DEVELOPMENT AND OPTIMIZATION OF TOPICAL ANTIMICROBIAL AGENTS BASED ON HUMAN ANTIMICROBIAL PEPTIDES

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A chi mi è così vicino da lontano

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ABSTRACT

New antimicrobial compounds are urgently needed to fight infective diseases caused by antibiotic-resistant human pathogens. Promising candidates are host defense peptides (HDPs), also known as antimicrobial peptides (AMPs), essential component of innate immunity in eukaryotes. Due to their biochemical properties (high content of basic and hydrophobic residues), they selectively bind and disrupt bacterial membranes that are rich in anionic lipids. In addition, many AMPs display immunomodulatory functions. This peculiar combination of biological activities makes AMPs very promising therapeutic agents. Surprisingly, many human proteins with functions not necessarily related to host defense can behave as sources of AMPs. Some examples are lactoferrin, lysozyme and thrombin. Since these AMPs are hidden in large proteins, they can be defined as “cryptic”. In order to identify by a rational approach further human proteins carrying cryptic AMPs, an *in silico* screening method that allows to localize antimicrobial regions hidden inside the primary structure of precursors was developed. A wide list of potential new AMPs was obtained using this method to analyze about 4’000 human extracellular proteins. This list represented the starting point of the present PhD project. The main aim was to select and characterize the most pharmacologically interesting potential AMPs in order to find candidates suited for the development of novel human antimicrobial and anti-inflammatory agents. Such peptides might be used for topical treatments, like treatment of surgical wounds and ulcers, and for the infections of skin, mouth and airways. Firstly, I developed a novel cost-effective fusion system, particularly well-suited for the production of toxic peptides in *E. coli*. To avoid AMPs toxicity toward bacterial host, I rationally designed a carrier protein starting from a *Rana pipiens* ribonuclease, named onconase (ONC), that is expressed at high level (200 mg/L of culture) as inclusion bodies in *E. coli*. In order to optimize this method, a well-known AMP derived from human thrombin, named GKY20 was used as model peptide. After acid cleavage of Asp-Pro bond inserted at carrier-peptide junction, the carrier protein was removed by selective precipitation at neutral pH. Peptide (P)GKY20 (so called as it has an additional proline at N-terminus) was recovered with yields of 10-15 mg/L of culture. Moreover, I demonstrated that this strategy also allows to prepare peptides with a N-terminal cysteine residues that could find many applications in basic and applied research like for example preparation of labeled peptides, protein ligation for the semi-synthesis of modified proteins, immobilization on supports etc. Using our improved carrier several newly identified putative AMPs from 20 to almost 50 amino acids were successfully produced with yields even higher than that of (P)GKY20 (up to 30 mg/L of culture in the case of a 47 residues long peptide derived from pepsinogen A3). Therefore, our method is complementary to chemical synthesis being particularly well-suited to prepare peptide longer than 20-25 residues. All these peptides showed, as expected, antimicrobial activity on GRAM-negative and GRAM-positive strains with MIC values in the micromolar range thus validating the efficacy of the bioinformatic screening method. Interestingly, some of them prevented *P. aeruginosa* biofilm formation at concentration lower than MIC values. Moreover, we found that four of our peptides possess very interesting anti-inflammatory properties (e.g. the ability to reduce release of pro-inflammatory cytokines). Very interestingly, they proved to be non-toxic for eukaryotic cells thus suggesting that these peptides could be used as drugs. Even if at the moment the panel of peptides and of the assays performed is limited, our data suggest that the *in silico*-derived list of potential AMPs is a rich source of peptides with pharmacologically relevant properties.

RIASSUNTO

La diffusione di ceppi patogeni per l'uomo dotati di resistenze multiple agli antibiotici convenzionali (fenomeno dell'antibiotico-resistenza) costituisce un grave problema per la salute umana, come evidenziato nell'ultimo rapporto dell'Agenzia Mondiale della Sanità (2015). Negli ultimi decenni a suscitare maggiore preoccupazione sono stati i ceppi resistenti alla meticillina o alla vancomicina appartenenti al genere *Staphylococcus* oltre che batteri Gram negativi appartenenti ai generi *Escherichia, Pseudomonas, Acinetobacter* ecc. Per combattere il fenomeno dell'antibiotico-resistenza, sono necessarie nuove classi di antimicrobici che garantiscano un'efficacia duratura nel tempo. Candidati ideali sono i Peptidi AntiMicrobici cationici (AMP). Tali molecole, sono presenti in quasi tutte le forme di vita -batteri inclusi- e rappresentano la più antica arma di difesa del sistema immunitario innato. Inizialmente scoperti per la loro attività antimicrobica ad ampio spettro, hanno suscitato sempre più attenzione anche per gli interessanti ruoli fisiologici che rivestono nei meccanismi dell'immunità innata. Per tale motivo sono spesso definiti, in senso più ampio, peptidi della difesa dell'ospite (HDP, dall'inglese Host defense peptides).

Sebbene siano una classe di molecole molto eterogenea in termini di lunghezza e struttura secondaria, gli AMP possiedono caratteristiche chimico-fisiche comuni, quali la carica netta positiva a pH fisiologico (di solito da +3 a +7), e l'elevato contenuto di residui idrofobici (≥ 30 %). Tali proprietà sono alla base del loro peculiare meccanismo d'azione. Funzionalmente, gli AMP differiscono dalla maggior parte degli antibiotici convenzionali per quanto riguarda il loro "bersaglio molecolare". Nel caso degli antibiotici convenzionali il bersaglio è generalmente una (macro)molecola specifica del batterio. Nel caso dei peptidi antimicrobici il principale, anche se non l'unico, "bersaglio" è rappresentato dalla membrana batterica (interna e/o esterna).

La prima fase di interazione peptide cationico/membrana è mediata dalla presenza di fosfolipidi anionici, come fosfatidilglicerolo e cardiolipina, distribuiti abbondantemente su entrambi i lati della membrana batterica, sia nei batteri Gram positivi che nei Gram negativi. In seguito, il peptide adotta una conformazione anfipatica che gli consente di inserirsi nella membrana. Si innescano così una serie di eventi che comportano la perdita delle funzioni fisiologiche della membrana (alterazioni di fluidità, di spessore, di permeabilità selettiva ecc.). Alcuni peptidi, tuttavia, danneggiano le cellule anche attraversando la membrana e attaccando bersagli intracellulari, come nel caso della buforina II capace di legare il DNA. La tossicità selettiva nei confronti dei batteri è legata alla differente composizione lipidica. Infatti negli eucarioti il foglietto esterno della membrana plasmatica contiene quasi esclusivamente lipidi zwitterionici mentre quelli anionici sono confinati nel foglietto citosolico, pertanto gli AMP non riescono a legarsi ed inserirsi efficientemente nelle membrane eucariotiche.

A causa del loro peculiare meccanismo di azione è improbabile che i batteri possano sviluppare una completa resistenza, ciò, infatti, richiederebbe un sostanziale cambiamento nella composizione di membrana che potrebbe avere "costi" metabolici troppo elevati per i batteri. Basti pensare che le membrane batteriche sono coinvolte in ruoli metabolici fondamentali, come le catene di trasporto degli elettroni e la produzione di ATP.

Oltre all' attività antimicrobica diretta, gli AMP fungono anche da molecole ponte tra i meccanismi dell'immunità innata e quella acquisita. Alcuni noti peptidi antimicrobici umani come α e β defensine e LL-37, l'unica catelicidina umana, sono dotati di diverse proprietà immunomodulanti che si aggiungono alla loro efficacia come antimicrobici. Ad esempio possono reclutare le cellule effettrici dell'immunità innata

nei siti di infezione (chemiotassi dei macrofagi) ed attivare le cellule specializzate coinvolte nei meccanismi dell'immunità adattativa (cellule dendritiche immature, cellule T immature).
In aggiunta ai già citati AMP convenzionali, negli ultimi decenni sono state descritte numerose proteine eucariotiche, non strettamente legate alla difesa dell'ospite, che si comportano come "contenitori" o vettori di AMP. Ne sono esempi il lisozima, la lattoferrina, le apolipoproteine, la trombina etc..
Ad esempio, la lattoferrina, esplica attività antimicrobica agendo come potente chelante del ferro, un nutriente essenziale per i microrganismi. Tuttavia nello stomaco del neonato essa viene proteolizzata dalla pepsina che causa il rilascio, dalla regione N-terminale, di un peptide antimicrobico di 25 residui denominato "lactoferricina" più attivo di quanto non lo sia la lattoferrina stessa nei confronti di svariati ceppi. Secondo un modello molto suggestivo quando tali proteine vengono a contatto con i batteri, l'azione delle proteasi batteriche, eventualmente combinata a quella di proteasi dell'ospite, permetterebbe il rilascio di regioni che, una volta libere dai vincoli conformazionali della proteina nativa, diventerebbero dei veri e propri AMP. Dunque, tali peptidi potrebbero essere definti "AMP criptici".
Preliminarmente alle attività del presente progetto di dottorato, al fine di ricercare con un approccio più razionale peptidi "criptici" presenti nel secretoma umano, è stato sviluppato dal Dott. Notomista, un metodo bioinformatico che consente di localizzare regioni antimicrobiche in precursori proteici e di fornire una predizione quantitativa dell'attività antibatterica associata ad esse. Mediante tale approccio, sono state analizzate circa 4'000 proteine extracellulari umane ed è stata così creata una lista di potenziali AMP. Il presente lavoro di tesi si colloca all'interno di una vasta linea di ricerca il cui obiettivo principale è l'isolamento e la caratterizzazione degli AMP umani più interessanti suggeriti dalla predizione bioinformatica, al fine di selezionare candidati ideali per lo sviluppo di nuovi agenti antimicrobici ed antinfiammatori ad uso topico impiegabili nel trattamento di ferite chirurgiche, ulcere cutanee e delle mucose, infezioni delle vie aeree come sinusiti ed otiti etc.
Pertanto, gli obiettivi del presente lavoro di tesi possono essere riassunti in tre punti fondamentali:

i) lo sviluppo ed ottimizzazione di un nuovo sistema di espressione di AMP in *E. coli*;
ii) la produzione e la caratterizzazione dei peptidi più interessanti presenti nella lista di potenziali AMP umani generata dal metodo bioinformatico;
iii) la caratterizzazione funzionale completa di alcuni dei peptidi più promettenti.

i) Sviluppo di un nuovo sistema di espressione di peptidi tossici in E. coli

L'accurata caratterizzazione delle proprietà farmacologicamente rilevanti dei potenziali AMP umani ha richiesto innanzitutto lo sviluppo di un metodo efficace e relativamente economico per la loro produzione. La sintesi chimica è particolarmente idonea per la produzione di peptidi di dimensioni ≤ 25 residui e quando sono necessarie soltanto piccole quantità (tra i 5-10 mg). Tuttavia, per la sintesi di peptidi relativamente lunghi (≥ 25 residui) e necessari in quantità maggiori, i metodi di produzione biologica posso rivelarsi più economici ed utili alla produzione su larga scala.
Il nuovo metodo di espressione sviluppato, si basa sull'utilizzo di una proteina *carrier* progettata razionalmente a partire da una ribonucleasi di *Rana pipiens* chiamata onconasi (ONC). Tale proteina presenta diverse caratteristiche che la rendono un *partner* ideale: i) elevati livelli di espressione (circa 200 mg/L di coltura); ii) capacità

di formare corpi d'inclusione mascherando quindi la tossicità del peptide nella proteina di fusione ONC-peptide; iii) piccole dimensioni (104 amminoacidi) che consentono elevate rese di peptide in seguito al rilascio dal *partner*; iv) solubilità dipendente dal pH. Infatti, la proteina ONC denaturata è solubile a pH inferiore a 4 ma a pH neutro forma aggregati macroscopici insolubili. Questa caratteristica rappresenta un grande punto di forza del metodo sviluppato, in quanto consente una purificazione semplice di peptidi solubili a pH 7.

L'efficienza del sistema di fusione è stata valutata clonando a valle della proteina *carrier* la sequenza di un AMP criptico già caratterizzato in letteratura, il peptide GKY20, derivante dall'estremità C-terminale della trombina umana.

Tra la proteina carrier ed il peptide è stato inserito come sito di taglio la sequenza acido labile Asp-Pro. Quindi, l'idrolisi acida rilascia un peptide dotato di un residuo di prolina addizionale all'estremità N-terminale.

Per evitare le reazioni indesiderate, come la modifica delle catene laterali, che possono verificarsi utilizzando acidi concentrati come l'acido formico (al 50-70%), è stato sviluppato ed ottimizzato un metodo di idrolisi acida in condizioni blande. L'ottimizzazione di tale sistema di taglio, ha rilevato risultati inattesi ed al contempo interessanti. Infatti, tutti i siti Asp-X presenti in ONC sono risultati sensibili all'idrolisi acida, seppure in misura differente. Di particolare interesse è stata la scoperta dell'elevata efficienza di idrolisi acida delle sequenze Asp-Cys, non ancora nota in letteratura.

In aggiunta ai frammenti aspecifici rilasciati dall'ONC, si è anche osservato che alcune proteine contaminanti di *E. coli* rilasciavano frammenti durante l'idrolisi acida che rendevano più complicata la successiva purificazione del peptide. Pertanto al fine di ottenere una versione migliorata della proteina *carrier* sono state introdotte diverse mutazioni. La versione finale della proteina, chiamata ONC-DC*less*-H6, è priva di residui di acido aspartico, cisteina e metionina ed è dotata di una sequenza di residui di istidina (His-Tag) che consente una più rapida purificazione del costrutto di fusione dalle proteine contaminanti di *E.coli*. Inoltre ONC-DC*less*-H6 si esprime con livelli simili o maggiori a ONC-(P)GKY20 ed è presente esclusivamente nella frazione insolubile di *E. coli*, dimostrando che le mutazioni scelte non alterano le proprietà caratteristiche dell'ONC. Mediante ONC-DC*less*-H6, è stato prodotto il peptide (P)GKY20 ad elevate rese (10-15 mg/L di coltura). Tale peptide, è stato separato dalla proteina di fusione attraverso idrolisi acida e purificato mediante precipitazione selettiva del *carrier*. Il grado di purezza di (P)GKY20, analizzato mediante HPLC è ≥ 95%, indicando che nessun ulteriore passaggio cromatografico era necessario. Va sottolineato che il peptide ricombinante (P)GKY20 ha attività antimicrobica identica a quella del peptide GKY20 sintetico (con valori MIC simili su 8 ceppi). Pertanto si può concludere che il residuo di prolina addizionale all'N-terminale del peptide ricombinante non altera la sua attività antimicrobica.

In base alle osservazioni ottenute nella fase di ottimizzazione dell'idrolisi acida, si è pensato di sostituire il sito di taglio Asp-Pro nella giunzione carrier-peptide con il sito acido labile Asp-Cys, per produrre il peptide ricombinante GKY20 dotato di un residuo di cisteina all'N-terminale. Il peptide (C)GKY20 è stato coniugato ad un fluoroforo dotato di gruppo maleimide sfruttando la peculiare reattività del gruppo tiolico della cisteina. Questo peptide marcato potrà essere utilizzato per studi di localizzazione cellulare, interazione con gli LPS e i liposomi ecc..

Incoraggiati dai risultati ottenuti con il nuovo sistema di espressione, alcuni degli AMP più promettenti sono stati prodotti in forma ricombinante. Tutti i peptidi sinora selezionati sono stati prodotti con rese simili o maggiori a (P)GKY20.

ii) Produzione di potenziali peptidi antimicrobici umani

L'analisi bioinformatica ha permesso di stilare una lista di potenziali AMP contenuti in proteine coinvolte nella coagulazione, proteine sieriche, della matrice extracellulare etc. In questa lunga lista ho selezionato due peptidi particolarmente interessanti per motivi differenti: il peptide di attivazione dell'isoforma A3 del pepsinogeno umano [PA-$3_{(1-47)}$] e gli ultimi 28 amminoacidi all'estremità C-terminale del β Fibrinogeno umano [β$Fib_{(420-447)}$].

PA-$3_{(1-47)}$ viene rilasciato dal pepsinogeno mediante proteolisi operata dalla pepsina. Questo taglio è essenziale per la produzione della pepsina matura. Finora l'unica funzione descritta in letteratura per PA-$3_{(1-47)}$ è infatti mediare la secrezione della pepsina.

Il peptide PA-$3_{(1-47)}$, è stato preparato in forma ricombinante [(P)PA $3_{(1-47)}$] mediante il metodo descritto per (P)GKY20, con rese anche più elevate (circa 30 mg/L di coltura). Con lo stesso metodo sono stati prodotti anche (P)PA-$3_{(1-25)}$ e (P)PA-$3_{(26-47)}$, due frammenti di PA-$3_{(1-47)}$, già descritti in letteratura, che sulla base dell'analisi bioinformatica potevano essere dotati di attività antimicrobica. Tutti e tre i peptidi, come predetto, hanno mostrato attività antimicrobica a concentrazioni micromolare verso ceppi batterici Gram negativi e Gram positivi.

Il potenziale peptide al C-terminale del β Fibrinogeno umano è stato prodotto in due varianti, β$Fib_{(420-447)}$ e β$Fib_{(425-447)}$, entrambi ottenuti con rese simili a (P)GKY20 ovvero 10 mg/L di coltura.

iii) Caratterizzazione funzionale degli AMP umani più promettenti

In aggiunta alla caratterizzazione dei peptidi ricombinanti è stata effettuata la caratterizzazione di cinque potenziali AMP prodotti mediante sintesi chimica: Apo $E_{(133-150),}$ frammento dell'apolipoproteina E; Apo $B_{(887-911)}$ frammento dell'apolipoproteina B umana; PA-$3_{(1-25)}$ e PA-$3_{(26-47)}$, i frammenti del pepsinogeno sopra descritti; β$Fib_{(420-447)}$, la regione C-terminale del fibrinogeno β prodotta anche in forma ricombinante.

Questi peptidi sono stati caratterizzati per quanto riguarda le loro proprietà antimicrobiche, antibiofilm ed immunomodulanti durante il periodo di ricerca all'estero della durata di cinque mesi nei laboratori del prof. Robert Hancock (Università della British Columbia, Vancouver, Canada) la cui esperienza nel campo della caratterizzazione dei peptidi antimicrobici è ben nota.

I risultati ottenuti hanno mostrato che tutti i peptidi in esame sono dotati di interessanti proprietà biologiche ma differenti da peptide a peptide. In particolare, il peptide derivato dall'apolipoproteina E mostra un ampio spettro di attività antimicrobica (con valori MIC al di sotto di 10 µM) mentre β$Fib_{(420-447)}$ risulta essere maggiormente attivo sui batteri Gram positivi, come ad esempio il ceppo MRSA di *Staphylococcus aureus*. Inoltre peptidi che possedevano moderata attività antimicrobica come quelli derivati da PA-$3_{(1-47)}$ e Apo $B_{(887-911)}$, hanno mostrato invece attività antibiofilm a concentrazioni alle quali non hanno avuto effetto sulle cellule in crescita planctonica. Inoltre, quasi tutti i peptidi analizzati presentano alcune proprietà tipiche degli AMP come per esempio la capacità di ridurre i livelli della citochina pro-infiammatoria TNF-α, innescati dalla stimolazione con i lipopolisaccaridi dei batteri Gram negativi. Tutti i peptidi analizzati ed in particolare il peptide β$Fib_{(420-447)}$, inducono il rilascio della chemochina MCP-1 suggerendo un ruolo nella chemiotassi dei leucociti in aggiunta all'attività antimicrobica diretta. Complessivamente, tutti i peptidi studiati hanno mostrato in varia misura caratteristiche tipiche degli AMP.

Applicazioni biotecnologiche del presente lavoro di tesi

Le peculiari caratteristiche degli AMP li rendono candidati particolarmente promettenti per lo sviluppo di nuovi agenti anti-infettivi:

i. Possiedono un ampio spettro d'azione poiché agiscono contro batteri, virus, lieviti e funghi;
ii. Sono selettivamente tossici per le cellule microbiche senza avere effetti dannosi per le cellule eucariotiche;
iii. Non possiedono bersagli molecolari definiti, ma in generale danneggiano le membrane batteriche riducendo quindi la probabilità che insorgano ceppi resistenti;
iv. Sono in grado di agire sia sui batteri in crescita planctonica che in forma sessile (biofilm).

Di particolare interesse è quest'ultimo punto, in quanto circa l'80% delle malattie infettive croniche per l'uomo sono provocate da batteri patogeni capaci di formare biofilm. Solo agenti battericidi specificamente attivi su cellule in stato stazionario o in lenta divisione come appunto gli AMP possono eradicare completamente il biofilm. In tal senso, possono rivelarsi utili anche "terapie combinate" basate sulla somministrazione di formulazioni contenenti un AMP ed un antibiotico convenzionale. Gli AMP trovano già impiego in vari campi inclusa l'industria alimentare, ne sono esempi la nisina noto conservante naturale, la gramicidina S e la polimixina utilizzati in creme ad uso topico e la lattoferrina presente nel latte in polvere. Tuttavia, il loro impiego in campo clinico rimane sicuramente una delle prospettive più interessanti. Ad oggi diversi AMP sono in fase di studio pre-clinico e clinico, principalmente come antimicrobici.

La ricerca di candidati antimicrobici umani dotati di proprietà biologiche addizionali (spesso dipendenti da interazione con recettori specie-specifici) rende alcuni dei peptidi analizzati più promettenti e interessanti per lo sviluppo di nuovi agenti antimicrobici ed antinfiammatori.

Il presente lavoro di dottorato ha contribuito alla validazione e ottimizzazione di due strumenti che potranno rivelarsi di grande utilità nello sviluppo delle applicazioni biotecnologiche degli AMP: un metodo bioinformatico utile per l'individuazione di potenziali nuovi AMP ed un sistema di espressione per AMP ricombinanti, efficiente e versatile che può essere considerato complementare alla sintesi chimica.

Chapter 1-Introduction

1.1 Antibiotic resistance

The development of antibiotics is possibly one of humanity's greatest achievements, contributing to significantly increased life expectancy compared to past generations.
The vast majority of antibiotic classes in use today have been isolated in the golden era of antibiotic discovery (between the 1940s and 1970s), mainly from soil *Actinomyces*. However, today, the evolution of antibiotic resistance by important human pathogens has rendered these original molecules and most of their successors largely ineffective [1-2].
The inappropriate and/or overuse of antibiotics, in agriculture and industry contributes to accelerate the development of antibiotic resistance. As a result, many pathogenic strains have become "multidrug resistant" (MDR) i.e. resistant to three or more antibacterial drug classes [3].
Antibiotic-resistant pathogens emerging as major threats to public health, according to the 2014 report of global antibiotic resistance, published by the World Health Organization (WHO), are the following [4]:

i) methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Staphylococcus aureus* (VRSA);
ii) multidrug-resistant (MDR) Gram-negative bacteria such as *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*;
iii) MDR strains of *Mycobacterium tuberculosis* which are a rising threat in the developing world [3].

The ability of these MDR bacteria to resist the effects of antibiotics generally depends on the following genetic strategies [5]:

- producing destructive enzymes to neutralize antibiotics;
- modifying antimicrobial targets, by mutations;
- removing antimicrobial agents by efflux pumps;
- preventing antibiotics from entering by creating a "biofilm" (i.e. a community of bacterial cells enclosed in a self-produced polymeric matrix which confers protection against environmental stresses).

The latter point is of particular interest since about 80% of infectious disease are complicated by involvement of biofilms [5].
Therefore, huge efforts to develop new anti-infective strategies, without overlook also biofilm level resistance, are urgently required. Promising candidates are AntiMicrobial Peptides (AMPs), more accurately termed "Host Defense Peptides" (HDPs) to describe the variety of their activities correlated to innate immunity [6].

1.2 General characteristics of host defense peptides

HDPs are present in almost every life form -bacteria included- as Nature's antibiotics [7-8] and represent the most ancient weapons of the innate immune system [9]. In mammals these peptides provide protection against a broad array of infectious agents: bacteria, viruses, fungi, yeasts and certain protists.
They are an extremely heterogeneous group of defense molecules that vary in sequence, length and secondary structure.
From the point of view of net charge, AMPs can be divided into anionic and cationic. Anionic AMPs are less common and generally not well characterized (noteworthy examples are dermcidin and SAAPs [10]).
On the other hand, cationic AMPs are very common and hundreds of them have been characterized [11].Generally, they are small (from 12 to 50 amino acids),

positively charged (under physiological condition from +5 to +8), with an unusual abundance of hydrophobic residues (≥30%) [7-8].
Based on their structure, AMPs can be classified into four different classes: α-helical peptides (for example, LL-37,magainins and mellitin), β-sheets peptides stabilized by two to four disulfide bridges (for example, human α and β defensins); loop peptides with one disulfide bridge (for example, bactenecin); peptides enriched in amino acids like proline, tryptophan, histidine or glycine without a well defined structure (for example, indolicidin) (Fig. 1) [12].
The majority of AMPs are disordered in solution but, upon contact with bacterial membranes, they are able to fold into an amphipathic conformation [7-13].

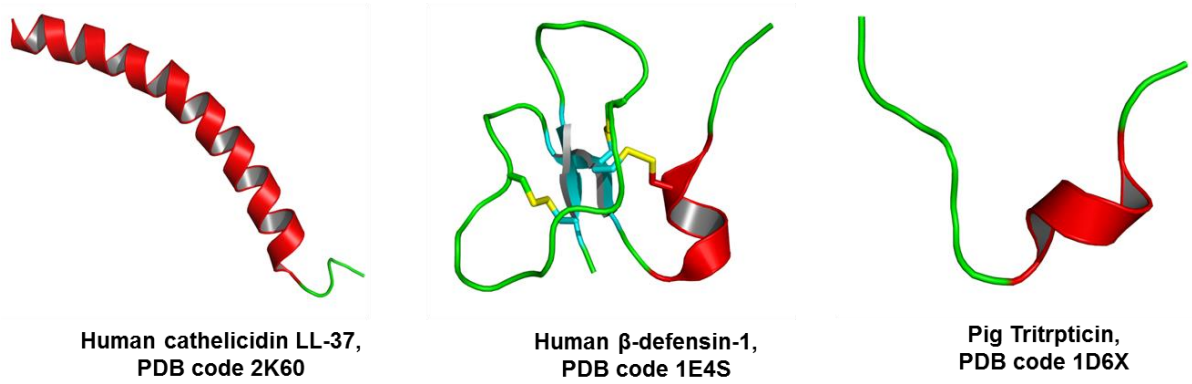


Figure 1: Examples of secondary structure of antimicrobial peptides.

1.3 Mechanism of action

The substantial differences existing between bacterial and eukaryotic cell membranes are the basis of AMPs interesting and selective mode of action. Indeed, in eukaryotic cell membranes, negatively charged phospholipids are sequestered in the inner leaflet of the lipid bilayer, whereas the outer leaflet is mainly composed of zwitterionic uncharged lipids [7-9-14]. In contrast, both Gram negative and Gram positive bacteria, have at least 15% anionic lipids in their membranes such as phosphatidylglycerol (PG) and cardiolipin (CL) (Fig.2) [14].

Although the exact nature of the mechanism of action of AMPs is still unclear, generally the overall positive charge is the driving force for the initial electrostatic interaction between cationic peptides and anionic microbial cell surface [7]. Successively, AMPs fold in an amphipathic conformation which allows their insertion into the membrane (Fig.3).

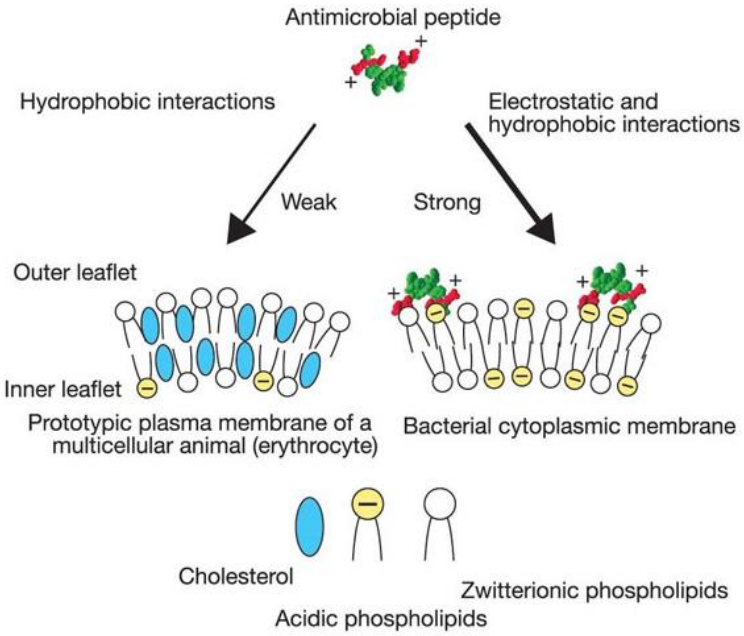


Figure 2: Schematic representation of selective mode of action of antimicrobial peptides (from Zasloff M.,2002).

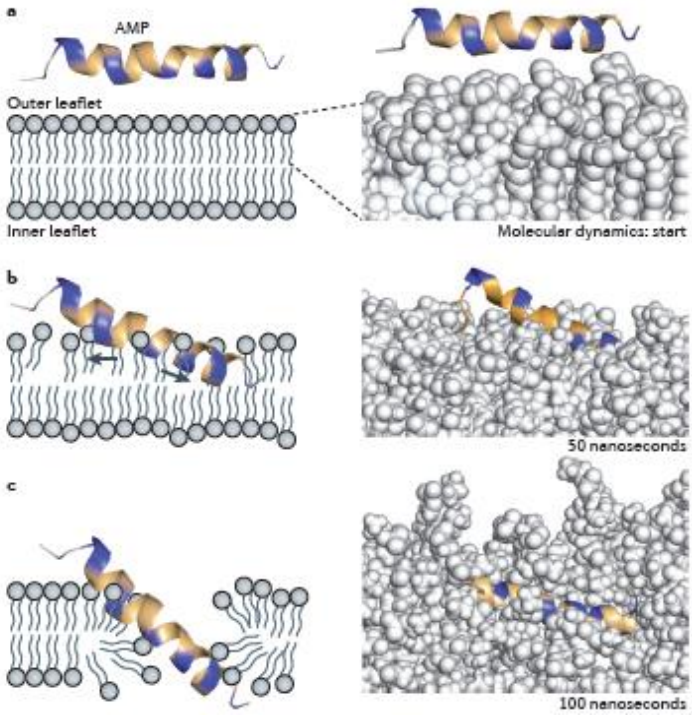


Figure 3: Potential mechanism of membrane disruption and/or translocation by antimicrobial peptides. On the left AMP is represented as a ribbon diagram, with positively charged residues indicated in blue (other residues are shown in yellow). (**a**) The initial stages for many AMPs include membrane attachment (**b**) and then disruption of the membrane (**c**) or form pores. The panels on the right illustrate hypothetical mechanisms of the AMP–membrane interaction that can be studied by molecular dynamics simulations (from Fjell CD. et al., 2011).

In contrast to conventional antibiotics, the development of AMPs resistance is very rare. It has been hypothesized that this depends on the fact that microbial cells might acquire resistance to AMPs only through modification of their membrane composition [7]. Such a change not only would require several mutations, but could also be detrimental to bacterial cells as their cytoplasmic membrane bears several essential metabolic functions including electron transport chains and ATP synthesis [7-16].
Although the acquisition of complete resistance against antimicrobial peptides is unlikely, some strains have acquired a limited resistance [17] by producing specific proteases like, for example, “omptins” of enterobacteria and V8 proteases of *Staphylococcus ssp* [18-19].

1.4 Biological roles of HDPs

Originally characterized as natural antimicrobial agents, it is increasingly appreciated that the main role of cationic peptides as host defense molecules may be to modulate the host immune response, a function complementary but independent from direct antimicrobial activity (Fig. 4).

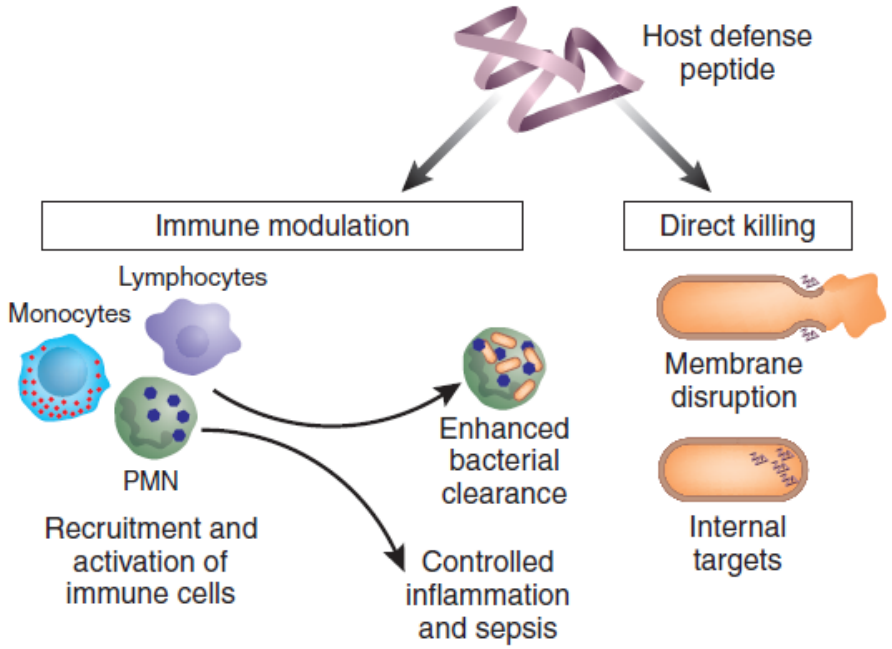


Figure 4: Biological roles of host defense peptide. Both direct antimicrobial killing and innate immune modulation occur with some peptides although certain peptides have one or the other activity preferentially (from Sahl G. and Hancock R.EW, 2006).

Peptides endowed with direct antimicrobial activity as primary function, are often properly termed antimicrobial peptides (AMPs) [20]. However, increasing evidences show that many AMPs, have multiple additional roles in innate immunity, such as anti-inflammatory activity [21], stimulation of wound healing [22], angiogenesis [23-24] and even anti-tumoral activity [25-26]. For this reason they are often referred to as host defense peptides (HDPs). In this regards, well-studied peptides are the two major classes of mammalian AMPs: defensins (which include the α, β and θ families) and cathelicidins with only one member in humans, the well-known peptide LL-37 [27-28]. As for the latter peptide, its anti-infective mechanism of action *in vivo*, although still not completely clear, seems to be related to its ability to modulate immune cell-functions rather than to its moderate direct antimicrobial activity [28].
Human major HDPs, are constitutively expressed in the granules of neutrophils (α defensins and LL-37) and in the body most susceptible to infection, such as β defensins and LL-37 expressed in the mucosal epithelia and the skin [9]. However, they may be inducibly expressed in response to pathogenic challenge such as β defensin 2 (hBD-2) up-regulated during inflammation disorders [28].

1.4.1 Examples of direct antimicrobial activity

Human defensins are an example of broad-range direct antimicrobial activity. Indeed they show antifungal activity against *Candida albicans* by lysing cells, in a manner similar to their antibacterial activity or exhibit strong viral-neutralizing activities avoiding viral entry or its intracellular shuttling [9]. There is no doubt that some peptides are present at concentrations suggesting that they act in a directly microbicidal fashion e.g. concentration of human α-defensins in neutrophil granules is estimated to be as great as 10 mg/ml. This would be sufficient to cause direct antimicrobial activity, despite the presence of divalent cations or other inhibitory substances [28]. On the other hand, it should be considered that body sites particularly sensitive to infections usually display a combination of several AMPs of different classes that may act additively or even synergistically. For example in human skin, at least four different classes of AMPs are co-expressed: LL-37, lysozyme, β-defensins and lactoferrin (Fig.5) [20-21].

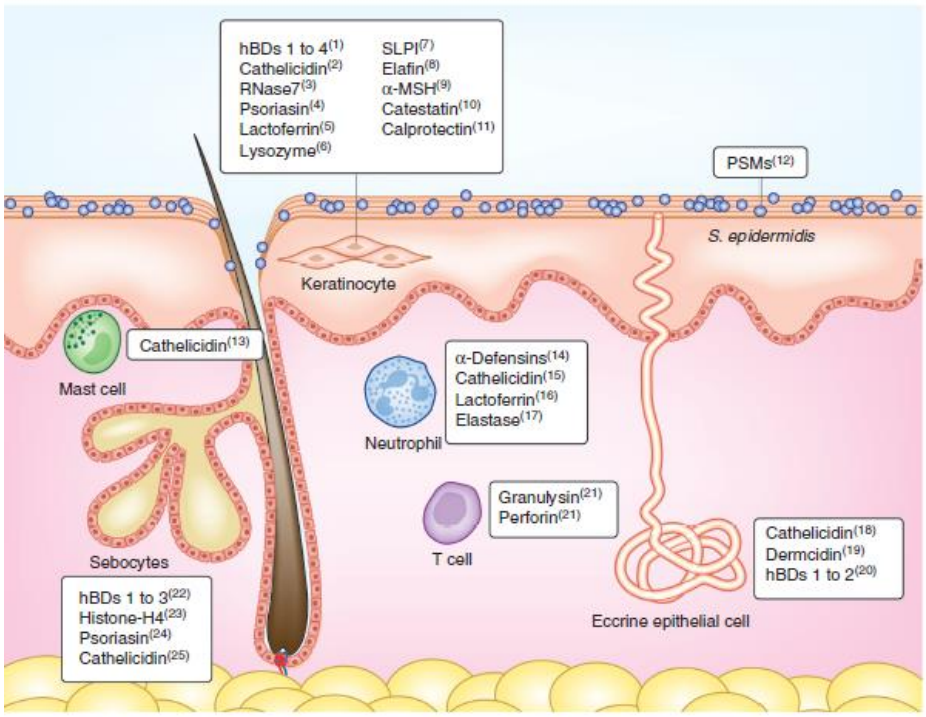


Figure 5: A representative partial list of antimicrobial peptides (AMPs) produced by skin-residing cells. hBD, human b-defensin; a-MSH, a-melanocyte- stimulating hormone; PSM, phenol-soluble modulin; RNase, ribonucleotidase; SLPI, secretory leukocyte peptidase inhibitor (from Nakatsuji T. and Gallo R.,2011)

1.4.2 Examples of immune modulatory activity

HDPs show additional biological properties involved in all stages of control and resolution of microbial infections and inflammation [21]. Studies on human and mouse cells have evidenced a variety of target cells stimulated by HDPs, including monocytes, macrophages, dendritic cells, epithelial cells, neutrophils, keratinocytes and others. The responses of these cells are often complex and dependent on the specific peptide, the type of cells, their activation state and the type of pathogen (Fig.6) [30]. For example, human α- and β-defensins can directly recruit leukocytes (by direct chemotaxis) or induce the expression of chemokines or cytokines including interleukin 8 (IL-8), MCP-1 (monocyte chemoattractant protein,) and interferon α (IFN-α), thereby indirectly promoting recruitment of effector cells (by indirect chemotaxis) [28-30]. HDPs direct chemotactic activity, is very interesting since it is based on receptor-dependent mechanisms, as it has been demonstrated for LL-37. The neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemo attract human peripheral blood neutrophils, monocytes, and T cells [31]. Furthermore, several HDPs act as anti-inflammatory compounds during sepsis [32], protecting the host from potentially lethal effects resulting from an excessive Toll-Like Receptor-mediated inflammatory response induced by bacterial endotoxins, such as lipopolysaccharide (LPS) and lipoteicoic acid (LTA) of Gram negative and Gram positive bacteria, respectively. HDPs act both directly by binding and sequestering LPS and indirectly by altering gene expression of various inflammatory cells [28]. The endotoxin-neutralizing activity is typical of many well-known HDPs (LL-37 and pig protegrin-39) [22] but is also found in recently identified AMPs, derived from human thrombin, GKY20 [33].
Finally, the biological activities of HDPs also include wound healing [22]. Very interestingly it has been demonstrated that cathelicidins can be used topically to stimulate wound healing in a diabetic mice model [29].

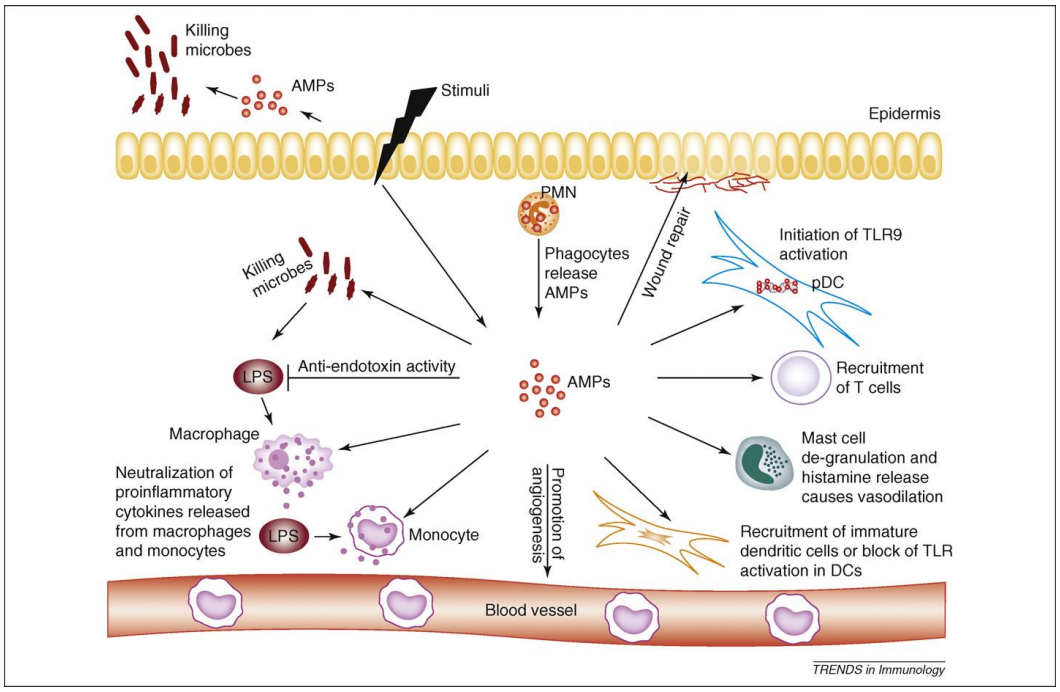


Figure 6: Schematic representation of multiple action of antimicrobial peptides in host defense. AMP, anti-microbial peptide; DC, dendritic cell; LPS, lipopolysaccharide; pDC, plasmacytoid dendritic cell; PMN, polymorphonucleocyte; TLR, Toll-like receptor (from Lai Y. and Gallo R.,2009).

1.5. AMPs advantages as novel anti-infective agents

The need of new antibiotics together with the knowledge of the broad and diverse biological functions of these endogenous peptides, have stimulated the interest in the development of AMPs as human therapeutics. They might be innovative anti-infective agents for several reasons:

1. broad spectrum of antimicrobial activity (including antifungal and antiviral);
2. ability to modulate immune cell functions often receptor-mediated;
3. low propensity for development of resistance;
4. ability to act either on planktonic cell and on resting cells (such as biofilm).

As regards point 4, it should be reminded that biofilm acts as a mechanical shield preventing antibiotic uptake, as shown for *Pseudomonas aeruginosa* biofilm, in the lung of immunocompromised patient of cystic fibrosis [34]. In this context, a promising potential application for AMPs is the enhancement of the potency of other antimicrobials: AMPs may facilitate access of conventional antibiotics into microbial cells, thus resulting in synergistic therapeutic effects [12-16]. A very interesting study demonstrated that biofilm of *P. aeruginosa* contains two subpopulations: a metabolically active subpopulation sensitive to tetracycline (an antibiotic) and a subpopulation with reduced metabolic activity sensitive to an AMP, colistin. Treatment with both tetracycline and colistin completely eradicate the biofilm [35].

1.6 Biotechnological application of AMPs

AMPs biotechnological applications span from pharmaceutical industry to food industry. They are gaining attention mainly as antimicrobial alternatives; food preservatives and recently as immune defense regulators. Some AMPs, are already in phase II/III clinical trial as antimicrobials restricted to topical applications [6-12].
For anti-infective purpose, the first AMPs to be commercially manufactured were gramicidins produced by *Bacillus brevis* [20] used as antibiotics for topical applications. Other examples are nisin, approved since 1969 [36], and lactoferrin added to infant milk formula [37], both used in food industry as natural preservatives or dietary supplements. Currently, application of antimicrobial peptides in the medical field is still one of the most interesting perspective. The ability of HDPs to modulate the innate immune system has made them promising candidates also as vaccine adjuvants [6]. The inclusion of appropriate adjuvants in vaccines helps to improve their efficacy. Evidences are based on several observations such as human neutrophil defensins that can enhance both humoral and cell-mediated antigen-specific immune response in murine models [38].
Moreover, using natural peptides as templates, synthetic peptides have been designed that retain the immunomodulatory properties but lack unwanted effects like mast cell degranulation exerted by certain natural HDPs as for example the bovine peptide bactenecin. The resulting molecules, named IDR peptides (Innate defense regulators) boost protective immunity against infections without possessing direct antimicrobial action [39]. Nowadays many IDR-peptides are in clinical trial for topical application. An example is IMX-492 that has recently completed phase I clinical trials in patients with cancer chemotherapy-induced immune suppression [6-12]. Other peptides, such as MX-226 and Hlf1-11, were originally developed as antimicrobial peptides but later showed interesting immunomodulatory activities [6]. Thus, there are ample opportunities for AMPs development for clinic use as anti-infective agents.

1.7 Proteins carrier of cryptic AMPs

Surprisingly, there are increasing evidences that many proteins whose functions are not necessarily related to host defense can act as sources of AMPs.

Some examples are haemoglobin, thrombin, lactoferrin, lysozyme, histone-like proteins and vertebrate secretory ribonucleases [40-41-42]. Lysozyme is a well-known example: it exerts bactericidal action by hydrolyzing peptidoglycan but, if inactivated by proteolysis, it releases AMPs [43]. Mammalian's lactoferrin, a multifunctional protein, possesses bactericidal determinants at the N-terminus. Proteolysis in the stomach of infants leads to the release of peptides, which are more bactericidal than the corresponding native protein on several strains, including antibiotic resistant strains [44-45]. Furthermore, it has been demonstrated that human thrombin, a key enzyme in the coagulation cascade, contains bactericidal determinants in the C-terminal region (Fig.7) [33-46]. Human fibrinogen, another key protein in coagulation process, after incubation with thrombin, releases from its β chain a peptide with antimicrobial effects [47]. Also proteins commonly known for their role in lipid transport into blood such as Apoliporotein B [48] and Apolipoprotein E [49] possess hidden AMPs. These AMPs, being "hidden" in sequence of large proteins, can be defined "cryptic" AMPs. The discovery of so many cryptic AMPs has led to propose a very interesting hypothesis: as some bacterial strains have acquired a limited resistance to AMPs by producing specific proteases, in response, multicellular eukaryotes, have developed a panel of "AMP-Releasing Proteins" (AMP-RPs) which release active peptides only after a partial proteolytic processing operated by bacterial and/or host proteases [50]. Thus, a bacterial strain that secretes proteases to protect from AMPs would "suicide" by releasing these "cryptic" AMPs from their precursors. This mechanism has been demonstrated for Zf-3, a ribonuclease from *Danio rerio* [51].

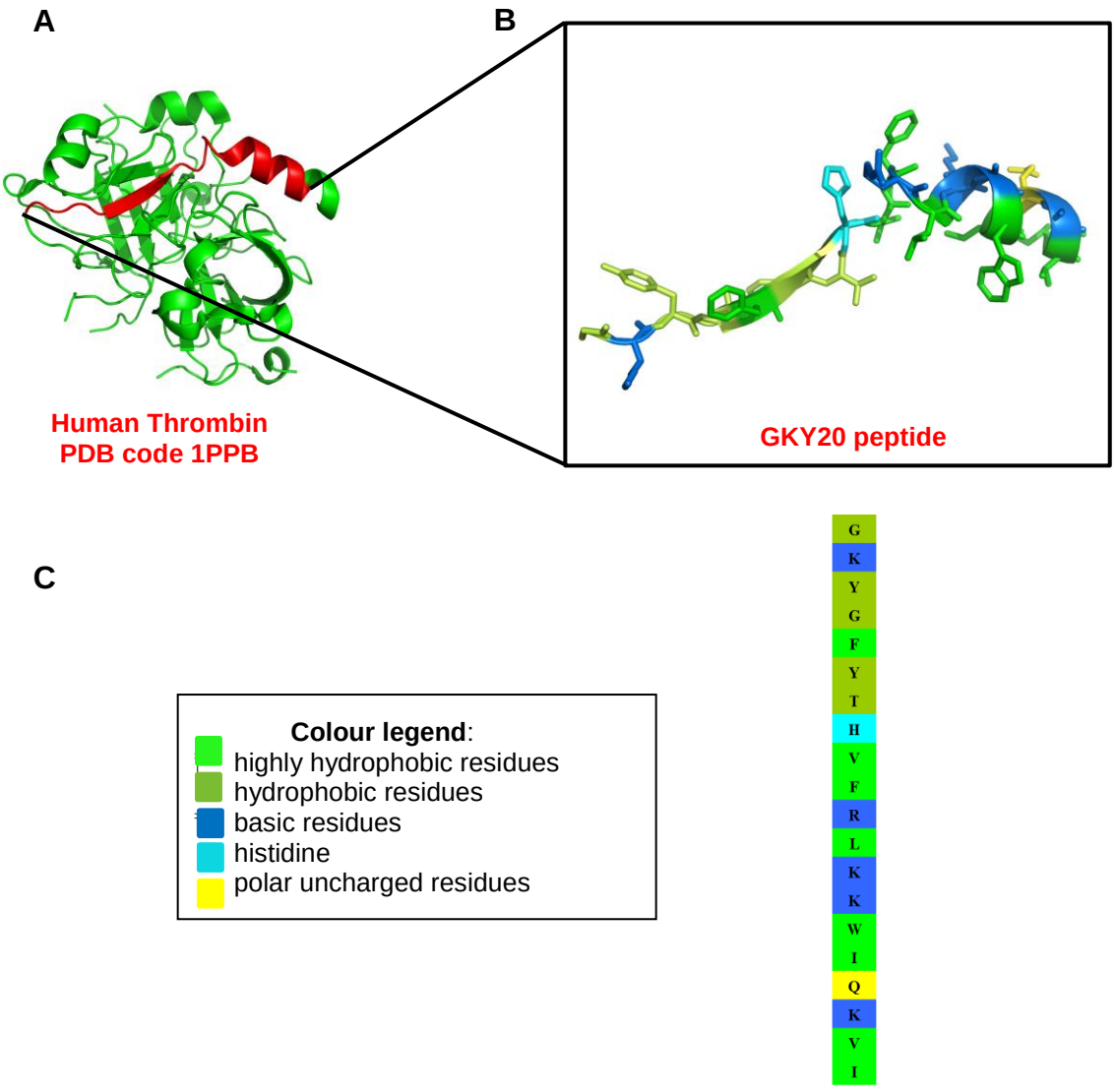


Figure 7: A) 3D structure of human thrombin protein, the antimicrobial C-terminal region is coloured in red. B) Secondary structure of GKY20 peptide. C) Amino acid sequence of GKY20 peptide.

1.8 A novel tool to identify new cryptic AMPs

Even if several cryptic AMPs are now known, their discovery often has been accidental rather than the result of a rational approach.
Moreover, it should be noted that the identification and the localization of a cryptic AMP inside its precursor is often carried out either through the analysis of peptide fragments generated by proteases, or by synthesizing a set of overlapping peptides which cover the entire sequence of the protein of interest.
Such experimental procedures are expensive and time-consuming, and thus faster bioinformatic approaches, capable of highlighting the presence of a putative antimicrobial region, could prove extremely useful.
In order to identify new hypothetical human AMPs by a rational approach, a novel bioinformatic method was developed by Dr. Notomista [52]. This method, based on a set of scoring functions, allows a semi-quantitative prediction of the antibacterial activity. This method was validated analyzing the sequence of known AMPs-Releasing proteins thus, it is a promising tool to identify new human AMP-RPs. Moreover, as the method includes some strain-specific variables, it can be calibrated to predict efficacy on any desired bacterial strain.
A first screening of about 4'000 human secreted proteins provided a list of potential new human AMPs derived from several proteins (including some proteins involved in coagulation cascade, some collagen and mucin isoforms, serum proteins etc.).
These new hypothetical human AMPs possess an high potential from a biotechnological point of view, indeed, being of human origin, likely they will be not immunogenic.

1.9 AMPs chemical and biological production methods

Chemical synthesis and biological production methods are the most popular techniques for production of peptides in general and of AMPs in particular.
Chemical synthesis is particularly well suited when a relatively small amount of peptide is required and when the peptide is shorter than about 30 residues [53]. However, as the biotechnological application of these molecules cannot overlook production costs, biological production methods can be potentially more attractive for large scale production.
Due to the well-known biology, availability of cloning vectors and cheap media for cell growth, *E. coli* is a common microbial platform for AMPs production [54-55].
The main concerns in developing an AMP production platform in bacteria expression system are: (i) AMP toxicity for the host, ii) intracellular degradation by proteases leading to low yields and iii) peptide purification.
These difficulties can be circumvented by fusing the desired peptides to a carrier protein that can protect peptides from proteases, neutralize possible toxic effects and provide a convenient route for their purification.
By now, more than 30 kinds of fusion tags with different sizes and functions have been used in AMPs expression. Some examples are: thioredoxin (Trx), glutathione S-transferase (GST), maltose binding protein (MBP), intein-mediated systems [56].
However, also fusion protein strategy has two main drawbacks. Firstly, the fusion protein has to be cleaved and peptide needs to be separated from the carrier, secondly, the desired peptide usually represents just a small percentage of the purified fusion protein. As for the cleavage of the peptide, it can be released from the fusion protein by enzymatic or chemical cleavage at a site suitably introduced at the carrier-peptide junction [55]. The efficiency and specificity of the cleavage is the main bottleneck of fused peptide production. Usually enzymatic proteolysis by factor Xa

[57-58], enterokinase [59] and thrombin [60-61], the most used enzymes, is less efficient than chemical cleavage [62]. On the other hand, the most popular cleavage reagents, like cyanogen bromide (CNBr), formic acid and hydroxylamine, [62], often produce unwanted cleavages and side-chain modifications due to the harsh reaction conditions needed for the cleavage [62].
The thorough characterization of new promising human AMPs requires an effective method for their production. For this reason, part of the work of this PhD project has been devoted to the development of an efficient and low cost method for the production of recombinant AMPs.

1.10 Aims
The main aims of this research work are:

I. Development and optimization of efficient strategies for the production of recombinant toxic peptides in *E.coli*.

II. Production of biologically interesting AMPs selected from the list of new potential AMPs suggested by the bioinformatic screening of human secreted proteins.

III. Functional characterization of human AMPs with promising pharmacological activities.

Chapter 2-Materials and Methods

2.1 Materials

Expression host strain E.coli BL21(DE3) genotype: 95 F^- *ompT hsdSB* (rB- mB -) *gal dcmrne* 131 (DE3) and plasmid pET22b(+) were purchased from Novagen (San Diego, CA, USA). E. coli strain TOP10F' was obtained from Invitrogen (San Diego, CA, USA). QIAprep spin miniprep kit was from Qiagen (Germantown, MD, USA). Wizard SV.Gel and PCR Clean-Up DNA Purification System for elution of DNA fragments from agarose gel was purchased from Promega (Madison, WI, USA). Enzymes and other reagents for DNA manipulation were from New England Biolabs (Ipswich, MA, USA). Ni Sepharose™ 6 Fast Flow was from GE Healthcare (Uppsala, Sweden). Difco Nutrient Broth, Mueller Hinton Broth, Tryptic Soy Broth and Yeast extract were from Becton-Dickenson (Franklin Lakes, NJ). Trypton was purchased from PanReac Applichem (Germany, UE). All other chemicals were from Sigma-Aldrich (Milano, Italy).

2.2 General procedures

Bacterial cultures, plasmid purifications and DNA manipulation were carried out according to Sambrook [63]. DNA sequences and oligonucleotide synthesis were performed by Eurofins MWG Operon service (Ebersberg, Germany). Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to Laemmli [64]. The percentage of polyacrylamide was 6% for the stacking gel and 15% in resolving gel (to separate proteins with a molecular weight up to 10'000 Da) and 20% to separate peptides (molecular weight lower than 8'000 Da). Cleavage efficiency was analyzed by SDS-PAGE 20%. Gels were stained by Coomassie Blue staining solution containing 4 % (v/v) formaldehyde to cross-link proteins and polypeptides [65]. *Gallus gallus* lysozyme (14.3 kDa), was used as molecular marker. When appropriate, the relative amount of bands was determined by densitometry performed using the Gel Doc™ XR system (Bio-Rad Laboratories, Inc.) equipped with the Quantity One Software. Protein concentration were determined using the Bradford Protein Assay (Sigma-Aldrich, St. Louis, MO, USA) with standard curves generated using bovine serum albumin. Concentrations of purified fusion proteins and peptides were determined by spectrophotometric analysis using the extinction coefficients calculated using the ProtParam tool (accessible to the address http://web.expasy.org/protparam/). Alternatively, Bicinconinic acid Assay was used for peptides without aromatic residues.

2.3 Peptides

Synthetic GKY20 peptide was from INBIOS s.r.l. (University of Naples, Italy), all other synthetic peptides used in this PhD thesis (Apo $E_{(133-150)}$; Apo $B_{(887-911)}$;PA $3_{(1-25)}$; PA $3_{(26-47)}$, $\beta Fib_{(420-447)}$, HHC-36, DJK-6 and IDR-1018) were from Peptide 2.0 Inc (Chantilly, USA). Peptides were synthesized using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and purified to a purity >95 % using reverse-phase high-performance liquid chromatography (HPLC). Peptides mass were confirmed by mass spectrometry.

2.4 Bacterial growth culture media

Luria Bertani Broth (LB) contained 1 % (w/v) sodium chloride, 1% (w/v) trypton and 0.5 % (w/v) yeast extract. The solid medium was obtained from the liquid one by adding agar to a final concentration of 1.5% (w/v) as a gelling agent [63].
Terrific Broth (TB) was prepared with 1.2 % (w/v) trypton, 2.4 % (w/v) yeast extract; 0.4 % (v/v) glycerol and potassium phosphate buffer 89 mM pH 7.4.

Basal medium 2 (BM2) composed of 62 mM potassium phosphate buffer pH 7.0, 7 mM $(NH4)_2SO_4$, 0.5 mM $MgSO_4$, 10 μM $FeSO_4$ containing 0.4% (w/v) glucose as a carbon source.

2.5 Antibiotics

Ampicillin (Amp) was used always at a concentration of 100 μg/mL.

2.6 Construction of the expression vectors

Synthetic genes coding for recombinant proteins reported in Table 1 in "Results and discussion" section were obtained by MWG-Biotech AG (Ebersberg, Germany). All codons were optimized for expression in E. coli and restriction sites.

2.7 Expression of recombinant proteins

E. coli strain BL21(DE3) was used to express recombinant proteins. Cells, transformed with pET recombinant plasmids, were grown in 10 mL of TB medium containing 100 μg/ml ampicillin, at 37°C up to an absorbance of 2 OD at 600 nm. These cultures were used to inoculate 1 L of TB/ampicillin medium supplemented with 4 g/L glucose. Cultures were incubated at 37°C up to $OD_{\lambda 600nm}$ of 3.5-4. Expression of recombinant proteins was induced by addition isopropylb- D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM.
Cells were harvested after overnight induction by centrifugation at 8000x g for 15 min at 4°C and washed with 50 mM Tris-HCl buffer, pH 7.4. The bacterial pellet was suspended in 50 mM Tris-HCl, pH 7.4, containing 10 mM EDTA, and sonicated in a cell disruptor (10 x 1 min cycle, on ice). The suspension was then centrifuged at 18,000 x g for 60 min at 4°C. Soluble and insoluble fractions were analyzed by SDS-PAGE. The insoluble fractions containing recombinant protein in the form of inclusion bodies, were washed three times in 0.1 M Tris-HCl, pH 7.4, containing 10 mM EDTA, 2% Triton X-100 and 2 M urea, followed by repeated washes in 0.1 M Tris-HCl, pH 7.4, to eliminate traces of Triton, urea and EDTA.

2.8 Purification of fusion proteins

All His6-tagged recombinant proteins reported in Table 1 in "Results and discussion" section, were purified by immobilized metal ion affinity chromatography (IMAC), using the Ni Sepharose™ 6 Fast Flow resin. 100 mg of fusion proteins were dissolved in 10 mL of denaturing buffer (6M guanidine/HCl in 50 mM Tris-HCl, pH 7.4) and incubated on a rotary shaker at 37°C for 3 h under nitrogen atmosphere. Soluble fractions were collected by centrifugation and incubated with 5 mL of Ni Sepharose™ 6 Fast Flow resin equilibrated in denaturing buffer. The resin was shaken at 4°C for 16 h and then collected by centrifugation. The supernatant, containing the unbound proteins, was discarded. The resin was washed three times with 25 ml of denaturing buffer at 4°C for 30 min and then packed in a glass column. The fusion proteins were eluted with 20 ml of 0.1M sodium acetate buffer, pH 5.0, containing 6M guanidine/HCl (elution buffer). The eluate was extensively dialyzed against 0.1 M acetic acid at 4°C. Samples were stored at -80°C under nitrogen atmosphere. Purified fusion protein concentrations were determined by spectrophotometric analysis using the extinction coefficients calculated using the ProtParam tool. Before purification ONC-DC*less*-H6-(C)GKY20 protein was modified by disulfide bond formation with cysteamine (CEA). 100 mg of proteins (inclusion bodies) were dissolved in modification and denaturing buffer (0.1 M Tris-HCl, pH 8.4, containing 0.5 M cystamine, 10 mM EDTA and 6 M guanidine/HCl). After that, soluble fraction was collected by centrifugation and

acidified to pH 4.0 by adding glacial acetic acid before to dialyze sample against 0.1 M acetic acid extensively at 4°C. This step produced a clear protein precipitation. Soluble and unsoluble fractions were lyophilized and then dissolved in denaturing buffer to purify recombinant protein by IMAC as described above. The modified product was named ONC-DC*less*-H6-(C)GKY20-CEA.

2.9 Acid cleavage of Asp-Pro and Asp-Cys peptide bond

Routinely acid cleavage of fusion proteins was obtained by using 0.1 M acetic acid at pH 2.0 (by addition of 18 mM HCl) with an incubation time of 24 h at 60°C in a water bath under nitrogen atmosphere. In the case of ONC-DC*less*-H6-(C)GKY20-CEA 5 mM of the reducing agent Tris(2-carboxyethy1)phosphine (TCEP) was added to the reaction to remove the cysteamine protecting group. In the case of ONCDCless-H6-(P)βFib$_{(420-447)}$ and ONC-DC*less*-H6-(P)βFib$_{(425-447)}$ to prevent methionine oxidation TCEP was added at molar ratio TCEP:methionine=5:1. Percentage of cleaved protein was determined by densitometric analysis of SDS-PAGE gels as described in "General procedures".

2.10 Purification of recombinant peptides

After acid cleavage of the fusion proteins, the pH of the mixtures was adjusted to 7–7.2 by adding NH_3. Samples were purged with N_2 and incubated at 28°C for 16 h in a water bath. Peptides soluble at pH 7 [(P)GKY20, (P)PA3$_{(1-47)}$, (P)βFib$_{(425-447)}$, (P)Apo E derived peptides and (P)Apo B derived peptides] were isolated from the insoluble carrier through repeated cycles of centrifugation at 18,000 x g for 60 min at 4°C (Table 1). Purified peptides were lyophilized and stored at -80°C. Peptide concentrations were determined by spectrophotometric analyses using the extinction coefficients calculated using the ProtParam tool or by BCA assay.

Peptides (C)GKY20 and βFib$_{(420-447)}$ that co-precipitated with Onconase carrier at neutral pH and (P)PA3 AB$_{(1-47)}$ and its derived peptides [(P)PA 3$_{(1-25)}$and (P)PA 3$_{(26-47)}$)] that were present as mixture of peptides after acid cleavage, were purified in RP-HPLC immediately after acid cleavage (Table 2).

2.11 HPLC

Peptides purification and purity analysis were performed by reverse-phase chromatography. HPLC carried out with a Waters (Milford, MA, USA) system (1525 binary pump and 2996 photodiode array detector). The column was a C18 (250 x 4,6 mm, 5µm particle size) Jupiter 5 µm C18 300Å (Phenomenex). The solvents were 0.05% trifluoracetic acid (TFA) in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). Solvent B and TFA were added to the sample before their loading, at a final concentration of 5% and 0.1%, respectively. The elution was monitored at the wavelength of 280 nm. HPLC gradients are reported in Table 1 and Table 2.

Table 1: Peptide purification method after acid cleavage and analysis of purity by RP-HPLC (with gradient programs used for each peptides).

Peptide	Peptide purification method after acid cleavage	Analysis of purity (HPLC method used)		
		Time (minutes)	Solvent A	Solvent B
(P)GKY20	Neutralization at pH 7 with NH_3 and peptide recovery by centrifugation	60'GRAD	5 %	95%
$\beta Fib_{(425-447)}$	Neutralization at pH 7 with NH_3 and peptide recovery by centrifugation	5' GRAD 40'GRAD 1' GRAD	70% 62% 5%	30% 38% 95%
(P)PA $3_{(1-47)}$	Neutralization at pH 7 with NH_3 and peptide recovery by centrifugation	5' GRAD 40'GRAD 1' GRAD	80 % 70% 5%	20% 30% 95%

Table 2: Peptide purification after acid cleavage and analysis of purity by RP-HPLC (with gradient programs used for each peptides).

Peptide	Peptide purification after acid cleavage (HPLC method)			Analysis of purity (HPLC method)		
(C)GKY20	**Analytical**			**Time (minutes)**	**Solvent A**	**Solvent B**
	Time (min)	**Solvent A**	**Solvent B**			
	10' ISO 5' GRAD 40' GRAD 1' GRAD	90% 80% 70% 5%	10% 20% 30% 95%	60'GRAD	5 %	95%
	Preparative					
	Time (min)	**Solvent A**	**Solvent B**			
	10' ISO 5' GRAD 60' GRAD 60' ISO 1' GRAD	90% 80% 70% 70% 2%	10% 20% 30% 30% 98%			
$\beta Fib_{(420-447)}$	**Analytical**			10' ISO 5' GRAD 40'GRAD 1' GRAD	95% 70 % 62% 5%	5% 30% 38% 95%
	Time (min)	**Solvent A**	**Solvent B**			
	10' ISO 5' GRAD 40' GRAD 1' GRAD	95% 70 % 62% 5%	5% 30% 38% 95%			
	Preparative					
	Time (min)	**Solvent A**	**Solvent B**			
	10' ISO 60' GRAD 60' ISO 1' GRAD	73% 70% 70% 5%	27% 30% 30% 95%			
$(P)PA3AB_{(1-47)}$ and its derived peptides	**Analytical and preparative**			10' ISO 5' GRAD 40'GRAD 1' GRAD	95% 80 % 70% 5%	5% 20% 30% 95%
	Time (min)	**Solvent A**	**Solvent B**			
	10' ISO 5' GRAD 40' GRAD 1' GRAD	95% 80 % 70% 5%	5% 20% 30% 95%			

2.12 PyMPO fluorophore conjugation to (C)GKY20 peptide

Purified (C)GKY20 peptide was conjugate to PyMPO fluorophore for the production of a fluorescently labelled peptide. The reaction was carried out as reported in Table 3 with a molar ratio of 5:5:1 respectively PyMPO:TCEP:thiol groups. Samples were incubated at 28°C in a water bath for 16 hours in the dark. Control reaction was carried out without adding peptide and TCEP in the reaction mixture. Reaction mixtures were analyzed by Reverse phase HPLC equipped with a photodiode array detector to determine the UV-Vis spectra of the chromatographic peaks. Solvent B and TFA were added to the samples at a final concentration of 5% and 0.1%, respectively. The conjugated peptide was purified by the gradient reported in Table 4. (C)GKY20-PyMPO peptide was lyophilized, dissolved in 5 mM ammonium acetate (AMAC) pH 5.0 and stored at -80°C under nitrogen atmosphere in the dark. Peptide concentration was determined by Bradford assay.

Peptide purity was verified by SDS-PAGE (20%) and RP-HPLC using the gradient reported in Table 4. Peptide identity was confirmed by mass spectrometry (MALDI).

Table 3: PyMPO fluorophore conjugation to (C)GKY20 peptide

Stock solution	Final concentration	Molar ratio
Tris HCl 1 M pH 7.2	0.1 M	
TCEP 100 mM in water	1 mM	TCEP:-SH 5:1
Peptide 1.78 mg/ml (680 µM) in AMAC 5 mM pH 5.0	191 µM (0.5 mg/ml)	
Guanidine-HCl (MW 95.53 g mol^{-1})	2 M	
EDTA 0.5 M pH 8.0	7 mM	
PyMPO 100 mM in DMF	1 mM	PyMPO:-SH 5:1 PyMPO:TCEP 1:1
Final volume	1 ml	

Table 4: (C)GKY20-PyMPO purification method and analysis of purity by RP HPLC.

Peptide	Purification method after conjugation reaction (HPLC method)			Analysis of purity (HPLC method)		
	Time (minutes)	Solvent A	Solvent B	Time (minutes)	Solvent A	Solvent B
(C)GKY20-PyMPO	10'ISO 5' GRAD 30' GRAD 10' GRAD 1' GRAD	95% 80% 60% 50% 5%	5% 20% 40% 50% 95%	10'ISO 5' GRAD 30'GRAD 10'GRAD 1' GRAD	95% 80% 60% 50% 5%	5% 20% 40% 50% 95%

2.13 Antibacterial and antibiofilm assay

Antibacterial activity assays were carried out by agar dilution plate viable-count method (see accompanying paper and [51]) and by broth microdilution method to determine the Minimal Inhibitory Concentration (MIC) [67]. Routinely assays were performed in Nutrient broth 0.5X. HHC-36 peptide was included as positive control in the experiment performed during the PhD period abroad. Antibiofilm assay were performed by 96-well plate assays and crystal violet staining of adherent biofilms in BM2 minimal medium to obtain the Minimal Biofilm Inhibitory Concentrations leading to 50% ($MBIC_{50}$) or 100 % ($MBIC_{100}$) decrease in biofilm growth as described previously [68] using DJK-6 peptide included as positive control. Biofilm cultivation in flow cell chambers for the microscopy analysis was performed as described previously [68] using *P. aeruginosa* PAO1 bacterial strain and peptides concentration of 10 µg/ml.

2.14 Bacterial strains used for antimicrobial susceptibility test

Escherichia coli O157:H7, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* MRSA (clinical isolate C622) and Bacillus subtilis (clinical isolate C626) are clinical strains isolated from patients in Vancouver Hospital, Canada. *Escherichia coli* DH5α, *Staphylococcus aureus* ATCC 6538P were from our laboratory strains collection. *Pseudomonas aeruginosa* PA14 and *Pseudomonas aeruginosa* KK27 clinical strains isolated from cystic fibrosis patients were kindly provided by Dr. Alessandra Bragonzi (San Raffaele Hospital, Milan).

2.15 Blood cell isolation and lactate dehydrogenase (LDH) assay

Venous blood from healthy volunteers was collected in Vacutainer collection tubes containing sodium heparin as an anticoagulant (BD Biosciences), in accordance with University of British Columbia ethical approval and guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated as described [69]. Peptides cytotoxicity was verified by LDH assay. Cells (2×10^5) were seeded into 96 well plates (Sarstedt, Newton, NC) and incubated at 37°C in 5% CO_2 overnight in presence of each peptides i.e. Apo $E_{(133-150)}$, $ApoB_{(887-911)}$, $PA3_{(1-25)}$, $PA3_{(26-47)}$, $\beta Fib_{(420-447)}$ or IDR-1018 (negative control) at concentrations up to 50 µg/ml. The release of cytosolic LDH in culture media was then measured using an enzymatic reaction that results in a red formazan product which can be measured spectrophotometrically (λ=490 nm). Cells treated with a final concentration of 2% Triton X-100 were used as positive control demonstrating 100 % of lysis. All experiments were done in triplicate on all blood samples used for cell isolation.

2.16 Detection of cytokines/chemokines

PBM cells were exposed to peptide at concentration of 50 µg/ml; 25 µg/ml and 5 µg/ml for 24 hours and/or 10 ng/ml of *P.aeruginosa* PAO1 LPS. IDR-1018 was used as positive control peptide. Chemokine MCP-1 and cytokines TNF-α and IL-1β secretion in the tissue culture supernatants were detected by sandwich ELISA kits (all from eBiosciences) as described [69]. All assays were performed in triplicate starting from at least three blood samples.

2.17 MTT assay

Human monocytic cell line THP-1 were seeded on 96-wells plates (10'000 cells in 100 µL of RPMI-1640 medium) and grown at 37°C overnight. Peptide (P)$PA3_{(1-47)}$, was added at concentrations up to 2-fold MIC value (on E.coli DH5α and S.aureus

ATCC6538P). After 24 hours of incubation, 10 µL of a MTT stock solution in PBS 1X were added to the cells to a final concentration of 0.5 mg/mL in Dulbecco's modified Eagle's medium without red phenol (final volume 100 µL). After 4h of incubation, cells were harvested by centrifugation at 1500 rpm for 5 minutes, the MTT solution was removed and MTT formazan salts were dissolved in 100 µL of 0.1 N HCl in anhydrous isopropanol. Cell survival was expressed as the absorbance of blue formazan measured at λ=570 nm with an automatic plate reader (Victor 3TM Multilabel Counter; Perkin Elmer, Shelton, CA, USA). Experiments were performed in triplicate and standard deviations were always <5% for each experiment.

2.18 Ellman assay

Ellman assay is a colorimetric assay based on Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid], DTNB). DTNB reacts with sulfhydryl groups to yield a colored (yellow) product, providing a reliable method to measure reduced cysteines and other free sulfhydryls in solution. This assay was used to estimate the percentage of free thiol cysteine group in ONC-DCless-(C)GKY20 by comparing the sample absorbance at λ=412 nm to a standard curve of a sulfhydryl-containing compound such as cysteine.

Chapter 3-Results and Discussion

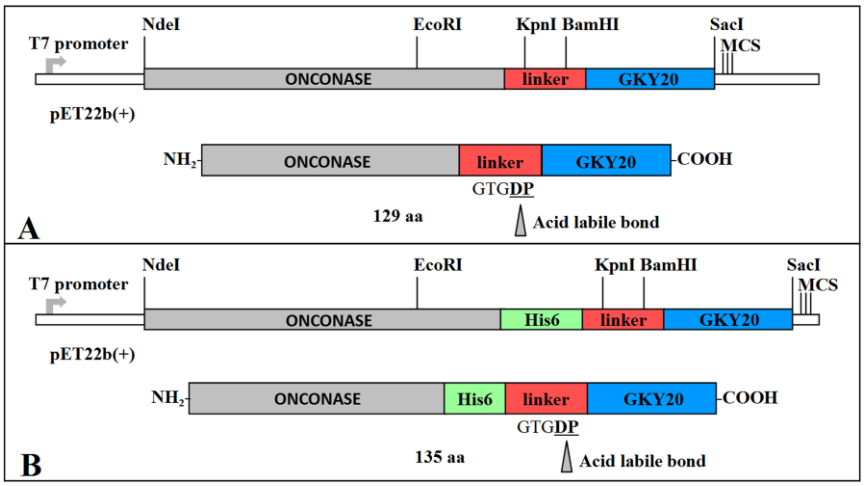
3.1 Development of a novel fusion system for recombinant AMPs production

AMPs are of great interest due to their potential applications in pharmacological and food industry. However, commercial uses of bioactive peptides require low cost and effective methods for their production. For this reason, part of this work was devoted to the development of a novel fusion system for high yield production of recombinant peptides in *Escherichia coli* very well suited for toxic peptides like antimicrobial peptides. This section describes briefly the method whereas a detailed description can be found in the paper "Rational design of a carrier protein for the recombinant production of toxic peptides in *Escherichia coli*" enclosed at the end of the thesis.

The denatured form of onconase (ONC), a RNase from *Rana pipiens* [70], was chosen as starting point to design the carrier protein. ONC is a very well suited carrier for several reasons: (i) it can be expressed at very high levels in inclusion bodies (about 200-250 mg/L in terrific broth); (ii) usually, no soluble ONC can be detected in cultures thus minimizing the risk of toxic effects of the ONC-peptide fusion proteins; (iii) it is a very small protein (104 aa) thus allowing high yields of the peptides after the cleavage; (iv) the solubility of denatured onconase is pH dependent – the denatured protein is soluble only at pH <4 – thus allowing the purification of peptides soluble at pH 7 by selective precipitation of the carrier.

Moreover, ONC does not contain Asp-Pro and Asn-Gly sequences and the mutant (M23L)-ONC [71] does not contain internal methionine residues, therefore onconase carrier will not be cleaved by all common chemical cleavage strategies such as formic acid, which cleaves the bond between aspartate and proline, cyanogen bromide, which cleaves the Met-X bonds and hydroxylamine, which cleaves the bond between asparagine and glycine [72].

We tested the efficiency of our strategy by producing GKY20, a short cationic antimicrobial peptide derived from the C-terminus of human thrombin [33] active on different strains of *E. coli* with a minimal inhibitory concentration (MIC) lower than 10 μM. In order to use denatured ONC as carrier for the production of recombinant peptide GKY20, (M23L)-ONC cDNA was fused to a linker sequence coding for the acid cleavable aspartyl-prolyl sequence, and an *in silico* designed sequence coding for GKY20 (Fig. 1).



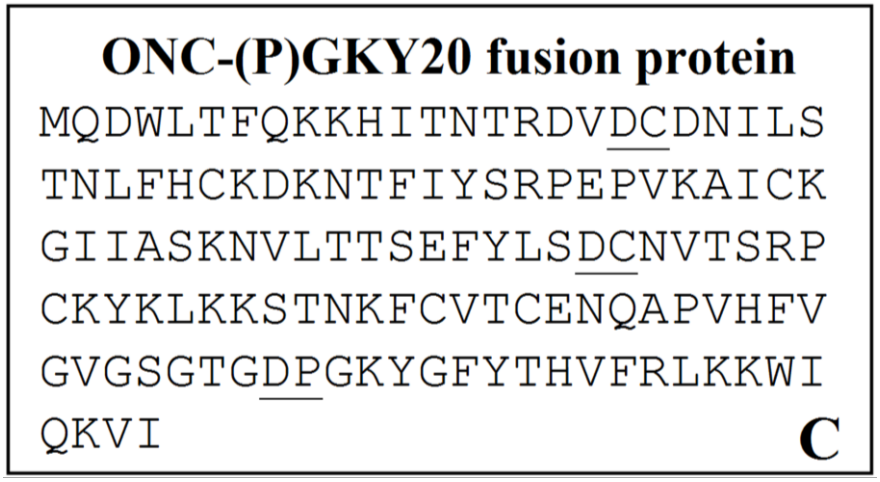


Figure 1: Schematic representation of expression vectors and recombinant proteins. Fusion proteins without (A) and with (B) His6-Tag; C) Amino acid sequence of ONC-(P)GKY20 fusion protein, the main cleavage sites are underlined.

The synthetic construct and the corresponding recombinant protein were named ONC-(P)GKY20 to underline that, by acid cleavage, the recombinant protein releases peptide (P)GKY20 i.e. peptide GKY20 with an additional proline at the N-terminus. The construct was cloned into pET22b(+) plasmid between NdeI and SacI restriction sites (Fig. 1) and expressed into BL21(DE3) *E. coli* cells.

SDS-PAGE analysis of induced cultures showed high expression levels of ONC-(P)GKY20 (200-250 mg/L) as inclusion bodies (Fig.2 in the accompanying paper). This sample was used to optimize the acidic cleavage of the DP sequence in order to find less harsh cleavage condition than concentrated formic acid (50-75% formic acid) at high temperature (from 55 to 80°C) commonly used in literature [73]. Several variables were gradually changed such as acid solution, its concentration, incubation temperature, time of incubation and pH, described in accompayning paper. These experiments showed that a solution of acetic acid 0.1 M adjusted at pH 1.8-2.0 with HCl allows to obtains cleavage efficiencies higher than 90-95%. Unfortunately, they also showed that hydrolysis of all the six Asp-X sites present in the sequence of (M23L)-ONC occurred (Fig 3 in the accompanying paper), although at different degree. Among these sites, two Asp-Cys sites were particularly sensitive to acid cleavage (see Fig.1), a result not described in literature. As cleavage efficacy was almost comparable to that of the Asp-Pro site this suggested the possibility to prepare recombinant peptides with a single additional cysteine at the N-terminus (see next section). In addition, some *E. coli* proteins, present in small amount as contaminants in the inclusion bodies, also underwent cleavage, thus releasing short fragments and making more complex the purification of (P)GKY20. On the basis of these observations we decided to improve the carrier sequence. First, to overcome the undesired cleavages observed, all Asp residues were mutated to Glu residues and then, to avoid the formation of short fragments, derived from the hydrolysis of contaminant *E. coli* proteins, an His6Tag sequence was added at ONC C-terminus to facilitate fusion protein purification and then peptide recovery. Moreover, all cysteine residues were mutated to Tyr, Ile or Leu. The choice of the mutations is described in detail in the accompanying paper.

The final carrier protein, ONC-DCless-H6-(P)GKY20, bearing 15 point mutations (see Table 1 in the accompanying paper), was produced in the form of inclusion bodies with a yield similar or higher than that observed for ONC-(P)GKY20 (Fig. 2 in the accompanying paper), thus demonstrating that the amino acid substitutions into the carrier sequence did not influence protein expression levels. After purification on Ni-sepharose, ONC-DCless-H6-(P)GKY20 showed about 98% purity as determined by SDS-PAGE densitometry scan (Fig. 6 in the accompanying paper), with a recovery of about 95%. After cleavage in 0.1 M acetic acid adjusted at pH 2.0, at 60°C for 24 h, SDS-PAGE analysis allowed to estimate 95% efficiency in the release of (P)GKY20 (Fig. 6 in the accompanying paper). Adjusting the pH to 7.2-7.4 with NH_3 and incubating the mixture at 28°C for 16 h the uncleaved ONC-DCless-H6-(P)GKY20 and ONC-DCless-H6 proteins formed macroscopic insoluble aggregates which were completely removed by centrifugation (Fig. 6 in the accompanying paper).

Peptide (P)GKY20 was recovered in the soluble fraction, lyophilized and used for the characterization described below without any further purification step. Mass spectrometry analysis (Fig. 7A in the accompanying paper), confirmed the peptide identity and showed that no modified form (e.g. oxidized or formylated peptides) was present in the sample. Final purity of (P)GKY20 peptide, as determined by reverse phase chromatography (Fig. 7B in the accompanying paper), typically ranged from 95% to 99%. The optimized procedure allowed the purification of about 10-11 mg of

peptide starting from 100 mg of purified fusion protein with 70-75% recovery efficiency (milligrams of purified peptide/expected milligrams of peptide) with respect to the theoretical amount of peptide in the fusion protein.
As the optimized carrier does not possess any methionine residue, cleavage of the desired peptides can also be achieved by introducing a methionine residue in linker sequence and using CNBr to cleave the Met-X bond. In order to verify this possibility, I prepared the fusion protein, ONC-DCless-H6-(PM)GKY20, which contains a Asp-Pro-Met sequence immediately upstream the GKY20 sequence. By acid hydrolysis it releases peptide (PM)GKY20 carrying two additional residues at the N-terminus whereas cleaved using CNBr it releases the peptide GKY20 without any additional residue at the N-terminus with an efficiency similar to acid cleavage (Fig. 8 in the accompanying paper).

3.2 Production of GKY20 with a N-terminal cysteine residue

Even if the sensitivity of Asp-Pro sequences to acid cleavage has been extensively reported in literature [73] the efficient acid cleavage of Asp-Cys peptide bonds, to the best of our knowledge, has never been described before. Therefore the observation that the two Asp-Cys bonds in ONC are very sensitive to acid cleavage suggested the intriguing possibility to prepare recombinant peptides with a cysteine residue at the N-terminus. Such peptides would possess several useful applications both in research and in the biotechnological field. For example, due to the high reactivity of the thiol group, cysteine residues can be irreversibly modified –through the use of alkylating reagents– and reversibly modified –through the formation of mixed disulfides with other thiols. Moreover, being a 1,2-aminothiol, a N-terminal cysteine residue can be used in cycloaddition reactions and in protein ligation reactions thus significantly expanding the variety of post-synthetic *in vitro* modifications.

To explore this possibility, the acid labile Asp-Pro sequence between ONC and GKY20 peptide, was replaced with a Asp-Cys sequence thus yielding ONC-DCless-H6-(C)GKY20 fusion protein in which the only cysteine residue is at the N-terminus of the peptide. The high reactivity of thiols however can make complex the purification of proteins/peptides with free cysteine residues. In order to prevent any undesired irreversible oxidation of the single cysteine residue at the acid cleavage site we decided to protect this residue by converting it to a mixed disulfide with cysteamine (CEA; 2-aminoethanethiol), a small and very soluble thiol that can be easily removed by the addition of any reducing agent (e.g. DTT, β-mercaptoethanol, TCEP ecc.) according to a procedure previously developed in our laboratory [70]. Inclusion bodies were dissolved in a denaturing buffer containing 0.5 M cystamine (CYA), the disulfide of cysteamine. By reacting with free cysteine residues in the proteins CYA provides cysteine-cysteamine mixed disulfides and free cysteamine [70]. As the reaction is reversible a large excess of cystamine is necessary to modify the majority of cysteine residues in the inclusion bodies. After the incubation at 37°C the reaction mixture was acidified to pH 4.0 to stop thiol exchange reaction and dialyzed vs 0.1 M acetic acid to remove cystamine and denaturants. After dialysis the desired protein was found partly in the soluble fraction (about 60%) and partly in the insoluble fraction (data not shown). The soluble fraction was lyophilized and both fractions were dissolved in denaturant buffer (without reducing or modifying agents). The fraction of free thiol groups with respect to the theoretical content of cysteine residues in the sample was determined by the Ellman assay.

The percentage of free cysteine residues was typically below 5% thus demonstrating that the protection reaction is efficient. The mixed disulfide-fusion protein, ONC-DCless-H6-(P)GKY20-CEA, was purified by IMAC as already described for ONC-DCless-H6-(P)GKY20 fusion protein. The yield of the purification process, was 90-95%. Sample was dialyzed against 0.1 M acetic acid pH 3, to remove guanidine. Acid cleavage was carried out on ONC-DCless-H6-(C)GKY20-CEA modified protein in the same conditions (pH 2 and 60°C) previously optimized for (P)GKY20 production.

The reaction was carried out either in the presence or in the absence of Tris 2-carboxylethil phosphine (TCEP) a strong reducing agent that, differently from conventional reducing agents like DTT, is active also at very acidic pH values. SDS PAGE analysis (Fig.2) revealed that TCEP, was essential to obtain an efficient cleavage. The cleavage efficiency of ONC-DCless-H6-(C)GKY20-CEA, was of 80%-85% as determined by densitometric analysis, thus only slightly less efficient than that of the Asp-Pro sequence.

Even if at the moment the mechanism of the acid promoted cleavage of the Asp-Cys peptide bond is not known, it is clear that a free thiol group on cysteine is required.

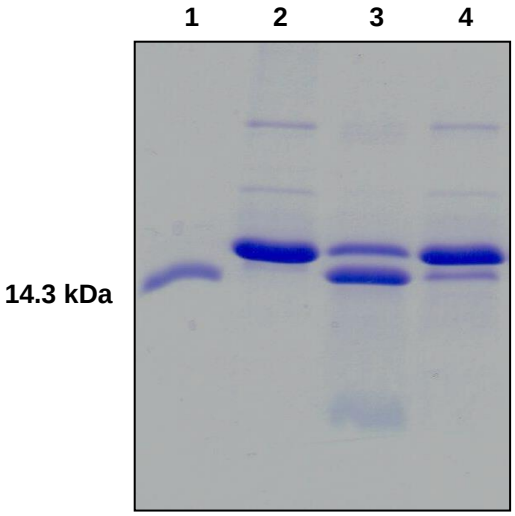


Figure 2: SDS-PAGE analysis of chemical cleavage carried out on ONC-DCless-H6-(C)GKY20-CEA protein at 60°C, pH 2 for 24 h, in the presence or absence of Tris (2-carboxyethy1) phosphine (TCEP) reducing agent. Lane 1: *Gallus gallus* lysozyme (14 kDa, 2 µg); Lane 2: ONC-DCless-H6-(C)GKY20-CEA in 0.1 M acetic acid; Lane 3: hydrolyzed ONC-DCless-H6-(C)GKY20-CEA with TCEP; Lane 4: hydrolyzed ONC-DCless-H6-(C)GKY20-CEA without TCEP.

After cleavage of ONC-DCless-H6-(C)GKY20-CEA in the presence of TCEP, in order to purify (C)GKY20 peptide, selective precipitation of ONC at neutral pH was attempted as described for the purification of the (P)GKY20 peptide. Unexpectedly, only 25% of (C)GKY20 peptide was found in the soluble fraction (data not shown).
To overcome this problem, after chemical cleavage, routinely (C)GKY20 was purified by reverse phase HPLC on C18 column (data not shown,). (C)GKY20 peptide was recovered with purity higher than 95% as determined by RP-HPLC (Fig.3). Interestingly, purified (C)GKY20 peptide was soluble at concentrations up to 1.7 mg/ml, thus suggesting that the relevant loss of peptide observed during the precipitation step of the ONC carrier could not be due to an intrinsic low solubility of (C)GKY20 but rather to the formation of co-precipitates with the ONC carrier.
Mass spectrometry analysis confirmed the peptide identity and purity (measured molecular weight value, 2613.4 Da; 2615 Da expected molecular weight). (C)GKY20 peptide final yield was of about 5 mg/L of culture.

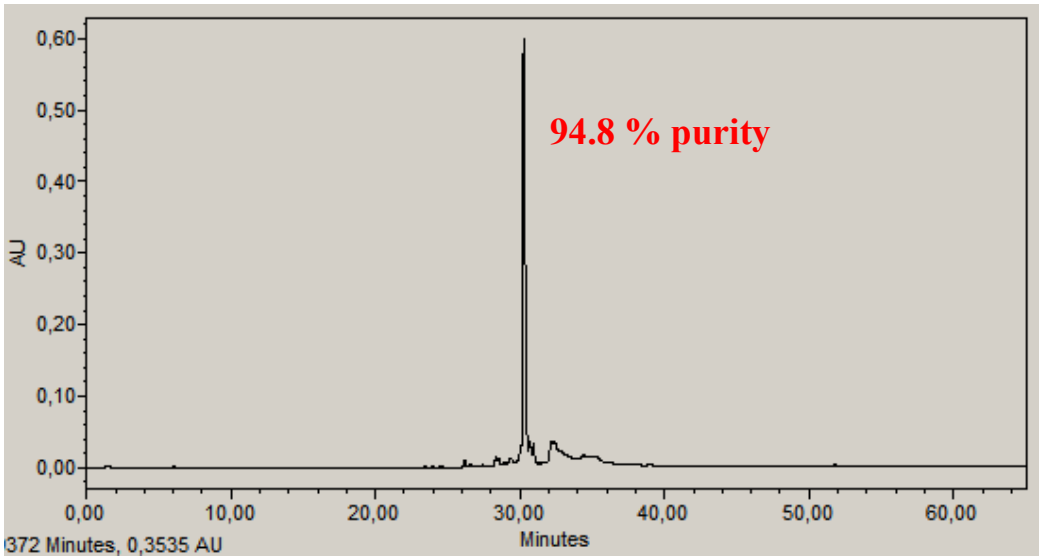


Figure 3: Reverse phase HPLC analysis on C18 column to verify (C)GKY20 purity. (C)GKY20 peptide purity was almost 95 %.

3.2.1 Selective labelling of (C)GKY20

Fluorescently labelled proteins and peptides have very wide applications in protein/membrane interaction studies, intracellular localization studies etc.. Although, lysine and cysteine residues are both common targets of chemical modifications, cysteine offers the possibility to perform more selective labelling reaction being less frequent in proteins/peptides and more reactive than lysine [74]. Moreover, in the specific case of antimicrobial peptides like GKY20, lysine residues are essential for their biological activity as they contribute to positive net charge and lipid binding. In this context the possibility to prepare recombinant peptides with a single additional cysteine residue at the N-terminus appears particularly well suited to the aim of preparing labelled AMPs. In order to test this possibility we decided to label peptide (C)GKY20 with a commercially available thiol reactive fluorophore. The selected fluorophore, 1-[2-(maleimido)etil]-4-[5-(4-metossifenil)-2-ossazolil]piridinio (PyMPO), is a relatively small, low polarity and positively charged molecule bearing a maleimide moiety which very specifically reacts with thiols under mild conditions (pH 6.5-7.5 at 37°C). (C)GKY20 peptide was incubated with PyMPO in guanidine 2 M at pH 7, in the presence of Tris 2-carboxylethil phosphine (TCEP) as reducing agent in order to prevent cysteine oxidation that could reduce modification efficiency.

Then (C)GKY20-PyMPO was purified by reverse phase HPLC on C18 column. Analytical HPLC analysis of labelling reaction mixture (Fig. 4A), showed 9 main peaks. The identity of these peaks was deduced from their spectral properties, SDS-PAGE analysis and by comparison with the HPLC analysis of control reactions lacking selected reagents. Peaks 1-4 are likely due to spontaneous degradation/hydrolysis of PyMPO. In fact peaks with similar retention times were found in a control mixture containing PyMPO but not containing the peptide and TCEP(Fig. 4B).

Peaks 5-7 likely are TCEP-PyMPO adducts. It should be noted that it is known that TCEP can react with maleimide groups [75].Peaks 8 and 9, eluted at 39.16 min and 40.48 min respectively, were identified as the desired (C)GKY20-PyMPO labelled peptide, as indicated by the spectroscopic properties (Fig.4A2 λ max 268 and 405 nm) and SDS-PAGE analysis (data not shown).

No peaks were recorded at 37.67 min, the elution time expected for (C)GKY20 peptide (Fig.5A), thus indicating that (C)GKY20 was completely modified (Fig.4A). Preparative RP-HPLC purification was performed using almost the same elution gradient with a peptide recovery of about 60%. Peptide was lyophilized and then suspended in 5 mM ammonium acetate buffer pH 5. RP-HPLC and SDS-PAGE analysis (Fig.5B and 5C respectively), allowed to estimate a peptide purity of about 99%. Under UV-light (λ=254 nm) the labeled peptide provided a strongly fluorescent yellow band in SDS-polyacrylamide gel (Fig. 5C).

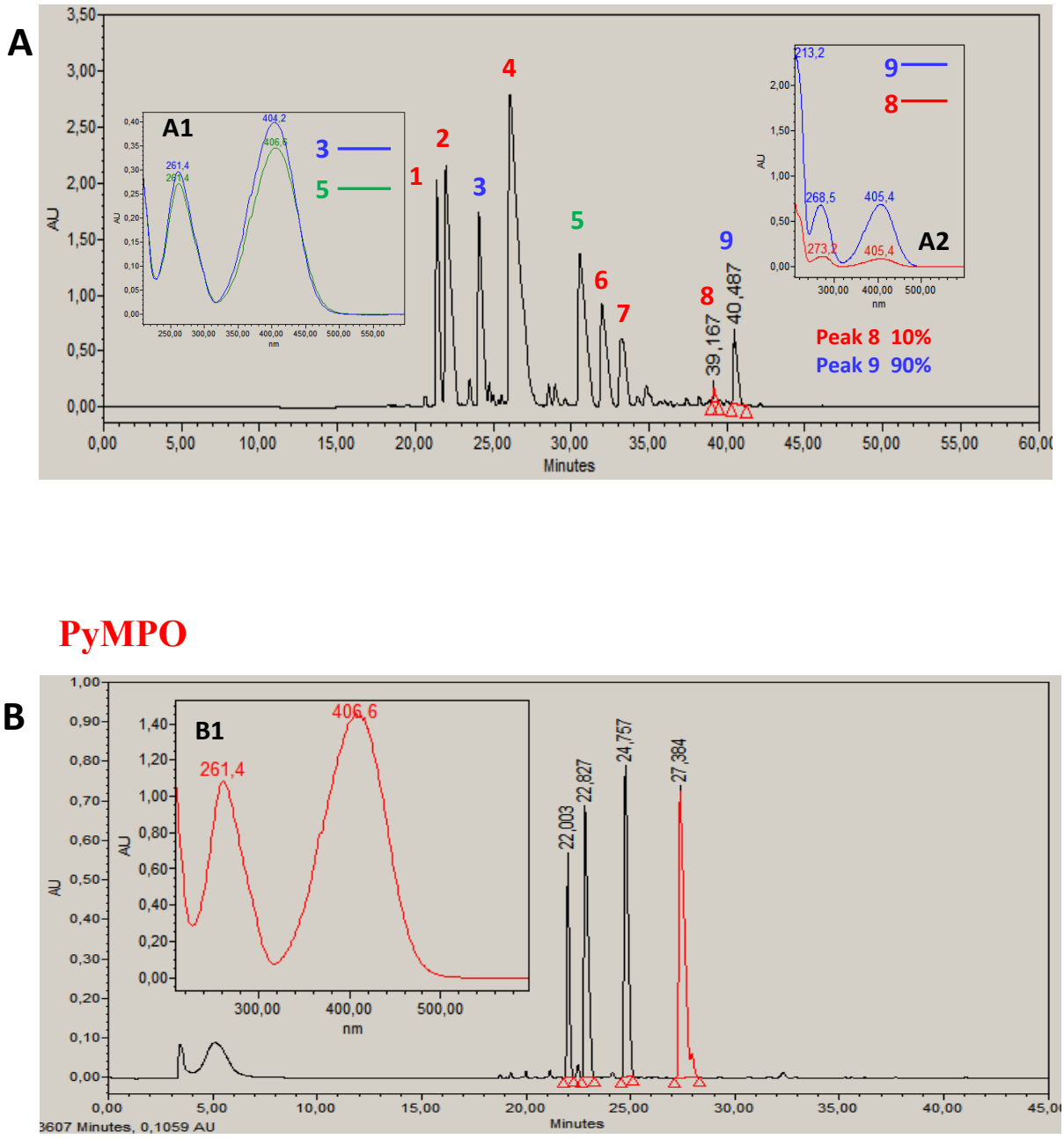


Figure 4: Reverse phase HPLC analysis on C18 column of (C)GKY20-PYMPO peptide modification reaction in the presence of TCEP reducing agent. A) Chromatogram recorded at 280 nm of (C)GKY20-PyMPO peptide purification, in A1 spectrum of peak 3 and 5 whereas in A2 spectrum of peak 8 and 9 B) Chromatogram of PyMPO fluorophore without peptide and TCEP (control reaction). In B1 panel, UV absorbance spectrum of (C)GKY20-PyMPO peptide (peak 8).

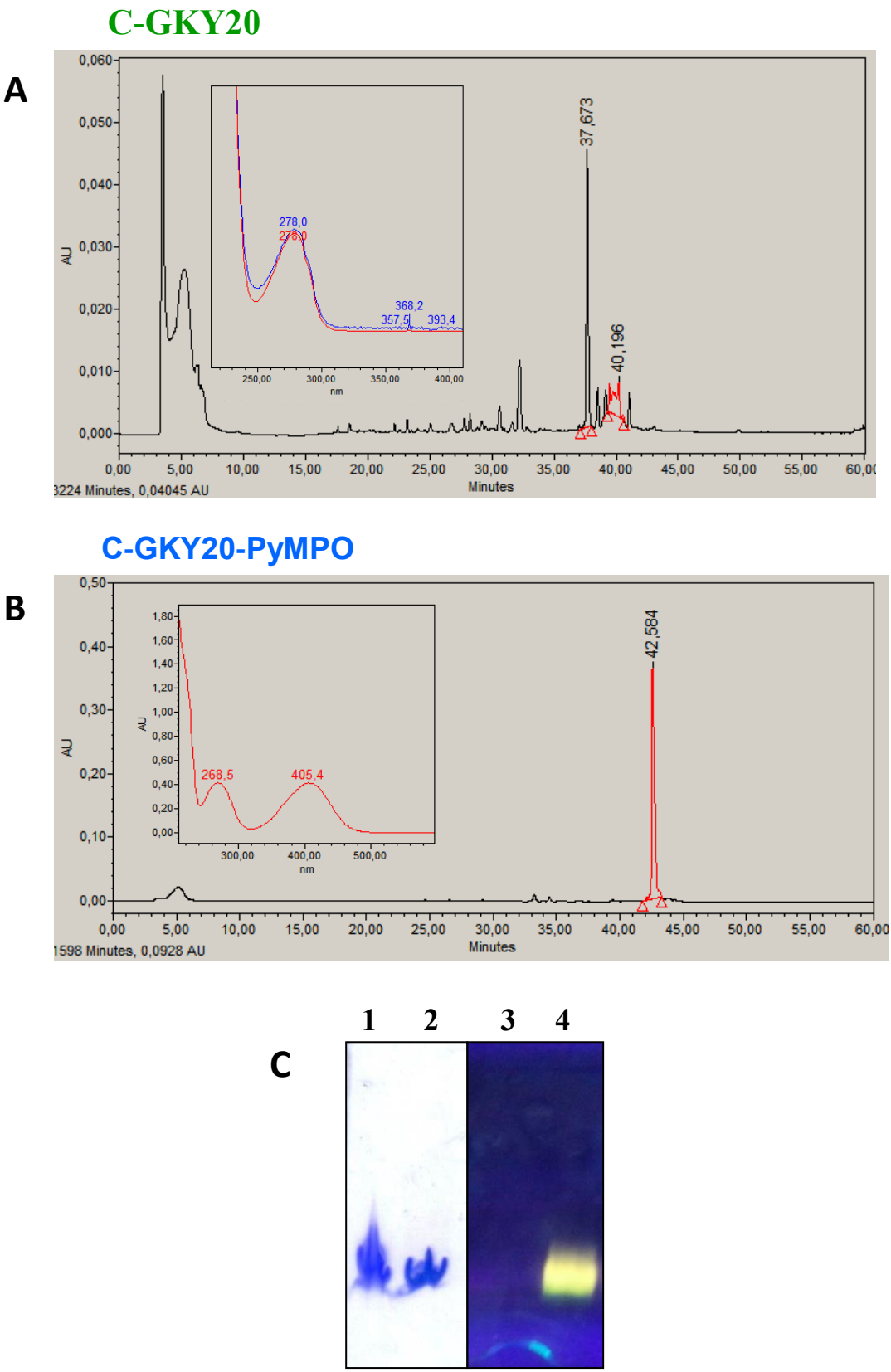


Figure 5: Reverse phase HPLC analysis of purity. A) Chromatogram recorded at 280 nm of (C)GKY20 peptide with peak spectrum of absorbance in the small panel B) Chromatogram recorded at 280 nm of (C)GKY20-PyMPO peptide with peak spectrum of absorbance in the small panel. C) 20% SDS-PAGE analysis of purified (C)GKY20 peptide (lane 1 and 3) and purified (C)GKY20-PyMPO peptide (lane 2 and 4). On the left gel stained in Coomassie crystal blue, on the right UV-light gel image.

3.3 Antimicrobial activity of thrombin derived peptides

In order to verify if recombinant peptides (P)GKY20, (C)GKY20, (C)GKY20-PyMPO possess antimicrobial activity comparable to that of the synthetic GKY20, all peptides were tested by Dr. Zanfardino (Department of Biology, University of Naples Federico II), by plate viable-count assay on a Gram-positive strain, *Staphylococcus aureus* ATCC 6538P, and a Gram-negative strain, *Pseudomonas aeruginosa* KK27 (a clinical strain isolated from a cystic fibrosis patient kindly provided by Dr. Alessandra Bragonzi, San Raffaele Hospital, Milan).

Dose-effect curves were carried out testing peptides at final concentrations of 0.1-0.5-1-2-3 μM. As shown in Fig. 6, (P)GKY20 and (C)GKY20 exhibited the same activity displayed by synthetic GKY20 on both strains, thus indicating that the two additional residues at the N-terminus did not affect the antimicrobial activity. The presence of PyMPO molecule on (C)GKY20, slightly decreased the bactericidal activity on both strains.

Moreover, antibacterial potencies of recombinant (P)GKY20 and synthetic peptide GKY20 were further compared by broth microdilution method on several Gram-positive and Gram-negative strains. Very similar MIC values (Minimal Inhibitory Concentration at which no planktonic growth is observed) were found for both peptides, (Table 2 in the accompanying paper) thus confirming that an additional proline at N-terminus of GKY20 did not affect its activity.

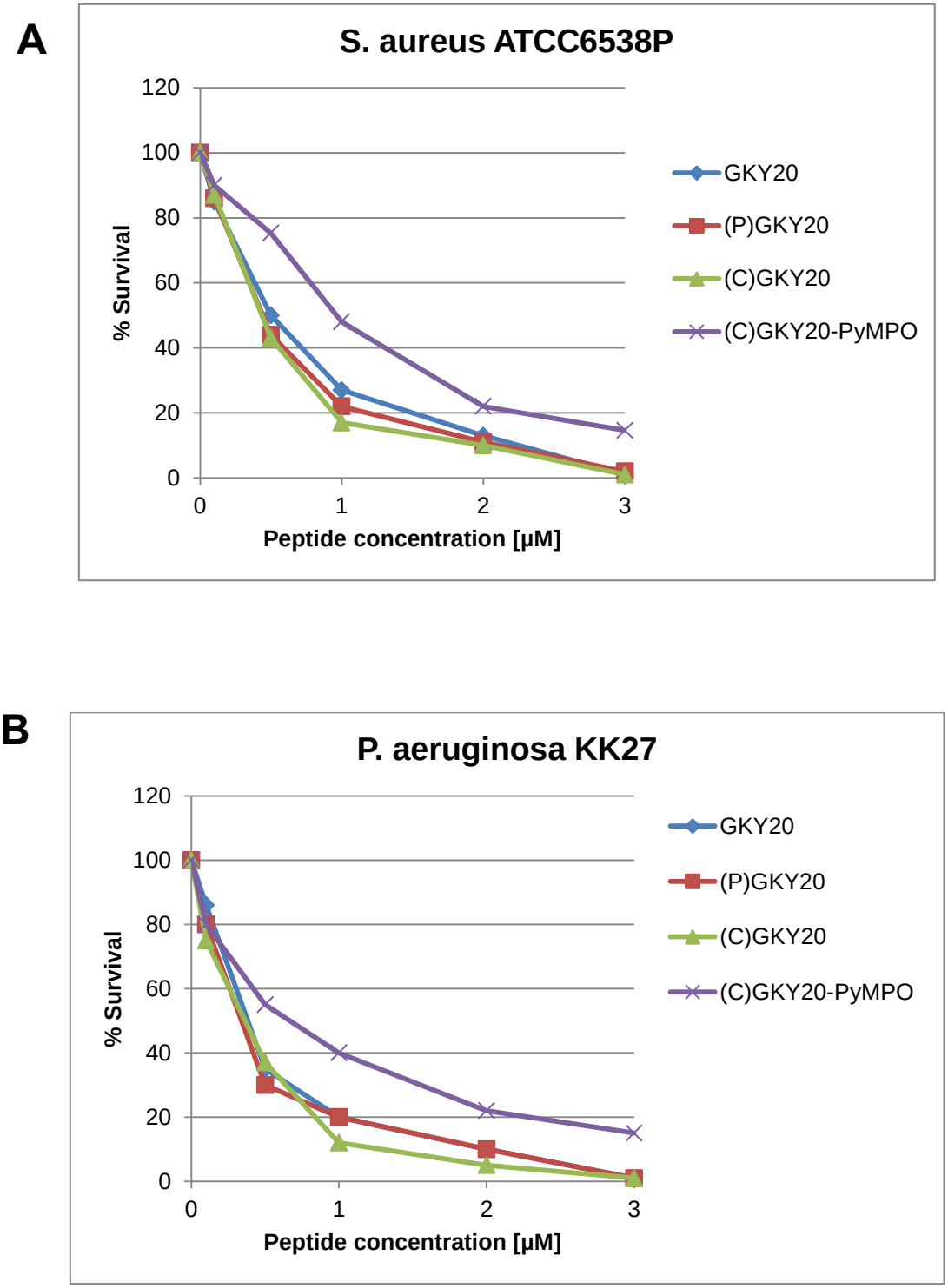


Figure 6: Comparison of antimicrobial potency between recombinant peptides and synthetic GKY20 by plate viable cell count antimicrobial assay. A) Gram positive strain *Staphylococcus aureus* ATCC 6538P and B) Gram negative strain *Pseudomonas aeruginosa* clinical isolate KK27.

3.4 Expression of further potential human AMPs

In order to verify the general applicability of our optimized carrier protein ONC-DCless-H6, several other hypothetical human AMPs (Table 1), were cloned and expressed as fusion proteins. All peptides were produced as recombinant peptides with an additional proline at the N-terminus and characterized in collaboration with the research group of Dr. A. Arciello and Dr. E. Pizzo.

As described in “Introduction” section 1.8, these peptides were chosen from a pool of hypothetical cryptic AMPs derived by analyzing about 4,000 human extracellular proteins, through the bioinformatic method developed by Dr. Notomista [52].

The recombinant fusion proteins, were expressed with yields similar to that of ONC-DCless-H6-(P)GKY20 (Table 1). Moreover, acid hydrolysis showed cleavage efficiency similar or higher than that of ONC-DCless-H6-(P)GKY20 (data not shown). Peptides yields ranging from 10 to 30-35 mg/L of culture were obtained, depending on the peptide. These results demonstrate that ONCDCless-H6 is well suited to produce as inclusion bodies peptides of length up to at least 50 residues.

The characterization of the biological activities of these novel human AMPs is currently ongoing in collaboration with the research groups of Dr. A. Arciello and Dr. E. Pizzo. During the activities of the present PhD project I have mainly focused the attention on pepsinogen A3 derived peptides [(P)PA $3_{(1-47)}$ and its fragments] and on peptides derived from β-fibrinogen [β $Fib_{(420-447)}$ and β $Fib_{(425-447)}$], described in details in the next sections.

Table 1: Peptides produced with ONCDCLess-H6 carrier protein so far.

Peptide	Primary structure[a]	Fusion protein (mg/L of culture)[b]
Apo $E_{(133-150)}$	LRVRLASHLRKLRKRLLR	120 mg/L
Apo $E_{(133-167)}$	LRVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAR	100 mg/L
Apo B (887-911)	HVALKAGKLKFIIPSPKRPVKLL	145 mg/L
Apo B (887-925)	HVALKAGKLKFIIPSPKRPVKLLSGGNTLHLVSTTK	220 mg/L
PA 3 (1-47)	IMYKVPLIRKKSLRRTLSERGLLKDFLKKHNLNPARKYFPQWKAPTL	200 mg/L
PA $3_{(1-25)}$[c]	IMYKVPLIRKKSLRRTLSERGLLKD	-[c]
PA $3_{(26-47)}$[c]	FLKKHNLNPARKYFPQWKAPTL	-[c]
α Fib (805-831)	GVVWVSFRGADYSLRAVRMKIRPLVTQ	150 mg/L
β Fib (420-447)	GVVWMNWKGSWYSMRKMSMKIRPFFPQQ	150 mg/L
β Fib (425-447)	NWKGSWYSMRKMSMKIRPFFPQQ	150 mg/L
γFib (366-395)	GIIWATWKTRWYSMKKTTMKIIPFNRLTIG	150 mg/L
h11 Beta (235-261)	GVFYPWRFRLLCLLRRWLPRPRAWFIR	200 mg/L

[a]Aminoacid color code: basic residues are colored in blue, hydrophobic residues in green, borderline residues in gray, hydrophilic residues in yellow, acidic residues in red.
[b] Fusion protein yield obtained as mean of at least three independent experiments carried out in a shake-flask culture in Terrific Broth medium.
[c] PA $3_{(1-25)}$ and PA $3_{(26-47)}$ were released after chemical cleavage of the *full length* peptide PA $3_{(1-47)}$ as its own N-terminal and C-terminal peptides.

3.5 Purification of recombinant pepsinogen A-3 derived peptides

Through the bioinformatic method [52] three fragments of human pepsinogen A3 were predicted to be endowed with antibacterial activity: PA $3_{(1-47)}$ corresponding to the activation peptide of human pepsin, and its N- and C- terminal portions here named, PA $3_{(1-25)}$ and PA $3_{(26-47)}$ respectively.

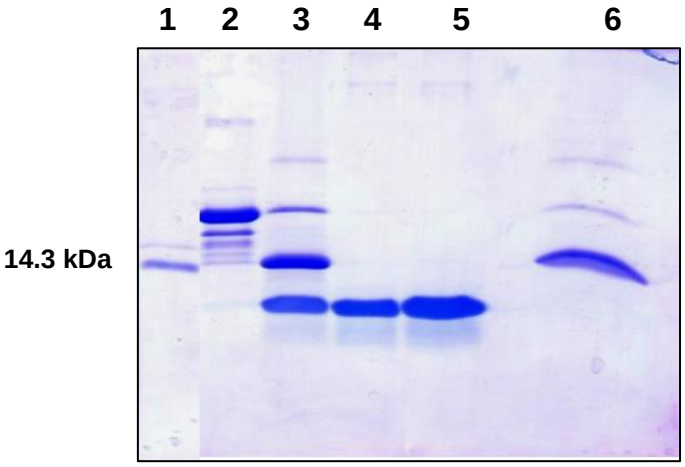
The two shorter peptides, 25 and 22 residues long, were also included in the panel of five peptides produced by chemical synthesis (see section 3.7 “Functional characterization of potential new human AMPs”) whereas PA $3_{(1-47),}$ 47 amino acids long, was prepared only as recombinant peptide.

Peptide PA $3_{(1-47)}$ is the activation peptide of human pepsin A3. *In vivo*, at neutral pH in the gastric mucosa of vertebrates, the precursor form of pepsin A-3 is synthesized and folded as a zymogen, pepsinogen A-3 (UniProtKB ID: P0DJD8). Maturation of pepsinogen, through the proteolytic cleavage release of the first 47 residues under acidic conditions, yields active pepsin A-3. [76].

Activation peptides of pepsinogen isoforms inhibit the proteolytic activity of pepsins by blocking the catalytic site thus allowing safe storage and secretion of the protein [76]. To the best of our knowledge, no other significant function has been attributed to activation peptides of pepsin. However it has been reported that the propeptide of pepsin isoform C from *Rana catesbeiana* shows antimicrobial activity against several microorganisms [77].

An *in silico* designed DNA fragment coding for PA $3_{(1-47)}$ was cloned and successfully expressed by using ONCDCLess-H6 as carrier protein (Fig.7A) as previously described for the production of (P)GKY20 peptide. The corresponding recombinant peptide named (P)PA$3_{(1-47)}$ for the additional proline at its N-terminus (48 amino acids) was produced with yield even higher than that of (P)GKY20, i.e. 30-35 mg/L of Terrific Broth. Moreover, final purity of (P)PA$3_{(1-47)}$, determined by RP-HPLC, typically was about 95% (Fig. 7B).

A



B

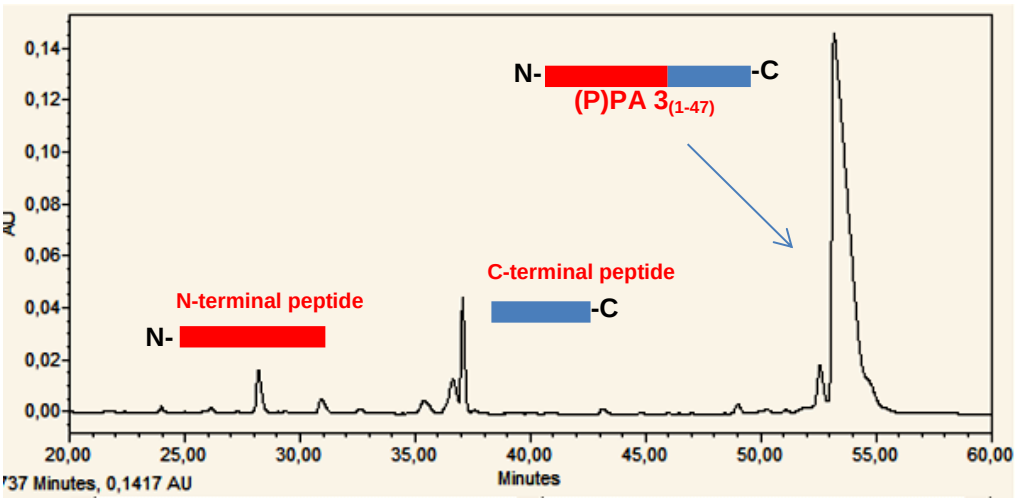


Figure 7:Purification of the recombinant activation peptide of human pepsinogen A3.
A) 20 % SDS-PAGE analysis. Lane 1: *Gallus gallus* lysozyme; Lane 2: ONC-DCless-H6-(P)PA $3_{(1-47)}$ purified in acetic acid 0.1 M; Lane 3: Acid cleavage of ONC-DCless-H6-(P)PA $3_{(1-47)}$ fusion protein; Lane 4 and 5: Soluble fraction after neutralization at pH 7 (5 µg and 10 µg peptide respectively); Lane 6: Insoluble fraction after neutralization at pH 7 (carrier protein and uncleaved fusion protein).

By SDS-PAGE analysis, RP-HPLC (Fig.7), and mass spectrometry (data not shown) we detected two peptides derived from the acid cleavage of the internal DF sequence (cleaved with an efficiency of about 5%). These peptides correspond to the N-terminal 26 residues [(P)IMYKVPLIRKKSLRRTLSERGLLKD] and to C-terminal 22 residues [FLKKHNLNPARKYFPQWKAPTL] of (P)PA $3_{(1-47).}$

In order to characterize these two smaller peptides, we prepared a variant of (P)PA $3_{(1-47)}$ in which, a proline residue is inserted between Asp 25 and Phe 26 to improve cleavage efficiency. This fusion protein called ONCDCLess-H6-(P)PA 3 $AB_{(1-47)}$ by acid cleavage yielded peptides (P)PA $3_{(1-25)}$ (26 aa) and (P)PA $3_{(26-47)}$ (23 aa). (Fig. 8A). The cleavage efficiency of internal DP was about 85% as determined by densitometric analyses. Peptides were purified by RP-HPLC (Fig. 8B), with a recovery of 80%. Peptides identity was confirmed by mass spectrometry with a measured molecular weight corresponding to that of the theoretical value i.e. (P)PA $3_{(1-25)}$ of 3111.8 Da and (P)PA $3_{(26-47)}$ of 2795.3 Da as determined by using Protparam tool.

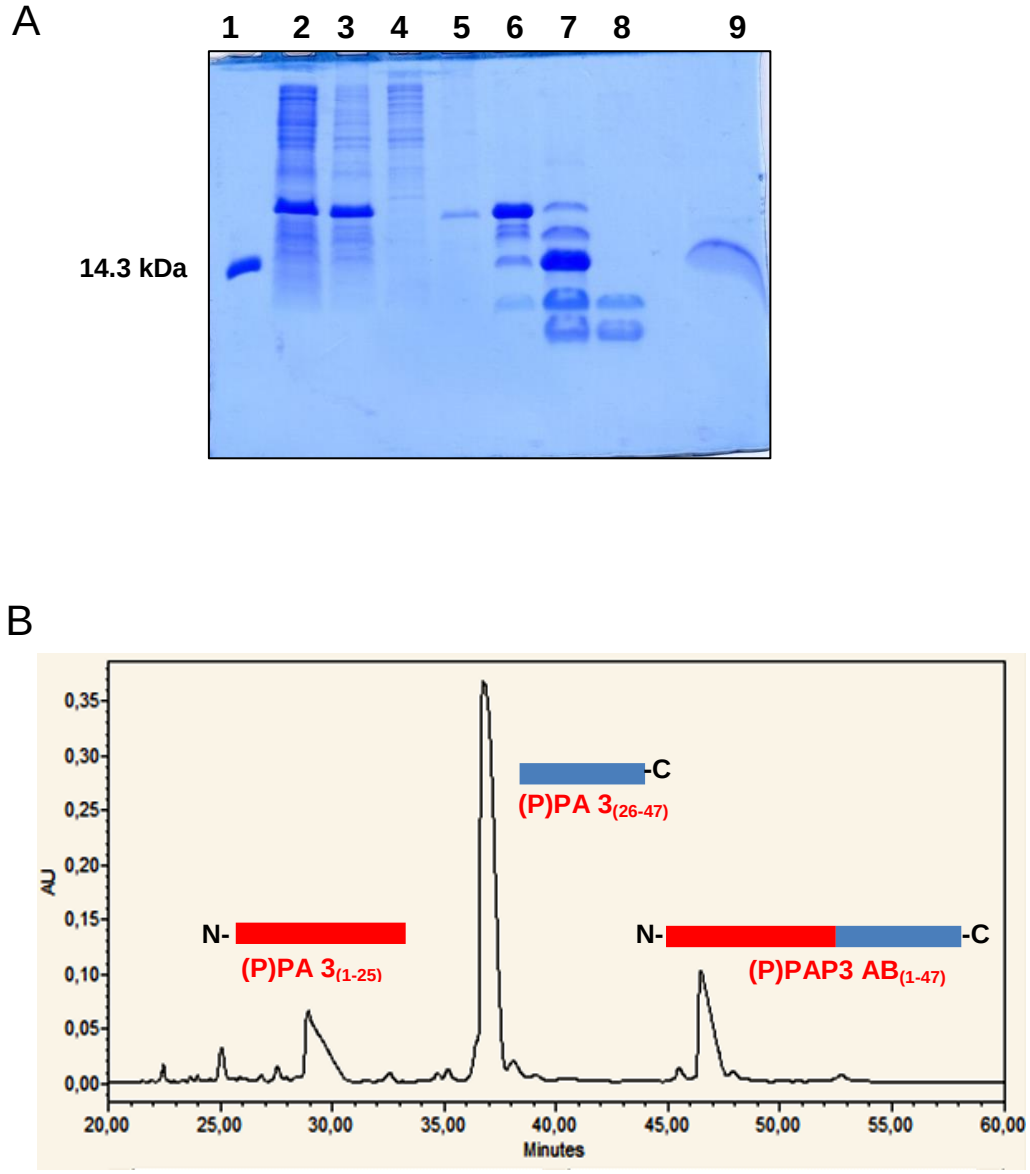


Figure 8: Purification of the recombinant peptides derived from activation peptide of human pepsinogen A3. A) 20 % SDS-PAGE analysis. Lane 1: *Gallus gallus* lysozyme; Lane 2: cell lysate 0.126 O.D; Lane 3: Inclusion bodies after Triton/urea wash 0.126 O.D. Lane 4: Soluble fraction 0.126 O.D.; Lane 5: ONC-DCless-H6-(P)PA3 $AB_{(1-47)}$ purified by IMAC; Lane 6: purified ONC-DCless-H6-(P)PA3 $AB_{(1-47)}$ in acetic acid 0.1 M; Lane 7: Acid cleavage of ONC-DCless-H6-(P)PA3 $AB_{(1-47)}$ fusion protein; Lane 8: Soluble fraction after neutralization at pH 7; Lane 9: Carrier protein at ph 7.
B) Reverse phase HPLC chromatogram of acid cleavage mixture. Peak 1: (P)PA $3_{(1-25)}$ peptide; Peak 2: (P)PA $3_{(26-47)}$ peptide; Peak 3: (P)PA 3 $AB_{(1-47)}$ peptide.

3.5.1 Antimicrobial activity

The antibacterial properties of the peptides derived from pepsinogen A3 were studied by viable count assay on plate and by the broth microdilution method for the determination of MIC values. Viable count assay were performed on Gram-negative strain *Escherichia coli* DH5α and on Gram-positive strain *Staphylococcus aureus* ATCC6538P at concentration of 1 µM for each peptide. (P)PA $3_{(1-47)}$ peptide showed similar activity on both strains (Fig. 9) whereas its fragments were both more effective on *Escherichia coli* DH5α. Interestingly, by using equimolar concentrations of both fragments, on *Escherichia coli*, the percentage of survived cells was very similar to that measured using (P)PA $3_{(1-47)}$ and lower than that expected for a purely additive effect. This suggests a cooperation between the two halves of (P)PA $3_{(1-47)}$.

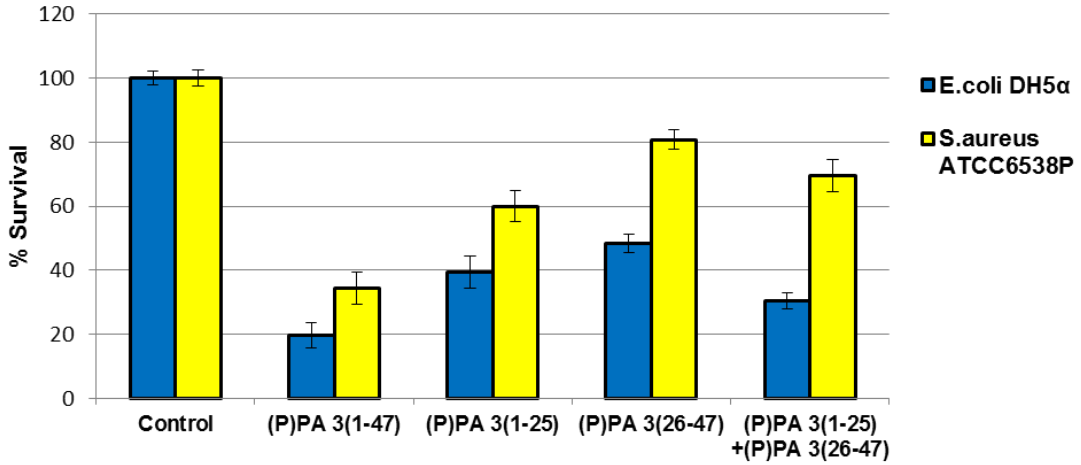


Figure 9: Comparison of antimicrobial potency between recombinant pepsinogen A 3 derived peptides by plate viable cell count antimicrobial assay treating cells with 1 µM of each peptide.

Furthermore, MIC values were determined on pepsinogen A3 recombinant peptides, using (P)GKY20 as positive control and antibiotic polymyxin B and vancomycin effective against most Gram negative and Gram positive bacteria, respectively. As shown in Table 2, *full length* (P)PA $3_{(1-47)}$ was active against all bacteria strains tested, including *Pseudomonas aeruginosa* PA14, a pathogenic antibiotic-resistant strain, with a MIC value of 5.5 µM.

As its synthetic counterpart (see section 3.7.1 Table 5), recombinant (P)PA $3_{(26-47)}$ did not inhibit planktonic growth at the concentrations used, whereas the N-terminal peptide (P)PA $3_{(1-25)}$ showed a potency similar to the *full-length* peptide, within micromolar range concentration (Table 2). Taken together, these data demonstrated that all three peptides are endowed with antibacterial activity as predicted.

Table 2: Antibacterial acitivity of pepsinogen A3 derived peptides by broth microdiluition broth assay and (P)GKY20 peptide (positive control). MIC value average of three independent experiments carried out in Nutrient Broth 0.5X.

		MIC (µM)		
Peptide	**Sequence**	***E.coli* *DH5α***	***PA14***	***S.aureus* *ATCC6538P***
(P)PA 3(1-47)	(P)IMYKVPLIRKKSLRRTLSERGLLKDFLKKHNL NPARKYFPQWKAPTL	5.5	5.5	6.13
(P)PA 3(1-25)	(P)IMYKVPLIRKKSLRRTLSERGLLKD	5.12	20.4	5.5
(P)PA 3(26-47)	(P)FLKKHNLNPARKYFPQWKAPTL	92	>92	>92
(P)GKY20	(P)GKYGFYTHVFRLKKWIQKVI	6.13	50	16
Polymyxin B		2 µg/ml	2 µg/ml	>32 µg/ml
Vancomycin		>32 µg/ml	>32 µg/ml	2 µg/ml

In addition, treatment with *full length* (P)PA $3_{(1-47)}$ up to 2 fold MIC value had no toxic effect on THP1 human monocyte cells viability after 24 hours of incubation (Fig. 10).

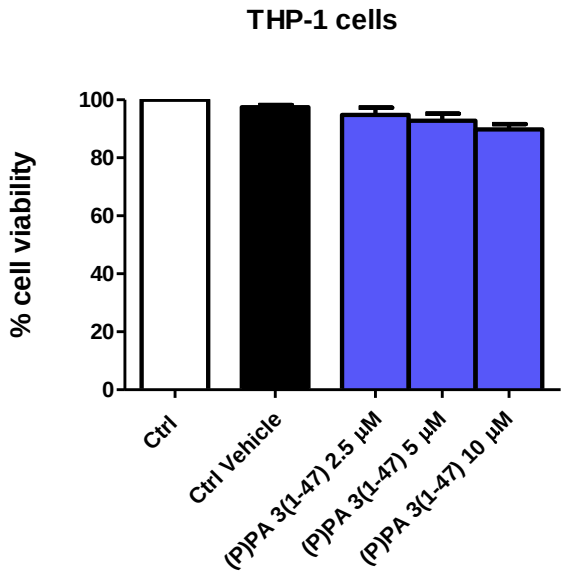


Figure 10: Dose response effect of *full length* (P)PA $3_{(1-47)}$ peptide on THP-1 human monocyte cells after 24 h of incubation (MTT assay).

3.6 Purification of recombinant β Fibrinogen derived peptides

Fibrinogen is a glycoprotein consisting of three pairs of polypeptide chains, α, β and γ, symmetrically interconnected through multiple disulfide bonds. Fibrinogen in plasma is heterogenous, being present as different forms characterized by different molecular weight, solubility and ability to form fibrin clot. This latter fibrinogen feature, constitutes its main physiological function. Indeed, through cleavage by thrombin and release of the fibrinopeptides A and B, is converted to fibrin in the last step of the coagulation process [78].

Relatively recent studies show that blood coagulation and inflammation are intimately connected and co-operate in the early response to infection [79]. Fibrinogen is a key player in acute phase response to trauma, wounds, bacterial infections, moreover it is also known to have pro-inflammatory properties.

In fact, recently, Pahlman et. al have demonstrated that thrombin-treated fibrinogen directly kills bacteria. They also reported that peptide fragment GHR28 released from the β chain of fibrinogen has broad antibacterial activity [47]. This is not completely surprising, taking into account that proteins homologous to fibrinogen-related are common also among invertebrates where they are not involved in coagulation but in immune defense reactions [80].

Very interestingly our bioinformatic method predicted the presence of a putative AMP at the C-termini of each chain of human fibrinogen (see Table 1).

For sake of brevity, only the production and characterization of the β fibrinogen derived peptide will be discussed in this thesis.

As described for thrombin and pepsinogen derived peptides we used ONC-DCless-H6 to prepare recombinant peptide (P)βFib$_{(420-447)}$.

Chemical cleavage efficiency of ONCDCLess-H6-(P)βFib$_{(420-447)}$ fusion protein was even higher than that observed for the previous peptides (≥98 %). However, after neutralization of the acids present in the cleavage buffer we found that peptide (P)βFib$_{(420-447)}$, was present essentially in the insoluble fraction with the ONC carrier. As the carrier protein ONCDCLess-H6, after cleavage, retains the His6 tag we attempted to separate ONC-DCless-H6 and (P)βFib$_{(420-447)}$ by IMAC under denaturing condition to avoid precipitation of carrier and peptide. Unfortunately even if peptide was recovered with an efficiency over 70%, a significant amount of the carrier protein (about 20%) was still present in sample (data not shown).

For this reason, I chose to purify directly by RP-HPLC the acidic cleavage mixture containing ONC-DCless-H6 and (P)βFib$_{(420-447)}$ (Fig. 11). In this case peptide recovery was about 50% but the final peptide purity was 96% as determined by RP-HPLC (Fig.12).

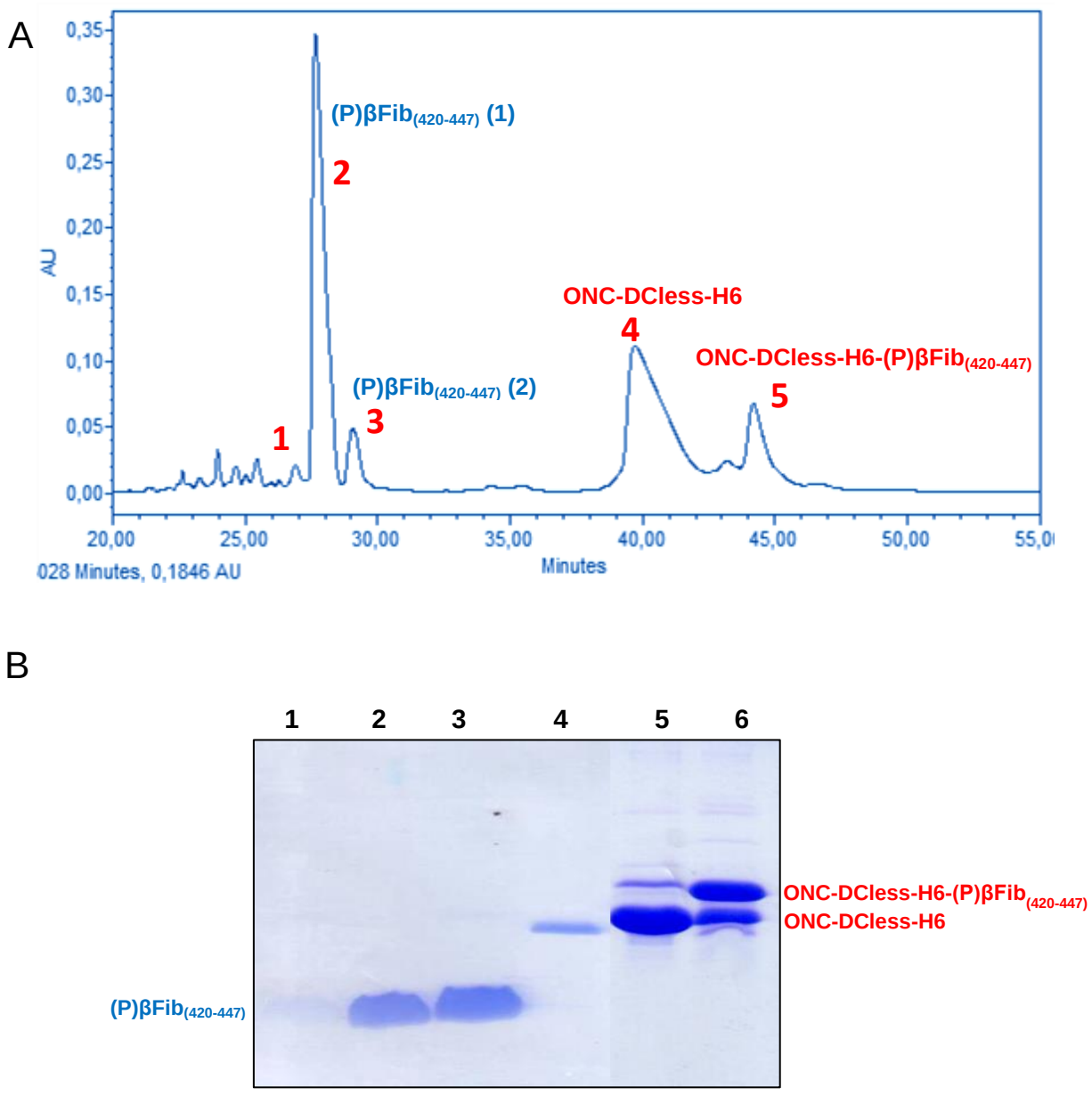


Figure 11 : Purification of the recombinant fibrinogen derived peptide βFib$_{(420-447)}$.
A) Purification of acidic cleavage mixture of (P) βFib$_{(420-447)}$ peptide performed by C18 RP-HPLC.
B) 20 % SDS-PAGE analysis for peak assignment. Lane 1: peak 1; Lane 2: peak 2 purified (P)βFib$_{(420-447}$ (1); Lane 3: peak 3 purified (P)βFib$_{(420-447)}$ (2); Lane 4: *Gallus gallus* lysozyme; Lane 5: peak 4 purified cleaved fusion protein ONC-DCless-H6; Lane 6: purified uncleaved fusion protein ONC-DCless-H6-(P)βFib$_{(420-447)}$.

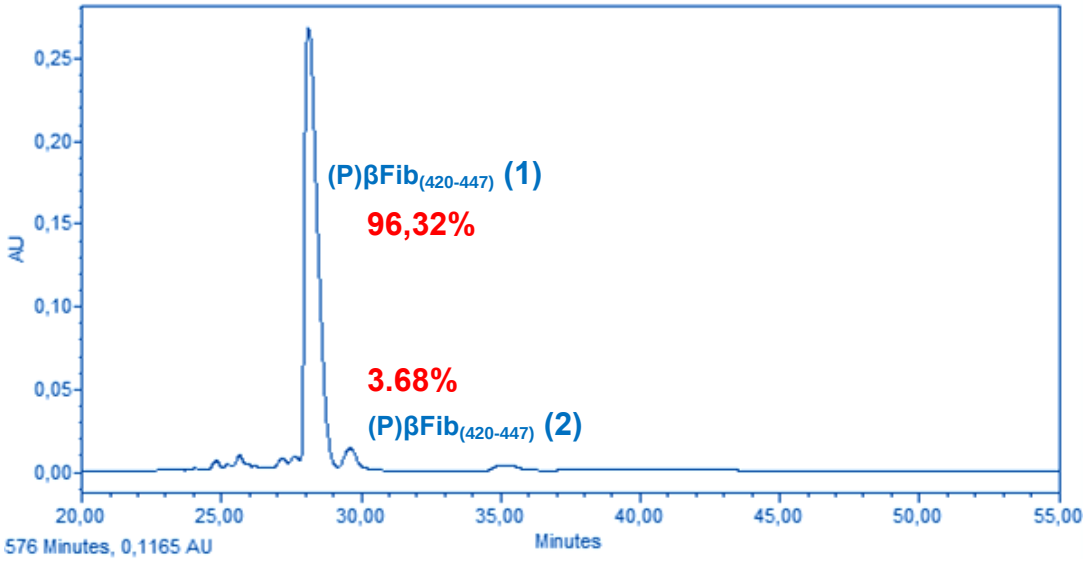


Figure 12: Analysis of purity of (P)βfib$_{(420-447)}$ by reverse phase HPLC chromatogram recorded at 280 nm.

The yield of the recombinant peptide (P)βFib$_{(420-447)}$, was about 10 mg/L of Terrific Broth, hence similar than that of the peptide previously described (P)GKY20.
It is interesting to note, that (P)βFib$_{(420-447)}$, was soluble in ammonium acetate 5 mM pH 5 and in water up to a concentration of 1 mg/ml. Higher concentrations lead to aggregates formation. A similar behavior was also observed for the corresponding synthetic peptide described in section 3.7.
These findings prompted us to analyze more accurately the sequence of βFib$_{(420-447).}$ We noticed that the N-terminus of this peptide contains four adjacent hydrophobic residue starting from residue 2: G**VVWM**NWKGSWYSMRKMSMKIRPFFPQQ. Moreover, “TANGO”, a server for automatic prediction of amyloidogenic regions in proteins (http://tango.crg.es/), confirmed that this hydrophobic sequence has a high propensity to form beta-aggregates.
Even if, without these four residues, the predicted antimicrobial potency of the peptide slightly decreases we decided to prepare a peptide five residues shorter, βFib$_{(425-447).}$ The coding sequence for βFib$_{(425-447)}$ was fused to ONC-DCless-H6 cDNA and expressed in *E.coli* (Fig.13) .
The solubility of (P)βFib$_{(425-447)}$ proved to be significantly higher that of (P)βFib$_{(420-447)}$, (at least up to 2 mg/ml in ammonium acetate at pH 5 or in water). This allowed to purify (P)βFib$_{(425-447)}$ by selective precipitation of carrier protein at pH 7 (Fig. 13), as described for (P)PA 3$_{(1-47)}$ and (P)GKY20.

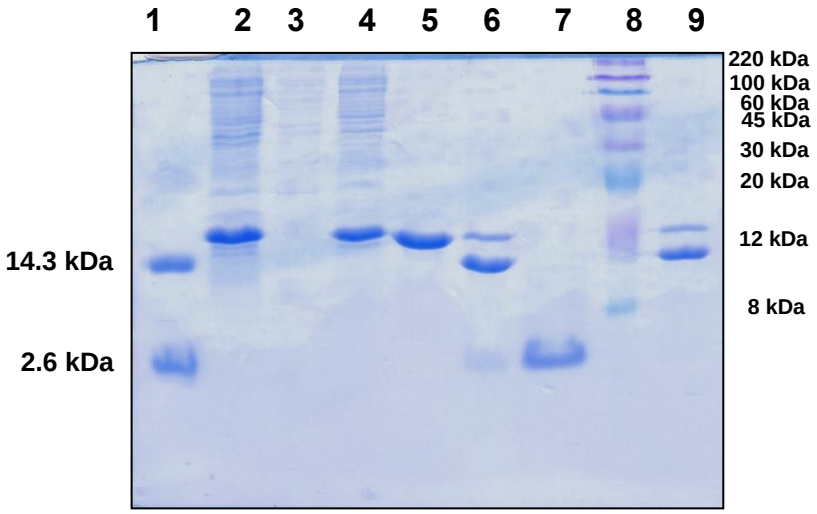


Figure 13: Purification process of (P)βFib$_{(425-447)}$. SDS PAGE analysis. Lane 1: *Gallus gallus* lysozyme and (P)GKY20 peptide; Lane 2: cell lysate of induced culture 0.126 O.D.; Lane 3: soluble fraction after cell lysis 0.126 O.D; Lane 4: insoluble fraction after cell lysis 0.126 O.D; Lane 5: purified ONC-DCless-H6-(P)βFib$_{(425-447)}$ recombinant protein after dialysis against 0.1 M acetic acid; Lane 6: acid cleavage of ONC-DCless-H6-(P)βFib$_{(425-447)}$. Lane 7: Soluble fraction after neutralization of the cleavage reaction [(P)βFib$_{(425-447)}$ peptide]; Lane 8: molecular weight marker Color Burst (220 -8 kDa); Lane 9: Insoluble fraction after neutralization of the cleavage reaction [cleaved and uncleaved ONC-DCless-H6-(P)βFib$_{(425-447)}$ recombinant protein].

3.6.1. Antimicrobial activity

Dose effect curve by viable count assay was performed on Gram-positive strain *Staphylococcus aureus* ATCC6538P in order to verify the antibacterial properties of the fibrinogen derived peptide. (P)βFib$_{(420-447)}$ was effective on this strain reducing to 40% cell survival at concentration of 3 µM (Fig.14).

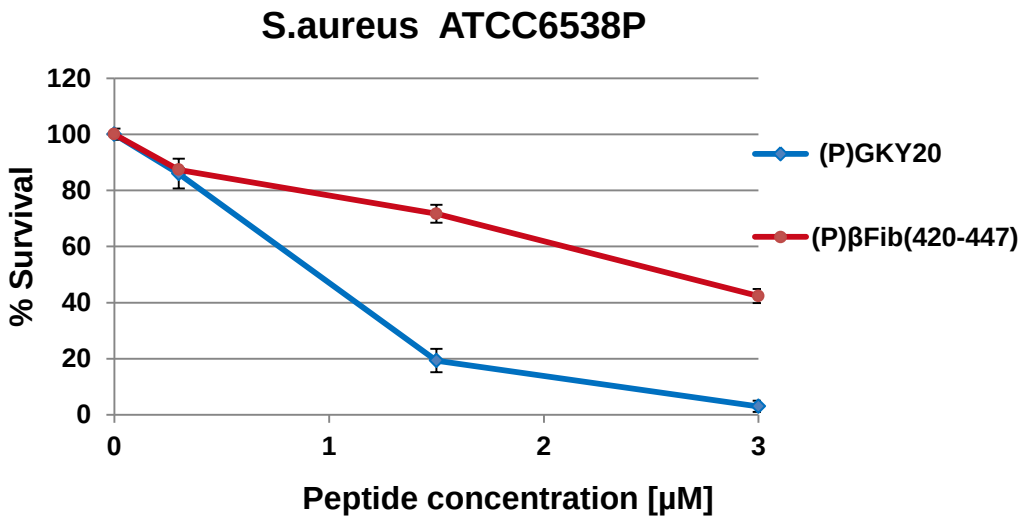


Figure 14: Antimicrobial potency of (P)βFib$_{(420-447)}$ peptide by plate viable cell count assay. compared to (P)GKY20 peptide, used as positive control.

MIC values were determined on three bacterial strains (*E. coli* DH5α, *P. aeruginosa* PA14 and *S. aureus* ATCC6538P) by broth microdilution method using (P)GKY20 as control peptide. Peptides showed antibacterial activity on all strains at concentration similar to that of (P)GKY20 peptide (Table 3). Interestingly, both β fibrinogen derived peptides, possess the same potency.

Table 3: Antimicrobial activity of recombinant β fibrinogen derived peptide and (P)GKY20 (positive control) by broth microdilution broth. MIC value average of three independent experiments carried out in Nutrient Broth 0.5X.

		MIC (µM)		
Peptide	**Sequence**	***E.coli* *DH5α***	***PA14***	***S.aureus* *ATCC6538P***
(P)βFib(420-447)	(P)GVVWMNWKGSWYSMRKMSMKIRPFFPQQ	10	40	10
(P)βFib(425-447)	(P)NWKGSWYSMRKMSMKIRPFFPQQ	10	40	10
(P)GKY20	(P)GKYGFYTHVFRLKKWIQKVI	10	40	5
Polymyxin B		2 µg/ml	2 µg/ml	>32 µg/ml
Vancomycin		>32 µg/ml	>32 µg/ml	2 µg/ml

3.7 Functional characterization of potential new human AMPs

Among our wide panel of novel AMPs, five particularly promising peptides - Apo $B_{(887-911)}$, Apo $E_{(133-150)}$, $\beta Fib_{(420-447)}$, and the two fragments of pepsinogen activation peptide (Table 1) - were prepared by chemical synthesis and characterized in the laboratory of prof. Robert Hancock (University of British Columbia, Vancouver, Canada) whose research group has extensive experience in the field of the characterization of AMPs.

Three types of biological activities were investigated: (i) antimicrobial activity; (ii) antibiofilm activity; (iii) immunomodulatory activity.

3.7.1. Antimicrobial activity

The five selected peptides were assayed on a panel of representative Gram-negative and Gram-positive bacteria: *Pseudomonas aeruginosa* strain PAO1 and clinical isolates of *Escherichia coli* O157:H7, methicillin-resistant *Staphylococcus aureus* (MRSA; clinical isolate C622) and *Bacillus subtilis* (clinical isolate C626). Minimal Inhibitory Concentration values (MIC_{100}), i.e. the minimal concentration that inhibits completely the bacterial planktonic growth, were determined by the broth microdilution method as described by Hancock et al. [67].

Three controls were included in assays: peptide HHC-36, a synthetic peptide whose antimicrobial activity has already been demonstrated [81], polimixyn B, a natural antibiotic peptide active only on Gram-negative bacteria and vancomicyn, an antibiotic active only on Gram-positive bacteria.

MIC assays were initially performed in Mueller Hinton Broth, an extremely rich medium, frequently used in the determination of antimicrobial activity. All MIC values measured in this medium were very high (Table 4). Moreover, some peptides (especially the β-Fibrinogen derived peptide) showed a significant tendency to form aggregates in Mueller Hinton Broth (MHB) as evidenced by turbidity. Since it has been demonstrated that very rich media can influence MIC values of peptides, I repeated the assay in different media. The lowest MIC values were obtained in Nutrient Broth and in 50% Nutrient Broth (Table 5).

With the exception of PA $3_{(26-47)}$ all the peptides showed antimicrobial activity but with significant differences from strain to strain. Apo $E_{(133-150)}$ was particularly active on *P. aeruginosa* and *S. aureus* with MIC values similar to those of the control peptide HHC-36. Apo $B_{(887-911)}$ and PA $3_{(1-25)}$ were active only on *E. coli* and *S. aureus*, respectively. $\beta Fib_{(420-447)}$ was active on *B. subtilis* and *S. aureus*.

Table 4: Antibacterial activity of five human peptides and HHC36 (positive control) by broth microdiluition broth. MIC value average of three independent experiments carried out in Mueller Hinton Broth. Antibiotics were used as control.

		MIC (µg/ml)			
Compound	**Sequence**	***E.coli***	***PAO1***	***B.subtilis***	***S.aureus***
Apo B(887-911)	HVALKAGKLKFIIPSPKRPVKLLSG	>256	>256	>256	>256
Apo E(133-150)	LRVRLASHLRKLRKRLLR	64	32	>256	256
PA 3(1-25)	IMYKVPLIRKKSLRRTLSERGLLKD	>256	>256	>256	>256
PA 3(26-47)	FLKKHNLNPARKYFPQWKAPTL	>256	>256	>256	>256
β Fib(420-447)	GVVWMNWKGSWYSMRKMSMKI RPFFPQQ	128	>256	>256	>256
HHC-36	KRWWKWWRR	8	8	64	8
Polymyxin B		1	1	>32	>32
Vancomycin		>32	>32	2	1

Table 5: Antibacterial activity of five human peptides and HHC36 (positive control) by broth microdiluition broth. MIC value average of three independent experiments carried out in Nutrient Broth 0.5X. Antibiotics were used as control.

		MIC (µg/ml)			
Compound	**Sequence**	***E.coli***	***PAO1***	***B.subtilis***	***S.aureus***
Apo B(887-911)	HVALKAGKLKFIIPSPKRPVKLLSG	16	256	256	>256
Apo E(133-150)	LRVRLASHLRKLRKRLLR	64	16	128	8
PA 3(1-25)	IMYKVPLIRKKSLRRTLSERGLLKD	128	>256	>256	64
PA 3(26-47)	FLKKHNLNPARKYFPQWKAPTL	>256	>256	>256	>256
β Fib(420-447)	GVVWMNWKGSWYSMRKMSMKI RPFFPQQ	128	128	64	32
HHC-36	KRWWKWWRR	16	16	16	8
Polymyxin B		2	1	16	16
Vancomycin		16	>32	0.5	0.5

3.7.2 Antibiofilm activity

As mentioned in the Introduction, biofilm is involved in more than 80% of infections. Moreover, antibiotics often are ineffective against biofilm. For this reason I decided to investigate the ability of human peptides to eradicate biofilm formed by the same Gram negative and Gram positive clinical isolates used for the antibacterial activity assay. This allowed to perform a comparison between antimicrobial and anti-biofilm activity on each selected strain.

Preliminarily to the anti-biofilm assays, I studied the conditions (in particular the broth) inducing the sessile phenotype in the selected strains. The best medium favouring biofilm growth of both Gram-negative bacteria was BM2 minimal broth (Fig. 15A). Unfortunately, in the case of Gram positive bacteria, even if several growth conditions were tested, no satisfactory biofilm growth was obtained, therefore antibiofilm assays were not performed on these two strains (Fig. 15B) .

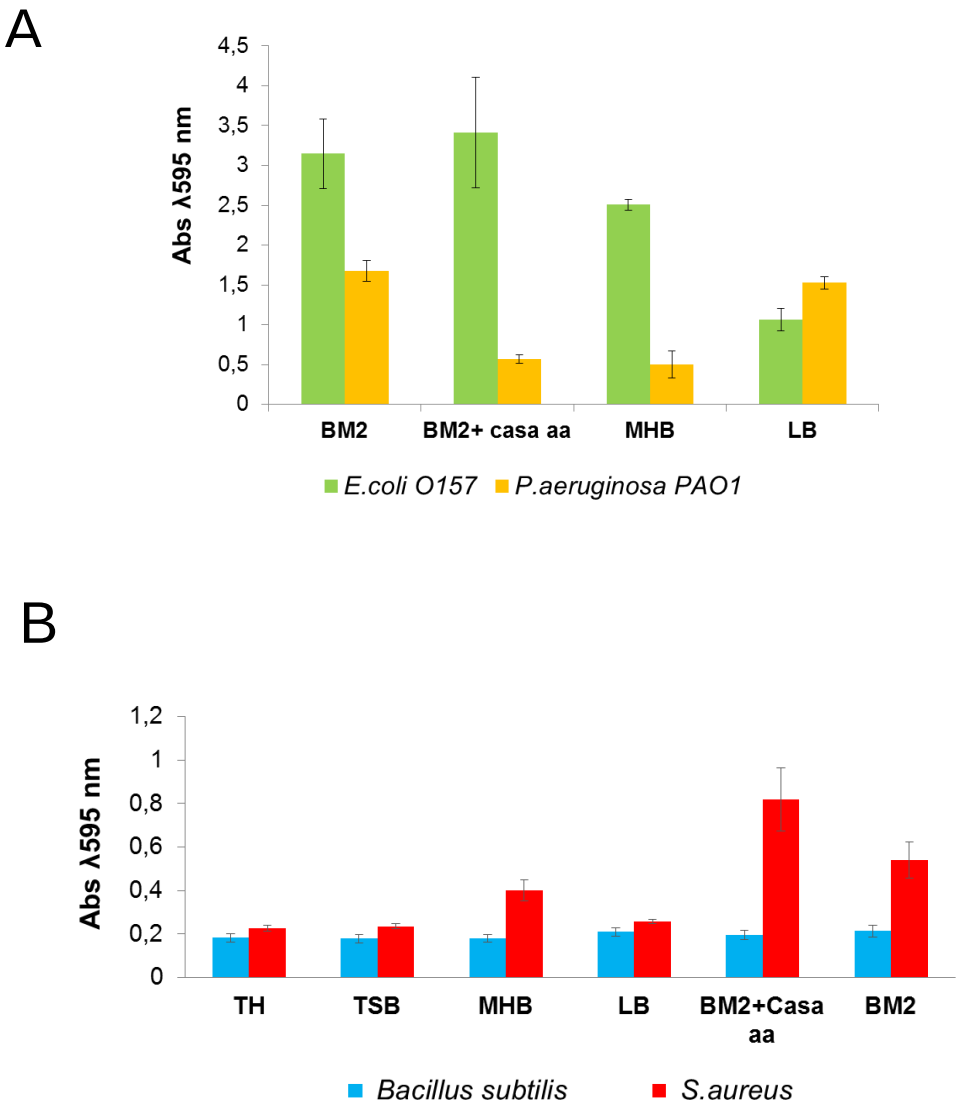


Figure 15: Selection of the best biofilm growth medium. A) Biofilm growth of Gram-negative bacteria. B) Biofilm growth of Gram-positive bacteria (clinical isolate strains).

The antibiofilm assay was performed following the protocol described in Materials and Methods [68]. The Minimal Biofilm Inhibitory concentration, MBIC, is the concentration required to fully prevent biofilm formation ($MBIC_{100}$) or to reduce to 50% biofilm growth ($MBIC_{50}$). Also in this case three controls were used: the D-peptide DJK-6, a very effective synthetic antibiofilm peptide [82], and the antibiotics polymyxin B and vancomycin.
Among the five human peptides, Apo $E_{(133-150)}$ and $\beta Fib_{(420-447)}$ exhibited a good antibiofilm activity in MBIC assay. In particular, $ApoE_{(133-150)}$ completely inhibited biofilm growth of both Gram-negative bacteria at concentrations similar to those required to inhibit planktonic growth (Table 6).
$\beta Fib_{(420-447)}$ peptide showed an antibiofim activity specific for *E.coli* ($MBIC_{100}$ is 64 µg/ml i.e. about 18 µM, Table 6).
Interestingly, peptide Apo $B_{(887-911)}$, was able to reduce at 50% biofilm growth of *P.aeruginosa* ($MBIC_{50}$ is 8-16 µg/ml, Table 6) at concentrations that had no effect on planktonic growth (MIC on *P. aeruginosa* is >256 µg/ml, Table 5). This behavior is not completely surprising. Indeed, in literature there are recent evidences that other peptides, like for example IDR-1018, inhibits biofilm growth at concentrations lower than those required to inhibit planktonic growth [83] thus suggesting that antimicrobial and anti-biofilm activity could depend on different mechanisms.

Table 6: Antibiofilm activity of five human peptide and DJK-6 peptide (positive control) by crystal violet stained 96 multiwell assay. MBIC value average of three independent experiments carried out in BM2 medium. Antibiotics were used as control.

	$MBIC_{100}$ (µg/ml)		$MBIC_{50}$ (µg/ml)	
Compound	***E.coli***	***P.aeruginosa***	***E.coli***	***P.aeruginosa***
Apo $B_{(887-911)}$	>64	>64	>64	8-16
Apo $E_{(133-150)}$	32-64	64	16-32	16-32
PA $3_{(1-25)}$	>64	>64	>64	>64
PA $3_{(26-47)}$	>64	>64	64	>64
β $Fib_{(420-447)}$	64	>64	32-64	>64
DJK-6	2	16	1-2	4-8
Polymyxin B	2	2	2	2
Vancomycin	>32	>32	>32	>32

A deeper characterization was performed in the case of peptides Apo $B_{(887-911)}$, PA $3_{(1-25)}$ and PA $3_{(26-47)}$ through experiments of biofilm eradication in a flow cell chamber. Such method allows to monitor morphological alterations of the biofilm even when a $MBIC_{100}$ value cannot be measured. Biofilm was allowed to develop for three days in flow cell chambers with a constant flow of BM2 minimal medium. Peptides were added to the flow medium during the entire three days of the experiment, then the resulting surface-adherent cells and biofilm were stained with stains that evidence total cells (green fluorescent dye Syto-9) or with stains selective for dead cells (red fluorescent dye propidium iodide) and visualized using confocal laser scanning microscopy [68]. As shown in Fig. 16A, treatment with 10 µg/ml of $ApoB_{(887-911)}$ led to a significantly decreased biofilm thickness, and triggered some cell death, as indicated by red dots. These data, confirmed that the $MBIC_{50}$ value on *P. aeruginosa* for $ApoB_{(887-911)}$ is in the range 8-16 µg/ml corresponding to 3 - 6 µM.
Interestingly, also biofilm treated with PA $3_{(26-47)}$ peptide showed a morphological alteration in terms of reducing biofilm thickness at concentration of 10 µg/ml (about 4 µM) (Fig. 16B), i.e. a concentration much more lower than MIC value toward planktonic PAO1 cells (MIC on PAO1 ≥ 256 µg/ml corresponding to about 95 µM, see Table 5). Finally, cells treated with PA $3_{(1-25)}$, did not show significant biofilm reduction (Fig. 16C).

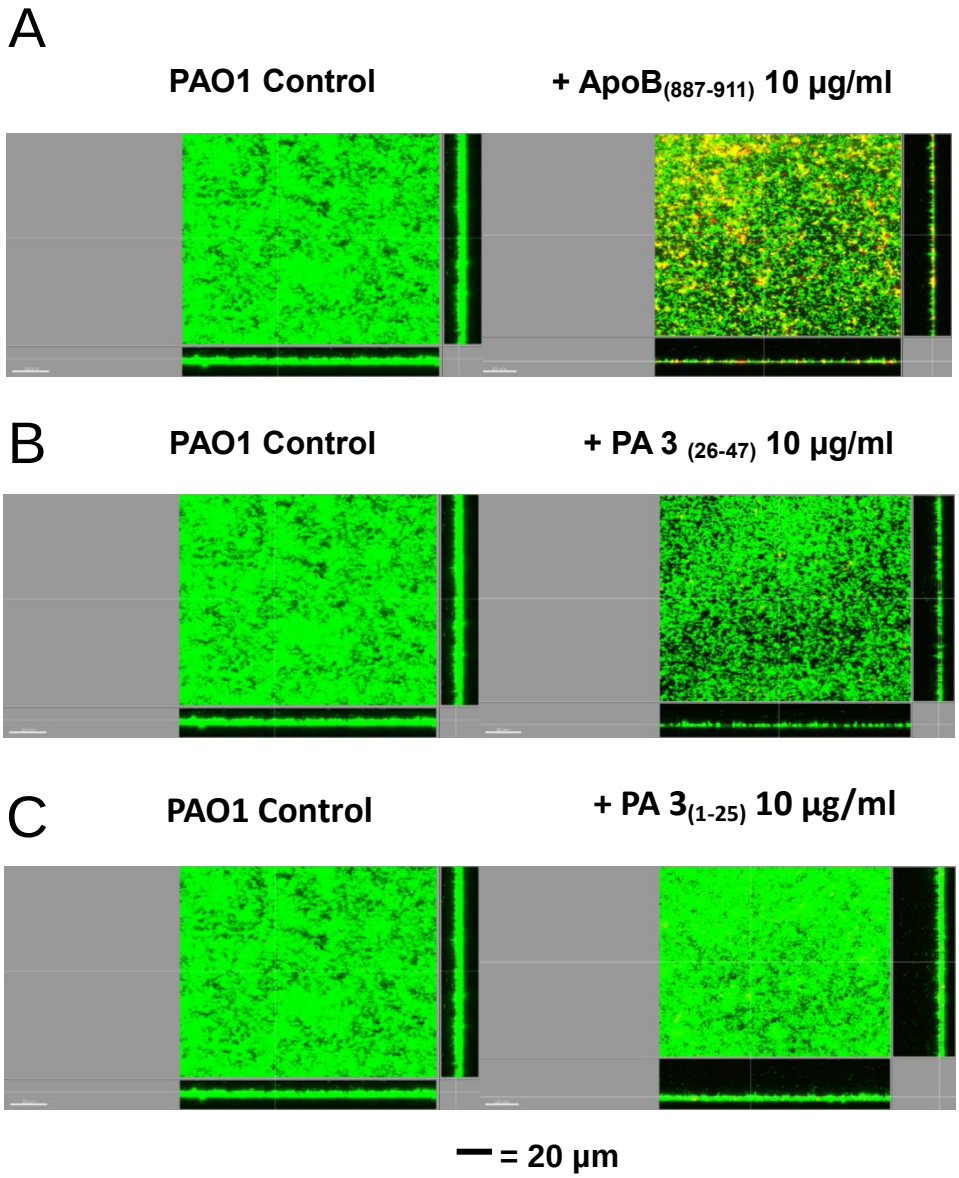


Figure 16: Biofilm eradication in flow cell system and microscopy. A) Treatment with Apo $B_{(887-911)}$ decreases biofilm thickness and triggers death of some cells, confirming that the $MBIC_{50}$ value on *P.aeruginosa* for Apo $B_{(887-911)}$ is about 8-16 µg/ml. B)The biofilm reduction of PA $3_{(26-47)}$ is at concentration much lower than MIC value.C) Treatment with 10 µg/ml of PA $3_{(1-25)}$ was not sufficient to significative alter biofilm structure.

3.7.3 Immunomodulatory activity and ability to enhance the innate immune system

In response to microbial invasion, immune cells release a complex network of cytokines to fight infection. It is the net effect of interactions between pro-inflammatory and anti-inflammatory molecules over time that determines the nature of the immune response in individual patients [23]. Host defense peptides can modulate this immune response. However, it should be reminded that, there is a huge variability of the immune response between individuals, therefore immunological studies to be significant should be conducted on a suited number of subjects.

The immunomodulatory properties I investigated included:

i) Immunomodulatory activity (suppression of potentially harmful excessive inflammatory response triggered by “microbial signature molecules” like lipopolysaccharides from Gram negative strains);
ii) Enhancement of innate immune response (by induction of chemokines involved in leukocyte activation).

i) Immunomodulatory activity

In vitro preliminary experiments were performed isolating peripheral blood mononuclear cells (PBMCs) from healthy blood adult volunteers. A PBMC is defined as any blood cell with a round nucleus (i.e. a lymphocyte, a monocyte, or a macrophage). They are a critical component of the immune system.

Preliminary to the immunomodulatory assay, the cytotoxic effect of all peptides was investigated using the lactate dehydrogenase (LDH) assay. As AMPs usually act via cell membrane damaging, this is a particularly well suited colorimetric assay to quantitatively measure lactate dehydrogenase (LDH) released into the media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis.

Even at the highest concentration tested for each peptide (50 µg/ml), the release of the cytosolic enzyme, LDH, was lower than 5% compared to cells treated with 0.2% triton X100, indicating that none of the selected peptides tested possesses lytic activity (Fig. 17).

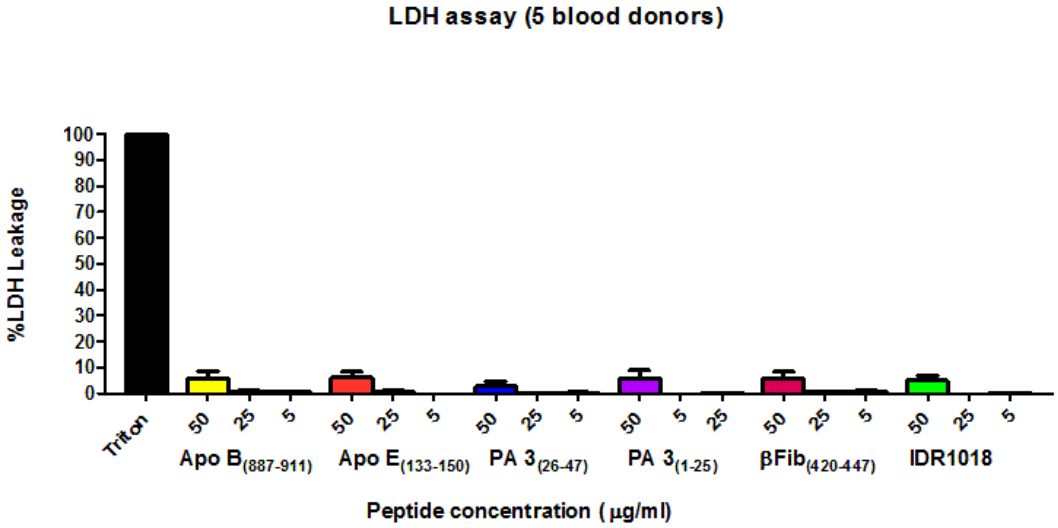


Figure 17: Cytolysis effect of the five studied human peptides on human PBMCs. Colorimetric assay based on the cytosolic enzyme activity LDH.

To investigate the immunomodulatory properties of human peptides, PBMCs were stimulated with *P. aeruginosa* LPS and treated with three different concentrations of each peptide (5-25-50 µg/ml). The immunomodulatory peptide IDR-1018, a synthetic versions of natural host defense peptides, was included as a positive control. Levels of two main proinflammatory cytokines, tumor necrosis factor–α (TNF-α) and interleuchin 1-β (IL-1β), were measured by ELISA.
Data collected by using PBMCs from three independent donors (Fig.18) showed that in response to LPS high levels of TNF-α were released from untreated cells whereas a general significant reduction of this pro-inflammatory cytokine was observed in cells treated with most of the peptides tested at the lowest concentration (Fig.18A). This indicates that most of the peptide tested possess immunomodulatory properties being able to suppress LPS-mediated inflammation. Indeed, treating PBMCs with increasing doses of peptides after LPS stimulation, the overall TNF-α reducing trend was confirmed for most peptides. Interestingly, dose-dependent response was mostly evident with $ApoB_{(887\text{-}911)}$ whereas an opposite trend with PA $3_{(1\text{-}25)}$ was observed, (Fig.18B). This behavior, is not completely surprising, since some peptides, like hβDF2, can act as alarmins i.e. with a pro-inflammatory trend, some others act by TNF-α reduction, like LL-37 [30].
On the other hand, no significant cytokine IL-1β reduction was observed on PBM cells, in response to LPS mediated inflammatory stimulation and treatment with each peptide (data not shown).

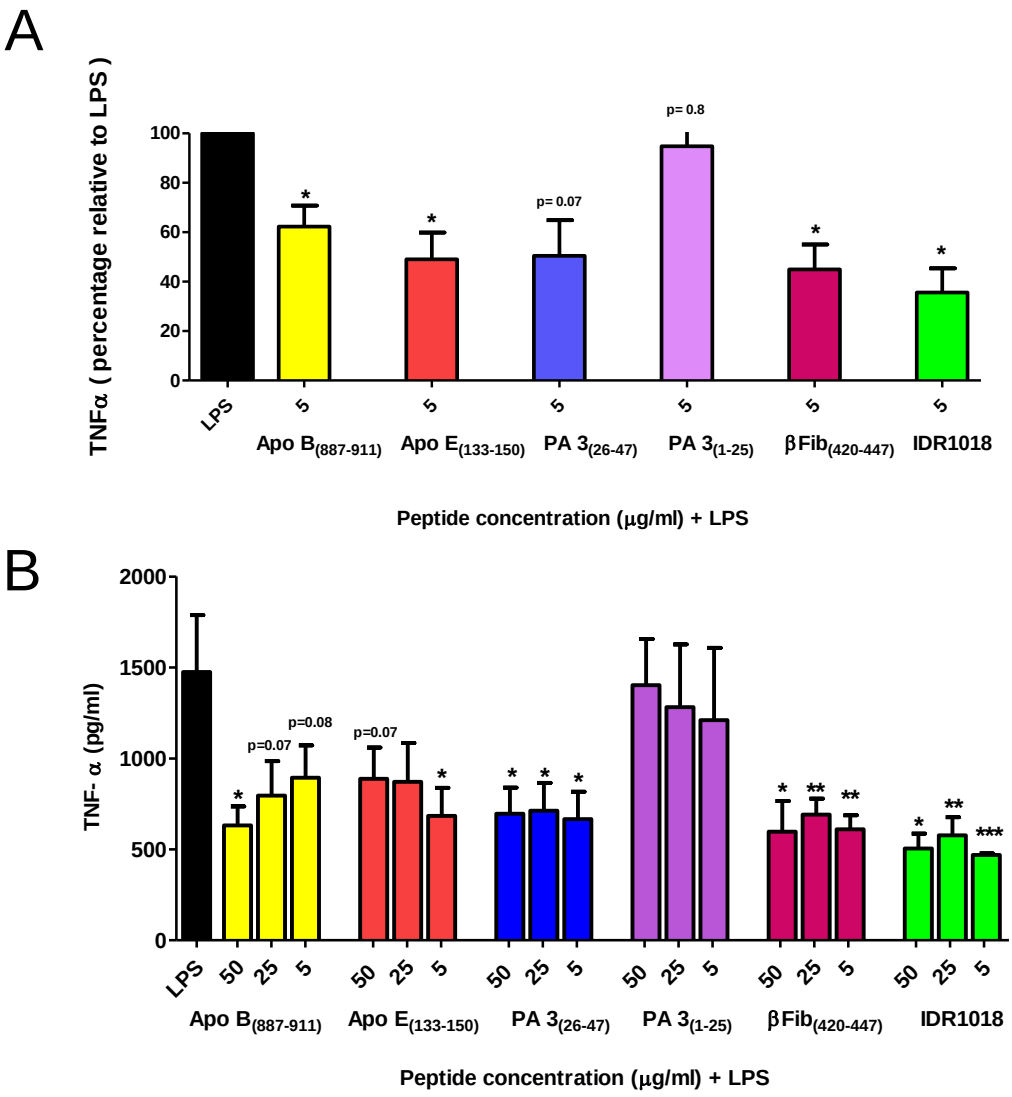


Figure 18: TNF-α reduction mediated by human peptides on PBMCs cells. A) Percentage reduction of TNF-α after stimulation with 20 ng/µl of *Pseudomonas aeruginosa* PAO1 LPS and treatment with the lowest peptide concentration 5 µg/ml. B) Dose response effect on LPS stimulated TNF- α concentration release after treatment with crescent doses (5-25-50) µg/ml of human peptides.

ii) Enhancement of innate immune response

As HDPs promote bacterial clearance by acting on the host immune response specifically enhancing the production of chemokines, I investigated the ability of human peptides to induce the release of MCP-1, monocyte chemotactic protein-1, a member of CC chemokynes subfamily mainly involved in the activation of leukocyte migration.

Data obtained using PBMCs of five independent donors (Fig.19) showed that in the absence of LPS-stimulation, PBMCs produce basal level of MCP-1 (about 1000 pg/ml). In response to LPS the release of MCP-1 is greatly enhanced (5000-20000 pg/ml).

Almost all human peptides increased levels of MCP-1 secretion compared to untreated cells (control). Apo $E_{(133-150)}$ showed a stimulating ability similar to that of the control peptide IDR-1018 (about 10000 pg/ml and 8000 pg/ml respectively) and with a dose dependent trend. Interestingly $\beta Fib_{(420-447)}$ showed the highest stimulation activity comparable to that of LPS (about 22000 pg/ml but with a great variability from donor to donor). PA $3_{(1-25)}$ increases MCP-1 release in a dose dependent manner. Thus, at least three of the selected peptides possess interesting immunomodulatory activity.

Overall, the analysis performed in the host laboratory indicate that the five potential AMPs, identified by the bioinformatic approach, show typical features of host defense (antimicrobial) peptides.

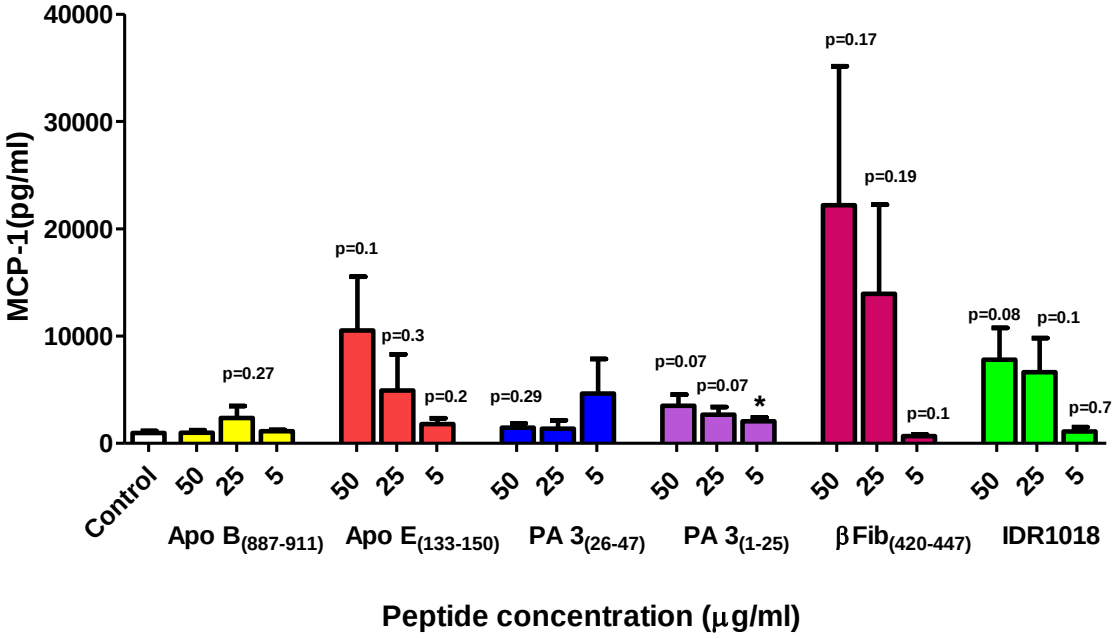


Figure 19: Induction of MCP-1 release on PBMCs treated with five human peptides compared to IDR-1018 (control peptide).

Chapter 4-Conclusions

Infections attributed to multi-drug resistant (MDR) microbes, are increasingly frequent. Therefore a huge effort to improve or develop molecules that can inhibit pathogens without incurring in pathogen resistance is urgently required. In this context, antimicrobial peptides (AMPs) are particularly well suited candidates being the most ancient weapon of innate immunity. In addition to their antimicrobial action, several AMPs show additional biologic properties usually correlated to the control of infections, a feature that makes them attractive therapeutic agents.
It is tempting to speculate that human proteome is a yet unexplored source of bioactive peptides with many potential pharmacological applications. In fact, a wide variety of human proteins whose primary functions are not necessarily related to host defence, contain AMPs hidden inside their sequence.
The activities of the present PhD thesis are framed in the context of a more complex research project whose main goal is the development of human AMPs-based antimicrobial drugs for topic applications like treatment of surgical wounds, of ulcers and of skin, mouth and airways infections. Being human peptides, their use will limit the risk of inducing immune response.
Preliminarily to the activities of the present PhD work, our research group has developed an *in silico* screening method that allows to localize antimicrobial regions hidden inside the primary structure of precursors, developed by Dr. Notomista. Using this method to screen about 4'000 human extracellular proteins, a wide list of potential new AMPs was obtained. These findings represented the starting point. The main aim of the present PhD project was to select and characterize the most pharmacologically interesting molecules suggested by the bioinformatic screening, in order to find candidates suited for the development of novel antimicrobial and anti-inflammatory agents. In line with this goal, the main tasks carried out were:

I. Development and optimization of efficient strategies for the production of recombinant toxic peptides in *E.coli*.

II. Production of biologically interesting AMPs selected from the *in silico*-defined list of potential AMPs.

III. Functional characterization of the new AMPs to find promising pharmacological activities.

Through a rational design strategy the suitability of onconase (ONC) as a carrier for the production of recombinant peptides in *E. coli* was progressively improved. The final version of the protein, ONC-DCless-H6, is a very efficient carrier particularly suited for the production of peptides potentially toxic for the host *E. coli*. It is expressed at high levels (>200 mg/L) efficiently forming inclusion bodies. Chemical cleavage of the fusion protein can be performed in diluted acids without the need to employ high concentrations of formic acid that is often chosen for its denaturing/solubilizing properties. After the cleavage at acidic pH, the ONC moiety, ONC-DCless-H6, can be easily removed due to its insolubility at neutral pH providing an easy procedure for peptide purification. Moreover, our strategy also allows to prepare peptides with a N-terminal cysteine residue that could find many applications in research and biotechnological procedure like for example preparation of labeled peptide, protein ligation in the semi-synthesis of modified proteins, immobilization on support etc. It should be noted that even if our method was developed specifically to prepare peptides toxic for the host it can be used for the production of other peptides. In particular, due to its simplicity and limited costs, this fusion system can be applied

to medium/large scale production of peptides with pharmacological activity required for example in the clinical trial phases. It is worth to underline that our method is complementary to chemical synthesis being particularly well suited to prepare peptide longer than 20-25 residues.
Using ONCDCless-H6 carrier protein several potential human AMPs, from 20 to almost 50 amino acids were successfully produced. All these peptides showed as expected antimicrobial activity thus validating the efficacy of the bioinformatic screening method.
In addition some of these peptides have been analyzed for their anti-inflammatory and immuno-modulating activity. Apo $E_{(133-150)}$ showed broad spectrum of activity on both Gram-negative and Gram-positive bacteria but also anti-biofilm activity on Gram-negative bacteria. Some peptides studied in this PhD work, led to a significant decrease of *P. aeruginosa* biofilm thickness and cell viability at concentration much lower than MIC values observed on planktonic growth [e.g. Apo $B_{(887-911)}$ and at a lower extent PA $3_{(26-47)}$]. Besides, our novel AMPs are able to modulate innate immune response, indeed, a general reduction of TNF-α pro-inflammatory cytokine was observed on PBMCs (innate immune cells) treated with most of the peptides tested at very low concentration (5 µg/ml). Furthermore, almost all human peptides increased levels of MCP-1 secretion compared to untreated cells suggesting a role in the control of infection.
Even if at the moment the panel of peptides and of assays performed is quite limited our preliminary data suggest that the *in silico*-derived list of potential AMPs is a rich source of peptides with pharmacologically relevant properties.

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Appendices

List of publications

1. Pane K, Durante L, Pizzo E, Varcamonti M, Zanfardino A, Sgambati V, Di Maro A, Carpentieri A, Izzo V, Di Donato A, Cafaro V, Notomista E. **Rational Design of a Carrier Protein for the Production of Recombinant Toxic Peptides in Escherichia coli.** PLoS One. 2016 Jan 25;11(1):e0146552. doi: 10.1371/journal.pone.0146552. eCollection 2016.

List of submitted manuscript

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2. Pane K, Durante L, Crescenzi O, Cafaro V, Pizzo E, Varcamonti M, Zanfardino A, Izzo V, Di Donato A, Notomista E. **Antimicrobial Potency of Cationic Antimicrobial Peptides can be Predicted from their Amino Acid Composition: Application to the Detection of "Cryptic" Antimicrobial Peptides.** Submitted on December 2015, revision under review. BBA General Subject.

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1. M. Verrillo, **K. Pane,** E. Pizzo, M. Varcamonti, A. Zanfardino, M. Siepi, A. Avitabile, A. Di Donato, E. Notomista, V. Cafaro. "*Efficient production and labeling of recombinant peptides endowed with a N-terminal cysteine residue"* Proteine 2016 Bologna. March 30th-April 1st 2016.
2. **K. Pane**, V. Sgambati, A. Zanfardino, A. Bosso, V. Cafaro, E. Pedone, S. Di Gaetano, E. Notomista, M. Varcamonti, G. Smaldone, A. Bragonzi, E. Pizzo. *"Identification and characterization of LPS-neutralizing human peptides:potential tools to control inflammation in cystic fibrosis lung disease (FFC 20#2014)"* XIII Convention of investigators in cystic fibrosis. Garda 26th - 28th November 2015.
3. **K. Pane**, V. Cafaro, A. Avitabile, A. Bosso, E. Pizzo, E. Notomista, A. Di Donato. *"A novel carrier protein for AMPs production*" 5th Antimicrobial International Meeting on Antimicrobial Peptides, Burlington House 7th-8th September 2015.
4. A. Avitabile, V. Cafaro , **K. Pane**, A. Bosso, E. Pizzo , E. Notomista, A. Di Donato. "*The activation peptide of human pepsinogen is an antimicrobial peptide*". 5th Antimicrobial International Meeting on Antimicrobial Peptides, Burlington House 7th-8th September 2015.
5. A. Bosso, V. Sgambati, **K. Pane**, A. Avitabile, A. Di Maro, E. Pizzo. "*A novel source of microbial agents: characterization of an antimicrobial peptide from PD-L4, a type 1 ribosome inactivating protein from Phytolaccadioica L*." 5th Antimicrobial International Meeting on Antimicrobial Peptides, Burlington House 7th-8th September 2015.
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Experience in foreign laboratories

Stage at Center for Microbial Diseases and Immunology Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada (13th September 2014- 15th February 2015). The work was supervised by prof. Robert E.W. Hancock, professor of Microbiology, Director of the Center.

Paper

PLOS ONE

RESEARCH ARTICLE

Rational Design of a Carrier Protein for the Production of Recombinant Toxic Peptides in *Escherichia coli*

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Citation: Pane K, Durante L, Pizzo E, Varcamonti M, Zanfardino A, Sgambati V, et al. (2016) Rational Design of a Carrier Protein for the Production of Recombinant Toxic Peptides in *Escherichia coli*. PLoS ONE 11(1): e0146552. doi:10.1371/journal.pone.0146552

Editor: Mark J van Raaij, Centro Nacional de Biotecnologia - CSIC / CIF Q2818002D, SPAIN

Received: September 22, 2015

Accepted: December 19, 2015

Published: January 25, 2016

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants FFC#9/2012, FFC#11/2013, and FFC#12/2014 from "Italian Cystic Fibrosis Research Foundation" (http://www.fibrosicisticaricerca.it) and "Programma F.A.R.O IV tornata" (CUP: E68C12000260003) funded by Polo delle Scienze e Tecnologie, Università di Napoli Federico II. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Commercial uses of bioactive peptides require low cost, effective methods for their production. We developed a new carrier protein for high yield production of recombinant peptides in *Escherichia coli* very well suited for the production of toxic peptides like antimicrobial peptides. GKY20, a short antimicrobial peptide derived from the C-terminus of human thrombin, was fused to the C-terminus of Onconase, a small ribonuclease (104 amino acids), which efficiently drove the peptide into inclusion bodies with very high expression levels (about 200–250 mg/L). After purification of the fusion protein by immobilized metal ion affinity chromatography, peptide was obtained by chemical cleavage in diluted acetic acid of an acid labile Asp-Pro sequence with more than 95% efficiency. To improve peptide purification, Onconase was mutated to eliminate all acid labile sequences thus reducing the release of unwanted peptides during the acid cleavage. Mutations were chosen to preserve the differential solubility of Onconase as function of pH, which allows its selective precipitation at neutral pH after the cleavage. The improved carrier allowed the production of 15–18 mg of recombinant peptide per liter of culture with 96–98% purity without the need of further chromatographic steps after the acid cleavage. The antimicrobial activity of the recombinant peptide, with an additional proline at the N-terminus, was tested on Gram-negative and Gram-positive strains and was found to be identical to that measured for synthetic GKY20. This finding suggests that N-terminal proline residue does not change the antimicrobial properties of recombinant (P)GKY20. The improved carrier, which does not contain cysteine and methionine residues, Asp-Pro and Asn-Gly sequences, is well suited for the production of peptides using any of the most popular chemical cleavage methods.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Peptides of length between five and fifty residues have many applications in research, diagnostic, medicine (e.g. antimicrobial [1, 2], anti-inflammatory [3], anti-cancer [4] and cell penetrating peptides [5] etc.) and industry (e.g. metal surface-binding [6] and graphite-binding peptides [7]). Solid phase chemical synthesis is well suited to obtain small amounts of peptides but it could be rather expensive when peptides longer than 30 amino acids or high quantities of peptide are required. On the other hand, recombinant production of peptides [8–11] entails several difficulties. For example, expression of some peptides can be toxic for the host and several peptides are very sensitive to proteases thus leading to low yields. These difficulties can be circumvented by fusing the desired peptide to a carrier protein that can protect peptides from proteases, neutralize possible toxic effects and provide a convenient route for their purification. However, also fusion protein strategy has two main drawbacks. Firstly, the fusion protein has to be cleaved and peptide needs to be separated from the carrier, secondly, the desired peptide usually represents just a small percentage of the purified fusion protein. As for the cleavage of the peptide, it can be released from the fusion protein by enzymatic or chemical cleavage at a site suitably introduced at the carrier-peptide junction ([10] and references therein). The efficiency and specificity of the cleavage is the main bottleneck of fused peptide production. Usually enzymatic proteolysis by factor Xa [12, 13], enterokinase [14] and thrombin [15, 16], the most used enzymes, is less efficient than chemical cleavage [17–19]. The lower efficiency of enzymatic methods is generally attributed to unfavorable surrounding residues and/or to steric hindrance which make the cleavage sites not fully accessible. Furthermore, enzymes usually require non-denaturing reaction conditions to work properly. Thus, enzymatic proteolysis cannot be used when the fusion protein is partly or completely insoluble. On the other hand, the most popular cleavage reagents, like cyanogen bromide (CNBr), formic acid and hydroxylamine, which cleave Met-X, Asp-Pro and Asn-Gly peptide bonds [10, 20, 21], respectively, often produce unwanted cleavages and side-chain modifications due to the harsh reaction conditions needed for the cleavage [22–28]. For example, cleavage of Asp-Pro sequences, usually performed at 60°C in 50–70% formic acid, often results in unspecific hydrolysis, formylation and oxidation reactions of side chains [28].

Here, we describe the rational development of a new carrier protein for high yield production of recombinant peptides in *Escherichia coli* very well suited for toxic peptides like antimicrobial peptides [10]. The denatured form of Onconase (ONC), a RNase from *Rana pipiens* [29], was chosen as starting point to design the carrier protein. ONC is a very well suited partner for several reasons: (i) it can be expressed at very high levels in inclusion bodies (about 200–250 mg/L in terrific broth); (ii) usually no soluble ONC can be detected in growth cultures thus minimizing the risk of toxic effects of the ONC-peptide fusion proteins; (iii) it is a very small protein (104 aa) thus allowing high yields of the peptides after the cleavage; (iv) the solubility of denatured Onconase is pH dependent—the denatured protein is soluble only at pH <4 –thus allowing the purification of peptides soluble at pH 7 by selective precipitation of the carrier. Moreover, ONC does not contain Asp-Pro and Asn-Gly sequences and the mutant (M23L)-ONC [30] does not contain internal methionine residues, therefore it is compatible with all common chemical cleavage strategies.

Starting from mutant (M23L)-ONC we have developed an improved carrier protein bearing 15 mutations and an improved cleavage procedure of Asp-Pro peptide bonds that does not require formic acid.

We tested the efficiency of our strategy by producing GKY20, a short cationic antimicrobial peptide derived from the C-terminus of human thrombin [31]. Like other similar antimicrobial peptides, GKY20 is endowed with broad spectrum antimicrobial activity being active both on Gram-positive and Gram-negative bacteria. Its bactericidal activity has been attributed to its

capacity to induce membrane lysis. Moreover, it shows potent anti-inflammatory activity due to its ability to bind LPS thus inhibiting macrophage responses to these components of the outer membrane [32]. Noteworthy, GKY20 is active on different strains of *E. coli* with a minimal inhibitory concentration (MIC) lower than 10 μM [33]. In spite of this high toxicity we were able to prepare recombinant GKY20 in *E. coli* with final yield of about 15–20 mg per L of culture and purity higher than 96–98%.

Results and Discussion

Expression vector design: ONC-(P)GKY20 fusion protein

Native ONC is a small protein (104 aa) stabilized by four disulfide bridges. When expressed in *E. coli*, wild type ONC and its mutants are produced at high yield as inclusion bodies [29, 30]. We have previously shown that once extracted by denaturation using guanidine chloride and dialyzed in acetic acid 0.1 M the denatured form is very soluble, whereas at pH 7 it is prone to aggregation and efficient renaturation can only be achieved by a special procedure based on reversible blocking of cysteine residues [29]. In order to use denatured ONC as carrier for the production of recombinant peptide GKY20, we prepared a chimeric open reading frame including codons 1–103 of (M23L)-ONC [34], five codons coding for a linker sequence (GTGDP) containing the formic acid cleavable aspartyl-prolyl dipeptide, and a sequence coding for GKY20 (Fig 1A and S1 File, panel A). This sequence was obtained by changing six codons in the human coding sequence to better match *E. coli* codon usage (S1 File, panel C). It should be reminded that ONC does not contain Asp-Pro and Asn-Gly sequences (S1 File, panel A). Moreover, mutant (M23L)-ONC does not contain any internal methionine residue. Thus, mutant (M23L)-ONC represents an ideal scaffold for the chimeric construct because it is not cleaved by the usual chemical cleavage strategies.

The linker sequence was chosen both to introduce a flexible region into the chimeric protein between the ONC moiety and GKY20 and to introduce two restriction enzyme sites, *KpnI* (codons coding for amino acids GT) and *BamHI* (codons coding for amino acids GDP). Moreover, C-terminal cysteine of the ONC moiety (Cys104) was deleted to avoid the presence of an oxidation prone residue near to the cleavage site. The synthetic construct and the corresponding recombinant protein were named ONC-(P)GKY20 to underline that, by acid cleavage, the recombinant protein releases peptide (P)GKY20 i.e. peptide GKY20 with an additional proline at the N-terminus. The construct was cloned into pET22b(+) plasmid between *NdeI* and *SacI* restriction sites (Fig 1A) and expressed into BL21(DE3) *E. coli* cells. Expression of recombinant protein was obtained as described in Materials and Methods.

SDS-PAGE analysis of induced cultures (Fig 2) showed high expression levels of ONC-(P)GKY20 (200–250 mg/L) as inclusion bodies. The recombinant protein was partially purified by denaturation of the inclusion bodies and dialysis in 0.1 M acetic acid pH 2.9 that causes the precipitation of most of the *E. coli* proteins. As shown in Fig 2 (lane 4), after dialysis the soluble fraction contained less than 15–20% of *E. coli* proteins as determined by SDS-PAGE densitometry scan. This sample was used to optimize the acidic cleavage of the DP sequence as described in the next section.

Chemical cleavage optimization of aspartyl-prolyl bonds in ONC-(P)GKY20 fusion protein

The cleavage of Asp-Pro sequences is usually performed at temperatures in the range 55–80°C in 50–75% formic acid. However, these reaction conditions often produce unwanted side reactions like formylation and unspecific hydrolysis [22–28].

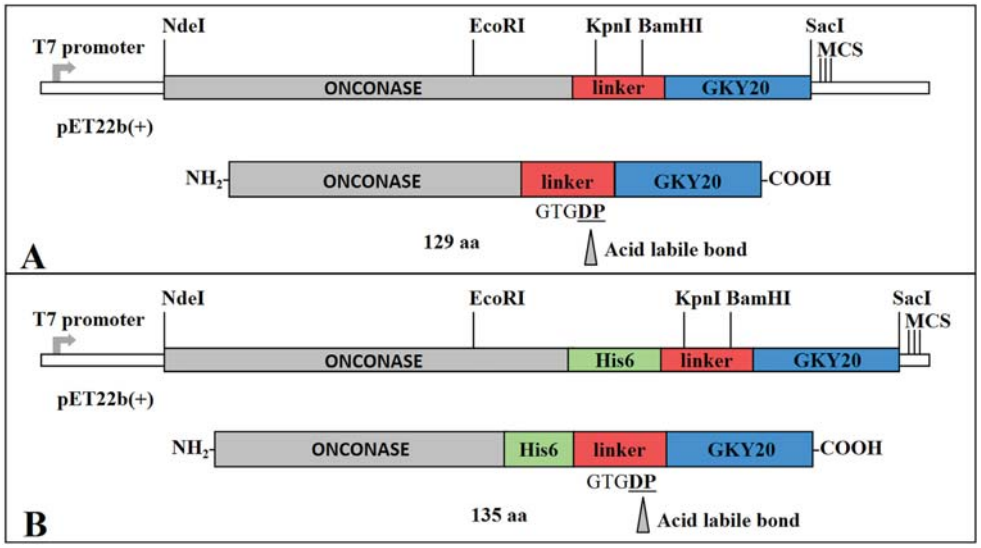

Fig 1. Schematic representation of expression vectors and recombinant proteins. Fusion proteins without (A) and with (B) His_6-Tag. The main restriction enzyme sites, *NdeI*, *EcoRI*, *KpnI*, *BamHI* and *SacI* were reported. MCS: multicloning site. Onconase: carrier protein (grey). Linker: DNA sequence coding for GTGDP amino acid residues (red). GKY20: human Thrombin derived peptide (blue).

doi:10.1371/journal.pone.0146552.g001

In order to find less harsh cleavage conditions, thermal incubations of ONC-(P)GKY20 fusion protein were carried out in different acid solutions: 50%, 60% or 70% formic acid (Fig 3A), 0.1 M acetic acid (pH 2.9), 0.1 M acetic acid adjusted at pH 2.0 with HCl (Fig 3B). Samples were incubated at 50° or 60°C for 3, 6, 9, 15 or 24 h. With the exception of 0.1 M acetic acid pH

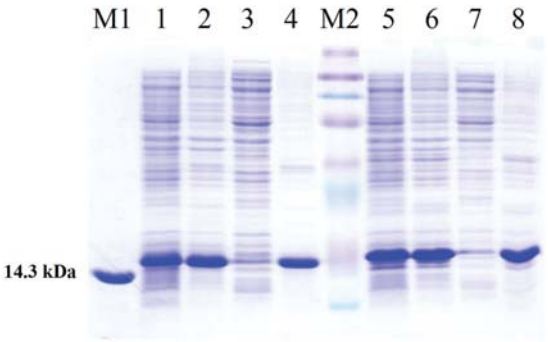

Fig 2. Protein expression. SDS-PAGE analysis of ONC-(P)GKY20 and ONC-DCless-H6-(P)GKY20 fusion proteins. Lane M1: marker (14.3 kDa). Lanes 1–4, ONC-(P)GKY20 protein: lane 1, cell lysate of induced culture; lane 2, insoluble fraction after cell lysis; lane 3, soluble fraction after cell lysis; lane 4, purified ONC-(P)GKY20 recombinant protein after dialysis against 0.1 M acetic acid. Lane M2: marker (8-12-20-30-45-60-100-220 kDa proteins). Lanes 5–8, ONC-DCless-H6-(P)GKY20 fusion protein: lane 5, cell lysate of induced culture; lane 6, insoluble fraction after cell lysis; lane 7, soluble fraction after cell lysis; lane 8, inclusion bodies after triton/urea wash.

doi:10.1371/journal.pone.0146552.g002

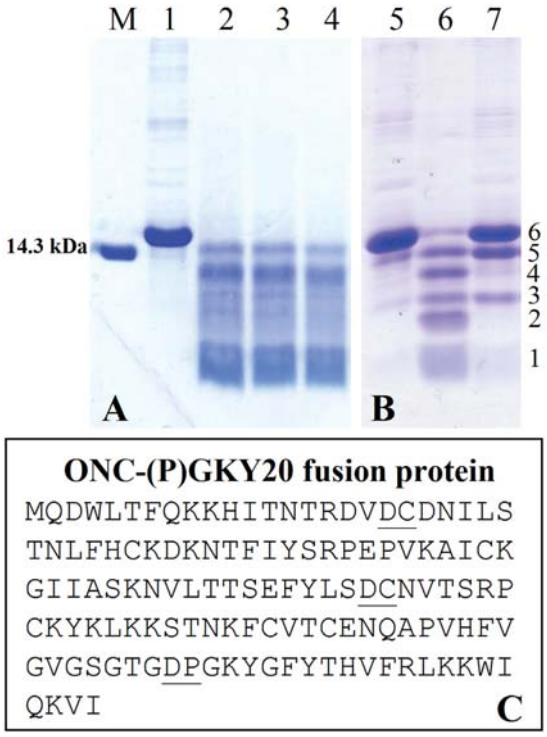

Fig 3. Hydrolysis pattern of ONC-(P)GKY20 protein. Samples were cleaved at acid pH for 24 h at 60°C and analyzed on 20% SDS-PAGE. Lane M: marker (14.3 kDa). Lanes 1, 5: purified fusion protein. (A) ONC-(P)GKY20 protein hydrolyzed in 50% (lane 2), 60% (lane 3) e 70% (lane 4) formic acid (60°C, 24 h). (B) ONC-(P)GKY20 protein hydrolyzed in 0.1 M acetic acid, pH 2.9, (lane 6) and in 0.1 M acetic acid adjusted at pH 2.0 with HCl (lane 7); the N—terminal sequences of the fragments obtained by hydrolysis of ONC-(P)GKY20 fusion protein (lane 6) were as follow: fragment 1, PGK [(P)GKY20 peptide)] and MQD (N-terminal peptide, less represented); fragment 2, X(Cys)NVT (D67-C68 cleavage site); fragment 3, X(Cys)NVT (D67-C68 cleavage site) and MQD (N-terminal peptide, less represented); fragment 4, X(Cys)DNI (D18-C19 cleavage site); fragments 5 and 6, MQD (N-terminal region) which represent the carrier protein and uncleaved fusion protein respectively. (C) Amino acid sequence of ONC-(P)GKY20 fusion protein; the main cleavage sites are underlined.

doi:10.1371/journal.pone.0146552.g003

2.9 (Fig 3B), all the acidic solutions resulted in the efficient hydrolysis (>90%) of the recombinant fusion protein by incubating samples at 60°C for times $\geq$ 15 h, but with a significant difference: in the case of the acetic acid solution at pH 2.0, a specific cleavage pattern with the formation of six discrete polypeptide bands was visible on SDS-PAGE (Fig 3B), whereas in the case of formic acid, in addition to the bands observed in the case of the acetic acid/HCl hydrolysis, a smear was clearly evident suggesting the occurrence of unspecific cleavages (Fig 3A).

This experiment indicated that it is possible to cleave the recombinant protein without using high concentrations of organic acids, but unfortunately they also revealed two unexpected problems: i) mild acidic hydrolysis cleaved ONC-(P)GKY20 in several fragments (Fig 3B) in spite of the fact that it contains a single Asp-Pro sequence between ONC and GKY20; ii) some *E. coli* proteins, present in small amount as contaminants in the inclusion bodies, also

underwent cleavage releasing shorter fragments as evidenced by the disappearance of high molecular weight bands.

N-terminal sequencing of the fragments separated by SDS-PAGE (Fig 3B), allowed to identify three main sites of cleavage in the fusion protein. In addition to the expected cleavage at the Asp-Pro sequence in the linker sequence, which releases the desired peptide (P)GKY20, two unexpected sites of hydrolysis were identified: Asp18-Cys19 and Asp67-Cys68. An incomplete hydrolysis at these sites accounts for the six bands observed in the SDS-PAGE (Fig 3B). Band 1 contained (P)GKY20 and a peptide corresponding to the N-terminus of ONC (including initial methionine). Intermediate bands were due to fragments starting at Cys19 and Cys68. Bands 5 and 6 contained fragments starting at the N-terminal sequence of ONC. On the basis of their migration they are likely the uncleaved fusion protein (band 6) and the ONC moiety resulting from a single cleavage at the Asp-Pro sequence (band 5). To the best of our knowledge this is the first report of efficient acid induced cleavage at Asp-Cys sequences.

On the basis of these observations we concluded that the acid cleavage with 0.1 M acetic acid pH 2.0 of ONC-(P)GKY20 fusion protein could not be used to obtain pure (P)GKY20 peptide. Thus, we decided to try to improve the carrier sequence (i) to overcome the undesired cleavages we have observed, and (ii) to avoid the formation of the short fragments derived from the hydrolysis of contaminant *E. coli* proteins.

Improvement of the carrier sequence: first generation mutants

Improvement of the Onconase carrier was performed by a trial and error procedure. For simplicity in Table 1 several different versions of the carrier are divided into "first generation" and "second generation" fusion proteins.

The mutations introduced into the first generation proteins were chosen mainly to remove the unexpected Asp-Cys cleavage sites and to obtain a high purity fusion protein in order to reduce the release of unwanted peptides during the acid cleavage step from contaminant *E. coli* proteins.

As for unexpected Asp-Cys cleavage sites, sequences Asp-Cys were mutated to Asp-Tyr and Glu-Tyr. Mutations were chosen to preserve the propensity of denatured ONC to form inclusion bodies and to aggregate at pH 7. Since protein solubility and propensity to form aggregates, like inclusion bodies, are generally related to net charge, hydrophobicity and to the presence of amphipatic secondary structures [35, 36], the amino acid substitutions were chosen in order to preserve these properties. Therefore, aspartic acid residues were replaced with glutamic acid residues in order to preserve the net charge. Cysteine residues were replaced with tyrosine residues as in several hydrophobicity scales these two residues show very similar hydrophobicity scores [37]. Moreover, the replacement of Asp-Cys with Glu-Tyr allows to keep unchanged secondary structure propensity since the replacement of aspartate with glutamate decreases the preference for loop structures, whereas the replacement of cysteine with tyrosine increases the preference for loop structures. Furthermore, the mutant bearing the two Glu-Tyr sequences was also prepared with a six histidine-tag sequence (H6) inserted between the ONC moiety and the flexible linker GTGDP (Fig 1B).

All the recombinant fusion proteins showed high expression levels as inclusion bodies, thus indicating that the chosen mutations did not interfere with the formation of inclusion bodies (Fig 4).

When Asp-Cys sequences were changed to Asp-Tyr [ONC(YY)-(P)GKY20 fusion protein] or to Glu-Tyr [ONC(EYEY)-(P)GKY20 fusion protein] a drastic reduction of unwanted acid cleavage in 0.1 M acetic acid pH 2.0 at 60°C for 24 h was observed (Fig 5A and 5B, respectively). The SDS-PAGE analysis of the cleavage products of ONC(YY)-(P)GKY20 and ONC

Table 1. Expression vectors, fusion proteins and peptides.

Expression vectors	Fusion proteins	Linker and Cleavage site	Peptides	Peptide (N-terminal end)
First generation fusion proteins				
pET22b(+)/ONC-(P)GKY20	ONC-(P)GKY20[a]	GTG**DP** *GKY*..	(P)GKY20	P-GKY...
pET22b(+)/ONC(YY)-(P)GKY20	ONC(YY)-(P)GKY20[b]	GTG**DP** *GKY*..	(P)GKY20	P-GKY...
pET22b(+)/ONC(EYEY)-(P)GKY20	ONC(EYEY)-(P)GKY20[c]	GTG**DP** *GKY*..	(P)GKY20	P-GKY...
pET22b(+)/ONC(EYEY)-H6-(P)GKY20	ONC(EYEY)-H6-(P)GKY20[d]	GTG**DP** *GKY*..	(P)GKY20	P-GKY...
Second generation fusion proteins				
pET22b(+)/ONC-DCless-H6-(P)GKY20	ONC-DCless-H6-(P)GKY20[e]	GTG**DP** *GKY*..	(P)GKY20	P-GKY...
pET22b(+)/ONC-DCless-H6-(PM)GKY20	ONC-DCless-H6-(PM)GKY20[e]	GTG**DP**M *GKY*..	(PM)GKY20	PM-GKY...
-	-	-	GKY20	GKY...

[a] ONC: Onconase mutant-M23L,C104 delete;

[b] ONC(YY): Onconase mutant-M23L,C19Y,C68Y,C104 delete;

[c] ONC(EYEY): Onconase mutant-M23L,D18E,C19Y,D67E,C68Y,C104 delete;

[d] ONC(EYEY)-H6: Onconase mutant-M23L,D18E,C19Y,D67E,C68Y,C104 delete; H6: Hexa-histidine tag at the C-terminal side of the onconase carrier.

[e] ONC-DCless-H6: Onconase mutant-M23L,D2E,D16E,D18E,C19Y,D20E,C30Y,D32E,C48L,D67E,C68Y,C75Y,C87I,C90I,C104 delete; H6: Hexa-histidine tag at the C-terminal side of the onconase carrier.

doi:10.1371/journal.pone.0146552.t001

(EYEY)-(P)GKY20 was similar but not identical. In particular only ONC(YY)-(P)GKY20 reproducibly showed a smear corresponding to bands 2 and 3 in the analysis of ONC-(P)GKY20 (Fig 5A), thus suggesting that only mutation Asp-Cys to Glu-Tyr abolished the cleavage at the bond Asp67-Cys68. On the contrary both mutants showed two close bands (Fig 5A and 5B, respectively) whose migration is similar to that of band 4, attributed to the cleavage at the bond Asp18-Cys19. As mentioned in the previous section the disappearance of high molecular weight bands suggested the release of unwanted peptides produced during the acid cleavage step from contaminant *E. coli* proteins (Fig 5B). Therefore, we decided to increase the purity of the protein sample subjected to acid hydrolysis by the addition of a H6 tag sequence to the chimeric proteins. ONC(EYEY)-H6-(P)GKY20 mutant was produced and purified as

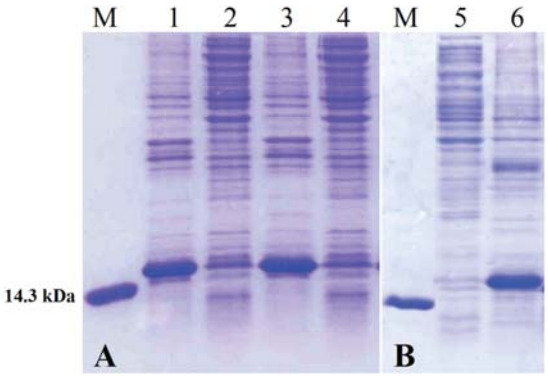

Fig 4. Protein expression of first generation mutants. (A) SDS-PAGE analysis of insoluble (lanes 1, 3) and soluble (lanes 2, 4) fractions after cell lysis of ONC(YY)-(P)GKY20 and ONC(EYEY)-(P)GKY20 fusion proteins, respectively. (B) SDS-PAGE analysis of soluble (lane 5) and insoluble (lane 6) fractions after cell lysis of ONC(EYEY)-H6-(P)GKY20 fusion protein. Lane M: marker (14.3 kDa).

doi:10.1371/journal.pone.0146552.g004

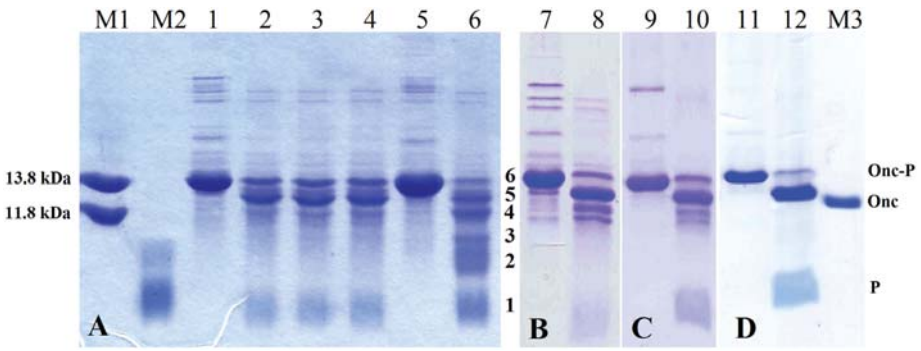

Fig 5. Comparison of hydrolysis patterns of first and second generation mutants. Chemical cleavage was carried out in 0.1 M acetic acid adjusted at pH 2.0 with HCl (60°C, 24 h). Samples were analyzed on 20% SDS-PAGE. Lane M1: markers (13.8 kDa, 11.8 kDa). Lane M2: RK-16 peptide (1961.25 Da). Lane M3: marker (14.3 kDa). (A) ONC(YY)-(P)GKY20 purified fusion protein (lane 1) and hydrolyzed protein (lanes 2–4, three independent experiments). ONC-(P)GKY20 purified protein (lane 5) and cleaved protein (lane 6). (B, C, D) ONC(EYEY)-(P)GKY20, ONC(EYEY)-H6-(P)GKY20 and ONC-DCless-H6-(P)GKY20 purified fusion proteins (lanes 7, 9, 11, respectively), and hydrolyzed proteins (lane 8, 10, 12, respectively). Onc-P: Onconase/Peptide fusion protein; Onc: Onconase carrier; P: (P)GKY20 peptide. Fragments obtained by hydrolysis of ONC-(P)GKY20 fusion protein (B, lane 6) are numbered as in Fig 3.

doi:10.1371/journal.pone.0146552.g005

described in Materials and Methods. As it is shown in Fig 5, the addition of the H6 tag significantly improved the purity of the chimeric protein from about 80% (Fig 5B) to more than 96–98% as determined by SDS-PAGE densitometry scan (Fig 5C), with a recovery of about 95% of the total fusion protein. Purified ONC(EYEY)-H6-(P)GKY20 was subjected to acid cleavage in 0.1 M acetic acid pH 2.0 at 60°C for 24 h as described above. SDS-PAGE analysis of ONC (EYEY)-H6-(P)GKY20 acid cleavage mixtures (Fig 5C) still evidenced the presence of the two faint bands observed in the case of ONC(EYEY)-(P)GKY20 thus demonstrating that these bands derive from cleavage of the fusion proteins and not from the contaminants. Taken together, our data suggested that, in addition to the bonds Asp18-Cys19 and Asp67-Cys68, further acid labile bonds are present in Onconase. Studies on protein and peptide aging have demonstrated that all Asp-X bonds can undergo spontaneous hydrolysis, even if at a lesser extent than the Asp-Pro bond [38–40]. For example, Joshi and co-workers [39, 40] demonstrated that at acidic pH glucagon undergoes degradation due to hydrolysis at the Asp9-Tyr10 and Asp15--Ser16 bonds and at a lesser extent at the Asp21-Phe22 bond. These findings are in agreement with our conclusion that Asp67-Tyr68 in ONC(YY)-(P)GKY20 fusion protein contributes to the release of ONC derived fragments and suggest that one or more of the additional Asp-X sequences present in the ONC moiety (Asp2-Trp3, Asp16-Val17, Asp20-Asn21 and Asp30-Lys31) could be sensitive to acid hydrolysis. In particular one or both the two close bands observed in the hydrolysis products of ONC(YY)-(P)GKY20 and ONC(EYEY)-(P) GKY20 fusion protein could be tentatively attributed to cleavage at the Asp16-Val17 and/or Asp20-Asn21 and/or Asp-30-Lys31 bonds.

Improvement of the carrier sequence: second generation mutants

Mutations introduced into the second generation protein ONC-DCless-H6-(P)GKY20 were chosen to remove all the potential unwanted cleavage sites and to reduce the possibility of unwanted formation of intra-chain disulfides without changing the expression level of the fusion protein, its propensity to form inclusion bodies in *E. coli* and the propensity of denatured Onconase to form large aggregates at pH higher than 5 as this feature is very useful to

remove the carrier after the acid cleavage by means of a simple centrifugation step (as described below). To this aim, as discussed for the first generation mutants, the four remaining aspartic acid residues in the ONC moiety were replaced with glutamic acid residues to preserve the charge and all the cysteine residues were mutated to other residues of similar or higher hydrophobicity chosen on the basis of the secondary structure of native ONC. Accordingly, the single cysteine residue (Cys48) present within alpha helix 3 (S2 File) was replaced with a leucine residue (a "helix-preferring" residue). The two cysteine residues located in beta strands (Cys87 and Cys90) were replaced with isoleucine (a "beta-preferring" residue). Finally, two cysteine residues located in loops (Cys30 and Cys75) were replaced with tyrosine residues. It should be noted that the mutation of cysteine to leucine or isoleucine increases significantly the hydrophobicity and, likely, the propensity of the protein to aggregate.

ONC-DCless-H6-(P)GKY20 (Table 1), was produced in the form of inclusion bodies with a yield similar or higher than that observed for ONC-(P)GKY20 (Fig 2), thus demonstrating that the amino acid substitutions into the carrier sequence did not influence protein expression levels. After purification on Ni-sepharose, ONC-DCless-H6-(P)GKY20 showed about 98% purity as determined by SDS-PAGE densitometry scan (Fig 5D), with a recovery of about 95%.

After cleavage in 0.1 M acetic acid adjusted at pH 2.0, at 60°C for 24 h, SDS-PAGE analysis allowed to estimate 95% efficiency in the release of (P)GKY20. It is worth noting that no unwanted cleavage was observed, thus confirming that aspartic acid residues were responsible for the low efficiency cleavages observed in the case of ONC(EYEY)-H6-(P)GKY20 protein (Fig 5D).

Adjusting the pH to 7.2–7.4 with NH_3 and incubating the mixture at 28°C for 16 h the uncleaved ONC-DCless-H6-(P)GKY20 and ONC-DCless-H6 proteins formed macroscopic insoluble aggregates which were completely removed by centrifugation (Fig 6). Peptide (P)GKY20 was recovered in the soluble fraction, lyophilized and used for the characterization described below without any further purification step. Mass spectrometry analysis (Fig 7A), confirmed the peptide identity and showed that no modified form (e.g. oxidized or formylated peptides) was present in the sample.

Final purity of (P)GKY20 peptide, as determined by reverse phase chromatography (Fig 7B), typically ranged from 95% to 99%.

The optimized procedure allowed the purification of about 10–11 mg of peptide starting from 100 mg of purified fusion protein with 70–75% recovery efficiency (actual milligrams of peptide/expected milligrams of peptide) with respect to the theoretical amount of peptide in the fusion protein.

Cleavage by cyanogen bromide of ONC-DCless-H6-(PM)GKY20 fusion protein

Starting from ONC-DCless-H6-(P)GKY20 we prepared mutant ONC-DCless-H6-(PM)GKY20. In this fusion protein the additional mutation, a single amino acid insertion, is located in the peptide moiety rather than in the ONC moiety which is identical to that of ONC-DCless-H6-(P)GKY20. By acid hydrolysis it releases peptide (PM)GKY20 carrying two additional residues at the N-terminus. However, as ONC-DCless-H6 does not contain methionine residues, ONC-DCless-H6-(PM)GKY20 fusion protein can also be cleaved using CNBr to release the peptide GKY20 which does not have any additional residue at the N-terminus.

By acid cleavage ONC-DCless-H6-(PM)GKY20 provided results identical to those obtained in the case of ONC-DCless-H6-(P)GKY20 (Fig 8), thus showing that the additional methionine residue following the Asp-Pro sequence has no influence on the acid cleavage efficiency.

ONC-DCless-H6-(PM)GKY20 fusion protein was also cleaved by using CNBr in 0.2 M HCl as described in details in the Material and Methods section. As shown in Fig 9 the efficiency of

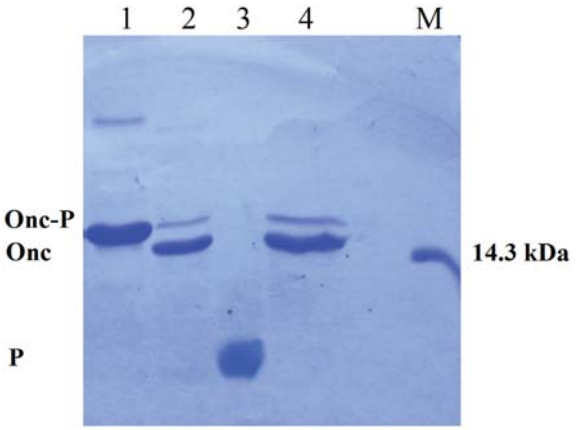

Fig 6. Purification of recombinant (P)GKY20 peptide. DS-PAGE (20%) analysis. Lane M: marker (14.3 kDa). Lane 1: ONC-DCless-H6-(P)GKY20 fusion protein purified by IMAC. Lane 2: cleaved fusion protein (0.1 M acetic acid adjusted at pH 2.0 with HCl, 60°C, 24 h). Lane 3: soluble fraction after neutralization of the cleavage reaction [(P)GKY20 peptide]. Lane 4: insoluble fraction after neutralization of the cleavage reaction (uncleaved fusion protein and Onconase cleaved carrier). Onc-P: Onconase/Peptide fusion protein; Onc: Onconase carrier; P: (P)GKY20 peptide.

doi:10.1371/journal.pone.0146552.g006

cleavage (about 80%, as determined by SDS-PAGE densitometry scan) was only slightly lower than that obtained with acid hydrolysis, thus demonstrating that ONC-DCless-H6-(PM) GKY20 fusion protein can be effectively cleaved by both chemical methods.

Antimicrobial activity

In order to verify if recombinant peptide (P)GKY20 possesses antimicrobial activity comparable to that of a synthetic GKY20, both peptides were tested by plate viable-count assay on a Gram-positive strain, *Staphylococcus aureus* ATCC 6538P, and a Gram-negative strain, *Pseudomonas aeruginosa* KK27 [a clinical strain isolated from a cystic fibrosis patient kindly provided by Dr. Alessandra Bragonzi (San Raffaele Hospital, Milan)]. As shown in Fig 10, the antimicrobial activity of (P)GKY20 was identical to that measured for synthetic GKY20.

Moreover, to further investigate if N-terminal proline of (P)GKY20 affected the potency of the peptide, we determined, by broth microdilution method, the MIC values (the lowest concentration of antimicrobial agent at which no growth is observed) of the two peptides. As shown in Table 2 and Fig 11, peptides exhibited the same MIC values towards several Gram-positive and Gram-negative strains. These data are very close to those determined against *E. coli* and *S. aureus* strains for synthetic C-terminal human thrombin peptides [33]. Taken together, these findings suggest that N-terminal proline residue does not change the antimicrobial properties of recombinant (P)GKY20.

Conclusions

Through a rational design strategy we have progressively improved the suitability of ONC as a carrier for the production of recombinant peptides. The final version of the protein, ONC-DCless-H6, may likely be a very efficient carrier particularly suited for the production of peptides expected to be very toxic for the host *E. coli* like antimicrobial peptides. It is expressed at high levels (>200 mg/L) efficiently forming inclusion bodies, and thus sequestering the hosted

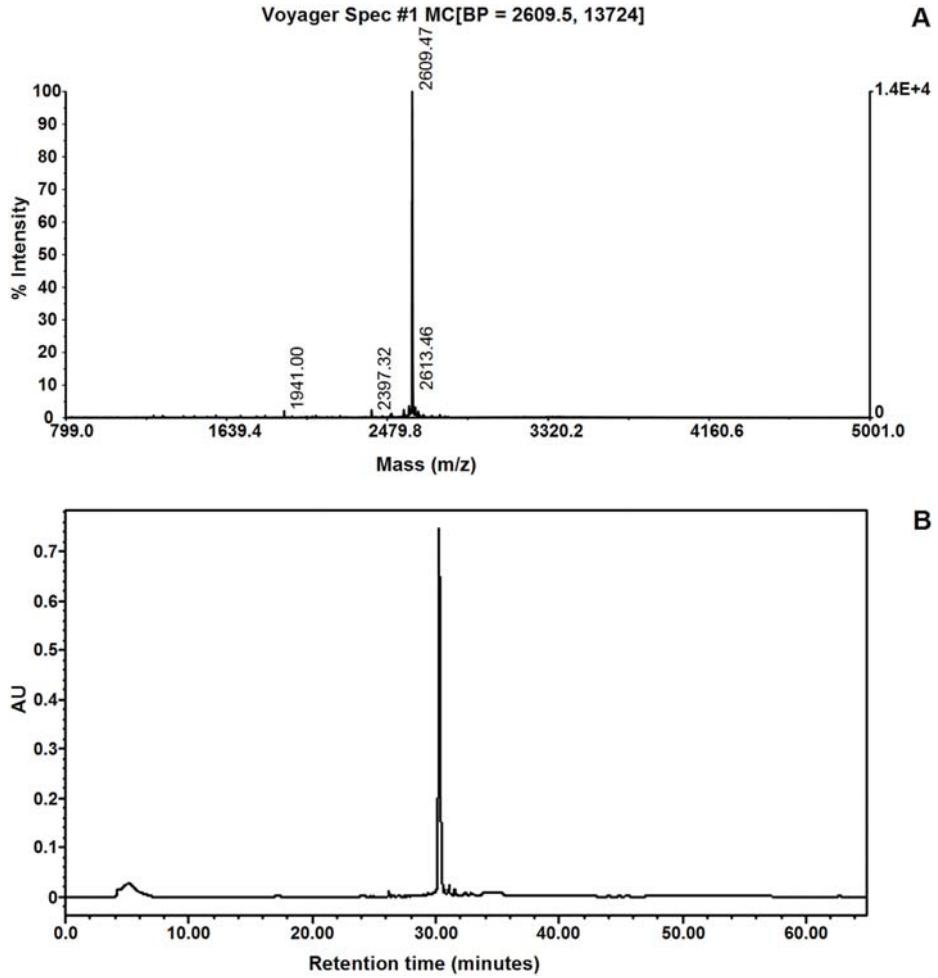

Fig 7. Characterization of purified recombinant (P)GKY20 peptide. (A) Mass spectrum of purified (P)GKY20 peptide. The measured molecular weight (2609.47 Da) is consistent with the theoretical value (2609.1 Da). (B) Reverse-phase HPLC chromatogram recorded at 280 nm wavelength. Purified peptide was applied to a C18 column (Jupiter 5u C18 300Å, 250 x 4.6 mm) and eluted with a linear gradient from 5% to 95% acetonitrile containing 0.05% trifluoroacetic acid, over 60 min at flow rate of 1 mL/min.

doi:10.1371/journal.pone.0146552.g007

peptide in an insoluble form. Once solubilized by chaotropic agents the chimeric protein can be easily purified by Ni-chelate chromatography without the need of removing the denaturing agent. At pH lower than 4 denatured ONC is very soluble, therefore chemical cleavage procedures that require an acidic environment (e.g. cleavage of aspartyl-prolyl bonds and CNBr cleavage) can be performed also in diluted acids such as 0.1 M acetic acid or HCl without the need to employ high concentrations of formic acid that is often chosen for its denturing/solubilizing properties [28, 41] but, as already mentioned, causes several unwanted reactions. After the cleavage at acidic pH, the ONC moiety, ONC-DCless-H6, can be easily removed due to its

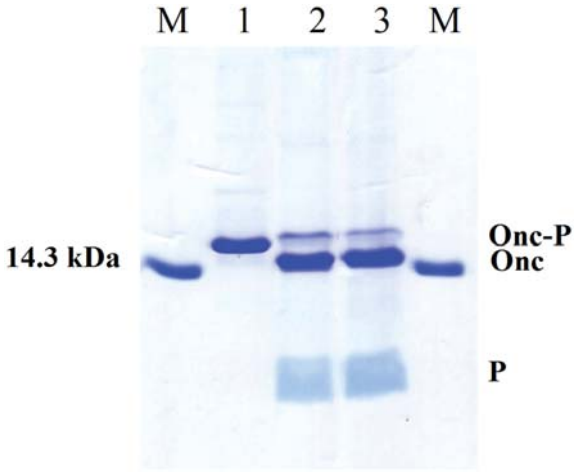

Fig 8. Asp-Pro cleavage efficiency comparison of ONC-DCless-H6-(P)GKY20 and ONC-DCless-H6-(PM)GKY20 fusion proteins. Samples were cleaved in 0.1 M acetic acid adjusted at pH 2.0 with HCl (60°C, 24 h) and analyzed on 20% SDS-PAGE. Lane M: marker (14.3 kDa). Lane 1: ONC-DCless-H6-(PM)GKY20 purified fusion protein. Lanes 2, 3: ONC-DCless-H6-(PM)GKY20 (lane 2) and ONC-DCless-H6-(P)GKY20 (lane 3) hydrolyzed proteins. Onc-P: Onconase/Peptide fusion protein; Onc: Onconase carrier; P: (P)GKY20 peptide.

doi:10.1371/journal.pone.0146552.g008

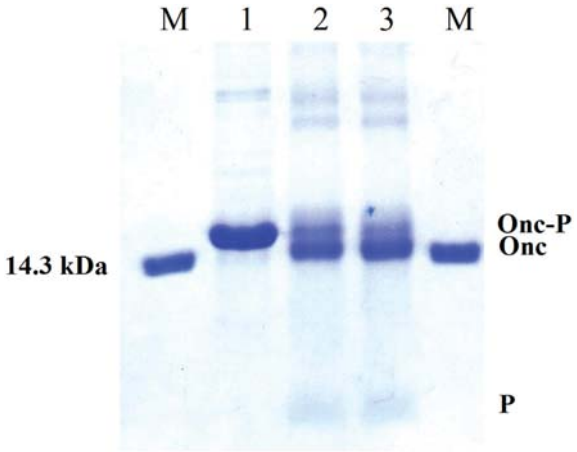

Fig 9. Cyanogen bromide cleavage of ONC-DCless-H6-(PM)GKY20 fusion protein. Samples were analyzed on 20% SDS-PAGE. Lane M: marker (14.3 kDa). Lane 1, purified fusion protein. Lanes 2, 3: cleaved recombinant protein in 0.2 M HCl with 100- (lane 2) and 400-fold (lane 3) molar excess of CNBr over methionine residues. Sample were incubated in the dark at room temperature for 24 h. Onc-P: Onconase/Peptide fusion protein; Onc: Onconase carrier; P: (P)GKY20 peptide.

doi:10.1371/journal.pone.0146552.g009

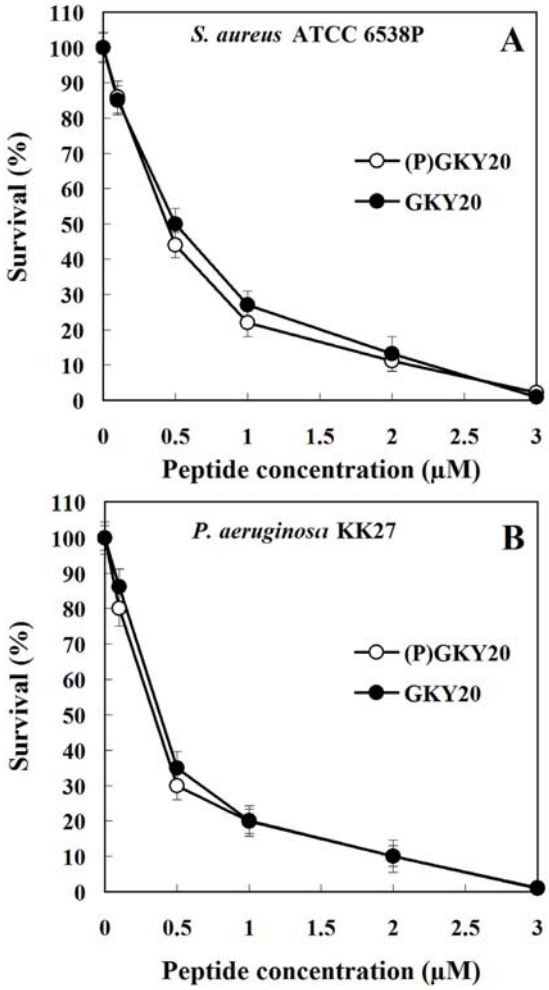

Fig 10. Antibacterial activity of recombinant (P)GKY20 and synthetic GKY20 peptides. Assays were carried out by viable-count method. (A) *Staphylococcus aureus* ATCC 6538P; (B) *Pseudomonas aeruginosa* KK27. Dose-effect curves: 0.1–0.5-1-2-3 μM final concentrations were tested. Error bars are standard deviations. Black circles: synthetic GKY20. White circles: (P)GKY20.

doi:10.1371/journal.pone.0146552.g010

complete insolubility at neutral pH. This feature may allow a simple and effective purification of any peptide that is soluble at pH 7.0. Alternatively, the carrier could be removed under denaturing conditions by Ni-chelate chromatography as after acidic cleavage it retains the H6 tag. Table 3 shows a comparison between our method and those reported in some recent papers describing the production of recombinant antimicrobial peptides.

As for the cleavage of the hosted peptide, ONC-DCless-H6 is very versatile. As it does not contain methionine residues nor Asp-Pro sequences or any other acid sensitive sequence,

Table 2. Antibacterial activity of Thrombin derived peptides.

	MIC (μM)[a]					
	(P)GKY20			GKY20		
Bacterial strain	Replicates[b]			Replicates[b]		
***E. coli* DH5α**	12.5	12.5	12.5	12.5	12.5	12.5
***E. coli* ATCC 25922**	12.5	12.5	25	12.5	12.5	25
***E. coli* ATCC 35218**	12.5	12.5	12.5	12.5	12.5	12.5
***P. aeruginosa* PAO1**	25	12.5	25	25	12.5	25
***P. aeruginosa* PA14**	50	50	25	50	50	25
***S. aureus* ATCC 6538P**	6.25	3.12	6.25	6.25	3.12	6.25
***K. pneumoniae* ATCC 700603**	12.5	12.5	12.5	12.5	12.5	12.5
***B. megaterium* SF185**	12.5	12.5	12.5	12.5	12.5	12.5

[a]Assays were carried out by broth dilution method in Nutrient Broth 0,5 X.
[b]Replicates were from three independent experiments.

doi:10.1371/journal.pone.0146552.t002

hosted peptides may be released either by CNBr or by acidic cleavage. Furthermore, as the ONC scaffold does not contain any cysteine residues, peptides may also be released by using 2-nitro-5-thiocyanatobenzoic acid, a reagent that very specifically modify thiols causing the cleavage of X-Cys bonds [20, 28], by replacing the DP or DPM sequences at the end of the flexible linker with a cysteine.

Our strategy, due to its simplicity and limited costs, is also suited for the scale up and could thus be useful for medium/large scale production of peptides needed, for example, in the clinical trial phases.

Materials and Methods

Materials

Expression host strain *E. coli* BL21(DE3) and plasmid pET22b(+) were purchased from Novagen (San Diego, CA, USA). *E. coli* strain TOP10F' was obtained from Invitrogen (San Diego, CA, USA). QIAprep spin miniprep kit was from Qiagen (Germantown, MD, USA). Wizard SV Gel and PCR Clean-Up DNA Purification System for elution of DNA fragments from agarose gel was purchased from Promega (Madison, WI, USA). Enzymes and other reagents for DNA manipulation were from New England Biolabs (Ipswich, MA, USA). Ni Sepharose™ 6 Fast Flow was from GE Healthcare (Uppsala, Sweden). The GKY20 peptide was chemically synthesized by INBIOS s.r.l. (University of Naples, Italy). Difco Nutrient Broth was from Becton-Dickenson (Franklin Lakes, NJ). All other chemicals were from Sigma-Aldrich (Milano, Italy).

General procedures

Bacterial cultures, plasmid purifications and DNA manipulation were carried out according to Sambrook [42]. DNA sequences and oligonucleotide synthesis were performed by Eurofins MWG Operon service (Ebersberg, Germany). Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to Laemmli [43]. *Gallus gallus* lysozyme (14.3 kDa), human pancreatic RNase A (13.8 kDa), recombinant Onconase from *Rana pipiens* (11.8 kDa) and RK-16 peptide from *Salmo salar* Ss-RNasi 1 [44] (16 aa: RYPHCRYRGSPPSTRK; theoretical molecular weight, 1961.25 Da) were used as molecular markers. When appropriate, the relative amount of bands was determined by densitometry performed

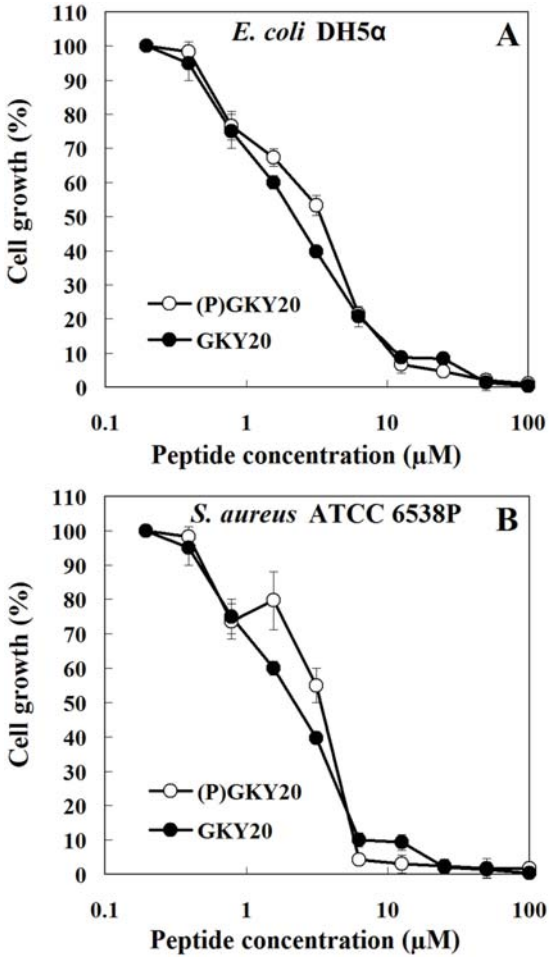

Fig 11. Inhibition growth assay of *E. coli* DH5α (A) and *S. aureus* ATCC 6538P (B). Assays were carried out by broth dilution method in Nutrient Broth 0.5x. Bacteria from overnight culture were inoculated (~$5x10^5$ CFU/mL) in the presence of different concentration of tested peptides (0.2–100 μM). After incubation at 37°C for 16 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration showing no visible growth. All measurements were carried out three times in independent experiments.

doi:10.1371/journal.pone.0146552.g011

using the Gel Doc™ XR system (Bio-Rad Laboratories, Inc.) equipped with the Quantity One® Software. Protein concentration was determined by the Bradford Protein Assay (Sigma-Aldrich, St. Louis, MO, USA) and standard curves were generated using bovine serum albumin (BSA). Concentrations of purified fusion proteins and peptides were determined by spectrophotometric analysis using the extinction coefficients calculated using the ProtParam tool (accessible to the address http://web.expasy.org/protparam/) [45]. Amino-terminal sequencing was carried out on polypeptides separated by denaturing gel electrophoresis and then electroblotted onto polyvinylidene difluoride (PVDF) membranes [46].

Table 3. Recently published papers reporting the production of recombinant antimicrobial peptides in *Escherichia coli* (*E.c.*) or *Bacillus subtilis* (*B. s.*).

Reference	Host	Carrier[a]	number of peptides	Carrier features	Purification of fusion proteins[i]	Cleavage method (efficiency)	Peptide Purification[i]	Peptide yield	Peptide purity
This paper	*E.c.*	ONC-DCless-H6	2[b]	Inclusion Bodies forming/pH dependent solubility	Inclusion Bodies isolation & I.M.A.C.	Diluted acid at 60°C for 18 h (>95%) or CNBr (~80%)[j]	Selective precipitation	~ 15 to 18 mg/L (~ 13 to 16 mg/g of dry cells)	>95%
[51]	*E.c.*	DAMP4var	2[c]	Highly soluble artificial thermostable protein	Two selective precipitation steps	Diluted acid at 60°C for 48 h (high)	Selective precipitation	Not reported	Not reported[m]
[52]	*E.c.*	His tagged ketosteroid isomerase	2[d]	Inclusion Bodies forming	IB isolation & I.M.A.C.	thrombin (80%-95%)[k]	I.M.A.C.	30 mg/g of dry cells	>95%
[53]	*E.c.*	His tagged SUMO[a]	1[e]	Soluble	I.M.A.C.	SUMO protease (complete)	I.M.A.C.	Not reported	>95%
[54]	*B.s.*	His tagged SUMO[a]	1[f]	Soluble	I.M.A.C.	SUMO protease cleavage	I.M.A.C.	5.5 mg/L (0.16 mg/g of wet cells[l])	>94%
[55]	*E.c.*	Chitin binding domain/Intein fusion[a]	1[g]	Soluble/chitin binding/auto-proteolytic	Adsorption to chitin beads (at 4°C)	auto-proteolysis at 25°C for 16 h (~50%)	RP-HPLC	1.7 mg/L	Not reported
[56]	*B.s.*	His tagged thioredoxin[a]	1[h]	Soluble/Secreted	selective precipitation/I.M.A.C	enterokinase (complete)	I.M.A.C./ Cation exchange	2 mg/L	~ 92%

[a]His tagged ketosteroid isomerase, SUMO, CBD/Intein and thioredoxin-based methods are commercially available; here we report the most recent applications based on these carriers.

[b]Sequences of peptides (P)GKY20 and (PM)GKY20 are in S1 File.

[c]PS-Pexiganan-HH (PS-Pex-HH; PS-GIGKFLKKAKKFGKAFVKILKK-HH) and omiganan (GILRWPWWPWRRK-HHHHHH). Omiganan was expressed as fusion protein but not further analysed.

[d]p53pAnt (GSRAHSSHLKSKKGQSTSRHKKWKMRRNQFWVKVQRG) and PNC27 (PPLSQETFSDLWKLLKKWKMRRNQFWVKVQRG).

[e]Scorpine (GWINEEKIQKKIDERMGNTVLGGMAKAIVHKMAKNEFQCMANMDMLGNCEKHCQTSGEKGYCHGTKCKCGTPLSY).

[f]Plectasin (GFGCNGPWDEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCY).

[g]P11-5 (GKLFKKILKIL)

[h]PR39, (RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP).

[i]Dialysis and lyophilization steps are not indicated.

[j]Measured only for ONC-DCless-H6-(PM)GKY20.

[k]Only peptide p53pAnt could be cleaved by thrombin. 80% cleavage efficiency was obtained by incubating for 48h the fusion protein with thrombin, whereas, 95% efficiency was reached by addition of fresh enzyme and further incubation.

[l]Calculated from data in figure 3 of reference 4.

[m]Authors state that after the selective precipitation "...most of the DAMP4var was removed by centrifugation (Fig 7A, lane 2), and relatively pure PS-Pex-HH was obtained for MIC testing (Fig 7A, lane 3 and Fig 7B)".

doi:10.1371/journal.pone.0146552.t003

Construction of the expression vectors

DNA sequences coding for fusion proteins reported in Table 1, were obtained by chemical synthesis (MWG-Biotech AG; Ebersberg, Germany) or by PCR mutagenesis.

Synthetic genes coding for recombinant proteins ONC-(P)GKY20, ONC-DCless-H6-(P)GKY20 and ONC-DCless-H6-(PM)GKY20 (Table 1) were obtained by MWG-Biotech AG (Ebersberg, Germany). All codons were optimized for expression in *E. coli* and restriction sites

NdeI and *SacI* were introduced at 5'- and 3'-end of the synthetic genes, respectively, for their cloning into pET22b(+) vector (Fig 1; S1 File).

All other DNA sequences coding for mutants reported in Table 1 were obtained by Overlap Extension PCR Mutagenesis or by PCR Mutagenesis [47] using the appropriate primers and *pET22b(+)/ONC-(P)GKY20* or *pET22b(+)/ONC(EYEY)-(P)GKY20* plasmids as templates. PCR reactions were carried out using the Phusion High Fidelity DNA Polymerase (Thermo Fisher, New England Biolabs). PCR reactions were started by preheating samples at 94°C for 2 min. Amplifications were performed as follows: 20 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, with a final elongation stage at 72°C for 10 min. Amplified products were digested with the appropriate restriction enzymes for their cloning into the pET22b(+) vector. *E. coli* strain TOP10F' was used for all cloning procedures.

Expression of recombinant proteins

E. coli strain BL21(DE3) was used to express recombinant proteins. Cells, transformed with pET recombinant plasmids, were grown in 10 mL of Terrific Broth medium (TB) containing 100 μg/ml ampicillin, at 37°C up to OD_{600nm} of 2. These cultures were used to inoculate 1 L of TB/ampicillin medium. Glucose at final concentration of 4 g/L was added to cultures to limit protein expression before induction with IPTG. TB medium was found to be able to induce protein expression over the early growth phase reducing the final optical density of cultures and therefore the recombinant protein yield [48]. Cultures were incubated at 37°C up to OD_{600nm} of 3.5–4. Expression of recombinant proteins was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.4 mM. Cells were harvested after overnight induction by centrifugation at 8000x *g* for 15 min at 4°C and washed with 50 mM Tris-HCl buffer, pH 7.4. The bacterial pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid (EDTA), and sonicated in a cell disruptor (10 x 1 min cycle, on ice). The suspension was then centrifuged at 18,000 x *g* for 60 min at 4°C. Soluble and insoluble fractions were analyzed by SDS-PAGE. The insoluble fractions containing the recombinant protein as inclusion bodies, were washed three times in 0.1 M Tris-HCl buffer, pH 7.4, containing 10 mM EDTA, 2% Triton X-100 and 2 M urea, followed by repeated washes in 0.1 M Tris-HCl buffer, pH 7.4, to eliminate traces of Triton, urea and EDTA. This procedure eliminated several contaminant proteins and cellular debris entrapped in inclusion body pellets.

Purification of fusion proteins

To purify fusion proteins lacking His_6-Tag, a procedure was developed to obtain partially purified protein without any chromatographic step. To this purpose, 100 mg of fusion proteins were dissolved in 10 mL of denaturing buffer (6 M guanidine/HCl in 50 mM Tris-HCl, pH 7.4) containing 10 mM β-mercaptoethanol. Mixtures were incubated at 37°C for 3 h under nitrogen atmosphere on a rotary shaker, and then centrifuged at 18,000 x *g* for 60 min at 4°C. Soluble fractions were collected by centrifugation and acidified to pH 3.0 by adding glacial acetic acid before extensive dialysis against 0.1 M acetic acid at 4°C. Soluble fusion proteins were separated from insoluble *E. coli* proteins by centrifugation and stored at -80°C under nitrogen atmosphere.

His_6-tagged recombinant proteins were purified by immobilized metal ion affinity chromatography (IMAC), using the Ni Sepharose™ 6 Fast Flow resin. 100 mg of fusion proteins were dissolved in 10 mL of denaturing buffer containing 10 mM β-mercaptoethanol and incubated on a rotary shaker at 37°C for 3 h under nitrogen atmosphere. Soluble fractions were collected by centrifugation and incubated with 5 mL of Ni Sepharose™ 6 Fast Flow resin equilibrated in

denaturing buffer. The resin was shaken at 4°C for 16 h and then collected by centrifugation. The supernatant, containing the unbound proteins, was discarded. The resin was washed three times with 25 ml of denaturing buffer at 4°C for 30 min and then packed in a glass column. The fusion proteins were eluted with 20 ml of 0.1 M sodium acetate buffer, pH 5.0, containing 6 M guanidine/HCl (elution buffer). The eluate was extensively dialyzed against 0.1 M acetic acid at 4°C. Samples were stored at -80°C under nitrogen atmosphere. Purified fusion protein concentrations were determined by spectrophotometric analysis using the extinction coefficients calculated using the ProtParam tool [ε_{280} = 24,410 M^{-1} cm^{-1}; ε_{280} (0.1%) = 1.53].

Cleavage optimization of Asp-Pro peptide bond

Fusion proteins were cleaved by thermal incubation in acid solutions under nitrogen atmosphere. To optimize chemical cleavage, formic acid and acetic acid, were tested. Purified fusion proteins in 0.1 M acetic acid were lyophilized and suspended in i) 50%, 60% and 70% formic acid or ii) 0.1 M acetic acid at pH 2.9 or at pH 2.0 (by addition of diluted HCl). The mixtures were incubated for 3-6-9-15-24 h, at 37°C, 50°C and 60°C. Cleavage efficiency was analyzed by SDS-PAGE using 20% gel. Formaldehyde was used to covalently link proteins and polypeptides in polyacrylamide gels [49]. To this purpose, 4% formaldehyde was added to the Coomassie Blue staining solution (0.2% Coomassie Brilliant Blue R-250, 25% isopropyl alcohol and 10% glacial acetic acid). Percentage of cleaved protein was determined by densitometric analysis of SDS-PAGE gels as described in "General procedures".

Identification of polypeptides obtained by chemical cleavage was carried out by N-terminal sequencing. Proteins and polypeptides were separated by denaturing gel electrophoresis, electroblotted onto polyvinylidene difluoride (PVDF) membranes, stained by Ponceau S dye and sequenced by the Edman method [46].

Chemical cleavage and purification of recombinant (P)GKY20 peptide

Cleavage of the peptide from fusion proteins was performed in 0.1 M acetic acid at pH 2.0, incubating samples at 60°C for 24 h in a water bath under nitrogen atmosphere. The mixtures were then neutralized to pH 7–7.2 by adding NH_3, purged with N_2 and incubated at 28°C for 16 h in a water bath. The soluble peptide was isolated from the insoluble carrier through repeated cycles of centrifugation at 18,000 x g for 60 min at 4°C. Purified peptide was lyophilized and stored at -80°C. Peptide concentrations were determined by spectrophotometric analysis using the extinction coefficients calculated using the ProtParam tool [ε_{280} = 8,480 M^{-1} cm^{-1}; recombinant (P)GKY20 peptide, ε_{280} (0.1%) = 3.250; GKY20 peptide, ε_{280} (0.1%) = 3.376].

The purity of (P)GKY20 peptide was verified by SDS-PAGE and reverse-phase HPLC using a Waters (Milford, MA, USA) HPLC system (1525 binary pump and 2996 photodiode array detector). The column was a C18 (250 x 4,6 mm, 5μm particle size) Jupiter 5u C18 300 Å (Phenomenex). The solvents were 0.05% trifluoracetic acid (TFA) in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). The column was equilibrated with 5% acetonitrile and the peptide was eluted by a linear gradient ranging from 5% to 95% solvent B over 60 min, at a flow rate of 1 ml/min. The elution was monitored at 280 nm. Purity was determined by measuring the relative area of peaks using the Empower software (Waters, Milford, MA, USA).

The identity of the peptide was determined by mass spectrometry. Positive Reflectron MALDI spectra were recorded on a Voyager DE STR instrument (AB Sciex, Framingham, MA). The MALDI matrix was prepared by dissolving α-cyano-hydroxycinnamic powder in 70% acetonitrile and 30% citric acid 50 mM. Typically 1 μL of matrix was applied to the metallic sample plate and 1 μL of analyte was added. The mixture thus obtained was then dried at room temperature. Acceleration and reflector voltages were set up as follows: target voltage at

20 kV, first grid at 65% of target voltage, delayed extraction at 400 ns to obtain the best signal-to-noise ratios and the best possible isotopic resolution. Mass calibration was performed using external peptide standards purchased from AB Sciex. Each spectrum represents the sum of 3,000 laser pulses from randomly chosen spots per sample position. Raw data were analyzed using the computer software provided by the manufacturers and are reported as monoisotopic masses.

Cyanogen bromide cleavage reaction

CNBr cleavage was performed on purified recombinant ONC-DCless-H6-(PM)GKY20 protein. Sample was lyophilized and then resuspended in 0.2 M HCl at final concentration of 1.5 mg/mL. Cleavage was started by the addition of 100- and 400-fold molar excess of CNBr (40 mg/mL in 0.2 M HCl stock solution) over methionine residues, and the mixtures were incubated in the dark at room temperature for 24 h. Reaction mixtures were dried under vacuum and washed three times in 0.1 M acetic acid to allow CNBr evaporation. Samples were resuspended in 0.1 M acetic acid and the efficiency of cleavage was estimated by densitometric analysis of SDS-PAGE.

Antimicrobial assay

Antibacterial activity assays were carried out by i) agar dilution plate viable-count method [44] and ii) broth microdilution method for antimicrobial peptides [50].

Agar dilution plate viable-count method was performed as previously described [44] with minor modifications. A single colony of *Pseudomonas aeruginosa* (KK27) or *Staphylococcus aureus* (ATCC 6538P) was suspended in 5 mL of Luria-Bertani (LB) medium (Becton, Dickinson) and overnight incubated at 37°C. When the cultures reached an OD_{600nm} of 1 unit, they were diluted 1:100 in 20 mM sodium phosphate buffer (NaP), pH 7.0. Samples were prepared by adding 40 μL of bacterial cells to peptides at 0.1, 0.5, 1.0, 2.0, and 3.0 μM final concentrations, in 1 mL of 20 mM NaP buffer, pH 7.0. Cells incubated with antibiotic (colistin 0.01 mg/mL and ampicillin 0.05 mg/mL) were used as positive control, whereas cells incubated without any antibiotics or peptide were prepared as negative control. Bovine serum albumin (BSA) was tested at the same concentration of the peptides. Samples were incubated at 37°C for 4 hours and dilutions (1:100 and 1:1000) of all the samples were placed on LB/agar medium and incubated overnight at 37°C. The following day survived cells were estimated by colonies counting on each plate and compared with controls. All compounds were tested in triplicate experiments. Standard deviations were always less than 5%.

Minimal Inhibitory Concentration (MIC) was determined by broth microdilution method following the protocol previously described for antimicrobial peptides [50] with minor modifications. Assays were carried out in Difco Nutrient Broth composed of 0.5% beef extract (w/v), 0.05% pepton and 0.25% NaCl, using sterile 96-well polypropylene microtiter plates (cat. 3879, Costar Corp., Cambridge, MA). Twofold serial dilutions of peptides were carried out in the test wells to obtain concentrations ranging from 100 μM to 0.2 μM. Bacteria were inoculated from an overnight culture at a final concentration of ~ $5x10^5$ CFU/mL per well and incubated overnight at 37°C. MIC value was taken as the lowest concentration at which growth was inhibited. Three independent experiments were performed for each MIC value. Controls included the peptide antibiotic polymyxin B and vancomycin (Sigma, St. Louis, MO). MIC values were measured on: *Escherichia coli* DH5α, *P. aeruginosa* PAO1, *P. aeruginosa* PA14, *B. megaterium* SF185, *S. aureus* ATCC 6538P from our laboratory strains collection and on *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *K. pneumoniae* ATCC 700603 (kindly provided by Eliana De Gregorio, University of Naples Federico II, Italy).

Supporting Information

S1 File. Nucleotide and amino acid sequences. (A) ONC-(P)GKY20 and (B) ONC-DCless-H6-(P)GKY20 fusion proteins. Onconase carrier (black); peptide (blue); linker region (red underlined); His_6-Tag (green). Amino acid substitutions in ONC-DCless-H6-(P)GKY20 protein sequence (B) were highlighted: glutamate residues (red), tyrosine residues (purple), leucine residue (light blue), isoleucine residues (orange). The main restriction enzyme sites were also reported: *NdeI* (turquoise); *EcoRI* (yellow); *KpnI* (grey); *BamHI* (green); *SacI* (pink). (C) Sequence alignment of human prothrombin gene (GenBank: M17262) segment coding for GKY20 (black) and the manually improved sequence (blue). Mutated codons were underlined and nucleotide substitutions were highlighted in yellow.
(DOCX)

S2 File. Onconase secondary structure. Alpha helices (h) and beta strands (e) from Onconase crystallographic structure [PDB:1ONC] are shown below the sequence. (A) Amino acid sequence of ONC mutant (Onconase M23L, C104 delete); (B) amino acid sequence of ONC-DCless mutant (Onconase M23L, D2E, D16E, D18E, C19Y, D20E, C30Y, D32E, C48L, D67E, C68Y, C75Y, C87I, C90I, C104 delete). The amino acid substitutions are pointed out: red, D2E, D16E, D18E, D20E, D32E, D67E mutations; grey, M23L mutation; turquoise, C19Y, C30Y, C68Y, C75Y mutations; light green, C48L mutation; dark green, C87I, C90I mutations; yellow, cysteine residues.
(DOCX)

Acknowledgments

The authors are grateful to M. Siepi for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: EN EP KP VC. Performed the experiments: AZ KP LD VC VS. Analyzed the data: EN EP KP VC. Contributed reagents/materials/analysis tools: AC ADM. Wrote the paper: ADD EN EP MV VC VI.

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