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Molecular Mechanisms Underlying the Anticancer Activity of Lactoferrin in Highly Metastatic Cancer Cell Lines

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Tese de Mestrado

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Trabalho efetuado sob a orientação de

Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real

Professora Doutora Lígia Raquel Marona Rodrigues

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MOLECULAR MECHANISMS UNDERLYING THE ANTICANCER ACTIVITY OF LACTOFERRIN IN HIGHLY METASTATIC CANCER CELL LINES

ABSTRACT

Cancer is currently one the most lethal disease worldwide and metastases remain the main cause of cancer-associated mortality, which reinforces the importance of developing more targeted and efficient cancer therapies. In this sense, lactoferrin (Lf) has emerged as a safe and effective agent in cancer therapy. Lf is a natural iron-binding protein derived from milk that is present in many tissues and biological fluids. Interestingly, it was found that Lf displays anticancer and anti-metastatic activities against several cancer cell lines. However, Lf cellular targets implicated in its mechanisms of action are poorly elucidated, which limits the usage of Lf in cancer therapy. Hence, unveiling the targets of Lf underlying its anticancer activity is of prime relevance and it will be explored in the current work.

Recently, results of our group showed that bovine Lf (bLf) is preferentially cytotoxic to highly metastatic breast cancer cells through inhibition of the plasmalemmal proton pump V-ATPase, while exhibiting no effect on non-tumorigenic cells. In the present study, we aim to ascertain whether this same mechanism of action could explain the anticancer/anti-metastatic activity of bLf against other types of highly metastatic cancer cells. To this end, three highly metastatic cancer cell lines, reported to display V-ATPase at the plasma membrane, were used: PC-3, MG-63 and MDA-MB-231, prostate, osteosarcoma and breast cancer cell lines, respectively. Results showed that the susceptibility to bLf of PC-3 and MG-63 cancer cell lines was similar to that of the breast cancer MDA-MB-231 regarding both inhibition of cell proliferation and induction of intracellular acidification. Moreover, we found that V-ATPase expression seemingly increased in the three cell lines in comparison to a non-tumorigenic cell line. These data encouraged us to implement several biochemical and analytical approaches, including flow cytometry, western blot, immunofluorescence and confocal microscopy in order to dissect the possible interplay between V-ATPase and bLf in these cell lines. Overall, the results herein obtained may be further explored to develop new cancer therapy strategies against highly metastatic cancers.

MECANISMOS MOLECULARES SUBJACENTES À ATIVIDADE ANTICANCERÍGENA DA LACTOFERRINA EM LINHAS CELULARES DE CANCRO ALTAMENTE METASTÁTICAS

RESUMO

Atualmente, o cancro é uma das doenças mais letais no mundo e as metástases constituem a principal causa de mortalidade associada ao cancro, o que reforça a importância do desenvolvimento de terapias mais direcionadas e eficazes. Neste sentido, a lactoferrina (Lf) tem surgido como um agente seguro e eficaz na terapia do cancro. A Lf é uma proteína natural com afinidade para o ferro, derivada do leite, que está presente em muitos tecidos e fluídos biológicos. Curiosamente, foi descoberto que a Lf exibe atividade anticancerígena e anti-metastática contra uma vasta gama de linhas celulares cancerígenas. Contudo, ainda não se conhecem os alvos celulares da Lf que estão implicados no seu mecanismo de ação, o que limita o uso desta proteína na terapia do cancro. Assim, a descoberta dos alvos subjacente à atividade anticancerígena da Lf é de extrema importância e será explorada no presente trabalho.

Recentemente, resultados do nosso grupo demonstraram que a Lf de origem bovina (bLf) é preferencialmente citotóxica para linhas de cancro da mama altamente metastáticas através da inibição da bomba de protões V-ATPase, não tendo qualquer efeito em células não tumorigénicas. No presente estudo, o nosso objetivo é entender se este mecanismo de ação poderá explicar a atividade anticancerígena e anti-metastática da bLf contra outros tipos de células de cancro também altamente metastáticas. Desta forma, para todos os ensaios foram utilizadas três linhas celulares de cancro altamente metastáticas, descritas como tendo V-ATPase na membrana plasmática, nomeadamente: PC-3, MG-63 e MDA-MB-231, linhas celulares de cancro de próstata, osteossarcoma e cancro da mama, respetivamente. Os resultados demonstraram que, à semelhança do que acontece na linha celular de cancro de mama, as linhas celulares PC-3 e MG-63 são também sensíveis à bLf, quer a nível da inibição da proliferação celular quer da indução de acidificação intracelular. Além disso, nas três linhas foi observado um aumento na expressão da V-ATPase em comparação com uma linha não tumorigénica. Estes dados impulsionaram a utilização de várias técnicas bioquímicas e analíticas, incluindo citometria de fluxo, *western blot* e microscopia de fluorescência e confocal, com o objetivo de entender a relação entre a V-ATPase e a bLf nestas linhas celulares. Em suma, os resultados aqui obtidos poderão ser explorados para o desenvolvimento de novas estratégias de terapia para cancros altamente metastáticos.

SCIENTIFIC OUTPUT

Scientific Publications

1. Cátia S. Pereira, Joana P. Guedes, Marília Gonçalves, Luís Loureiro, Lisandra Castro, Hernâni Gerós, Lígia R. Rodrigues, Manuela Côrte-Real (2016) "Lactoferrin selectively triggers apoptosis in highly metastatic breast cancer cells through inhibition of plasmalemmal V-H^+-ATPase". *Oncotarget*. DOI 10.18632/oncotarget.11394

2. Joana P. Guedes, Cátia S. Pereira, Lígia R. Rodrigues, Manuela Côrte-Real (2016) "V-ATPase as a target of lactoferrin in highly metastatic cancer cell lines". *In prep*.

Panel Communications

1. Joana P. Guedes, Cátia S. Pereira, Lígia R. Rodrigues, Manuela Côrte-Real (2016) "Insights on the Molecular Mechanisms Underlying the Anticancer Activity of Lactoferrin in Highly Metastatic Cancer Cell Lines". XIX National Congress of Biochemistry, 8-10/12, Guimarães, Portugal.

2. Cátia S. Pereira, Joana P. Guedes, Marília Gonçalves, Luís Loureiro, Lisandra Castro, Hernâni Gerós, Lígia R. Rodrigues, Manuela Côrte-Real (2016) "Lactoferrin selectively triggers apoptosis in highly metastatic breast cancer cells through inhibition of plasmalemmal V-H^+-ATPase". XIX National Congress of Biochemistry, 8-10/12, Guimarães, Portugal.

3. Cátia S. Pereira, Joana P. Guedes, Marília Gonçalves, Luís Loureiro, Lisandra Castro, Hernâni Gerós, Lígia R. Rodrigues, Manuela Côrte-Real (2016) "Lactoferrin selectively triggers apoptosis in highly metastatic breast cancer cells through inhibition of plasmalemmal V-H+-ATPase". 24th congress of the European Cell Death Organization - "Cell Death in Health and Disease", 28-30/09, Barcelona, Spain.

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List of Abbreviations and Acronyms

ANOVA	**AN**alysis **O**f **VA**riance
ATCC	**A**merican **T**ype **C**ulture **C**ollection
ATP	**A**denosine **T**ri**P**hospate
BafA1	**Baf**ilomicin **A1**
BCECF-AM	2',7'-**B**is-(2-**C**arboxy**E**thyl)-5-(and-6)-**C**arboxy**F**luorescein - **A**cetoxy**M**ethyl Ester
bLf	**b**ovine **L**actoferrin
BSA	**B**ovine **S**erum **A**lbumin
Caspase	**C**ystein-dependent **a**spartate **s**pecific **p**rote**ase**
Cdk	**C**yclin-**d**ependent **k**inase
CF	Carboxyfluorescein
CFSE	**C**arboxy**F**luorescein diacetate **S**uccinimidyl **E**ster
ConcA	**Conc**anamycin **A**
DMEM	**D**ulbecco's **M**odified **E**agle's **M**edium
DMSO	**D**i**M**ethyl **S**ulf**O**xide
DNA	**D**eoxyribo**N**ucleic **A**cid
DRMs	**D**etergent-**R**esistant **M**embrane**s**
ECM	**E**xtra**C**ellular **M**atrix
EFSA-NDA	**E**uropean **F**ood **S**afety **A**uthority – Panel on **D**ietetic Products**,** **N**utrition and **A**llergies
FBS	**F**etal **B**ovine **S**erum
FDA	**F**ood and **D**rug **A**dministration
FITC	**F**luorescein **I**so**T**hio**C**yanate
GPI	**G**lycosyl**P**hosphatidyl**I**nisotol
HBSS	**H**ank's **B**alanced **S**alt **S**olution
hLf	**h**uman **L**acto**f**errin
Lf	**L**acto**f**errin
MCT	**M**ono**C**arboxylate **T**ransporter
MMP	**M**atrix **M**etallo**P**roteinase

MβCD	**M**ethyl-**β**-**C**iclo**D**extrin
NK	**N**atural **K**iller
NPC	**N**aso**P**haryngeal **C**ancer
PBS	**P**hosphate **B**uffered **S**aline
PBST	**P**hosphate **B**uffered **S**aline **T**ween
PFA	**P**ara**F**orm**A**ldehyde
pHcyt	**cyt**osolic **pH**
pHe	**e**xtracellular **pH**
pHi	**i**ntracellular **pH**
PI	**P**ropidium **I**odide
PS	**P**hosphatidyl**S**erine
PVDF	**P**oly**V**inylidene **D**i**F**luoride
rLf	**r**ecombinant **L**acto**f**errin
RNA	**R**ibo**N**ucleic **A**cid
ROS	**R**eactive **O**xygen **S**pecies
rpm	**r**otations **p**er **m**inute
RT	**R**oom **T**emperature
S.D.	**S**tandard **D**erivation
S.E.M.	**S**tandard **E**rror of the **M**ean
SDS	**S**odium **D**odecyl **S**ulfate
shRNA	**S**hort **H**airpin **R**ibo**N**ucleic **A**cid
TME	**T**umor **M**icro**E**nvironment
TNT	**T**unneling **N**ano**T**ubes
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
V-ATPase	**V**acuolar proton-translocating **ATPase**

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Chapter I

Introduction

I.1. CANCER, METASTASIS AND ANTICANCER DRUGS: A BRIEF OVERVIEW

Cancer has been defined as a disease that is characterized by a wide range of features, which are summarized in the hallmarks of cancer. These hallmarks include unregulated cell proliferation due to the loss of growth control, escape to signals that control the normal cell behaviour, activation of invasion and metastasis, induction of angiogenesis, among others. Recently, genome instability, deregulation of cellular energetics and escape to immune destruction have been added to those hallmarks (Hanahan and Weinberg, 2011).

Currently, cancer is the most lethal disease worldwide, which supports the great need for more accurate and targeted cancer therapies. In 2012, about 14.1 million new cancer cases and 8.2 million deaths due to cancer were reported (World Health Organization, 2014). A recent study performed by Ferlay and colleagues showed that the most frequently diagnosed cancers are lung, breast, colorectal and prostate with incidence rates of 1.8, 1.67, 1.36 and 1.1 million, respectively. Lung, liver and stomach cancers are responsible for the most common cancer-associated deaths (Ferlay et al., 2015). On the other land, metastases are the main cause of cancer-associated mortality. The metastatic process involves two major steps: (i) physical translocation of cancer cells to a distant organ, and (ii) colonization of the translocated cells in this organ (Chaffer and Weinberg, 2011). In the first step, after acquisition of the invasive phenotype, the cancer cells invade the matrix and enter into the bloodstream, transiting to distant organs. Afterwards, when out of circulation, cells invade the foreign tissue, after overcoming resistance by the immune system, and are able to survive in the secondary site as a single cell, promoting the colonization. Therefore, for the development of metastases, cancer cells require not only the integration of multiple events, such as invasion and angiogenesis, but also the adaptation to a foreign microenvironment (Martin et al., 2013).

Combined therapies may be required to overcome cancer. Cancer therapy has been mainly focused on the use of different anticancer drugs, from natural to natural-derived products, attempting to set aside therapeutic measures such as surgery, chemotherapy, radiation and hormones. These conventional treatments are costly, undesirable due to severe side effects and can only extend the patient's lifespan by a few years. Hence, there has been a continuous search for less expensive and non-toxic natural drugs (Reddy et al., 2003; Prasad and Tyagi, 2014). Indeed, in the recent decades, 78% of the drugs approved by the Food and Drug Administration (FDA) are natural or natural-derived drugs, from which 48,6% are currently in use in cancer therapy (Prasad and Tyagi, 2014) (Table I.1).

Table I.1. Food and Drug Administration-approved drugs that include natural and natural-derived compounds.

Drug	Origin	Target	Cellular mechanism affected	Type of Cancer (Reference)
Docetaxel Paclitaxel	Plant	Phosphorylation of Bcl-2	Cell survival	Breast Cancer (Nobili et al., 2009)
Etoposide	Plant	Arrest at S/G2 phase DNA topoisomerase II	Cell cycle	Prostate cancer (Gao et al., 2013)
Vinorelbine	Plant	Tubulin disruption	Cell cycle	Breast cancer (Nobili et al., 2009)
Genistein	Plant	Inhibition of NF-kB	Tumor cell invasion	Breast cancer (Valachovicova et al., 2004)
Gambogic acid	Plant	Downregulation of VEGFR-2 and AKT	Tumor cell angiogenesis	Prostate cancer (Yi et al., 2008)

Several drugs, as specified in Table I.1, derived from natural compounds have been used in the treatment of several types of cancers including prostate, colorectal, lung, breast, stomach, lymphoblastic leukemia and brain tumours and have different targets, such as tubulin, microtubules and DNA topoisomerases(Prasad and Tyagi, 2014). The interaction between the drug and the respective target culminates in one or more of the following consequences: suppression of invasion, inflammation, angiogenesis, proliferation and metastasis of cancer cells (Gupta et al., 2010).

Among the different natural compounds with anticancer activities, some are present in food or are food-derived compounds, commonly referred as nutraceuticals, either of plant or animal origin or produced by microorganisms. Particularly, throughout the years researchers have explored the anticarcinogenic activity of individual milk compounds such as fat components (e.g., linoleic acid, lipids and sphingomyelin, among others) or proteins (e.g., caseins and lactoferrin) (Håkansson et al., 1995; Parodi, 1999; Tsuda et al., 2000; Sah et al., 2015). However, this issue is somewhat controversial. Several authors have reported that it is not clear the beneficial role associated to milk consumption, particularly in women. In 2014, a study demonstrated that high milk administration seems to be associated to the higher mortality incidence in women (Michaëlsson et al., 2014). Moreover, the effect of milk consumption in cancers is also a controversial issue since some evidences indicate its association with an increased risk of prostate cancer (Qin et al., 2004) but a decreased risk of colorectal cancer

(Larsson et al., 2015). On the other hand, other authors reported that milk administration does not affect prostate tumour progression, namely inflammation, fibrosis and invasiveness (Bernichtein et al., 2015). Nevertheless, despite this controversy, some milk compounds and/or milk-derived bioactive peptides have been identified not only as an important part of a normal daily diet, but also as potential agents for cancer prevention and management (Mohanty et al., 2015; Sah et al., 2015). In particular, lactoferrin (Lf), an important protein in the milk whey fraction, was found to have an inhibitory potential against chemically-induced and hereditary carcinogenesis and metastasis (Tsuda et al., 2000; Sah et al., 2015; Pereira et al., 2016). Since this thesis is focused on the study of this natural protein and its anticancer activity, these topics will be further detailed in the following sections.

I.2. LACTOFERRIN: AN IRON-BINDING GLYCOPROTEIN

The transferrin family is a group of single-chain glycoproteins found in both vertebrates and invertebrates with the ability to bind tightly iron and transport it from plasma to cells. Therefore, this family of proteins has a crucial role in the iron metabolism, controlling its levels in biological fluids (Lambert, 2012; Hughes and Friedman, 2014). In vertebrates, transferrins bind and release ferric ions (Fe^{3+}) to respond to receptor binding and changes in pH (Lambert, 2012). This group includes several proteins with different tissue distribution namely: serum transferrin, ovotransferrin, lactotransferrin, also known as lactoferrin (Lf), and melanotransferrin (Table I.2) (Lambert, 2012; Hughes and Friedman, 2014).

Table I. 2. Characteristics of transferrin family members (adapted from Lambert, 2012)**.**

Protein	Gene	Iron binding	Tissues	Taxonomic groups
Serum transferrin	*TF*	Yes	Liver, serum, brain, pancreas, bone marrow	Mammals
Ovotransferrin	*OTF*	Yes	Serum, egg, yolk	Birds and reptiles
Lactotransferrin	*LTF*	Yes	Milk, saliva, tears, bile, other secretions	Mammals

Melanotransferrin	*MFI2*	N-lobe only	Melanomas, saliva, sweat, liver, intestine	Vertebrates

Lf is an 80 kDa protein containing around 700 amino acids. Its three-dimensional structure is composed by a single polypeptide chain folded into two highly homologous iron-binding lobes, namely the C-lobe (carboxyl) and the N-lobe (amino). Each lobe is further divided in two domains known as N1 and N2, C1 and C2 that enclose a deep cleft where the iron-binding site is located. Therefore, each Lf protein is capable of binding two iron ions with the concomitant binding of two carbonate ions (CO_3^{2-}) (González-Chávez et al., 2009). The two lobes are connected through a region containing parts of α-helix responsible for the flexibility of the molecule (Fig. I.1) (Öztafl and Özgünefl, 2005; Baker and Baker, 2012).

I.2.1. Structure and Synthesis of Lactoferrin

Lf is an 80 kDa protein containing around 700 amino acids. Its three-dimensional structure is composed by a single polypeptide chain folded into two highly homologous iron-binding lobes, namely the C-lobe (carboxyl) and the N-lobe (amino). Each lobe is further divided in two domains known as N1 and N2, C1 and C2 that enclose a deep cleft where the iron-binding site is located. Therefore, each Lf protein is capable of binding two iron ions with the concomitant binding of two carbonate ions (CO_3^{2-}) (González-Chávez et al., 2009). The two lobes are connected through a region containing parts of α-helix responsible for the flexibility of the molecule (Fig. I.1) (Öztafl and Özgünefl, 2005; Baker and Baker, 2012).

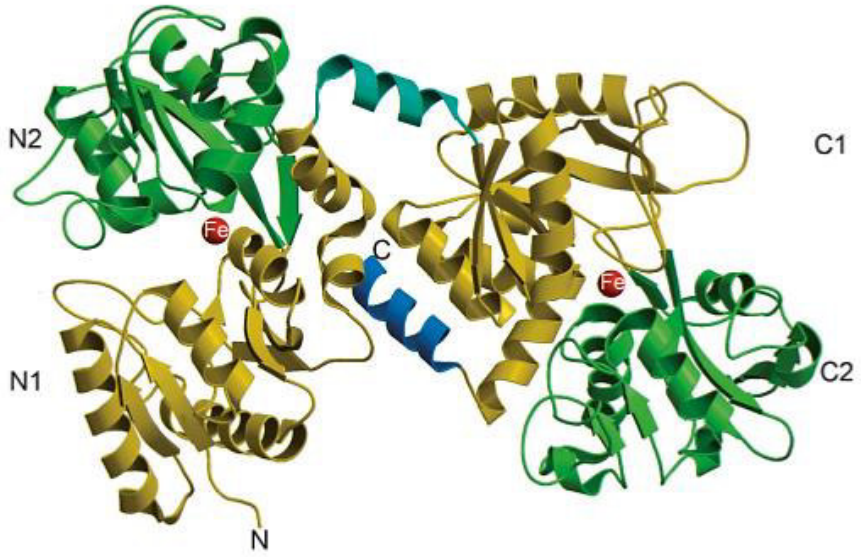

Figure I.1. Structure of lactoferrin. Crystal structure of Lf that illustrates its N1 and C1, N2 and C2 domains in yellow and green, respectively. The α-helix that binds the two domains is shown in turquoise, the C-terminal α-helix in blue, and the bound iron in red (adapted from Baker and Baker, 2012).

As previously mentioned, Lf has affinity to Fe^{3+}, but this binding is reversible. Accordingly, Lf can exist free or associated with Fe^{3+} as apo-Lf and holo-Lf, respectively. Consequently, the Lf molecule can adopt two different three-dimensional conformations: apo-Lf exhibits an open conformation while holo-Lf presents a closed conformation (Wally and Buchanan, 2007; González-Chávez et al., 2009; García-Montoya et al., 2012). Hence, large-scale conformational changes are associated with the iron binding and release, in which the domains close over the bound ion or open to release it (Baker and Baker, 2012).

Lf is synthesized by mucosal epithelial cells and neutrophils during inflammatory processes. As referred before, this protein is present in many tissues and body fluids as well as in many mammalian secretions. Indeed, it can be found in tears, saliva, semen, urine, pancreatic, gastric and vaginal fluids, bronchial and nasal secretions, and at higher concentrations in milk and colostrum, being the second most abundant milk protein after casein (González-Chávez et al., 2009; Alexander et al., 2012; García-Montoya et al., 2012).

I.2.2. Biological Functions and Applications of Lactoferrin

The Lf structure endows it multiple biological activities including: strong antimicrobial activity (Yamauchi et al., 2006; Yen et al., 2011), regulation and maintenance of iron absorption, anti-

inflammatory and anticarcinogenic activities (González-Chávez et al., 2009; García-Montoya et al., 2012). Figure I.2 illustrates some of activities that have been proposed for Lf.

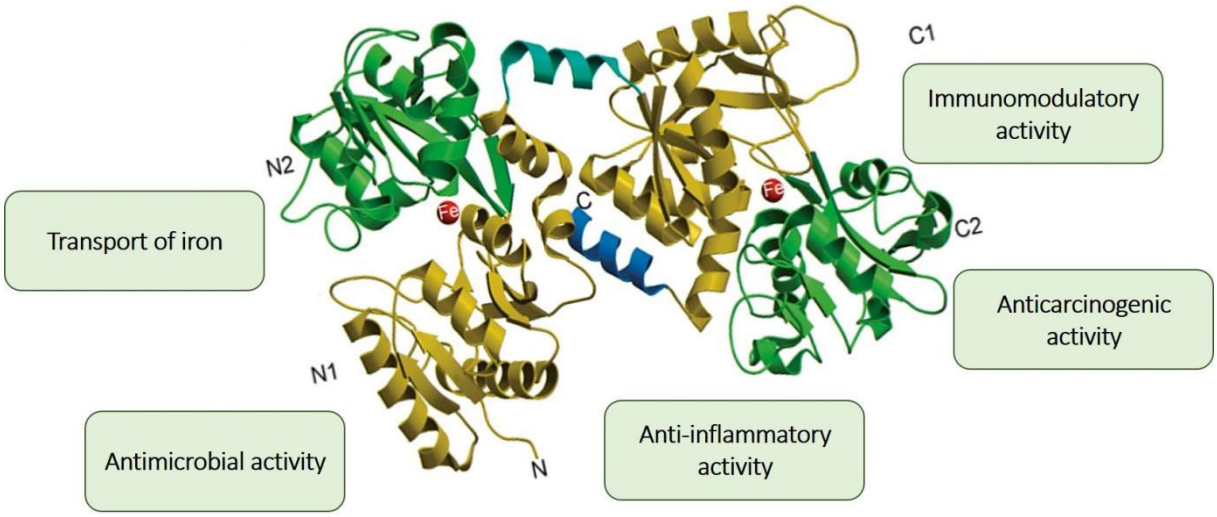

Figure I.2. Main biological functions and activities described for Lf.

Regarding its antimicrobial activity, Lf exhibits a strong toxic effect against a wide spectrum of bacteria (Gram + and Gram -), fungi, yeasts, viruses, protozoa and parasites (Yamauchi et al., 2006; González-Chávez et al., 2009; García-Montoya et al., 2012). There are some mechanisms proposed to explain the Lf antibacterial activity such as iron sequestration from bacterial pathogens, which inhibits their growth (Zarember et al., 2007); direct interaction with bacterial cell surface by an iron-independent mechanism (Farnaud and Evans, 2003; Valenti and Antonini, 2005); and weakening the attachment of certain bacteria to the host cells (Diarra et al., 2003). On the other hand, the anti-viral activity of Lf relies on the inhibition of the internalization and replication of certain viruses in the host cell and the stimulation of the host immune system (Welsh et al., 2011).

Lf is also well known by its immunomodulatory and anti-inflammatory properties. Indeed, Lf is a major component of the secondary granules of neutrophils, being released upon neutrophil activation, which leads to an increase of its levels (Masson et al., 1969). This increase in Lf levels, in turn, promotes the recruitment of leukocytes, cytokines, natural killer (NK) and adaptive cells, and the activation of dendritic cells that allow the control of the excessive inflammation and stimulate the immune response (González-Chávez et al., 2009; García-Montoya et al., 2012). The role of Lf in regulating innate immune responses confirms its crucial function as a first line host defence mechanism against invading pathogens (Puddu et al., 2009).

Anticancer and anti-metastatic activities are two other relevant biological activities of Lf that are the subject of the present study and therefore will be discussed in more detail in the next subtopic.

Based on its multiple biological activities, Lf has been used in several applications. In particular, clinical trials have been conducted to evaluate the effectiveness of Lf administration in the treatment of a large variety of human diseases (González-Chávez et al., 2009; Tomita et al., 2009; García-Montoya et al., 2012). The first clinical application of Lf was in infant formulas to improve the intestinal microbial flora (Roberts et al., 1992), enhance serum ferritin (Chierici et al., 1992) and haematocrit levels and to prevent nosocomial infections (Manzoni et al., 2009). Besides, the activity of Lf and its bioactive peptides have been proved in *in vitro* an *in vivo* studies against several pathogens, in the treatment of infections, inflammatory diseases and cancer (González-Chávez et al., 2009; Kozu et al., 2009; García-Montoya et al., 2012). Concerning its administration, the oral intake of Lf has been suggested to be the most promising option as it is easy and safe, and also because more than 60% of the ingested bLf remains intact after the passage through adult human stomach and entrance in the small intestine (Troost et al., 2001; Mayeur et al., 2016).

Lf can also be used as a reliable disease biomarker because its increased levels can be used as a clinical indicator of inflammatory syndromes. In fact, increased levels of fecal Lf have been found in inflammatory bowel disease and allow distinguishing it from irritable bowel disease (Kane et al., 2003; Zhou et al., 2014). Furthermore, it was shown that Lf is downregulated in nasopharyngeal carcinoma (NPC) cells, and its increased expression has therefore been associated with a good prognosis (Zhang et al., 2015b).

The form of Lf that has been mostly used in the health care field, both in prevention and treatment of diseases, is the one from bovine origin. Indeed, bLf has already been approved by the European Food Safety Authority (EFSA) as a safe ingredient for various applications, including for medical purposes (Efsa, 2012; Mayeur et al., 2016). bLf can easily be isolated from cow's milk by several purification methods and it is produced in large scale by manufacturing companies mainly using a cation-exchange chromatography system (Tomita et al., 2009). However, taking into account the multiple applications of bLf, distinct processes to produce it at a large scale have been exploited. These alternative processes include the production of recombinant Lf (rLf) in bacterial, viral and fungal expression systems, or production using transgenic plants and animals able to express rLf (González-Chávez et al., 2009; García-Montoya et al., 2012).

I.2.2.1. Anticancer and Anti-Metastatic Activities of Lactoferrin

As previously mentioned, anticancer and anti-metastatic activities are among the many different biological activities assigned to Lf. Indeed, the Lf anticancer activity has been observed against a wide range of human cancers in different cell lines, animal models and even in clinical trials (Gibbons et al., 2011; Yin et al., 2013; Zhang et al., 2014a). The Lf anticancer activity was demonstrated for the first time in 1994 in mice. Using this model, this protein was found to strongly decrease the solid tumour growth and inhibit the metastasis occurrence. This study proved the potential of Lf as an anticancer compound, and since then it has been explored for therapeutic purposes (Bezault et al., 1994). Also, in 1995, it was established that the whey fraction of bovine milk could significantly inhibit the development of colon tumours in rats (McIntosh et al., 1995). These pioneer studies and others encouraged scientists to investigate the protein effects on different types of cancer like bladder (Masuda et al., 2000), colon (Fujita et al., 2004), head and neck (Xiao et al., 2004; Wolf et al., 2007), stomach (Xu et al., 2010), lung (Tung et al., 2013) and breast (Duarte et al., 2011; Zhang et al., 2015; Pereira et al., 2016).

Some studies addressed the effect of bLf on colorectal cancer demonstrating that the oral intake of 3 g of bLf per day during 1 year induced a significant delay of the growth of colorectal adenomatous polyps in human patients. These data suggest that bLf inhibits colorectal carcinogenesis and serves as an alternative to the surgical removal of polyps (Kozu et al., 2009; Tsuda et al., 2010). Moreover, due to the *in vitro* high resistance of non-tumorigenic cell lines to Lf (Gibbons et al., 2015; Pereira et al., 2016) and absence of secondary effects in humans (Gibbons et al., 2011; Yin et al., 2013; Zhang et al., 2014a), bLf exhibits a great potential to be safely used in cancer therapy.

I.3. MECHANISMS UNDERLYING LACTOFERRIN ANTICANCER ACTIVITY

Several researchers along the last years have already provided some mechanistic insights on the anticancer activity of Lf based on its ability to interfere with the cell membrane and its receptors, cell cycle progression and induction of apoptosis, as well as on its anti-metastatic and anti-angiogenesis potential, immunostimulation and iron sequestration capacity (Fig. I.3).

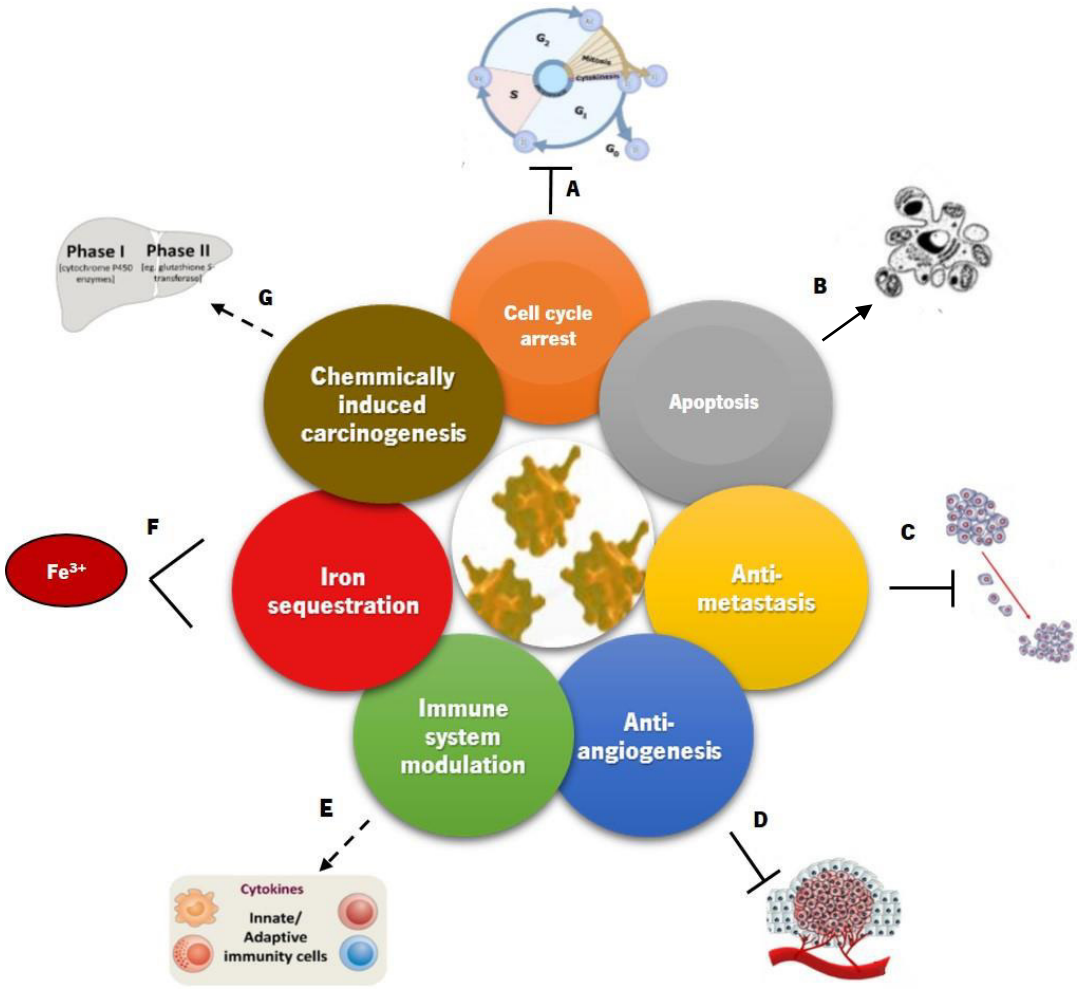

Figure I.3. Proposed mechanisms for the anticancer activity of Lf.

Legend: → Induction ⊣ Inhibition - → Modulation < Sequestration

Regarding Lf membrane receptors, many studies have demonstrated its presence in cancer cells and each cell type seems to have its specific Lf receptors. The cancer cells that overexpress Lf receptors may be more susceptible to Lf, thus creating an opportunity for the development of cancer-specific drug carriers coated with Lf that may be administered intravenously Moreover, it has been demonstrated an association between Lf and heparan sulfate, in which Lf exhibits conformation-dependent uptake efficiency correlated with efficient binding to heparan sulfate and lipid-induced conformational changes. Accordingly, when heparan sulfate chains are removed, membrane binding and cellular uptake of the peptide are reduced (Duchardt et al., 2009). Furthermore, Lf selective cytotoxicity for cancer cells can be explained by differences in the cellular membrane. The membrane of cancer cells has higher levels of negatively charged molecules when compared with normal cells, which attracts the cationic Lf/Lf-derived peptides. This membrane feature favours a preferentially binding of Lf to cancer cells than to normal cells

(Szachowicz-Petelska and Dobrzynska, 2010). These studies suggest that the membrane can be one of the Lf targets through which it exerts its anticancer activity.

The Lf anticancer activity also relies on its ability to trigger cell cycle arrest (Fig. I.3 A), which occurs predominantly at the G1 phase (Wolf et al., 2007; Wang et al., 2011). Lf can induce this process by decreasing phosphorylated Akt to increase the expression of p21 and p27, which are known inhibitors of the cell cycle, and by stopping the transition of G1 to S of MDA-MB-231 breast cancer cells with decrease in the levels of proteins Cdk2, Cdk4 and cyclin E, and increase in the expression of Cdk inhibitor p21 (Damiens et al., 1999; Xiao et al., 2004). Moreover, this Lf inhibitory effect was reported as cell type-dependent, since in MDA-MB-231 cells the arrest was observed at G2 phase, whereas in MCF-7 cells there was an arrest at G1 phase with low doses of Lf and an arrest at G2 phase with higher doses (Zhang et al., 2014b).

Apoptosis is a highly regulated and conserved process of cell death that involves several events namely cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation culminating in cell demise and self-destruction without the release of cytosolic contents that trigger an inflammatory response. The regulation of apoptosis is crucial for cell homeostasis and therefore, deregulation of apoptosis can be associated with many pathologies and diseases (Meier and Vousden, 2007; Redza-Dutordoir and Averill-Bates, 2016). Lf-treated cancer cells showed alterations in the proteins involved in apoptosis, such as Bcl-2 family members and p53 (Fig. I.3 B). In human stomach and breast cancer cell lines, the levels of the anti-apoptotic protein Bcl-2 were found to be downregulated by Lf (Xu et al., 2010; Zhang et al., 2014b). Another study showed that, in mice bearing EMT6 breast cancer, apoptosis was induced by decreasing the expression of Bcl-2 and increasing the expression of the pro-apoptotic Bax and the executioner caspase-3 (Wang et al., 2011). Indeed, diet supplemented with bLf decreased carcinogenesis as it increased the expression of fatty acid synthase, caspase-3 and caspase-8, leading to DNA fragmentation in rat colon mucosa (Fujita et al., 2004). Additionally, bLf was found to induce apoptosis and inhibit colony formation of MCF-7 cells, which was associated with mitochondria membrane depolarization, decrease of Bcl-2 levels, cell cycle arrest at G1/G0 phase, and downregulation of CDC25c (Zhang et al., 2015c). Recently, we showed that bLf preferentially inhibits proliferation and induces apoptosis in the highly metastatic breast cancer cell lines MDA-MB-231 and Hs 578T, as well as decreases the extracellular acidification rate and increases intracellular acidification in these two highly metastatic cancer cells (Pereira et al., 2016).

Furthermore, the anticancer activity of Lf also stems from its anti-metastatic potential (Fig. I.3 C), as already mentioned. Several authors demonstrated that Lf is able to reduce or inhibit lung colonization in melanoma and lymphoma cells and colon carcinoma (Bezault et al., 1994; Iigo et al., 1999). According to Iigo and colleagues (Iigo et al., 2004), increased production of IL-18 and activation of NK and T cells to enhance immunity is a consequence of the protective role of Lf against metastasis (Iigo et al., 2004). Recently, Gibbons and colleagues evaluated the proliferation, migration and invasion of two breast cancer cell lines namely MDA-MB-231 and MCF-7 cells when treated with apo-Lf and holo-Lf. Both Lf forms induced a reduction of cell proliferation, as well as the invasion in both cell lines and the inhibition of the expression of survivin, an inhibitor of apoptosis (Gibbons et al., 2015). Therefore, Lf can inhibit tumour metastasis by inhibiting growth and decreasing the number of tumour-induced blood vessels, which indicates that bLf also exhibits anti-angiogenic activity (Fig. I.3 D) (Yeom et al., 2011). Recently, it was shown that bLf inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis since oral administration of bLf in transgenic mice overexpressing the human VEGF-A165 gene suppressed the formation of tumours. This suggests that these effects of Lf might be attributed at least in part to the inhibition of new blood vessel formation. Lf anti-angiogenic properties have important implications in prevention or treatment of angiogenesis-related diseases, such as cancer and chronic inflammatory diseases (Tung et al., 2013; Mayeur et al., 2016).

The ability of Lf to modulate the immune system is another capacity of this protein that may underlie its anticancer activity (Fig. I.3 E). The immunomodulatory activity of Lf is considered a key factor for the *in vivo* anticancer effects of Lf and involves the modulation of the production of cytokines, such as TNF-α, IL-1b, IL-6 and IL-8, encompassing both innate and adaptive immunity (Yeom et al., 2011; Legrand and Mazurier, 2010). In fact, several studies in animal models established that Lf is capable of modulating the production and activation of cytokines in immune cancer cells (Wolf et al., 2007). On the other hand, when it is released from activated neutrophils, Lf was shown to inhibit lymphocytes proliferation and granulopoiesis, to suppress antibody production and to regulate NK cells. Moreover, at low concentrations, Lf can stimulate NK cells and macrophages against cancer cells by activating a strong Th1 response (Fischer et al., 2006) and promote the recruitment of tumour-infiltrating lymphocytes, such as $CD4^+$ and $CD8^+$, which greatly inhibit the proliferation of cancers (June, 2007).

Another mechanism involved in the anticancer activity of Lf is dependent on Lf iron-binding ability (Fig. I.3 F). Free iron has been found to act as a mutagenic promoter by inducing oxidative damage to nucleic acids and in the gastrointestinal tract (Toyokuni, 2009). Hence, since Lf can tightly bind iron and

hold it in a stable nonreactive form, it can mitigate the local reactive oxygen species (ROS) production thus reducing locally the risk of oxidant-induced carcinogenesis (Rodrigues et al., 2009; Mayeur et al., 2016).

Finally, another mechanism underlying the anticancer activity of Lf was established in models of chemically induced carcinogenesis (Fig. I.3 G). This induction process encompasses two stages, namely initiation and post-initiation. The first stage includes phase I enzymes like cytochrome P450, responsible for the liver detoxification metabolism and activation of carcinogens leading to DNA damage in the specific organs. Liver phase II enzymes, also known as "blocking agents", suppress this activation and are responsible for detoxication and excretion – post-initiation stage. The compounds that can inhibit this phase by suppressing the proliferation of pre-malignant cells are called "suppressing agents". Several types of cancers (colon, lung, bladder, among others) in rat and hamster models of chemical carcinogenesis have been inhibited by oral administration of bLf, meaning that bLf acts as a blocking and suppressing agent by inhibiting phase I enzymes and stimulating the phase II enzymes (Tsuda et al., 2002; Mohan et al., 2006).

Although all the mechanisms referred above point to a clear anticancer role of Lf, its cellular targets are still elusive (Rodrigues et al., 2009; Zhang et al., 2014b; Mayeur et al., 2016). More recently, our group identified V-ATPase as a target of Lf. Currently, it is recognized that V-ATPase plays a prominent role in maintaining the tumour microenvironment (TME) acidosis, as well as in the metastatic process. It has therefore been suggested as a target in cancer therapy, as it will be discussed below.

I.4. V-H^+-ATPASE: A MOLECULAR TARGET FOR CANCER THERAPY

The vacuolar-type H^+-ATPase (V-H^+-ATPase) is an ATP-driven enzyme of the rotary ATPases family that transforms the energy of ATP hydrolysis in a proton electrochemical potential across different biological membranes, thus mediating a primary active transport of H^+ (Beyenbach and Wieczorek, 2006; Cipriano et al., 2008). A diverse collection of physiological processes depend on V-ATPases, and a number of diseases have been associated with anomalies of these pumps (Bowman and Bowman, 2005).

At the structural and functional level, V-ATPase is a multi-subunit complex comprising two major functional domains known as V_1 and V_0. The V_1 domain (cytoplasmic domain) is soluble and interacts with ATP, ADP and inorganic phosphate through several subunits identified with capital letters (A-H). Also, this domain contains three catalytic sites for ATP hydrolysis formed by A and B subunits. The V_0 domain

consists of a H^+-binding ring structure formed by six or more c subunits and at least more three different subunits identified with small letters (a, d and e) that mediate the transport of H^+ across the membrane, as shown in Fig. I.4 (Forgac, 2007; Marshansky et al., 2014; Rawson et al., 2015).

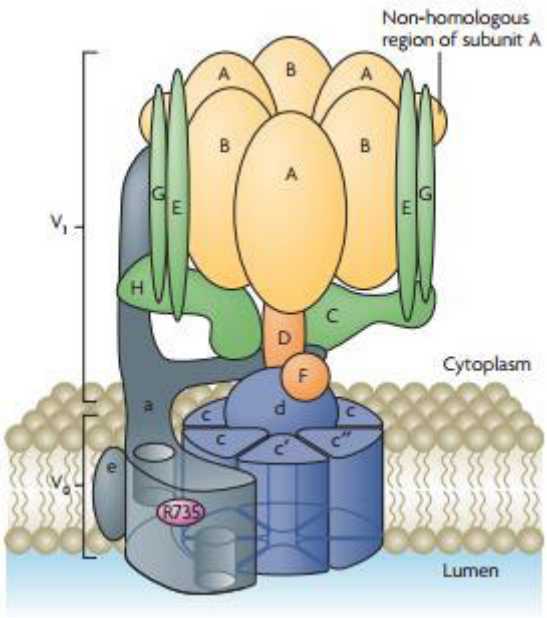

Figure I.4. Model of the structure of the vacuolar V-ATPase expressed in an eukaryotic cell membrane. Molecular model: cytoplasmic V_1 domain (shown in yellow and orange) with eight different subunits identified by capital letters (A-H) responsible for ATP hydrolysis; and membrane V_0 domain (shown in blue and grey) with at least four subunits identified by small letters (a, c, d and e), responsible for proton translocation across the membrane (Forgac, 2007).

V-ATPase was first discovered in lysosomes and vacuoles of plants and fungi and, later on, in secretory vesicles, endosomes, Golgi-derived, among others (Saroussi and Nelson, 2009). Besides this intracellular localization, V-ATPase is also present at the plasma membrane of cancer cells and specialized cells like osteoclasts, macrophages, etc (Cipriano et al., 2008).

Concerning its physiological role, V-ATPase has been reported to be essential in the acidification of intracellular compartments like endosomes, lysosomes and Golgi apparatus (Sun-Wada et al., 2004; Rawson et al., 2015). Moreover, V-ATPase is essential in cancer cells, namely in metastasizing cells, allowing the invasion process by acidifying the tumour extracellular environment (Sennoune et al., 2004), as it will be discussed below.

I.4.1. The role of V-H$^+$-ATPase in the tumour microenvironment and metastasis

Cancer cells often thrive in a hypoxic microenvironment with an acidic extracellular pH (pHe) when compared with normal cells. Under normal conditions, the low pHe associated with the hypoxic environment is not favourable for cell growth, promoting apoptosis (Gottlieb et al., 1995). To survive in these conditions, cancer cells must display a cytosolic pH (pHcyt) regulatory system, which is crucial to normal cell function providing the optimal pH for many cellular processes like cell growth, while promoting tumorigenesis, metastasis, cell motility (Rofstad et al., 2006) and drug resistance in cancer cells (Martínez-Zaguilán et al., 1999). In fact, pHe in tumours ranges from 6.5 to 6.9, while the intracellular pH (pHi) remains neutral to alkaline, creating an acid-outside pH gradient not observed in normal cells (Wojtkowiak et al., 2011). V-ATPase are normally present in various intracellular organelles, however, in some metastatic cancer cells, they are also functionally expressed at the plasma membrane where they contribute to the acidosis of the TME, playing pivotal roles in tumour invasion and metastasis (Martinez-Zaguilan et al., 1993; Pereira et al., 2016). The link between V-ATPase and tumour metastasis is apparent since the abnormal acidic pH in the TME contributes to the activation, secretion and distribution of proteases like metalloproteinases involved in the digestion of the complex extracellular matrix (ECM) (Fais et al., 2007). In this way, understanding the mechanisms regulating pHi and tumour acidity is of prime importance to develop V-ATPase targeting strategies for cancer therapy.

I.4.2. Relevance and features of V-H$^+$-ATPase in highly metastatic cancer cell lines

Studies in human breast cancer cell lines with distinct metastatic potential have been performed to ascertain the distribution and functional activity of the V-ATPase and relate it with the migration and invasion of these cell lines (Sennoune et al., 2004; Hinton et al., 2009; Capecci and Forgac, 2013). The immunocytochemical results reported by Sennoune and co-workers (2004) demonstrated that highly and lowly metastatic breast cancer cells display a different localization of V-ATPase. Indeed, in the highly metastatic cancer cells, this proton pump was preferentially located at the plasma membrane while in the lowly metastatic cancer cells it had predominantly an intracellular localization. The same authors found that migration and invasion, two hallmarks of cancer, were related with V-ATPase localization/activity in breast cancer cell lines. Moreover, the migration kinetics of highly and lowly metastatic cancer cells was studied in the absence and presence of bafilomycin A1 (BafA1), a specific V-

ATPase inhibitor, to evaluate the physiological relevance of this pump at the cell surface. In the absence of this inhibitor, invasion and migration were significantly faster in highly metastatic cancer cells, while in its presence a decrease of these processes was observed. Additionally, no significant effect was found in lowly metastatic cancer cells (Sennoune et al., 2004). Similar results were obtained in human prostate carcinoma cell line PC-3M-2B4 (Xu et al., 2012b). Notably, the a and c subunits seem to be the V-ATPase subunits that more significantly influence the metastasis and proliferation of cancer cells, thus being important factors in the regulation of cancer metastasis (Hinton et al., 2009; Capecci and Forgac, 2013). These studies suggest that the greater migratory and invasive ability of the highly metastatic cells as compared to the lowly ones can be suppressed by V-ATPase inhibitors. Furthermore, at the cell surface this proton pump plays a role in maintaining an alkaline intracellular microenvironment – pHi - favourable for cell growth, conferring them a competitive advantage over normal cells; while maintaining an acidic extracellular microenvironment - pHe - essential for invasion (Xu et al., 2003). In this way, the acquisition of a metastatic and invasive phenotype is associated to the localization of V-ATPase in the plasma membrane.

Recently, in HCT116 and SW480 colon cancer cell lines, Lozupone and colleagues found a new transmembrane protein - TM9SF4 - related with invasive behaviour of metastatic cells and implicated in the V-ATPase activation through the interaction with the ATP6V1H subunit of the V_1 domain. TM9SF4 suppression and silencing with small interfering RNAs resulted in a series of cellular alterations related with a reduced V-ATPase activity: (i) decrease of pHi; (ii) alkalization of the intracellular vesicles and (iii) reduction of extracellular acidity through a decrease of proton extrusion. These effects promoted a significant inhibition of the invasive behaviour reported in colon cancer cell lines (Lozupone et al., 2015).

Given the contribution of V-ATPase to the acidity of the TME and its recognized importance in breast cancer, inhibitors of this pump have emerged as excellent candidates for breast cancer therapy. The most-well known V-ATPase inhibitors are concanamycin A (ConcA) and BafA1, and treatment with these compounds results in the neutralization of TME. However, several studies have shown that ConcA and BafA1 are extremely toxic *in vivo* and therefore not suitable for clinical use (Pérez-Sayáns et al., 2009; Spugnini et al., 2015). New V-ATPase inhibitors have then been developed such as indole derivatives (Supino et al., 2008), macrolacton archazolids (Von Schwarzenberg et al., 2013), among others. A recent study from our group with highly metastatic breast cancer cell lines showed that bLf, like ConcA, inhibits V-ATPase in sub-cellular fractions. Thus, bLf was proposed as a V-ATPase inhibitor as it was found to

decrease the extracellular acidification rate and to promote intracellular acidification (Pereira et al., 2016) and selectively induce apoptosis of cancer cells.

Since the preferential expression and activity of V-ATPase at the cell surface has an important role for the development of metastasis and invasiveness of the cancer cells, V-ATPase is considered a potential target in chemotherapy and may be an excellent candidate for anticancer drugs such as bLf.

I.5. LIPID RAFTS: STRUCTURE AND INTERPLAY WITH V-H^+-ATPASE

In 1972, for the first time, a model about the organization and structure of the proteins and lipids in the biological membranes was proposed by Singer and Nicholson, and it was named the fluid mosaic model. According to this model, biological lipid bilayers are two-dimensional fluids favourable to lateral motility allowing free diffusion of proteins in the phospholipid bilayer and distribution along the membrane surface. This fluidity leads to the creation of highly organized structures or membrane domains differing in lipid/protein composition (Singer and Nicolson, 1972). Over time, several experiments indicated that some lipids are essential for creating these levels of order, leading to an "organization of the lipid components of membranes into domains", supporting the concept of lipid membrane domain (Karnovsky et al., 1982). In 1988, Simons and van Meer explained the different lipid composition providing the basis for the "lipid rafts hypothesis" (Simons and van Meer, 1988). After a decade, intracellular trafficking and signal transduction mechanisms were hypothetically associated with lipid rafts (Simons and Ikonen, 1997). Later on, stabilization of the lipid rafts was associated with the presence of cholesterol, its key lipid component, within a liquid-ordered phase (Simons and Sampaio, 2011). Therefore, lipid rafts were initially described as ordered domains created by lateral separation of sphingolipids with different molecular composition and properties relative to the surrounding membrane (Sonnino and Prinetti, 2012). This notion has then emerged in several studies in cell biology being involved in a great variety of cellular and biological functions. Such ordered lipid microdomains are enriched in glycosylphosphatidylinisotol (GPI)-anchored proteins as well as in lipids, especially cholesterol and sphingolipids (sphingomyelin and gangliosides), in which the cholesterol tightly interlaces the saturated fatty acyl chains of the sphingolipids forming these organized microdomains. These structures are small, dynamic and heterogeneous and have affinity to several proteins such as Src family kinases (SFKs) and transmembrane proteins such as CD44 (Murai, 2012; Mollinedo and Gajate, 2015). Lipid rafts are resistant to extraction with non-ionic detergents, being normally isolated as detergent-resistant

membranes (DRMs) (Staubach and Hanisch, 2011; Sonnino and Prinetti, 2012). However, some experimental evidences appear to contradict the principles underlying the lipid rafts notion, leading to some controversy around this subject. For example, a recent study in live cell plasma membrane demonstrated that GPI-anchored proteins do not reside in ordered domains, since it is not observed the formation of a connective phase with altered membrane fluidity (Sevcsik et al., 2015). For these reasons, the concept of lipid rafts, mainly its true nature and role, still generates some debate among scientists and it is currently being redefined. Nevertheless, lipid rafts have been implicated in various cellular processes, namely associated to cancer and V-ATPase.

At the functional level, these lipid structures represent authentic signalling platforms responsible for signal transduction and protein trafficking. Therefore, they are implied in many signalling pathways namely related with cancer progression like cell migration and adhesion. Indeed, the deregulation of raft-dependent signalling was shown to favour tumour progression (Murai, 2012; Wang et al., 2013; Mollinedo and Gajate, 2015). In particular, a study with breast cancer cells showed that lipid rafts may regulate several processes involved in cancer progression, namely at the initial stages of tumour growth and progression to a migratory and metastatic phenotype (Babina et al., 2011). Murai and colleagues demonstrated that lipid rafts play a critical role in the localization and functionality of CD44, a major cell adhesion molecule expressed in cancer cells. When cells were treated with a lipid-raft-disrupting agent methyl-β-cyclodextrin (MβCD) or filipin, CD44 shedding increased suggesting that low lipid rafts levels disturbs the CD44 membrane localization, which is essential for enhanced cancer cell adhesion and migration (Murai, 2012). Moreover, a recent study performed in human melanoma cells confirmed these evidences through the evaluation of the actin cytoskeleton and cell adhesion. In this case, lipid rafts not only regulate the dynamics of actin cytoskeleton, but also the disruption of its integrity with MβCD, promoted alterations on cell morphology such as actin fiber formation and inhibition of the adhesion disassembly (Wang et al., 2013). In our laboratory, we have also studied breast cancer cell lines treated with bLf and MβCD in which cholesterol-rich lipid rafts are re-distributed (Pereira C, 2014 master thesis).

Importantly, V-ATPase has been identified as a component of the lipid rafts in highly metastatic melanoma cells (Baruthio et al., 2008), osteoclasts (Ryu et al., 2010) and coronary arterial epithelial cells (Xu et al., 2012a). Focusing on melanoma cells, Baruthio and colleagues demonstrated that V-ATPase is present in the lipid rafts fraction of highly metastatic cells, while appearing to be inconspicuous in non-metastatic cells, thus suggesting that there is an interplay between this proton pump and the lipid rafts (Baruthio et al., 2008). This interplay is still unknown but there are some studies that explain the V-

ATPase transport into lipid rafts. In coronary arterial epithelial cells, V-ATPase was found to provide an acidic microenvironment around the lipid rafts when it is assembled and transported into these microdomains, promoting the formation of ceramide-enriched signalling platforms and amplification of the lipid raft-associated signals. In the presence of BafA1, V-ATPase was inhibited impeding the lipid rafts clustering. Given this evidence, it was concluded that when V-ATPase co-localizes with lipid rafts it has a crucial role in the structural and functional stability of these domains (Xu et al., 2012a). However, additional evidence support that this interaction is reciprocal since V-ATPase activity is regulated by the interaction with specific lipidic environments (Lafourcade et al., 2008).

In this work, the interplay between V-ATPase and lipid rafts will be explored in different highly metastatic cancer cell lines after treatment with bLf.

I.6. AIMS

Our previous results showed that the selective anticancer activity of bLf against highly metastatic breast cancer cells, in comparison with lowly metastatic and non-tumorigenic cell lines, occurs through inhibition of the plasmalemmal V-ATPase (Pereira et al., 2016). Herein, we aimed to ascertain whether this same mechanism of action of bLf could underlie its activity against other types of highly metastatic cancer cells. To accomplish our goal, we selected three highly metastatic cell lines: a prostate cancer cell line (PC-3), an osteosarcoma cell line (MG-63), and the previously characterised breast cancer cell line (MDA-MB-231), the latter used as a positive control, and defined the following specific aims to:

- compare the susceptibility of these three cell lines to bLf in terms of inhibition of cell proliferation and uncover the mechanism underlying bLf-induced cell death;
- evaluate a possible relation between cell susceptibility and the levels of V-ATPase of the three cell lines and compare them with the non-tumorigenic breast cell line MCF-10-2A;
- assess whether the susceptibility of PC-3 and MG-63 cell lines to bLf is associated with alteration in the intracellular pH;
- study the effect of bLf on F-actin arrangement;
- unravel the effect of the pre-treatment of cells with the lipid rafts disruption agent methyl-β-cyclodextrin (MβCD) in the response to bLf treatment;
- determine the effect of bLf on the distribution of lipid rafts and understand the interplay between bLf, V-ATPase and lipid rafts.

Chapter II

Materials and Methods

II.1. Chemicals and Solutions

Bovine lactoferrin (bLf) was obtained from DMV (Veghel, The Netherlands). The protein was dissolved in phosphate buffered saline (PBS) (1.37 M NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) to obtain the different concentrations used throughout this study. According to the manufacturer, the protein purity is about 80% with 3.5% moisture and 21% iron-saturated.

Concanamycin A (ConcA), methyl-β-ciclodextrin (MβCD), paraformaldehyde (PFA), cisplatin, etoposide, filipin and LC3B, β-actin and caspase-3 antibodies were purchased from Sigma-Aldrich. BCECF-AM (2', 7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) and Alexa Fluor 488-Phalloidin were obtained from Molecular Probes. CFSE probe (Carboxyfluorescein diacetate succinimidyl ester), and FITC Annexin V apoptosis detection kit were acquired from BD Bioscience. Secondary antibodies - goat anti-rabbit IgG or goat anti-mouse IgG were obtained from Jackson ImmunoResearch. Beclin-1 antibody was acquired from Cell Signalling and V-ATPase C1 antibodies were purchased from Santa Cruz Biotechnology. Vectashield mounting medium was acquired from Biosystems.

II.2. Cell lines and culture conditions

Human prostate cancer cell line PC-3 (CRL-1435; ATCC), human osteosarcoma cell line MG-63 (CRL-1427; ATCC) and human breast cancer cell line MDA-MB-231 (HTB-26; ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM), purchased from Biochrom - Merck Millipore, supplemented with 10% fetal bovine serum (FBS), acquired from the same company, and 1% zell shield (Minerva Biolabs). MCF-10-2A (CRL-10781), a normal breast cell line, was grown in DMEM-F12 medium supplemented with 5% horse serum, 1% zell shield, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor and 0.01 mg/mL insulin.

Cells were kept in cryovials with a freezing mixture containing 5% dimethyl sulfoxide (DMSO), 10% fetal bovine serum (FBS) and DMEM without supplements, and stored in liquid nitrogen. In order to perform the experiments bellow described, frozen cells were thawed and maintained in culture in a 37 °C incubator with a humidified atmosphere containing 5% CO_2.

For each experiment, cells were collected from the culture flask, washed with PBS 1× and incubated with trypsin 0.05% (v/v). Trypsin was inactivated by fresh medium and the cell suspension was harvested to sterile falcon tubes. Cell concentration was estimated using a Neubauer chamber. For each

experiment, cells were seeded in 6-well plates at an appropriate concentration: 1×10^5 at 24 h and 7.5×10^4 cell/ml at 48 h for PC-3 cell line, 9×10^4 at 24 h and 5×10^4 cell/ml at 48 h for MG-63 and MDA-MB-231 cell lines, and 7.5×10^4 cell/ml at 48 h for MCF-10-2A cell line. All the compounds under study were added to the wells only when cells reached at least 80% confluence.

II.3. Assessment of cell proliferation by carboxyfluorescein diacetate succinimidyl ester (CFSE)

The CFSE probe was used to assess cell proliferation. This non-fluorescent compound is metabolized by cellular esterases and the resulting fluorescent carboxyfluorescein (CF) binds covalently to long-lived intracellular molecules emitting green fluorescence when excited at 488 nm. The cellular fluorescence is diluted in each cell division. The daughter cells exhibit half the fluorescence intensity of their mother cells (Lyons et al., 2013).

II.3.1. Preparation of CFSE dye solution in DMSO 2%

To prepare a 10 mM stock solution of CFSE dye, 90 µL of DMSO 2% were added to a CFSE vial. Afterwards, the CFSE working solution (1.25 µM) was distributed into single-use aliquots that were frozen at -20 °C protected from light.

II.3.2. CFSE labelling of cells

CFSE labelling was performed before cells' seeding. Briefly, cells were collected from the culture flask and washed with PBS 1× to remove all the serum proteins to avoid probe inactivation in the presence of those proteins (Quah et al., 2007). Cell concentration was estimated using a Neubauer chamber. Next, 1×10^5 cells/ml were resuspended in PBS 1× and were further incubated with the CFSE dye (final concentration: 20 µM) for 15 min in a 37 °C water bath. Afterwards, cells were rinsed with PBS 1×, the correct amount of complete culture media to obtain the same cell concentration was added and cells were plated in 6-well plates. After 24 h of adherence, cells were treated solely with medium (negative control), 50 µM cisplatin (MG-63 and MDA-MB-231 cell lines) or 60 µM etoposide (PC-3 cell line) as positive controls, and 175 µM bLf. Cisplatin and etoposide were the selected controls for these cell lines because they induce apoptosis and are anti-tumour agents currently used in clinical use for the treatment

of this type of cancers (Carrle and Bielack, 2006; Gao et al., 2013, respectively). Cells were harvested and the CF median fluorescence intensity was analysed by flow cytometry using the FL1 channel at time points 0, 24, 48 and 72 h after treatment. At the moment of seeding, a sample from the labelled cell suspension was also collected and analysed to ensure the correct staining of the cells. In the assays with MβCD, cells were pre-treated with 0.5 mM MβCD 2 h before incubation with bLf.

The FlowJo 7.6 software was used to analyse the data, namely to obtain the medians and the histograms. All data (medians) were normalized to the time point 0 h, which corresponds to the maximum fluorescence intensity. GraphPad Prism version 6.0 was used to elaborate the graphics.

II.4. FITC-Annexin V/Propidium Iodide apoptosis assay

The Annexin V/Propidium iodide (AV/PI) apoptosis assay was performed to characterize bLf-induced cell death. Cells were seeded in 6-well plates, two wells per condition – negative control (cells without treatment), positive control (treated with 60 μM etoposide for PC-3 cell line, and 50 μM cisplatin for MG-63 and MDA-MB-231 cell lines), 175 μM bLf and 10 nM ConcA (used as positive control for V-ATPase inhibition). After an adherence period of 24 h, cells were incubated in the absence or presence of the different compounds. After 24 and 48 h of treatment, cells were harvested, rinsed with PBS 1× and centrifuged at 2500 rpm for 5 min. Pellets were resuspended in PBS 1× for cell counting to obtain a final concentration of 2×10^5 cells per condition. The corresponding amount of cellular suspension was transferred to microtubes correctly identified – autofluorescence, AV or PI mono-stainings, and AV/PI double staining – and centrifuged at 2500 rpm for 7 min. The pellets were resuspended in Binding Buffer 1× (BD Pharmigen™), transferred to cytometer tubes and incubated with 1 μL of AV-FITC and 1 μL of PI for 15 min in the dark. Binding buffer 1× was added to the cells in order to obtain a final volume of 500 μL. Acquisition was performed in a flow cytometer using the FL1 and FL4 channels. Data were analysed using the FlowJo 7.6 software.

II.5. Protein Extraction and Quantification

Cells seeded in 6-well plates were harvested by trypsinization, centrifuged at 2000 rpm for 5 min, and resuspended in 40-60 μL ice-cold RIPPA buffer [50 mM Tris HCl pH=7.4, 150 mM NaCl, 2 mM EDTA, NP-40 1% (v/v)] supplemented with inhibitors [20 mM sodium fluoride (NaF), 20 mM sodium

orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 4% protease cocktail inhibitor], and kept on ice for 20 min. Then, cell extracts were centrifuged at 14000 rpm for 15 min and the supernatant containing the total proteins was transferred to new microtubes, previously identified. The soluble protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories). The protein concentration of each sample was determined using a calibration curve obtained with solutions of bovine serum albumin (BSA) with increasing concentrations – from 5 mg/mL to 0.25 mg/mL.

II.6. Western Blot analysis

For western blot analysis, 35 µg or 50 µg (depending on the assay) of protein were separated by a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Afterwards, protein bands were transferred onto PVDF (polyvinylidene difluoride) membranes. Next, to avoid non-specific interactions, membranes were blocked in 5% non-fat milk in PBS-Tween 0.1% solution (PBST 1×) for 1-2 h with agitation at RT. Membranes were then incubated overnight at 4 °C with the primary antibodies, namely monoclonal anti-β-actin, anti-active caspase 3, anti-LC3B, anti-Beclin-1 and anti-V-ATPase (Table II.1).

Table II.1: List of the antibodies and incubation conditions used in the western blot analysis.

Antibody (Host)	Size of the target (kDa)	Dilution	Temperature (ºC)	Incubation time	Manufacturer
Anti-β-Actin (Mouse)	42	1:1000	4	Overnight	Sigma Aldrich
Active anti-caspase 3 (Rabbit)	17-19	1:2000	4	Overnight	Sigma Aldrich
Anti LC3B (Rabbit)	16-18	1:1000	4	Overnight	Sigma Aldrich
Beclin-1 (Rabbit)	60	1:1000	4	Overnight	Cell Signalling
V-ATPase C1 (Mouse)	43	1:25	4	Overnight	Santa Cruz Biotechnology
Anti-Rabbit (Goat)	-	1:2000	RT	1 hour	Jackson ImmunoResearch
Anti-Mouse (Goat)	-	1:2000	RT	1 hour	Jackson ImmunoResearch

Next, membranes were incubated with secondary antibodies Peroxidase AffiniPure goat anti-rabbit IgG or goat anti-mouse IgG (1:2000; Jackson ImmunoResearch). Chemiluminescence detection was performed using the ECL detection system (Millipore-Merck) in a ChemiDOC™ XRS system (BioRad).

II.7. Intracellular pH measurement

Measurements of pHi were performed with the pH-sensitive probe BCECF-AM. This non-fluorescent ester is a substrate of intracellular esterases and the fluorescence of BCECF varies according to the pH. Changes in the cytosolic pH were monitored by changes in the ratio of green/red fluorescence intensities (FL1/FL4), since this ratio value is independent of the probe concentration and exclusively dependent of pHi (Ozkan and Mutharasan, 2002).

Cells were seeded in 6-well plates and treated with only medium, 175 μM bLf and 10 nM ConcA, for 24 h and/or 48 h. After this time, cells were tripsinized, washed with HBSS – Hank's Balanced Salt Solution (10 × concentrated solution: 1379 mM NaCl, 53.33 mM KCl, 3.36 mM Na_2HPO_4-$7H_2O$, 55.55 mM D-glucose and 4.41 mM KH_2PO_4) and centrifuged. The pellets were resuspended in HBSS 1× and incubated with 50 μM BCECF-AM from a stock solution of 161.3 μM for 30 min at 37 °C, and protected from light. After incubation, samples were analysed in a flow cytometer. For the assays with MβCD, cells were pre-treated with 0.5 mM MβCD during two hours before the additional treatments. The % of cells exhibiting intracellular acidification were estimated from the percentage of cells displaying a FL1/FL4 ratio lower than control cells. Data were analysed using the FlowJo 7.6 software.

II.8. Flow cytometric analysis

Flow cytometry analysis was performed in an Epic® XLTM (BeckmanCoulter) flow cytometer equipped with an argon-iron laser with emission of a 488 nm beam at 15 mW. Red fluorescence was collected through a 560 nm short-pass dichroic, a 640 nm long-pass and another 670 nm long-pass filter. Green fluorescence was collected through a 488 nm blocking filter, 525 nm band-pass filter and a 550 nm long-pass dichroic. For each experiment, 20.000 events were evaluated for each sample and data were analysed using the FlowJo 7.6 software.

II.9. Immunofluorescence and confocal microscopy

For Alexa Fluor 488-Phalloidin staining, cells were seeded in 6-well plates containing three glass coverslips per well. After 48 h, cells were fixed with 4% paraformaldehyde (PFA) for 40 min. After rinsing with PBS 1× (five times during 5 min each), cells were incubated with ammonium chloride 50 mM for 10 min, rinsed again with PBS 1× and permeabilized with PBS-SDS 0.1% for 10 min. Subsequently, cells were blocked with PBS-BSA 3% during 20 min and washed with PBS-BSA 0.1%. Following this procedure, cells were incubated with conjugated antibody - Alexa Fluor 488-Phalloidin (0.5 µL per glass coverslip) in PBS-BSA 0.1% (50 µL per coverslip) for 1 h at 4°C in a humidified chamber in the dark. Cells were then washed with PBS-BSA 0.1% and the coverslips were mounted in Vectashield mounting medium. Samples were maintained at 4°C, protected from light, until visualization. Images were acquired in a sequential mode by Confocal Scanning Laser Microscope OLYMPUS BX61/FLUOVIEW1000, using a 40× objective and the specific filter settings for Alexa Fluor 488 (green fluorescence).

II.10. Evaluation of lipid rafts distribution by filipin staining and fluorescence microscopy

For rafts visualization, a polyene antibiotic with fluorescent properties that binds cholesterol, the major constituent of lipid rafts, called filipin was used. For filipin staining, cells were seeded in 6-well plates containing glass coverslips and allowed to attach for 24 h. Next, cells were incubated with fresh medium or fresh medium containing MβCD or bLf. After 48 h incubation, cells were fixed with 4% PFA, for 40 min. Then, cells were rinsed with PBS 1× and the aldehyde residues left by PFA were quenched by incubation of the fixed cells with a 1.5 mg glycine/ml PBS solution for 15 min at room temperature. Cells were then stained with 0.01 mg/mL filipin dissolved in PBS 1× supplemented with 0.5% BSA for 2 h at room temperature in the dark. Finally, cells were washed with PBS 1× and the coverslips were mounted together with the anti-fading mounting medium to overcome the instability of the dye. Samples were visualized in a fluorescence microscope Leica DM 5000B (Leica Microsystems) with the specific filter settings for this dye (blue channel).

II.11. Statistical analysis

Experimental values are expressed as means or medians ± S.D. of at least three independent experiments. Statistical analysis was performed using one-way ANOVA followed by the Bonferroni post-test (it assumes that all the tests are independent from each other) using the GraphPad Prism version 6.0.

Chapter III

Results

III.1. bLf inhibits the cell proliferation of highly metastatic cancer cell lines

The global aim of this study was to ascertain whether inhibition of the plasmalemmal V-ATPase by bLf, as observed for the highly metastatic breast cancer cells (Pereira et al., 2016), could be a generalized mechanism underlying the anticancer activity of this natural protein against other highly metastatic cancer cells. To this end, we assessed the effects of bLf on the proliferation of the prostate cancer PC-3 and the osteosarcoma MG-63 cell lines, and compare it with the effect previously found for the highly metastatic breast cancer cell line MDA-MB-231, using the CFSE probe. In each cell division, the fluorescence of the CFSE stained cells is reduced to half of the initial fluorescence, thus when proliferation is inhibited a reduction in the cell fluorescence decrease is observed. We found that after 24 h of incubation with bLf, similarly to what happens in the cells incubated with etoposide or cisplatin - used as positive controls (C+) for PC-3 and MG-63, respectively - the cell fluorescence was not significantly different from the negative control cells (C-), thus indicating that cell proliferation of the three cell lines was not affected in any of the conditions (Figure III.1 A and B). However, after 48 h and 72 h of incubation with bLf, as well as with the positive controls, cell proliferation was inhibited since the cell fluorescence decreased much slower than that observed in the control cells. The percentage of cell proliferation inhibition by bLf or by etoposide/cisplatin, for each cell line was estimated and no significant differences between the three cancer cell lines were found (see Table VI.1. Supplementary Material). These results indicate a similar susceptibility to bLf and cisplatin or etoposide. In accordance with the inhibition of cell proliferation after a 48 h exposure, a decrease in the cell number and changes in cell morphology were observed by phase contrast microscopy (Figure III.1 C).

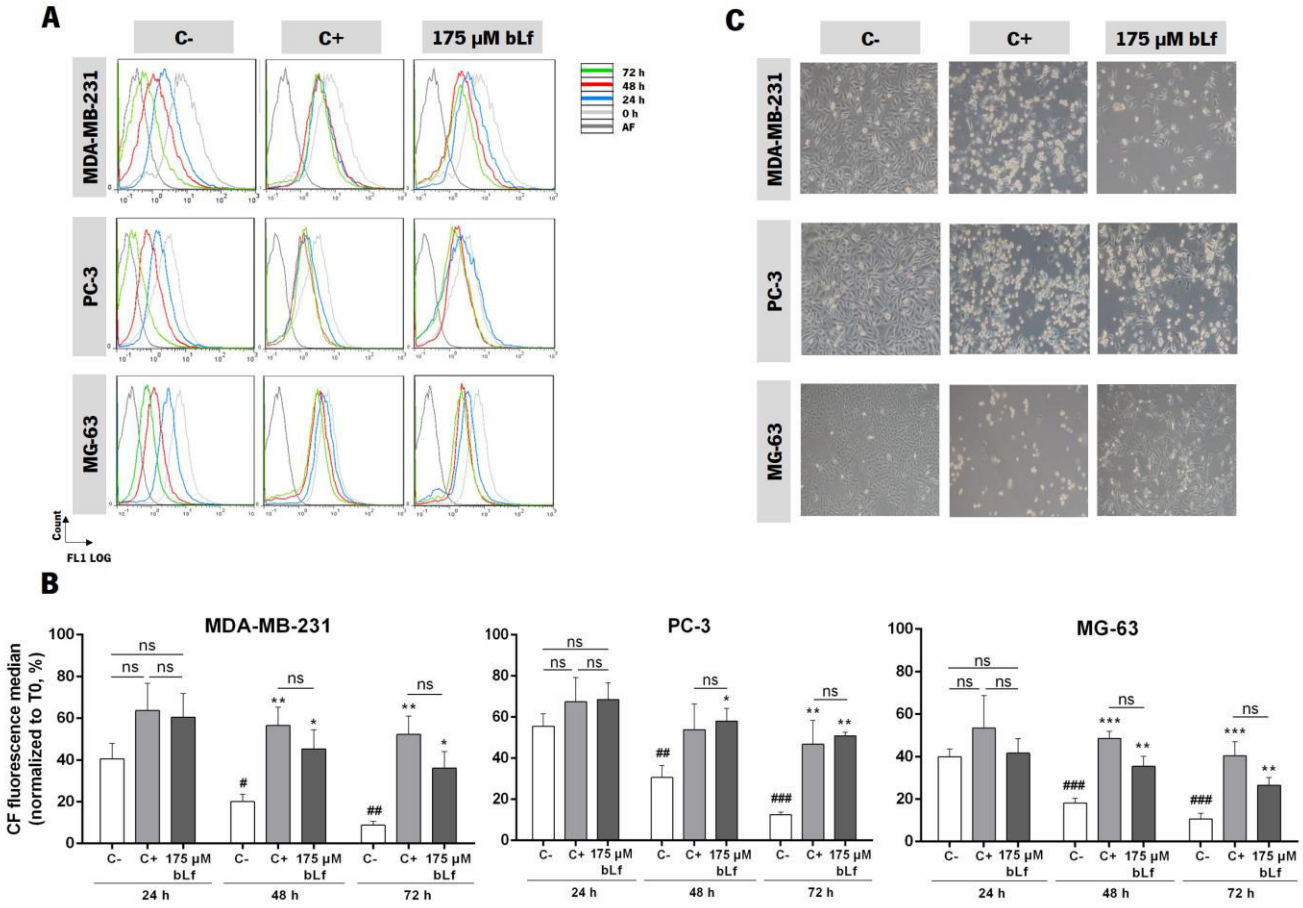

Figure III.1. bLf inhibits the cell proliferation of highly metastatic cancer cell lines. A. Representative histograms of cell proliferation analysis with CFSE probe of the different conditions after 0, 24, 48 and 72 h exposure to bLf in three cell lines. Unexposed cells were used as a negative control (C-) and cisplatin (for MDA-MB-231 and MG-63 cell lines) or etoposide (for PC-3 cells) were used as a positive controls (C+). **B.** Representative graphics of the CF median fluorescence values normalized to T0 after 24, 48 and 72 h of exposure. Values represent median ± S.D. of three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0,001$ compared with C- of each time point and $^{\#}P<0.05$; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0,001$ compared with 24 h. **C.** Representative images of cell cultures after 48 h of treatment with bLf (175 µM) and C+ (cisplatin or etoposide) in MDA-MB-231, PC-3 and MG-63 cell lines (phase contrast 10×).

III.2. Highly metastatic cancer cell lines display different V-H^{+}-ATPase expression levels in comparison with a non-tumorigenic breast cell line

As aforementioned, V-ATPase was found at the plasma membrane of several highly metastatic cancer cells, namely in the three cell lines under study (Sennoune et al., 2004; Bermudez, 2010; Perut et al., 2014). To find if V-ATPase is the molecular target of bLf in the highly metastatic cancer cell lines MDA-MB-231, PC-3 and MG-63, we attempted to establish a relation between cell susceptibility and the

levels of V-ATPase expression and compared them with those of the normal breast cell line MCF-10-2A, which is not susceptible to bLf (Pereira et al., 2016).

Results from the western blot analysis of total cell extracts and the respective quantification of the bands by ImageJ Software (Fig. III.2) suggested that PC-3 and MDA-MB-231 cells display higher levels of V-ATPase expression in comparison with the non-tumorigenic breast cell line MCF-10-2A. On the other hand, the MG-63 cell line displays V-ATPase expression levels closer to those of the non-tumorigenic cell line. However, since the levels of actin and V-ATPase expression for the MDA-MB-231 cells are quite low and only one independent assay was performed, these results are still preliminary and further assays are required to establish a relation between cell susceptibility to bLf and the levels of V-ATPase expression.

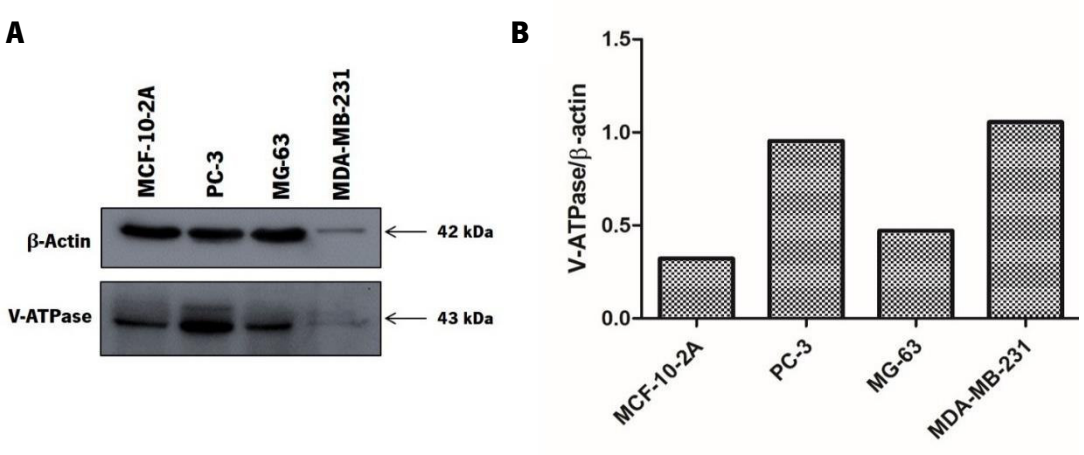

Figure III.2. V-ATPase expression levels in highly metastatic cancer cell lines MDA-MB-231, PC-3 and MG-63 in comparison with the non-tumorigenic breast cell line MCF-10-2A. A. Western blot images of V-ATPase expression in each cell line using 35 μg protein extract in each lane. **B.** Quantification of the levels of V-ATPase expression in the four cell lines normalized to the levels of β-actin. The values represent one independent assay.

III.3. bLf induces cell death of highly metastatic cancer cell lines

The above reported bLf-induced inhibition of the proliferation of the cell lines under study may be associated or not with the induction of cell death. To address this question, we first evaluated whether bLf was able to induce apoptosis as it was previously reported in different studies (Amiri et al., 2015; Gibbons et al., 2015; Zhang et al., 2015c). For this purpose, we performed two different experiments. In one hand, we monitored by flow cytometry the exposure of phosphatidylserine (PS) in the outer leaflet of the plasma membrane and its relation with the plasma membrane integrity (Brauchle et al., 2014; Rieger et al., 2011) by double staining with Annexin V and propidium iodide (AV/PI). On the other hand, we

assessed the activation of caspase-3 by western blot. Annexin V is a phospholipid-binding protein with high affinity for PS, which is externalized in early apoptotic cells that still preserve their plasma membrane integrity. Propidium iodide stains cells that have lost the integrity of their plasma membrane. Therefore, the AV/PI assay allows discriminating, viable/live cells (AV^-/PI^-) from early apoptotic cells (AV^+/PI^-) and late apoptotic/necrotic cells (AV^+/PI^+ and AV^-/PI^+). Representative histograms of AV/PI assays with MDA-MB-231, PC-3 and MG-63 cells are shown in Fig. III.3 A. Cells were incubated with only medium (C-), with 50 μM of cisplatin for breast and osteosarcoma cancer cells, or 60 μM of etoposide for prostate cancer cells (C+), with 175 μM bLf, or with 10 nM of ConcA (a V-ATPase inhibitor). The exposure of PS in the MDA-MB-231 cells could be detected after incubation with bLf, as well as with cisplatin and ConcA. Indeed, the percentage of AV^+/PI^- cells for this cell line was significantly different after 48 h incubation with bLf, cisplatin or ConcA in comparison with untreated cells. Moreover, the percentage of AV^-/PI^+ cells was very low. This suggests that apoptosis underlies bLf-induced cell death in the breast cancer cell line MDA-MB-231. A similar behaviour was found in PC-3 cells, in which the percentage of AV^+/PI^- cells after treatment with etoposide (C+) and bLf was also statistically different from the negative control cells (Fig. III.3 A and B). On the other hand, the analysis of the MG-63 cells indicates that, although there is a decrease of the percentage of viable cells and an increase of the percentage of AV^+/PI^- cells, after 48 h of treatment with bLf, cisplatin or ConcA, the changes are not significantly different from the negative control cells (Fig. III.3 A and B). In order to explain the amount of cells in suspension (dead cells) observed by microscopy (see Fig. III.1 C) and to ascertain whether death induction was occurring, an analysis of the population of viable cells (AV^-/PI^-) against dead cells (AV^+/PI^- and AV^+/PI^+ or AV^-/PI^+ and AV^+/PI^+) was also performed (see Fig. VI.1 Supplementary Material). The results showed that, at least for the concentration and incubation times used in this work, bLf does not induce cell death in MG-63 cell line.

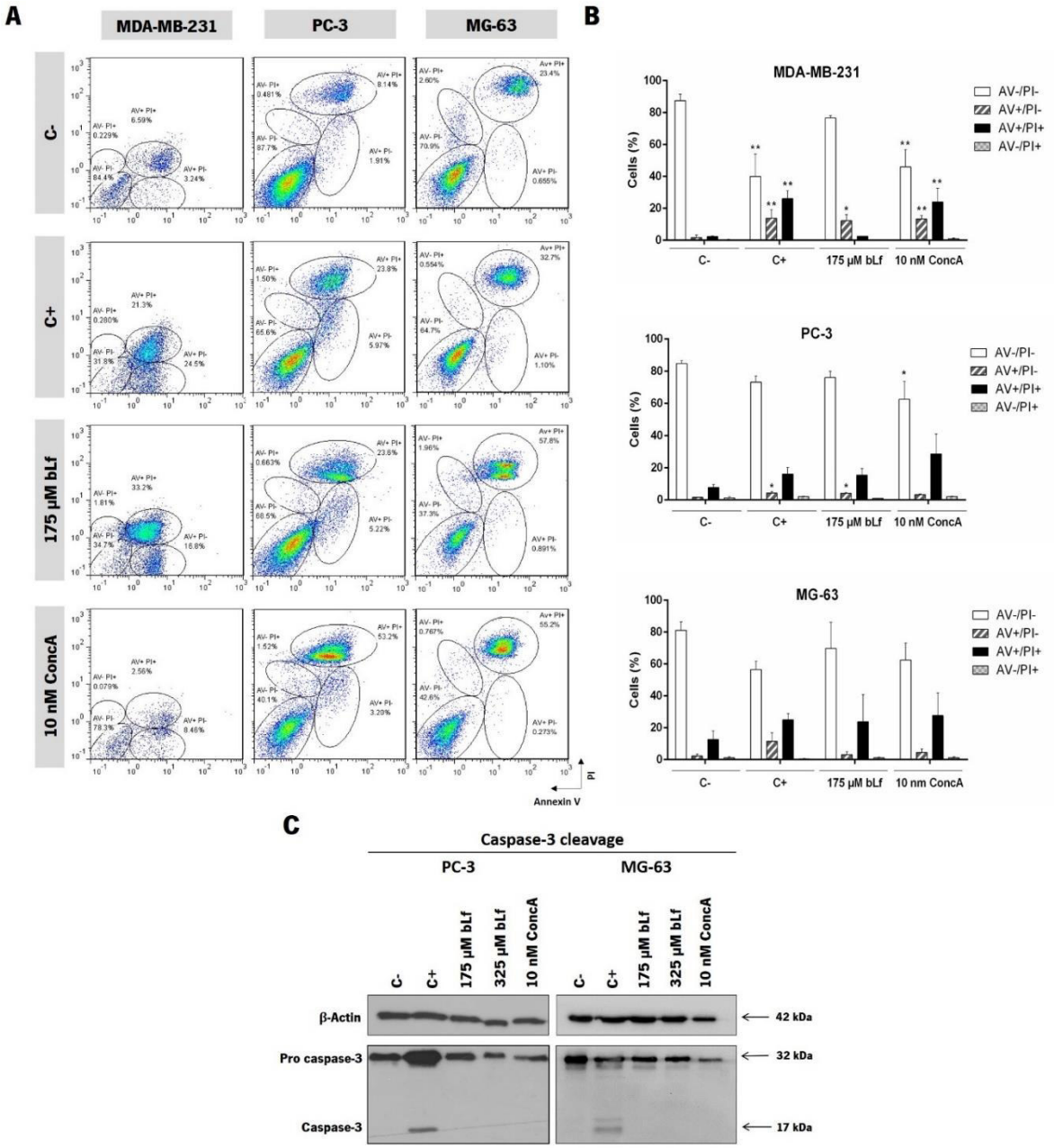

Figure III.3. bLf induces cell death of the highly metastatic cancer cell lines MDA-MB-231and PC-3 but not of MG-63. A. Exposure of phosphatidylserine induced by bLf or ConcA was monitored by AV-FITC/PI staining. Representative histograms of cells untreated (negative control, C-) or treated during 48 h with 175 µM bLf or 10 nM ConcA, or with 50 µM cisplatin, 60 µM etoposide (used as positive controls, C+, for MG-63 and PC-3 cells, respectively). **B.** Quantitative analysis of the AV/PI assays in A. Values represent mean ± S.D. of three independent experiments, $^{*}P<0.05$ and $^{**}P<0.01$ when compared to negative control cells. **C.** Western blot analysis of caspase-3 activation in PC-3 and MG-63 cell lines. After 48 h treatment. Cells were harvested and cell extracts were prepared for western blot analysis. 100 µg protein extract was added to each lane. β-actin was used as a loading control. A representative experiment, of two independent experiments with similar results, is shown.

Since caspase-3 is a common effector of most apoptotic pathways, its cleavage and activation are used as markers of apoptosis induction. This protein is expressed as a proenzyme of 32 kDa (pro-caspase 3) that in response to various stimuli undergoes cleavage by caspase-8 and caspase-9, generating two subunits of 17 and 12 kDa. Several methods can be used to detect active caspase-3 in cells, including immunostaining, immunoblotting and colorimetric assays using fluorochrome substrates (Liang et al., 2001; Nestal De Moraes et al., 2011). In the current study, caspase-3 activity was determined through immunoblotting to better characterize death cytotoxicity induced by bLf in PC-3 and MG-63 cells. In order to understand if different concentrations of bLf could lead to different apoptosis levels, incubation with 325 μM of bLf was also tested. Western blot analysis showed that both PC-3 and MG-63 cells did not display caspase-3 activation in response to the two different bLf concentrations or to 10 nM ConcA, in contrast to the cells treated with cisplatin/etoposide (C+) (Fig. III.3 C).

Next, we addressed whether autophagy is involved in the response of PC-3 and MG-63 cells to bLf. Autophagy is a catabolic process that digests cellular contents within lysosomes, stimulated by a several cellular stressors like nutrient starvation, DNA and organelle damages. In this sense, autophagy serves as a protective mechanism promoting the degradation of damaged cellular constituents, however, the hyperactivation of autophagy can lead to cell death (Shimizu et al., 2014). To this end, we assessed the expression of two autophagic markers, LC3B and Beclin-1, to understand if these cells can alter their basal level of autophagy in response to bLf. LC3B is the microtubule-associated protein light chain 3 distributed ubiquitously in mammalian tissues and cells, with a molecular mass of ~18 kDa, widely used to monitor autophagy. During this process, a cytosolic form of LC3B (LC3B-I) is conjugated to phosphatidylethanolamine and modified by mammalian Atg7 and Atg3 to form LC3B-II, which is recruited to autophagosomal membranes. The amount of LC3B-II is correlated with the number of autophagosomes (Mizushima and Yoshimori, 2007). Beclin-1, which has a molecular mass of 60 kDa, is another protein with a crucial role in autophagy because it intervenes in the autophagosome formation and maturation, and its upregulation can be correlated with induction of autophagy (Kang et al., 2011). Therefore, LC3B and Beclin-1 were herein used to monitor autophagy along treatment (12, 24 and 48 h) with bLf (175 and 325 μM) or with the V-ATPase inhibitor ConcA (10 nM). Etoposide (60 μM) and cisplatin (50 μM) were again used as positive controls (C+) in the assays with PC-3 and MG-63 cells, respectively (Yoo et al., 2012; Leisching et al., 2015). ConcA, like BafA1, used as an inhibitor of V-ATPase essential for the acidification of lysosomes and to inhibit the formation of the autophagosome. Moreover, avoiding LC3B-II degradation allows estimating the autophagic flux through its accumulation (Tanida and Waguri, 2010).

The western blot analysis of the PC-3 and MG-63 cell extracts from untreated cells subjected to different treatments is shown in Fig. III.4. In order to facilitate the analysis, optical density of the bands was measured using ImageJ Software and the results will be analysed in detail bellow:

- regarding the PC-3 cell line, the results showed differences not only between the time points, but also between the proteins LC3B and Beclin-1. After 12 h of treatment, no LC3B-II is detected in C-, but the representative plots of band density [LC3B-II/(LC3B-II+LC3B-I)] normalized to β-actin showed that cells treated with 325 μM of bLf or with C+ display higher levels of LC3B-II. At 24 h of treatment, C- already exhibits LC3B-I and LC3B-II, and a significant increase of these levels is observed for bLf and ConcA when compared with C-, except for C+ cells. After 48 h of treatment, C- becomes the condition with higher levels of LC3B-II, while the treatments display similar levels between them (Fig. III.4 A1). As for Beclin-1, its expression levels increased in a bLf dose-dependent way until 24 h and then decreased at 48 h. A similar behaviour was observed for the treatment with ConcA. The highest increase of Beclin-1 at 24 h was in accordance with the observed highest increase of LC3B-II at this same time point (Fig. III.4 A2). Therefore, after 12 h and 24 h of treatment, higher levels of both proteins are detected after treatment with 175 μM or 325 μM of bLf and with ConcA as compared to the C-, while for 48 h this does not occur, suggesting an induction of autophagy in the two first time points followed by a decrease at 48 h.

- the autophagy assays with MG-63 cell line revealed an expression pattern of LC3B-II and Beclin-1 upon treatments similar to the PC-3 cell line. For all treatments, except for cisplatin treatment (C+), there was an increase in the amount of LC3B-II after 12 h. After 24 h, all treatments exhibited higher levels of LC3B-II expression in comparison with the C-, but when the two concentrations of bLf were compared, 175 μM was found to display a greater expression than 325 μM bLf. After 48 h, C- and ConcA were the conditions with higher LC3B-II levels, while the lowest detection of the protein is observed for C+ cells (Fig. III.4 B1). Beclin-1 expression levels, like LC3B-II, displayed an evident increase after 12 h of treatment. At 24 h, only 325 μM of bLf displayed higher levels of expression compared to C- cells. For 48 h of treatment, as for LC3B-II levels, Beclin-1 decreased in comparison with 24 h (Fig. III.4 B2). Therefore, the expression levels of both proteins are very similar for 12 h and 48 h of treatment. Overall, similarly to the PC-3 cell line, an increase in these autophagy-related proteins was only detected after 12 h and 24 h of treatment with bLf and ConcA, with particular relevance after 12 h. This suggests that, at this time point, autophagy is induced. At 48 h of treatment, a downregulation of the proteins levels occurs,

which points to an inactivation of autophagy. In contrast to PC-3 cells, the expression of Beclin-1 for ConcA treatment was identical to C- cells for all the time points studied.

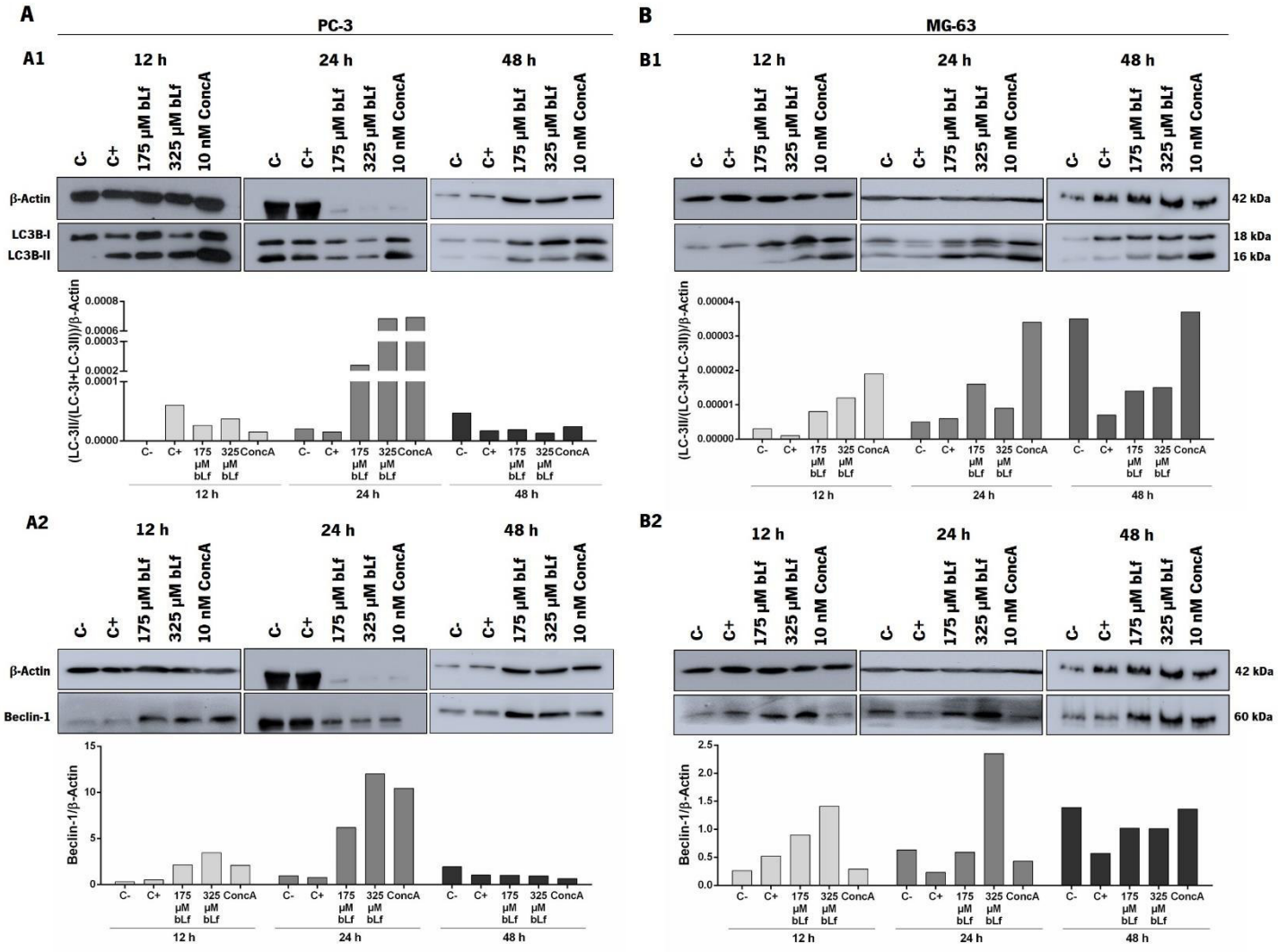

Figure III.4. bLf induces an increase in the levels of LC3B-II and Beclin-1 in PC-3 and MG-63 cell lines after 12 h and 24 h of treatment which decreases after 48 h. Western blot analysis and detection of the autophagic markers LC3B and Beclin-1 after 12, 24 and 48 h of treatment, and representative graphics of density of bands normalized to β-actin in PC-3 and MG-63 cell lines. **A.** Western blot images for the detection of LC3B **(A1)** and Beclin-1 **(A2)** using total extracts of PC-3 cells incubated with 60 µM etoposide (C+), 175 and 325 µM bLf, 10 nM ConcA and without treatment (C-). **B.** Western blot images for MG-63 line of the detection of LC3B **(B1)** and Beclin-1 **(B2)** using extracts of cells incubated in the same conditions as the PC-3 cell line, except for C+ (50 µM cisplatin). For each western blot, 35 µg protein extract were added to each lane.

III.4. bLf induces intracellular acidification in highly metastatic cancer cell lines

Once established that 175 μM of bLf inhibits cell proliferation and induces cell death of the three cell lines under study, we next evaluated whether these cytotoxic effects could be associated to an intracellular acidification as a consequence of V-ATPase inhibition by bLf. To this end, pHi measurements were performed using the pH-sensitive probe BCECF-AM, widely used to determine this cell functional parameter. Excitation by visible light and its stable retention from labelled cells are two advantageous characteristics that make BCECF-AM a common probe used for pHi measurements (Franck et al., 1996). As previously mentioned, this non-fluorescent probe rapidly diffuses into cells where intracellular esterases cleave it to the unesterified form, which emits fluorescence according to the pHi (Ozkan and Mutharasan, 2002). Data were obtained as a ratio of green/red fluorescence intensities (FL1/FL4), which is independent of the probe concentration and exclusively dependent on the pHi.

The results are expressed by the percentage of cells that exhibit a decrease in the ratio of FL1/FL4 in comparison with untreated cells (C-). Fig. III.5 A shows representative histograms of cells incubated in the absence or presence of 175 μM of bLf or of 10 nM ConcA for 24 h. For each histogram, the linear cursors designed to estimate the percentage of cells exhibiting intracellular acidification are shown. We found an increase of the percentage of MDA-MB-231, PC-3 and MG-63 cells exhibiting an intracellular acidification after 24 h and 48 h of treatment with bLf and ConcA, when compared with C- cells (Fig. III.5 B). Since bLf exhibits a similar behaviour as the V-ATPase inhibitor, these results strongly suggest that bLf inhibits V-ATPase, and that the consequent intracellular acidification underlies its cytotoxicity against the three cell lines.

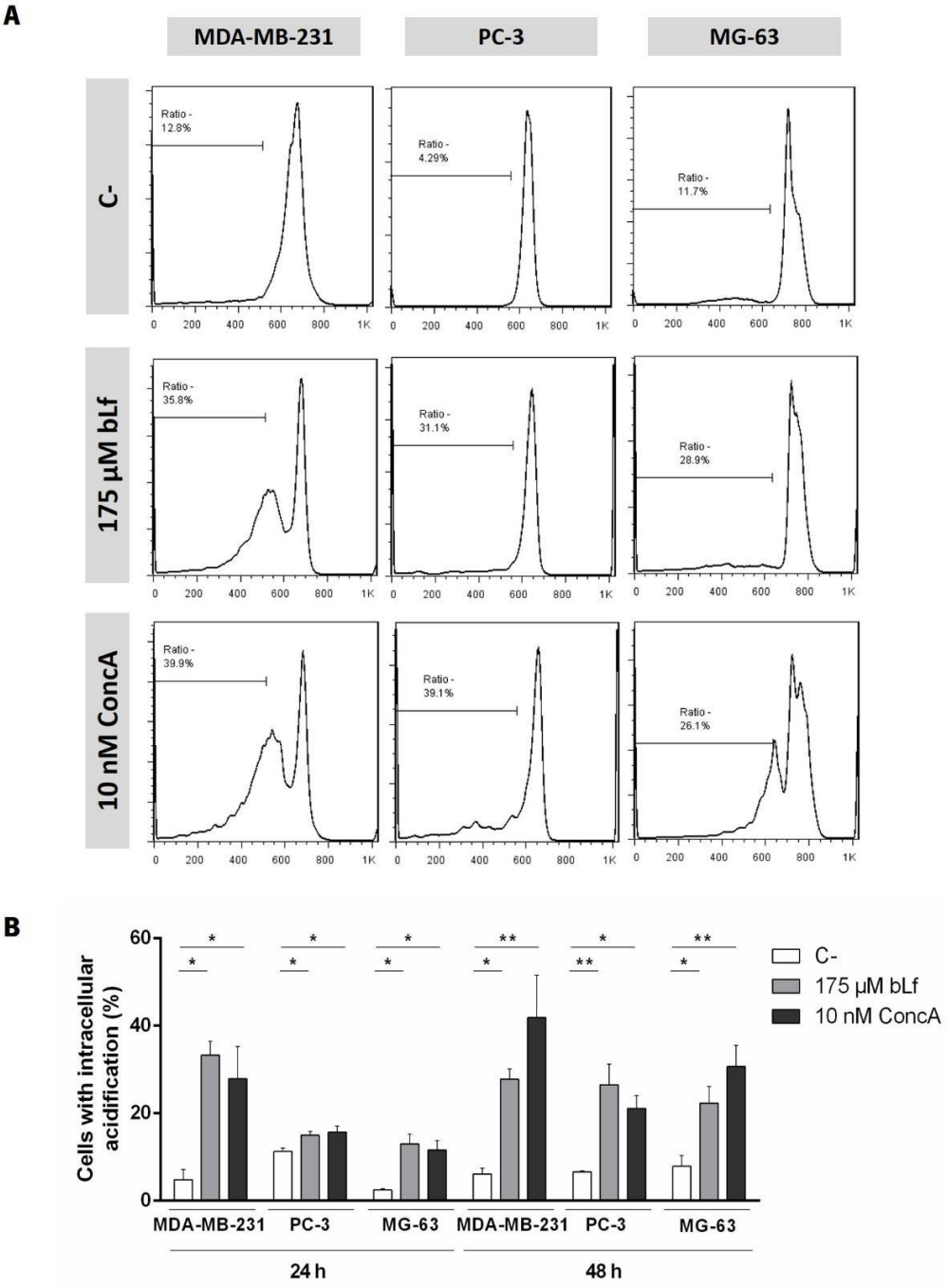

Figure III.5. bLf and ConcA induce intracellular acidification in MDA-MB-231, PC-3 and MG-63 highly metastatic cancer cell lines. A. Representative histograms of the percentage of BCECF-AM-loaded cells displaying intracellular acidification assessed through the decrease of FL1/FL4 median fluorescence intensity ratio in comparison to negative control cells. **B.** Percentage of MDA-MB-231, PC-3 and MG-63 cells treated with 175 µM bLf or 10 nM ConcA, for 24 and 48 h, displaying intracellular acidification (identified under the cursor on A). Values represent the mean ± S.D. of three independent experiments; $^{*}P<0.05$ and $^{**}P<0.01$ compared with the negative control of each cell line.

III.5. bLf alters F-actin arrangement of highly metastatic cancer cell lines

Some studies have reported that plasmalemmal V-ATPase binds to F-actin through its C1 subunit facilitating cancer metastasis by modulation of actin cytoskeleton rearrangement. Therefore, the loss of this subunit affects the normal rearrangement required for invasion (Feng et al., 2014). Since bLf is cytotoxic for the three metastatic cancer cell lines and it triggers an intracellular acidification, we aimed to determine whether V-ATPase inhibition by bLf could affect the actin rearrangement. For this purpose, we treated the cells with two different concentrations of bLf (175 μM and 275 μM) and monitored cell morphology and actin rearrangement by staining with Alexa Fluor 488-Phalloidin that stains the F-actin and delineates the cell cytoskeleton.

The results showed differences between the three metastatic cell lines (Fig. III.6). In the MDA-MB-231 breast cancer cell line there were no evident alterations in the actin cytoskeleton rearrangement when the cells were treated with bLf. However, the protrusions observed in C- cells appeared more visible in the treated cells. Still, some cells with a rounded form seem to undergo cell shrinking after bLf treatment. However, these cells could also be dead cells. For the PC-3 and MG-63 cells, significant alterations on F-actin arrangement were visualized since actin cytoskeleton is completely modified when cells are treated with bLf. In contrast with the untreated PC-3 cells (C-) that show a normal epithelial morphology, after treatment with bLf these cells acquire a more elongated morphology - cell stretching - with formation of lamellipodia (head arrows) and filopodia-like extensions (wide arrows). Another interesting characteristic of the treated cells is the formation of long structures or extensions (thin arrows) that appear to bind different and distant cells. In MG-63 cells, it was also possible to observe the protrusions that connect to extracellular matrix via invadopodia, but these are not so obvious. In these cells, lamellipodia were more evident than filopodia-like extensions upon the bLf treatment. Thus, PC-3 and MG-63 cell lines undergo more prominent alterations on F-actin arrangement than MDA-MB-231. As V-ATPase binds to F-actin, these alterations in the actin cytoskeleton can be related with alterations in the activity of this proton pump.

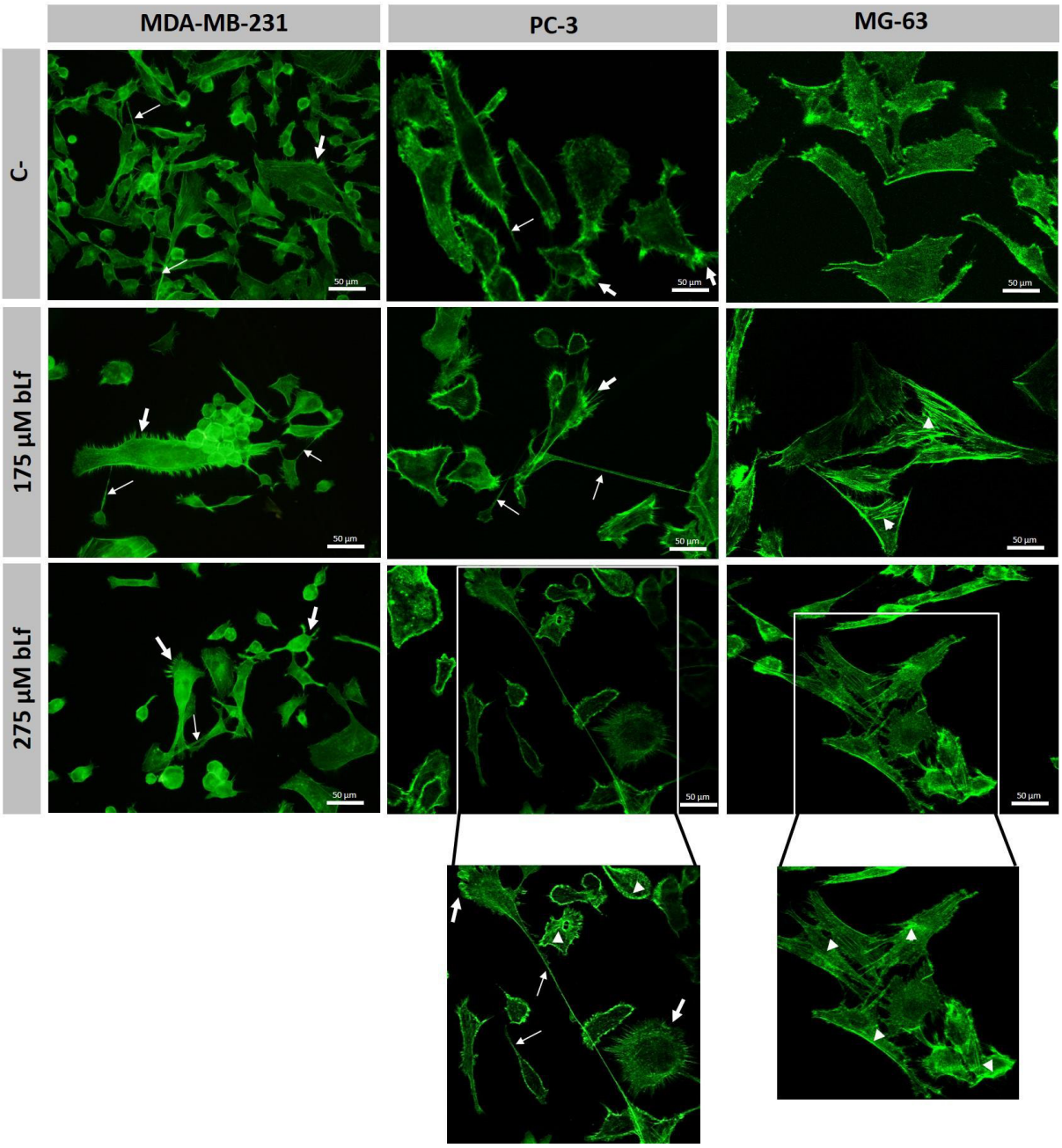

Figure III. 6. bLf can alter F-actin rearrangement and/or produce cellular extensions in highly metastatic cancer cell lines. Representative images of immunofluorescence using fluorescence microscopy for MDA-MB-231 cell line and confocal microscopy for PC-3 and MG-63 cell lines upon 48 h of treatment with 175 and 275 µM bLf. Cytoskeleton was labelled with Alexa Fluor 488-Phalloidin (green fluorescence). Alterations on F-actin arrangement were observed in the three metastatic cell lines, namely lamellipodia (head arrows), filopodia (wide arrows) and extensions (thin arrows).

III.6. bLf perturbs lipid rafts distribution of highly metastatic cancer cell lines

As above mentioned, V-ATPase has been associated to the cholesterol-rich lipid rafts, namely in melanoma cells (Baruthio et al., 2008). In the highly metastatic breast cancer cell line Hs 578T, the treatment with bLf led to a re-distribution of the lipid rafts (Pereira C, master thesis). In this way, we speculated if bLf treatment could also affect the lipid rafts distribution through interaction with V-ATPase in the three cancer cell lines under study. Cells were incubated with 175 μM of bLf or 10 nM of ConcA for 48 h or pre-incubated with 0.5 mM of MβCD for 2 h followed by incubation in fresh culture medium for 48 h. As previously mentioned, MβCD is a lipid microdomain disrupting agent and cholesterol chelator that was herein used as a positive control of lipid raft disruption. Rafts were visualized using filipin, a polyene antibiotic with fluorescent properties that binds cholesterol, the major constituent of lipid rafts (Paffett et al., 2011).

Using filipin labelling, we found that untreated and treated cells display a different distribution of lipid rafts (Fig. III.7). Indeed, untreated cells (C-) exhibited a discrete plasma membrane fluorescence corresponding to cholesterol localization at the lipid rafts, whereas cells treated with MβCD showed an increased intracellular fluorescence and absence or a considerable decrease of plasma membrane fluorescence, indicative of internalization of these microdomains (white arrows). Although this was observed for the three cancer cell lines, in the MDA-MB-231 cells there was already some intracellular filipin stained structures in C- cells. Cells treated with bLf or ConcA, displayed an increase of intracellular filipin staining in the form of larger and brighter clusters in comparison with C- cells.

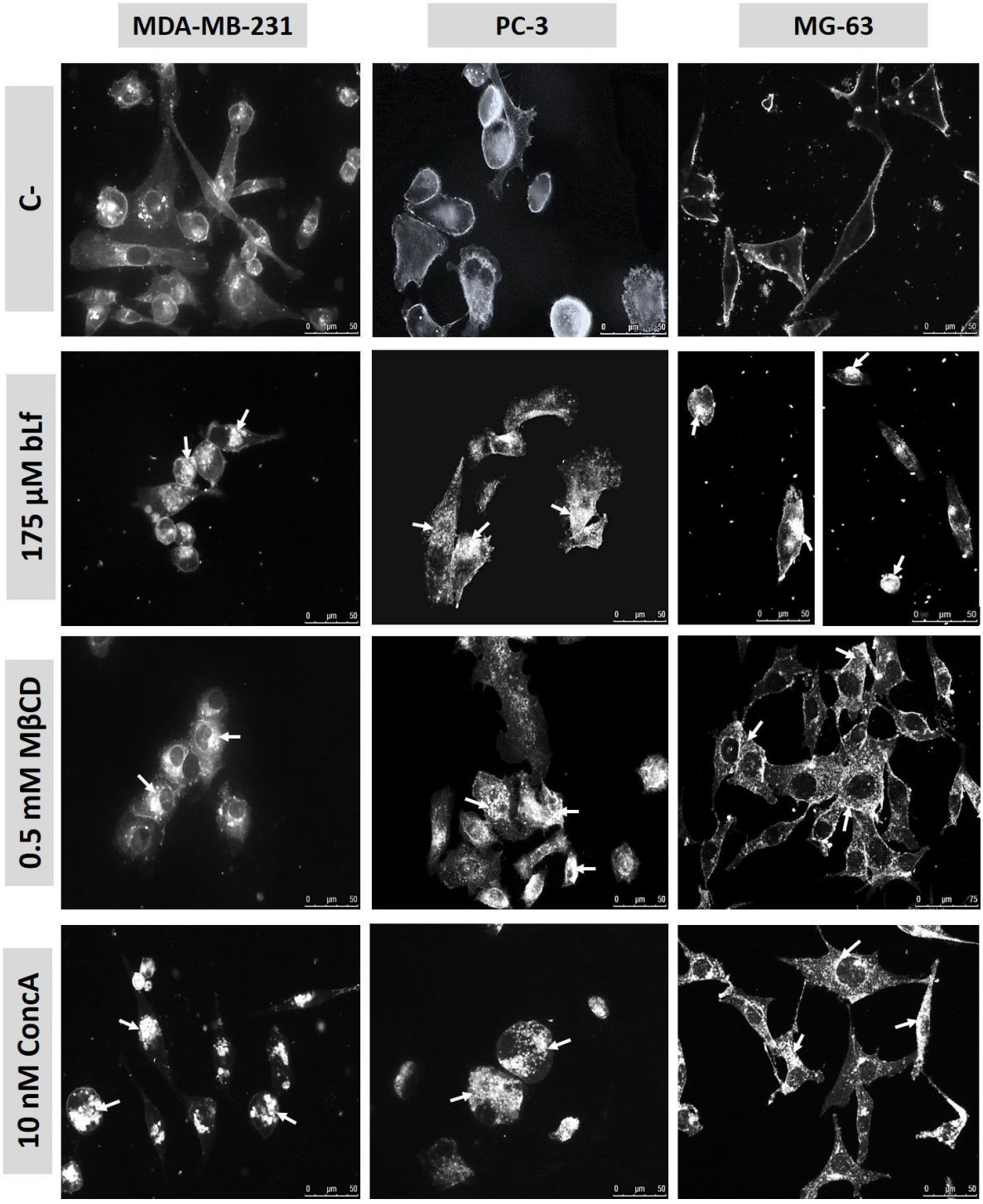

Figure III.7. bLf perturbs cholesterol-rich lipid rafts distribution in the highly metastatic cancer cell lines. Representative images of immunofluorescence of MDA-MB-231, PC-3 and MG-63 cells upon treatment with 175 µM bLf, 10 nM ConcA or after pre-treatment with 0.5 mM MβCD for 2 h and incubation in fresh culture medium for 48 h. Lipid rafts were stained with filipin (0.01 mg/mL) (white fluorescence). All the three compounds led to an internalization of the lipid rafts (white arrows).

III.7. Methyl-β-ciclodextrin protects highly metastatic cancer cells against bLf-induced intracellular acidification and inhibition of cell proliferation

Taking into account that the three cell lines under study were previously reported to display V-ATPase at the plasma membrane and that V-ATPase has been found to localize at the cholesterol-rich lipid rafts, we sought to investigate whether cells pre-treated with MβCD were equally susceptible to bLf-induced intracellular acidification and inhibition of cell proliferation.

Intracellular acidification was assessed with the pH sensitive probe BCECF-AM as described above. The results are expressed as the percentage of cells displaying a decrease in the ratio of green/red median fluorescence intensities (FL1/FL4) in cells pre-treated or not with 0.5 mM MβCD during 2 h, as described in Fig. III.7, and then incubated with 0 μM or 175 μM bLf, for 48 h (Fig. III.8 A). While the percentage of cells exhibiting intracellular acidification in cell suspensions pre-treated with MβCD and non-exposed to bLf was not statistically different from untreated cells (C-) cells, pre-treatment with MβCD and subsequent exposure to bLf led to a percentage statistically lower than the one observed for cells that were solely exposed to bLf. These results demonstrate that MβCD protects cells from bLf-induced intracellular acidification.

When cell proliferation was evaluated with the CFSE probe under these same conditions, pre-treatment with 0.5 mM MβCD did not affect cell proliferation comparatively with C- cells, but this pre-treatment reverted completely the inhibition of cell proliferation by bLf after 48 h and 72 h (Fig. III.8 B). However, after 24 h of treatment, cell proliferation of the three cell lines was not affected in any of the conditions tested. bLf treated cells, after 48 h, inhibited cell proliferation, being its effect more evident in the PC-3 cell line. Also, after 72 h, bLf exhibited a similar inhibitory effect in all cell lines, when compared with C- cells, as reported above (III.1). On the other hand, while the three cells lines treated with MβCD displayed a similar behaviour to the untreated cells (C-), cells pre-treated with MβCD and then treated with 175 μM bLf were found to proliferate similarly to the C- cells. In conclusion, the pre-incubation of cells with MβCD displays a cell protective effect since it prevents bLf-induced inhibition of cell proliferation.

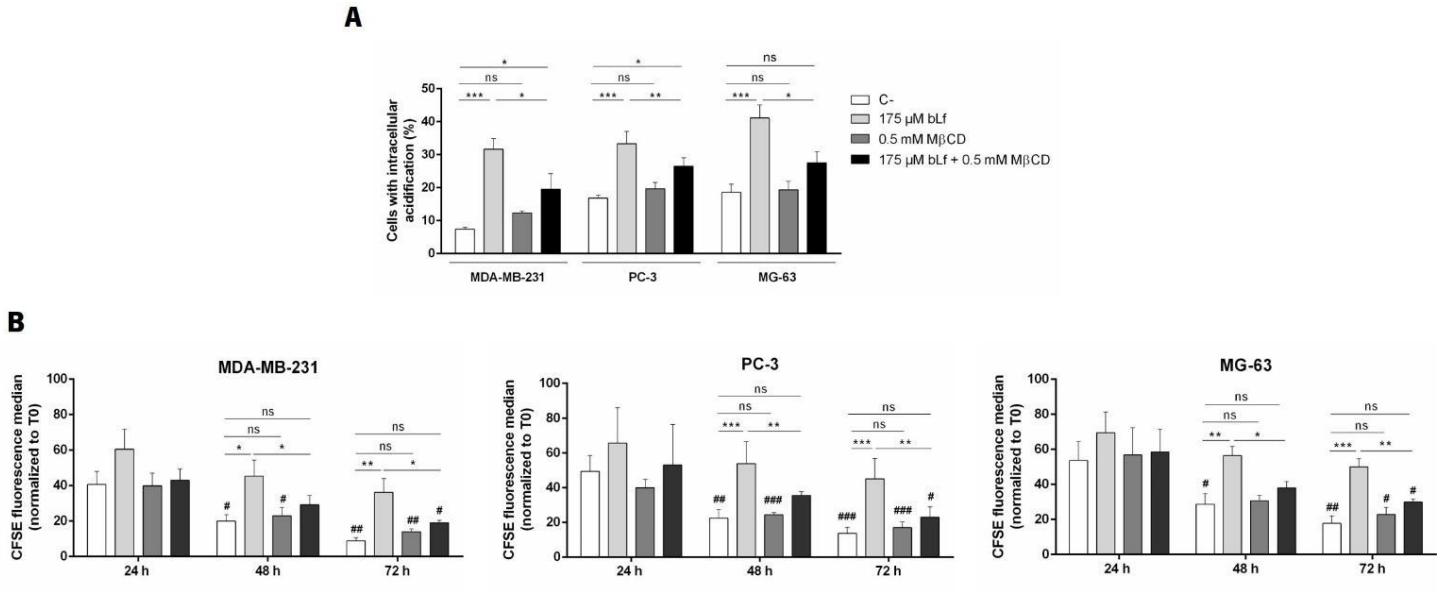

Figure III.8. MβCD protects cells against bLf-induced intracellular acidification and inhibition of cell proliferation in MDA-MB-231, PC-3 and MG-63 cells. A. Effect of MβCD on the bLf-induced intracellular acidification. The percentual values were estimated using the pH-sensitive probe BCECF-AM. **B.** Effect of MβCD on bLf-induced cell proliferation inhibition after 48 h, assessed with the CFSE probe. The fluorescence values were estimated for cells untreated (C-) or treated with 175 µM bLf for 48 h after incubation with 0.5 mM MβCD (2 h) and normalized to T0. The values represent the median (A) or mean (B) of 3 independent assays. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0,001$ compared with C- of each time point and $^{\#}P<0.05$; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0,001$ compared with 24 h.

Chapter IV

Discussion

As previously referred, multiple biological activities have been attributed to the iron-binding glycoprotein Lf (García-Montoya et al., 2012), such as anticancer, antimicrobial, anti-inflammatory and immunomodulatory. Anticancer activity is among the protective effects displayed by orally administered Lf (Giansanti et al., 2016). Furthermore, many of the defensive activities of Lf that have been discovered may be ascribed to peptides derived from proteolysis of the orally ingested protein. Lf from bovine milk, compared to other sources, is cheaply produced and commercially available, besides being well tolerated after ingestion (Tomita et al., 2009). Altogether, these features make bLf an ideal nutraceutical. Indeed, bLf has been used in immune system-enhancing nutraceuticals, cosmetics, drinks, fermented milks, toothpaste, among others (Manzoni et al., 2009; García-Montoya et al., 2012). Meanwhile, EFSA has also approved bLf as a safe ingredient for various applications (EFSA-NDA, 2012). Moreover, due to the established bLf anticancer activity against a wide range of human cancers using different cell lines and animal models, and even in clinical trials, this biodrug emerged as highly promising for the prevention and therapy of cancer, as well as for further research (Mayeur et al., 2016). Although, some mechanisms underlying bLf anticancer activity have already been elucidated, its cellular/molecular target(s) had not been identified.

Recently, it was reported by our group that the highly metastatic breast cancer cell line Hs 578T exhibits a higher susceptibility to bLf than the poorly invasive breast cancer cell line T-47D, and that no cytotoxic effect was found in the non-tumorigenic MCF-10-2A cell line. These results indicate that bLf displays a preferential cytotoxicity against highly metastatic breast cancer cells that have V-ATPase at the plasma membrane. Moreover, we found that another highly metastatic breast cancer cell line - MDA-MB-231, previously reported to possess V-ATPase at the plasma membrane, is also susceptible to bLf. Besides, the results showed that bLf displays a similar behaviour as ConcA, inhibiting extracellular acidification rate and inducing intracellular acidification by inhibiting this proton pump (Pereira et al., 2016). As a whole, a large body of evidence was gathered supporting that this selective activity occurs through inhibition of the plasmalemmal V-ATPase (Pereira et al., 2016).

In the present study, we question whether bLf also targets V-ATPase in highly metastatic cancer cells other than breast cancer cells, which would allow establishing a common mechanism and a more rational administration of bLf in the prevention of different cancers. To address this issue, we selected two other highly metastatic cancer cells, namely the prostate cancer (PC-3) and the osteosarcoma (MG-63) cell lines to compare with the previously studied MDA-MB-231. Our results show that the three highly metastatic cancer cells exhibit a similar susceptibility to bLf regarding inhibition of cell proliferation and

induction of intracellular acidification. Localization of V-ATPase at the plasma membrane is another reported feature that is shared by the three cell lines under study (Sennoune et al., 2004; Bermudez 2010; Perut et al., 2014). This common cellular trait together with our previous data on highly metastatic breast cancer cell lines support the notion that bLf also targets the plasmalemmal V-ATPase of these two non-breast highly metastatic cancer cell lines hindering its activity. This inhibition causes an intracellular acidification and subsequent reduction of cell proliferation, and likely will underlie the selective anticancer activity of bLf against other highly metastatic cancer cells. In fact, there are same studies attesting the anti-metastatic role of Lf against other types of cancers. For example, in human nasopharyngeal carcinoma (NPC) cells, the overexpression or treatment with Lf inhibited the proliferation these cells *in vitro*. Importantly, the analysis of human NPC tissues derived from primary tumours and metastasis sites revealed that Lf may negatively regulate the development of metastasis in NPC (Zhou et al., 2008).

To further support that V-ATPase is the molecular target of bLf in MDA-MB-231, PC-3 and MG-63 cancer cells we attempted to establish a relation between cell susceptibility and the cellular levels of V-ATPase as compared to the non-tumorigenic breast cell line MCF-10-2A which is not susceptible to bLf (Pereira et al., 2016). Though the three cell lines display a higher V-ATPase total level than the non-tumorigenic breast cell line, this is less evident for MG-63 cells. However, since these data are from only one independent assay further experiments are required to support this conclusion. Moreover, the comparison of the levels of V-ATPase in plasma membrane cellular sub-fractions would reinforce our proposed mechanism of action and V-ATPase as the molecular target of bLf in highly metastatic cancer cells.

Recently, our group discovered that the inhibition of Hs 578T and MDA-MB-231 cell proliferation by bLf was accompanied by the induction of cell death associated with the exposure of phosphatidylserine (Pereira et al., 2016). Other authors also found that different cancer cell lines, namely the gastric cancer cell line AGS (Amiri et al., 2015) and the breast cancer cell lines MCF-7 and MDA-MB-231 (Gibbons et al., 2015; Zhang et al., 2015c), display this apoptotic marker in response to bLf treatment. In accordance with our previous study, we found that bLf-induced inhibition of cell proliferation in MDA-MB-231 cells is associated with an increase in AV^+/PI^- indicating induction of apoptosis. In contrast, bLf-induced inhibition of cell proliferation in PC-3 cells is associated only with a slight but statistically significant increase of AV^+/PI^- cells. A similar increase of AV^+/PI^- cells is detected in PC-3 cells treated with etoposide (C+). While the exposure of phosphatidylserine in C+ of these cells is associated with caspase-3 cleavage, the same is not observed in response to bLf, which indicates that the apoptotic pathway is caspase-3 independent.

On the other hand, for the time points and bLf doses tested, cell proliferation inhibition of the MG-63 osteosarcoma cancer cell line appears to not be associated with cell death. Indeed, the analysis of the viable cells (AV^-/PI^-) *versus* dead cells (either AV^+/PI^- plus AV^+/PI^+ plus AV^-/PI^+; or total apoptotic, early AV^+/PI^- plus late AV^+/PI^+; or total necrotic AV^-/PI^+ plus AV^+/PI^+) revealed that only exposure to cisplatin induces a statistically significant increase of dead cells or of total apoptotic cells. Though these observations are consistent with caspase-3 activation by cisplatin, but not by bLf treatment, they are in disagreement with the visible amount of cells in suspension (dead cells) observed in the microplate wells corresponding to cells treated with bLf (see Fig. III.1 C). This discrepancy may be due to the high standard deviations of the results, and therefore more independent assays are required to confirm if cell death is induced. Anyway, it appears that MG-63 cells are more resistant to apoptosis induction by bLf and ConcA, and that higher concentrations should be tested. In fact, for these two cell lines no cell death studies with bLf have been previously performed. Furthermore, PC-3 cells can undergo apoptosis when exposed to others compounds like chemotherapeutic agent piplartine with up-regulation of procaspase-3 and PARP-1 cleavage (Kong et al., 2008). Interestingly, in androgen-independent prostate tumours, such as the one from which PC-3 cell line derives, a much higher level of expression of Bcl-2, an anti-apoptotic protein, was detected, making the cells resistant to apoptosis and insensitive to cytotoxic chemotherapeutic agents (Pilat et al., 1998). Curiously, PC-3 cells have been reported to not be capable of producing any p53 protein (Russell and Kingsley, 2003). Some authors have reported that the absence of *P53* gene may be important for cell proliferation and consequently, the restoration of this tumour suppressor gene may decrease the growth potential of PC-3 cells (Carroll et al., 1993). This evidence may be relevant for understanding certain behaviour of this cell type. Other studies with the osteosarcoma MG-63 cell line also showed apoptosis induction by high concentrations of proflavin and curcumin through the activation of the intrinsic caspase pathway (Zhang et al., 2015a) and through accumulation of intracellular ROS (Chang et al., 2014), respectively.

Currently, autophagy has caught particular attention in cancer research. Indeed, this cell survival and stress response process, which is engaged in the removal of damaged organelles, protein aggregates and long-lived proteins, and is responsible for cellular homeostasis, has been proposed to play dual opposite roles in cancer (Galluzzi et al., 2015). It appears to have a tumour suppressor role in the early phases of cancer formation, while in a later phase it may provide and/or aid tumour growth, spread and contribute to treatment resistance. We found increased expression levels of LC3 and Beclin-1 after 12 h and 24 h of treatment with bLf and ConcA for both PC-3 and MG-63 cell lines followed by a decrease,

more prominent in PC-3 cells, after 48 h. Although Yoo et al., 2012 and Leisching et al., 2015 suggested etoposide and cisplatin, respectively, as autophagy inducers in PC-3 and MG-63 cell lines, they were not the most indicated controls for autophagy induction, and other conditions, such as starvation and hypoxia or treatment with rapamycin (Gozuacik and Kimchi, 2004; Jiang et al., 2013; Li et al., 2013) should have been tested as positive controls. Curiously, after 12 h, the C- cells already display increased levels of expression of the proteins used as autophagic markers. As referred above, under physiological conditions, autophagy proceeds at basal levels to ensure the continuous removal of damaged organelles potentially dangerous, mediating a key homeostatic function that operates as an intracellular control system (Galluzzi et al., 2015). However, the two highly metastatic cancer cell lines PC-3 and MG-63 may have high basal levels of autophagy. To ascertain this hypothesis, we should have determined the levels of LC3B and Beclin-1 at 0 h of treatment, i.e., in the moment of the treatment. It is recognised that the accumulation of autophagosomes is not always indicative of autophagy induction representing either the increased generation of autophagosomes or a block in autophagosomal maturation and the completion of the autophagy pathway. This also applies to the increase in the expression levels of LC3B and Beclin-1 (Mizushima et al., 2010). In this line, though the increase in the expression levels of LC3B and Beclin-1 suggests that autophagy is induced in PC-3 and MG-63 cells in response to bLf, the determination of the autophagic flux, through the conversion of LC3-I to LC3-II in the presence and absence of BafA1 or ConcA should have been determined (see chapter V).

Since it is known that V-ATPase binds to F-actin, we next sought to investigate whether inhibition of this proton pump by bLf could affect the actin rearrangement of the three metastatic cancer cell lines. Recently, it was shown that the C subunit of V-ATPase is responsible for the reversible dissociation of the V_0 and V_1 domains. When this subunit was knocked down by shRNA, the regular arrangement of F-actin observed in MDA-MB-231 cells was lost, suggesting that C subunit regulates actin cytoskeleton arrangement (Feng et al., 2014). Also, in highly metastatic cells, V-ATPase plays an important role in invasion since treatment with V-ATPase inhibitors prevents *in vitro* invasion (Cotter et al., 2015). In another cancer cell line, 4T1, in normal conditions, a spear-shaped elongated cell morphology, essential for cell migration, is acquired, but in the absence of the C subunit of V-ATPase the cells lose this regular orientation acquiring a rounded shape (Feng et al., 2014). In this work, we found that bLf significantly promotes a prominent F-actin cytoskeleton rearrangement in PC-3 and MG-63 cell, though less significant in MDA-MB-231 cells, as well as cell stretching or shrinking. We anticipate that the adhesive structures such as the protrusions and extensions observed in the three highly metastatic cancer cells may be

related with cell migration and invasion, but also can act as a defence mechanism against the bLf treatment. Indeed, Lf was already described as a promoting factor for the migration of MCF-7 breast cancer cell line (Ha et al., 2011; Zhang et al., 2015c). Furthermore, lamellipodia and filopodia-like extensions connected to the extracellular matrix (ECM) via invadopodia and podosomes were already found in PC-3 cells. These structures were implicated in migration and invasion of metastatic tumour cells to surrounding tissues (Desai et al., 2008), and therefore were considered as attractive therapeutic targets (Jacquemet et al., 2015). Another hypothesis to explain the appearance of these structures upon bLf treatment can be an interference of this protein with the actin-binding proteins, for instance Profilin I, stimulating or inhibiting the actin polymerization. In MDA-MB-231 cells, inhibition of Profilin I expression promotes hypermotility and metastatic spread, contrasting with its role in enhancing polymerization (Lorente et al., 2014). Besides, bLf treatment can cause compressive stress or instability in the cells during the tumour growth stimulating formation of leader cells, stress fiber and cell-substrate adhesion (Tse et al., 2012). Nonetheless, these studies generate a controversy about the role of bLf, since this protein has been associated with an opposite role in the process of invasion (Yin et al., 2013). However, cell migration assays may clarify whether these protrusions and extensions are necessary but not sufficient for cell migration. This hypothesis is conceivable since cell proliferation is inhibited upon treatment with bLf and ultimately should inhibit cell invasion/metastasis. The protrusions observed upon bLf treatment can also correspond to the so-called tunnelling nanotubes (TNTs) – structures of intercellular communication – that allow the transfer of signals, molecules, organelles, membrane contents, Golgi vesicles and mitochondria between cells (Gurke et al., 2008). These structures have been reported as potential therapeutic targets with a crucial role in cancer progression (Lou et al., 2012a), since they mediate the transfer of factors that enhance chemoresistance (Pasquier et al., 2013) and the transport of miRNAs between tumour-tumour cells and tumour-stromal osteosarcoma cells (Thayanithy et al., 2014). The recent discovery of antibiotic resistance of bacteria following communication via TNTs (Dubey and Ben-Yehuda, 2011) provides further support for the idea that highly metastatic cancer cell lines, such as those under study in this work, may also propagate chemoresistance by acquiring and sending genetic codes or signals which induce resistance to chemotherapy via TNTs (Lou et al., 2012a, 2012b). In summary, the highly metastatic prostate PC-3 and osteosarcoma MG-63 cancer cell lines undergo more alterations on F-actin arrangement than the highly metastatic breast cancer cell line MDA-MB-231. Moreover, as V-ATPase binds to F-actin, alterations in the actin cytoskeleton can be related with

alterations in this proton pump activity. These results, together with our previously data, reinforce that bLf targets V-ATPase at the plasma membrane.

Another key outcome from our work concerns the interplay between bLf, V-ATPase and cholesterol-rich lipid rafts. V-ATPase has been identified as a component of the lipid rafts in highly metastatic cancer cell lines, namely in melanoma cells (Baruthio et al., 2008). Therefore, we evaluate whether inhibition of V-ATPase by bLf perturbed these membrane lipid microdomains. We found that, as expected, MβCD, alters the membrane cholesterol-rich lipid rafts distribution, and leads to the internalization of cholesterol and of its depletion from the plasma membrane, while pre-incubation with MβCD antagonizes the effect of bLf in the cell lines under study. Indeed, the induction of intracellular acidification and inhibition of cell proliferation by bLf are reverted. Remarkably, bLf induces an internalization of lipid rafts similar to that induced by MβCD. Also the anti-tumour compound edelfosine, which exhibits affinity for cholesterol, induced, in HeLa cells, a similar re-distribution of the lipid membrane microdomains (Mollinedo and Gajate, 2015; Mollinedo et al., 2011). Our hypothesis for this effect of bLf, similarly to what occurs in melanoma cells (Baruthio et al., 2008) and in the highly metastatic breast cancer cell line Hs 578T (Pereira et al. 2014, master thesis), is that this destabilization and internalization of the lipid rafts is a consequence of V-ATPase inhibition that leads to intracellular acidification and consequently, to the inhibition of cell proliferation. On the other hand, pre-incubation with MβCD prevented bLf-induced inhibition of cell proliferation in the three cell lines. Two possible explanations can be proposed. Firstly, cholesterol depletion and perturbation of lipid rafts may lead to the removal of the bLf molecular target (V-ATPase) from the plasma membrane, blocking the inhibitory action of bLf and the subsequent intracellular acidification. However, this is not consistent with the recovery of pHi by MβCD, unless cells find a V-ATPase independent mechanism to regulate its pH_i. As a second hypothesis, V-ATPase is not removed from the plasma membrane and the alterations in the lipid environment mediated by MβCD counteract the inhibitory effect on V-ATPase by bLf preserving its activity and allowing the recovery of the pH_i. However, V-ATPase may in turn remain in the plasma membrane but inactive, and in this case, cells have to rely on another alternative mechanism to maintain the pH_i. A study has confirmed that V-ATPase activity is regulated by the association/dissociation of its domains and by the interaction with the specific lipid environment (Lafourcade et al., 2008). All subunits of this proton pump were reported to show great affinity for DRMs isolated from late endosomes, creating a possible association of this proton pump with cholesterol-rich lipid rafts domains. Accordingly, evidences established not only that several V-ATPase inhibitors incorporate into the lipid bilayer, but also affect its

structural flexibility (Lafourcade et al., 2008). In yeast cells, the treatment with C2-phytoceramide as with MβCD causes a decrease of fluorescent dot staining of rafts at the plasma membrane, and an increase in fluorescent intracellular structures. Pre-treatment of yeast cells with MβCD lead to an increased resistance to C2-phytoceramide indicating that ergosterol is a C2-phytoceramide target (Pacheco et al., 2013). Furthermore, after C2-phytoceramide exposure, a uniform rather than a punctuated pattern at the plasma membrane of the yeast plasma membrane ATPase (Pma1p) was observed (Pacheco et al., 2013). This proton pump is localized in the lipid rafts (Bagnat et al., 2001) and is essential for the modulation of the pH_i (Mollinedo, 2012). In light of these data, the protective effect of bLf cytotoxicity by MβCD could suggest the lipid raft component cholesterol, the ergosterol mammalian counterpart, as a target of bLf. However, this hypothesis would not explain the preferential selectivity of bLf to cancer cells and the non-cytotoxicity to non-tumorigenic cells. Immunofluorescence assays are required to assess whether bLf affects the localization of V-ATPase at the plasma membrane of the three highly metastatic cancer cells, and if MβCD interferes with it. If in the presence of MβCD, V-ATPase still remains in the plasma membrane, it is conceivable that the MβCD protective effect relies on the perturbation of V-ATPase lipidic environment that is critical for the pump's activity and, in this way, counteracts the intracellular acidification and cell proliferation induced by bLf. In summary, these data indicate that membrane lipid microdomains are re-distributed in the highly metastatic cancer cell lines upon bLf treatment, suggesting that bLf perturbs lipid rafts distribution likely through inhibition of the V-ATPase. Since this proton pump has been reported to be present at the plasma membrane of the three metastatic cell lines, all these evidences further support that V-ATPase is a molecular target of bLf. Moreover, MβCD displays a protective role against bLf-treated cells, avoiding inhibition of the proton pump by bLf and subsequently the induction of intracellular acidification and the inhibition of cell proliferation.

Overall, our data demonstrate that there is a clear relation between bLf and V-ATPase and that, the molecular mechanism underlying the selective Lf anticancer activity against highly metastatic cancer cells relies on its targeting. Therefore, V-ATPase may be considered a molecular target for bLf in cancer therapy of highly metastatic cancers.

Chapter V

Final Remarks & Future Perspectives

V.1. FINAL REMARKS

In this thesis project, we aimed to ascertain whether inhibition of the plasmalemmal V-ATPase by bLf, as previously observed for two highly metastatic breast cancer cells (Pereira et al., 2016), could be a generalized mechanism underlying the anticancer activity of this natural protein against other types of highly metastatic cancer cells. Given the well-known importance of V-ATPase in cancer progression and lack of mechanistic studies concerning bLf, this could be a step forward to a more rational use of bLf in cancer therapy, in particular in highly metastatic cancers.

We showed that the susceptibility to bLf of the two highly metastatic cancer cell lines derived from prostate cancer (PC-3) and osteosarcoma (MG-63) was similar to that derived from breast cancer (MDA-MB-231) regarding both inhibition of cell proliferation and induction of intracellular acidification. These observations supported the notion that, as previous shown in the highly metastatic breast cancer cell line MDA-MB-231, inhibition of V-ATPase in the prostate cancer and osteosarcoma cells also determines their susceptibility to bLf. We were therefore encouraged to proceed with a deeper investigation on our hypothesis, as V-ATPase has been reported to localize at the plasma membrane of these highly metastatic cancer cells (Sennoune et al., 2004; Bermudez 2010; Perut et al., 2014), and we found that its expression seemingly increases in comparison to a non-tumorigenic cell line. Notably, while inhibition of cell proliferation and induction of intracellular acidification by bLf is apparently associated with the induction of apoptosis for MDA-MB-231, and with a caspase-independent apoptotic pathway for PC-3 cells, no cell death was detected in MG-63 cells. This may suggest that this cell line is more resistant to cell death induced by bLf.

Some studies reporting that V-ATPase, located at the plasma membrane, binds to F-actin through its C subunit to facilitate cancer metastasis by modulation of actin cytoskeleton rearrangement motivated us to search whether V-ATPase inhibition by bLf could affect actin rearrangement. In fact, we observed several alterations on F-actin cytoskeleton in the three cancer cell lines after bLf treatment, namely the formation of lamellipodia/filopodia-like protrusions and large extensions between cells. However, PC-3 and MG-63 cell lines undergo more alterations on F-actin arrangement than MDA-MB-231 cells. The alterations in the cytoskeleton in response to bLf likely reflect a perturbation of the interaction between V-ATPase and F-actin, and further reinforce that V-ATPase is a molecular target of bLf.

Other evidence that inspired us in our study was that bLf perturbed the lipid rafts distribution, since cholesterol-rich lipid rafts are re-distributed in the three highly metastatic cancer cell lines when

exposed to bLf treatment. Moreover, we evaluated the effect of MβCD on the bLf-induced intracellular acidification and inhibition of cell proliferation. We found that the cholesterol chelator and lipid raft perturbing agent MβCD counteracted these cytotoxic effects likely through the perturbation of the V-ATPase lipidic environment that determines its activity and, in some way counteracts the inhibition of the proton pump by bLf.

Overall, our data strongly support that bLf selectively targets highly metastatic cancer cells exhibiting V-ATPase at the plasma membrane, while having no effect on non-tumorigenic cells. Besides, since bLf is a natural drug, it contributes to the inhibition of cell proliferation and tumour growth and progression, and also surpasses multidrug resistance, a severe problem of current cancer therapies. In summary, the main outcome of this study is the finding that bLf can be used in the therapy of highly metastatic cancers through the targeting of plasmalemmal proton pump V-ATPase, and consequent perturbation of the tumour microenvironment crucial for the survival of the cancer cell.

As bLf is a commercially available non-toxic and low-cost dietary natural protein, it is expected that its application in healthcare and cancer therapy will increase extensively. Therefore, for its safe and appropriate application in humans it is very important to know its mechanism of action. In this sense, we believe that the work developed in this master thesis and the finding that bLf targets V-ATPase in highly metastatic cancer cells is of great scientific impact and brought novel important data useful for further *in vitro* and *in vivo* researches on the anticancer role of bLf.

V.2. FUTURE PERSPECTIVES

Following this study, new questions emerged on the interaction between bLf and V-ATPase that still need to be answered towards a full exploitation of the bLf beneficial effects in the therapy of highly metastatic cancer cells. First, it will be important to perform all assays, including the effect of bLf on cell proliferation and intracellular pH, in a non-tumorigenic cell line like the immortalized fibroblasts BJ-5ta. Though we have shown that a non-tumorigenic breast cell line is insensitive to bLf, the use of other non-tumorigenic cell lines is essential for validating the protein non-cytotoxicity and for comparison purposes with the highly metastatic cancer cell lines under study. It will be also important to verify the localization of bLf in the cell in order to better understand if the protein is internalized by the cell lines herein used. In fact, if bLf is internalized, even in a small amount, it may trigger some events, which could be a consequence of its interaction with other targets. On the other hand, if bLf is not internalized, the target(s)

reside(s) at the cell surface and all the ensuing events are a consequence of the interactions of bLf and component(s) of the cell surface. Immunofluorescence microscopy assays with a bLf antibody can be performed to elucidate this question.

Regarding the cell death pathway triggered by bLf, several experiments should be performed to better characterize the death process. It would be interesting to monitor other cellular events such as release of cathepsins and mitochondrial pro-apoptotic effectors, as well as lysosomal and mitochondrial membrane permeabilization. Moreover, detection of other apoptotic markers such as DNA fragmentation, mitochondrial ROS accumulation and chromatin condensation, will be required to further characterize cell death induced by bLf. To address the involvement of autophagy in the response to bLf, other autophagic markers should be evaluated. For instance, the number of autophagosomes can be determined through the subcellular localization of LC3 and counting the number of punctate LC3 structures per cell, by electron microscopy or fluorescence microscopy. As referred in the previous chapter, an accumulation of autophagosomes is not always indicative of autophagy induction and, in this sense, it is important to monitor the autophagic flux. This can be determined by assessing the conversion of LC3-I to LC3-II or the LC3 turnover based on LC3-II degraded in autolysosomes through western blot and immunofluorescence microscopy with GFP-LC3B fusion protein, respectively, in the absence and presence of ConcA or BafA1.

Wound healing assays allow assessing the migratory ability of cells with or without treatments. This assay could be used to unravel the relation of bLf with the production of cellular protrusions and extensions, and to evaluate whether these structures are associated or not with cell motility and migration.

In the present study, novels aspects on the interplay between lipid rafts, V-ATPase and bLf emerged. Indeed, it was found that bLf perturbs cholesterol-rich lipid rafts. Moreover, MβCD displays a protective role against bLf-treated cells, avoiding inhibition of the proton pump by bLf, and subsequently the induction of intracellular acidification and inhibition of cell proliferation. In order to better comprehend the behaviour of the cells during this combined treatment, the filipin staining should be performed to visualize its effects on lipid rafts distribution. Moreover, it would be pertinent to assess, through filipin staining and immunofluorescence fluorescence assays whether V-ATPase, as described for some cell lines, is located in the lipid rafts and also whether bLf co-localises with the proton pump in the lipid rafts. Finally, it would be important to find if V-ATPase is re-localized after bLf treatment in cells pre-treated or not with MβCD. Additionally, as lipid rafts are important signalling platforms, upon rafts perturbation by bLf, intracellular cascades can be explored, constituting an innovative research topic that may reveal a

new mechanism underlying the anticancer activity of Lf. Basal extracellular rate (ECAR) of the three cell lines under study, reflecting V-ATPase activity, should also be monitored before and after exposure to bLf in cells pre-treated or not with MβCD.

Answering all these questions with the help of methods and techniques previously mentioned, will surely improve our knowledge on the interaction between bLf and V-ATPase in highly metastatic cancer cell lines. These studies are extremely relevant for clarifying the anticancer activity of bLf, and ultimately to validate this emergent strategy and turn out the administration of bLf widespread in cancer therapy of highly metastatic cancers.

Chapter VI

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Chapter VII

Supplementary Material

Table VII.1. bLf exhibits similarly cytotoxicity against the three highly metastatic cancer cell lines MDA-MB-231, PC-3 and MG-63 regarding inhibition of cell proliferation. Inhibition of cell proliferation by bLf, cisplatin or etoposide follows an exponential kinetics. The percentage of inhibition (%) was estimated through the mean of the slopes of the linearised exponential functions of the CF mean fluorescence intensity of cells treated with 175 μM of bLf, cisplatin or etoposide (used as C+ for MDA-MB-231/MG-63 and PC-3 cells, respectively) *versus* time, normalized to the mean of the slopes of the linearised exponential functions of the CF mean fluorescence intensity of untreated cells (C-), for each cell line. The values presented are the relative means expressed in percentage ± S.D. of three independent experiments: $^{\#}$ cisplatin and etoposide susceptibility comparison between the 3 cell lines**;** $^{\$}$ susceptibility comparison of each cell line to bLf or cisplatin or etoposide; and * bLf susceptibility comparison between the 3 cell lines.

		Cell Proliferation Inhibition (%)	SD
MDA-MB-231	Cisplatin	11,56$^{\#\$}$	3,668
	bLf	34,30$^{*\$}$	3,396
PC-3	Etoposide	30,45$^{\#\$}$	23,29
	bLf	19,79$^{*\$}$	10,63
MG-63	Cisplatin	20,15$^{\#\$}$	14,15
	bLf	34,00$^{*\$}$	13,03

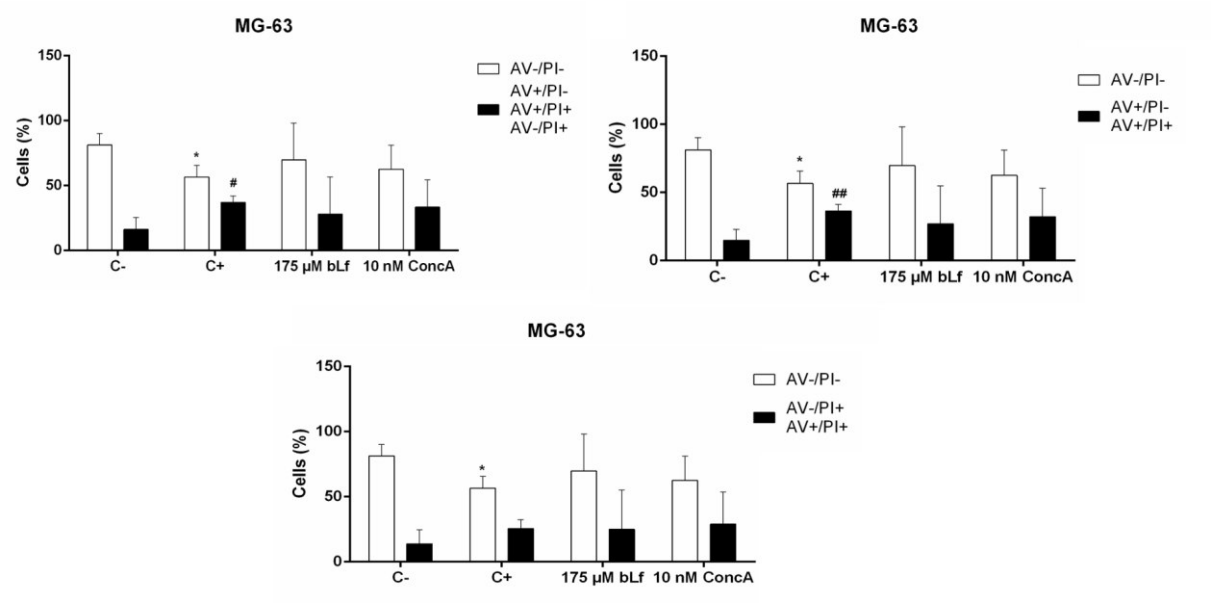

Figure VII.1. bLf appears to not induce cell death in MG-63 cell line. Quantitative analysis of the AV/PI assays of cells untreated (negative control, C-) or treated with 175 µM bLf or 10 nM ConcA, or with 50 µM cisplatin (C+) during 48 h. Values represent mean ± S.D. of three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ comparing with to AV^{-}/PI^{-} cells of C- cells; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ in comparison with death cells of C- cells.