# UNIVERSITY OF NAPOLI FEDERICO II

**Doctorate School in Molecular Medicine**

**Doctorate Program in**
**Genetics and Molecular Medicine**
**Coordinator: Prof. Lucio Nitsch**
**XXIV Cycle**

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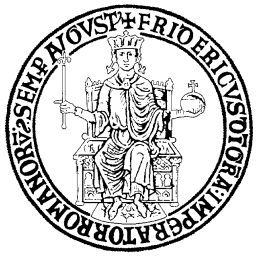
**Ersilia Nigro**

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# *"Antimicrobial Activity and Cell Toxicity of Human Beta Defensins and Their Newly Synthesized Analogs"*


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**Abstract**

Human Beta Defensins (hBD-s) are a class of antimicrobial peptides (AMPs) that play an important role both in innate and adaptive immunity. In fact, they directly kill bacteria (Gram+, -), viruses and fungi and exert chemotaxis against dendritic cells, lymphocytes and monocytes. Thanks to their antimicrobial and chemotactic activity, hBD-s represent a good target to develop new therapeutic molecules (Scudiero et al. 2010).

Previously, Scudiero et al. synthesized the wild type hBD-1, hBD-3 and designed eight analogs selecting the sequences of hBD-1 and -3 that are crucial tracts for their activity. They demonstrated that the C-terminal domain of hBD-3 and the internal portion of hBD-1 are necessary for the antibacterial, antiviral and chemotactic activity; they demonstrated that 1C and 3N are the only two analogs with higher activity respect to the wild type confirming that this is a potent strategy to develop new pharmacological molecules.

In the present study, we tested the possibility to find a new defensin analog with enhanced antimicrobial properties. We designed a new peptide, 3NI, with the following features: (a) shorter total sequence in order to provide greater stability and ability to penetrate bacteria and viruses; (b) presence of the C-terminus portion of hBD-3 selected for the antimicrobial activity; (c) presence of inner portion of hBD-1 selected for the chemotactic activity. At this aim, we used 3NI to perform antibacterial tests on *E.coli, P. aeruginosa, E. faecalis* and demonstrated that it suppresses bacterial growth already at a concentration of 12.5 μM. We also tested the antiviral activity on herpes simplex virus type 1 and found that 3NI suppresses the infectivity at a concentration of 50 μM. Therefore, our results indicate that 3NI analog represents a new attractive target for therapeutic approaches.

In addition, we investigated two others pharmacological properties of these peptides: the stability in human serum and the potential cytotoxic effects using human cell lines. We demonstrated that hBD-3, used as a reference, is a peptide with pronounced stability; in fact it resulted not degraded after 1 hour of serum incubation and with a degradation rate of 45% after 24 hours.

Successively, to evaluate the cytotoxic effects of hBD-1, hBD-3 and 1C, 3N and 3NI analogs, we tested cell viability, apoptosis and DNA damage using three human epithelial cell lines: lung carcinoma (A549), colon carcinoma (CaCo-2) and pancreas adenocarcinoma (Capan-1). In all cell lines, we demonstrated that the peptides cause 30% reduction of cell viability and 20% of apoptosis only after 72 hours treatment. In addition, we demonstrated that the peptides do not induce DNA damage.

Finally, in order to investigate whether defensins interact with plasma membranes and penetrate into human epithelial cell lines, we labeled our peptides with NBD fluorochrome and performed confocal microscopy

experiments. We observed that peptides bind to plasma membranes and penetrate into A549 and CaCo-2 cells through active mechanisms involving early endosomes. We observed that they are partially digested through lysosomes.

Altogether our results confirm that the beta defensins analogs are attractive factors to develop new antimicrobial molecules with therapeutic applicability for the treatment of infectious diseases. In fact, 1C, 3N and 3NI are analogs that show an increased antimicrobial activity compared to wild type defensins, do not exert relevant cytotoxic effects and have pronounced stability in human serum.

# 1 Introduction

## 1.1 Innate and adaptive immune system

Although microorganisms surround and vastly outnumber us, very few succeed in causing illness. To cause infections, pathogens must entire our organism and counteract the complex system of tissues, cells and molecules responsible for the defence of our body, collectively known as immune system (Reddy et al. 2004; Lehrer et al 2004).

The immune system is a network of tissues, organs, cells, and chemicals that protects the body against infections. In humans, two types of immunity are known: innate and adaptive. Innate immunity, given to us by birth, provides the first barrier against microorganisms. Adaptive immunity, instead, refers to antigen-specific defense mechanisms that take several days to become protective; it is acquired later in life, such as after an immunization or successfully fighting off an infection. The two immune systems tightly cooperate (Iwasaki et al. 2010): in fact, the innate immune system can initiate adaptive immune responses (Medzhitov et al. 2000).

The innate immune system is commonly considered to be a mechanism of immunity primitive and highly conserved during the evolution. It is activated immediately (few minutes) after infection and rapidly controls replication of the infecting pathogen. Its activation is based on the recognition of molecules considered non-self through receptors, called pattern-recognition receptors (PRRs). PRRs are expressed on several immune cells including macrophages, dendritic cells and lymphocytes B and are able to recognize non-self molecules, antigens. The strategy of the innate immune response is to identify some highly conserved structures associated to pathogens as lipopolysaccharide, peptidoglican and double strand RNA, collectively called pathogen-associated molecular patterns (PAMPs). PAMPs are existing structures in large groups of microbes (Medzhitov et al. 1997; Janeway et al. 2002). In this way, the innate immune activity mechanism is direct against whatever bacteria, fungi or viruses.

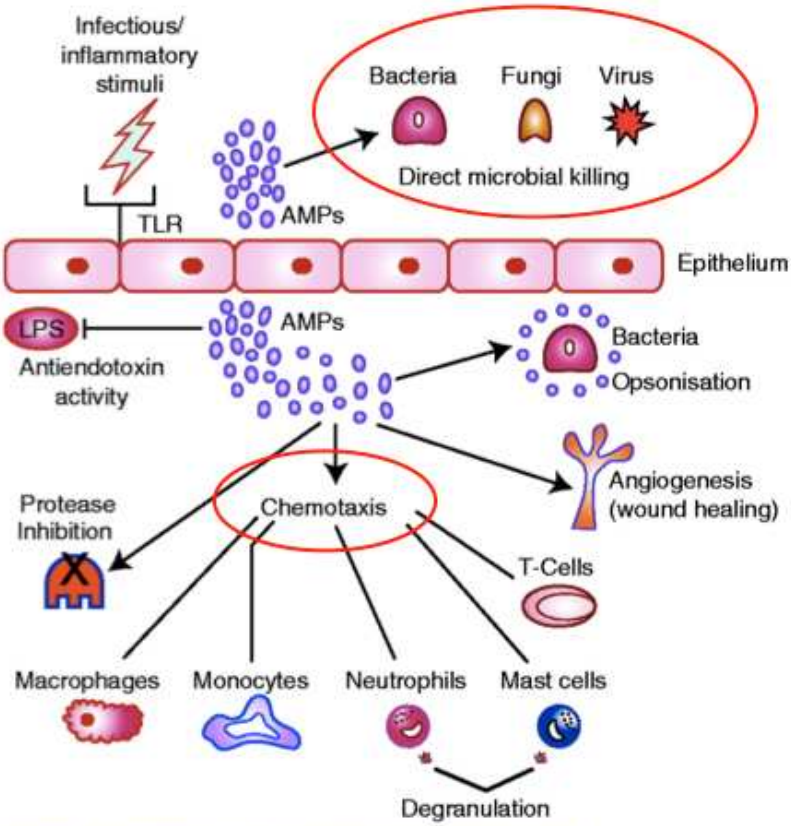
The innate immune system displays three types of barriers: anatomical, cellular and humoral. The first includes skin, internal and external epithelial layers which act as physical barriers to invasion. The cellular barrier is activated after the invasion of pathogens. The main cellular components are macrophages, neutrophils, lymphocytes etc. that, after activation, secrete cytokines and chemokines to control host replication. The humoral barrier is constituted by peptides and small proteins with antimicrobial activity, collectively called antimicrobial peptides (AMPs). AMPs are secreted by epithelial cells after microbial stimuli.

Altogether, these barriers participate to the defense of our organism (Medzhitov 2010; Dartois et al. 2005).

### 1.2 Antimicrobial Peptides

AMPs are effector molecules of the innate immune system; they have a broad antimicrobial spectrum and lyse microbial cells by interaction with biomembranes. Besides their direct antimicrobial function, they have multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells influencing diverse processes such as cell proliferation, immune induction, wound healing, cytokine release, chemotaxis and protease-antiprotease balance (Fig. 1). In this way, AMPs activate the adaptive immune system (Cederlund et al. 2011; Wiesner et al. 2010). To date, over 1500 peptides have been identified; they have antimicrobial activities from amoeba, plants, birds to humans. Usually, AMPs are classified based on secondary structural features, such as cathelicidins (linear α-helical peptides), defensins (β-strand peptides connected by disulfide bonds), and bactenecins (loop peptides) (Cederlund et al. 2011). The majority of these small peptides (fewer than 100 amino acids) share a common feature: the presence of a positive charge due to an excess of positively amino acids (i.e. arginine and lysine). The mechanism of action is based on their net positive charge. AMPs interact electrostatically with negative charges of microbial cell membranes (i.e. phospholipids), thereby increasing the membrane permeability and finally resulting in cell death (Bals et al. 2000; Brogden 2005).
In humans, two main categories of antimicrobial peptide play a key role in the defence of our organism: cathelicidins and defensins (Cederlund et al. 2011). They are both secreted by epithelial cells of many tissues as respiratory, urogenital and intestinal tracts.

Actually, the antibiotic treatment of bacterial infections is still one of the mainstays of human medicine. It is thus a challenge to design new antimicrobial molecules with novel structure and mechanism of action. Antimicrobial peptides represent good templates for a new generation of antibiotics. AMPs qualify as prototypes of innovative drugs that may be used as antimicrobials and or reducing the inflammation. Several strategies have been used to design new molecules candidates for drug development, and to produce sufficient amounts for clinical studies. Many studies are now focused on the design and development of synthetic analogs of AMPs in order to find active molecules on viruses, fungi and bacteria (Reddy et al. 2004).




Frew L. et al "Antimicrobial peptides and pregnancy". *Reproduction*. 2011;141(6):725-35.

**Fig. 1:** Antimicrobial peptides (AMPs) have two functions: they are able to direct kill microbes; they activate the adaptive immune system through chemotaxis toward several cells of immune system.

### 1.3 Defensins

Defensins are a class of cationic AMPs arginine rich. They are small peptides highly conserved during the evolution from plants to humans (Lehrer 2004). They are secreted by immune cells as neutrophils, monocytes and epithelial cells of gastro-intestinal, pulmonary, urogenital epithelia. Moreover skin and placenta produce defensins (Ganz 2001; Selsted et al. 2005). They have antimicrobial activity against a broad spectrum of microorganisms, Gram-positive, - negative, fungi and viruses (Hazlett et al. 2010) and exert chemotaxis toward dendritic cells, monocytes and lymphocytes.
In humans three defensin subfamilies are known: $\alpha$, $\beta$ and $\theta$ defensins
These peptides are produced as pre-pro-peptides of 94-95 amino acids that undergo two cleavage processes releasing the mature peptides of 29-34 amino acids (Lehrer 2004).
The mature defensins of the three subfamilies share some common features:

- Short sequence: 18-50 amino acids (5-7 KDa)
- Net cationic charge
- Presence of 6 cystein residues
- three intramolecular disulphide bonds due to the presence of 6 cysteins
- Absence of post-traductional modifications

They differ for:

- The length of peptide segment between the 6 cysteines
- The pairing of the cysteins that are connected by disulfide bonds
- Tridimensional conformation.

In humans, $\alpha$ and $\beta$ defensins are the produced by two distinct genes evolved by a common ancestral gene; this process probably is probably due to the evolutionary response of the immune system to the continuous ecological and environmental changes (Selsted et al. 2005). The two genes are nearby and located on the chromosome 8.
Moreover, $\theta$ defensins, identified and expressed only in primates, are cyclic peptides.

The core of $\alpha$ and $\beta$ defensin molecules consist of three beta strands arranged in anti-parallel sheets, which are constrained by the 3 intramolecular disulfide bridges (Lehrer 2004). In addition, in $\beta$ defensin, but not in $\alpha$ class, the beta sheet is flanked by an alpha helical segment of variable length, corresponding to the N-terminal domain (Fig. 2).

The tridimensional conformation of both α and β defensins is of particular interest; in fact, it is possible to identify a C-terminal domain with a high cationic charge and an amphipatic domain at the N-terminus.

This particular conformation and charges distribution are probably responsible for the antimicrobial activity of defensins (Selsted et al 2005).

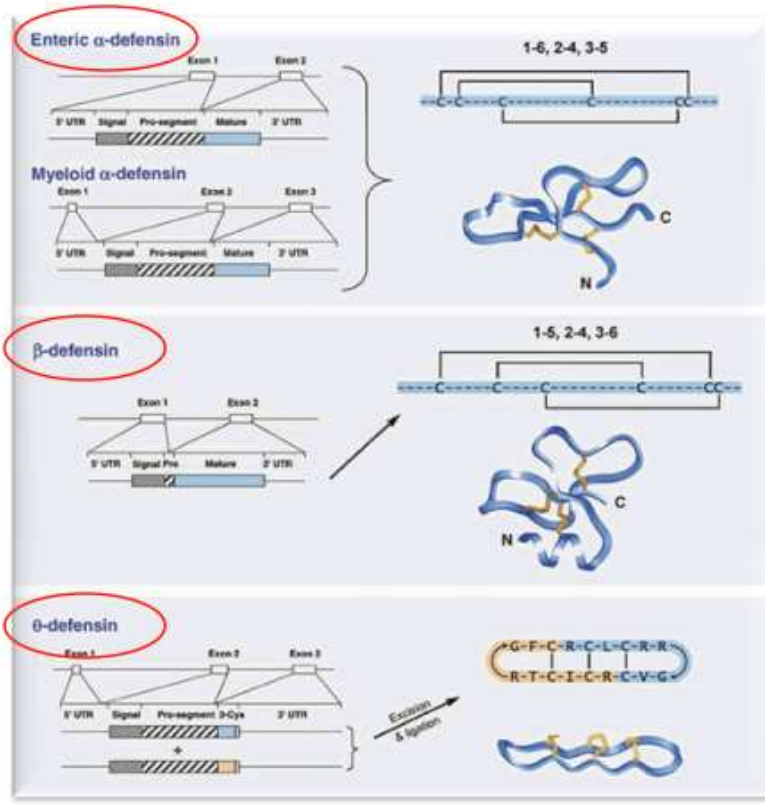



**Fig. 2:** Tridimensional representation of α, β and θ defensins.

### 1.4 Human Alpha Defensins

α defensins are peptides of 29-35 amino acids. In humans, six α defensins have been isolated: HPN1, HPN3, HPN4, HD5, and HD6 HPN2; the latter is, however, a peptide truncated without proven activity. The genes encoding these six defensins are in the same region of the chromosome 8.
The α defensins have been isolated from neutrophils and, more specifically, within the azurophilic granules in which HPN1-3 represent about the 50% of protein content while HNP4 is present at low concentrations (Patil et al. 2004). Defensins HNP1-3 are also present in B and natural killer lymphocytes. The two defensins HD5-6 are defined as enteric defensins because they have been found in Paneth cells of the small intestine and in the epithelial cells of the female urogenital tract (endometrium, and fallopian tubes) (Cunliffe 2003).
From a functional point of view, the α defensins play a key role in oxygen-dependent destruction of phagocytised microorganisms.

### 1.5 Human Teta Defensins

θ defensins are expressed in different species of monkeys and orangutans, but not in humans (Selsted 2004). The θ defensins are structurally different from α and β defensins: they are in fact cyclic peptides. Moreover, the gene encoding for θ defensins originates from the mutated gene of α defensins (Selsted 2004). The precursor of the θ defensins is a shorter paralogous of the α defensins because it is truncated by the insertion of a stop codon. The resulting mature peptide of 18 amino acids presents the classical 6 cysteines and 3 disulfide bridges that, making the structure very rigid, give rise the typical cyclic structure.

### 1.6 Human Beta Defensins

Human β Defensins (hBD-s) are a family of small cationic peptides with a molecular weight ranged between 3-7 kDa (Pazgier et al. 2006). Three domains characterize the primary sequence:

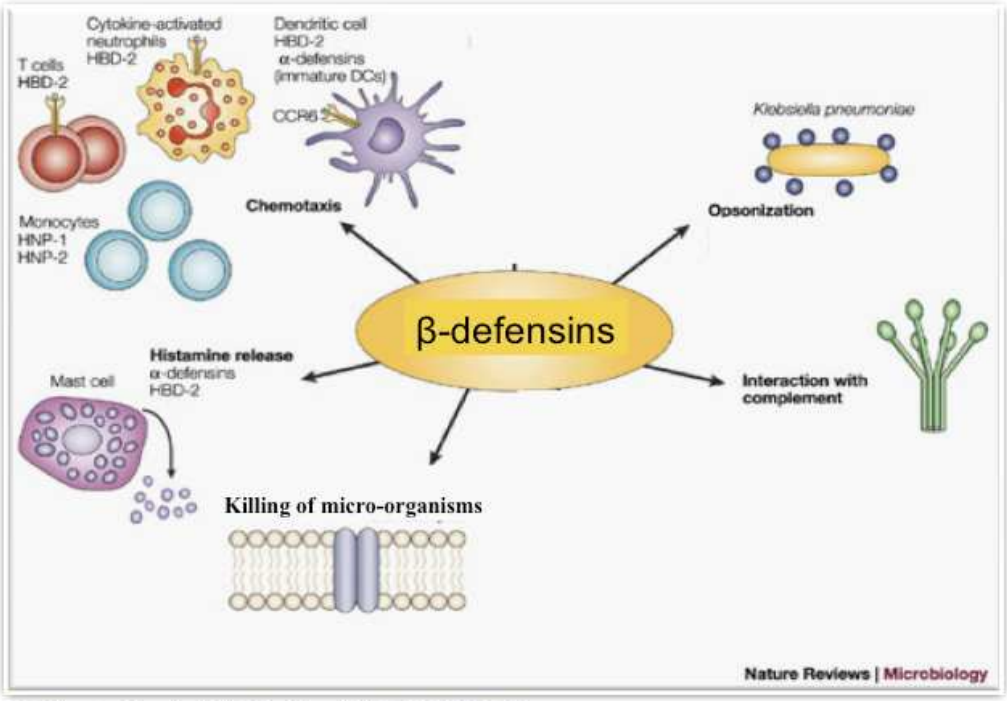
1. Six cysteins domain. These amino acids, highly conserved, are responsible for the formation of three disulfide bonds between residues 1-5, 2-4, 3-6. The disulphide bonds generate, in the tridimensional structure, three beta sheets and an alpha helix. If the presence of cysteins is fundamental for the antimicrobial activity is not yet clear (Chandrababu et al. 2009, Hoover et al. 2003, Kluver et al. 2005, Wu et al. 2003).
2. Cationic domain, constituted by several cationic amino acids (expecially arginine, lysine, histidine). In the tridimensional conformation, this domain is the most exposed on the surface because it is involved in the antimicrobial activity of the peptide interacting with microorganism surface. On the contrary of the six cysteines, these residues are not highly conserved to allow defensins to adapt to pathogens.
3. Leader domain, highly hydrophobic. This domain in the tridimensional conformation of the peptide is located diametrically opposite to the cationic residues and contributes to the mechanism of elimination of pathogens provoking the formation of pores in the plasma membranes of pathogens.

In the last years, a number of beta defensins have been studied; the best characterized are hBD-1, hBD-2, hBD-3 and hBD-4 (Schneider et al. 2005).

hBD-1 was firstly isolated from a patient undergoing hemofiltration dialysis; later it has been later found in a large variety of tissues (Bensch et al. 1995, Valore et al. 1998). hBD-1 is expressed in that epithelia directly exposed to the external environment such as lung, mammary glands, salivary glands, kidney, pancreas and prostate (Pazgier et al. 2006, Hoover et al. 2001). hBD-1 is the only one defensin that can be expressed both in a constitutive or inducible manner. The latter, is consequent to exposure to bacteria or pro-inflammatory cytokines (as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon- γ (IFN-γ) (King et al. 2002).

A second family member, hBD-2, was firstly isolated from psoriatic scale extracts; successively it was found in lung, stomach, urogenital system and intestine (Seo et al. 2001, Wehkamp et al. 2002). hBD-2 is not expressed at basal level but it is inducible after exposition to bacteria, lipopolysaccharide and pro-inflammatory cytokines as TNF-α and IL-1β (King et al. 2002).

hBD-3 was initially isolated from epidermal keratinocytes from patients with psoriasis. It is induced by IFN-γ, TNF-α and bacteria in lung and intestine (Harder et al. 1997, Harder et al. 2001).
Finally, hBD-4 was first isolated in lung tissues. It is not expressed at basal level but can be up-regulated by Gram-negative and Gram-positive bacteria and by IFN-γ and TNF-α (Yanagi et al. 2005). hBD-3 and hBD-4 are expressed also in the endometrium (King et al. 2003).
hBD-s possess two main activities: antimicrobial and chemotactic.
hBD-s manifest antimicrobial activity against several microbes, including Gram-positive and Gram-negative bacteria, fungi and viruses (Pazgier et al. 2006; Weinberg et al 2006). However, different hBDs are selective in their activity. hBD-1 is more active against Gram-negative bacteria, while hBD-2, hBD-3 and hBD-4 are also active against Gram-positive bacteria and yeast (Pazgier et al. 2006, Maisetta et al. 2006). Furthermore, hBD-1 and hBD-2 are sensitive to salt whereas hBD-3 is the only beta defensin salt-resistant. The salt resistance is a very important aspect in patients affected by Cystic Fibrosis: several studies have demonstrated that the system of beta defensins in the airway epithelium of these patients is completely inactivated by the high salt concentrations (Goldman et al. 1997).
Thank to their chemotactic activity, beta defensins have been described as molecules that provide a link between innate and adaptive immune responses. Chemo attractant activity toward dendritic cells, memory T cells and mast cells has been reported for hBD-2 and hBD-3 (Pazgier et al. 2006) and both hBD-3 and hBD-4 are chemotactic toward monocytes (Garcia et al. 2001; Harder 2001). The chemotactic activity of hBDs towards immune cells seems to be mediate by the binding of defensins to the chemokine receptors CCR6 and CXCR4 (Lehrer 2004, Yang et al. 1999).
In addition to their antimicrobial and chemotactic activity, the beta defensins exhibit other properties as stimulation of the expression of cytokines and adhesion molecules by epithelial cells, histamine release from mast cells, interaction with complement and opsonisation of bacteria (Chaly et al. 2000; Durr et al. 2002).
Thanks to their antimicrobial and chemotactic power, beta defenisns are attracting great attention for their potential use in the treatment of infections. In particular, the attention is to the design and the development of analogs of the wild type defensins with a higher activity (Antcheva et al. 2009, Cole et al. 2003, Hoover et al. 2003, Jung et al. 2011, Krishnakumari et al. 2009, Scudiero et al. 2010, Taylor et al. 2007).




Lehrer RI. "Primate defensins". *Nat Rev Microbiol.* 2004; 2(9):727-38.

**Fig. 3:** Human Beta Defensins. Representation of hBD-s functions.

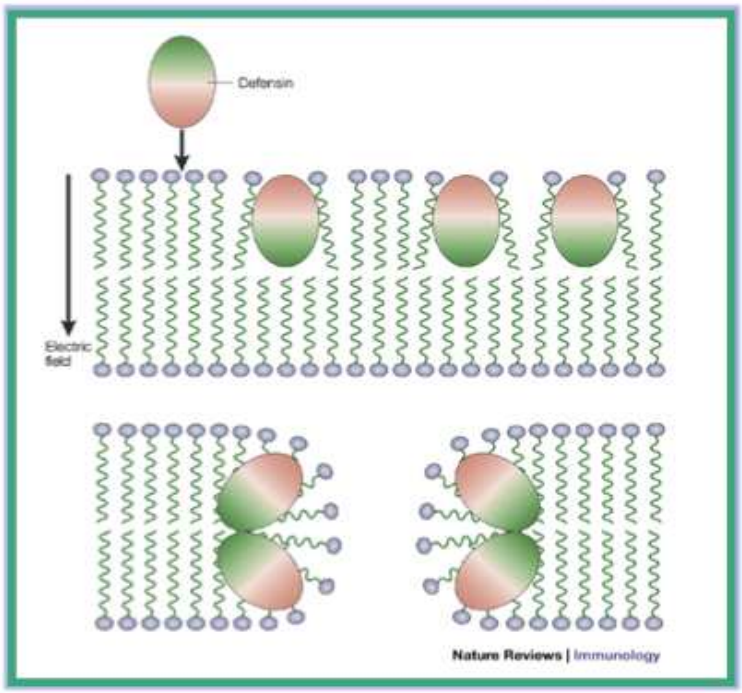
### 1.7 Mechanism of Action

As already said, AMPs are able to directly kill pathogens. In particular, defensins have antimicrobial activity against bacteria, fungi and viruses, especially when tested under low ionic strength conditions. The antimicrobial activity of defensins is mainly carried out on metabolically active bacteria, while their action is weaker in regard to bacteria inactivated by the lack of nutrients or by the action of metabolic inhibitor. Although the killing mechanism of defensins is not yet clear, it seems that the permeabilization of target membranes is the crucial step in defensin-mediated antimicrobial activity.

In fact, it has been shown that bacteria treated with defensins can be permeabilized by small molecules. Moreover, the conditions which interfered with permeabilization of bacterial membranes prevent the loss of viability of bacteria, indicating permeabilization mechanism for microbial killing (Lichtenstein 1991). Further, in experiments with artificial membranes characterized by the presence of negative charges, defensins formed channels (Wimley et al. 1994). All these observations are consistent with the idea that the insertion of defensin molecules into the membranes depends on electrostatic forces (Fujii et al. 1993). Moreover, the activity of defensins against artificial membranes was diminished in the presence of increased salt concentrations, supporting the importance of electrostatic forces between the anionic phospholipid headgroups and the cationic defensins (Wimley et al. 1994).

Considering these data, the most reliable mechanism of antimicrobial activity is the following: the positive residues of hBD-s interact with negative charges of plasma membranes of microorganisms through an electrostatic recognition. Successively, with the amphipathic portion, defensins penetrate into the lipid bilayer inducing the permeabilization of membranes (Fig. 4). This appears to be the lethal event (Sahl et al. 2005; Ganz 2003).

However, stable pore formation is not the only mechanism of defensin pathogen killing. Probably, hBD-s are able to inhibit RNA, DNA and protein synthesis and induce the synthesis in epithelial cells of cytokines, as INFγ, that contribute to bacteria and viruses elimination. It is also possible that several mechanisms cooperate to induce pathogen death (Sass et al. 2010).




Ganz T. "Defensins: Antimicrobial peptides of innate immunity". *Nature Rev Immunol 2003; 3:710-20*

**Fig. 4:** Mechanism of killing of Human Beta Defensins. A model of the most accreditated mechanism of microorganisms killing by defensins. The positive charges of hBD-s (pink) bind negative residues of plasma membrane of pathogens. Successively, amphipathic portion of the peptide (green) intercalate the lipid bilayer, causing pores formation and cell death.

### 1.8 Aim of the Thesis

Human Beta Defensins (hBD-s) are small cationic peptides that play an important role in immunity killing bacteria, viruses and fungi and exerting chemotaxis against dendritic cells, lymphocytes and monocytes. Thanks to their antimicrobial and chemotactic activity, hBD-s represent a good target to develop new therapeutic molecules.

Previously, wild type, hBD-1, hBD-3 have been chemically synthesized and eight analogs have been designed selecting the tracts crucial for their activity (Scudiero et al, 2010). In fact, Scudiero et al demonstrated that the C-terminal domain of hBD-3 and the internal portion of hBD-1 are sequences necessary for the antibacterial, antiviral and chemotactic activity; furthermore 1C and 3N resulted the analogs with higher activity respect to that of the wild type (Fig. 5).

In the present work, we studied and tested a new analog as a potential pharmacological molecule with enhanced antimicrobial properties. To this aim, we designed 3NI, a new peptide carrying out the following features: (a) shorter total sequence (in order to provide greater stability and ability to penetrate bacteria and viruses); (b) the C-terminus portion of hBD-3 (crucial for the antimicrobial activity); (c) the inner portion of hBD-1 (crucial for the chemotactic activity).

In addition, we investigated the three most relevant pharmacological properties of defensins: the antimicrobial activity, the stability in human serum and the potential cytotoxic effects.

For the antimicrobial activity, we performed a Colony Forming Unit assay (CFU), and estimated the Minimum Inhibiting Concentration (MIC) of peptides on three bacteria strains, *E. coli, P. aeruginosa, E. faecalis.* Furthermore we tested the inhibition of infectivity of peptides on the Herpes simplex Virus Type 1.

For the stability in human serum, we incubated peptides in human healthy serum and performed HPLC and MALDI-TOF analysis to evaluate the degradation rate after 35 min, 1h, 2 h, 4 h, 6 h and 24 h.

For the cytotoxicity, we tested the effects of peptides on three human epithelial cell lines (A549, CaCo-2, Capan-1). We evaluated cell viability using MTT test, cell apoptosis using Annexin-V Propidium Iodine assay and DNA integrity using single cell gel electrophoresis assay.

Finally, we labeled peptides with the fluorochrome NBD and performed confocal microscopy experiments on human epithelial cell lines to elucidate the mechanisms of interaction of peptides with plasma membranes.

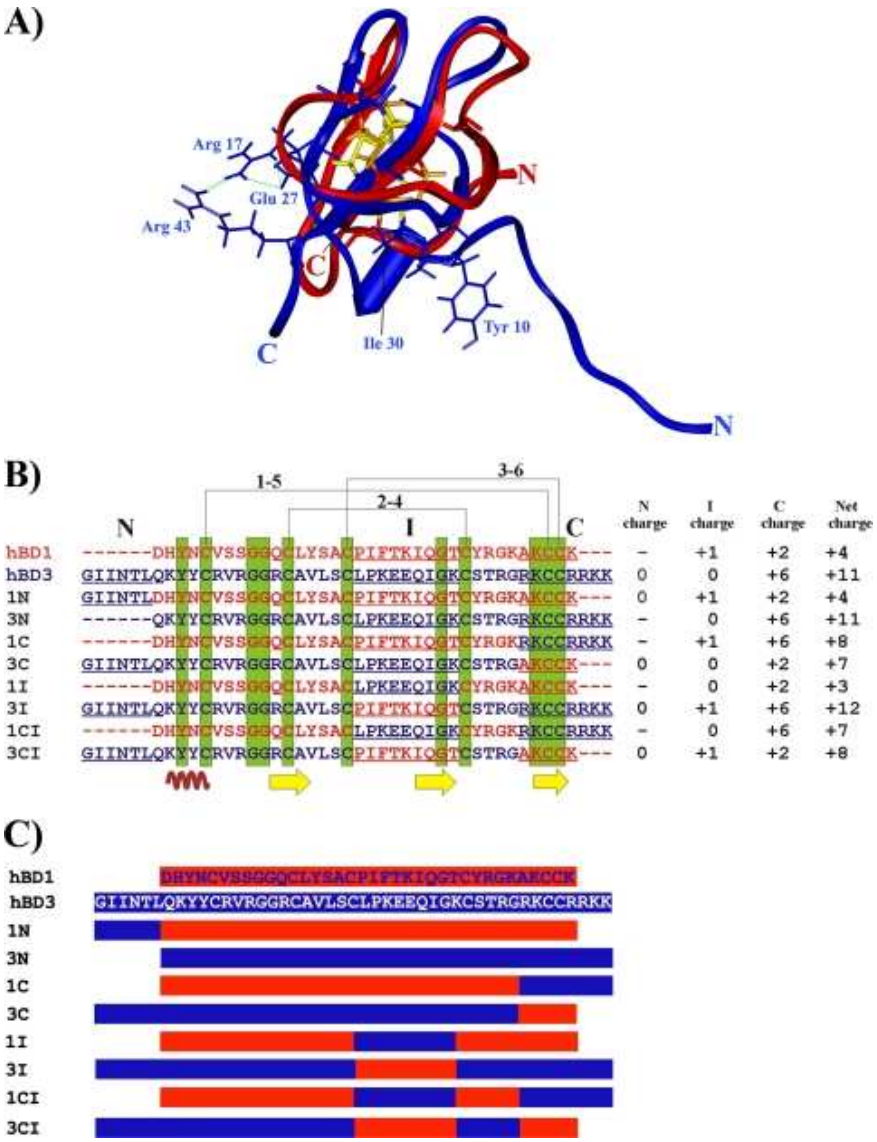



**Fig. 5**: (A) Superimposition of hBD-1 (red) and hBD-3 (blue) polypeptide chains. The side chains of some charged residues (blue) and disulfide bonds (orange and yellow) are also shown. (B) Sequence alignments and net charges of hBD-1, hBD-3, and synthetic analogs. The disulfide connectivities among the consecutively numbered cysteins are shown at the top. The hBD-1 and hBD-3 regions that have been substituted in the analogs and that are underlined are N (amino-terminal peptide segment), I (internal domain between Cys3 and Cys4), and C (C-terminal peptide segment). The net charges for these segments are reported on the right. Conserved residues are marked by the vertical green bands; secondary structure elements are shown at the bottom in brown (alpha helix) and yellow (beta sheet). The coloured segments of sequences derive from hBD-1 (red) or hBD-3 (blue). (C) The coloured drawings in the boxed bars indicate the regions exchanged between hBD-1 and hBD-3.

## 2 Materials and Methods

### 2.1 Design of Synthetic Peptides

Peptides were synthesized using the standard solid-phase-9-fluorenylmethoxycarbonyl (Fmoc) method as previously reported (Scudiero et al. 2010). The NovaSyn TGA (Merck, Darmstadt, Germany) resin (substitution 0.25 mmol/g) was used as solid-phase support, and syntheses were performed on a scale of 100 μmol. Peptides were fully deprotected and cleaved from the resin with trifluoroacetic acid (TFA) with 5% thioanisole, 3% ethandithiol and 2% anisole as scavengers. The crude peptides were precipitated with ice-cold ethyl ether, filtered, dissolved in water, lyophilized, and reduced with dithiothreitol (DTT). Peptides were purified to homogeneity by preparative reverse phase-high pressure liquid chromatography (RP-HPLC). The samples were injected on a Phenomenex (Phenomenex, Torrance, CA, USA) C18 column (22 mm x 25 cm, 5 mm) eluted by H2O/0.1% TFA (A) and CH3CN/0.1% TFA (B) solvent mixture. A linear gradient from 5 to 50% of B over 17 minutes (min) at a flow rate of 20 ml/min was used. The collected fractions were lyophilized to dryness and analyzed by analytical RP-HPLC using a Phenomenex C18 analytical column (4.6 x 250 mm, 5 mm). The identity of purified peptides was confirmed by electron spray ionisation liquid chromatography-mass spectrometry (ESI LC-MS) using a Thermo Electron MSQ Surveyor.

*NBD labelling of peptides*: Labelling was performed on resin-bound peptides as previously reported by Rapaport & Shai (Rapaport and Shai 1991). Briefly, 30-70 mg of resin-bound peptide (10-25 mmol) was treated with piperidine in DMF in order to remove the Fmoc protecting group of the N-terminal aminoacid of the linked peptide. The resin-bound peptide was then reacted with 4-chloro-7-nitrobenz-2-oxa-1, 3-diazole (NBD-Cl) in DMF (3-4 equiv.). After 24 hours (h), the resin-bound peptides were washed thoroughly with methylene chloride, and the peptides were then cleaved from the resin and purified as previously reported for the non-labelled peptides. Under these conditions, a major product was obtained, possessing an NBD moiety attached to the peptide's N-terminal amino group. The identity of this compound was confirmed by LC/MS.

### 2.2 Antibacterial Assay

A CFU assay of the antibacterial activity of hBDs against *Pseudomonas aeruginosa* ATCC (American Type Culture Collection, Manassas, VA, USA) 27853, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 was performed. The selection of these strains arose from the need to find

substances with increased activity of those endogenous and able to be active even in extreme conditions of ionic strength, as occurs for patients with Cystic Fibrosis. In fact, CF patients have lung fluid with high concentrations of NaCl. For this reason, tests were performed on the antimicrobial activity in normal and high salt concentration. The strains were grown in aerobic conditions in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 37°C. The next day she applies a refreshment rate of the culture overnight with fresh medium and placed in incubation at 37° C with stirring until the exponential phase (determined by absorption spectrophotometer). At this point, 100 µl of this culture are centrifuged and subjected to a series of washes with 1 mM phosphate buffer in order to ward off the ground. After the last step, the cells were resuspended in 1 mM phosphate buffer 1000 µl. The experiments were carried out by taking 10 µl of this solution containing the cells, adding the solution of NaCl, water or phosphate buffer and defensins in order to reach a final volume of 200 µl for 2 h at 37*C in agitation. Controls were prepared as the experiments except the absence of defensins. We used two peptides concentrations, i.e., 2.5 µM and 12.5 µM. For salt dependence assay, NaCl in a range of concentrations i.e., 0, 50, 100 and 200 mM NaCl, were included in the incubation buffer as previously described (Scudiero et al, 2010). Each assay was performed in triplicate. At the end of incubation, serial dilutions were plating onto agar plates. The count of the colony-forming units (CFU) was executed the next morning. Bactericidal activity (mean and SD of three assays) is expressed as the ratio between colonies counted and the number of colonies on a control plate. The minimal inhibitory concentration (MIC) of the new molecule 3NI was determined with a modified version of the microbroth dilution assay of the National Committee for Clinical Laboratory Standards using a final inoculum of $10^5$ CFU/ml. The following peptide concentrations were used: 100.0, 50.0, 25.0, 12.5, 6.25, 3.12 and 1.56 mM.

### 2.3 Antiviral Assay

Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum. HSV-1 carrying a lacZ gene driven by the cytomegalovirus (CMV) IE-1 promoter to express beta-galactosidase was propagated as described (ref). All experiments were conducted in parallel with no-peptide controls. The effect of peptides on inhibition of HSV infectivity on cell monolayers were assessed: i) For "co-exposure" experiments, the cells were incubated with increasing concentrations of the peptides (1,5, 10.0, 20.0, 50.0, 100.0 and 250.0 µM) and with the viral inoculum for 45 min at 37°C. Non-penetrated viruses were inactivated by

citrate buffer at pH 3.0. Monolayers were fixed, stained with 5-bromo- 4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and plaque numbers were scored. Experiments were performed in triplicate and the percentage of inhibition was calculated with respect to no-peptide control experiments; ii) For "virus pre-exposure" experiments, approximately 2 x $10^4$ PFU of HSV-1 were incubated in the presence of 20 µM of peptides for 45 min at 37°C, then titrated on Vero cell monolayers; and iii) For "cell pre-exposure" experiments, Vero cells were incubated with 20 µM of peptides for 30 min at 4°C and infected with serial dilutions of HSV-1 for 45 min at 37°C.

### 2.4 Cell Culture and Viability Assay

Cell lines were obtained from the Bank of Human and Animal Continuous Cell Lines-CEINGE Biotecnologie Avanzate – Napoli- Italy.
Parental A549 cells (human lung carcinoma) and parental CaCo-2 (human colon adenocarcinoma) stored in liquid nitrogen were thawed by gentle agitation of their vials for 2 min in a water bath at 37°C. After thawing, the content of each vial was transferred to a 75 $cm^2$ surface area, tissue culture flask, diluted with 90% Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) (Sigma-Aldrich St. Louis. MO USA) supplemented with 10% foetal bovine serum (FBS) (Lonza Basel, Switzerland) and 1% L-glutamine (Sigma-Aldrich St. Louis, MO USA). Parental Capan-1 cells (human pancreas adenocarcinoma) were growth with 80% RPMI 1640 Medium (Sigma-Aldrich St. Louis, MO, USA) supplemented with 20% FBS 1% L-glutamine. Then the cells were incubated for 24 h at 37°C in a 5% $CO_2$ to allow them to grow and form a monolayer in the flask. Cells grown to 80–95% of confluence were washed with phosphate buffer saline (PBS), trypsinized with 3 ml of trypsin-EDTA (ethylenediaminetetraacetic) solution (1X) (Sigma-Aldrich St. Louis. MO. USA), diluted, counted and seeded (4x$10^3$ cells/200 µl per well) into a 96-well ml tissue culture plates for 24 h in triplicate.
The reduction of the proliferation of the cells was evaluated by the 3- [4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich St. Louis, MO, USA) assay, which allows measurement of metabolic changes (Liu et al. 1997). This assay is an excellent gauge of the cellular response to external factors such as synthetic molecules because it is used to test molecules that interfere with cell viability (Ferrari et al. 1990). The assay is based on the use of yellow tetrazolium salt (MTT), which are processed only in metabolically active cells by the action of enzymes dehydrogenase. The result is the production of NADH/NADPH and an intracellular purple precipitate (formazan). The solubilisation of the purple precipitate allows the quantisation of active cells by reading at a spectrophotometer.

Cells were incubated with or without beta-defensin peptides wild type 1 and 3 and their analog molecules (1C, 3N and 3NI) according to different schemes: after 4, 8, 12, 16, 20, 24, 48 and 72 h of incubation at 37 °C at different concentrations i.e. 2.5, 12.5 and 25.0 µM. Adherent cells were stained with MTT dye solution i.e. 20 µl of 1:10 diluted MTT stock solution (5 mg/ml) and incubated for 4 h. After the incubation, the presence of the purple crystals that normally indicate the occurred metabolization of the MTT was verified. Then the medium was removed (180 µl) and 180 µl of Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich St. Louis, MO, USA) was added to dissolve the MTT crystals. The eluted specific stain was measured by a spectrophotometer (550 nm). The experiments were performed in triplicate. The proliferation index of the untreated cells was compared to the negative controls (cell + medium without peptides) that represent 100% viability and were included as a control in all experiments to allow estimation of the percentage viability of all the cell samples.

### 2.5 Apoptosis Assay

To identify the number of cells that undergo programmed cell death after incubation with the wild type and chimeric peptides, we performed the apoptosis assay using the kit "Annexin V-FITC Apoptosis Detection Kit 1" (BD bioscience). The cells used for this test were A549, Caco2 and Capan-1. The apoptotic program is characterized by several functional and morphological changes including:

- Disruption of the plasma membrane
- Condensation of the cytoplasm and the nucleus
- DNA fragmentation

The loss of the plasma membrane integrity is a phenomenon observed from the early stages of apoptosis: phosphatidylserine (PS), a component of the membrane, is translocated from inside to outside the cell. Annexin-V is a protein that bind phospholipids in a calcium-dependent manner and that has a high affinity for PS. Consequently, it is able to bind with good specificity of the apoptotic cells that have exposed PS on the surface. Because the exposition of PS on the plasma membrane is an early stage of apoptosis, it is certainly possible to identify cells that have programmed apoptotic death even before the phenomenon is complete. The revelation of the "convicted" cells take place through a fluorescent staining (V-FITC), which guarantees a high sensitivity for the test followed by the analysis of fluorescence by flow cytometry. In association, another staining is used to identify death cells or in late apoptosis (Propidium Iodine (PI)). The viable cells with intact cell membranes exclude the PI; on the contrary damaged cells are instead

permeable. Specifically, therefore, the test allows to use a double standard for cell selection, one positive and one negative, which allows to distinguish viable cells from those in early apoptosis and those death. The pattern of reactivity is as follows:

- Living cells: V-FITC and PI negative
- Early apoptotic cells: Annexin V-FITC positive and PI negative
- Necrotic or apoptotic cells, terminal: V-FITC and PI positive

The test cannot distinguish between cells that have died by apoptosis or necrosis. The result of the assay is quantitative, as a percentage.
Exponentially growing A549, CaCo-2 and Capan-1 cells were trypsinized and plated at density of 1 x $10^5$ cells per ml per 24-well plate and allowed to attach for 24 h. On the next day, cells were exposed to three different concentrations (i.e. 2.5, 12.5 and 25.0 µM) of wild type peptides hBD1 and hBD3 and their analogs 1C, 3N and 3NI for 24, 48 and 72 h. After incubation times, cells were spinned 5 min at 1300 rpm and then resuspended in 100 µl of Annexin-V Binding Buffer 1X (Becton Dickinson), 5 µl of Annexin-V-FITC (Becton Dickinson) and 5 µl of PI (50 µg/ml) were added. Cells were stained 15 min at room temperature and then 400 µl of Annexin-V binding buffer were added before acquisition. The results were interpreted as follows: cells negative for both PI and Annexin-V-FITC staining were considered live cells; PI-negative, Annexin-V-FITC-positive stained cells were considered in early apoptosis. Negative controls (cells+medium without peptides) were included as a control in all experiments to allow estimation of the percentage of apoptotic and death cells.

### 2.6 DNA Damage Assay

The Comet assay is an excellent gauge of the cellular response to external factors such as synthetic molecules because it is used to test molecules that interfere with DNA integrity (McKelvey-Martin et al. 1998; Speit et al. 2004). The DNA strand breakage was defined using comet assay kit form Trevigen (Gaithersburg MD). The cells used for this test are A549, Caco2 and Capan-1. For the determination of DNA strand breakage, exponentially growing cells were trypsinized and plated at density of 1 x $10^5$ cells per ml per 24-well plate and allowed to attach for 24 h. On the next day, cells were exposed to two different concentrations (i.e. 2.5 and 12.5 µM) of wild type peptides hBD1 and hBD3 and their analogs 1C, 3N and 3NI for 4 and 24 h. At the end of incubation time, cells are resuspended in 50µl of 1x PBS. The suspension is then mixed with 500 µl of agarose melted at 37° C; 75 µl of this mixture must be immediately entered in special slides, the "Comet Slide". The slides are left for 10 min at 4 ° C in the dark, and then immersed in a lysis solution for 30-60

min. The next step consists of immersing the slides in an alkaline solution for 20-60 min. Finally after electrophoresis (electric field of 1 volt per cm for 10 min), the slides are stained with SYBR green and read the fluorescence microscope. The maximum absorption and emission of SYBR green are 494nm and 521nm respectively. In intact cells the fluorescence emission is confined to the nucleus: the undamaged DNA is supercoiled and this allows only a weak migration of the nucleic acid within the nucleus. In cells in which DNA is damaged, treatment with an alkaline solution allows the relaxation of DNA fragments and therefore a greater degree of migration. All the steps were conducted under yellow lamp in the dark to prevent additional DNA damage. Stained slides are viewed under automated robotic epifluorescent microscope. Experiments were performed in triplicate.
We compared our data with a positive control that cause DNA breakage (A549, CaCo-2 and Capan-1 cell lines treated with $H_2O_2$ 250 µM for 30 min and Etoposide 60 µM for 1 hour) and a negative control (untreated cells).

### 2.7 Confocal Microscopy

A549 and CaCo-2 cells were seeded at 25% confluence in IBIDI 6 wells chambers (Ibidi GmbH) in DMEM containing FBS 10%, at 37°C and 5% CO2 ("growth conditions"), and used for the experiments after 36 h of growth.
In pulse and chase confocal based experiments, cells were incubated in 15 µM of each NBD-labelled defensin for 10 min at 37°C and 5% CO2 ("pulse"); subsequently, defensin-containing medium was substituted with fresh growth medium ("chase") and cells were incubated at 37°C, 5% CO2, for variables times. After chase times, cells were immediately fixed with PFA 3.7% in PBS and stained without permeabilization steps. WGA-Al555 and DAPI were used as marker of plasma membrane (PM) and nuclei, respectively. In experiments, FM4-64FX (Invitrogen) was used as vital/fixed cell marker of plasma membranes. "Mild pre-fixation" of A549 cells consisted of 1.8% PFA in PBS, 2 min at room temperature.
Immunofluorescence assays were performed with the primary antibody anti EEA1 (Early Endosome Antigen 1; Affinity bioreagent), which is a marker of early endosomes. A549 cells and CaCo-2 were treated with 15 µM each defensin for 10 min, followed by wash with growth medium and incubation for 0, 15 min, 30 min, 1 hour, 1.5 h and 2 h in growth conditions. Cells were then fixed with PFA 3.7% in PBS, permeabilized by PBS/triton x100 0.1% 10 min at room temperature, blocked by BSA1%/PBS for 30 min, and treated with EEA1 antibody 1:100 in BSA1%/PBS for 30 min at room temperature, followed by secondary Alexa 546 conjugated antibody treatment (Invitrogen). LysoID (Enzo Life Sciences) was used as follows: A549 and CaCo-2 cells were

treated with 15µM of each defensin for 10 min in growth conditions, and then washed with growth medium and incubated in growth conditions for 1 and 4 h. Cells were then incubated with LysoID working solution for 15 min, washed once with growth medium, and immediately imaged by confocal microscopy. Experiments were performed in triplicate.
Microscope was LSM 510 meta confocal microscope (Carl Zeiss, Jena) equipped with an oil immersion planapo 63x objective 1.4 NA. Used settings were the following: green channel for detecting NBD, excitation 488nm argon laser, emission bandpass filter 505-550nm; red channel for detecting WGA-Alexa555, FM4-64FX and LysoID, excitation 543 nm Helium/Neon laser, emission bandpass filter 560-700nm (by using the meta monochromator); blue channel for detecting DAPI, excitation 405nm blue diode laser, emission bandpass 420-480 nm.

### 2.8 Serum Stability

50 µg of wild type hBD3 were incubated with 250 µl of untreated human serum from healthy donors [25% (v/v) in PBS] at 37°C for 24 h. Aliquots of 25 µl, corresponding to 5µg of peptide, were added to 65 µl of cold 0.5% (v/v) of TFA (trifluoroacetic acid) in water at different time intervals (0, 0.5, 1, 2, 4, 6 and 24 h), kept on ice for 5 min and then centrifuged at 16200 g for 5 min. The supernatants were analyzed using a reverse phase high performance liquid chromatography, RP-HPLC (Perkin Elmer), followed by MALDI-TOF mass spectrometry analysis with a Voyager DE-STR (Applied Biosystem). The components were separated using a Jupiter C18 column 5 µm particle size, 300 Å pore size, 250x2 mm (Phenomenex) with a flow rate of 0.2 ml/min and a linear gradient from 20 to 60% of solution B (0.1% TFA in acetonitrile) and solution A (acidified water 0.1% TFA) for 25 min. The percentage of the intact peptide (P) was calculated from the area of the corresponding peak in the chromatogram (P=At/A0 x100); At is the peak area of the peptide at any given time; A0 is the peak area at time zero. The MALDI-TOF was operated in the linear mode using a matrix solution of α-Cyano-4-hydroxycinnamic acid 10 mg/ml (TFA 0.2% in 70% ACN). Externally mass spectrometer calibration was performed using a protein mixture of Insulin, Myoglobin and Cytocrome C.

## 3 Results and Discussion

### 3.1 Design of the Novel Synthetic Peptide 3NI

We previously synthesized and reported the antimicrobial and chemotactic activities of eight analogs obtained from hBD1 and hBD3 (Scudiero et al. 2010). We were able to identify the peptides and/or their domains that exerted the most potent antibacterial, antiviral and chemotactic activities. Two of these peptides, i.e., analogs 1C and 3N, were the best suited, in terms of maximizing antimicrobial activity also in the presence of high NaCl concentrations. This finding showed that both the charged C-terminal domain of hBD3 and the internal domain of hBD1 played a fundamental role in the analog's antimicrobial activity. In the present study we designed a novel chimeric molecule 3NI analogue to hBD3 without the N-terminal domain of hBD3 and comprising the internal domain of hBD1 (Fig. 6). Successively, we tested the antibacterial and antiviral activity of the novel molecule 3NI.


**Fig. 6:** Sequence alignments of hBD1, hBD3 and synthetic analogs 3NI, 3N and 1C. The coloured segments of sequences derived from hBD-1 (red) and hBD-3 (bleu). The coloured drawings in the boxes bars indicate the regions exchanged between hBD1 and hBD3.
3NI sequence: KYYCRVRGGRCAVLSCPIFTKIQGTCSTRGRKCCRRKK

### 3.2 Antibacteial Assay

In order to investigate the activity of the new analog 3NI, we performed an antibacterial assays (CFU and MIC) using 3NI analog against *P. aeruginosa*, *E. coli* and *E. faecalis*. We previously reported the antibacterial activity of hBD1 and hBD3 (Scudiero et al. 2010). Furthermore, in the present study, in order to test the antibacterial activity of 3NI, hBD1 and hBD3 have been considered as positive controls. We used two concentrations of each peptide (2.5 and 12.5 μM) and four concentrations of NaCl (0, 50, 100 and 200 mM). The antibacterial activities of all peptides against *P. Aeruginosa* (Fig. 7, panel A), *E. coli* (Fig. 7, panel B) and *E. faecalis* (Fig. 7, panel C) were comparable. At a concentration of 2.5 μM, the antibacterial activity of hBD1 was strongly inhibited at a NaCl concentration as low as 50 mM ($p < 0.001$). On the contrary, the antibacterial activity of hBD3 was not inhibited within 200 mM NaCl ($p<0.001$). Considering hBD3 as reference wild type defensin because of the high salt-resistance, our new analog 3NI at a concentration of 2.5 μM maintained good antibacterial activity up to a 100 mM of salt. At 12.5 μM, 3NI is active up to 200 mM NaCl.

In addition, we performed the MIC of 3NI with the conventional micro-broth dilution assay. The MIC values ranged between 12.5–25.0 mM for all the tested microorganisms. Peptides hBD1, hBD3 and the 3NI analog exerted a strong antibacterial effect against *P. aeruginosa*, *E. coli* and *E. faecalis* (12.5 mM).

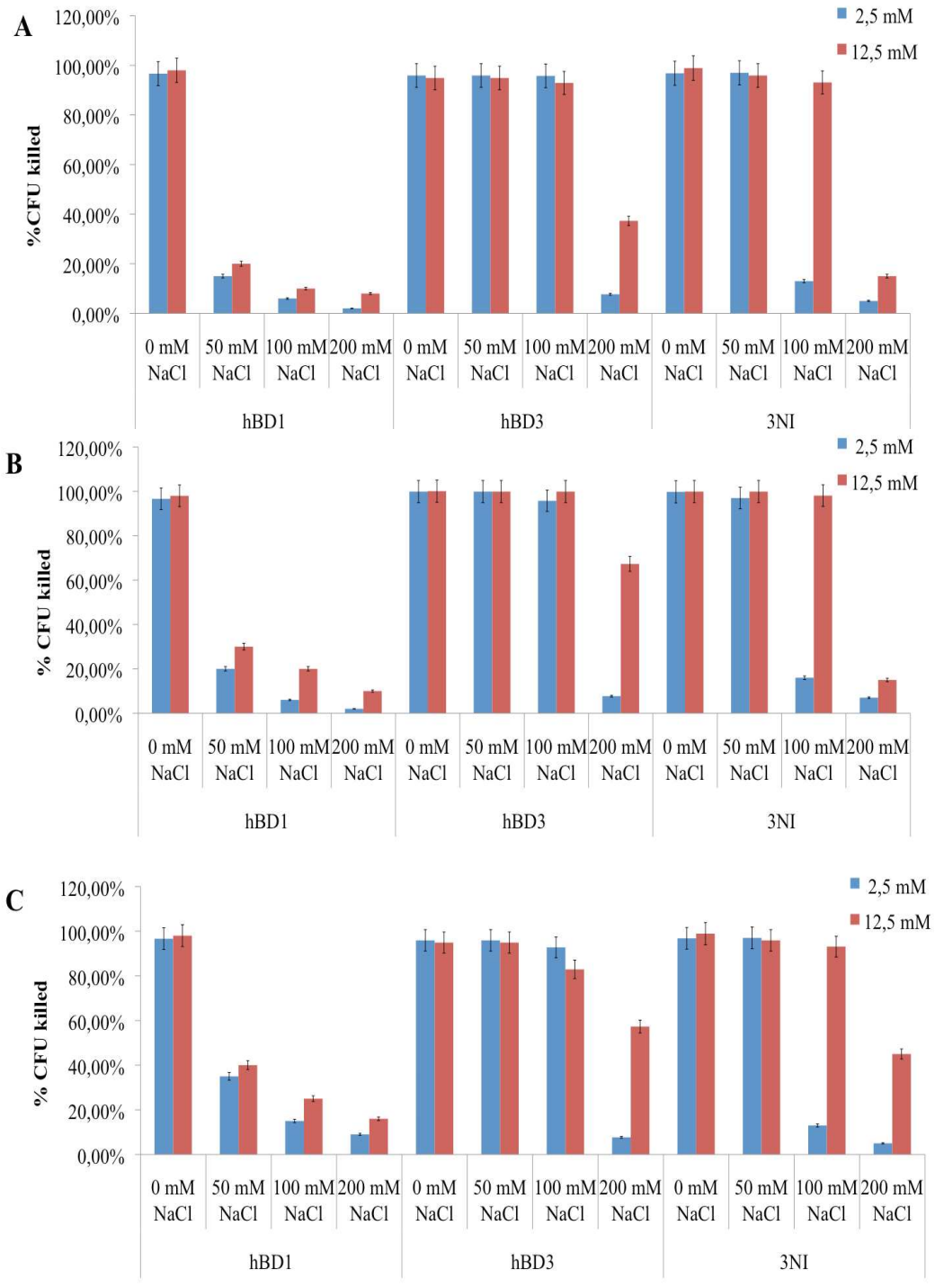



**Fig. 7:** Antibacterial activity of wild type hBD-1 and hBD-3 and the novel analog 3NI. The results are mean and SD of three replicates for each assay; all peptides were tested at two concentrations (2.5 and 12.5 µM) against P.aeruginosa (A), E. coli (B) and E. faecalis (C) at different NaCl concentrations (0, 50, 100, 200 mM). Error bars show the SD of experiments performed in triplicate.

### 3.3 Antiviral Assay

In order to investigate the activity of the new analog 3NI, we performed an antiviral assay of 3NI. We measured and compared the activity of 3NI with previously determined activities obtained for hBD1, hBD3, 1C and 3N. All peptides dose-dependently inhibited HSV infectivity (Fig. 8). Analog 3NI had no residual infectivity at 50 µM; the data are comparable with those obtained for hBD3 (no residual infectivity at 50 mM). At the same concentration, the residual infectivity for analogs 3N and 1C was 10% and 34 %, respectively. Peptide hBD1 displayed a lower compared to hBD3 and 3NI antiviral activity at 50 µM.

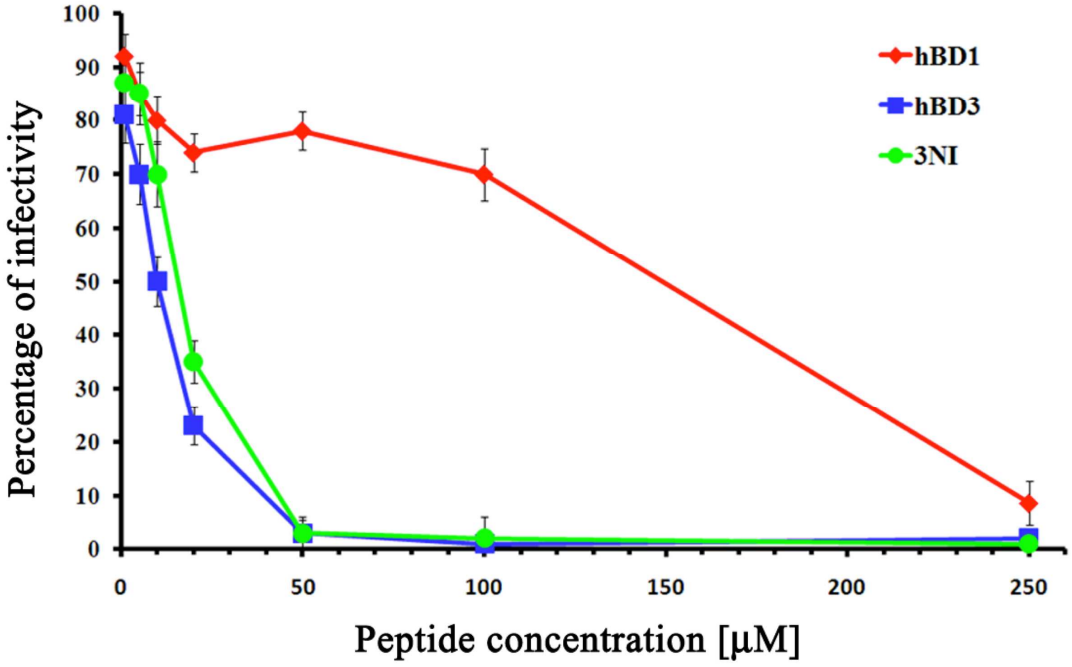



**Fig. 8:** Antiviral activity of wild type hBD-1 and hBD-3 and the novel analog 3NI. All peptides were tested against herpes simplex virus type 1 at a concentration of 20.0 µM after different treatments (see also materials and methods). Error bars show the SD of experiments performed in triplicate.

### 3.4 Viability Assay

The MTT test was performed to evaluate the cytotoxic effect of hBD wild type 1 and 3 and their analogs 1C, 3N, 3NI on three human epithelial cell lines: lung carcinoma (A549); colon carcinoma (CaCo-2); pancreas carcinoma (Capan-1). We tested three concentrations of peptide (2.5, 12.5 and 25.0 μM) and eight incubation times (0, 4, 8, 12, 16, 20, 24, 48 and 72 h) (Fig. 9, panles A-I).

The results obtained for A549 cells are shown in Fig. 4 (panels A, B, C). During exposure between 4 h and 24 h, cell viability unchanged. After 24 h of exposure, the percentage of reduction of the proliferation induced by hBD1 and hBD3 wild type is comparable to that of their analogs 1C, 3N and 3NI. The percentage of the reduction of proliferation was < 10% at all concentrations tested (2.5, 12.5 and 25.0 μM). After 48 h of exposure, peptides reduced cell viability between 4-12% for the three concentrations tested. After 72 h cells hBD1, hBD3 wild type and their analogs 1C, 3N, 3NI at 2.5 μM reduced cell viability of 30%, while for the concentrations of 12.5 and 25.0 μM, the reduction ranged between 28-30%. Between 4 h and 72h of incubation of cells with the peptides, a cell viability reduction higher than 30% was never observed.

Figure 9 (panels D, E, F) shows the effects of the peptides on CaCo-2 cells. As observed for A549 cells, there was no reduction in cell viability between 4 and 24 h of exposure of cells to the peptides. After 24 h of exposure, the peptides at a concentration of 2.5 μM, caused a reduction in cell viability of 2%, while at a concentration of 12.5 and 25.0 μM, the reduction of viability was between 5 and 10%. After 48 h until 72 h of incubation, all the peptides tested reduced viability of 4-12%. During the time course between 4 h and 72 h of incubation of the cells with the peptides a reduction of cell viability greater than 20% was never observed.

The MTT results of hBD-s wild type 1 and 3 and their analogs 1C, 3N, 3NI on Capan-1 cells is shown in Fig. 9 (panels G, H, I). As observed for A549 and CaCo-2 cells, there was no reduction in cell viability between 4 and 24 h of exposure of cells to the peptides. After 24 h of exposure, the reduction in viability ranged between 8-16% for all the concentrations tested (2.5, 12.5, 25.0 μM). After 48 h of incubation, the reduction in cell viability was between 11-17%, while after 72 h a reduction ranged between 18 and 24%.

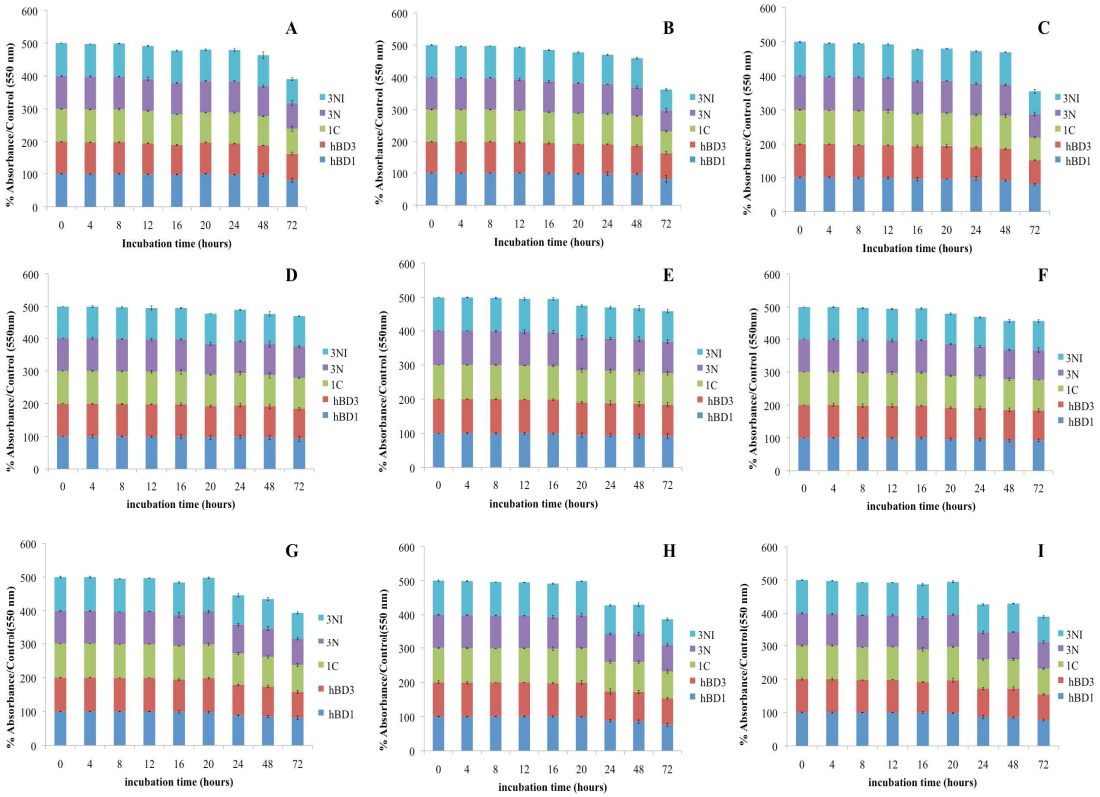



**Fig. 9:** Effect of hBD-1, hBD-3 wild type and their analogs 1C, 3N, 3NI on cell viability. Three different concentrations tested: 2.5 µM (panel A, D, G); 12.5 µM (panel B, E, H); 25.0 µM (panel C, F, I) on the proliferation of the three different cell lines: A549 (panel A, B, C), CaCo-2 (panel D, E, F) and Capan-1 (panel G, H, I). After 0, 4, 8, 12, 16, 20, 24, 48 and 72 h of incubation, adherent cells were stained with MTT dye solution. The eluted specific stain was measured by a spectrophotometer (550 nm). The data are expressed as peptide proliferation index compared to negative controls values. The data are expressed as the means +/- SD of two experiments performed in triplicate.

In conclusion, all the peptides behaved similarly for all the concentrations tested (2.5, 12.5 and 25.0 µM). During the time course between 4 and 72 h of incubation of the cells with the peptides we never observed a reduction of cell viability greater than 30% for all the tested human cell lines (a549, CaCo-2, Capan-1).

### 3.5 Apoptosis Assay

In order to evaluate if the reduction of cell viability observed by MTT test was mediated by apoptotic events, we performed the AnnexinV-Propidium Iodine assay. The flow cytometric analysis of the test gets two plots: the first allows assessing the size (FSC-A) and the degree of cell complexity (SSC-A) (Fig. 10); the second plot allows estimating the percentage of apoptotic cells.
Looking at the first plot, it is important to note that the lower part of the plot represents the cells of small size and low cytoplasmatic complexity. In this region the number of recorded events, increases proportionally to the increase of the phenomenon of apoptosis and necrosis. This increase is caused by the presence of cellular debris produced by necrotic and apoptotic cells.

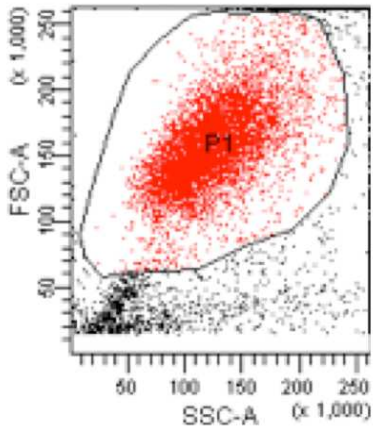



**Fig. 10**: Dot plot resulting from the flow cytometry. The plot allows assessing the size (FSC-A) and the complexity of the cells (SSC-A).

The second plot allows estimating the percentage of apoptotic cells induced by wild type and chimeric peptides (Fig. 11).
The chart is divided into four quadrants:

1. Q1 represents cellular artefacts;
2. Q2 represents the late apoptotic cells, positive for the staining with both Annexin and Propidium Iodine;
3. Q3 represents living cells, negative for the binding of both Annexin-V and Propidium Iodine;
4. Q4 represents the early apoptotic cells, positive for Annexin-V binding.

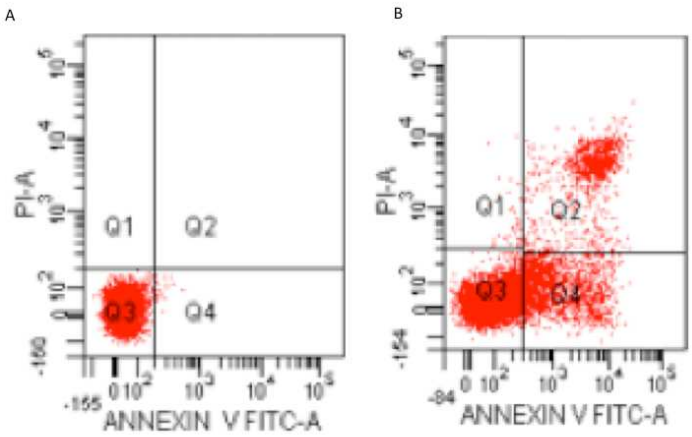



**Fig. 11:** Dot plot resulting from the flow cytometry. Dot plots of untreated cells (a) and cells treated with the peptides (b).

The analysis of the obtained plots allows to determine the percentage of cells in early apoptosis (Q4) and late (Q2). Flow cytometric analysis did not reveal any relevant apoptotic effect after the incubation of the hBD-s wild type 1, 3 and their analogs (1C, 3N, 3NI) with the three human cell lines: A549 (Table 1), CaCo-2 (Table 2) and Capan-1 (Table 3) at the three concentrations i.e. 2.5µM, 12.5µM and 25.0 µM for 24, 48, and 72 h.

| **Q2: %** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **[peptides]** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** |
| ***NC*** | 1,2 | 1,2 | 1,2 | 1,4 | 1,4 | 1,4 | 2,3 | 2,3 | 2,3 |
| ***hBD1*** | 2 | 2,2 | 2,7 | 1,4 | 2,2 | 2,1 | 0,8 | 1 | 0,9 |
| ***hBD3*** | 3,6 | 2,9 | 4,5 | 1,5 | 2,2 | 3,6 | 0,8 | 2 | 3,7 |
| ***1C*** | 2,2 | 1,7 | 2,3 | 3,2 | 2,5 | 2,9 | 1,6 | 1,6 | 1,2 |
| ***3N*** | 2,2 | 1,6 | 3,4 | 4,2 | 3,3 | 4,8 | 1,3 | 2,1 | 1,5 |
| ***3NI*** | 1,9 | 1,8 | 1,1 | 4,7 | 5,7 | 14,3 | 2 | 2,2 | 2,7 |
| **incubation time (h)** | **24** | | | **48** | | | **72** | | |

**Table 1:** Effect of synthetic peptides (hBD-1, hBD-3, 1C, 3N, 3NI) on apoptosis in A549 cells. Cells were incubated for 24, 48, 72 h with all peptides at different concentrations: 2.5, 12.5 and 25 µM. Apoptosis was characterized by exposure of phosphatidylserine with Annexin-V labelling. Data are expressed as a percentage of Annexin-V positive and Propidium Iodine negative cells (Q2 on the scatter) after subtraction of negative control (untreated cells). All the synthetic peptides do not reveal a significant increase in the number of apoptotic cells when compared to untreated cells. NC: negative control. Experiments performed in triplicate.

| Q2: % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **[peptides]** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** |
| ***NC*** | 4,2 | 4,2 | 4,2 | 6,6 | 6,6 | 6,6 | 6,3 | 6,3 | 6,3 |
| ***hBD1*** | 7,1 | 10,3 | 9,6 | 6,1 | 6,2 | 7 | 8,2 | 7,5 | 4,1 |
| ***hBD3*** | 12,6 | 12,8 | 20,8 | 8,7 | 6 | 34,1 | 9,2 | 7,6 | 9,8 |
| ***1C*** | 9 | 12,3 | 11,7 | 7,8 | 13,6 | 20,6 | 5,2 | 10,9 | 8 |
| ***3N*** | 8,2 | 12,3 | 12,9 | 10,8 | 10,3 | 10,1 | 6 | 5,9 | 9,1 |
| ***3NI*** | 6,6 | 9,2 | 14,3 | 7,5 | 6,2 | 14,2 | 7,3 | 6,3 | 6,2 |
| **incubation time (h)** | **24** | | | **48** | | | **72** | | |

**Table 2:** Effect of synthetic peptides (hBD-1, hBD-3, 1C, 3N, 3NI) on apoptosis in CaCo-2 cells. Cells were incubated for 24, 48, 72 h with all peptides at different concentrations: 2.5, 12.5 and 25 µM. Apoptosis was characterized by exposure of phosphatidylserine with Annexin-V labelling. Data are expressed as a percentage of Annexin-V positive cells and Propidium Iodine negative (Q2 on the scatter) after subtraction of negative control (untreated cells). All the synthetic peptides do not reveal a significant increase in the number of apoptotic cells when compared to untreated cells. NC: negative control. Experiments performed in triplicate.

| Q2: % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **[peptides]** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** | ***2,5 µM*** | ***12,5 µM*** | ***25,0 µM*** |
| ***NC*** | 15,2 | 15,2 | 15,2 | 10,2 | 10,2 | 10,2 | 19,6 | 19,6 | 19,6 |
| ***hBD1*** | 14,4 | 12,6 | 12,9 | 17,8 | 11,3 | 10,7 | 15,1 | 8,1 | 17,2 |
| ***hBD3*** | 14 | 8,3 | 9,2 | 10,9 | 14,8 | 18,7 | 10,8 | 15,9 | 26,2 |
| ***1C*** | 9,2 | 12,3 | 14 | 16,1 | 16,2 | 16,2 | 21 | 18 | 22 |
| ***3N*** | 8,4 | 7,4 | 16,7 | 14,7 | 12,5 | 11,2 | 12,8 | 25,2 | 22,3 |
| ***3NI*** | 10,7 | 15,5 | 13,1 | 15,4 | 16,9 | 23,4 | 25,6 | 24,5 | 31,8 |
| **incubation time (h)** | **24** | | | **48** | | | **72** | | |

**Table 3:** Effect of synthetic peptides (hBD-1, hBD-3, 1C, 3N, 3NI) on apoptosis in Capan-1 cells. Cells were incubated for 24, 48, 72 h with all peptides at different concentrations: 2.5; 12.5 and 25 µM. Apoptosis was characterized by exposure of phosphatidylserine with Annexin-V labelling. Data are expressed as a percentage of Annexin-V positive cells and Propidium Iodine negative (Q2 on the scatter) after subtraction of negative control (untreated cells). All the synthetic peptides do not reveal a significant increase in the number of apoptotic cells when compared to untreated cells. NC: negative control. Experiments performed in triplicate.

### 3.6 DNA Damage Assay

The comet assay is routinely used to study the extent DNA damage in drug-treated cells. We tested the effect of the wild type peptides hBD1 and hBD3 and their analogs 1C, 3N, 3NI on A549 cells (Fig. 12, panel A), CaCo-2 cells (Fig. 12, panel D) and Capan-1 cells (Fig. 12, panel G). As positive controls, we incubated cells with $H_2O_2$ (250 µM for 30 min) and observed the characteristic comate. After 4 h and 24 h of exposure to a peptide concentration of 2.5 and 12.5 µM, we observed intact nuclei in each cell line tested. Our results demonstrated that all peptides do not induce DNA damage.

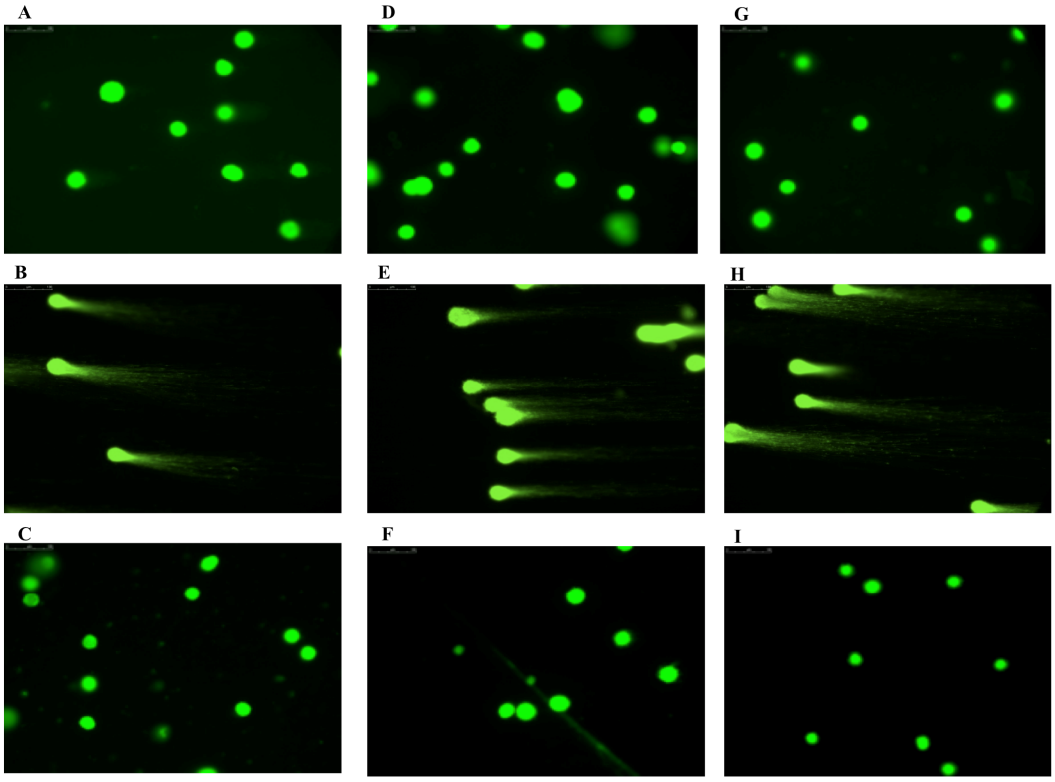



**Fig. 12:** Comet assay on A549, CaCo-2, Capan-1 cells. Panels A, D, G: representative image for the results obtained for the wild type hBD-1, hBD-3 and analogs 1C, 3N, 3IN at two different concentrations (2.5 and 12.5 µM) and two different incubation times (4, 24 h) of A549, CaCo-2 and Capan-1 cells respectively. Panels B, E, H: positive control of A549, CaCo-2 and Capan-1 cells respectively (cells treated with $H_2O_2$ 250 µM for 30 min). Panels C, F, I: negative control of A549, CaCo-2 and Capan-1 cells respectively (untreated cells). One representative image obtained in three independent experiments. All the synthetic peptides do not induce DNA damage.

### 3.7 Confocal Microscopy

To study the interactions of our molecules with eukaryotic cells, we used two cell lines, A549 and CaCo-2. Capan-1 cells were not included in these experiments because of their autofluorescence. The confocal microscopy experiments were performed using NBD-labelled molecules. NBD was chosen because it is a small fluorescent molecule and therefore we supposed that it would have a minimal effect on the activity of defensins. A set of confocal microscopy experiments in fixed and in vivo cells were performed to investigate the mechanisms of interaction and internalization into cell plasma membranes. Cells were treated with peptides and then chased at several time points. After each chase time, cells were fixed and stained with WGA-Al555 and DAPI, as markers of plasma membrane and nuclei, respectively. All the fluorescent NBD-labeled defensins displayed a comparable behaviour in these time-course experiments. Similarly, no significant differences were found between the two cell lines. Immediately after incubation with peptides, cells were washed to remove unbound molecules. At time 0 fluorescence was clearly localized only on the plasma membrane as indicated by the colocalization with the plasma membrane marker (Fig. 13, panel A). After 2 h, fluorescence appeared in cytosolic vesicles as shown by the ring-shaped fluorescence pattern obtained from the optical slices (Fig. 13, panel B). After 8 h, the vesicle pattern did not change significantly (Fig. 13, panel C). After 18 h, a reduction of peptide fluorescence and of the number of vesicles occurred (Fig. 13, panel D). These results indicate that within 10 min, defensins were able to bind cell plasma membranes, in the subsequent 2 h, they were internalized in vesicles, and within 8 and 18 h, clearance of peptides had started.

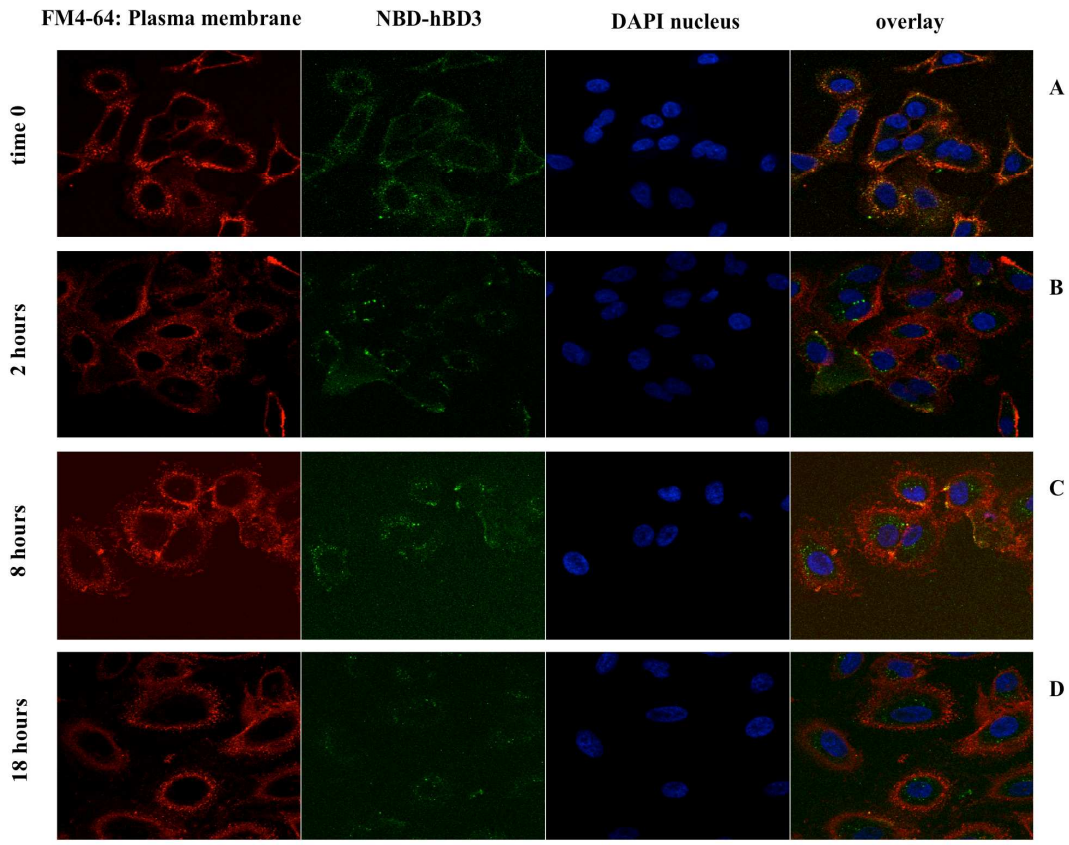



**Fig. 13:** Interaction of A549 and CaCo-2 cells with defensins. Cells were treated with 15 µM of each defensin for 10 min; upon wash with fresh growth medium, they were incubated for 0, 2, 8 and 18 h. Immediately after the proper times, cells were PFA fixed and stained with WGA-Al555 and DAPI to localize plasma membrane and nuclei. Images represent single optical slices 1µm thick. Defensin is green, PM is red and Nuclei are blue; colocalizing regions appear orange-yellow in the overlay image. The five defensins in A549 and CaCo-2 cells behaved very similarly, therefore we show representative images of A549 treated with hBD-3. At time zero, defensins localized exclusively to the plasma membrane (panel A). After 2 and 8 h (panels B and C), defensins were found mainly internalized as vesicles. After 18 h (panel D), some vesicles are still apparent but reduced in number and intensity respect to previous times. Scale bar: 5µm. Experiments were repeated three times and images were acquired from at least 100 cells per experiment.

Successively, to evaluate the mechanisms of internalization, we conducted a set of in vivo experiments using FM4-64FX, a marker of the plasma membranes and endocytosis. Our peptides colocalized with FM4-64FX (Fig. 14). Because FM4-64FX binds immediately to the outer leaflet of plasma membrane and is internalized by endocytosis, our data suggest that cells by means of this process internalized defensins.

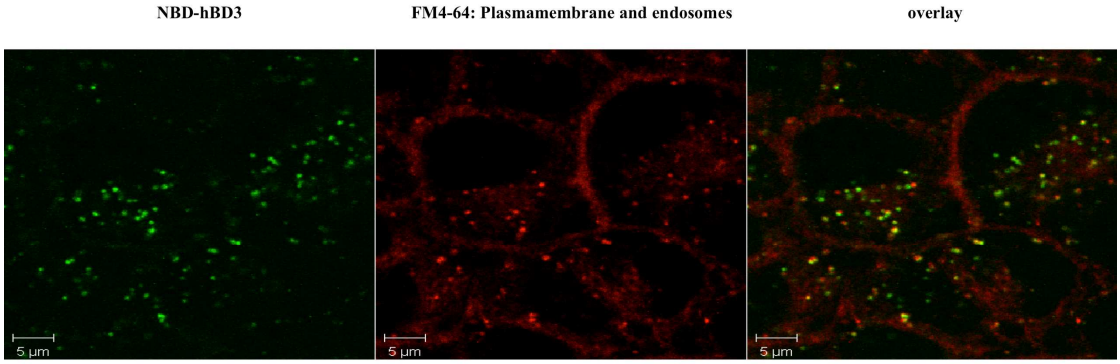



**Fig. 14:** Defensins are internalized by endocytosis. Cells were treated with 15 µM of each defensin and FM4-64FX, which is a marker of plasma membrane, for 2 h. Upon wash with PBS, they were immediately PFA fixed and imaged by confocal microscope. Images represent single optical slices 1µm thick. Defensin is green, Plasma membranes and endosomes are red; colocalizing regions appear orange-yellow in the overlay image. The five defensins in A549 and CaCo-2 cells behaved very similarly, therefore we show representative images of A549 treated with hBD-3. Defensins colocalized extensively with endosomes. Scale bar: 5µm. Experiments were repeated three times and images were acquired from at least 100 cells per experiment.

To clarify which kind of mechanism contribute to defensins internalization, if an active or a passive mechanism occur. To this aim, we carried out experiments with a mild "pre-fixation" of cells before the incubation of the cells with peptides. Within 10 min, our molecules localized with the plasma membrane (data not shown) and remained in this compartment also after 2 h (Fig. 15). These data indicate that, association to the plasma membrane did not require an active mechanism while the internalization required it.

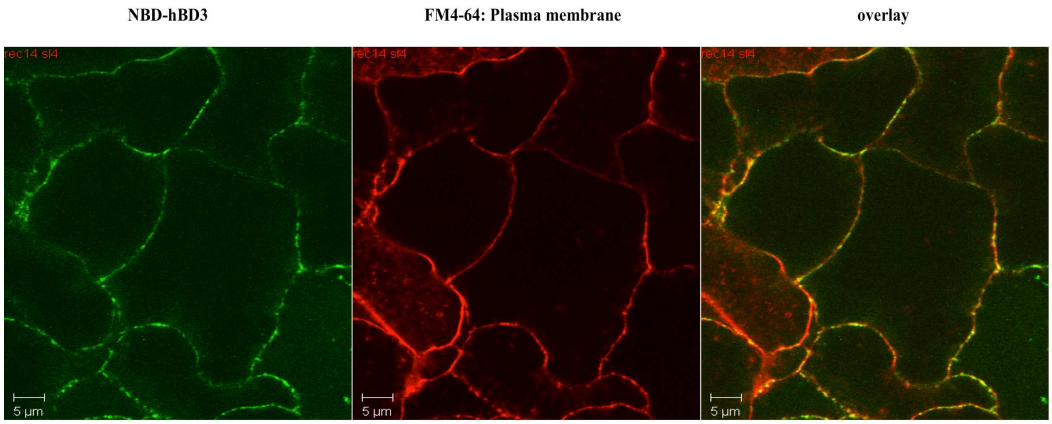


**Fig. 15:** When active mechanisms are blocked defensins remain in the plasma membrane. Cells were mildly PFA prefixed to kill active mechanisms and then treated with 15 µM of each defensin and FM4-64FX, a marker of plasma membrane for 2 h. Confocal images represent single optical slices 1µm thick. Defensin is green, FM4-64 is red; colocalizing regions appear orange-yellow in the overlay image. The five defensins in A549 and CaCo-2 cells behaved very similarly, therefore we show representative images of A549 treated with hBD-3. Defensin colocalized with FM4-64 which was found exclusively on the plasma membrane. No defensin and FM4-64 fluorescence was detected in the cell interior. Scale bar: 5µm. Experiments were repeated three times and images were acquired from at least 100 cells per experiment.

To further verify that endocytosis mechanisms are required for defensins internalization, we carried out experiments at 4°C that block active mechanisms. After 2 h, no fluorescence was observed inside the cells (Fig. 16), which suggests that endocytosis was indeed the mechanism involved in internalization.

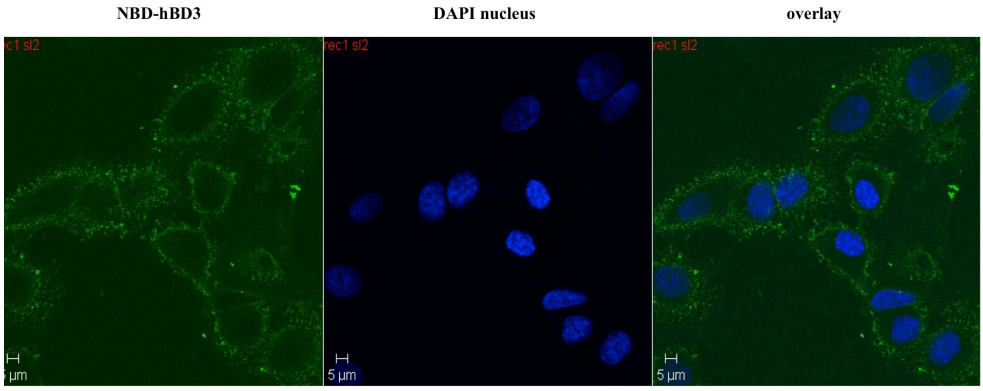



**Fig. 16:** When endocytosis is blocked, defensins are retained on the plasma membrane. Cells were treated with 15 µM of each defensin for 2 h on ice. Ice temperature is known to block endocytosis. Cells were PFA fixed immediately after ice incubation. Confocal images represent single optical slices 1µm thick. Defensin is green, DAPI is blue. The five defensins in A549 and CaCo-2 cells behaved very similarly, therefore we show representative images of A549 treated with hBD-3. Defensin localized exclusively on the plasma membrane. No defensin fluorescence was detected in the cell interior. Scale bar: 5µm. Experiments were repeated three times and images were acquired from at least 100 cells per experiment.

Since we demonstrated that active mechanisms of endocytosis are responsible for defensins internalization in the cells, we investigated if early endosomes are involved in this process. To this aim, we performed immunofluorescence assays using the antibody anti EEA1, a marker of early endosomes, upon pulsing of cells with each peptide, and chasing for several times. Peptide fluorescence partially colocalized with EEA1 already after 15 min (Fig. 17, panel B) and the extent of colocalization increased within 2 h

(Fig. 17, panel A), suggesting that early endosomes are involved in peptide processing.

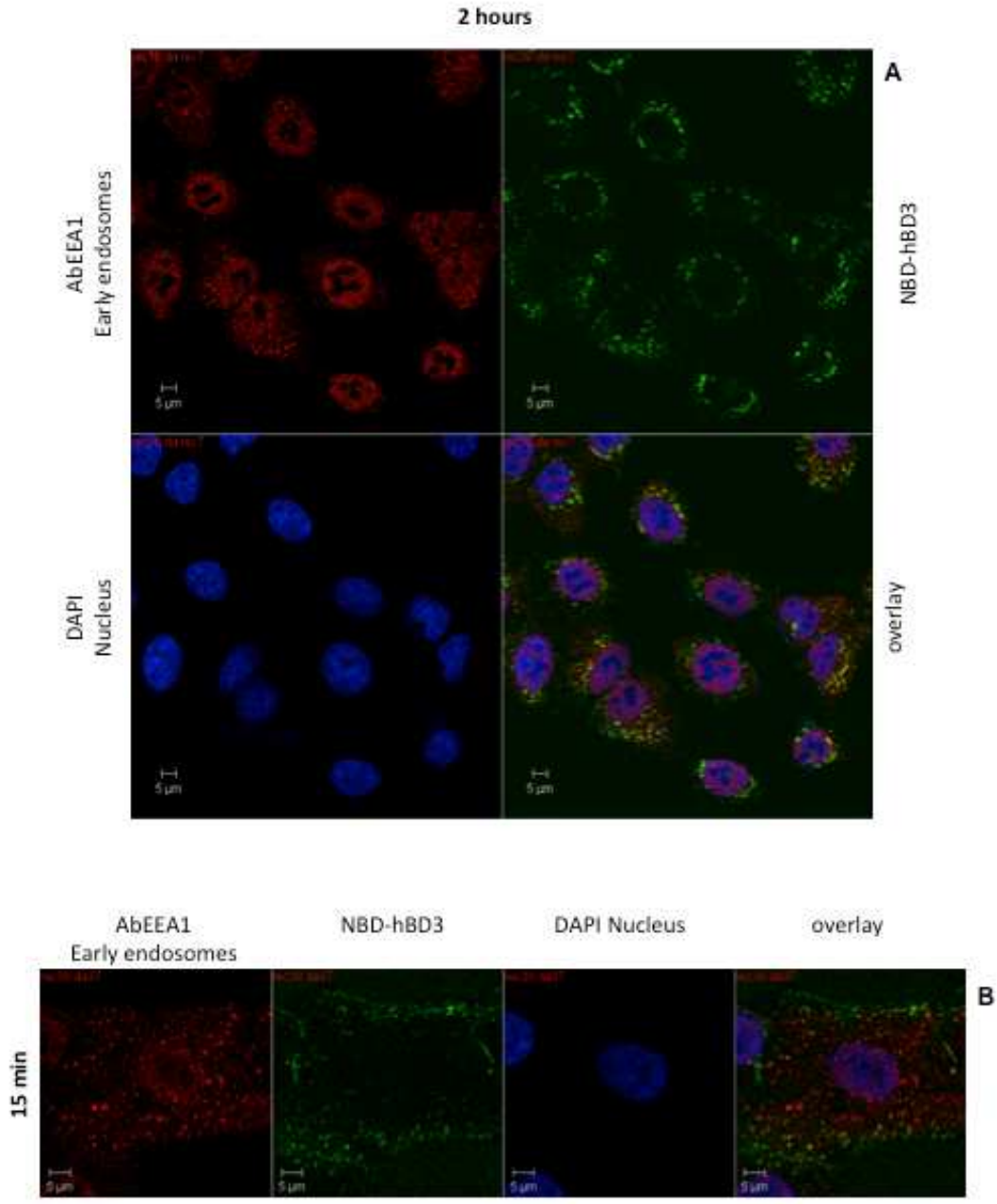



**Fig. 17:** Defensin containing endosomes docks on early endosomes. Cells were treated with 15 µM of each defensin for 10 min; upon wash with fresh growth medium, they were incubated for 2 h, PFA fixed, permeabilized and stained by immunofluorescence with antibody anti EEA1, marker of the early endosome compartment, and DAPI for nuclei. Confocal images represent single optical slices 1µm thick. Defensin is green, AntiEEA1 is red, DAPI is blue; colocalizing regions appear orange-yellow in the overlay image. The five defensins in A549 and CaCo-2 cells behaved very similarly, therefore we show representative images of A549 treated with hBD-3. Defensin colocalized partially with early endosomes

already after 15 min (panel B) and with higher extent after 2 h (panel B). Scale bar: 5µm. Experiments were repeated three times and images were acquired from at least 100 cells per experiment.

In addition to further discuss our data, LysoID have been used to mark lysosomes in vivo experiments; the cells have been pulsed with each peptide, chased and then stained for lysosomes. LysoID fluorescence was partially colocalizing with that of our molecules after 1 and 4 h, indicating that at least some of the peptide-containing vesicles were targeted to lysosomes (Fig. 18). Therefore, degradation processes of our molecules partially occur by means of lysosomes. We cannot exclude that others mechanisms participate too defensins disposal from the cells.

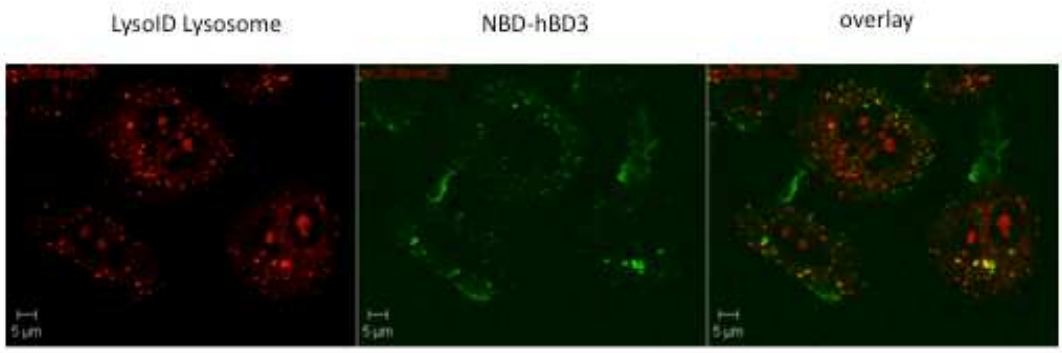



**Fig. 18:** Defensin vesicles localize to lysosomes. Cells were treated with 15 µM of each defensin for 10 min; upon defensin removal, cells were incubated for 4 h in fresh growth medium. Before observation cells were treated with LysoID, which stains lysosomes. Confocal images represent single optical slices 1µm thick of live cells. Defensin is green, LysoID is red; colocalizing regions appear orange-yellow in the overlay image. The five defensins in A549 and CaCo-2 cells behaved very similarly, therefore we show representative images of A549 treated with hBD-3. Defensins colocalized partially with lysosomes already after 4 h. Scale bar: 5µm. Experiments were repeated three times and images were acquired from at least 100 cells per experiment.

### 3.8 Serum Stability

Since we proposed these peptides as therapeutic target for the treatment of infectious diseases, our attention focused on the study of the stability of these peptides in human serum. In fact, demonstrated their antimicrobial activity, and assuming an employment in the treatment of infectious state, we wanted to understand firstly the stability of wild type hBD-3.
We found from 0 min until 1 h, no degradation of peptide. After 2 h, hBD-3 is degraded of about 30% and finally from 4 h until 24h around 45% (Fig. 19).

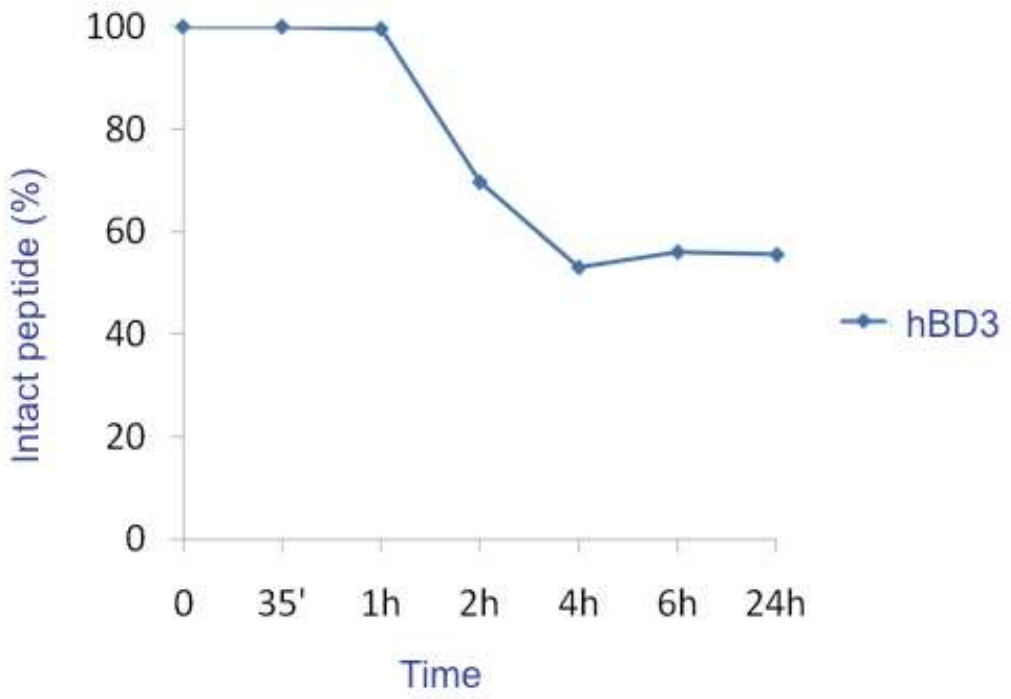



**Fig. 19:** Stability profiling of hBD-3 in human serum from an healthy donor. 25% (v/v) of untreated human serum in PBS incubated with hBD-3.

### 3.9 Discussion

The production of AMPs plays a fundamental role in defense against infections (Wiesner et al. 2010). We previously designed and characterized the biological activities of two hBD-1 and hBD-3 analogs, namely, 1C and 3N (Scudiero et al. 2010). We demonstrated a higher salt resistance of the two newly designed analogs compared to the wild type.

The superimposition of the three-dimensional structures of hBD-1 and hBD-3 showed that the main differences between the two molecules were in the N-terminal region, the C-terminal region and the internal domain between the third and the fourth cysteines (Kluver et al. 2006, Krishnakumari et al. 2006). The C-terminal positively charged region in hBD-3 is packed against the internal domain, which contains two positive residues while the internal domain of hBD1 contains only one positive residue (Krishnakumari et al. 2006). Moreover, the N-terminal helix in hBD-3 starts from Lys8, with residues 1-7 being disordered (Conejo-Garcia et al. 2001). It has been previous showed that the C-terminal domain of hBD-3 and the internal domain of hBD-1 are fundamental for antimicrobial and chemotactic activity and also that the N-terminal disordered domain of hBD-3 is not necessary for activity (Scudiero et al. 2010, Taylor et al. 2008). To verify these results we synthesized a novel molecule without the N-terminal domain of hBD-3 and with the internal domain of hBD-1, 3NI. Analog 3NI consists of the hBD-3 sequence plus the internal domain of hBD-1 and without the N-terminal domain of hBD-3. Furthermore, the C-terminal domain (8 amino acids) of hBD-3 has more positive charges (net charge: + 6) than the C-terminal domain (5 amino acids) of hBD-1 (net charge: + 2). The charged residues in the internal domain between the third and fourth cysteines residues also differ between hBD-3 and hBD-1. In particular, hBD-3 has two glutamines and two lysines residues, whereas hBD-1 has only one lysine residue. Finally, hBD-3 has more net total positive charges (+ 11) than hBD-1 (+ 4) and the analogs all present a higher net positive charge compared to hBD-1 (3NI: +12; 3N: +11; 1C: +8). Moreover, it has been recently suggested that the antimicrobial activity of β-defensins is probably attributable to their reduced unstructured form (Scudiero et al. 2010, Schroeder et al. 2011).

We tested analog 3NI and found that it exerts good antibacterial activity against *E. coli, P. aeruginosa, E. faecalis* at 2.5 μM and up to 100 mM NaCl, which confirms that the C-terminus of hBD3 and the internal domain of hBD-1 are crucial for antibacterial activity and salt-resistance. (Krishnakumari et al. 2006, Scudiero et al. 2010). The antiviral activity of the analog 3NI against HSV was similar to that of hBD-3 with a reduction of infectivity of 99% at 50 μM,

which is in accordance with previous studies of HIV (Conejo-Garcia et al. 2001, Weinberg et al. 2006, Cole et al. 2003).
We used three epithelial cell lines (A549, CaCo-2 and Capan -1) derived from tissues normally expressing β defensins as a model to investigate the potential cytotoxic activity of our peptides. Interestingly, exposure of the cells to the wild type and chimeric peptides between 4 and 24 h did not result in a reduction of cell viability. Neither was the percentage of apoptotic cells affected by this treatment. Similarly, our analogs do not cause DNA damage, as demonstrated by the results of single-cell gel electrophoresis. After 48 h and within 72 h of exposure of the cells to the peptides, there was a small decrease in the percentage of cell proliferation (30%). These results coincide with the results of the apoptotic assay in which there was a minimal increase in the percentage of apoptotic cells (20%). In addition, we found, by confocal microscopy experiments, that the cells partially degrade the peptides between 24 and 72 h. We suggest that the reduction of cell proliferation within 72 h (30%) occurs via two mechanisms: a mild induction of apoptosis by peptides and digestion of peptides.
It is also critical to note that the three cell lines showed different behaviour in proliferation and apoptotic tests: CaCo-2 cells were less susceptible to the effects of peptides than the other two cell lines. Moreover, proliferation and apoptotic tests revealed that Capan-1 cells were more sensitive than the other two cell lines to the effects exerted by the peptides. Additionally, our results obtained through confocal microscopy experiments, confirm that these peptides are not lethal for human epithelial cell lines. We also demonstrated that peptides are able to bind plasma membrane and be internalized into the cells by active mechanisms that involve early endosomes. However, we cannot exclude that other mechanisms participate to the internalization of defensins. Similarly, we suggest that cells are able to partial eliminate peptides within 18 h through lysosomes but we cannot exclude that others mechanisms participate to the removal of defensins from the cells. Finally, our results obtained from serum stability experiments demonstrated that hBD-3 is stable until 1 h of incubation in human serum and is degraded of about 45% in 24 h of incubation. Our results are partially in accordance with Antcheva et al. 2009 that reported a degradation rate of hBD-3 of around 20% just after 2 h.

## 4 Conclusions and Perspectives

The goal of the present work was to find defensin analogs with high antimicrobial activity without cell toxicity, giving the potential to these peptides to be exploited as therapeutic targets in infectious diseases.
To this aim, we designed and synthesized a new defensin analog 3NI and characterized its antibacterial and antiviral activity. We found that 3NI possess a higher antimicrobial activity and a higher salt resistance compared to the wild type hBD-1 and hBD-3. Moreover, the results of cytotoxicity studies on three human epithelial cell lines of hBD-1, hBD-3, 1C, 3N and of 3NI analogs, supported by the confocal microscopy data, suggest that these peptides have a low effect on the reduction of cell viability (30% after 72 hours of incubation with cells), do not damage DNA and cause a minimum induction of apoptosis (20% after 72 hours of incubation with cells). Moreover, our experiments of stability in human serum demonstrated a good stability of hBD-3 (degradation of 45% after 2 hours).
Altogether our results show that 1C, 3N the new molecule 3NI are characterized by a significant antimicrobial activity and salt resistance and they are not toxic for the three human epithelial cell lines tested.
Our results allow us to candidate our analogs as potential therapeutic molecules to be tested *in vivo*. In addition, our results can be validated *in vitro* through experiments on co-culture of eukaryotic cells and bacteria.
Then our results confirm the hypothesis that is possible to design new analogs with increased antimicrobial activity. In fact, an improved serum stability would be obtained designing analogs with a modified C-terminus or a cyclic structure; molecules with an increased antimicrobial activity would be achieved synthetizing smaller peptides and/or dimeric peptides.
Moreover, since peptides are active against bacteria and viruses and do not exert relevant toxic effects on human cells, it would be of great interest to analyse directly *“in vivo”* the properties of wild type and analogs peptides in mice models of infectious diseases. It would possible to consider severe infections in several epithelia and organs due to pathogen viruses and bacteria. This approach will allow us to evaluate the most relevant pharmacological properties of peptides: drug efficacy (in physiological conditions and in condition of high salt concentrations), stability in serum, half-life and side effects.

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