IN THE BACKSTAGE OF LACTOFERRIN DERIVED PEPTIDES' ANTIVIRAL ACTIVITY

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ABSTRACT

Introduction: Lactoferrin (LF) is a transferrin family protein present in blood and in almost all of the exocrine secretions of mammals (milk, saliva, bile, tears, etc.) which exhibits a variety of biological functions, such as influencing iron homeostasis, modulating the immune response, and exerting anti-infectious properties against a broad range of Gram-positive and Gram-negative bacteria and viruses.

Objectives: LF and its derivatives can represent effective alternatives or adjuvants to the current antiviral therapies that carry the risk of numerous side effects and resistance emergence.

Methods: This review summarizes the data gathered up to date on the antiviral activity of lactoferrin and peptides derived from different domains of this multifaceted molecule.

Results: Antiviral effects reported for peptides derived from N-terminal domain (Lactoferricin, Lactoferrampin, LF1-11, HLP1-23, LF-33) and peptides derived from C-terminal domain (corresponding to sequences 506-522, 418-429, 553-563 and 600-632) were analyzed and compared with the properties of the native molecule; the results indicated the capacity of LF and a series of LF-derivates to interfere with the early stages of the life cycle of a wide variety of viruses. Short synthetic peptides have the advantages of a low molecular weight and offer the possibility of *in vitro* molecular simulation studies that can potentially improve their antiviral activity.

Conclusion: The LF-derived peptides could represent promising candidates for prophylaxis or treatment of a broad spectrum of viral infections.

Keywords: Lactoferrin, Lactoferrin-derived peptides, antiviral activity.

REZUMAT

Introducere: Lactoferina umană (LF) este o glicoproteină din familia transferinei, prezentă în sânge și în majoritatea secrețiilor mamiferelor (lapte, salivă, bilă, lacrimi etc.), care exercită numeroase funcții, precum modularea homeostaziei fierului, dar și activități imunomodulatoare, antibacteriene și antivirale.

Obiective: LF și peptidele derivate din Lactoferină ar putea reprezenta alternative eficiente sau adjuvanți importanți în tratamentul infecțiilor virale, mai ales din cauza reacțiilor adverse asociate medicamentelor antivirale.

Metode: Acest articol sumarizează datele recente legate de acțiunea antivirală a Lactoferinei și a peptidelor derivate din diferite domenii ale acestei glicoproteine multifuncționale.

Rezultate: Acțiunile antivirale ale peptidelor derivate din domeniul N-terminal al Lactoferinei (Lactofericina, Lactoferampin, LF1-11, HLP1-23, LF-33) și a peptidelor derivate din domeniul C-terminal al acesteia (corespunzatoare secvențelor 506-522, 418-429, 553-563 și, respectiv, 600-632) au fost comparate cu efectele moleculei native; rezultatele au indicat faptul că pentru o varietate largă de virusuri, LF și peptidele derivate pot interfera în principal cu fazele incipiente ale ciclului replicativ viral, inhibând astfel replicarea virală. Aceste peptide scurte au avantajul unei greutăți moleculare reduse, oferind posibilitatea îmbunătățirii proprietăților lor antivirale prin studii de simulare moleculară *in vitro*.

Concluzii: LF și peptidele derivate din Lactoferină reprezintă o strategie terapeutică promițătoare în profilaxia și tratarea unui spectru larg de infecții virale.

Cuvinte-cheie: Lactoferină, peptide derivate din Lactoferină**,** acțiune antivirală.

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INTRODUCTION

Natural and synthetic peptides with biological activity against bacteria, fungi, protozoa, viruses and yeast infections are promising broad spectrum antimicrobial therapies, as well as immunomodulatory, anti-inflammatory, angiogenic and anti-tumoral agents.

Lactoferrin (LF) is a protein of the transferrin family present in the blood and in almost all of the exocrine secretions of mammals (milk, saliva, bile, tears, etc.) [1]. LF exhibits a variety of biological functions such as influencing iron homeostasis, modulating the immune response, and exerting anti-infectious properties against a broad range of Gram-positive and Gram-negative bacteria and viruses [2-7]. Some studies have also reported antiviral activities of LF-derived synthetic peptides containing amino-acids from different parts of the native molecule, and detailed their modes of action. Therefore, LF and its derivatives can represent effective alternatives or adjuvants to the current antiviral therapies that carry the risk of numerous side effects and resistance emergence.

This review summarizes the data gathered up to date on the antiviral activity of lactoferrin and peptides derived from different domains of this multifaceted molecule.

LACTOFERRIN IN PREVENTION OF FREQUENT VIRAL INFECTIONS

X-ray crystallography indicates that human lactoferrin (HLF) is a polypeptide chain of about 700 amino acids, containing two homologous globular domains named N-lobe (amino acid residues 1–333) and C-lobe (amino acids 345–692), connected by a short α-helix. Each lobe consists of two subdomains and contains one iron-binding site and one glycosylation site [8]. The structure is highly similar in different mammalian species and the two symmetrical lobes have high sequence homology, possibly as a result of an ancestral gene duplication [9].

The antimicrobial properties of lactoferrin are mainly mediated by a sequence located in the N-terminus region of the protein which contains high amount of basic residues allowing interaction with surface components of the microbial structure [2]. Antimicrobial and antiviral activities have also been attributed to peptides derived from the C-lobe of LF [10].

In vivo and *in vitro* studies have shown that ***human lactoferrin*** can inhibit the early phase of viral replication, its antiviral activity being reported for a wide range of enveloped and non-enveloped DNA and RNA viruses.

- Alpha and Beta Herpesviridae [11, 12]
- Hepatitis B virus (HBV) [13] and C virus (HCV) [14]
- Adenoviruses [15]
- Retroviruses: Feline Immunodeficiency Virus [16], and Human Immunodeficiency Virus (HIV) [12]; HLF polymorphisms correlate with the risk of HIV-1 mother to child transmission [17]
- Respiratory syncytial virus and Rotaviruses [18]
- Enteroviruses: poliovirus [19] and Enterovirus 71 [20].

Other mammalian lactoferrins have also been documented as strong viral inhibitors for the above mentioned, as well as other viral families [12, 19, 21-28], such as human echovirus 5 [29], influenza A H1N1 [30], Papillomaviruses [31], Norovirus [32], and Arboviruses Sindbis and Semliki Forest viruses [33], Dengue virus [34], Toscana virus [35], Hantavirus [36], Mayaro virus [37], Chikungunya and Zika viruses [38].

MECHANISMS OF THE ANTIVIRAL ACTIVITY

In most cases, LF acts in the early phase of viral life cycle, either by blocking viral attachment to receptors on the cell membrane, or by direct binding to viral particles [39-41] – Fig. 1.

One particular molecular feature of LF is the high positive surface charge which is crucial for most of its properties. LF prevents viral attachment to target cells by binding to Glycosaminoglycans (GAGs), such as heparan sulphate or chondroitin sulphate. Thus, the interference of LF with virus-GAGs interactions was reported to be mediated through the

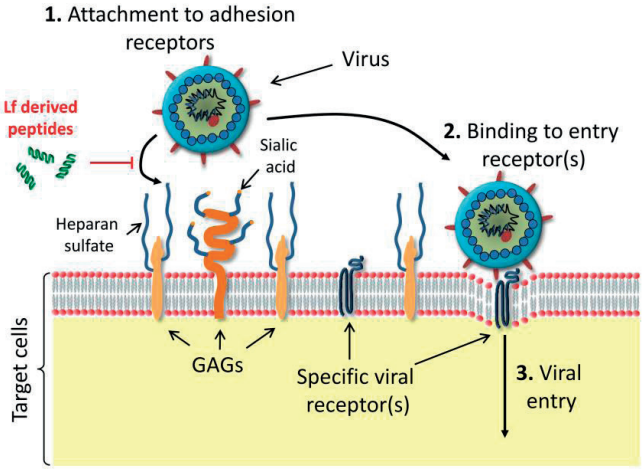

Fig. 1. Schematic presentation of the mechanisms of antiviral effect

cationic clusters GRRRR and RKVRVG located in the N-terminus region of protein [42-44].

This antiviral mechanism was demonstrated *in vitro* on cell cultures with selective modified expression of GAG molecules [40] and shown to be independent of the iron withholding, since both iron saturated and apo-LF were equally effective [45].

LF can prevent viral infections by using additional non-glycosaminoglycans pathways, such as binding to dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) [46] and low-density lipoprotein receptors (LDLR) [47].

LF can also target proteins from the viral surface, for example capside III and IIIa structural polypeptides of adenoviruses [42], and envelope E1 and E2 of HCV [48].

An intracellular activity involving direct inhibition of viral replication and/or activation of different inflammatory pathways were also suggested [49]. Additional protective effects were associated with activation of natural killer cells, monocytes, and granulocytes which can enhance the clearance of the viral particles [50].

Despite its antiviral potential, the use of LF in protein-based products for clinical application is limited, due to its short half-life. To overcome this disadvantage, LF-derived peptides that contain only active sequences from its N- or C-terminal regions are being tested in *in vitro* and *in vivo* studies. These chemically synthesized peptides have low molecular weight and increased solubility. Moreover, their structure-function relationship can be easily studied by molecular simulation in order to improve the antiviral properties.

PRODUCTION AND ISOLATION OF LACTOFERRIN DERIVED PEPTIDES

Production and isolation of peptides derived from human or bovine LF was accomplished by either the use of various enzymatic hydrolysis methods, or more recently, of heterologous expression in different systems.

Enzymatic hydrolysis of bovine LF with gastric pepsin generated a cationic peptide - lactoferricin B (LFcin B) with a broad spectrum antibacterial activity, being 8-fold more potent than the native molecule [51, 52]. Over the past few years, the concept of plant-made biopharmaceuticals was introduced in practice to obtain heterologous peptides [53], while the *E. coli*-based system was used to obtain lactoferricin [54] and different other antimicrobial peptides [55]. The heterologous expression of cationic peptides is a practical and sometimes better option for peptides production [52] as it eliminates safety concerns related to animal-derived products, and provides consistency across production lots. Additionally, liposomes can be used as a drug delivery vehicle; encapsulation of lactoferrin or its derivates into liposomes was reported to improve the stability and enhance the efficacy of the bioactive molecule [56].

PEPTIDES DERIVED FROM N-TERMINAL DOMAIN

1. Lactoferricin (LFcin)

In 1991, Tomita *et al*. found that the pepsin-hydrolysate of LF has potent **antimicrobial activity**, and the purified active peptide named lactoferricin (LFcin) is the most studied LF-derived compound [51] – Fig. 2. LFcin-B derived from bovine LF (aa residues 17- 41 of LF) and LFcin-H from human LF (aa residues 1 - 47 of LF) are highly hydrophobic peptides, with 36% sequence similarity [57], and containing multiple positively charged residues.

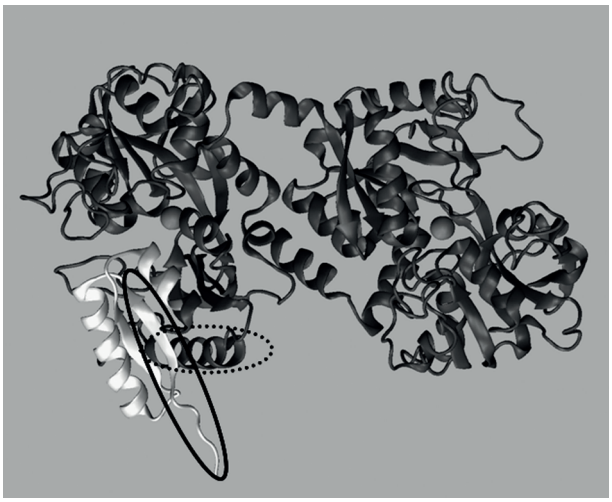

Fig. 2. Location of Lactoferricin (white), Lactoferampin (dotted line), LF1-11 (solid line) in the N-terminal lobe of human Lactoferrin - ribbon diagram representation of the secondary structure of iron saturated human Lactoferrin

The secondary structure of LFcin-B includes a single β-sheet strand instead of the long α-helix present in LF molecule [58]; this alteration seems to promote the binding with the bacterial membranes, explaining its increased antimicrobial effect compared to the entire molecule. LFcin has strong antimicrobial and immunological properties, but exerts only weak antiviral activities [11, 59] suggesting that the antiviral effect is either dependent on the secondary structure stabilized by a disulfide bond formation of the native molecule, or it is mediated by other LF regions.

The antiviral effects of LFcin were demonstrated on ***HSV-1 and 2*** and were attributed to viral entry blocking through its interaction with heparin sulphate GAGs present on host cell surface. The human peptide seems to exert lower antiviral activity than the bovine one, a feature probably explained by the interference with intracellular trafficking of the virus exhibited by LFcin-B and bovine LF [60]; additional characteristics, such as size, quaternary structure, hydrophobicity and distribution of charged amino acids were also incriminated [41].

LFcin-B, LFcin-H, and the cyclic form of LFcin-H were active on ***CMV infection,*** inhibiting the *in vitro* viral entrance into the host cell, but the antiviral activity is more than seven-fold weaker than that of the native molecule [61].

Only a moderate inhibition was obtained with lactoferricin B in ***HIV infection*** as reported by Berkhout *et al.* [26]. However, a 4-fold increase in the anti-HIV potency was detected when negatively-charged groups were added to the LF molecule by succinylation [12], allowing a more efficiently binding to the V3 loop from gp120 envelope protein [62].

In the recent years, LFcin has been reported as an active antitumoral peptide, with proposed underlying mechanisms, such as the formation of transmembrane pores, depolarization of mitochondria or activation of the caspases cascade [63], mechanisms that can also interfere with the viral life cycle.

2. Lactoferrampin (LFampin) – Sequence: WNLLRQAQEKFGKDKSP; Ip = 9.70

Lactoferrampin contains residues 268–284 from the N-domain that plays a key role in the membrane-mediated activities of lactoferrin – Fig. 2; it has good antimicrobial and anti-fungal activities [64], but only modest antiviral effects. Its properties seem to be associated with binding and disruption of the bacterial membrane following a two-step model of action, similar to that reported for LFcin [65]. Recent studies indicate that the amino acid composition is crucial for the biological effects of both LFampin and LFcin. Thus, Morten *et al.* indicated that a high amount of tryptophan residues promote the interactions with membrane functioning as anchor for membrane proteins [66], while Sijbrandij T *et al*. found arginine residues to be important, as lysine to arginine substitutions produced an increased antimicrobial activity especially in the case of LFampin [67].

In humans, LFampin and Lactoferrin-derived peptide LF1-11 have been tested on HIV, as potential inhibitors of viral integration [68]. Both peptides inhibit the activity and the nuclear translocation of the HIV-1 integrase. The proposed mechanism is that the LF-derived peptides block the interaction between HIV-integrase and Lens Epithelium-Derived Growth Factor, p75/LEDGF [69]. p75/

LEDGF is a host-cell cofactor that favors viral integration in latently infected $CD4^+$ T cells from reservoirs, by binding the HIV-1 integrase in the C-terminal domain and reading chromatin through the PWWP domain – a conserved member of the "Royal Family" of domains that function as chromatin methylation readers [70].

3. Lactoferrin derived peptide LF1-11 – Sequence: GRRRSVQWCAV; Ip = 11.70

LF1-11 consists of the first eleven aminoacid residues of the N-terminus of LF – Fig. 2. The peptide has been shown to be highly effective against several bacterial strains and a number of Candida species, and was recently tested in HIV infection, as mentioned above [68].

4. Human Lactoferrin derived Peptide 1-23 (HLP 1-23). Sequence: GRRRRSVQWCAVSQPEATKCFQW; Ip = 11.2

HLP1–23 is a synthetic peptide comprising the first 23 amino acids residues from the N-terminal domain of the LF molecule, reported to prevent HBV infection and replication. HLP1–23 contains the GRRRR cationic cluster of the native HLF, which is one of the two glycosaminoglycan binding sites involved in its antiviral activity. *In vitro* studies on HepaRG and HepG2.2.2.15 cell lines suggested that HLP1-23 binds to the viral particle, using the cationic cluster that allows peptide interaction with the negatively charged residues from the virion envelope, while the absence of the second glycosaminoglycan binding site limits the possibility of attachment to the cells [71].

5. Human Lactoferrin derived Peptide 33 (LF-33). Sequence: GRRRRSVQWCAVS QPEATKCFQWQRNMRKVRGP

LF-33, a synthetic peptide containing the amino acids 1-33 from the N-terminal domain, was recently reported to be involved in LF binding to HIV *env* protein. The derived peptide also inhibits early steps of HIV mucosal transmission. Both HLF and LF-33 can inhibit the *in vitro* attachment of HIV strains from early infection (R5-tropic) and from late infection (X4-tropic) to CD4-negative epithelial cells cultures [72]. Nevertheless, only the native HLF seems to exert an efficient effect in the transfer of HIV-1 strains from immature dendritic cells to CD4 T lymphocytes, probably in a DC-SIGN-dependent manner.

LACTOFERRIN C - LOBE DERIVATES

Lactoferrin C-lobe derivates seem to have a more limited-spectrum inhibitory effect, demonstrated only on influenza and hepatitis C virus infections. Bovine lactoferrin was shown to inhibit all major ***influenza virus subtypes*** including the currently circulating H1N1 and H3N2, by C-lobe binding to the HA2 region of the viral hemagglutinin [22]. This part of the viral envelope is a highly conserved region containing the fusion peptide that is under intense scrutiny for the design of a universal influenza vaccine [73]. Effectively targeting of HA2 could potentially be used to obtain broad anti-influenza drugs, as this constant region, embedded in the HA head domain does not suffer the unpredictable changes characteristic of the HA1 subunit. Recent reports indicate that lactoferrin derived C-lobe fragments can inhibit viral hemagglutination and influenza infection even at very low concentrations [22]. Three bovine Lactoferrin-derived peptides that correspond to the C-lobe sequences 506-522 (SKHSSLDCVLRP), 418-429 (AGDDQGLDKCVPNSKEK) and 553-563 (NGESSADWAKN) are patented and have demonstrated good antiviral activities [10].

Both human and bovine LF can inhibit ***Hepatitis C virus*** attachment to human hepatocytes *in vitro* [24], possibly through binding to the C-terminal part of E1 and E2 proteins from the HCV envelope [48]. Early *in vivo* studies have shown that oral administration of BLF for 2 - 6 months can produce a significant decrease in serum alanine transferase and HCV-RNA viral load in HCV infected patients [14, 74].

The anti-HCV effects are thought to be associated with the interaction of HCV envelope proteins and a region of 93 carboxyl amino acids of the lactoferrin molecule that shows partial homology to the HCV receptor CD81, indicating a potential interference with

the viral life cycle [74]. Further deletion analysis and production of tandem repeats identified 33 amino acid residues (amino acids 600-632) as the minimum binding site in the carboxyl region of LF that can prevent HCV infection in cultured human hepatocytes; moreover, site-directed mutagenesis studies indicated that Cys at amino acid 628 was critical for the binding of E2 viral protein [75].

CONCLUSIONS

Taken together, these studies have demonstrated the capacity of LF and a series of LF- derivates to interfere with the early stages of the life cycle of a wide variety of viruses. Short synthetic peptides have the advantages of a low molecular weight and offer the possibility of *in vitro* molecular simulation studies that can potentially improve their antiviral activity. Based on these properties, the LF-derived peptides could represent promising candidates for prophylaxis or treatment of a broad spectrum of viral infections.

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