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PhD Thesis

THERAPEUTIC POTENTIAL OF BAC7(1-35), A PROLINE- RICH ANTIMICROBIAL PEPTIDE: *IN VITRO* AND *IN VIVO* STUDIES AND PEGYLATION STRATEGY TO IMPROVE ITS BIOAVAILABILITY

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*Benincasa M, **P**elillo C, Zorzet S, **G**arrovo C, **B**iffi S, **G**ennaro R, **S**cocchi M.* **The proline-rich peptide Bac7(1-35) reduces mortality from Salmonella typhimurium in a mouse model of infection.** BMC Microbiol. (2010) 10:178-83.

***P**elillo C, **B**enincasa M, **P**acor S, **R**unti G, **G**ennaro R, **S**cocchi M.* **Evaluating the *in vitro* efficacy of the proline-rich peptide Bac7(1-35) in human blood components and its effects on human immune cells** (in preparazione).

***P**elillo C, **Z**ahariev S, **B**enincasa M, **S**cocchi M, **G**ennaro R.* **PEGylation via thioether ligation enhances the pharmacokinetics properties of Bac7(1-35), a proline-rich antimicrobial peptide (**in preparazione)**.**

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***D**onati I, **T**ravan A, **P**elillo C, **S**carpa T, **C**oslovi A, **B**onifacio A, **S**ergo V, **P**aoletti S.* **Polyol synthesis of silver nanoparticles: mechanism of reduction by alditol bearing polysaccharides.** Biomacromolecules.(2009)10: 210-3.

LIST of ABBREVIATIONS

AMPs - antimicrobial peptides
CCCP - carbonyl cyanide 3-chlorophenylhydrazone
CD - cluster of differentiation
CFU - colony-forming units
CSF - colony stimulating factor
DC - dendritic cells
DCM - dichloromethane
$DiOC_6$ - dihexylocarbocyanide iodide
DMF - dimethylformamide
DMSO - dimethyl sulfoxide
ECM - extracellular matrix
EDTA - ethylenediaminetetraacetic acid
ESI-MS - electrospray ionization mass spectrometry
FBS - fetal bovine serum
FDA - food and drugs administration
GFP - green fluorescent protein
HDPs - host-defence peptides
HRP - horse radish peroxidase
IFN - interferon
IL - interleukin
LC-ESI/MS - liquid chromatography electrospray ionization mass spectrometry
LPS - lipopolysaccharide
MFI - Mean Fluorescence Intensity
MH - Mueller-Hinton (microbiological medium)
MIC - minimal inhibitory concentration
m-PEG - metoxy- Poly(ethylene glycol)
MW - molecular weight
NADPH - dihydro-nicotinamide-adenine-dinucleotide
NMR - nuclear magnetic resonance
OD - optical density
PBS - phosphate-buffered saline (microbiological medium)
PEG - Poly(ethylen glicol)
PI - propidium iodide
PMA - phorbol-12-myristate-13-acetate
PR-AMPs - proline-rich antimicrobial peptides
ROS - reactive oxygen species
RP-HPLC/ELSD - reversed phase high liquid chromatography/ Evaporative Light Scattering Detector
RPMI - Roswell Park Memorial Institute (cell culture medium)
SCVs - Salmonella-containing vacuoles
SDS - sodium dodecyl sulphate
SIF - Salmonella-induced filament
SPI - salmonella pathogenicity island
TB - tripan blue
TFA - trifluoroacetic acid
TLR - Toll-like receptor
TNF - tumor necrosis factor
T3SSs - type III secretion system

Introduction

1. HOST DEFENSE and ANTIMICROBIAL PEPTIDES (AMPs)

1.1 Host defense

The immune system is composed of an innate (non-specific) and an adaptive (specific) response. Innate immunity is constitutively present, acts immediately following an infection and is termed non-specific because the protective response is the same regardless of the initiating factor. This is in contrast to the adaptive immune system, which is slower, responds specifically, and generates an immunological memory.

The primary response of innate immunity is inflammation. Injured cells release cytokines and other pro-inflammatory factors (e.g., bradykinin, histamine, leukotrienes, prostaglandins, serotonin, etc) to control the spread of infection and promote healing. These pro-inflammatory mediators induce vasodilatation and attract phagocytes: initially neutrophils, which participate in the response by also attracting other immune cells. The innate response involves a wide number of cell types but is particularly dependent on neutrophils and macrophages (phagocytosis), and basophils and mast cells (inflammation). Another important function of the innate immune system is to stimulate the adaptive immune response via antigen presentation.

The elements of the innate immune system are:

- **Anatomical barriers to infections**. The epithelial surfaces act as first line of defense against invading organisms forming a physical barrier that is impermeable to most infectious agents. Movement due to cilia or peristalsis, the flushing action of tears and saliva, and the trapping effect of mucus help prevent microbial colonization of different organs.
- **Humoral components**. Once infectious agents have penetrated tissues, humoral factors play an important role in inflammation. These factors are found in plasma and at the site of infection. They include: i) the complement system, which once activated can lead to increased vascular permeability, recruitment of phagocytic cells, and opsonization and lysis of bacteria; *ii*) the coagulation system, which contributes to the increased vascular permeability; in addition, some of its products act as chemotactic agents for phagocytic cells or as antimicrobial molecules (e.g., the beta-lysin protein that is produced by platelets during coagulation); *iii*) other molecules, such as

lactoferrin, transferrin, interferons, lysozyme, cytokines, lactic and fatty acids and antimicrobial peptides (AMPs).

- **Cellular components**. The main cell lines of defense involved in the non-specific innate response are dendritic cells, monocytes, macrophages, granulocytes, and natural killer cells, as well as the skin, pulmonary and gut epithelial cells that form the interface between an organism and its environment.

Among the innate immune components, this PhD thesis will focus on the role of antimicrobial peptides and macrophages during bacterial infections.

1.1.1 Macrophages

These cells are important elements of immune defence due to their capacity to phagocytose foreign material, to produce cytokines and to present antigens to T cells (Gordon and Taylor 2005).

Macrophages derive from blood monocytes by maturation upon migration from the capillary bed to tissue sites, where they are exposed to environmental signals. Macrophages are more numerous than circulating monocytes due to their long life span, which can last from several weeks to years (van Furth and van Dissel 1989). They can be found in many tissues, including connective tissue, liver, lung, lymph nodes, spleen, bone marrow, serous fluids, skin, pleural and alveolar cavities. Local populations of macrophages are maintained by proliferation of resident progenitor cells and influx of monocytes from blood. Traditionally, macrophages have been described as antigen-presenting phagocytes that secrete pro-inflammatory and antimicrobial mediators (Gordon 1999).

Mounting evidence, however, describes a more complex model involving multiple macrophage phenotypes carrying out different functions and eliciting divergent effects on surrounding cells and tissues (Hallam *et al.* 2009).

Classically, the activation of macrophages requires a priming signal in the form of IFN-gamma via the IFN-gamma receptors. When the primed macrophage subsequently encounters an appropriate stimulus, such as bacterial LPS, it becomes classically activated (Krutzik *et al.* 2005) and exhibits a Th1-like phenotype, promoting inflammation, extracellular matrix (ECM) destruction, and apoptosis. Alternatively, activated macrophages do not require any priming. IL-4 and/or IL-13 can act as sufficient stimuli (Stein *et al.* 1992; Doherty *et al.* 1993). The binding of these factors to

their respective receptors is followed by fluid-phase pinocytosis of soluble antigen (Brombacher 2000; Conner and Schmid 2003). The internalized soluble antigen is then loaded onto MHC class II and subsequently presented to T cells. Alternatively, activated macrophages display a Th2-like phenotype, promoting ECM construction, cell proliferation, and angiogenesis.

In vitro maturation of monocytes to macrophages is induced by serum factors or artificial stimuli such as phorbol-12-myristate-13-acetate (PMA), a potent tumor promoter, and colony stimulating factor (CSF), a glycoprotein that binds specific receptors and activates intracellular pathways of proliferation and differentiation. These processes are accompanied by a characteristic change in the secretory repertoire of macrophages and typical morphological and biochemical transformation. They are larger than monocytes because of their abundant cytoplasm, and the nuclear chromatin appears looser. Macrophages also show an increased number of cytoplasmic organelles, but the content of lysosomes may vary with the stage of cellular maturation, as well as with location and activity of the cells. Macrophages are metabolically and functionally more active than monocytes, showing an increase in protein content, glucose utilization, pinocytic and phagocytic activities and synthesis of enzymes (Jain 1993).

1.1.2 Phagocytosis

Phagocytosis is defined as a mechanism for internalizing and destroying particles (Cardelli 2001). Many mammalian cells can perform phagocytosis. For example, non-professional phagocytes contribute to clearance of apoptotic cells, which is a process essential in tissue homeostasis (Birge and Ucker 2008). Nevertheless, professional phagocytes, which include neutrophils, dendritic cells, and especially macrophages, play a major role during host defence since they have the ability to recognize pathogens via immuno-receptors on their surface. Phagocytosis is initiated by adherence of a particle to the surface of the plasma membrane of a phagocyte. This step usually involves several types of pathogen-related molecules recognized by surface receptors on the membrane of the phagocyte.

Bacterial lipopolysaccharide (LPS) is known as one of the most effective inducers of macrophage activation. LPS is initially bound by soluble LPS binding protein (LBP) found in plasma and then by either soluble or membrane-bound CD14. This protein delivers LPS to the LPS recognition complex (Yang *et al.* 1999), which consists of the

Toll-like receptor 4 (TLR4). The TLRs are important pathogen receptors that are expressed on immune system-related cells and are involved in direct killing of pathogens, as well as in the production and/or release of pro-inflammatory cytokines, chemokines, cell surface markers and anti-microbial peptides by the stimulated host cells (Aderem and Ulevitch 2000, Takeda *et al.* 2003). Binding of a pathogen to a TLR initiates a signalling pathway that leads to the activation of a transcription factor that turns on cytokine genes such as those for tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and chemotactic compounds that attract white blood cells to the site of infection and enhance phagocytosis.

Pathogens are subsequently taken up into vacuoles known as phagosomes. Once inside the cytoplasm, these vesicles fuse with lysosomes forming a phagolysosome. The killing and digestion of the bacterial cell takes place in the phagolysosome as a consequence of the presence of different factors: degradative enzymes (proteases, lysozyme, glycosydases, phospholipases, and nucleases), antimicrobial cationic peptides from the lysosome-like granules, the acid pH, and the reactive oxygen species derived from activation of the NADPH oxidase, which promotes the respiratory burst event leading to lethal modifications of engulfed microbes. Dead microbes are rapidly degraded in phagolysosomes to low molecular-weight components. Macrophages digest debris and allow insertion of microbial antigenic components into the plasma membrane for presentation to lymphocytes and induction of adaptive immune responses (Fig. 1.1). Suitable antigens are in fact processed and loaded onto MHC class I and class II molecules in late endocytic compartments. The peptide-loaded MHC I complexes are transported to the cell surface, where they may be recognized by T-cell receptors on CD8 T cells. Following transport to the cell surface, peptide-loaded MHC II may be recognized by T-cell receptors on CD4 T cells.

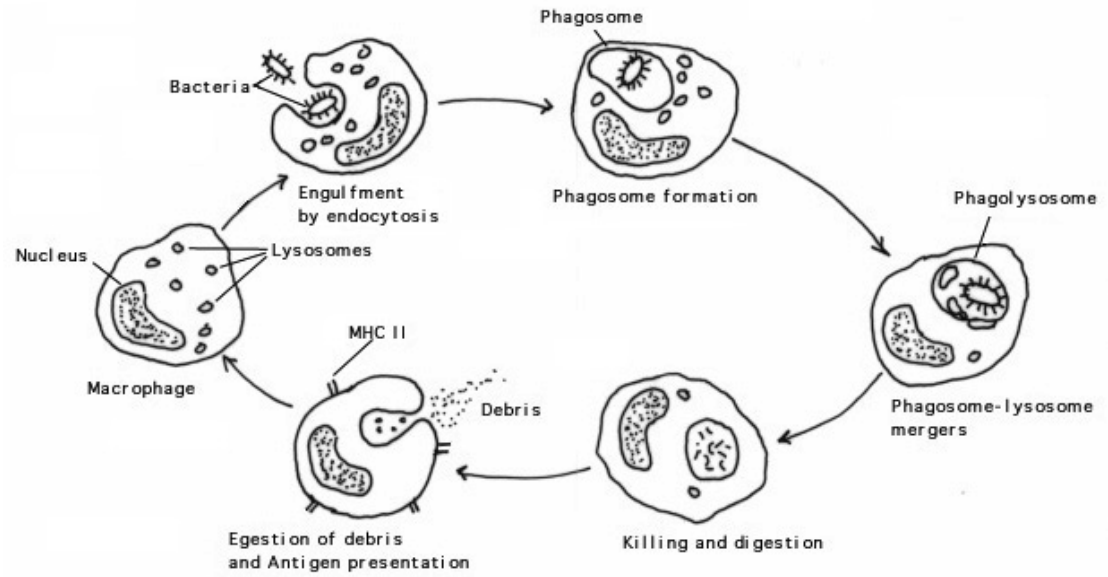


Fig. 1.1 Phagocytosis by a macrophage. A bacterium, which may or may not be opsonized, is phagocytosed and ingested in a membranous vesicle called the phagosome. Digestive granules (lysosomes) fuse with the phagosome, to form a structure called the phagolysosome, and release their contents. The killing and digestion of the bacterial cell takes place in the phagolysosome. The macrophage egests debris while processing the antigenic components of the bacterium, which return to its surface in association with the MHC II for antigen presentation to T cells (Todar 2008).

1.2 Antimicrobial peptides (AMPs)

During the immune response, antimicrobial peptides (AMPs) are expressed and released to limit the replication and viability of bacteria at the site of infection. Because of their multifunctional roles both in the innate and adaptive immunity (Yang *et al.* 2001), a new and wider definition of these peptides has been introduced: Host Defense Peptides (HDPs).

AMPs are evolutionary ancient members of the host defense system. Originally characterized in plants and insects, AMPs have been found in all classes of organisms and are a widely used mechanism of biochemical defence against pathogens. Their emerging diverse functions in immune response are stimulating a strong interest on

these molecules also for the future development of multipurpose anti-infective drugs. So far, over 1200 AMPs have been already identified or predicted from genome sequencing.

They are typically cationic and amphipathic molecules, with a relatively short length (less than 100 residues, mostly between 12 and 50). Based on their primary structure and conformation, they may be classified as:

a. linear peptides with amphipathic α-helical domains;
b. linear peptides with extended conformations and rich in specific amino acids;
c. β-hairpin peptides stabilized by 1 or 2 disulphide bonds;
d. β-sheet structures stabilized by 3 or more disulfide bridges.

In addition to this variety of structures, they are also expressed by a variety of producing cell types and, *in vivo*, multiple AMPs are usually co-expressed by the same cells during infection (Lai *et al.* 2009). Their expression is commonly induced by microbes, microbial components or inflammatory signals, and can be regulated both at the transcriptional and post-transcriptional levels.

The constitutive expression of AMPs is rare and under strict developmental control and is influenced by age and sexual maturation. In adults, AMPs can be stored in a highly concentrated form within cellular granules or vesicles, and then rapidly released (Lai and Gallo 2009). Their release at the site of infection can involve signalling by the pattern recognition receptors, such as Toll-like receptors (TLRs), or be induced by specific cytokines.

AMPs act against a wide spectrum of Gram-positive and Gram-negative bacteria, as well as fungi and some viruses. They show a multimodal mechanism of action (Fig. 2). In most cases they interact and disrupt membrane integrity (Shai 2002): depending on their amino acid composition, amphipathicity, cationic charge and size, they can cover the surface of the membrane in a carpet-like manner and act as a detergent upon the membrane bilayers (carpet model); otherwise, they can produce transient cavitation of the membrane (toroidal-pore model); the peptides can also associate with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (barrel-stave model). In other cases, translocated peptides can alter the cytoplasmic membrane septum formation and interact with intracellular targets thus inhibiting cell-wall synthesis, nucleic-acid and protein synthesis or enzymatic activity (Brodgen *et al.* 2005).

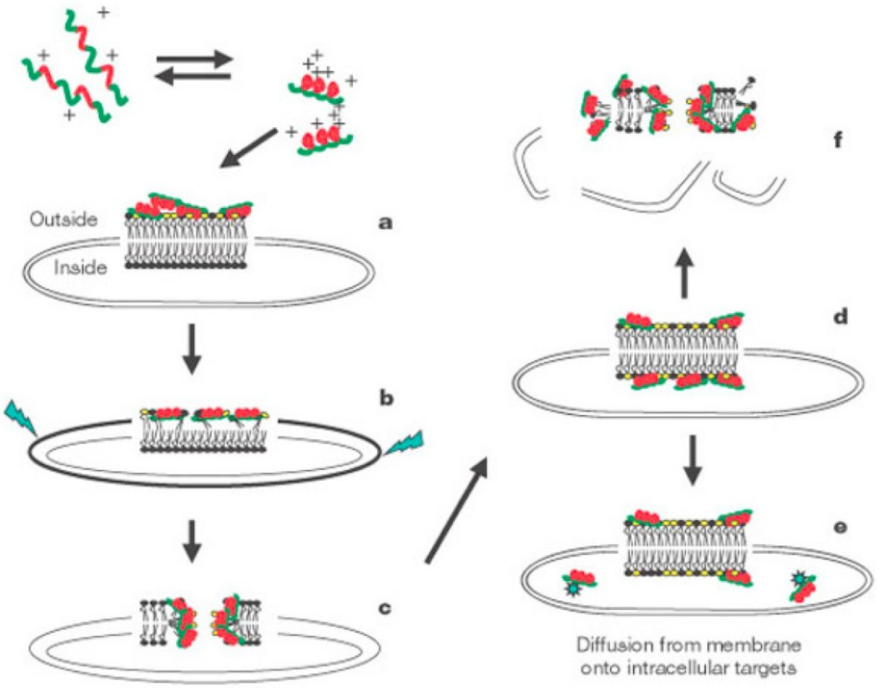


Fig.1.2. The Shai-Matsuzaki-Huang model of the mechanism of action of membranolytic antimicrobial peptides. a. Electrostatic interaction of the peptide with the outer bacterial membrane. **b**. Integration of the peptide into the membrane and thinning of the outer leaflet. **c**. Phase transition and 'wormhole' formation. Transient pores form at this stage. **d**. Transport of the peptide into the inner leaflet. **e**, Diffusion of the peptide into the cytosol to reach intracellular targets. **f**. Collapse of the membrane into fragments and physical disruption of the target cell (Shai 2002).

AMPs have also the ability to invade the barrier of the bacterial cell wall, thereby permitting passage of various molecules into the cells (van't Hof *et al.* 2001). In addition to their direct antimicrobial activity, they also show an active role in the transition to the adaptive immune response by exerting chemotactic activity for human monocytes, neutrophils, and T-cells and by activating the release of different immune factors such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and interferon-γ (IFN-γ) (Yang *et al.* 2001). Moreover, it has been found that they can also promote wound healing and angiogenesis. *In vivo* animal models indicate that host defence peptides are crucial for both prevention and clearance of infection.

In mammals, AMPs have been found in the granules of phagocytes, in mucosal or skin secretions from epithelial cells, or as the degradation products of proteins. The most extensively investigated families of AMPs in vertebrates are the alpha- and beta-

defensins (Ganz and Lehrer 1998), and the cathelicidin peptides (Zanetti *et al.* 1995, Zanetti *et al.* 2002, Zanetti 2004). Considering the whole array of AMPs, one of the most intriguing groups is that of the proline-rich antimicrobial peptides, whose members have attracted considerable interest for their distinct mode of action, which is independent on membrane lysis.

1.3 Proline-rich antimicrobial peptides

Proline-rich antimicrobial peptides (PR-AMPs) are a group of peptides widespread in nature that have been found in insects (Otvos 2002), mammals (Gennaro *et al.* 2002), amphibians (Li *et al.* 2004), crustaceans (Destoumieux *et al.* 1999, Rolland *et al.* 2010) and molluscs (Gueguen *et al.* 2009). Although diverse in sequence, they show some common features: *i*) a high content of proline residues, *ii*) a net cationic charge mainly due to the presence of arginine residues, and *iii*) a mode of action that does not depend on membrane damage, at least at low concentrations (Scocchi *et al.* 2011).

All mammalian PR-AMPs belong to the cathelicidin family. The members of this family are characterized by a highly conserved proregion (the cathelin domain), which is their hallmark. This region shares sequence identity to cathelin, a protein that was isolated from porcine neutrophils as an inhibitor of cathepsin L (cathelin is an acronym for *cathepsin L inhibitor*) (Kopitar *et al.* 1989), and which is likely the proregion of a cathelicidin. The cathelin-like proregion is immediately followed by a highly variable peptide domain, which exerts antimicrobial activity once released by proteolytic cleavage (Fig. 1.3). The cathelicidins are synthesized as pre-propeptides and stored within the cells as propeptides. The proregion likely protects the peptide from proteolytic degradation in a protease-rich environment (Zaiou *et al.* 2003) and the cell from the potential toxic effects of the free peptide (Scocchi *et al.* 1992).

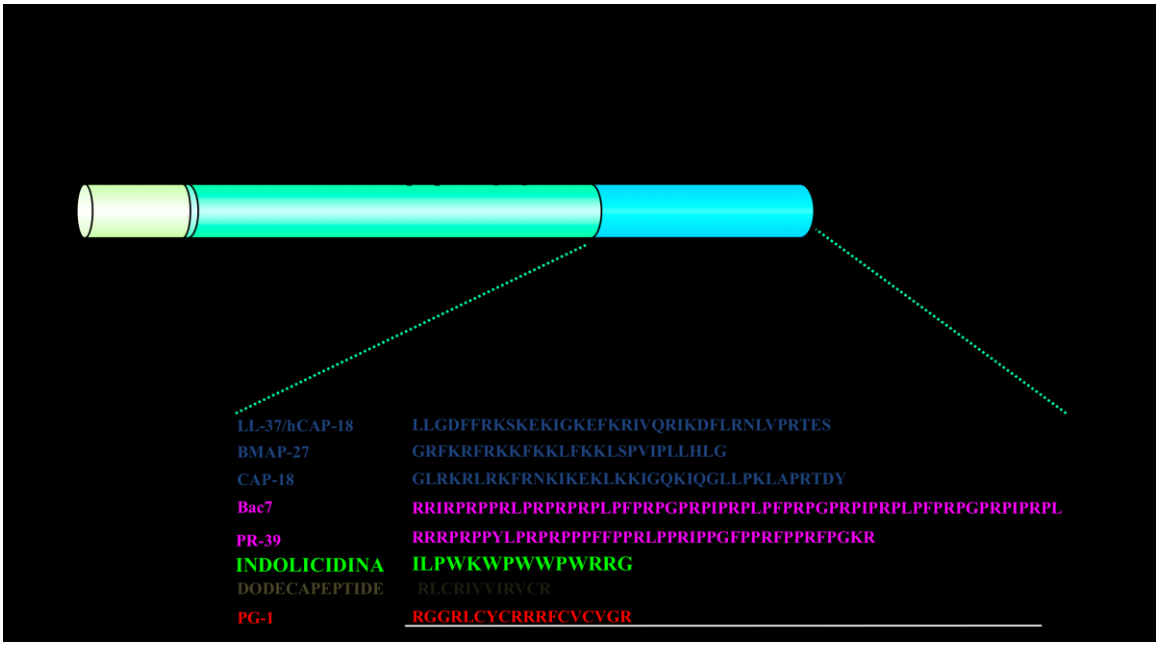


Fig. 1.3. Schematic representation of a member of the cathelicidin family with examples of sequences corresponding to active cathelicidin-derived AMPs.

The presence of conserved regions such as the 5' region spanning the 5' UTR, the sequence coding for the signal peptide and the propiece in the cathelicidin mRNAs, allowed the identification of many congeners in other species by applying the RACE protocol (Zanetti *et al.* 1995). Interestingly, the number of different cathelicidins varies substantially among species with one member present in humans and primates and over ten in pig and cattle.

The presence of PR-AMPs in mammals has only been found in artiodactyls. The first two members of this group were isolated from circulating bovine neutrophils and named bactenecin 5 (Bac5) and 7 (Bac7) (Gennaro *et al.* 1989), later followed by a putative pseudogene containing the sequence of a third bovine PR-AMP, Bac4 (Scocchi *et al.* 1998). Their orthologues have been identified in other bovids, including sheep and goat (Shamova *et al.* 1999), in addition to additional members (OABac11 and OABac6) detected in the former species (Huttner *et al.* 1998). Other types of PR-AMPs have been identified in pig leukocytes: PR-39, whose name derives from the high content of Pro (P) and Arg (R) and from a length of 39 residues (Agerberth *et al.* 1991), and prophenins (Pungercar *et al.* 1993).

Despite a similar amino acid composition, and apart from the similarity of orthologues in evolutionarily related species, they show different length and sequences and are

characterized by the presence of typical, but differently repeated proline motifs, of the type PPRX or PRPX, where X is most often a bulky hydrophobic residue or Gly (Table 1). Apart from cathelicidins, other proline-rich AMPs are not common in mammals. In fact, the only other member found is a weakly cationic peptide named basic proline-rich peptide or SP-B, which was isolated as the main component of porcine salivary gland granules (Cabras *et al.* 2008). This peptide has many Proline residues and possesses a good antifungal activity and a negligible antibacterial activity (Cabras *et al.* 2008). Numerous well-characterized PR-AMPs have been identified in insects and are divided into short-chain and long-chain proline-rich peptides (Bulet *et al.* 1999). The short-chain peptides include drosocin, a 19-residue peptide isolated from *Drosophila melanogaster* (Bulet *et al.* 1993), and the apidaecins, from the lymph fluid of honeybees (Casteels *et al.* 1989) and other hymenoptera (Otvos 2002) Pyrrhocoricin, a well studied PR-AMP isolated from the hemypteran insect *Pyrrhocoris apterus*, belongs to the long-chain proline-rich peptides (Cociancich *et al.* 1994). An interesting structural feature of some Pro-rich peptides of insects is the presence of an *O*-glycosidic substitution at the level of a conserved, centrally located threonine residue, which is relevant for the antimicrobial activity. PR-AMPs were also found in invertebrates other than insects, such as crabs (Schnapp *et al.* 1996) and shrimps (Destoumieux *et al.* 2000).

Concerning the antimicrobial activity of mammalian PR-AMPs, this was tested against a wide panel of Gram-negative and Gram-positive species. These peptides are active in the micromolar range of concentration and the most common susceptible microorganisms are the *Enterobacteriaceae* (e.g., *Escherichia coli, Salmonella typhimurium, Enterobacter cloacae and Klebsiella pneumoniae*) (Skerlavaj *et al.* 1989, Shi *et al.* 1996, Shamova *et al.* 1999). The activity of these peptides decreases by increasing the salt concentration in the medium and by lowering the pH to acidic values. PR-AMPs from insects are also active against Gram-negative bacteria, with only a few Gram-positive species that are affected (Casteels *et al.* 1989).

1.3.1 Mode of action of PR-rich peptides

The PR-AMPs thus far identified are all characterized by a non lytic mechanism of action that explains their slow killing kinetics (from several minutes to some hours) compared to lytic AMPs, which generally are fast acting compounds (they kill susceptible bacteria within few minutes upon exposure).

The relevance of peptide stereochemistry for the antimicrobial activity supports the

hypothesis that they act by a mechanism different from that of lytic peptides. In fact, at variance with the all-*D* enantiomers of these latter AMPs, which display the same activity of the all-*L* counterparts, indicating a non-stereospecific mode of interaction with the bacterial membranes (Wade *et al.* 1990; Bassell *et al.* 1990), the all-*D* enantiomers of PR-AMPs show in general a marked loss of activity (Podda *et al.* 2006; Otvos 2002; Casteels *et al.* 1994), indicating that at least one step in their mechanism is stereospecific. Interestingly, it has been shown for both mammalian and insect PR-AMPs that they have the ability to penetrate into bacterial cytoplasm without any apparent membrane damage (Podda *et al.* 2006) and that this step is necessary to exert their microbicidal activity, at least at about the MIC concentrations. In addition, the peptides' uptake into bacterial cells appears to be energy-dependent and to involve a permease/transporter-mediated process (see below) (Mattiuzzo *et al.* 2007; Benincasa *et al.* 2009). All these results suggest that the PR-AMPs act by interacting with one or more intracellular targets and inhibiting their activity.

Table 1. Proline-rich antimicrobial peptides from vertebrate animals (Scocchi 2011)

Name	Origin	Sequence[a]	N° AA	Z [c]	Pro (%)	Repeated motifs
PR-39	**pig**	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP-NH2	39	+11	49	PPRX (× 4)
PR-39[b]	**pig**	RLRRQAFPPPIFPGPGFRPPIFPPPPFRPAPFGPPRFP-NH2	(38)[d]	+7	42	PPRX (× 1)
prophenin	**pig**	AFPPPNFPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPG PPFPPPRIFPGPWFPPPPPFRPPPFGPPRFP-NH2	79	+7	53	PPPX (× 8)
Bac3.4	**goat**	RFRLPFRRPPIRIHPPPFYPPFRRFL-NH2	26	+8	31	PPXR (× 2)
Bac5	**cow**	RFRPPIRRPPIR---PPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP-NH2	43	+10	47	PPXR (× 7)
Bac5	**sheep**	RFRPPIRRPPIR---PPFNPPFRPPVRPPFRPPFRPPFRPPIGPFP-NH2	43	+11	47	PPXR (× 7)
Bac5	**goat**	RFRPPIRRPPIR---PPFRPPFRPPVRPPIRPPFRPPFRPPIGPFP-NH2	43	+12	47	PPXR (× 8)
Bac4[b]	**cow**	RRLHPQHQRFPRER--PWPKPLSLPLPRPGPRPWPKPL-OH	(36)[d]	+6	33	PRPX (× 2)
Bac6	**sheep**	RRLRPRHQHFPSER-RPWPKPLPLPLPRPGPRPWPKPLPLPLPRPGLRPWPRPL-OH	53	+12	38	PRPX (×4)
Bac7	**cow**	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPL-OH	60	+17	47	PRPX (× 11)
Bac7	**buffalo**	RRFRPRRPRLPRPRPRPLPLPWPRPRPIPRPLPLPWPGPRPIPRPLPLPRPGPRPIPL-OH	58	+16	45	PRPX (× 8)
Bac7	**sheep**	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQPRRIPRPILLPWRPPRPIPRPQPQPIPRWL-OH	60	+20	38	PRPX (× 7)
Bac7	**goat**	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQPRRIPRPILLPWRPPRPIPRPQPQPIPRWL-OH	60	+20	38	PRPX (× 7)
Bac 11	**sheep**	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRPLPLPRPRPKPIPRPLPLPRPR PRRIPRPLPLPRPRPRPIPRPLPLPQPQPSPIPRPL-OH	94	+28	45	PRPX (× 15)
PRP-SP-B	**pig**	APPGARPPPGPPPPGPPPPGP-OH	21	+1	67	PPPGP (× 3)
PR-bombesin		ŒKKPPRPPQWAVGHFM [e]	16	+3	25	PP ((2)

a) Tandem repeats present in some sequences are underlined with alternating solid and dotted lines.
b) Sequence is from a putative pseudogene.
c) Net charge, His residues are considered neutral.
d) Putative peptide size. Charge and percentage of proline residues are based on this sequence.
e) Œ = pyroglutamic acid.

1.3.2 Intracellular bacterial killing.

Until now, few intracellular bacterial processes have been identified with which PR-AMPs interfere. One of the killing mechanisms observed for AMPs such as PR-39 (Boman *et al.*, 1993) and indolicidin (Subbalakshmi and Sitaram, 1998) is the inhibition of cell division, which may be the consequence of DNA, RNA and/or protein synthesis inhibition.

Disruption of respiratory mechanisms leading to the generation of reactive oxygen species is another killing mechanism, employed by histatins against fungi (Bellomio *et al.* 2007). Another significant antibacterial mechanism common to different Pro-rich peptides, such as drosocin, pyrrhocoricin, apidaecin (Otvos 2000), and Bac7 (Scocchi *et al.* 2009) is the inhibition of the chaperone protein DnaK.

This chaperone assists a large variety of protein folding processes, so that the inactivation of susceptible bacteria by insect and mammalian PR-AMPs may depend on their ability to inhibit protein folding and refolding by binding to this protein. However, it has been reported, at least for Bac7, that its *in vitro* interaction with DnaK does not translate into an altered susceptibility of a *dnaK*-knock out strain of *E. coli* (Scocchi *et al.*, 2009), suggesting that other internal targets involved in bacterial killing are present and have still to be identified.

1.3.3 Additional biological activities of PR-AMPs peptides

Some PR-AMPs exert other biological activities in addition to bacterial inactivation. In fact, they can modulate innate immune responses and boost processes that resolve infection by directly interacting with cells of the innate immune system (monocytes, dendritic cells, T-cells, epithelial cells) or by influencing the bioactivities of such cells in an indirect manner (Bowdish *et al.* 2006; Brown and Hancock 2006). For example, it has been shown that PR-39 modulates angiogenesis (Li *et al.* 2000) and wound healing (Heilborn *et al.* 2003), and displays chemotactic activity (Eilsbach 2003; Yang *et al.* 2004; Beisswenger 2005). Moreover, it also shows efficacy in limiting myocardial damage after experimental ischemia in rodent models (Hoffmeyer *et al.* 2000).

Among the PR-AMPs, the one used in the experiments reported in this PhD thesis is the N-terminal fragment 1-35 [Bac7(1-35)] of the cathelicidin-derived peptide bactenecin 7 (Bac7), isolated from bovine neutrophils (Gennaro *et al.* 1989).

1.4 Bactenecin 7

The bactenecins Bac7 and Bac5 were isolated from bovine neutrophils over twenty years ago (Gennaro *et al.* 1989) and played a major role in the elucidation of their biosynthesis and maturation (Zanetti *et al.*1990).

Bac7 is a cationic 60-residue peptide characterized by an Arg-rich cationic N-terminal region followed by three identical hydrophobic tandem repeats of 14 residues (Fig. 1.4).

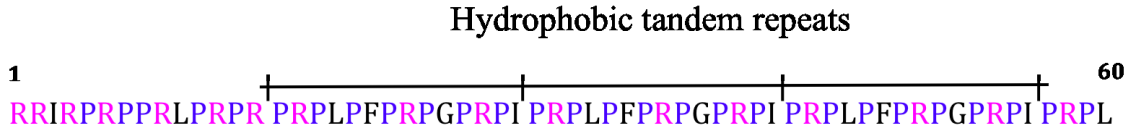


Fig. 1.4. **Schematic representation of Bac7 sequence**

It exerts *in vitro* a potent bactericidal activity against several Gram-negative species such as *E. coli, S. typhimurium, K. pneumoniae,* and *E. cloacae* at concentrations from 1 to 10 μM (Gennaro *et al.* 1989). It also arrests the growth of some strains of *Pseudomonas aeruginosa* and is active against a panel of Gram-negative, antibiotic-resistant clinical isolates (Table 2) (Benincasa *et al.* 2004). In addition, it neutralizes the human herpes simplex virus (Zerial *et al.* 1987).

Table 2 Antibacterial activity of the Bac7 fragments Bac7(1-35) and Bac7(1-16) against Gram-negative clinical isolates. MIC value is expressed in μM.

Bac7 is synthesized as pre-propeptide by neutrophil precursors in the bone marrow. After removal of the signal sequence, the proBac7 form is stored in the large granules of the bovine neutrophils and the active antimicrobial peptide is released upon proteolytic cleavage by elastase, which is stored in the azurophil granules (Scocchi *et al.* 1992). This event occurs when the contents of both large granules and azurophils are discharged into the phagosome after phagocytosis by neutrophils (Zanetti *et al.* 1990, Scocchi *et al.* 1992).

Previous structure-activity relationship (SAR) studies with chemically synthesized fragments of Bac7 from 13 to 35 residues in length and covering the whole sequence of the peptide, have shown that the fragment Bac7(1–35) maintained an antibacterial activity comparable to that of the native peptide. In addition, these investigations showed that the N-terminal part of the peptide is necessary for activity, as indicated by the severe decrease in antibacterial activity upon removal of the first four N-terminal residues (RRIR), and that a minimum length of 16 N-terminal residues is required for biological activity (Fig 1.5) (Benincasa *et al.* 2004).

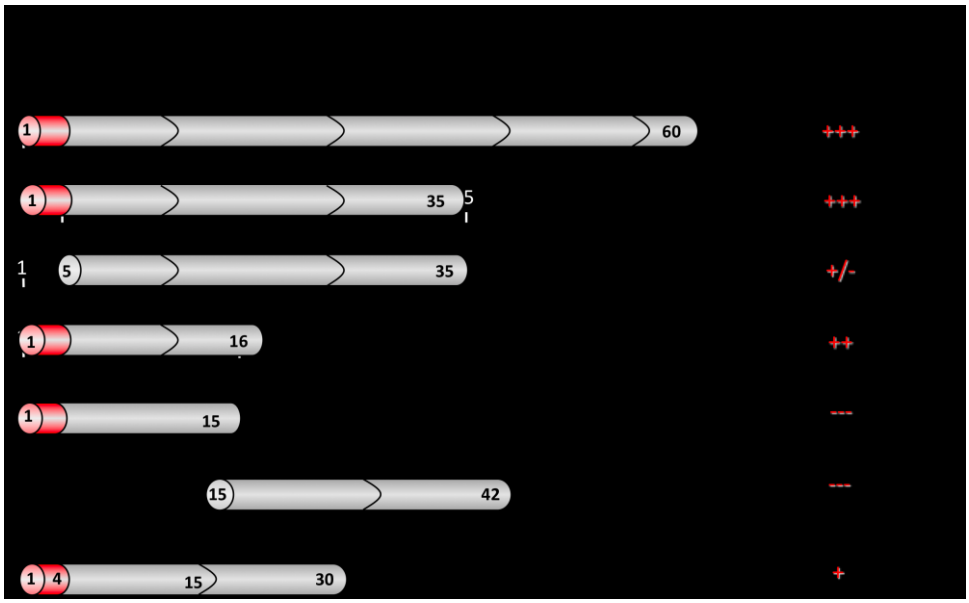


Fig.1.5. SAR studies: antibacterial activity of different Bac7 fragments.

Due to its positive charge, Bac7 interacts electrostatically with the lipid bilayer of the outer membrane of Gram-negative bacteria. After this event, it may inactivate the target cell via two different modes of action, depending on its concentration: *i)* at near-MIC concentrations it acts via a mechanism based on a stereospecific and energy-dependent uptake that is likely followed by its binding to unknown intracellular targets; *ii)* at concentrations several times the MIC value, the peptide acts via a non-stereoselective, membranolytic mechanism common to most of the AMPs (Podda *et al.* 2006).

A putative membrane interactor of the N-terminal Bac7(1-35) active fragment has been described in *E. coli*. This interactor is SbmA, an inner membrane protein responsible for the uptake of the peptide into the bacterial cells. In fact, mutations affecting the *sbmA* gene lead to a partial resistance of *E. coli* and *S. typhimurium* strains to Bac7(1-35) and to other proline-rich peptides (Mattiuzzo *et al.* 2007, Prantig *et al.* 2008).

Bac7 is also able to interact with mammalian cells and to affect cellular functions. It has been demonstrated that Bac7(1-35) is rapidly internalized by cells mainly via macropinocytosis and, in part, via direct translocation across the plasma membrane (Tomasinsig *et al.* 2006) without cytotoxic effects up to a concentration of 50 μM. Once inside the cell, the peptide was detected in the cytoplasm as well as in the nucleoli. The uptake process is energy-dependent and temperature-sensitive and, also in this case, the first four N-terminal amino acids are essential for Bac7(1-35) internalization. Focusing on its biological effects on eukaryotic cells, the peptide stimulates DNA synthesis and proliferation of fibroblasts (Tomasinsig *et al.* 2006).

2. *IN VIVO* THERAPEUTIC POTENTIAL OF AMPs

2.1 *In vivo* applications of AMPs

The spread of antibiotic-resistant bacterial strains is driving researchers and pharma industry to find new treatment options for infective diseases. As a potential new class of antibiotics, AMPs combine several attractive intrinsic properties that make them good candidates for the development of antibacterial drugs, although coupled to weaknesses, such as poor pharmacokinetics, susceptibility to proteases and high production costs. Their broad spectrum of activity and their reduced propensity to select resistant strains are however of particular clinical significance, and this features induced many groups to investigate the *in vivo* efficacy of AMPs.

For example, PR-39, at a dose of 40 mg/kg, significantly improved animal survival when tested in a septic shock mouse model induced by LPS (Mahdani *et al.* 2002, James *et al.* 2003).

The efficacy of the human LL-37 peptide was widely investigated in different animal models of infection. In rat models of Gram-negative sepsis induced by *E. coli*, it significantly decreased the plasma levels of endotoxin and cytokines as well as the bacterial load (Cirioni *et al.* 2006). The positive protective effects of LL-37 and of its mice orthologue CRAMP were also proved when the peptides were endogenously expressed in a mouse model of urinary *E. coli* infection and in human mucosal immunity of the urinary tract (Chromek *et al.* 2006). The efficacy of the human peptide was also demonstrated in a *Candida albicans* skin infection mouse model (Lopez-Garcìa *et al.* 2005). Finally, it was demonstrated that topically added LL-37 strongly accelerated delayed-excision wound closure in mice, further supporting the therapeutic potential of the peptide in the promotion of wound healing in a mouse model of type II diabetes (Carretero *et al.* 2008).

The therapeutic efficacy of Bac7 as an antiendotoxic compound was investigated in a rat model of Gram-negative septic shock. Treatment of the animals with 1 mg/kg of the active fragment Bac7(1-35) resulted in a significant decrease in plasma endotoxin levels and greatly improved survival (Ghiselli *et al.* 2003).

The potent and broad spectrum antimicrobial activity of SMAP-29, an α-helical cathelicidin with a lytic mechanism, and its ability to protect animals from infection led to a conspicuous number of studies aimed at assessing its potential as a novel anti-

infective agent. Clear evidence for host defence capacity of SMAP- 29 was obtained in a lamb pneumonia model of a bovine respiratory disease associated with *Mannheimia haemolytica,* in which intra-tracheal administration of this ovine peptide at the concentration of 37 mg/Kg, significatly reduced the bacterial concentration in the bronchoalveolar fluid and in consolidated pulmonary tissues, as well as the severity of the lung lesions (Brogden *et al*. 2001).

Efficacy studies, in a rat model of staphylococcal device-related infection, were performed using the bovine cathelicidin peptide BMAP-28. Catheters pre-treated with this peptide showed a drastic reduction in their development of bacterial biofilms (Cirioni *et al.* 2006). The therapeutic potential of BMAP-28 and of its inversed (D) and retro-inversed (RI) isomers was also tested in a mouse model of peritoneal infection and each peptide conferred protection at the dose of 20 mg/kg, significantly reducing the bacterial load (Kindrachuk *et al.* 2010).

A promising aspect in improving antimicrobial therapy is the combination of conventional antibiotics with AMPs. To mention a few examples, the application of the frog peptide temporin L (1 mg/kg) from *Rana temporaria*, combined with β-lactams in a murine model of Gram-negative sepsis, led to a lower plasma endotoxin level and to a higher antimicrobial activity , which resulted in an improved survival rate (Giacometti *et al.* 2006); Similarly, the combination of two α-helical AMPs (cecropin A and magainin II) and vancomycin resulted effective against *S. aureus* in a murine sepsis model (Cirioni *et al.* 2006). Numerous clinical trials have been also applied to many AMPs.

2.2 Clinical trials and applications of AMPs

Antimicrobial peptides are currently in clinical use for disease treatment as well as prophylactically, to prevent infections in neutropenic or cystic fibrosis patients (Marr *et al.* 2006). Colomycin, a derivative of polymyxin, is used systemically in intravenous therapy of lung infections in cystic fibrosis patients (Li *et al.* 2006). Topical applications of polymyxins and gramicidin S are safely used in the treatment of infections caused by *P. aeruginosa* and *A. baumannii* and often associated with bacitracin for generic wound creams, eye drops and ear drops (Marr *et al.* 2006).

Currently, the starting point for development of new drugs is the identification of natural antimicrobial peptides followed by their modification and optimization. A few

of these "new generation" AMPs, which differ from their natural progenitor by a limited number residues, are currently undergoing clinical trials. For instance, P-113, a derivative of histatin 5, a human salivary peptide, was reported for HIV patients with oral candidiasis. The benefits of P-113 over current treatments are its safety profile, which has been demonstrated in previously conducted clinical trials, and the reduced risk of drug resistance due to its unique mechanism of action (Helmerhorst 2007). A peptide derived from bovine indolicidin, MX-226, developed by Migenix, is currently under confirmatory Phase III study for the treatment and prevention of catheter-associated infections, under a special protocol assessment with the FDA. The most promising candidate is omiganan, an indolicidin analogue, used in preventing catheter related bloodstream infections and various cutaneous infections (Fritsche *et al.* 2008; Sader *et al.*, 2004). This peptide is now awaiting registration.

Finally, the lantibiotic nisin was developed commercially by Astra and Merck for treatment of gastric helicobacter infections and ulcers, while other nisin variants (nisin-A and Z) have entered preclinical trials for treating vancomycin-resistant enterococci. (Field 2010).

2.3 Improvement of AMPs as therapeutic agents

As observed above, AMPs possess features that make them good candidates for development of antibacterial therapeutics. However, there are issues yet to be solved to pursue therapeutic implementation of the antimicrobial peptides.

One of the main problems related to the limited clinical use of AMPs is their weak *in vivo* stability. Strategies to prolong physiological half-life of such compounds are highly on demand for their clinical use. Short systemic half-life (from few minutes to few hours) of the AMPs is commonly due to fast renal clearance, which is related to the hydrophilic properties of most of these agents as well as to their small size, and/or to enzymatic degradation caused by proteases present in blood, liver and kidney (Reddy *et al.* 2004).

Orally administered drugs, which are absorbed from the stomach or the intestine, are transported in the venous blood via the vena porta through the liver to enter systemic circulation. Parenterally administered drugs have to pass liver and kidney, which are very well supplied with blood. In the kidneys, glomerular ultrafiltration occurs and the overall effect of the filter is to completely pass anything less than 5-7 kDa (Werle *et al.*

2006) and to (almost) completely exclude anything greater than 70 kDa. Less and less of the substance will pass through the filter moving in size between the lower and upper limit. In the case of small molecules such Bac7(1-35) (4.2 kDa), the half-life of the compound can be prolonged increasing their size.

Pegylation, the covalent coupling of polyethylene glycol (PEG) chains to drugs, is one of the most promising and extensively studied strategies with the goal of improving the pharmacokinetic properties of a potentially therapeutic agent with a short half-life (Pasut and Veronese 2006).

The main advantages conferred by PEG conjugation include: (1) enhanced solubility and, as a consequence, an improved drug bioavailability; (2) decreased antigenicity leading to a lower inmmunological response by the organism; (3) reduced rates of kidney clearance and improvement in pharmacokinetics; (4) enhanced selective targeting to the site of action of the drug; (5) the possibility to form an advanced complex drug delivery system, which, in addition to drug and polymer carrier, may include several other active components that enhance the specific activity of the main drug (Khandare *et al.* 2005).

A large number of scientific articles have already been published in which the effectiveness of pegylation to improve half-life of drug candidates was clearly demonstrated (Lee *et al.* 2005; Ramon *et al.* 2005; Kozlowsky *et al.* 2001).

As for stability of AMPs in biological fluids, enzymatic degradation has to be taken into account. A broad variety of peptidases and proteases occur in the blood and in tissues. Aminopeptidases cleave peptides at the N-terminus whereas carboxypeptidases cleave them from the C-terminal site. Endopeptidases often exhibit narrow cleavage specificity (Werle and Bernkop-Schnurch 2006). Methods successfully verified and now routinely used to improve resistance of peptides to proteases involve the modification of their N- and C-termini by acetylation and amidation, respectively, and/or the replacement of amino acids at predicted cleavage sites with other amino acids or non-natural residues (e.g., D-amino acids) (Stromstedt *et al.* 2009).

Protease resistance can also be achieved by “head to tail” cyclization of peptides via bond formation between the N- and C-termini (Rosengreen *et al.* 2004; Dathe *et al.* 2004). This type of modification has been shown to improve the stability of human kallikrein-2-specific peptide in human plasma for up to 24 h compared to the 4 h of the linear form. (Pakkala 2007). Synthetic cyclic peptides not only have favourable *in vitro* and *in vivo* stability profiles, but also show a greater selectivity for bacterial rather than

mammalian cells (Dartois *et al.* 2005; Oren and Shai 2000). A new, promising strategy that confers resistance to proteolysis (Falciani *et al.* 2007; Pini *et al.* 2007; Bracci *et al.* 2003), in addition to decreased cytotoxicity (Kolar *et al.* 2010) and increased antimicrobial activity (Liu *et al.* 2006), is also represented by the synthesis of peptides in the form of dendrimers.

Finally, also in this context, pegylation is an efficient strategy to improve peptide stability, especially in the case of multi-branched PEG use, where the PEG chains are able to "hide" the peptide sequence to the activity of proteases (Veronese and Mero 2008).

A strategy to improve the availability of AMPs is their conjugation to an efficient slow release drug delivery system, which may reduce the need for extensive modifications. Liposomal systems offer a number of benefits including increased circulation time of the encapsulated therapeutic. These systems have been widely investigated for delivery of anticancer drugs, while the liposome encapsulation of peptides is still under development. Cationic, cell-penetrating peptides have also been extensively studied for the delivery of molecules to intracellular compartments (Vives *et al.* 2008). However, despite being able to deliver a variety of molecule types *in vitro,* their *in vivo* application requires further development. Recently, the use of microspheres composed of polymers that allow controlled release of their cargo at specific physiological conditions (e.g., temperature and pH), led to promising results (Fogueri and Singh 2009).

In summary, physical-chemical modifications are likely required to widen the pharmacokinetic properties of an AMP to make it more effective *in vivo*. Due to the increased interest of researchers in this field, the therapeutic application of AMPs is likely to expand to include novel peptides and peptidomimetics and a wider range of infections.

3. *SALMONELLA ENTERICA* INFECTION

Salmonella enterica is a facultative intracellular Gram-negative species that causes a wide array of diseases in a multitude of hosts. It is a facultative anaerobic bacterium that belongs to the family of Enterobacteriaceae and is made up of nonspore-forming rods, usually motile with peritrichous flagella (Warrell *et al.* 2003).

The genus *Salmonella* contains over 2,000 sero-species and is one of the most important

pathogens in the Enterobacteriaceae family. Taxonomically, all strains of *Salmonella* fall within one species, *S. enterica.* The Typhimurium is among the most common *Salmonella* serovars causing salmonellosis in humans. In mice, *S. typhimurium* causes a systemic disease and provides a murine model for human typhoid fever that allowed investigating the various aspects of host-pathogen interaction in *S. enterica* infection (Santos *et al.* 2003). Typhoid fever disease is an important example of a severe systemic disease responsible for approximately 21 million cases per year worldwide (Crump *et al*. 2004). Typhoid fever disease occurs when *Salmonella* escapes the host immune defence and disseminates in different organs (Watson *el al.* 2010). In fact, as a facultative intracellular pathogen, *Salmonella enterica* serovar Typhimurium is able to invade and replicate inside a variety of phagocytic and non-phagocytic cells *in vivo*. Following intestinal colonization, salmonellae enter enterocytes, M cells and dendritic cells (DCs) in the intestinal epithelium. Afterwards, they reach the submucosa and can be internalized by resident macrophages and rapidly disseminate through the blood stream, accumulating in mesenteric lymph nodes and finally in liver and spleen (Salcedo *et al.* 2001) (Fig. 3.1).

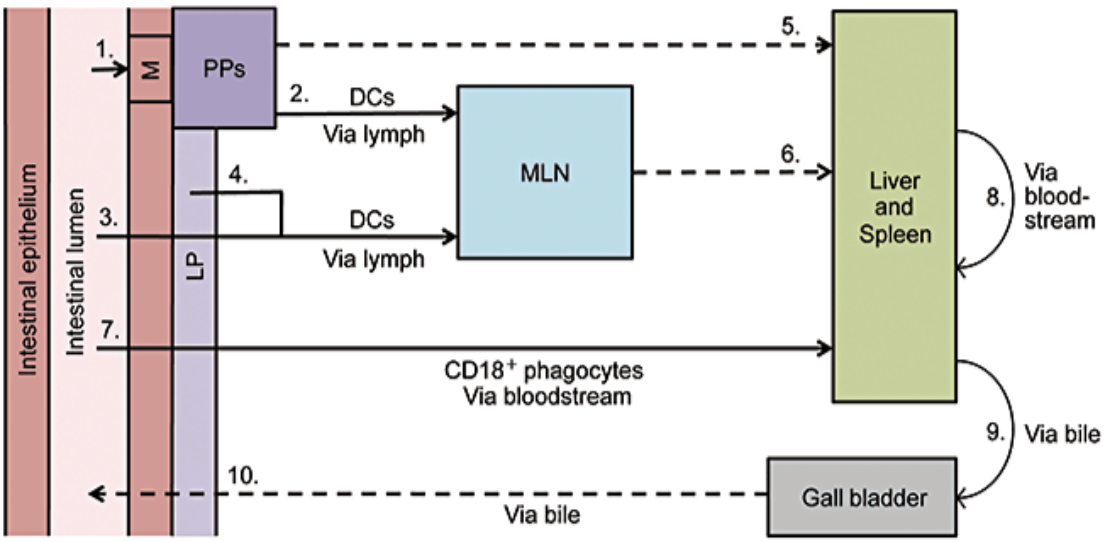


Fig. 3.1. Dissemination of *Salmonella* from the intestinal lumen to extra-intestinal sites in mouse.

(1) After reaching the distal ileum, the majority of *Salmonella*e invade M cells that overlie Peyer's patches (PPs). (2–4) Most bacteria are likely to reach the mesenteric lymph nodes (MLN) by carriage within DCs via the lymphatic system. DCs transport *Salmonella* from the PPs (2), intestinal lumen (3) or lamina propria (LP) (4). (5) It is possible that *Salmonella* can be transported directly from the PPs to the liver and spleen, but large numbers of bacteria are

unlikely to migrate out of the MLN (6). (7) *Salmonella* can also be carried directly from the intestine to the liver and spleen via a hematogenous route involving carriage within $CD18^+$ phagocytes, most likely monocytes or DCs. (8) After prolonged infection, *Salmonella* can be transported between organs via the bloodstream. (9) Bile excretion from the liver also carries bacteria to the gall bladder. (10) Gall bladder colonization presumably leads to re-infection of the intestine through bile secretion (Watson *et al.* 2010).

The ability of salmonellae to survive in a variety of host cells is essential to its virulence and depends, at least in part, on mechanisms of resistance to AMPs. Genetic studies have led to the identification of a large number of genes necessary for intracellular survival, and among them, the phoP/Q genes have great relevance. They encode a two-component regulatory system, which regulates the expression of over 40 genes (Miller and Mekalanos 1990) and are also involved in resistance to AMPs (Gunn and Miller 1996). PhoQ is an inner membrane sensor kinase with a periplasmic domain that is responsible for sensing specific signals in the environment, and a cytoplasmic domain responsible for intermolecular autophosphorilation upon activation and subsequent phoshotransfer to PhoP. PhoP is a regulator protein involved in the activation of the expression of important virulence genes. Most of them are clustered at specific sites of the *Salmonella* chromosome called pathogenicity islands (SPI). Two SPIs are important for virulence of *Salmonella*, SPI-1 and SPI-2. Both encode two different type III secretion systems (T3SSs), needle-like structures on the bacterial surface. T3SSs are used by many Gram-negative pathogens to deliver bacterial effector proteins into host cells (Hueck 1998) through formation of pores in host cell membranes (Blocker *et al.* 1999). The T3SS encoded in *Salmonella* SPI-1 mediates the internalization of *Salmonella* into host cells by translocating inside them effector factors that induce membrane ruffling and cytoskeleton rearrangement in order to force *Salmonella* internalization (Schaible *et al.* 2010; Ibarra *et al.* 2009).

Inside eukaryotic cells, T3SS1 effectors are implicated in the biogenesis of *Salmonella*-containing vacuoles (SCVs), niches in which *Salmonella* replicates and protects itself from host cell antimicrobial activities (Buchmeier *et al.* 1991; Rathman *et al.* 1997; Vazquez-Torres *et al.* 2000) (Fig. 3.2). After the early stages of infection, the SPI-1 system is down-regulated (Drecktrah *et al.* 2005; Ericksson *et al.* 2003), and the expression of the SPI-2 T3SS is induced, mainly due to the acidic conditions present inside the SCVs (Coombes *et al.* 2004). The effector proteins translocated by the T3SS-2 system are necessary to continue modification of the SCV, allow intracellular

bacterial growth and finally induce host cells to apoptosis (Monack *et al.* 1996; Hersch *et al.* 1999). SPI-2, with its T3SS-2, is especially involved in *Salmonella* survival into macrophages. In this scenario, bacteria are chosen by a professional phagocyte which is duly programmed to eliminate them. Under this condition, SPI-2 appears to prevent the phagocyte NADPH oxidase from trafficking toward SCVs, both reducing the oxidant stress encountered by bacterial cells and potentially enhancing collateral oxidative damage to host tissues (Vazques-Torres *et al.* 2000).

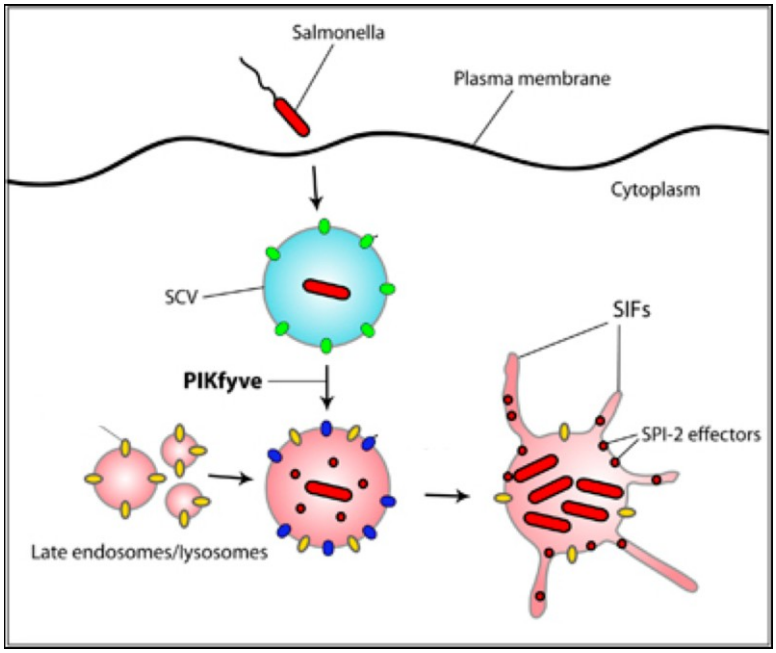


Fig. 3.2. *Salmonella* internalization into host cells. Once internalized, *Salmonella* cells are located in vacuoles (SCV). As a consequence, the SCV becomes fusogenic with lysosomal/late endosomal compartments. This fusion allows acidification, SPI-2 effector induction and *Salmonella*-induced filament (SIF) formation. The result is an appropriate niche for *Salmonella* replication (Gutierrez *et al.* 2010).

In addition to its ability to invade and replicate inside host cells, a large subpopulation of intracellular *Salmonellae* in splenic macrophages was surprisingly found to persist for long periods of time without undergoing a single division. Further characterization of these bacterial cells revealed a gradual loss in metabolic activity that appeared to be triggered soon after uptake by macrophages and was not influenced by the bactericidal activities of the host cell. This suggested entry into a dormant state; it is possible that these bacteria represent a reservoir that persists in chronic infections. Other potential reservoirs have also been proposed, such as the mesenteric lymph nodes (MLN) (Monack *et al.* 2004), the liver (Nath *et al.* 2010) and the gall bladder (Sinnott *et al.* 1987), perhaps by forming biofilms on existing gallstones (Crawford *et al.* 2010).

Aim of the PhD project

The rapid increase in the number of bacterial strains resistant to conventional antibiotics is a serious threat to public health. For this reason, the development of novel antibiotics with a mechanism of action different from that of the antibiotics in use is in high demand by clinicians. In this respect, AMPs are increasingly recognized as potential candidates for the development of new classes of antimicrobial drugs.

They combine several attractive intrinsic properties, such as direct killing of invading pathogens, modulation of the immune response and, last but not least, a poor propensity to induce microbial resistance. In addition, successful preclinical studies with AMPs indicate that clinical evaluation of these agents is worth doing.

The encouraging *in vitro* results already obtained with Bac7(1-35), a fragment of the bovine cathelicidin Bac7, its activity against Gram-negative bacteria of clinical relevance, its peculiar mode of action, and its low toxic effects, suggested us to test its *in vivo* therapeutic efficacy, to ascertain its potential as a lead compound for the development of new antimicrobial agents.

In this context, the experiments carried out for this PhD thesis aimed at extending the preclinical studies thus far performed with the peptide and exploiting its therapeutic potential. In short, the studies performed had the scope to test:

i) the peptide's activity and stability *in vitro* in the presence of biological components that mimic the *in vivo* environment; both murine and human serum and plasma were used so as to compare the potential of Bac7(1-35) in different organisms;

ii) the *in vivo* efficacy of the peptide in a murine model of infection: a mouse model of *S. enterica* infection resembling typhoid fever in humans was developed to investigate the *in vivo* activity of Bac7(1-35);

iii) modifications of the peptide aimed at improving its pharmacokinetic properties; to this aim, several different pegylation strategies have been set up to optimize Bac7(1-35) bioavailability;

iv) the effects of the peptide on cells of the human immune system involved in *S. enterica* infection, such as macrophages; these studies were designed to investigate the effects of Bac7(1-35) on the phagocytosis process and, once extracellularly added, its antimicrobial activity against pathogens that proliferate inside the macrophages, such as *S. enterica*.

Materials and Methods

1. SYNTHESIS AND LABELING of BAC7(1-35)

1.1 Synthesis and purification of Bac7(1-35)

The N-terminal fragment 1-35 of Bac7 was synthesized by the solid phase on a CEM Liberty microwave-assisted peptide synthesizer using a 2-Chlorotrityl chloride resin (substitution 0.20-0.25 meq/g). Loading of the first amino acid was carried out manually.

On synthesis completion, the peptide was cleaved from the resin by incubation for 4 h on a shaker in the following mixture: 87% trifluoroacetic acid (TFA), 8% 3,6-dioxa-1,8-octane-dithiol (DODT), 2% triisopropylsilane (TIPS), 3% water. The cleaved peptide was washed several times with *tert*-butyl methyl ether (TMBE) and dried with N_2.

The crude peptide was then purified using a preparative C18 RP-HPLC column (Waters Delta-Pak^{TM} C18, 5 μm, 300 Å, 25 x 100 mm). The quality of the peptide was checked by mass spectrometry (ESI-MS Bruker Esquire 4000) after purification.

All the peptide solutions used in the assays described below were prepared from a stock solution of the purified peptide dissolved in water. The peptide concentration was determined by at least three methods:

I. by weighing the peptide used to prepare the solution;
II. by using the Waddell method (Waddell 1956);
III. by considering the excinction coefficients (ε_{215}) of Gly and Pro residues and of the peptide bonds (Kuipers and Gruppen 2007).

1.2 Fluorescent labelling of Bac7(1-35)

In order to fluorescently-label Bac7(1-35) via linkage of the thiol-reactive dye, ALEXA FLUOR® 680 C_2maleimide or BODIPY C_2maleimide, a C-terminal cysteine residue was specifically added. Labelling of Bac7(1-35) was performed in solution with the purified peptide and not directly on resin because the dye is not stable under the peptide's cleavage conditions (acidic pH of the trifluoroacetic acid solution).

To label Bac7(1-35) with the ALEXA FLUOR 680, 1 mg of the fluorescent dye, was dissolved in 100 μl of DMSO, and added dropwise to 30 ml of 10 mM Na-phosphate buffer, pH 7, under nitrogen bubbling in the dark. Four milligrams of Bac7(1-35)-Cys were then dissolved in this solution; after 1 h incubation at room temperature with stirring, a new 4 mg aliquot of peptide was added and the solution was left overnight at 4°C. At the end of the incubation time, an excess of cysteine (10 mg) was added to

scavenge the excess of thiol-reactive reagent. The solution was left with stirring for 1-2 h and the labelled peptide was then purified with semi-preparative RP-HPLC (Waters Delta-Pak^{TM} C18, 5 μm, 10 x 100 mm) and analyzed by ESI-MS. The fraction containing the pure Bac7(1-35)-Cys_{36}ALEXA FLUOR® 680 was lyophilized and the concentration determined using the above mentioned methods. To label Bac7(1-35) with BODIPY C_2 maleimide, a 20 ml peptide solution (0.1 mg/ml in 30% AcCN in 10 mM Na-phosphate buffer, pH 8) was used with a 10-fold excess of the fluorescent dye (2mg in 2 ml AcCN) added drop wise under nitrogen bubbling in the dark. After 2-3 h of incubation at room temperature under stirring, a new aliquot of 2 mg peptide was added to the same solution and incubated overnight in the dark at 4 °C under stirring. The reaction was quenched by addition of a 10 to 20-fold excess of cysteine an incubated for 1 h at room temperature under stirring. The labelled peptide was then purified by using a semi-preparative RP-HPLC (see above), analyzed by ESI-MS and lyophilized. Its concentration was determined using the above mentioned methods.

2. Bacterial strains

Salmonella enterica serovar Typhimurium ATCC 14028 was used for the antibacterial assays. *Salmonella enterica* serovar Typhimurium LT-2 and GFP-expressing *S. typhimurium* (strain LT-2) were used for the phagocytosis assays.

2.1 Preparation of GFP-expressing *S. typhimurium*

GFP-expressing bacteria were obtained by transforming *S. typhimurium* LT-2 with the pGLO plasmid carrying the inducible *gfp* gene under control of the arabinose operon and the antibiotic resistance for ampicillin. Competent cells of *S. typhimurium* were firstly prepared. In brief, a standard overnight bacterial culture in MH broth was prepared and refreshed the day after in 200 ml of medium, up to an OD_{600} of 0.5-0.7. The culture was transferred into four 50 ml tubes, cooled down on ice for 30 min and centrifuged at 1800 x g and 4°C for 15 min. After the medium was discarded, the pellet of bacteria was washed twice in 25 ml of sterile water and collected by centrifugation. The cells were resuspended in 10 ml of sterile 10% glycerol and centrifuged. Finally, the bacteria were resuspended in 1 ml of sterile 10% glycerol, distributed into several 1.5 ml tubes and stored at -80°C.

The percentage of competent cells was verified with a test transformation with the pUC18 plasmid following standard methods.

To obtain GFP-expressing bacteria, competent cells were mixed with 100 μg of pGLO plasmid and exposed to an electric discharge (2.5 kV, 5 ms). Immediately afterwards, 950 μl of SOC medium (20 g of bacto-tryptone, 5 g of yeast extract, 10 mM $MgCl_2$ and 20 mM glucose dissolved in water), and 0.5 g NaCl were added and the cell suspension was incubated at 37°C for 1 h. Finally, the cells were diluted and plated on MH agar with ampicillin (50 μg/ml). Colonies were collected and prepared for storage at -80°C by adding 140 μl of DMSO to 1.5 ml of overnight culture in MH supplemented with ampicillin (50 μg/ml).

3. Bacterial growth conditions

Mid-logarithmic phase *S. typhimurium* ATCC 14028 and *S. typhimurium* LT-2 cells were obtained by diluting 1:50 an overnight-grown culture, followed by incubation at 37°C with shaking up to an OD_{600} of 0,3-0,4 and diluting the suspension at the desired concentration.

GFP-expressing *Salmonella* were grown in MH broth supplemented with ampicillin (50 μg/ml) at 37°C. After overnight incubation, an aliquot of the culture was diluted in fresh medium supplemented with ampicillin (50 μg/ml) and the cells left to grow for 90 min at 37°C. Arabinose (3 mg/ml) was then added to switch on the *gfp* gene expression and the culture incubated for 2 additional hours at 37°C. At the end of the incubation time, the GFP-expressing *S. typhimurium* cells were washed and resuspended in PBS at the desired density. Expression of GFP was verified cytofluorimetrically.

4. ANTIBACTERIAL ACTIVITY OF BAC7(1-35) IN SERUM and PLASMA

4.1 Antibacterial activity of Bac7(1-35) in human and murine serum and plasma

Killing kinetics assays

The antimicrobial activity of Bac7(1-35) was first determined by performing killing kinetics assays against *Salmonella enterica* serovar Typhimurium ATCC 14028 in the

presence of 66% murine serum or plasma.

Murine serum and plasma were drawn from a pool of mice. Plasma was obtained using 2% (v/v) Na-citrate as an anticoagulant. Fluid samples were then centrifuged at 13000 g for 5 min and stored at -20°C until use.

Mid-logarithmic phase *S. enterica* cultures were diluted in murine serum or plasma (66% or 25% v/v final concentration) to give approximately $1x10^6$ cells/ml, and incubated with 10 μM Bac7(1-35) at 37 °C in a shaking water bath. Samples were withdrawn at different times, diluted, plated on MH agar and incubated at 37°C for 16-18 h to allow colony counts (Podda *et al.* 2006).

Broth microdilution susceptibility assays

Minimum inhibitory concentration (MIC) values of Bac7(1-35) against *S. typhimurium* ATCC 14028 in the presence of 25% human and murine serum or plasma were also determined.

Murine serum and plasma were prepared and stored as described above.

Human serum and plasma were drawn from a pool of healthy donors. Plasma was obtained using 2% (v/v) Na-citrate as an anticoagulant. Fluid samples were then centrifuged at 13000 g for 5 min and stored at -20°C until use.

The broth microdilution susceptibility assay was performed following the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2000).

Two-fold serial dilutions of Bac7(1-35) were prepared in 96-well microtitre plates under different medium conditions (human and murine serum or plasma diluted in MH broth to reach the final concentration of 25% and 100% MH broth as control) in a final volume of 50 μl. Each series included a well without peptide as a control. A total of 50 μl of a mid-logarithmic phase *S. enterica* culture prepared in MH broth was added to each well to a final density of 5×10^4 cells/ml. Samples were then incubated at 37°C for 24 h. The MIC was taken as the lowest concentration of antimicrobial peptide resulting in the complete inhibition of visible growth after 24 h of incubation.

4.2 Peptide stability in serum and plasma

Western blot analysis

To test the peptide stability in human and murine biological fluids, Bac7(1-35) (120 μg) was incubated in 200 μl of murine or human serum and plasma [25% (v/v) in PBS] at 37°C up to, respectively,24 h and 72 h. At different times, aliquots were withdrawn,

diluted 1:5 in sample buffer (12% SDS, 6% dithiothreitol, 40% glycerol, 0.05% bromophenol blue, 150 mM Tris-HCl, pH 7), incubated for 15 min at 60°C and analyzed on a 16% Tricine/SDS gel. Proteins were then blotted onto nitrocellulose membrane and incubated overnight with shaking in 40 mM Tris-HCl, pH 7.5, 5% non-fat milk, 0.05% Tween 20, 200 mM NaCl (blocking solution) at 4°C. Samples were incubated for 90 min with rabbit anti-Bac7(1-35) IgG diluted 1:1000 in blocking solution, followed by a HRP-conjugated anti-rabbit IgG. The ECL detection system was used to develop the Western blots.

LC-MS analysis

Bac7(1-35) was incubated in 250 μl of human or murine serum and murine plasma [25% (v/v) in PBS] at 37°C for 24 h. Aliquots of 25 μl (5 μg of peptide) were withdrawn at different time intervals (0, 1, 4, 8, and 24 h) and added to 65 μl of cold TFA (0.5% v/v) in H_2O, kept on ice for 5 min and then centrifuged at 10000 x g for 5 min. The supernatants were analysed by using an LC-ESI/MS instrument in which an Amersham Pharmacia Biotech HPLC is coupled to an ESQUIRE 4000 spectrometer (Bruker Daltonics). Components in each sample were separated by using a Jupiter C18 analytical column [5 μm, 300 Å, 2 x 150 mm (Phenomenex, USA)] with a 30 min linear gradient from 25 to 45% of water/acetonitrile in 0.05% TFA. The percentage of the intact peptide was calculated from the area of the corresponding peak in the chromatogram (% of intact peptide = A_t/A_0 x 100, where A_t is the pea k area of the peptide at any given time and A_0 is the peak area at time zero) (Antcheva *et al.* 2009).

5. *IN VIVO* STUDIES

5.1 Animals

Male Balb/c and CBA/Ca mice of approximately 20 g and 6 weeks of age were obtained from Harlan Laboratories (Udine, Italy) and maintained under pathogen-free-conditions. All the experimental procedures were performed according to the guidelines of the European (86/609/EEC) and the Italian (D.L. 116/92 and subsequent addenda) laws and approved by the Italian Ministry of University and Research as well as by the Animal Experimentation Committee of the University of Trieste Animal House.

5.2 *In vivo* toxicity

To test the toxicity of Bac7(1-35), increasing amounts of peptide dissolved in apyrogen PBS (0.2 ml per mouse) were injected in mice via i.p. The control animals received the vehicle alone. Animal behaviour and survival were monitored over a 14-day period. During this period mice were observed daily for signs of toxicity: their weight and the aspect of the hair were controlled.

5.3 *Salmonella* infection and protective effect of Bac7(1-35)

Inocula containing 10^2 CFU/mouse of *S. enterica* ATCC 14028, expected to result in 90-100% mortality in 4-6 days for Balb/c (Campoy *et al.* 2002) and 10-18 days for CBA/Ca, were prepared by diluting log-phase bacterial cultures in sterile PBS. Ten mice were infected intraperitoneally and monitored for survival over a 60-day period after infection. The peptide (30 mg/kg) was injected via i.p. after bacterial challenge. The choice of dose was based on preliminary data obtained with lower doses (data not shown) and on results reported in the literature for AMPs belonging to other structural groups. Control mice were given 0.2 ml of PBS or gentamicin (10 mg/kg). The experiment was repeated two times and comparable results were obtained. The analysis of survival curves was conducted by using the Kaplan-Meyer method and subsequent statistical evaluation by the Logrank test. Significance of percentage differences among groups was calculated by using the Fisher exact test. Values of $p < 0.05$ were considered statistically significant.

5.4 Viable colony counts in murine liver and spleen homogenates after *Salmonella* infection and treatment with Bac7(1-35)

Three days after bacterial infection, a group of 3 untreated and 3 peptide-treated mice were killed by cervical dislocation, and the liver and spleen were removed. The organs were weighed, homogenized separately and dissolved in PBS. Suitable dilutions of 50 μl of the homogenate in PBS were plated in duplicate on MH agar. The plates were then incubated at 37°C overnight to allow colony counts. Results are expressed as number of CFU/g of organ. This assay was repeated twice.

5.5 *In vivo* time-Domain Optical Imaging and Bac7(1-35) biodistribution.

Biodistribution of Bac7(1-35) in living mice was analyzed by using the *in vivo* imaging

technology.

5.5.1 Optical imaging technique

Optical imaging is a non invasive diagnostic technology that allows visualization of molecular events in real-time and over time in living animals. It is increasingly used as a leading technology in bio-pharmaceutical research and development, both in the preclinical and clinical phases of research and drug development process. Traditional survival-based endpoints are fast going out of fashion as a way of measuring drug efficacy, in favour of *in vivo* imaging. Non-invasive mapping of cellular and molecular events *in vivo* drastically enhances the quality and quantity of information available to researchers, accelerating drug discovery and development and the path towards ever improving precocious diagnosis. This new attractive technology has been also approved by FDA to track drug "microdoses" in preclinical studies in order to allow pharmaceutical companies to enter into phase I faster. This technique is based on detecting the fluorescent lifetime of the fluorophore bound to the target molecule (Fig. 1).

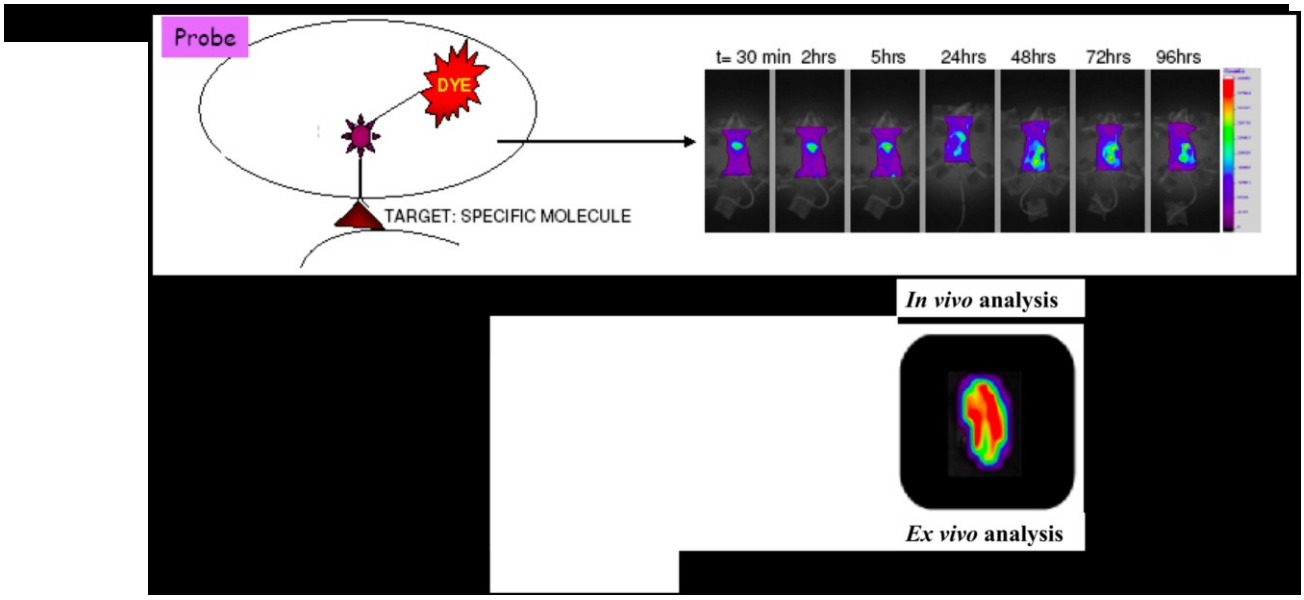


Fig. 1. **Schematic representation of the Optical imaging technique, and example of analysis.** This technique acquires the fluorescent signal of the dye used to target the molecule of interest once inside a living mouse, giving information about its biodistribution and half-life.

The lifetime is the average value of time spent by the dye in the excited state before

returning to the ground state and emitting a fluorescent photon (Gallant *et al.* 2004; Zhang *et al.* 2001). The instrument works in the Near Infrared (NIR) because of its good tissue penetration (~1 cm), low absorbance and low background signal. The time domain technology also allows studying several biological events at the same time, detecting multiple fluorophores simultaneously by their lifetimes. Moreover, it is able to discriminate bound and unbound dyes by comparing their different fluorescence lifetime characteristics .

The benefits of the fluorescence lifetime imaging system are:

- the estimation of the relative concentration of the target molecule based on detection of the light intensity as a function of arrival time in nanoseconds, considering that the signal from deeper tissues arrives later;
- the recognition of different environments in which the fluorophore could be found and determination of the location of the source (Truong *et al.* 2001).

5.5.2 Bac7(1-35) biodistribution in mice

The day before the treatment, healthy CBA mice were anesthetized by an intramuscular injection of a diluted mixture (1:5 in PBS) composed by 0.4 ml Zoletil 100 and 0.25 ml Rompun 2% (3 μl/g body weight), and shaved in the regions of interest to avoid laser scattering caused by hair. The following day, mice were anesthetized using a gaseous anaesthesia chamber and the animals were then placed inside an eXplore Optix instrument in the presence of 1% isoflurane. Two mice were then injected intraperitoneally with 36.6 μg/mouse of Bac7(1-35)-Alexa 680, corresponding to 6.9 nmol ALEXA FLUOR©680. One mouse was monitored in the abdominal region and the other in the renal region for 24 hours. A blank image was acquired before treatment of each animal and this was subtracted to the images of the treated animal. The experiment was repeated twice. The small-animal time-domain eXplore Optix preclinical imager (GE Healthcare) was used in this study. In all imaging experiments, a 670 nm pulsed laser diode with a repetition frequency of 80 MHz and a time resolution of 12 ps light pulse was used for excitation. The fluorescence emission at 700 nm was collected and detected through a fast photomultiplier tube and a highly sensitive time-correlated single-photon counting system. Two-dimensional scanning regions of interest (ROI) were selected and the laser power, integration time and scan step were optimized according to the signal emitted. The data were recorded as temporal point-spread functions, and the images were reconstructed as fluorescence intensity and lifetime.

6. PEGYLATION STRATEGIES

6.1 Pegylation of Bac7(1-35) via amide bond

Metoxy-PEG20K-NH_2 was used to pegylate the Carboxy terminal of Fmoc-Bac7(1-35). Fmoc-Bac7(1-35) (8.8 mg) dissolved in 500 μl of DMF was added to 200 mg Metoxy-PEG-NH_2 dissolved in 1 ml of DCM (1:5 ratio of Bac7:PEG) in the presence of 0.38 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxybenzotriazole (HOBt), and triethylamine (TEA) added dropwise. The reaction was left to occur at pH 8, under nitrogen stream at 50°C for 4 h and carried on overnight at room temperature. At the end of the incubation time, the mixture was precipitated by using an ether solution followed by heated ethanol. The mixture was filtered, dried and then separated by RP-HPLC using a strong cation exchange column (HITRAP SP, Waters Delta-Pak^{TM} C4, 5 μm, 2x150 mm).

6.2 Pegylation of Bac7(1-35) via ester bond using a Glycine residue as linker

Metoxy-PEG20K-OC(O)-Gly-NH_2 was prepared as described (Ballico *et al.* 2005). Briefly, the metoxy-PEG20K-OH was dissolved in 1 ml of DCM with the addition of Boc-Glycine-OH (8 equimol) and 4-Dimethylaminopyridine (DMAP) (10 equimol). DIPEA was then added dropwise and the reaction was left to proceed overnight at room temperature and under stirring. The mPEG-Gly-Boc obtained was precipitated by using ethyl ether and the Boc-group was then removed by dissolving the compound in 10 ml of a TFA/DCM (1;1 v/v) solution. After 2 h of incubation, the mixture was precipitated with ethyl ether and dried under vacuum. The purity and the identity of the m-PEG20K-OC(O)-Gly-NH_2 obtained were then determined by H-NMR analysis (NMR 400 MHz). *m*-PEG20K-OC(O)-Gly-NH_2 (120 mg) was then used to pegylate 5 mg of Fmoc-Bac7(1-35) (1:5 ratio of Bac7 to PEG) at the C-terminus by following the same procedure described in section 6.1 of Materials and Methods. At the end of the incubation time, the mixture was precipitated by using ether followed by heated ethanol, filtered, dried and then separated by RP-HPLC using a strong cation exchange column (HITRAP SP, Waters Delta-Pak^{TM} C4, 5 μm, 2x150 mm).

6.3 Pegylation of Bac7(1-35)-Cys via thioether ligation

Metoxy-PEG20K-OC(O)CH_2Br was prepared by dissolving 2 g mPEG20K-OH in 10 ml of dry DCM and by adding 200 mg $BrCH_2COBr$ (10:1 ratio) dissolved in 2 ml DCM, under N_2 at -20°C. DIPEA was then added dropwise. After 1 h of incubation at room temperature, the solution was directly passed through a SiO_2 Column (10 x 10 mm) and washed with 10 ml of DCM. Isopropyl alcohol (15 ml) was then added and the DCM was evaporated under vacuum at 30 °C. The recovered compound was precipitated with ethanol, cooled in ice bath for 30 min, filtered, washed with ethanol and dried under vacuum. The identity and the purity of the product were determined by RP-HPLC/ELSD (Evaporative Light Scattering detector) detection (Phenomenex Jupiter C4, 4.6 x 150 mm) and ^{1}H-NMR analysis (NMR 400 MHz,), respectively.

Pegylation was performed by adding $BrCH_2COOPEG$-OMe to H-Bac7(1-35)-Cys(H)-OH. Before use, the peptide (5 mg) was reduced to remove oxidized Cys-Cys dimers. To this aim, it was treated for 30 min with 5 µl of a 62 mg/ml solution of tris(2-carboxyethyl)phosphine-HCl (TCEP.HCl) in 145 µl of phosphate buffer (0.3 M) at pH 7.6, and mixing from time to time. $BrCH_2COOPEG$-OMe (23 mg) was then added to the solution. After 3 h of incubation at room temperature, the pH was adjusted to ~3 with 50% TFA to quench the reaction, and the product was then separated by RP-HPLC by using a Jupiter C4 column Jupiter, Phenomenex.

6.4 Tricine SDS-PAGE to detect pegylation efficiency

Tricine SDS-PAGE was performed to verify the efficiency of the pegylation reaction and to identify the fractions containing the pegylated peptide after the purification procedure.

According to the protocol described by Shagger (2006), samples, mixed with SDS-containing reducing sample buffer, were loaded onto the 4% stacking gel by applying a potential difference of 50 V and then resolved in the 16% acrylamide running gel at 100 V. To visualize the peptide and the band corresponding to PEG, the gel was respectively stained with Coomassie Blue and with $BaCl_2$ and Iodine solution (Morpurgo and Veronese, 2004). Both staining procedures were done on the same gel using the Coomassie staining first.

6.5 Determination of the concentration of the pegylated peptide

The concentration of the pegylated peptide was determined by three methods:

I. by weighing the pegylated peptide after lyophilization
II. by using the Waddell method
III. by analytical RP-HPLC using an internal standard.

6.6 Detection of Bac7(1-35)-Cys-CH_2COOH release from mPEG-OH in human fluids

To investigate the kinetic of release of the peptide in blood components by proteolytic cleavage of the ester bond between the mPEG-OH and the CH_2COOH group, western blot analyses of the Bac7(1-35)-Cys-$CH_2CO(O)$-PEGm incubated at different times in 25% of human serum or plasma were performed. Bac7(1-35)-Cys-$CH_2CO(O)$-PEGm (172 μg) was incubated at 37°C in 150 μl of PBS containing 25% (v/v) human serum or plasma. At different times, aliquots were withdrawn, diluted 1:5 in sample buffer, treated and then analysed as described in section 4.2 (Western blot analyses) of the current chapter.

6.7 Antibacterial activity of the pegylated peptides

To investigate the antibacterial activity of the Bac7(1-35) peptide pegylated via amide bond and thioether ligation, the broth microdilution susceptibility assay was perfomed in the presence of 25% of human serum or plasma as described in section 4.1.

6.8 Biodistribution of the pegylated Bac7(1-35)-CAM-PEG

BAC7(1-35)-Cys(Alexa Fluor® 680 C2-maleimide-)-CO-OPEG-OMe was prepared by native chemical ligation between Bac7(1-35)-COSPhNHAc and H-Cys(H)-COOPEG-OMe, followed by thioether ligation with Alexa Fluor® 680 C2-maleimide.

6.8.1 Synthesis of Bac7(1-35)-Cys-CO-OPEG-Ometoxy

Bac7(1-35)-SPhNHAc was firstly prepared. In brief, Boc-Bac7(1-35)-OH (212.5 mg) and HS-Ph-NHAc, (2.25 mg) were dissolved in 1 ml of DCM/DMF (1:1, v/v), followed by addition of 31.2 mg of DIEA. The solution was cooled in an ice bath for 15 min and then 62 mg of PyBop were added. After mixing for 10 min at 4°C and for 35 min at room temperature, the DCM was evaporated under a stream of N_2. To cooled in ice bath

and vigorously stirred reaction mixture of Boc-Bac7(1-35)-SPhNHAc were added 9 ml Ethanol/Petroleum ether (1:2, v/v). The oil formed was separated by centrifugation, washed with Ethanol/Petroleum ether (1:2, v/v) and dried under vacuum. Bac7(1-35)-SPhNHAc was then purified by preparative RPHPLC (RCM C18 100 x 40 mm).

H-Cys-CO-OPEG-Ometoxy was then prepared by dissolving 585 mg of Boc-Cys(Trt)-OH in 1.4 ml of dry DMF and 0,1 ml of dry pyridine and by adding 2 g of 20 kD mPEG-OH dissolved in 8 ml of dry DCM and shaken overnight. The product was precipitated and washed with ethanol. After drying, it was redissolved in a mixture of 5 ml DCM and 15 ml of isopropanol, precipitated in Ethanol and cooled for crystalization. The crystals formed were filtered, washed with Ethanol and dried in vacuo. The identity of the product was determined by RP-HPLC/ELSD detection (Phenomenex Jupiter C4, 4.6 x 150 mm). Boc-Cys(Trt)-CO-OPEG-Ometoxy was then deprotected by dissolving it in 0,2 ml of DCM/(TFA/H_2O/TIPS 90:5:5 v/v). After 1 h the solvent was evaporated under a stream of N_2, the rest was mixed with Ethanol, precipitated and separated by centrifugation.

To obtain Bac7(1-35)-Cys(H)-COOPEGOmetoxy, native chemical ligation of Bac7(1-35)-SPhNHAc and H-Cys(H)-COOPEGOmetoxy occurred. H-Cys(H)-COOPEG-Ometoxy (80 mg) was dissolved in 1 M phosphate buffer-EDTA pH 7.3 containing 0.05 mg TCEP.HCl and the solution was flushed with N_2; H-Bac7(1-35)-SPhNHAc (10 mg) was then added and the reaction was left to occur. After 28 h of incubation at room temperature, the reaction was quenched by adding cysteine (2.4 mg) and TCEP (60 μg) and mixed for addition 2 h. Then the solution was diluted with 20 ml water, acidified to pH 3.5 with 10% H_3PO_4 and applied for purification by IE chromatography and RP-HPLC/ELSD (Phenomenex Jupiter C4, 4.6 x 150 mm).

6.8.2 Labelling of the pegylated peptide

Alexa Fluor®680 C_2-maleimide was coupled to H-Bac7(1-35)-Cys(H)-COO-PEG-Om via thioether ligation in order to obtain H-Bac7(1-35)-Cys(AlexaFluor680 C_2maleimide)-COOPEG-Om.

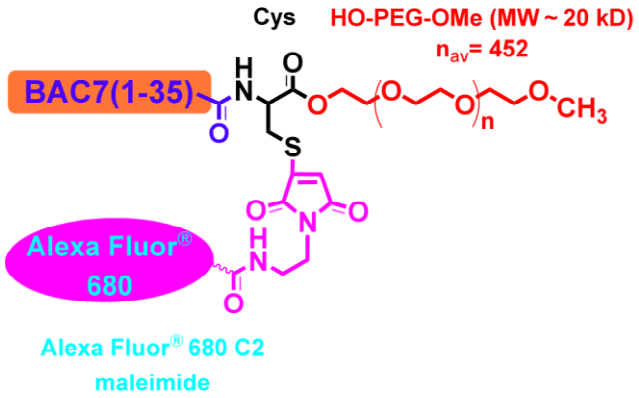


Fig. 2. Structure of the pegylated Bac7(1-35) labelled with Alexa Fluor 680 C2.

Briefly, Bac7(1-35)-Cys(H)-CO-OPEG-Om (25 mg) was dissolved in 0.5 M posphate buffer pH 7 (0.3 ml) and water/TCEP/EDTA (0.3 mg /0.1 ml water in total 0.4 ml) and mixed for 15 min. One mg of Alexa680 was then added and the mixture was stirred under N_2 for 2 h, quenched with 10% H_3PO_4 to pH 4. The mixture was diluted with water (21 ml) and the product separated on semi-preparative RP-HPLC by using a C4 column (Phenomenex 250 x 10 mm).

The concentration of the labelled pegylated peptide was determined by at least three methods:

I. by weighing the peptide used to prepare the solution;
II. by using the Ellmann's reagent method to detect free SH (Ellmann 1959);
III. by considering the extinction coefficient (ε_{679}) of Alexa Fluor®680.

6.8.3 Bac7(1-35)CAM(Alexa680)PEG20k biodistribution

Biodistribution analyses of Bac7(1-35)CAM(Alexa680)PEG20k in mice were performed as described in section 5.5.2 of the current chapter. In this case, three Balb-c male mice 5-week old were used. The background signal intensity, recorded with the baseline image for each animal before the injection of the probe, was subtracted from each post contrast image. Twentyfour hours after sample injection, the animals were sacrificed and *ex vivo* optical imaging of specific organs was performed.

7. EFFECTS OF Bac7(1-35) ON HUMAN MACROPHAGES

7.1 Mammalian cells

The human macrophage-like cell line U937 was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum (FBS). For induction of differentiation, the cells ($1x10^6$ per ml) were seeded in the same medium and treated with 50 ng/ml of PMA at 37 °C for 72 h in a 5% CO_2atmosphere. After incubation, non-adherent cells were removed by aspiration and the adherent ones were washed with PBS. The cells were then incubated with trypsin solution [0.5% trypsin (w/v) in PBS containing 0.2 mM EDTA] for 5 minutes at 37°C in 5% CO_2, to detach them from the tissue culture flask.

The human monocytes were isolated from a pool of buffy coats from different donors. The buffy coats were diluted 1:1 with PBS and added to an equal volume of Histopack. After centrifugation for 30 min at 400 x g without brake, the white band at the interface between the plasma and the Histopack fractions was soaked up, transferred into a Falcon sterile tube and washed twice with PBS. The cell pellet was then resuspended in fresh medium and transferred to a cell culture flask. After the monocytes were left to adhere for 1 h, the lymphocytes were washed away. The differentiation of the seeded monocytes was induced by addition of 50 ng/ml of *S. typhimurium* LPS followed by incubation at 37 °C for 5 days in 5% CO_2.

7.2 Citotoxicity assay

The U937 cell line was grown and differentiated to macrophages as described above.

The cells were seeded in 12-well tissue culture plates at a density of $4x10^5$ cells per well and incubated with or without peptide [10, 30, 50 and 150 μM Bac7(1-35)] at 37 °C for 3 h in 5% CO_2. Two identical plates were prepared. At the end of the incubation time, the samples of the first plate were immediately analyzed to evaluate the cytotoxic effect of the peptide, while the analysis of the other plate was performed after replacement of the medium with fresh RPMI 1640, and extension of the incubation time up to a total of 24 h.

At the end of each incubation time (3 or 24 h), the cells were stained in the dark at 37

°C for 15 min with 50 nM of 3,3'-dihexylocarbocyanide iodide ($DiOC_6$), a fluorescent probe used to measure the mitochondrial transmembrane potential in intact cells. A positive control for collapse of mitochondrial transmembrane potential was obtained by incubation of the cells with 50 μM of the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 15 min at 37 °C.

To test their viability after treatment with $DiOC_6$, the cells were washed twice with 2 ml of PBS, stained at room temperature and in the dark with 10 μg/ml of propidium iodide (PI) for 10 min, and analyzed by flow cytometry, acquiring at least 10,000 events for each sample.

The double staining with $DiOC_6$ and PI allows distinguishing three subsets of cells: viable cells ($DiOC_6$ positive and PI negative), apoptotic cells ($DiOC_6$ negative and PI negative), and necrotic cells ($DiOC_6$ negative and PI positive).

7.3 Peptide uptake by differentiated U937 cells

PMA-differentiated U937 monocytic-like cells (3×10^4 cells per well) were incubated for 3 h with or without 1 μM Bac7(1-35)-BODIPY. The cells were then washed with PBS, to remove the non-internalized peptide, and analysed by fluorescence spectroscopy. The analyses were also performed by using flow cytometry before and after addition of 1 mg/ml Trypan blue, an extracellular quencher that is not internalized in intact cells.
The cells were also analysed by confocal laser microscopy (see section 3.6 of Materials and Methods).

7.4 Phagocytosis assay

The gentamicin-protection invasion assay was used to test phagocytosis (Su *et al* 2010). The *S. enterica* LT2 suspensions were seeded to each well of a flat-bottom well tissue culture plate to achieve the desired cells to bacteria ratio (multiplicity of infection, MOI). PMA-differentiated U937 monocytic-like cells in RPMI supplemented with 10% FBS were then added to the plate. After allowing phagocytosis to occur for 1 hour, gentamicin was added to each well at 18 μg/ml and left at 37 °C in 5% CO_2 for 1 hour. The added gentamicin kills extracellular bacteria without affecting the viability of intracellular bacteria and of macrophages.

7.4.1 Effects of Bac7(1-35) on *Salmonella* phagocytosis by use of GFP-expressing bacteria

Phagocytosis assays were performed as described above (section 7.4) by incubating PMA-differentiated U937 monocytic-like cells (1 x 10^5 per well) and GFP-expressing *S. typhimurium* cells in a 24-well plate, at a MOI value of 50,. The experiments were performed in duplicate by pre-treating the cells with 50 μM Bac7(1-35) at 37 °C for 1 h, or, *vice versa*, by pre-treating the bacteria with 0.5 μM peptide. At the end of the incubation time, samples were collected, centrifuged at 400 x g for 5 min, and the cells resuspended in PBS to allow flow cytometric analysis.

7.4.2 Phagocytosis assay to evaluate the protective effect of Bac7(1-35) against intracellular *Salmonella* survival and replication

Phagocytosis assays were performed as described above (section 3.4) by using PMA-differentiated U937 monocytic-like cells (4 x 10^5 per well) or macrophages derived from human monocytes (MDM) after LPS treatment (50 ng/ml) and *S. typhimurium* at a MOI of 10.

At the end of the phagocytosis assay, the medium was removed and replaced with fresh RPMI containing 10% FBS with or without peptide added at 50 or 150 μM. For each sample, three identical wells were prepared. The cells of the first well were collected and plated after phagocytosis and immediately before peptide's addition. Those of the second well were treated with the peptide for 3 h and then plated. The cells of the third well were treated with the peptide for 3 h and then washed with abundant PBS to remove the peptide, supplemented of fresh medium and allowed to incubate for additional 21 hours at 37°C in 5% CO_2. A control without peptide addition was prepared for each sample (first well).

At the end of the incubation time, PBS was added to each well and the cells were collected, centrifuged at 400 x g for 5 min, and counted. To recover phagocytosed bacteria, the cells were then lysed by treatment with 1 ml of 0,1% Triton X-100 in PBS for 30 min at room temperature. The bacteria were then diluted, plated on MH agar plates, incubated overnight at 37 °C to allow colony counts. Significance of percentage differences among the number of bacteria within the peptide-treated and untreated cells

at 3 h and 24 h was calculated by using the t-test. Values of $p < 0.05$ were considered statistically significant.

7.4.2.1.1 Flow cytometer and data analysis

Flow cytometry is a powerful technique that is used in a range of applications for the analysis of multiple parameters of individual cells within a heterogeneous population. The flow cytometer performs this analysis by passing thousands of cells per second through a laser beam and capturing the light that emerges from each cell as it passes through. The data acquired can be analyzed statistically by dedicated software that reports cellular parameters such as size, complexity, phenotype and viability. As shown in Fig. 3, the flow cytometer is generally composed of:

- a fluidic system which presents cells to the interrogation point and takes away the waste;
- the lasers, which are the light source for scatter and fluorescence;
- the optics, which gather and direct the light
- the detectors (photomultipliers), which receive the light
- the electronics and computer system that convert the signals from the detectors into digital data.

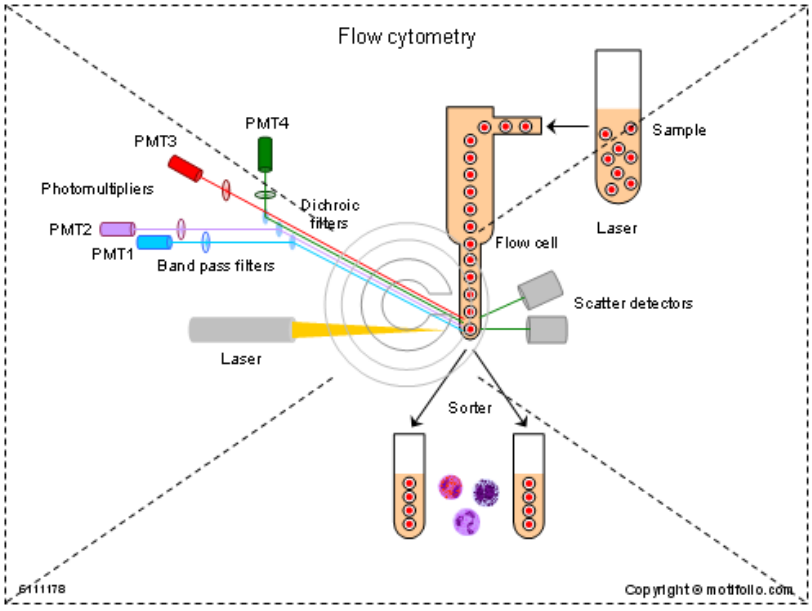


Fig. 3. Schematic representation of a flow cytometer.

A cell that passes through the laser beam scatters light at all angles. Forward scatter, or

low-angle light scatter, is the amount of light that is scattered in the forward direction and is dependent on the cell size. Side scatter is the large-angle light scattering dependent on the granularity and structural complexity inside the cell. The scatter plot created using the forward and side scatter data, gives a distribution of the cells in the analyzed population based on their size, granularity and structural complexity (Fig. 4).

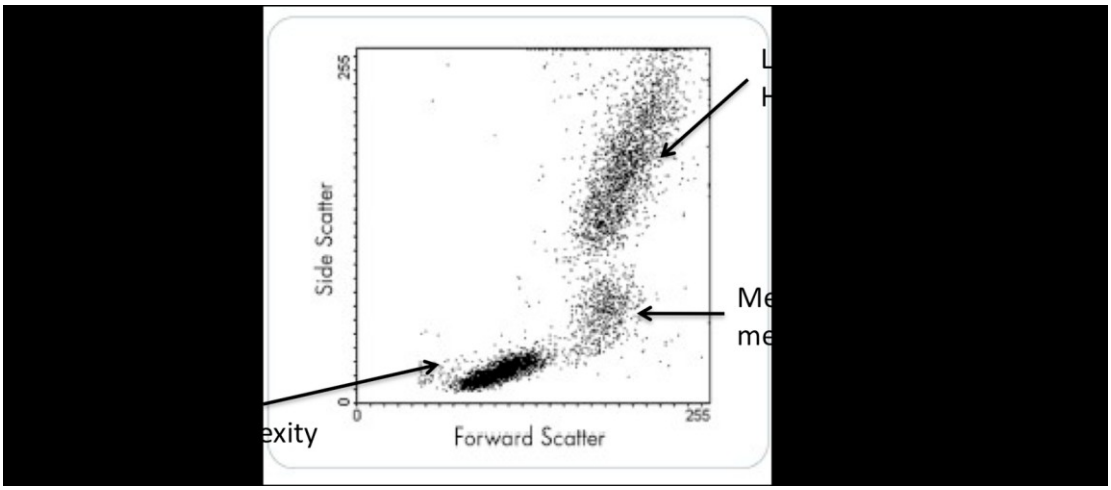


Fig. 4. Scatter plot: the plot represents the distribution of a cells population and is obtained by plotting the forward (x-axis) and side (y-axis) scatter values of each individual cell.

One of the most common ways to study cell characteristics using flow cytometry involves the use of fluorescent molecules. When fluorescent cells pass through the laser beam and the laser light of the right wavelength strikes the fluorophore, a fluorescent signal is emitted and directed to the appropriate detector. This translates the signal in a voltage pulse proportional to the amount of fluorescence emitted by each cell. The fluorescence intensities of all the cells analysed are described by a normal distribution and the Mean Fluorescence Intensity (MFI) of the cell population can be calculated (Fig. 5).

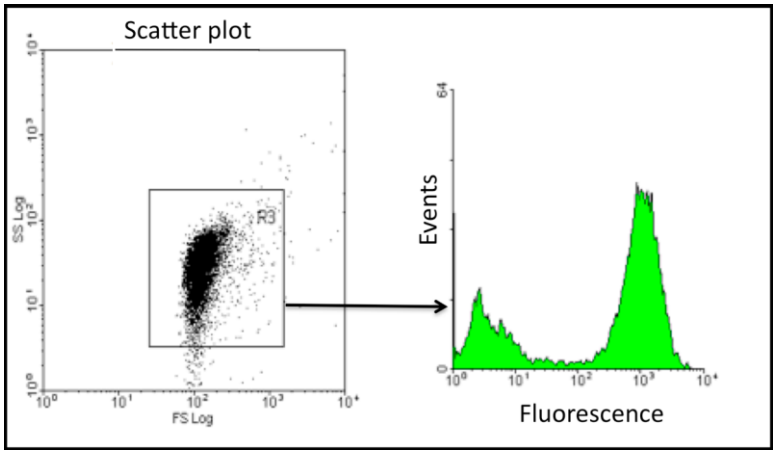


Fig. 5. Fluorescence Histogram: the histogram, on the right, represents the normal distribution of the fluorescence intensities of all the cells counted (events), which were selected among the cell population analysed (R3 in the scatter plot on the right).

7.5 Confocal Laser analysis

Confocal Laser Microscopy analyses were performed to evaluate the peptide uptake into the cells and its biodistribution after phagocytosis. For the former type of evaluation, the U937 cells (1 x 10^6) were seeded on coverslips in complete medium supplemented with 50 ng/ml PMA to induce differentiation into macrophages. After 3 days of incubation at 37°C and in 5% CO_2, the culture medium was removed. The cells were then washed with abundant PBS and stained at 37 °C and 5% CO_2 with FAST DiI (0.5 mM) diluted in 0.5 ml of fresh complete medium. After 20 min of staining, the cells were washed with PBS before adding medium supplemented with 10% FBS and Bac7(1-35)-Alexa680 (1 μM) for 1 h and 3 h. The peptide solution was then aspirated; the cells were washed several times with PBS, and then fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. After washing three times, the fixed cells were treated with 0.1% glycine and 0.02% NaN_3 in PBS for 5 min. After permeabilization with 0.01% Triton X-100 for 5 min, the cells were rinsed three times with PBS and once with deionised water. The slides were mounted with Mowiol and conserved in the dark at -20°C until examination by fluorescence microscopy.

To analyse the uptake and biodistribution of the peptide after phagocytosis, cells were stained with FAST DiI solution as described above. After washing, the cells were resuspended in RPMI medium supplemented with 10% FBS, and the GFP-expressing salmonellae, prepared as described in section 2.1, were added at a MOI 50. Phagocytosis was left to occur for 2 h. Coverslips were then washed three times with PBS and treated with 1 μM Bac7(1-35)-Alexa for 3 h. The peptide solution was then aspirated and the cells were washed several times with PBS, fixed, treated as described above, and stored in the dark at -20°C. All the samples were examined with a Nikon C1-SI confocal microscope with an oil immersion objective lens.

Results

1. THERAPEUTIC POTENTIAL of Bac7(1-35): *in vitro* and *in vivo* ACTIVITY

1.1 Antibacterial activity of Bac7(1-35) in the presence of serum and plasma

Before testing Bac7(1-35) *in vivo*, its antibacterial activity was assayed *in vitro* in the presence of serum and plasma. Killing kinetics assays were firstly performed in the presence of 66% (v/v) murine serum or plasma towards *S. enterica*. The results showed that murine serum and plasma exert a time-dependent inhibitory effect on the bactericidal activity of Bac7(1-35), reducing significantly its efficacy (Fig. 1.1).

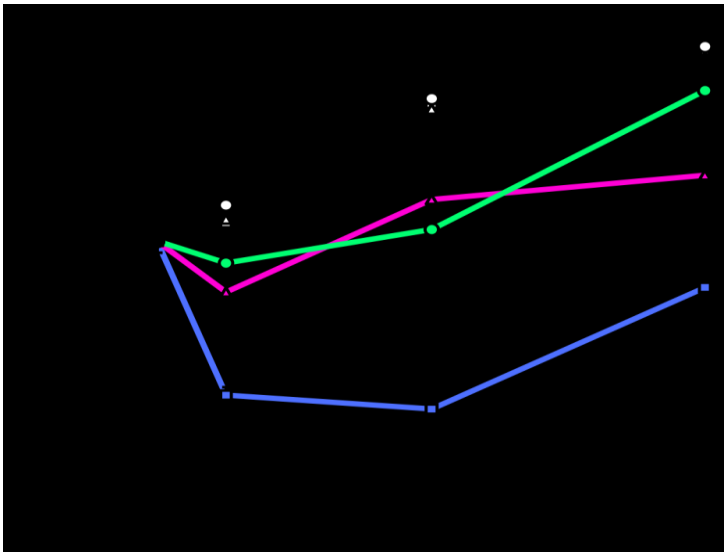


Fig. 1.1. Antimicrobial activity of Bac7(1-35) in the presence of murine serum or plasma

Kinetics of the bactericidal activity of 10 μM Bac7(1-35) against *S. enterica* ATCC 14028 in the absence (blue line) or in the presence of 66% murine serum (pink line) or 66% murine plasma (green line). Bacterial growth without peptide in the absence (□) or presence of 66% serum (△) or plasma (○) is shown by black lines. Results represent the mean ± SD of three independent determinations performed in triplicate.

In particular, after 1h-incubation with serum or with plasma, 10 μM Bac7(1-35) reduced the number of CFU by 0.5-1 log *vs* 2.5 log detected in the absence of the blood components. After 4h-incubation, Bac7(1-35) was able to reduce the number of CFU both in serum and plasma by 2 log compared to the control (i.e., bacteria without peptide in the absence or presence of 66% biological fluids) but its efficacy decreased if we compare it with that shown by the peptide in culture medium. At longer incubation

times, the activity of the peptide was further inhibited especially by murine serum. In fact, after 8 h incubation, the number of CFUs in the presence of peptide and serum, increased of 2 log with respect to the peptide in MH. Killing kinetics assays were also performed in the presence of BSA at physiological concentrations (40 mg/ml) so as to investigate its possible effect on Bac7(1-35) antibacterial activity. Results showed that this serum protein does not alter the activity of the peptide (data not shown).

In order to investigate whether these inhibitory effects are also observed in the presence of human blood components, microdilution susceptibility assays were performed to determine the minimum inhibitory concentration (MIC) of Bac7(1-35) in the presence of 25% human serum and plasma. In parallel, the MIC values were also tested in 25% murine serum or plasma, to compare the effect of fluids from the two species.

Table 1.1 Antimicrobial activity of Bac7(1-35) in human or murine biological fluids.

	MIC (μM)				
	Human		Murine		Mueller-Hinton
	Plasma	Serum	Plasma	Serum	
S. enterica ATCC 14028	0.06-0.12	0.5	32	64	0,5

MIC values of Bac7(1-35) towards *S. enterica* (10^5 CFU/ml) in the presence of 25% murine serum or plasma, human serum or plasma diluted in MH broth or in 100% MH. Data shown are representative of three independent experiments with comparable results.

As expected from the kinetics results previously reported, the MIC value of Bac7(1-35) in murine blood components drastically increased. In serum and in plasma the peptide was active respectively at concentrations 128- and 64-fold higher than in culture broth alone (Table 1.1). Interestingly, when the same assay were repeated with human serum or plasma, the results were quite different. Bac7(1-35) in human serum was active at the same concentration showed in Mueller-Hinton broth: no inhibitory effects were detected and the peptide inhibited the bacterial growth at 0.5 μM. To evaluate the involvement of the complement in enhancing the antibacterial effect of the peptide, the assays were also repeated using heat-inactivated serum (30 min at 56 °C). Results showed that there was no difference in the MIC values (data not shown), indicating that the effect was not dependent on complement activation.

In human plasma, Bac7(1-35) seemed to potentiate its activity: the MIC value increased

4-8 folds with respect to the MIC value in MH or in the presence of human serum. This effect was also observed with other peptides, such as BMAP-27, and the Bac7 fragments 1-15 and 1-16, that were tested in the presence of human plasma (Table 1.2). Since we observed that in human plasma the kinetics of bacterial growth is slowed down (data not shown), it can be hypothesized that there is a synergistic effect between peptide and serum that leads to an improve activity.

Table 1.2. Comparison of the antimicrobial activity of different peptides in human plasma or in culture medium

	MIC (μM) ***S. enterica*** ATCC 1402	
	Human Plasma	Mueller Hinton
BMAP-27	0,5	1
Bac7(1-16)	0,5	1
Bac7(1 -15)	16	32

MIC values of BMAP-27, Bac7(1-16) and Bac7(1-15) towards *S. enterica* (10^5 CFU/ml) in the presence of 25% human plasma diluted in Mueller Hinton broth or in 100% Mueller Hinton. Data shown are representative of three indipendent experiments with comparable results.

1.2 Stability of Bac7(1-35) in serum and plasma

One of the main causes of short *in vivo* half-life of peptides and proteins is their enzymatic degradation (Werle and Bernkop-Schnurch 2006). Following this consideration, the degradation of Bac7(1-35) by blood components was studied to verify if the reduced activity of the peptide in mouse fluids was due to this reason.
Western blot analyses were performed after incubation the peptide in human or murine serum or plasma up to 72 h. In murine serum, immunodetection indicated a slow and progressive reduction of the band corresponding to intact Bac7(1-35), which disappeared completely after 24 h-incubation (Fig. 1.2A). In plasma, where proteases of the coagulation cascade are not activated, the degradation level of Bac7(1-35) appeared slightly lower than in serum. In contrast, in human fluids, the effects of proteolytic

degradation were much slower. In fact, a visible decrease in the amount of intact peptide could only be detected after 48 h of incubation and a significant amount of it was still present after 72 h (Fig. 1.2B).

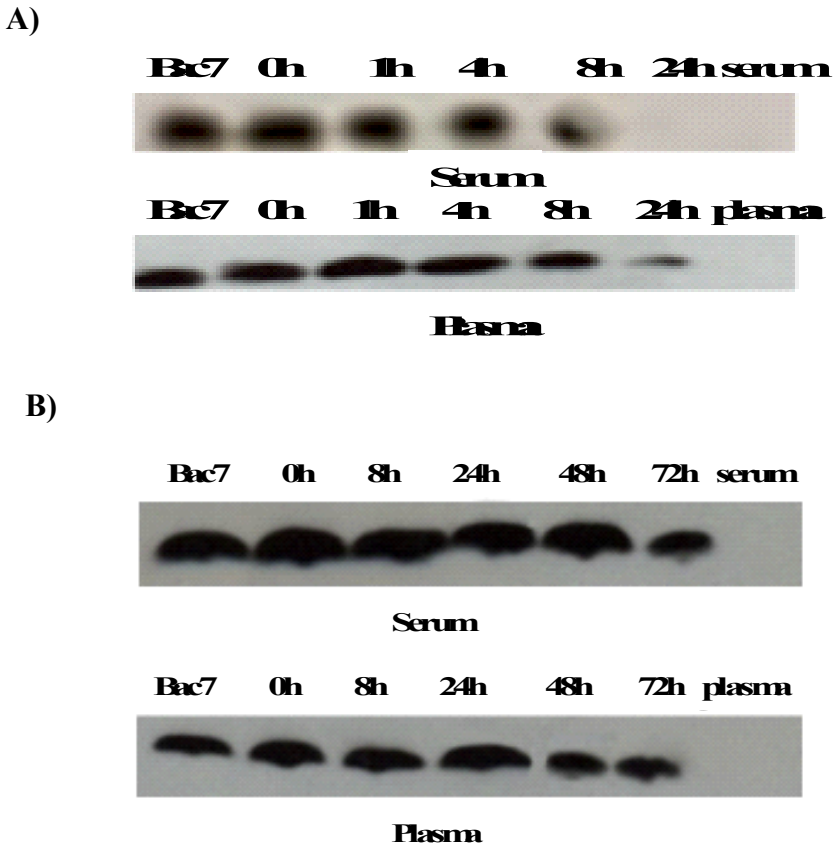


Fig. 1.2. Western blot analyses of Bac7(1-35) degradation by biological fluids. The peptide was incubated for different times at 37 °C in 25% (A) murine and (B) human serum and plasma.

A quantitative evaluation of Bac7(1-35) degradation was carried out by using LC-MS analysis. Results were in agreement with those obtained by Western Blot analysis. The amount of intact peptide decreased of 40% after 4 h of incubation in murine serum and disappeared almost completely after 8 h. In human serum, the reduction was much lower as already indicated by Western blot analysis. (Fig. 1.3).

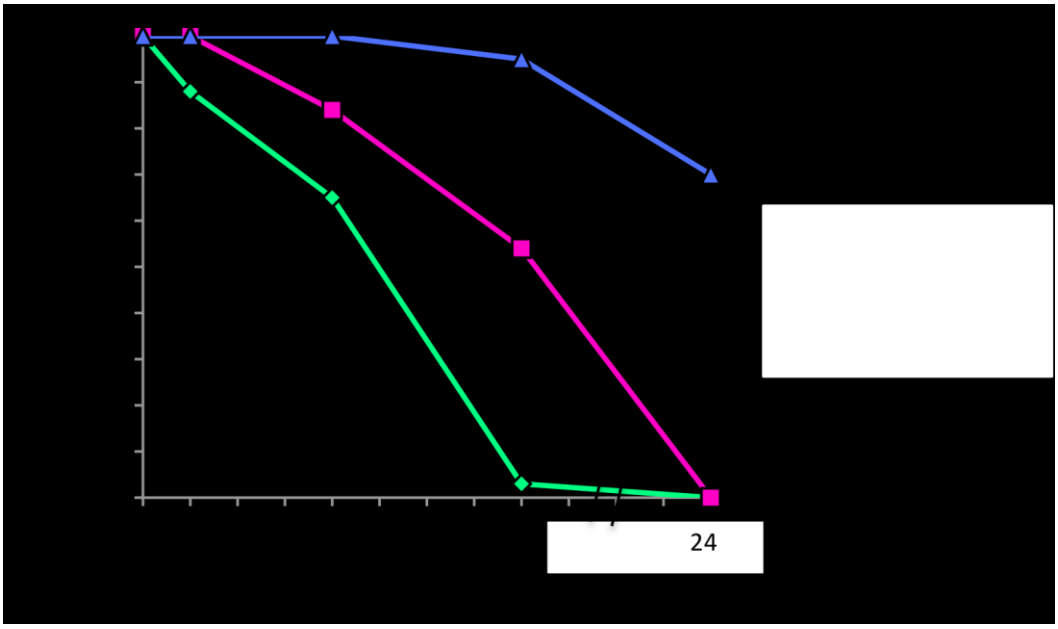


Fig. 1.3. Bac7(1-35) degradation by blood fluids analyzed by LC-MS. The percentages of intact Bac7(1-35) at the different incubation times in human serum (blue line) and murine serum (green line) or plasma (pink line) were calculated with respect to an untreated control. Data are from an experiment representative of three independent experiments with comparable results.

The lack of detection of fragments of Bac7(1-35) by LC-MS has to be pointed out and likely depends on generation of small, undetectable hydrophilic fragments. This fragmentation likely depends on the trypsin-like serine proteases of blood, which could cleave the peptide in correspondence of the many Arg residues present in its sequence.

1.3 *In vivo* toxicity of Bac7(1-35)

The determination of acute toxicity represents the first step in a preliminary evaluation of the therapeutic potential of a drug candidate. To this aim, Bac7(1-35) was injected via i.p. at increasing doses in mice of two different strains, Balb/c and CBA/CA, and mice survival was monitored up to 14 days. According to standard procedures, we also monitored the weight and the aspect of the hair of treated mice as sign of toxicity or pain. Up to the dose of 75 mg/kg mice did not show any apparent sign of suffering, the weight remained stable within the 14 days and their hairs that did not show any change. At the highest dose tested, 150 mg/kg, all mice died within 3 days. From these data and using the Wilcoxon and Mann-Whithey test, it could be calculated that the LD_{50} value for Bac7(1-35) is 105 mg/kg. This result confirms that Bac7(1-35) is much less toxic than other cathelicidin-derived peptides as BMAP-27 and BMAP-28 [LD_{50} respectively

10 and 15 mg/kg (Benincasa *et al.* 2004)].

1.4 *In vivo* Bac7(1-35) activity in a mouse model of typhoid fever

The capacity of Bac7(1-35) to protect mice from a bacterial challenge was tested in a mouse model of *Salmonella* infection. Animals infected with this pathogen develop a systemic disease characterized by rapid bacterial multiplication in the liver and spleen that resembles typhoid fever caused by *Salmonella* serovar Typhi in humans (Santos *et al*. 2001). CBA/CA mice were chosen for this assay because Balb/c mice were extremely susceptible to *Salmonella* infection, due to an intrinsic defect in cell-mediated immunity (Hormaeche *et al*. 1981, Takahashi *et al*. 1990).

Previous studies performed to determine the lethal dose of *S. enterica* ATCC 14028 in the CBA/CA strain, indicated that injection via i.p. of a bacterial suspension of $1x10^2$ CFU/mouse was sufficient to cause death of untreated mice at around 10 days. Gentamicin, which was chosen as control to validate the experimental model, gave 100% of protection against infection at the dose of 10 mg/kg.

Bac7(1-35) at a dose of 30 mg/kg was injected via i.p. immediately after infection. This dose was chosen in light of preliminary *in vivo* experiments in which the use of 15 mg/Kg Bac7(1-35) did not protect the infected animals (only a minor improvement in survival was observed with respect to untreated controls, data not shown). The number of survivors was monitored for 60 days and compared to that of control mice that only received the lethal bacterial challenge. The survival curves of untreated and peptide-treated mice are significantly different ($p < 0.05$); the mean survival time of control mice was 10 days, while the treatment of infected mice with Bac7(1-35) increased the mean survival time to 24.5 days (Fig. 1.4A). A group of peptide-treated mice was also analysed for bacterial load at 3 days post-infection and, at this time, mice did not show any visible sign of disease. Viable bacterial cells were counted in murine liver and spleen of infected mice. These are in fact the main organs that are infected by *Salmonella* once in the systemic circle (Gulig 1996; Falcow *et al*. 1996). Results showed a great reduction in the number of viable bacterial cells in liver and spleen of treated mice compared to those detected in untreated mice, despite the high variability in each group (Fig. 1.4 B).

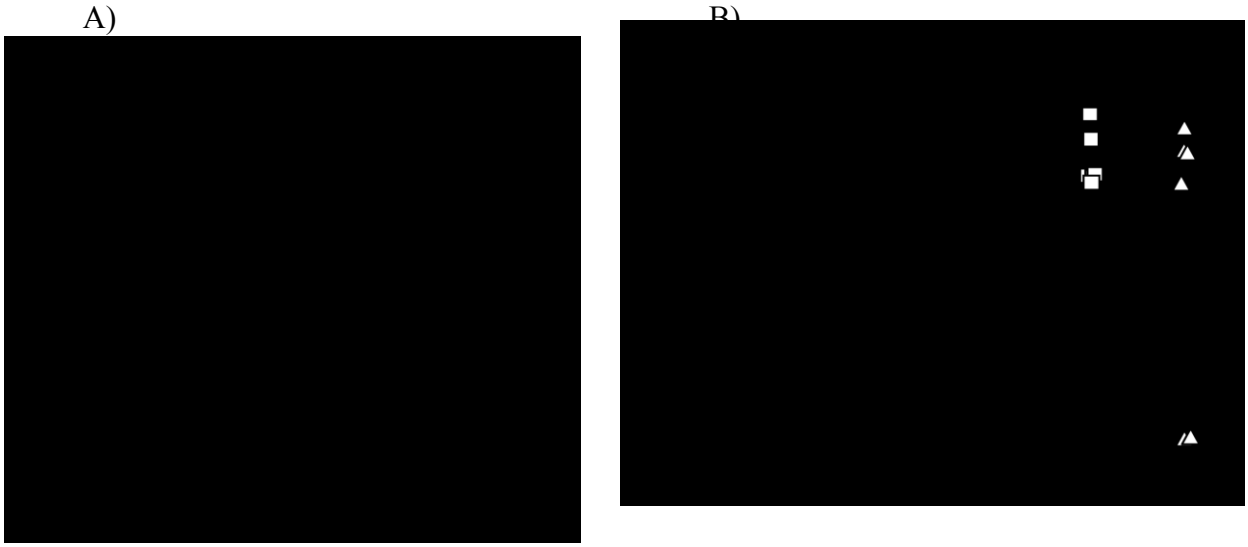


Fig. 1.4. *In vivo* activity of Bac7(1-35) in a salmonellosis animal model. (A) Survival curves of mice infected with *S. enterica* ATCC 14028 and treated with Bac7(1-35) (dotted line). Control mice were given 0.2 ml of PBS (continuous line). Mice were monitored over a period of 60-days after infection. *$p<0.05$ treated *vs* untreated mice. **(B)** Viable bacterial counts in liver and spleen homogenates. Three days after infection, untreated (squares) and peptide-treated (triangles) mice were killed, and liver (full symbols) and spleen (empty symbols) were collected and homogenized in order to allow bacterial colony counts Results are expressed as number of CFU/g organ, horizontal bars represent the mean value for each group.

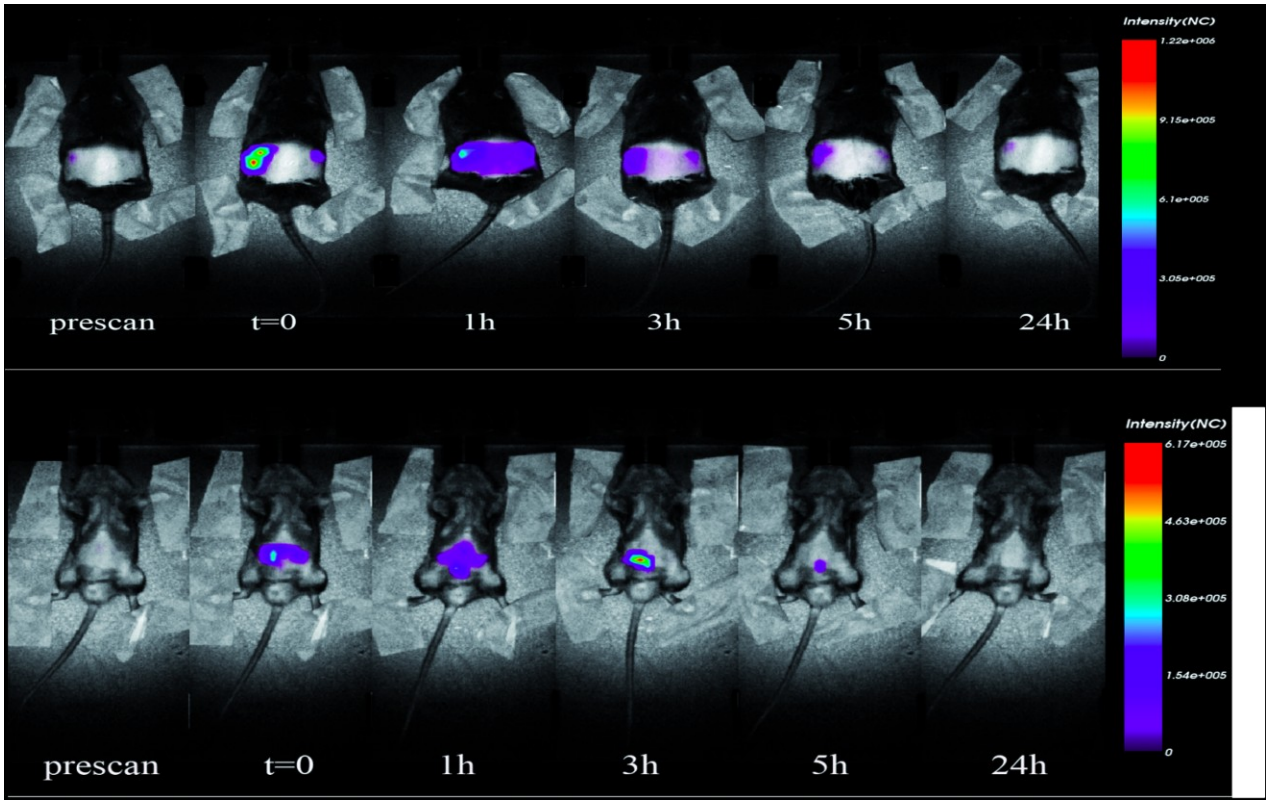
In 3 out of 10 of the animals, bacteria were undetectable in both the spleen and liver. This result is in keeping with the percentage of mice completely cured, as extrapolated by the survival curve (Fig. 1.4 A). Given that the model of infection tested is quite aggressive (i.p. injection of as few as 100 salmonellae is lethal for mice), the increased survival times and the eradication of the infection in 35% of the peptide-treated animals is a promising result.

1.5 *In vivo* Bac7(1-35) biodistribution

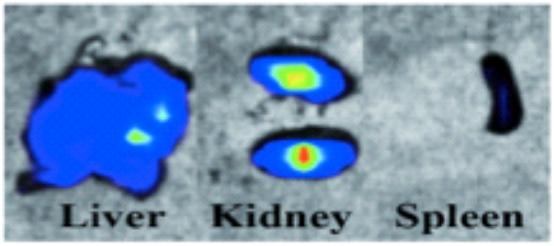
To better understand the behaviour of Bac7(1-35) *in vivo* and with the aim to modify it so as to improve its *in vivo* performance, we investigated the biodistribution and rate of clearance of the peptide in mice. To this aim, time-domain optical imaging analyses

were performed by using a derivative of Bac7(1-35) that was fluorescently labelled with the dye Alexa680, a modification that did not alter its antimicrobial efficacy. Time killing assays performed by incubating *S. enterica* with Bac7(1-35)-Alexa680 up to 8 h, revealed that the reduction of bacterial CFU differs of only half a log from that obtained with unlabeled Bac7(1-35)(data not shown). The peptide was thus injected via i.p. in mice at the dose used for *in vivo* studies, and its biodistribution was monitored up to 24 h by acquiring the fluorescence intensity signal (lifetime) of the dye linked to the peptide as described in the Materials and Methods 5.5.1 section. To be certain that the fluorescence signal was derived from the conjugated peptide and not from the free dye, lifetimes of the two forms of dye were compared and resulted different. The fluorescence signal of the Bac7(1-35)-Alexa680 was detected in the kidney 1 h after i.p injection and in the bladder after 3 h. During the following hours, the fluorescence decreased and completely disappeared at 24 h (Fig. 1.5A). *Ex vivo* analysis performed on liver, kidney and spleen at 5 and 24 h confirmed these data: at 5 h the peptide was still present in the kidney but not in liver and spleen (data not shown); at 24 h post-injection the fluorescent signal was not detectable in any of the organs collected (Fig. 1.5B). These results indicate that Bac7(1-35) does not accumulate in any particular organ except those involved in the elimination processes and that in mice the peptide is characterized by a fast renal clearance after i.p. injection. In addition, *ex vivo* analyses allowed monitoring of the morphological aspect of the organs and no visible alteration due to the presence of the peptide was detected, suggesting that the peptide did not cause any macroscopic side effect.

A .



B.



5 h post-injection

Fig. 1.5 Biodistribution of Bac7(1-35)-Alexa680 in healthy mice after i.p. injection. (**A**) The animals were placed in prone position (upper panel) and supine position (lower panel); fluorescence emission in the regions of interest encompassing respectively the kidneys, the thorax and abdomen, were acquired at indicated times post-injection and normalized with respect to the prescan acquired before peptide injection. (**B**) *Ex vivo* images of organs at 5 h after i.p. injection of the labelled peptide. At 5 h post injection, the signal of Bac7(1-35)-Alexa was detected only in the kidney (not shown). At 24 h the signal of Bac7(1-35)-Alexa was not present. Images of the organs were acquired immediately after sacrifice: laser power and integration time were optimized while keeping constant scan step to compare fluorescence intensities after normalization. The images are representative of two independent experiments with comparable results.

2. PEGYLATION STRATEGIES TO IMPROVE Bac7(1-35) BIODISTRIBUTION

Due to its small size, Bac7(1-35) is easily cleared by kidneys, as also indicated by the *in vivo* imaging experiments. To overcome this problem and increase the pharmacokinetic profile of the peptide, it was decided to modify it by conjugation with PEG. Molecules are generally conjugated to PEG via amino-groups (Veronese *et al.* 2005). However, the internalization of Bac7(1-35) in the bacterial cytosol and its activity are strictly dependent on the presence of a cationic and free N-terminus (Benincasa *et al.* 2004). For this reason, conjugation of PEG was pursued to the C-terminus of the peptide. Different strategies were used in order to find the best one in terms of efficiency of reaction and biological activity of the pegylated peptide.

2.1. Bac7(1-35) pegylation via amide bond

In a first attempt, the pegylation of Bac7(1-35) was performed *via* formation of a stable amide bond involving the C-terminal carboxylic group of the peptide and an amino-PEG (NH_2-PEG20k-metoxy) with a MW of 20000 (Fig. 2.1).

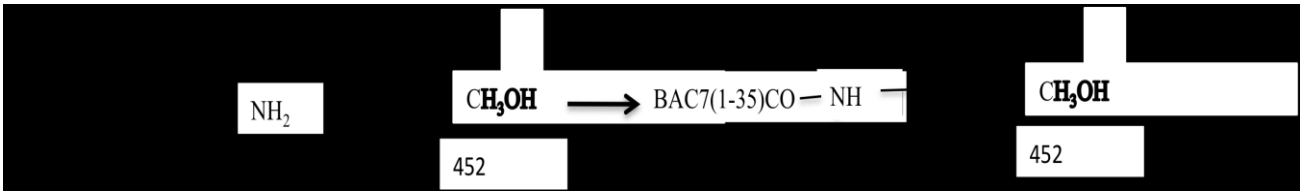


Fig. 2.1. Reaction of the C-terminal carboxylic group of Bac7(1-35) with an amino-PEG (NH_2-PEG20k-metoxy).

At the end of the reaction, the pegylated peptide Bac7(1-35)PEG20k was purified using cation-exchange chromatography and RP-HPLC, and its purity was checked by SDS-PAGE analysis (Fig. 2.2).

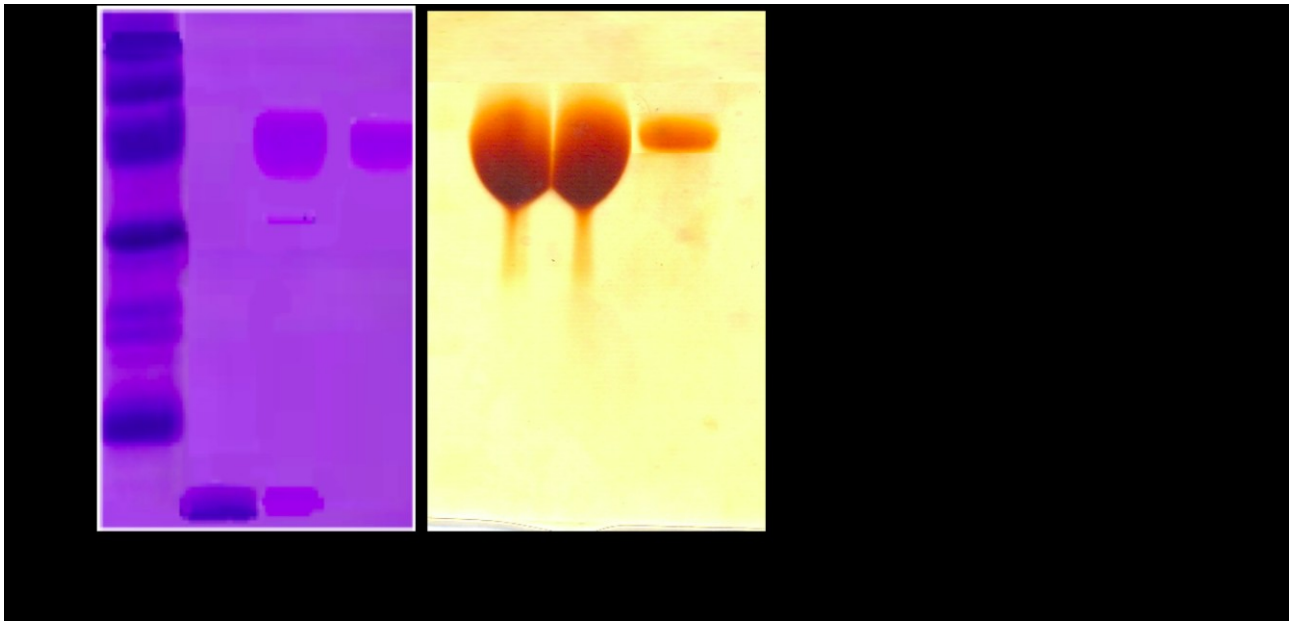


Fig. 2.2. SDS-PAGE analysis of Bac7(1-35)-PEG20k.

Tricine SDS-PAGE of the pegylated peptide before and after purification. Two different methods of staining were used specifically allowing the detection of the peptide and of PEG. The gel on the left is stained with Coomassie Blue to detect the band corresponding to the peptide and the gel on the right is stained with barium chloride/iodine solution to visualize the band corresponding to PEG. For each sample, 3 μg were loaded. The apparent mass of the bands corresponding to PEG is higher than expected (40k *vs* 20k) due to the high capacity of water to bind the polymer.

As shown, this reaction allowed to obtain the pegylated form of Bac7(1-35) in good yield (≈ 30%) and pure to homogeneity after appropriate chromatographic steps. The double staining with Coomassie and barium chloride/iodine solutions allowed detection of both PEG and peptide. The purified compound showed a distinct profile with respect to samples containing unpurified Bac7(1-35)PEG20k or the commercial PEG alone (Fig. 2.2). Unfortunately, Bac7(1-35) conjugated to amino-PEG showed a great loss in antibacterial activity. In fact, the MIC value against *S. enterica* increased from 0.5 μM for the free peptide to 32 μM for the conjugate (Table 2.1).

As for the unpegylated form, the activity of Bac7(1-35)-PEG20k decreased further in the presence of murine blood components and remained unchanged in the presence of human serum or plasma (Table 2.1).

Table 2.1. Antimicrobial activity of Bac7(1-35) and Bac7(-35)PEG20k in human and murine biological fluids

	MIC (μM) *S. enterica* ATCC 14028				
	Human		Murine		Mueller-Hinton
	Plasma	Serum	Plasma	Serum	
Bac7(1-35)	0.06 –0.12	0.5	32	64	0.5
Bac7(1-35)-PEG20K	32	32	64	128	32

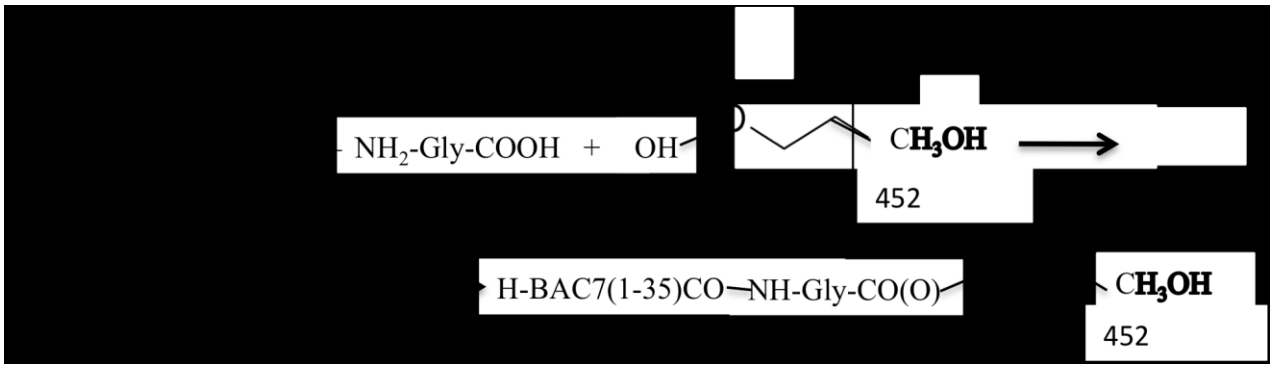
MIC values of Bac7(1-35) and Bac7(1-35)-PEG20K towards *S. enterica* (10^5 CFU/ml) in the presence of 25% of murine serum and plasma, human serum and plasma diluted in Mueller Hinton broth, or in 100% Mueller Hinton. Data shown are representative of three independent experiments with comparable results.

2.2 Bac7(1-35) pegylation via ester bond and thioether bond

To overcome the lack of Bac7(1-35) activity when pegylated *via* a stable bond, as the amide bond above described, we used a different conjugation strategy based on the presence of a cleavable ester bond that could be the substrate of blood esterases, to allow a slow peptide release.

To this aim, a glycine linker was first attached to 20k PEG via reaction of its carboxy group with the alcohol function of the polymer (OH-PEG20k-metoxy), to give a cleavable ester bond (Fig. 2.3). The C-terminus of Bac7(1-35) was then conjugated to the Gly amino group by forming an amide bond. The glycine residue was used to increase the efficiency of the conjugation between the peptide and PEG, which would otherwise be very low if the ester bond would be formed between the C-terminus of the peptide and the OH of a high molecular weight PEG molecule. When the ester bond of the obtained conjugate is cleaved, the Bac7(1-35)-Gly_{36} form of the peptide is released (Fig. 2.3 B).

A)



B)

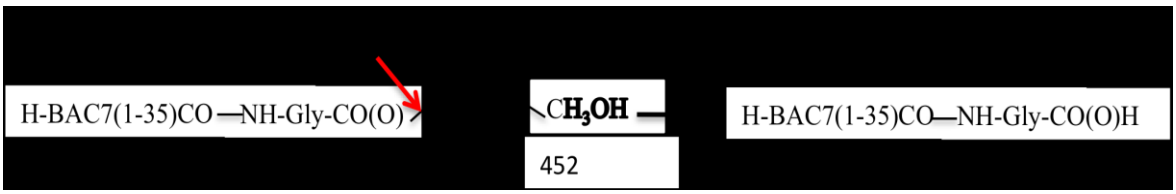
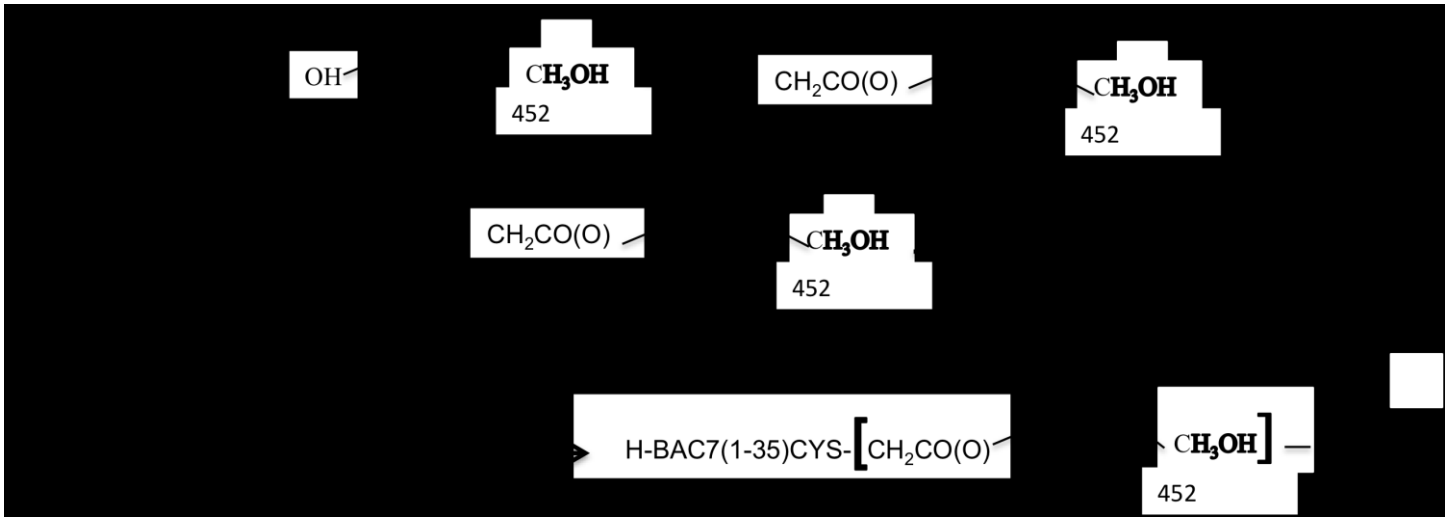


Fig. 2.3. A) Scheme of the synthesis of the conjugate between Bac7(1-35) and OH-PEG20k-metoxy via a glycine residue whose amino group forms an amide bond with the alcohol function of PEG. B) The arrow indicates the cleavable bond that can be hydrolyzed by esterases.

As for the peptide pegylated via an amide bond, Bac7(1-35)-Gly-PEG20k was purified and analyzed by SDS-PAGE (Fig. 2.5 A). Despite a purified product has been detected, unfortunately the yield of the reaction was very low (≈ 1%), leading to an insufficient amount of conjugate to test its biological activity.

To increase the yield of the reaction, a quite different coupling strategy was adopted, based on a cleavable thioether bond, which makes use of Bac7(1-35) with a Cys residue added at its C-terminus and of the PEG derivative bromoacetyl-PEG-MeO. This procedure, known as thioether ligation or native chemical ligation, is a chemoselective approach that allows obtaining a very high yields of reaction (Brunel *et al.* 2005). Also in this case, a cleavable bond is obtained between the peptide and the PEG molecule in order to ensure a slow release of Bac7(1-35) in the systemic circle. In fact, the thioether bond formed between the acetyl group and the PEG molecule can be cleaved by blood esterases (Fig. 2.4 B).

A)



B)

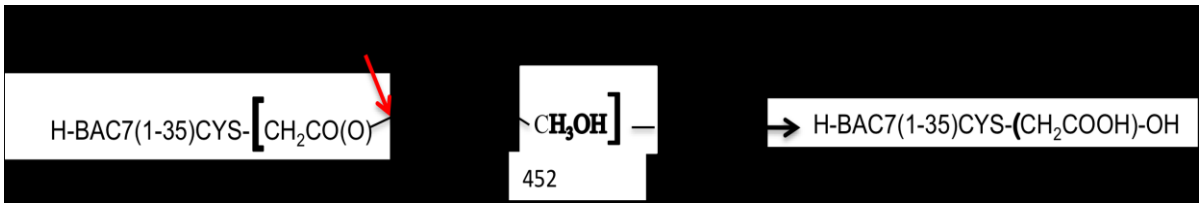


Fig. 2.4. A) Scheme of the synthesis of the conjugate between Bac7(1-35) and acetyl-PEG20k-metoxy via a cysteine residue by using the thioether ligation strategy. B) The arrow indicates the cleavable bond that can be hydrolyzed by esterases.

The pegylated Bac7(1-35) compound was purified following the same procedures used previously and the purity of Bac7(1-35)-CAM-PEG20k was checked by SDS-PAGE (Fig. 2.5 B).

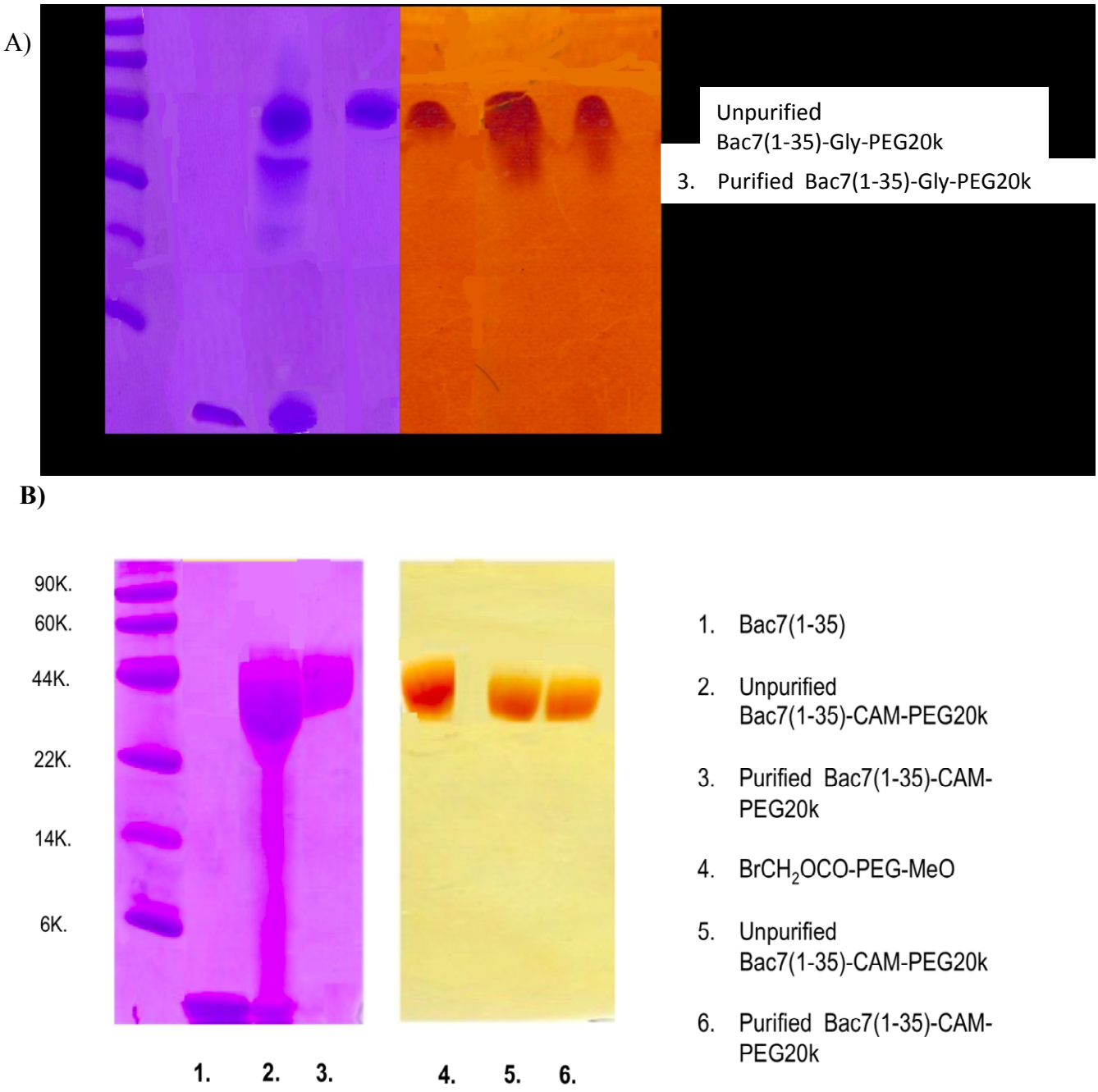


Fig. 2.5. Evaluation of the purity of the conjugates Bac7(1-35)-Gly-PEG20k (A) and Bac7(1-35)-CAM-PEG20k (B). Tricine SDS PAGE of the pegylated peptide before and after purification. The gel on the left is stained with Comassie Blue to detect the band corresponding to the peptide; the gel on the right is stained with Iodine solution to detect the band corresponding to PEG. For each sample, 3 μg of peptide were loaded.

This strategy allowed to obtain a pegylated Bac7(1-35) with a high yield of reaction

(~ 70%). As can be inferred from Fig. 2.5, the presence of non-reacted free peptide in the unpurified sample suggested that the reaction conditions were not yet fully optimized. Published data underline the importance of pH in the thioether ligation reaction (Tsutsumi *et al.* 2000). Taking into account this aspect, in a recent preparation a yield of ~ 99% of the desired product was obtained.

MIC assays, both in culture medium alone and in the presence of human and murine serum or plasma, were then performed to evaluate the antimicrobial activity of purified Bac7(1-35)-CAM-PEG20k. The results showed that the compound maintained a good antibacterial activity, although its potency was lower than that of unconjugated Bac7(1-35) (Table 2.2).

Table 2.2. Antimicrobial activity of Bac7-PEG complexes against *S. enterica* in the presence or absence of human and murine biological fluids.

	MIC (μM) *S.enterica* ATCC 14028				
	Human		Murine		Mueller-Hinton
	Plasma	Serum	Plasma	Serum	
BAC7(1-35)	0.06-0.12	0.5	32	64	0.5
Bac7(1-35)-CAM-PEG20k	2	8	32	64	8
BAC7(1-35)-CAM	0.25	1	64	64	1
BAC7(1-35)-CAM-PEG20k 48 h*	0.25	1	nd	nd	1

MIC values of Bac7(1-35), Bac7(1-35)-CAM and Bac7(1-35)-CAM-PEG20k towards *S. enterica* (10^5 CFU/ml) were determined in the presence of 25% murine serum and plasma, human serum and plasma, diluted in Mueller Hinton broth, or in 100% Mueller Hinton. * incubated for 48 h in serum or plasma before the MIC assay. Data shown are representative of three indipendent experiments with comparable results. Nd: not determined.

To further investigate on the efficacy of Bac7(1-35)-CAM-PEG20k, the MIC values against *S. enterica* in MH broth alone and in the presence of blood components were determined with the released form Bac7(1-35)-CAM from PEG molecule obtained after blood proteases activity (Fig.2.4 B). It showed an activity comparable to that of the unmodified Bac7(1-35) (table 2.2). The following step was to test the kinetics of

release of Bac7(1-35)-CAM from the complex with PEG: the conjugate was incubated with human serum or plasma up to 72 h and the product analyzed by Western blot (Fig. 2.6).

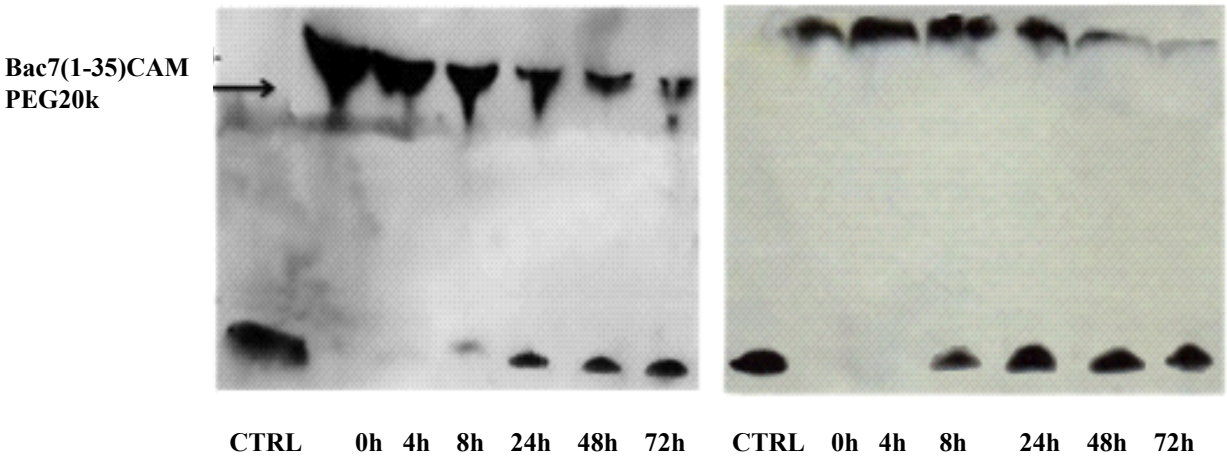


Fig. 2.6. Evaluation of Bac7(1-35)-CAM release from PEG conjugates in the presence of human biological fluids. Western blot analyses of Bac7(1-35)-CAM-PEG20k in human serum (right panel) and human plasma (left panel). Bac7(1-35)-CAM-PEG20K was incubated in 25% human serum or plasma up to 72 h at 37 °C. Samples were then loaded on the gel to allow peptide detection with a specific anti-peptide antibody.

In human serum the release of Bac7(1-35)-CAM was already detectable after 8 h-incubation and was almost complete at 72 h. The band corresponding to the released peptide increased along time in spite of the peptide degradation process that is detectable in human serum from 48 h of incubation (see section 1.2 of the results).
These results indicate that the kinetics of Bac7(1-35)-CAM release in human serum is slow as in human plasma and proteolytic degradation seemed to be negligible (Fig. 2.6).
In order to verify whether the released product had an antimicrobial potency comparable to that of synthetic Bac7(1-35)-CAM, microdilution susceptibility assays were repeated with Bac7(1-35)-CAM-PEG20K after 48 h incubation in 25% human serum or plasma. The MIC value under these conditions improved 8-fold with respect to that of the non-preincubated, pegylated peptide, reaching the same potency exerted by Bac7(1-35)-CAM. This indicates that the peptide was completely released during the incubation time.

Overall, these results indicate that an active peptide can be released from Bac7(1-35)-CAM-PEG20K by exposure of the conjugate to blood components for 48-72 h, a period of time in which the degradation of the peptide is negligible.

2.3 *In vivo* Bac7(1-35)-CAM-PEG20K biodistribution

To better understand the advantages of Bac7(1-35) pegylation via thioester ligation, *in vivo* biodistribution analysis were performed. To this purpose a derivative of Bac7(1-35)-CAM-PEG20k, fluorescently labelled with the dye Alexa680 has been recently synthesized. Two chemical strategies were followed to maintain the same conformation of the Bac7(1-35)-CAM-PEG20k molecule: the PEG molecule was linked to Bac7(1-35) via native chemical ligation while the Alexa Fluor680© with a maleimide group was linked to the thiol group of the cysteine residue present between Bac7(1-35) and CH_2COO-PEG-OMe (Fig. 2.7).

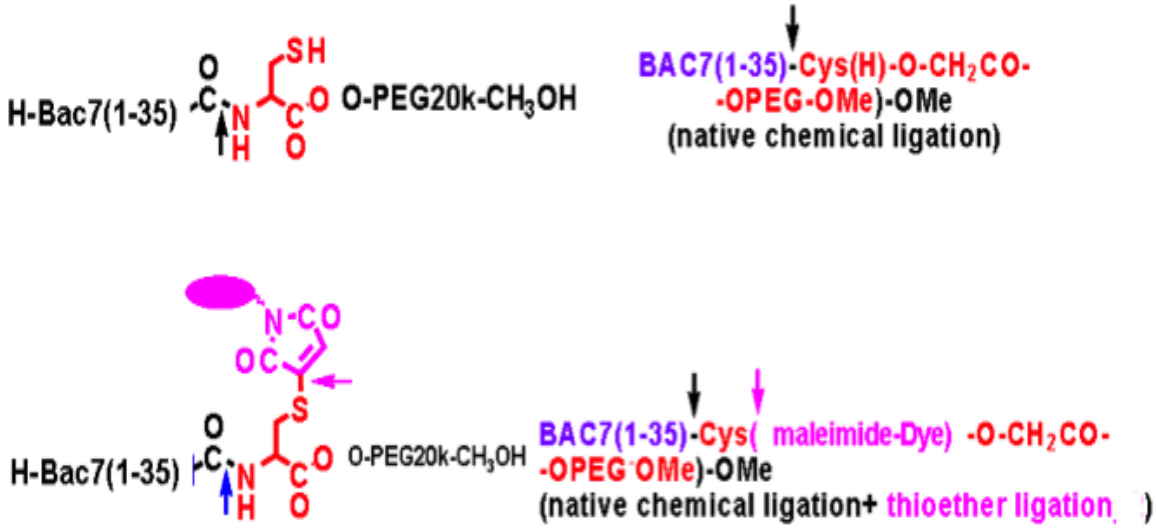


Fig. 2.7. Scheme of the synthesis of the conjugate between Bac7(1-35) and acetyl-PEG20k-metoxy via a cysteine residue by using the native chemical ligation strategy followed by thioether ligation strategy. The obtained compound was then labelled with maleimide-Alexa FLUOR680©.

Biodistribution analyses in healthy mice were performed as previously described for Bac7(1-35)-Cys_{36}Alexa680, by monitoring the availability of the labelled, pegylated peptide up to 24 h after its intraperitoneal administration and performing *ex vivo* analysis at 24 h. Preliminary results showed that the pegylated peptide had a much wider distribution than the unpegylated one and was detected in different organs (Fig. 2.8 A and B). Furthermore, it was still present at 24 h post-injection (Fig. 2.8 B), showing a slower renal clearance compared to the unmodified peptide (Fig. 1.5). *Ex vivo* analysis showed a significant concentration of the pegylated Bac7(1-35)-Alexa680 in liver even at 24 hours after injection, in addition to kidney (Fig. 2.8 C).

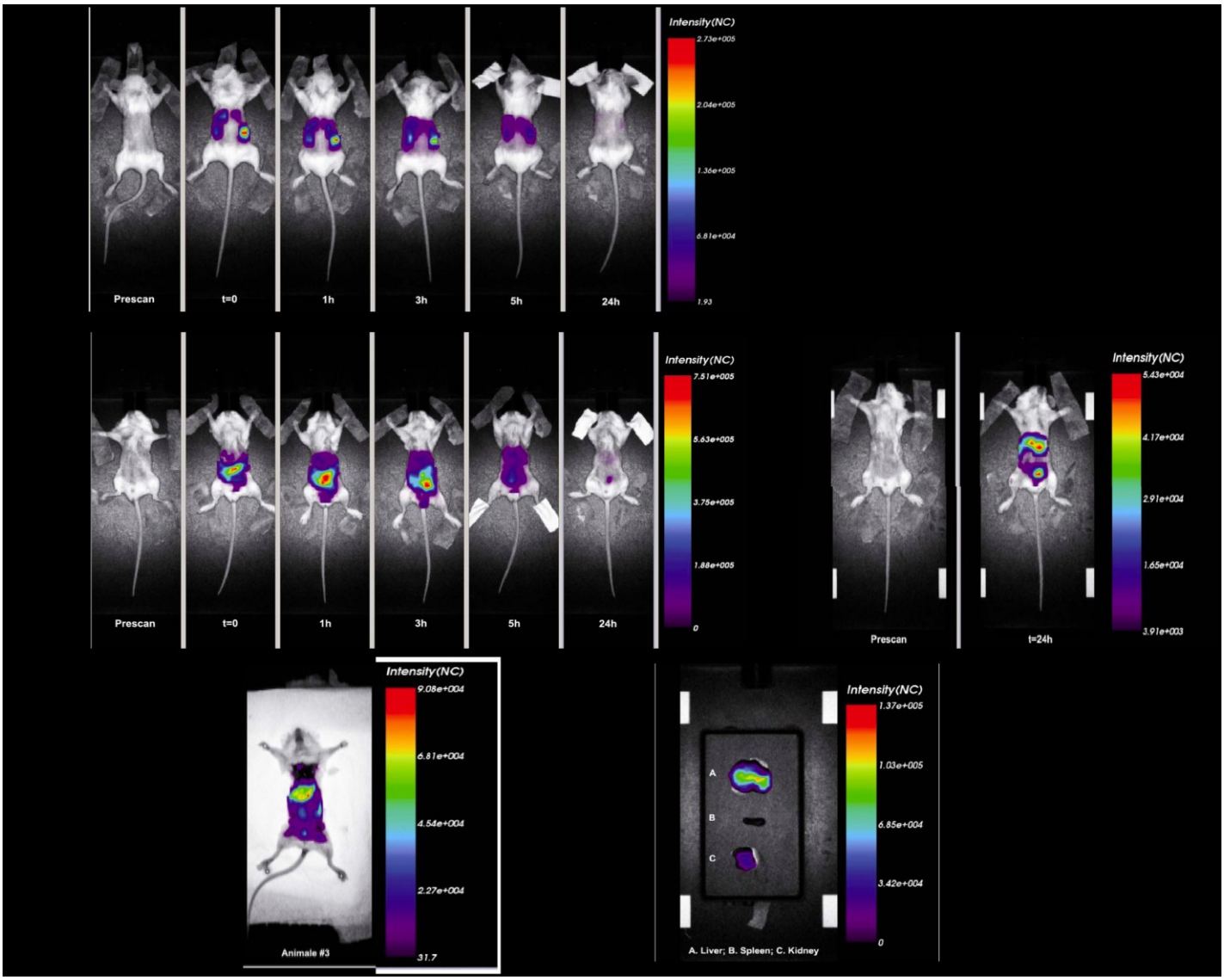


Fig. 1.5 Biodistribution of Bac7(1-35)-CAM(Alexa680)PEG20k in healthy mice after i.p. injection. The animals were placed in prone position (**A**) and supine position (**B**); fluorescence emission in the regions of interest encompassing respectively the kidneys, the thorax and abdomen, were acquired at indicated times post-injection. The panel B on the light shows the fluorescence emission of the mouse in supine position at 24 h post injection and normalized with respect to the prescan acquired before peptide injection. (**C**) *Ex vivo* images: post-mortem images of mice at 24 hours after i.p. injection of PEGylated Bac7(1-35)-Alexa680. Imaging of the mice was performed immediately after sacrifice, after removal of the abdominal wall and normalized. A specific signal was detected in kidney, liver and peritoneal cavity (panel on the left); *Ex vivo* images of organs at 24 hours after i.p. injection of PEGylated Bac7(1-35)-Alexa680. Imaging of the organs was performed immediately after sacrifice and normalized. A specific signal was detected in kidney and liver. No specific signal was detectable in spleen**.** The images are representative of one of three mice analysed that showed comparable results.

3. EFFECTS of Bac7(1-35) ON CELLS OF THE HUMAN IMMUNE SYSTEM DURING *Salmonella typhimurium* INFECTION

The aim of this part of the study was to analyze the effects of Bac7(1-35) on the interaction between cells of the immune system and *S. typhimurium*, trying to mimick what happens during an *in vivo* infection. To this purpose, experiments were carried out setting up a cellular model of *Salmonella* infection to evaluate *i)* the effect of the peptide on phagocytosis after pre-treatment of bacterial or of phagocytic cells with Bac7(1-35); *ii)* the effect of externally added peptide on survival of *S. enterica* phagocytized by human macrophage-like cells.

3.1. Cytotoxicity of Bac7(1-35) on PMA-differentiated U937 cells

Before investigating the effects of Bac7(1-35) on biological activities of phagocytic cells, we determined the maximum concentrations of peptide that could be used on cell lines without significant toxic effects. Previous studies performed by Zanetti and co-workers, showed that Bac7(1-35) is not toxic to a fibroblast cell line after 60-min incubation up to 50 μM concentration (Tomasinsig *et al.* 2006). Following these observations, the viability of PMA-differentiated U937 cells was tested after a long-term treatment (24 h incubation) using 10, 30 and 50 μM Bac7(1-35). For this assay we used a combination of two fluorescent probes, DiOC6 and PI, to investigate the type of cell response induced by the peptide. The former probe measures the mitochondrial transmembrane potential and is used to detect apoptotic cells, while the latter detects permeabilized, necrotic cells. Results indicated that treating the cells with 50 μM peptide for 24 h decreased the cell viability mainly inducing necrosis in half of the cellular population; in contrast, the percentage of damaged cells was comparable to that of the control at lower peptide concentrations (Fig. 3.1).

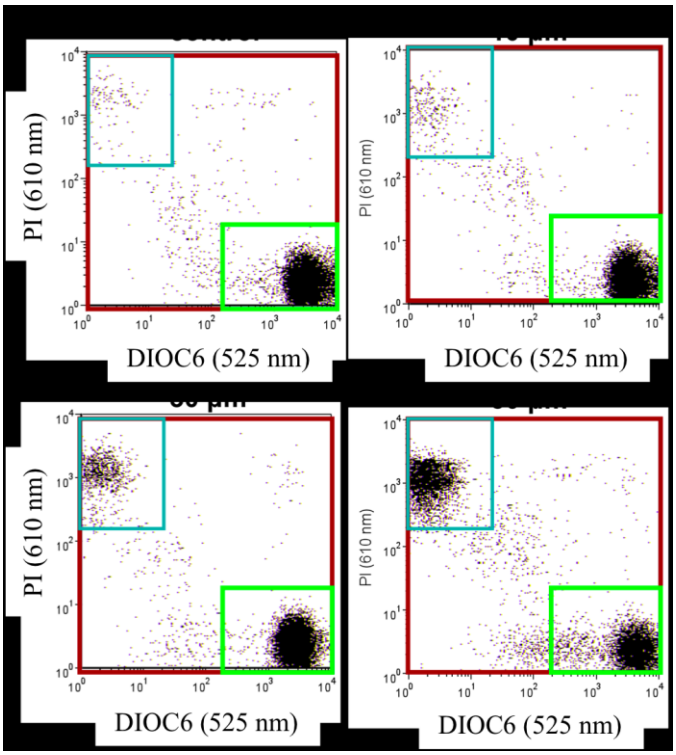
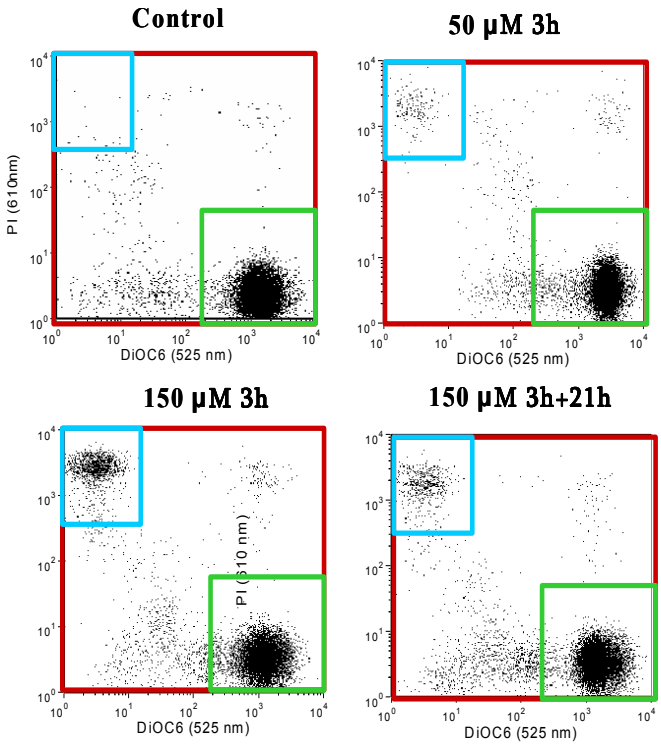


Fig. 3.1 Flow cytometric analysis of PMA-differentiated U937 cells treated for 24 h with different concentrations of Bac7(1-35). Bi-parametric dot plot of U937 cell red fluorescence due to Pi uptake vs green fluorescence due to $DiOC_6$ uptake. The dyes $DiOC_6$ and PI were added before analysis. Normal, apoptotic and necrotic cells are respectively enclosed in boxed regions with green, red, and cyan colours.

However, considering the intracellular antimicrobial activity of Bac7(1-35), up to 50 μM the peptide did not exert any effect on phagocytosed salmonellae. On the light of these considerations, two concentrations of Bac7(1-35) were chosen for the following assays (after 3 h of peptide-treatment, or 21 h later after peptide removal by cell washing) 50 and 150 μM and the incubation time that maintains cell viability at these concentrations was investigated. The results showed that after 3 h of treatment with 50 μM peptide only 9 % of the cells were damaged compared with the 6% of the peptide-untreated control; conversely, treatment of the cells with 150 μM peptide significantly increased the percentage of necrotic cells (12% *vs* 2% of untreated cells,) indicating that the peptide starts to exert a not negligible toxicity at this higher concentration.

A)



B)

	Apoptotic cells (%)		Necrotic cells (%)	
	3 h	**3 h+21h**	**3 h**	**3h+21h**
no peptide	4±2	8 ± 3	2.2±1	3.83±3
50 µM Bac7(1-35)	6 ±3	7.5±2	3.2±1	3.5±2
150 µM Bac7(1-35)	8.5±4	14±4	12±4 (*)	5±2

Fig 3.2. Flow cytometric analysis of U937 cells treated with Bac7(1-35) at high concentration (A) Bi-parametric dot plot of fluorescence emission by U937 cell due to Pi uptake (red fluorescence) vs $DiOC_6$ uptake (green fluorescence). U937 cells were treated for 3 h with 50 or 150 µM Bac7(1-35) and analysed immediately thereafter or after additional 21 h of incubation following peptide removal by cell washing. The dyes $DiOC_6$ and PI were added before analysis. Normal, apoptotic and necrotic cells are respectively enclosed in green, red, and cyan regions. (**B**) Percentages of necrotic and apoptotic cells in peptide-untreated controls and after treatment with 50 and 150 µM peptide for 3 h without or with further 21 h incubation after peptide removal by washing. Statistical analysis carried out by the T-*student* test: $p < 0.05$ (*) treated *vs* untreated cells.

When the cells were analyzed 21 h later after removing the peptide by washing, no

further increase in the percentage of dead cells was detected by expositing the cells to both 50 and 150 μM peptide (Fig. 3.2). This indicates that the toxic effect of Bac7(1-35) does not increase upon removal of the peptide.

3. 2 Bac7(1-35) internalization into monocytic cell lines

To investigate the capacity of Bac7(1-35) to inhibit the growth and kill intracellular salmonellae, we first evaluated the efficiency of peptide internalization and its cellular localization into the human U937 monocytic cell line. Alexa-tagged Bac7(1-35) was added at a concentration of 1 μM to cultures of PMA-differentiated U937. After incubation for 3 h the cells were then examined by confocal laser scanning microscopy. Bac7(1–35) appeared to diffuse in the cytoplasm as well as in the nucleus (Fig 3.3).

Confocal analysis performed with Bac7(1-35)-ALEXA680 These analyses indicated that the peptide was able to penetrate the cells showing a diffused distribution in the cell soma (Fig. 3.3).

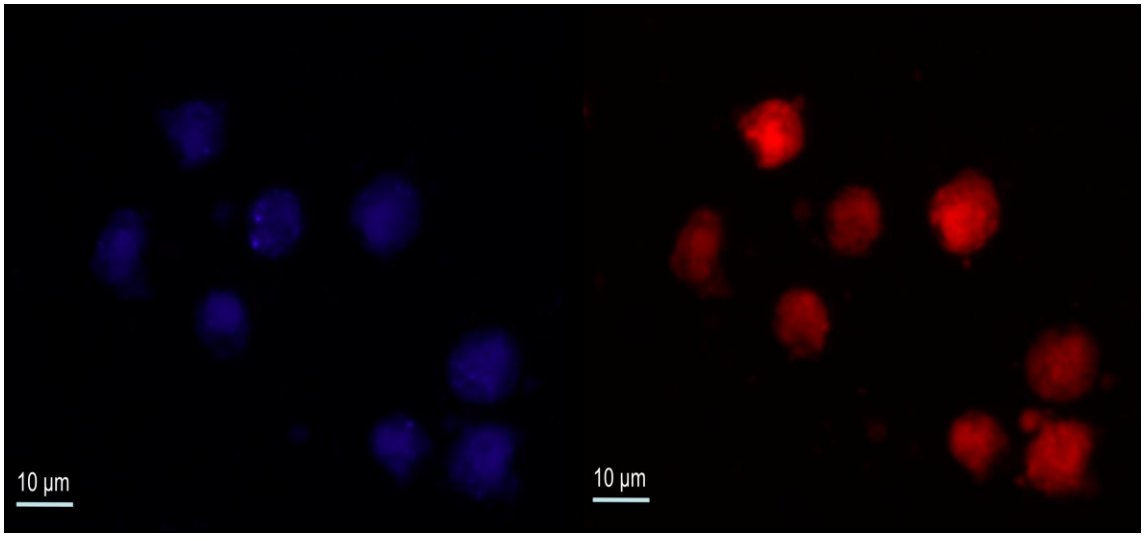


Fig. 3.3. Confocal analysis: Bac7(1-35) distribution into U937 cells.
Confocal images of U937 cells showing that Bac7(1-35)-ALEXA is internalized and homogenously diffused. The cells (blue) were first stained with FAST DiI solution to decorate the cytoplasmic membrane and than treated with Bac7(1-35)-ALEXA (red) for 3 h.

Internalization of fluorescently-labelled peptide (in this case by conjugation with BODIPY) by U937 cells was further analyzed by flow cytometry. Addition of the quencher trypan blue, which does not penetrate within intact cells, just before analysis confirmed that 60% of the cell-associated fluorescence was not quenchable and thus due to intracellular, non-exposed peptide. The remaining 40% of the fluorescence signal was extracellular, as indicated by its quenching on addition of trypan blue (Fig. 3.4).

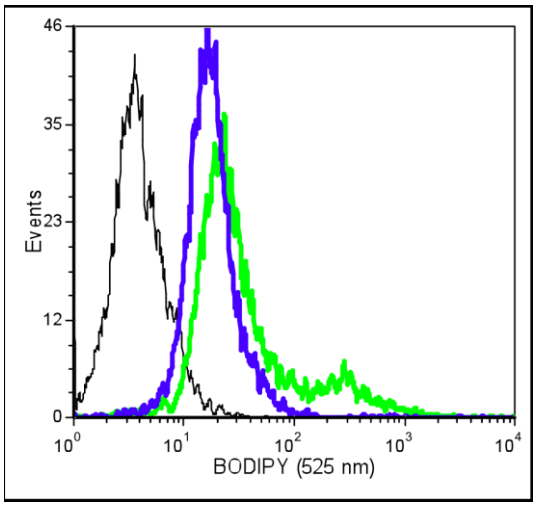


Fig. 3.4. Bac7(1-35) uptake into U937 cells.
Flow cytometric analyses of the cell-associated fluorescence, which indicates the presence of Bac7(1-35)-BODIPY. The peptide was added to the cells for 3 h and then the samples were treated (blue trace) or not (green trace) with 1 mg/ml Trypan Blue, which quenches extracellular fluorescence. The auto-fluorescence of the cells is indicated by the black trace. The reported data are representative of two experiments with comparable results.

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3.3 Effect of Bac7(1-35) on phagocytosis

To investigate if Bac7(1-35) can modulate the phagocytic uptake of bacteria by macrophages, a set of phagocytosis assays was carried out by using a *S. enterica* strain expressing the green fluorescent protein (GFP), which allows detection of the engulfed bacteria. First of all, we determined the bacteria to cell ratio (MOI) giving the best number of phagocytosing cells, as evaluated by flow cytometric analysis. Despite the high variability of the cell response among different experiments, at MOI 50 the number of cells phagocytosing varied from 20% to 50%, while at MOI 100 it ranged from 25 to 70%, but at this MOI the amount of engulfed bacteria was approaching a plateau (Fig. 3.5 A). Taking into account the mean intensity of fluorescence per phagocytosing cell as a parameter of the number of bacteria engulfed, it appears that at MOI 100 the fluorescence intensity increased only slightly compared to the intensity of fluorescence detected at MOI 50 (Fig. 3.5 B). For this reason, a MOI 50 was chosen as bacteria to cells ratio for the subsequent studies.

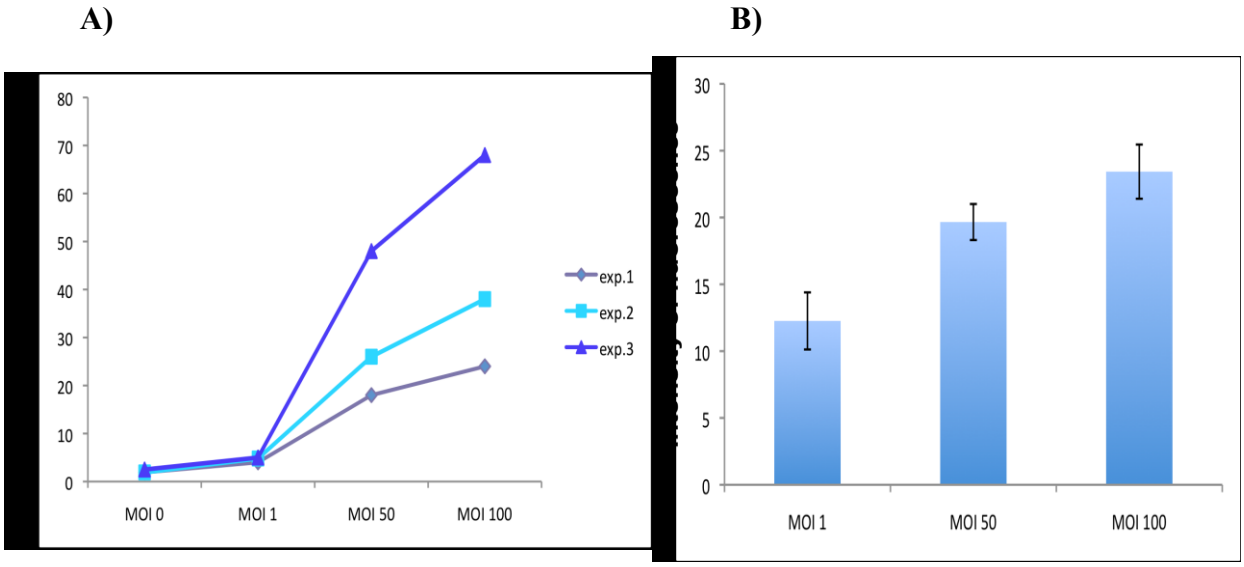

Fig. 3.5. Percentages of phagocytosing cells (A) and amount of engulfed bacteria (B) at different bacteri to cells ratio (MOI). Bacteria were left in contact to U937 cells for 2 h to allow phagocytosis (1 h plus 1 h in the presence of gentamicin, to kill non-phagocytosed bacteria). Flow cytometric analysis was then performed. The percentage of phagocytosing cells was determined on the basis of specific morphological parameters and by measuring the fluorescence due to the engulfed bacteria expressing GFP. The individual results of three experiments are reported in panel A), while the mean the mean ± SD of three independent experiments is shown in panel B).

The experiments of phagocytosis were carried out either by pre-incubating the cells with Bac7(1-35) followed by addition of untreated bacteria or, *vice versa*, by pre-incubating the bacteria with the peptide followed by addition to the PMA-differentiated U937 cells.

The results showed that pre-treatment of macrophages with Bac7(1-35) led to an increase of the percentage of phagocytosing cells and of the number of bacteria engulfed per cell. The percentage of cells involved in phagocytosing was 48% after pretreatment with peptide, significantly higher with respect to peptide-untreated cells (36%). In this set of experiments it was also observed a slight increase (+14%) in the number of engulfed bacteria per cell (Fig. 3.6).

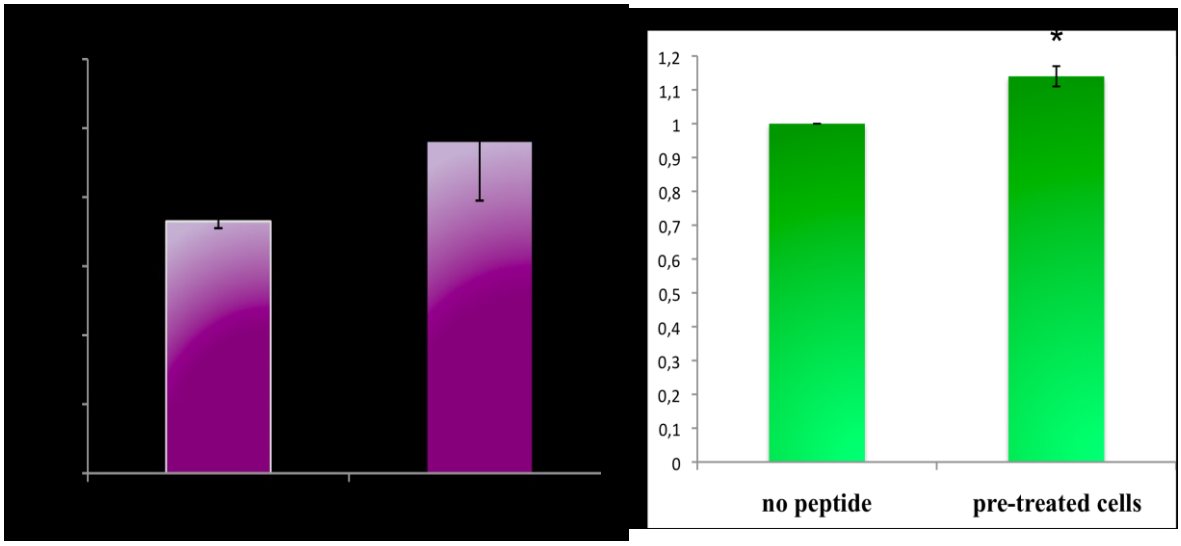

Fig. 3.6. Evaluation of the effect of Bac7(1-35) on pre-treated U937 cells on *S. typhimurium* phagocytosis

U937 cells were pre-treated for 1 h with 50 μM Bac7(1-35). Peptide-treated cells were then challenged with untreated bacteria at MOI 50. The cells were collected for flow cytometric analysis. The percentage of phagocytosing cells and the number of engulfed bacteria were determined as described above. The mean ± SD of three independent experiments is reported. Statistical analysis was carried out with the T-*student* test: p <0.05(*); 0.05> p >0.01 (**) for treated *vs* untreated cells.

In contrast, pre-treatment of bacteria with Bac7(1-35) at sublethal concentrations for 1 h, led to a lower percentage of phagocytosing cells (-15%) also accompanied by a decreased amount of engulfed bacteria (Fig. 3.7).

These results suggest that Bac7(1-35) added to PMA-differentiated U937cells has a positive effect on phagocytosis of salmonellae. Future studies are necessary to understand the mechanism responsible for this effect. In contrast, pretreatment of bacteria with the peptide makes them less suitable to be phagocytosed by the same cells.

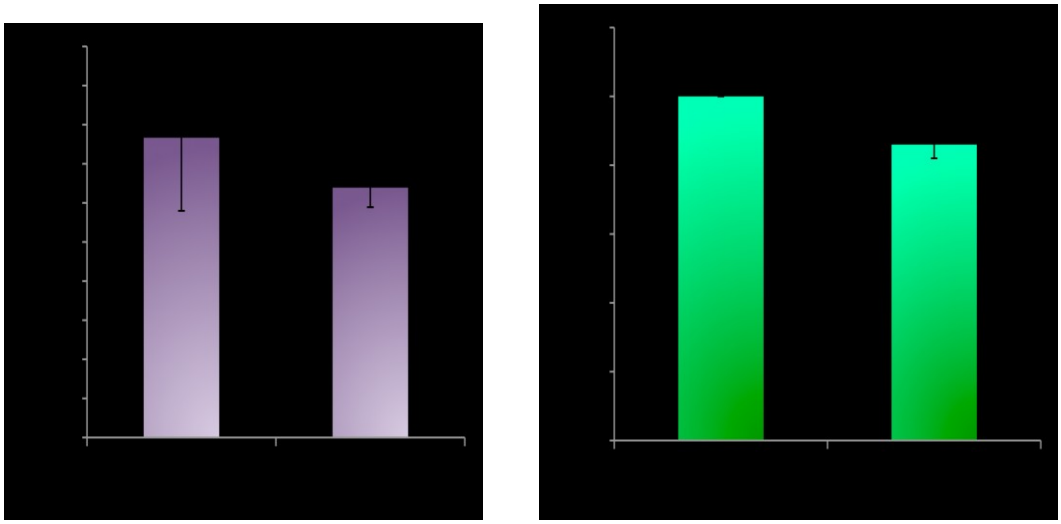

Fig. 3.7. Evaluation of the effect of Bac7(1-35)-pretreatment of *S. typhimurium* on phagocytosis by U937 cells. *Salmonella* cells were pre-treated for 1 h with 0.5 μM Bac7(1-35). Peptide-treated bacteria at MOI 50 were then added to untreated cells. At the end, gentamicin was added for 1 h to kill extracellular bacteria. The cells were then collected for flow cytometric analysis. The percentage of phagocytosing cells and the number of engulfed bacteria were determined as described above. The resulta are the mean ± SD of three independent experiments. Statistical analysis was carried out with T-*student* test: $p < 0.05$(*); $0.05 > p > 0.01$ (**) treated *vs* untreated cells.

3.4 Exogenously added Bac7(1-35) inhibits intracellular *Salmonella* survival and replication

We then investigated the effects of exogenously-added Bac7(1-35) on survival and replication of bacteria which had infected macrophage-like cells. To this aim, PMA-differentiated U937 cells were first incubated for 1 h with cultures of *S. typhimurium* cells (MOI 10) to allow phagocytosis, before the addition of gentamicin for 1 h to kill non-phagocytosed bacteria. Then PMA-differentiated U937 cells were exposed to non-toxic concentrations of Bac7(1-35) to evaluate its capacity to reduce the number of intracellular vital bacteria. Preliminary experiments, carried out by using fluorescent GFP-expressing bacteria and Bac7(1-35)-ALEXA680, showed that bacteria were present in the cells and that the peptide had a diffused localization within the cells and could co-localize in the same cellular compartments, as established by confocal microscopy analyses (Fig. 3.8). The number of intracellular bacteria was counted by lysing the cells after 3 h incubation with 50 μM Bac7(1-35). As shown in Fig. 3.9 (panel A), the peptide caused a 43% reduction of viable bacteria (CFU/ml) rescued

from the lysed cells compared to cells not exposed to the peptide. This effect was more marked after an additional 21 h incubation of the cells after removal of the external peptide by washing (Fig. 3.9 panel B). When the peptide concentration was augmented to 150 μM, a dose with initial toxic effects (see fig. 3.2) the reduction of viable bacteria rescued from the U937 cells was even higher.

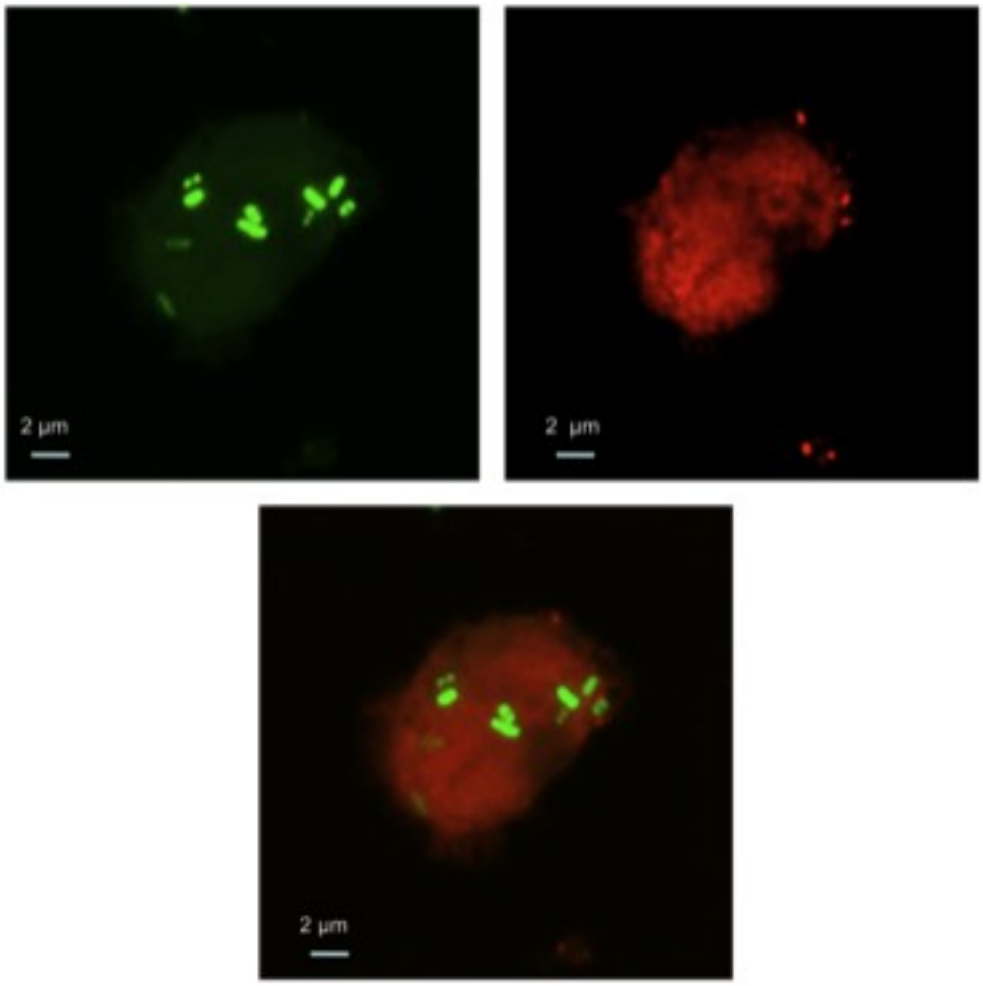

Fig. 3.8 Co-localization of *S. enterica*-GFP and **Bac7(1-35)-ALEXA680 in U937 cells.** Confocal fluorescence microscopy of *S. enterica*-GFP (green, upper left), Bac7(1-35)-ALEXA (red, upper right), in U937 cells and image of the merged two signals (lower right).

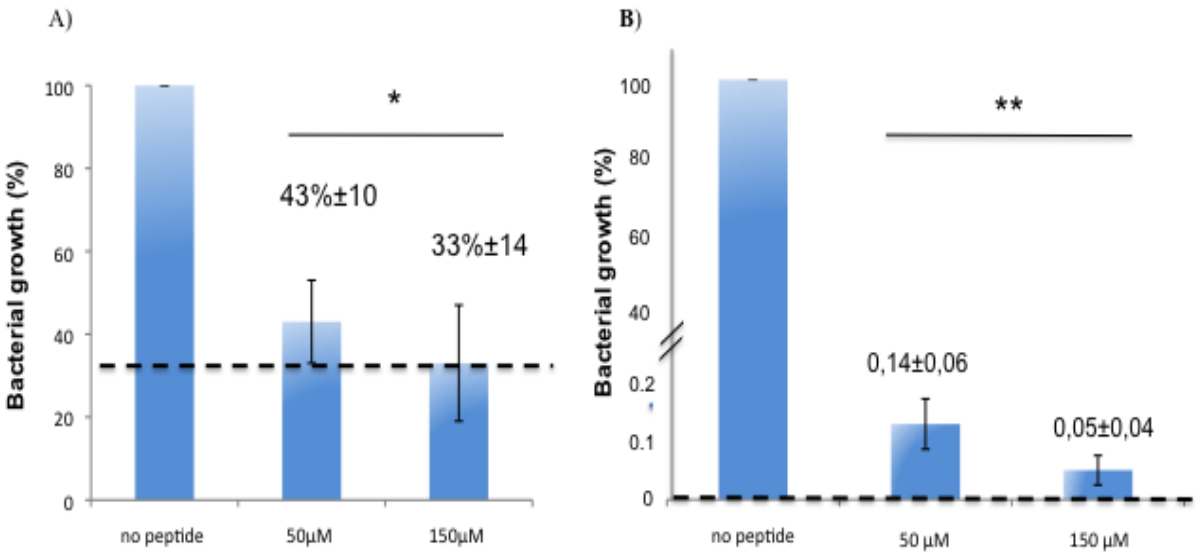

Fig. 3.9. Effect of Bac7(1-35) on intracellular survival and growth of *Salmonella* in U937 cells. U937 cells were infected with *S. typhimurium* at a MOI of 10 for 2 h (the second hour in the presence of gentamicin to kill extracellular bacteria), and then incubated with Bac7(1-35) at 50 or 150 µM for 3 h (A) and for additional 21 h (B) after removal of the peptide. At the end, the cells were collected, counted, lysed and the lysate plated to count the number of viable bacteria. The percentage of *Salmonella* surviving in peptide-treated cells is referred to the value found in control, peptide-untreated cells taken as 100% of survival/growth. The horizontal dashed line indicates the percentage of intracellular viable bacteria after 2 h of phagocytosis, taken as 100%. Results are mean ± SD of six independent experiments. Statistical analysis was carried out with the T-*student* test: p <0.05(*); 0.005> p >0.01 (**) for treated *vs* untreated cells.

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The same type of assay was repeated using primary cells derived from human monocytes (MDM*)* differentiated with *Salmonella* LPS. The results obtained were similar to those with the U937 cells: the number of viable intracellular bacteria was reduced by 60% with respect to peptide-untreated control after 3 h of cell treatment with the peptide (Fig. 3.10, panel A). Additional 21 h of treatment after extracellular peptide washing, showed that *Salmonella* cells replicated considerably in untreated cells, while the bacterial growth in Bac7-(1-35) treated cells was almost completely inhibited at both concentrations tested (Fig. 3.10 panel B).

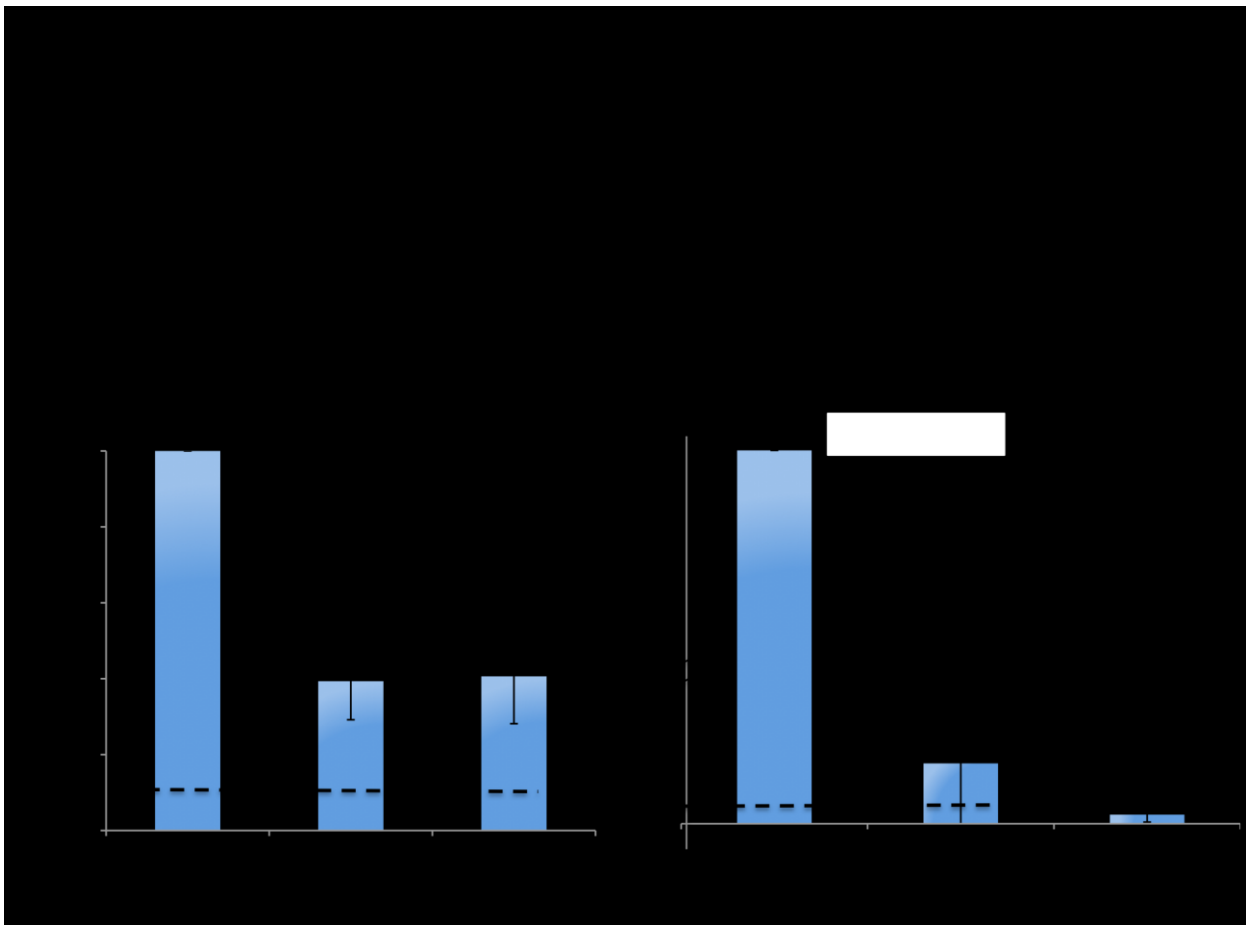

Fig.3.10. Effect of Bac7(1-35) on intracellular survival and growth of *Salmonella* cells in macrophages derived from monocytes (MDM)

MDM cells were infected with *S. typhimurium* at a MOI of 10 and the experiment was performed as described above. The percentage of *Salmonella* surviving in peptide-treated cells is referred to the value found in control, peptide-untreated cells taken as 100% of survival/growth. The horizontal dashed line indicates the percentage of intracellular viable bacteria after 2 h of phagocytosis, taken as 100%. Results are the mean ± SD of three independent experiments. Statistical analysis was carried out with the T-*student* test: $p < 0.05$ (*) for treated *vs* untreated cells.

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These results suggest that Bac7(1-35), during *Salmonella* infection of pahgocytic cells, can penetrate the cells and exert its antibacterial activity once inside them.

DISCUSSION

1. THERAPEUTICAL POTENTIAL OF BAC7(1-35) IN A MOUSE MODEL OF *S. ENTERICA* INFECTION

The starting point for the development of a new class of drugs is the identification of a lead compound that is significantly effective not only *in vitro* but also *in vivo* in specific animal models. In this respect, AMPs have the potential to be developed into a new class of clinically useful anti-infective agents. However, in spite of their potent *in vitro* activity and modest or nul propensity to select resistant mutant strains (Nizet *et al.* 2006, Zhang *et al.* 2005), many AMPs perform poorly in *in vivo* models of infection.

In view of the promising results obtained by the *in vitro* antibacterial activity of the bactenecin fragment Bac7(1-35) against several Gram-negative bacterial strains, including clinical isolates (Benincasa *et al.* 2005), here its activity was also evaluated in a more physiological context. In order to mimic the blood environment, antimicrobial assays were performed in the presence of serum or plasma. Serum and plasma from both mice and humans were diluted in the culture medium up to 25% (v/v), so as to use an intermediate condition with respect to the full potential of these fluids to inhibit the peptide activity (Powell *et al.*1992). Results showed that in the presence of murine blood components, Bac7(1-35) partially loses its antibacterial activity and its inactivation is more rapid in serum than in plasma. In an attempt to identify the inhibiting factor, we hypothesized that albumin could have a role in sequestering Bac7(1-35). In fact, it is well documented that albumin is able to bind antibiotics reducing their potency *in vivo* by decreasing their effective concentration (Krag-Hansen 1990; Sudlow *et al.* 1978). Time-killing assays performed in the presence of bovine serum albumin, which is homologous to the human albumin (Gelamo *et al.*2002) and has a high sequence similarity in the drug binding site I with the rat serum albumin (Kosa *et al.*1997), demonstrated that this is not the case, as the activity of Bac7(1-35) was not inhibited. This indicates that the peptide binds very weakly or not at all to serum albumin. We then investigated the role of proteolytic degradation as an event that could inactivate the peptide. Blood is rich of proteases with trypsin-like activity that can cleave at Arg residue like those present in the Bac7(1-35) sequence. In addition, amino- and carboxyproteases, which are also present in blood, can cleave the peptide at the N- and C-termini, respectively.

Incubation of Bac7(1-35) with murine serum and plasma demonstrated that the peptide is in fact degraded in both fluids, with the former showing a more rapid inactivation, as expected from a fluid in which the proteases of the coagulation cascade are activated.

It is worth noting that the effects of human blood components on the peptide are quite different from those of mouse. In fact, Bac7(1-35) maintains most of its activity in both human serum and plasma. This is due to a greatly reduced degradation of the peptide. In fact, as shown by Western blot analysis, the peptide is still present after 72 h of incubation in human fluids. This is confirmed by LC-MS analyses, which showed that the peptide degradation kinetics in the human system is slower than in murine fluids. This difference between human and mouse fluids could be explained by differences in activity and specificity of the proteases there present (Lin *et al.* 2005).

The high stability of Bac7(1-35) in human serum and plasma for several days is interesting also in the light of the behaviour of other AMPs in these media. Human β-defensin hBD3 shows a reduced antibacterial activity in the presence of serum proteins and is partially degraded by human serum proteases after only 90 min of incubation (Maisetta *et al.* 2008); the cathelicidin-peptide LL-37 is also inhibited by human serum (Johansson 1998), and the insect antibacterial peptide drosocin is completely degraded within 4 h in both diluted human and murine sera (Hoffmann *et al.* 1999). Similar results were also obtained with pyrrhocoricin, an insect AMP that is degraded relatively quickly by some batches of human serum (Hoffmann *et al.* 1999).

An unexpected observation is that the activity of Bac7(1-35) seems to be potentiated in human plasma. This effect, although less evident, was also observed, with other peptides such as BMAP-27, which has a membranolytic mode of action, and with two shorter fragments of Bac7. This observation suggests that this improvement in the activity of structurally different peptides is not specific and probably is related to some antibacterial component contained into the plasma, whose activity is detectable only in synergy with the exogenously added AMP. For example, the human plasma contains the platelet microbicidal proteins (PMPs) (Yeaman *et al.* 1999), which could in part be released during the plasma preparation (Tammen *et al.* 2005) and then potentiate the antibacterial activity of the AMPs tested. Taken together, these results were encouraging and prompted us to carry out some *in vivo* studies for a

first evaluation of the therapeutic potential of Bac7(1-35).

Before testing the protective effect of the peptide in an animal model of infection, its acute toxicity was investigated in mice. Bac7(1-35) shows an LD_{50} of 105 mg/Kg, and is much less toxic than cathelicidin-derived helical peptides such as BMAP-27 and -28 (LD_{50} of 10-15 mg/kg) (Benincasa *et al.* 2003). In this respect it behaves similarly to other proline-rich AMPs, such as pyrrhocoricin (LD_{50} of 50 mg/kg) and drosocin (LD_{50} of 100 mg/kg) (Otvos 2002). This result indicated that the peptide can be used safety in the host even at high concentrations.

The *in vivo* antibacterial activity of Bac7(1-35) was tested in a mouse model of infection. To this aim, we used a protocol based on intraperitoneal injection of *S. enterica*, which gives an infection resembling a typhoid fever in humans (Santos *et al.* 2001). The CBA/CA strain of mice was preferred to the more common strain Balb/c because of the extremely high susceptibility of the latter to *Salmonella* infection, due to lack of response by resident macrophages (Takahashi *et al.*1990). Nevertheless, the infection revealed to be very aggressive also in the CBA/CA host, since an injection of as few as 10^2 CFU/ml of *Salmonella* caused the death of 50% of the animals in 10 days.

Bac7(1-35) at a dose of 30 mg/kg showed a significant level of protection, prolonging the mean survival time of the treated mice and completely curing 1/3 of them. In addition, the peptide significantly reduced the amount of viable bacteria in the liver and spleen of infected mice.

Subsequently, a preliminary pharmacokinetic study was performed using a fluorescent-derivative of Bac7(1-35) with an antibacterial activity comparable to that of the unlabelled peptide, and using the Time-Domain Optical Imaging technique. Optical Imaging analysis in mice suggested that the protective role showed by the peptide *in vivo* is not due to its systemic diffusion but most probably by its direct action in the peritoneal cavity. In fact, due to its low molecular weight, the peptide is rapidly excreted through the renal route, greatly decreasing its systemic availability. The conjugation of the peptide with the fluorescent dye Alexa to allow biodistribution analyses did not significantly increase its molecular weight and presumably does not interfere significantly with its biodistribution and efficacy. It is well known that mice eliminate drugs through kidney much more quickly than humans (Mathe *et al.* 2006). As no nefrotoxic compounds causing renal dysfunction were used to alter pharmacokinetic parameters (Mathe *et al.* 2006), the very rapid

clearance of the peptide may likely have limited its activity against pathogens after injection in the animals.

However, in spite of the drawbacks of the decreased activity of Bac7(1-35) in mouse blood and the rapid renal clearance, the *in vivo* results obtained are promising and indicate that the antibiotic activity of Bac7(1-35) might be much higher in animal infection models less aggressive than that here used and might be improved by slowing the kinetics of its renal excretion, thereby enhancing its therapeutic index.

2. BAC7(1-35) PEGYLATION TO IMPROVE ITS AVAILABILITY

The half-life of a small peptide can be prolonged by increasing its size. Pegylation of target molecules offers this opportunity; each ethylene oxide unit that composes PEG associates with two to three water molecules, which results in a molecule behaving as if it were five to ten times as large as a polypeptide of comparable molecular weight (Kozlowski *et al.* 2001).

Poly(ethyleneglycol) (PEG) exhibits several properties that are of relevance for pharmaceutical applications (Delgado *et al.*1992, Harris *et al.* 1997): *i)* high water solubility, *ii)* high mobility in solution; *iii)* lack of toxicity and immunogenicity. For these reasons, pegylation is one of the most promising and used strategies to improve the pharmacokinetic behaviour of a drug. A multitude of scientific articles have been published in which the effectiveness of PEG to improve half-life of a drug molecule was clearly demonstrated. For example, mono-PEGylation of GLP-1 (Glucagon-like peptide) led to a 16-fold increase in plasma half-life in rats (Lee *et al.* 2005); PEG-Intron and PEGylated α-interferon, marketed by Schering-Plough and approved by FDA in 2001 for the treatment of hepatitis C, are characterized by a renal clearance of 77 hours (Kozlowsky *et al.* 2001); recently, it has been demonstrated that INF-α-2b exhibited a 330-fold prolonged plasma half-life in rats compared to the native protein (Ramon *et al.* 2005). On the light of these observations, pegylation of Bac7(1-35) was chosen as a tentative solution to improve its *in vivo* availability.

The most used method to link PEG to proteins or peptides takes advantage of reactive electrophiles at the terminal end of methoxy-PEG that are suitable for reaction with nucleophiles in proteins. This is the so-called first generation PEGylation chemistry (Roberts *et al.* 2002), which commonly involves the amino

group at the N-terminus of the proteins. As the antibacterial activity of Bac7(1-35) is dependent on a free N-terminus (Podda *et al.* 2006), it was necessary to carry out the pegylation of the molecule via its C-terminus. To ensure a lower renal excretion of Bac7(1-35) and, at the same time, to maintain a good antibacterial activity, PEG with a molecular mass of 20k was used.

Different strategies of pegylation were pursued to overcome difficulties that have been encountered with some of them. For example, for the high costs of peptide synthesis, it was necessary to set up pegylation strategies that allowed the use of low amounts of Bac7(1-35) and also, at the same time, the possibility to detect small amounts of the conjugate. Each pegylation strategy required several RP-HPLC and SDS-PAGE analyses, to control the reaction efficiency, the purification level and the concentration of the pegylated peptide.

The first strategy adopted was the above mentioned first generation pegylation chemistry via formation of an amide bond involving the C-terminus of Bac7(1-35) and the amino group of an amino-methoxy-PEG. Although a discrete yield of product (30% of the peptide conjugated) was obtained, Bac7(1-35)-PEG20k was active against Salmonella only at concentrations much higher than the peptide alone. The presence of a long polymer chain coupled to the peptide through a very stable bond likely interferes with Bac7(1-35) uptake into bacterial cells, so that its residual antibacterial activity was probably exerted through a secondary lytic mechanism, a mode of action that becomes relevant at peptide concentrations in the range 32-64 μM and that does not require penetration into the cytoplasm. This loss of activity of the peptide was not completely unexpected as it is well documented for other biomolecules after stable covalent pegylation (Veronese 2008).

In order to allow Bac7(1-35) uptake into the bacterial cytosol, the peptide was pegylated via a cleavable bond that can ensure its release from the PEG molecule. The polymer was coupled to Bac7(1-35) via formation of an ester bond potentially cleavable by the esterases that are present in the blood. After a first unsuccessful attempt to form an ester bond with an *ad hoc* added C-terminal glycine residue to enhance the reactivity of PEG, we moved to a different strategy. This was based on the introduction at the C-terminus of the peptide of an additional cysteine residue whose SH group can selectively react by thioether ligation with the acetyl group of a methoxy-PEG-acetyl molecule.

As predicted from the literature, the yield of reaction was very high (almost 80%)

and the pegylated peptide, Bac7(1-35)CAM-PEG20K, maintained a good antibacterial activity although lower than that shown by the unmodified molecule. To reach an efficacy comparable to the unpegylated peptide, it was necessary to pre-incubate the conjugate with human serum to ensure the release of Bac7(1-35). Western blot analysis demonstrated that Bac7(1-35) started to be released from the PEG molecule within 8-24 h and was completely released within 72 h incubation both in human serum and plasma, without significant peptide degradation. These results demonstrate that pegylation of Bac7(1-35) may allow to obtain a compound that is more stable in blood, shows a reduced sensitivity to degradation by proteases and, at the same time, is active as the unmodified peptide after the slow release that occurs in the presence of serum or plasma.

These promising *in vitro* results were completed by *in vivo* imaging experiments aimed at verifying if pegylation improves the Bac7(1-35) pharmacokinetic parameters. According to the data published in the literature on the advantages of pegylation (Zahnd *et al.* 2010; Chang *et al.* 2009), Bac7(1-35)CAM-PEG20K showed (i) a slower clearance compared with that shown by the unpegylated peptide, as a consequence of its increased molecular weight, (ii) an increased biodistribution due to its ability to join the blood circulation and (iii) a prolonged availability due to slow release of Bac7(1-35) from the PEG complex.
Future investigations will provide data on the efficacy of the pegylated peptide in the animal model of *Salmonella* infection.

3. EFFECTS OF BAC7(1-35) ON CELLS OF THE IMMUNE SYSTEM DURING INFECTION WITH *S. ENTERICA*

We then investigated the effects of Bac7(1-35) on immune cells such as macrophages during a *S. enterica* infection, and its ability to exert antibacterial activity once having penetrated the cells.
Initially, we extended previous observations on the effects of the peptide on mammalian cells, by showing that it is weakly cytotoxic to PMA-differentiated U937 cells even after long-term incubation and at concentrations that are much higher than those antibacterial (3 h at 50 μM). In addition, we confirmed that the peptide, added to U937-derived macrophages, was efficiently internalized into the

cells. In particular, Bac7(1-35) seemed to induce necrosis of the cells rather than apoptosis, suggesting that it probably induced surface changes that led to plasma membrane disruption and did not interfere with molecular mechanisms or signal pathways involved in the apoptotic process.

Our attention focused on the effects of Bac7(1-35) on macrophages, since these cells play a central role in host defence against pathogenic microorganisms through their ability to rapidly recognize bacterial components, phagocytose them, and activate an arsenal of antimicrobial effectors. At the same time, macrophages also appear to be one of the preferred sites for bacterial replication in the murine model of human typhoid fever (Wijburg *et al.* 2000). Salmonellae reside intracellularly within macrophages (Richter-Dahlfors *et al.* 1997) in a specialized vacuole (see Introduction, paragraph 3) in which they escape from killing by components of the innate and humoral immune defenses and replicate starting from 4 to 8 h after infection (Rosenberg and Finlay 2002).

During a systemic *Salmonella* infection, when the peptide is given, different possible situations can occur: macrophages that get first in contact with the peptide and then phagocytose the bacteria, macrophages that phagocytose bacteria which have previously come into contact with the peptide or macrophages that had already engulfed bacteria and then internalized Bac7(1-35).

In the light of this scenario, our attention focused on how the presence of the peptide could interfere or modulate *i)* phagocytosis by interacting with both the macrophages and the bacteria *ii)* survival and replication of intracellular salmonellae.

The effects of Bac7(1-35) on phagocytosis were studied using cytofluorimetric analysis and GFP-expressing Salmonellae. PMA-differentiated U937 cells and *S. enterica* cells were treated with Bac7(1-35) and the effects of the peptide studied in terms of variation in the percentage of phagocytosing cells and in the number of engulfed bacteria. In the case of macrophages pre-treated with the peptide at non-toxic concentrations, a stimulation of the phagocytosis was clearly observed. Pre-treating cells with Bac7(1-35) increased by 12% the percentage of phagocyting cells and, as consequence the number of engulfed bacteria (+ 14%). A possible explanation of the mechanism of activation is related to the capacity of the peptide to be rapidly internalized into the cells. Once inside it could activate molecular processes involved in the earlier phases of phagocytosis. In this context, one of the

main processes involved in bacterial engulfment is the activation of actin polymerization. Cytoskeletal changes in fact cause the cells to extend pseudopods that then engulf the bacteria (Anderem *et al.* 1999). Recent findings suggest interactions between the actin cytoskeleton and cell membrane rafts that regulate membrane raft functions (Chichili and Rodgers 2009). As described by Zanetti and coworkers (Tomasinsig *et al.* 2006), Bac7(1-35) is able to interact with the lipid bilayers and inserts itself deeply into the lipid core of the cell membrane. In the light of these findings, it is possible that Bac7(1-35) could take part in the interplay between membrane rafts and actin cytoskeleton, promoting actin polimerization.

In contrast to what reported above, when bacteria were exposed to Bac7(1-35) before phagocytosis, the percentage of phagocytosing cells and the number of engulfed bacteria per cell slightly but significantly decreased, suggesting that in this case the peptide interferes negatively with phagocytosis. This effect could be explained taking into consideration that *Salmonella* plays an active role in cell invasion, a role that could in someway be hampered by pre-treatment with peptide. There are many bacterial processes involved in the infection of host cells that could be modulated and could explain this result. It is known, for example, that in the first stage of infection, the bacterial cell assembles a needle-like structure belonging to the type III secretion system (see paragraph 3 of the Introduction), to deliver some virulence proteins into the host cells, a process that is crucial for *Salmonella* infection (Yu *et al.* 2010). The presence of Bac7(1-35) could interfere or delay this early process. Another hypothesis involves the initial recognition of microorganisms by macrophages, an event that is mediated by Toll-like receptors that recognize specific bacterial surface structures (e.g., flagella and membrane vesicles) and other specific antigens (Bergman *et al.* 2005). The interaction between the peptide and salmonellae cells could interfere with this recognition.

The *in vivo* protective role of Bac7(1-35) against *S. enterica* infection suggests that the peptide might exert its bactericidal action also within infected cells. In this respect, Segall and coworkers recently demonstrated that an exogenously added hexapeptide inhibited *Salmonella* growth in a human macrophage-like cell line and in murine peritoneal macrophages (Su *et al.* 2010). Since *S. enterica* is a facultative intracellular pathogen able to invade host cells and survive within them (Richter-Dahlfors *et al.* 1997), and Bac7(1-35) is able to penetrate host cells through macropinocitosys without killing them (Sandler *et al.* 2002, Tomasinsig *et al.* 2006),

we set up a series of experiments aimed at evaluating if the proline-rich peptide maintains its bactericidal activity against engulfed bacteria once inside the cells.

The results obtained indicate that PMA-differentiated U937 cells that had phagocytosed *S. enterica* cells and then treated with 50 μM peptide for 3 h, showed a reduced number of viable bacteria recovered after lysis of the host cells with respect to untreated controls. This effect is even more evident when the count of the surviving bacteria was performed 21 h after removal of the peptide. These results suggest that Bac7(1-35) at 50 μM enters the cells and effectively inhibits the growth of engulfed salmonellae within 3 h, while it presumably affects their survival at a longer time period (3 + 21 h) inhibiting almost completely their replication.

The effect of the peptide on intracellular bacterial growth is dose-dependent and appears to be increased at a Bac7(1-35) concentration of 150 μM. Although at this concentration the peptide starts to be toxic, the percentage of viable U937 cells remained high during the experiment.

Similar results have been obtained using a second cells type: MDM cells. These are LPS-differentiated cells derived from monocytes isolated from human healthy donors and for this reason they are a model likely resembling the *in vivo* macrophages. Bac7(1-35) exerted antibacterial activity also in MDM cells and considerably reduced replication of intracellular salmonellae.

What remains to be established is the mechanism of bacterial inhibition and/or killing. It is likely that the peptide acts directly on intracellular bacteria. However, it cannot be ruled out that the peptide could activate or potentiate cellular processes known to be involved in bacterial killing, such as production of reactive oxygen species and of nitric oxide, or release in the phagolysosomes of microbicidal substances produced by macrophages.

Some clues support the first hypothesis. Considering the intracellular volume of macrophages and the exposition of the cells to 50 μM peptide, it can be estimated that the intracellular concentration of Bac7(1-35) is well above its microbicidal concentration. Although it may be that a large part of the internalized peptide is not in direct contact with the intracellular bacteria, it is possible that once inside cells, an aliquot of Bac7(1-35) enters the lysosomal vesicles and contributes to kill the entrapped bacteria. This hypothesis is supported by confocal analysis showing that the peptide seems not to localize into specific cellular compartments but is ubiquitously distributed inside U937 cells.

Overall these data indicate that Bac7(1-35) can play a role in the phagocytic process of macrophage-like cells and, most relevant, that it maintains its antimicrobial activity also once inside infected macrophages.

Conclusions

The studies here reported allowed the evaluation of some aspects of the therapeutic potential of Bac7(1-35), an active fragment of the natural proline-rich AMP Bac7, to understand if it could be a lead compound for future development of novel antimicrobial agents. In this respect, our results have emphasized some desirable properties of the peptide: a poor toxic effect on animals and a significant protective effect *in vivo*, when used in a mouse model of infection resembling typhoid fever in humans. However, it also showed some weaknesses that are common to peptides when they are tested as drugs: a rapid excretion and a poor stability in biological fluids that can lead to decreased activity. A great effort was thus made to try to overcome these weaknesses by modifying the peptide with PEG. The first results were encouraging. A pegylated compound, prepared via a cleavable thioester bond, showed that the peptide can be released in a fully active form by esterases present in biological fluids increasing its *in vivo* biodistribution and half-life. Protection experiments in mice are planned to verify the therapeutic efficacy of the pegylated Bac7(1-35).

In addition to pegylation, it would also be useful to evaluate other biochemical modifications, such as the use of unnatural amino acids, with the aim of overcoming the peptide degradation observed for instance in murine serum and plasma. This is particularly important, since many mouse models of infection are well established and are useful to evaluate the therapeutic potential of the peptide.

Finally, we demonstrated that Bac7(1-35) is effective in killing salmonella cells after their uptake by macrophages. It could thus be interesting to evaluate if the presence of the PEG molecule could interfere or potentiate the activity of the peptide inside host cells. In this context, it would be useful to investigate the molecular mechanisms by which the peptide leads to killing intracellular bacteria and obtain, at the same time, additional information on its interaction with immune cells.

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