, 31.25, and 37.5 µM etoposide, respectively, decreased the viability of Jurkat cells to 9 to 0% ($p < 0.05$) (Fig. 9).

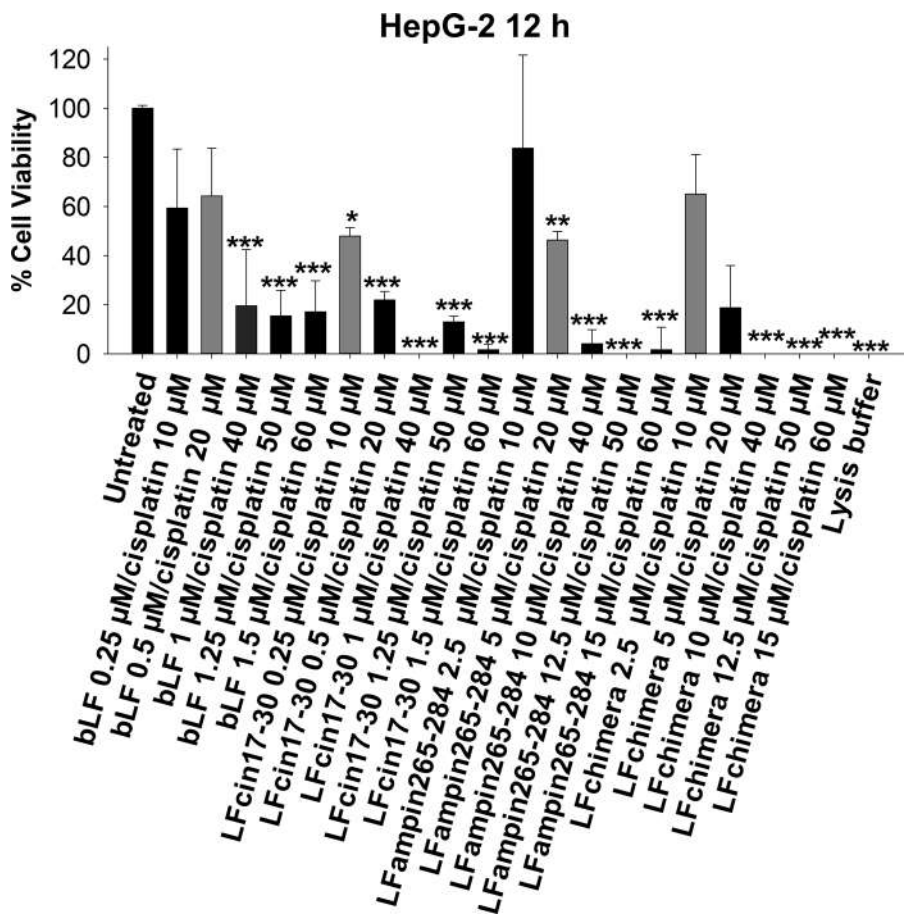

Fig. 8 bLF and LF-peptides have additive and synergistic interaction with cisplatin in hepatocellular cancer cells. The HepG-2 cells were treated with combinations of bLF and LF-peptides with cisplatin for 12 h, using constant ratios of EC50 of each. Cell viability was quantified by MTT assay. After identify the combination with around 50% of effect, the interaction was established using the combination index (CI). CI < 1, synergism; CI = 1, additivity; CI > 1, antagonism. bLF and LFampin265-284 presented an additive effect with cisplatin in HepG-2 cells (CI = 1), however, LFchimera and LFcin17-30 showed a synergistic interaction with cisplatin (CI = 0.5). Columns, percentage of viable cells; bars, standard deviation (SD); *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001

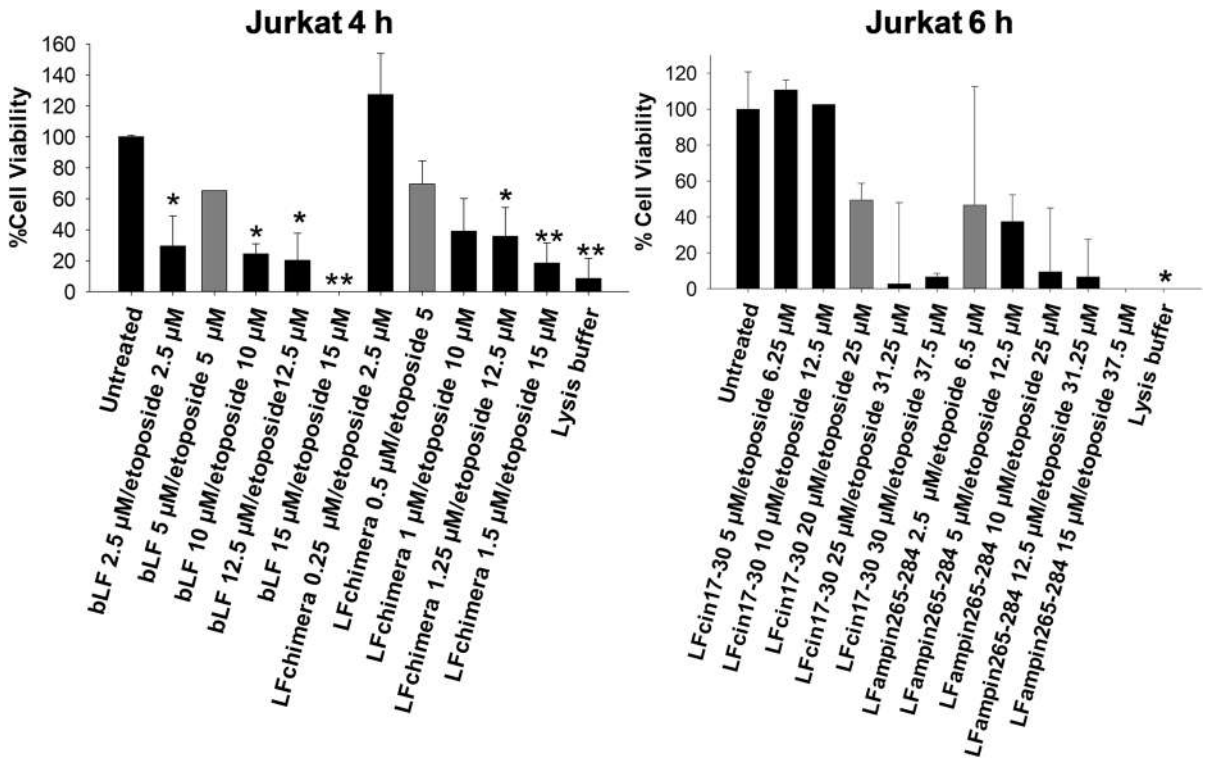

Fig. 9 bLF and LF-peptides have additive and synergistic interaction with cisplatin in leukemia cells. Jurkat cells were treated with combinations of bLF and LF-peptides with etoposide for 4 or 6 h, using constant ratios of EC_{50} of each. Cell viability was quantified by MTT assay. After identify the combination with around 50% of effect, the interaction was established using the combination index (CI). CI < 1, synergism; CI = 1, additivity; CI > 1, antagonism. bLF and LFchimera showed an additive effect (CI = 1) and LFampin265-284 presented a synergistic interaction in combination with cisplatin in Jurkat cells (CI = 0.5). Nevertheless, LFcin17-30 showed an antagonistic interaction with cisplatin (CI = 2.5). *Columns,* percentage of viable cells; *bars*, standard deviation (SD); $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$

The results in HepG2 cells showed that bLF and LFampin265-284 presented additive effects, while LFchimera and LFcin17-30 had synergistic effects when used in combination with cisplatin. An additive effect is described as an interaction in which both treatments can be used together and produce an effect equal to the sum of the individual effects, while synergism indicates a higher effect than the sum of the individual effects. Because the additive and synergistic effects are not just an arithmetic sum of the effect of both treatments, it is necessary to analyze other factors, i.e., the dose−response curve of both treatments, which is considered in the median-effect equation and combination index (CI). Chou (2010) stated that synergism is more than an additive effect and antagonism is less than an additive effect (Chou 2010) (Table 1).

In Jurkat cells, bLF and the LFchimera showed additive effects when used with etoposide. LFampin265-284 and etoposide exhibited synergistic effects in Jurkat cells. In contrast, LFcin17-30 and etoposide presented antagonistic effects in Jurkat cells (Table 1).

Discussion

In the present study, we investigated the anticancer properties and explored the mechanism of action of bLF and LF peptides (LFcin17-30, LFampin265-284 and LFchimera) against hepatocellular cancer and leukemia cell lines to identify new alternatives to prevent or treat these diseases, and we explored their use as adjuvants in combination with chemotherapeutics.

Table 1 Effect of bLF and LF-derived peptides in combination with cisplatin in leukemia and hepatocellular cancer cell lines

Cell line	Individual EC_{50}		Combination Treatments (50% effect)*	CI	Type of interaction
	bLF and LF-peptides	Chemotherapeutic drugs			
HepG-2[a]	bLF 1 μM	Cisplatin 40 μM	bLF 0.5 μM + Cisplatin 20 μM	1	Additivity
	LFcin17-30 1 μM		LFcin17-30 0.25 μM + Cisplatin 10 μM	0.5	Synergism
	LFampin265-284 10 μM		LFampin265-284 5 μM + Cisplatin 20 μM	1	Additivity
	LFchimera 10 μM		LFchimera 2.5 μM + Cisplatin 10 μM	0.5	Synergism
Jurkat	bLF 10 μM[b]	Etoposide 10 μM	bLF 5 μM + Etoposide 5 μM	1	Additivity
	LFcin17-30 20 μM[c]	Etoposide 25 μM	LFcin17-30 20 μM + Etoposide 25 μM	2.5	Antagonism
	LFampin265-284 10 μM[b]	Etoposide 25 μM	LFampin265-284 2.5 μM + Etoposide 6.25 μM	0.5	Synergism
	LFchimera 1 μM[c]	Etoposide 10 μM	LFchimera 0.5 μM + Etoposide 5 μM	1	Additivity

*Constant ratios of the individual concentrations at 50% effectiveness (EC_{50}) were used in the different combinations to find the combination with 50% effect. The type effect in combination was established with the Combination Index (CI). CI < 1, synergism; CI = 1, additivity; CI > 1, antagonism

[a]The individual treatments and combinations were incubated for 12 h

[b]The incubation period was 4 h

[c]The incubation period was 6 h

The HepG2 and Jurkat cell lines, derived from liver cancer and leukemia, respectively, were used in the present study. We found that bLF and LF peptides inhibited the proliferation of HepG2 and Jurkat cells, reducing the cell viability of both cell lines. bLF induced apoptosis. LFchimera induced necrosis, and LFcin17-30 and LFampin265-284 induced both apoptosis and necrosis. Moreover, bLF and LF peptides had additive and synergistic effects in combination with chemotherapeutics. The results were similar to our previous work, in which we explored the anticancer properties of bLF and LF peptides (LFcin17-30, LFampin265-284 and LFchimera) against endometrial and cervical cancer, demonstrating inhibition of cell viability and cell morphological changes (Ramirez-Sanchez 2020).

Additionally, bLF has been shown to have anticancer activity against different types of cancer, such as breast cancer, colon cancer and squamous cell carcinoma. (Gibbons et al. 2015; Ma et al. 2013). However, there are only a few reports for the effects of bLF in liver cancer and leukemia. bLF modified in a liposome system has been demonstrated to have anticancer activity against hepatocellular carcinoma in vitro, decreasing the cell viability of HepG2, BEL7402 and SMMC7721 cells (Wei et al. 2015). Regarding the LF peptides, some studies have reported the activity of LF peptides against cancer (Onishi et al. 2008; Meng et al. 2017; Mader et al. 2005; Roy et al. 2002); however, there is only one study that has reported the anticancer activity of the synthetic LF peptides used in the present study (Ramirez-Sanchez 2020).

In the present study, bLF and LF peptides caused cell morphological changes and damage in HepG2 cells, which agreed with previous studies. For instance, Luzi et al. (2017) observed morphological changes characteristic of LF-induced apoptosis in HeLa tumor cells. In addition, Mader et al. (2005) observed that LFcinB (bLFcin17-41) induces apoptosis in Jurkat cells (Luzi et al. 2017; Mader et al. 2005). These studies prompted us to perform additional experiments to confirm the mechanism of action of bLF and synthetic bLF peptides through Annexin V and PI staining (Mader et al. 2005; Farkas et al. 2010; Berghe et al. 2010).

In the present study, we demonstrated that bLF and the synthetic LF peptides, LFcin17-30 and LFampin265-284, induced cell death by apoptosis in HepG2 and Jurkat cells. In addition, LFcin17-30 and LFampin265-284 induced not only apoptosis but also necrosis to some degree, which was consistent with previous research (Ramirez-Sanchez 2020). In other reports, Zhang et al. (2015) and Guedes et al. (2018) also determined that bLF triggers apoptosis in cancer cells, which agreed with the observations of the present study, in which bLF and LF peptides displayed

signs of apoptosis or even necrosis depending on the treatment (Guedes et al. 2018; Zhang et al. 2015). However, the concentrations of bLF in their studies were as high as 175 μM with treatment times of 48 and 72 h, obtaining between 50 and 70% inhibition percentages, similar to the results found in this work using lower concentrations. These results may be due to the highly metastatic and aggressive characteristics of the cell lines used in those reports, displaying more resistance to the treatments (Guedes et al. 2018; Zhang et al. 2015).

Treatment with LFchimera revealed different characteristics as it induced necrotic events in HepG2 and Jurkat cells. However, further research is needed. Previous studies have reported that synthetic peptides, such as a mutant of hLFcin17-41, induces necrosis in leukemic cells with good tolerability and low cytotoxicity, depending on the concentration used, and side effects in vivo have not been reported (Lu et al. 2016; Onishi et al. 2008). In particular, for liver cancer and leukemia, it has been reported that the fragment of bLF 17–41, called LFcin17-41 or LFcinB, inhibits the proliferation of cell lines derived from leukemia, lymphoma, breast, colon and ovary cancer (Mader et al. 2005). The fragment of bLF 17–38, called Pep1, also affects cell viability in HL-60 leukemia cells, inducing cell death by apoptosis or necrosis, depending on the concentration used (Onishi et al. 2008). Similarly, a synthetic mutant derived from hLFcin 21–31, called LF11-322 or PFR, has anticancer activity against HL-60 and MEL cell lines, reducing tumor growth induced by leukemia cells (MEL) in mice (Lu et al. 2016). Although the aforementioned studies used different peptide fragments, they provide a background and reaffirm the results observed in this study.

In Jurkat cells, we observed the effect of bLF and LF peptides more clearly, which may be due to the specific characteristics and/or receptors in their membranes or to the suspension conditions of this cell line, providing more surface available and movement in the media to increase the interaction and activity of the treatments. Thus, we used Jurkat cells to elucidate the specific pathway of apoptosis that is activated by bLF and LF peptides by expression analysis of genes related to this process. There are two main pathways of apoptosis, namely, the extrinsic and intrinsic pathways (Fujita et al. 2004; Mader et al. 2005). In the present study, LFcin17-30, LFampin265-284 and LFchimera increased the expression of *CYCS* and *BLID*, which are genes related to the intrinsic or mitochondrial pathway. These genes are triggered in the early stages of apoptosis; however, caspase activity was not considerably altered. Nevertheless, the ability of LF peptides to trigger the intrinsic pathway of apoptosis agreed with the results of Mader et al. (2005), who reported that bLFcinB activates this pathway in Jurkat cells (Mader et al. 2005). Similarly, Meng et al. (2017) reported that the LFcinB P-13 peptide triggers apoptosis and caspase activation via the intrinsic pathway in hepatocellular carcinoma in vitro and in vivo (Meng et al. 2017). However, the molecular mechanisms of the anticancer activity of bLF and LF peptides need to be elucidated to develop new strategies to combat these types of cancer.

A challenge for oncology research is the development of more effective treatments to improve the patient's quality of life. bLF and LF peptides can contribute to the improvement of chemotherapeutic treatments. bLF can be used in combination with chemotherapeutics, such as 5-fluorouracil, against colorectal cancer, stimulating the immune response, increasing serum IFN-γ and activating NK, CD4 + and CD8 + cells (Moastafa, et al. 2014). In addition, previous studies have observed an additive interaction between cisplatin and bLF or LF peptides in endometrial cancer, indicating that dose reduction of either component in combination may reduce the cytotoxicity of the treatment and thus side effects (Ramirez-Sanchez 2020).

Because liver cancer is difficult to diagnose, the disease is already in advanced stages when treatment is given. Consequently, the strategies implemented for its management are usually toxic with poor prognosis. Thus, it is important to have strategies to enhance the mechanism of action of treatments. In the present study, bLF and LFampin265-284 presented additive effects in HepG2 cells, while LFchimera and LFcin17-30 presented synergistic effects in combination with cisplatin in HepG2 cells. The combination treatment may be involved in the mechanism of action or the entry of one or both treatments when used together; specifically, the synergistic effect gives an additional effect, which is necessary in chemotherapeutic treatments because it may reduce the toxicity of the drugs.

Leukemia, which has a higher incidence in children, has both short- and long-term side effects, indicating the importance of providing effective

treatments. In Jurkat cells (leukemia), bLF and LFchimera showed additivity with etoposide, and LFampin265-284 and etoposide showed synergistic effects. However, LFcin17-30 and etoposide exhibited antagonistic effects in Jurkat cells; the concentrations of LFcin17-30 and etoposide used in the treatments of Jurkat cells were both higher than the other combinations (20 and 25 μM), respectively, which may have hindered the entry or action of one or both treatments due to saturation of the uptake system in general (cellular receptors or endocytosis processes). These findings contribute to progress in cancer research and provide new alternatives.

Nevertheless, to our knowledge, this is the first report exploring the anticancer activity and synergism of chemotherapeutics with bLF and LF peptides (LFcin17-30, LFampin265-284 and LFchimera) in Jurkat and HepG2 cells. Regarding the limitations of the present study, we did not quantitatively analyze the observed cell death. Further research is required to describe the expression of genes involved in the mechanism of action of the different treatments.

Conclusions

The present study provided evidence that bLF and LF peptides are effective against hepatocellular cancer and leukemia cell lines by reducing cell viability, producing morphological changes, inducing apoptosis and inducing necrosis. In the present study, we found that bLF triggers apoptosis and that LFchimera induces cell necrosis, whereas LFcin17-30 and LFampin265-284 promote apoptosis and induce necrosis to some degree. However, further investigations are needed to elucidate the complete mechanism of action of these treatments. Additionally, bLF and LF peptides can act in combination with commonly used chemotherapeutics, such as cisplatin and etoposide, with additive and synergistic effects. These results suggested that bLF and LF peptides can be used to improve chemotherapeutic treatments, suggesting that additional studies should be performed.

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Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose. The authors declare no conflicts of interest.

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