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Antimicrobial activity of the indolicidin-derived novel synthetic peptide In-58

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Natural peptides with antimicrobial activity are extremely diverse, and peptide synthesis technologies make it possible to significantly improve their properties for specific tasks. Here, we investigate the biological properties of the natural peptide indolicidin and the indolicidin-derived novel synthetic peptide In-58. In-58 was generated by replacing all tryptophan residues on phenylalanine in D-configuration; the α-amino group in the main chain also was modified by unsaturated fatty acid. Compared with indolicidin, In-58 is more bactericidal, more resistant to proteinase K, and less toxic to mammalian cells. Using molecular physics approaches, we characterized the action of In-58 on bacterial cells at the cellular level. Also, we have found that studied peptides damage bacterial membranes. Using the *Escherichia coli* luminescent biosensor strain MG1655 (*pcolD'::lux*), we investigated the action of indolicidin and In-58 at the subcellular level. At subinhibitory concentrations, indolicidin and In-58 induced an SOS response. Our data suggest that indolicidin damages the DNA, but bacterial membrane perturbation is its principal mode of action. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; indolicidin; synthetic peptides; mode of action; biosensors; bioluminescence; SOS response

Introduction

All organisms have features to protect themselves against exterior invasion. One such feature is the production of various nonspecific factors of resistance among which host-defense peptides play an essential role [1]. Host-defense linear peptides [or antimicrobial peptides, (AMPs)] are short, mainly cationic, amphipathic molecules involved in killing bacteria invading an inappropriate habitat [2].The properties of the AMPs influence their biological activity. For example, summary charge determines the electrostatic interaction between peptides and bacterial surface; a peptide with a larger positive charge is bactericidal at the lower concentrations [3]. Amphipathicity, the spatial separation of hydrophobic and polar residues, is also important [4,5].

Some peptides require high concentrations to achievement the antimicrobial effect, and these are characterized by cytotoxic properties. These features of peptides limit their usage in the therapy of microbial infections. Methods of peptide design and synthesis were firstly developed in the 1980s and may overcome most of the undesirable properties associated with some AMPs [6].

Indolicidin is a naturally occurring peptide (tridecapeptide with the following sequence: Ile–Leu–Pro–Trp–Lys–Trp–Pro–Trp–Trp–Pro–Trp–Arg–Arg–NH_2) isolated from the cytoplasmic granules of bovine neutrophils [7], and these demonstrate bactericidal activity against a wide range of bacteria [8] and fungi [9]. Indolicidin, regarding to its properties, may be considered as a candidate to the development of antibacterial pharmcomposition. Unfortunately, indolicidin also has an undesirable cytotoxicity and susceptibility to proteinases that reduces their practical application. Indolicidin modifications can overcome some of these undesirable traits [10].

We have previously designed and synthesized a large number of indolicidin analogues [11]. By screening the number of artificial

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analogues, we selected compounds with minimum inhibitory concentrations (MICs) against pathogens of <10 μg mL^{-1} and causing the hemolysis of erythrocytes (MIH $_{50}$) at a concentration higher than 200 μg mL^{-1} [12]

Thus, the aim of this work was to investigate the biological activity of indolicidin and its best synthetic analogue In-58. In-58 is characterized by replacement of all tryptophan residues on phenylalanine in D-configuration; α-amino group in the main chain also was modified. These modifications reduce the affinity of In-58 for the membranes of eukaryotic cells and improves its resistance to proteolytic enzyme.

It is important to evaluate the biological effects of new peptide analogues not only by MIC but also a comprehensive study at cellular and subcellular levels in a more appropriate approach. The antimicrobial action of indolicidin has been related to cell membrane permeabilization [13]. Also, indolicidin can inhibit DNA replication leading to *Escherichia coli* filamentation [8]. However, the exact circumstances of producing antimicrobical effect of indolicidin remains unclear.

The achievement of the stated aim was performed by using various research tools, such as microscopy, fluorescence spectroscopy, bacterial biosensor, and structure–function analysis of the native and modified target peptides based on molecular modeling results. Also, using the bioluminescent strain of *E. coli* MG 1655 allowed the investigation of the genotoxic effects of indolicidin and In-58 *in vivo*.

Material and Methods

Synthesis of the Indolicidin and In-58

The AMPs In-58 and indolicidin were prepared by solid-phase chemical peptide synthesis using fluorenylmethoxycarbonyl technology as described in previous work [11]. The deblocked crude product was purified to homogeneity by preparative reversed-phase high performance liquid chromatography. The final product was characterized by analytical reversed-phase high performance liquid chromatography and matrix assisted laser desorption ionization-time of flight mass spectrometry.

Evaluation of Hemolytic Activity of the Antimicrobial Peptides

Erythrocytes were isolated by centrifugation from the heparin-treated human blood. The erythrocytes were washed with an isotonic solution of NaCl (150 mM, pH 7.0). Erythrocyte suspension was placed into wells of a 96-well plate that already contained a peptide. The tested concentrations of the erythrocytes were 0.5 %; the peptide solutions were started at 200 μg mL^{-1} followed by serially twofold dilution. The phosphate buffered saline buffer (the absence of hemolysis, negative control A_{NC}) and the saponin solution (100% hemolysis, positive control A_C) were placed into the control wells. The plates were incubated for 1 h at 37°C and centrifuged. The supernatants were transferred to a new plate, and optical absorption was measured at 415 nm (A_P). Each experiment was repeated three times in three different plates. The resulting values of optical absorption were processed according to the following equation:

$$\text{Hemolysis} = \left(A_P{}^{415} - A_{NC}{}^{415}/A_C{}^{415}A_{NC}{}^{415}\right)\text{x}100\% \qquad (1)$$

Evaluation of Antimicrobial Peptides-Resistance to Proteolysis

Protease susceptibility assessment was carried out by incubation of 100 μg mL^{-1} of indolicidin and In-58 with serial twofold dilutions of protease K (biotechnology grade, Sigma-Aldrich, USA) at 37°C for 1 h in buffer [5 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), pH 7.1]. AMPs in buffer without enzyme were used as positive control; enzyme in buffer solution and buffer without any additives were used as negative controls. The inhibitory activity of AMPs after treatments was determined using the well agar diffusion assay.

Evaluation of Antibacterial Activity of the Antimicrobial Peptides and the Time-Killing Assay

The antimicrobial activity of the AMPs and conventional antibiotics (vancomycin, gentamycin, and norfloxacin purchased from Sigma-Aldrich, USA) against bacteria was determined by the broth microdilution assay [14]. The bacterial strains were cultured in Muller-Hinton medium (HiMedia, India) for 18 h, and bacterial suspensions were prepared with an optical density that corresponded to 10^6 per mL^{-1} colony forming units (CFU). The wells were filled by 80 μl Muller-Hinton medium and 10 μl bacterial suspension; in each well, 10 μl of twofold dilutions of a tested substance (AMPs or conventional antibiotics) was added. Bacteria were incubated in a 96-well flat bottom microplate (Eppendorf, Germany) at 37°C, and growth was monitored by absorbance at 620 nm using a spectrophotometer IEMS MF (Labsystems, Finland). Antimicrobial activity was expressed as the MIC that was defined as the lowest peptide dose possesses no visible growth detection.

To evaluate the dynamics and mode of action of indolicidin and its analogue In-58, the time-killing assay was performed. For the time-killing assay, we used the *E. coli* MG 1655 and the *Bacillus cereus* ATCC 14893, which are represented a model Gram-negative and a model Gram-positive bacterial cells, respectively. Preparation of bacteria for the test was performed as described earlier, but twofold dilutions of In-58 were added at the mid-log phase of bacterial growth.

The Bioluminescence Assay

Deoxyribonucleic acid damage and SOS response induced by AMPs were assessed using the *E. coli* reporter strain MG1655 (*colD::lux*). This strain carries the *pcolD '::* luxCDABE-Amp^R plasmid which includes the *lux* genes of the soil luminescent bacteria *Photorhabdus luminescens* Zm 1 driven by the promoter of the *E. coli colD* gene.

The bacterial strain was cultivated in Luria–Bertani liquid medium-containing ampicillin (100 μg mL^{-1}). The overnight culture of the biosensor was washed and resuspended in Milli-Q water to obtain a cell density corresponding to 10^6 CFU mL^{-1}.

Ninety microliters of bacterial suspension were mixed with an appropriate volume of twofold dilutions of peptide and placed into wells of a 'Microlite 2+' microplate with non-transparent side walls (ThermoScientific, USA) up to the final volume of 100 μl. Wells filled with sterile Milli-Q water and containing an appropriate amount of bacterial biosensor were used as the negative control. Wells containing biosensor and the 0.5 MIC of norfloxacin (Sigma-Aldrich, USA) was used as the positive control. Bioluminescence measurements were carried out using an Infinite F200 pro microplate reader (Tecan, Austria) which dynamically registered

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the luminescence intensity of the samples for 4 h and were estimated in relative light units.

Bacterial Permeabilization Assays

Permeabilization of the cytoplasmatic membrane

The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA) was used to evaluate the cytoplasmatic membrane integrity of *E. coli* according to the manufacturer's protocol. Water and 70% isopropanol were used as negative and positive controls, respectively.

The fluorescence microscopy of stained bacteria was performed using a Mikromed-3-Lum fluorescent microscope (Lomo, Russia) equipped with filter sets useful for simultaneous viewing of the SYTO 9 and propidium iodide (PI) stains; images for cell quantification then were acquired with a digital camera. Fluorescence detection was performed using the fluorescent spectrometer Fluorat-02 Panorama (Lumex, Russia). The percentage of bacteria with intact/damaged membranes was calculated according to the green/red fluorescence signal ratio.

Measurement of SYTO 9 fluorescence kinetic was performed using a plate reader Infinite F200 pro (Tecan, Austria) at 485 nm emission and 535 nm of excitation wavelength. Norfloxacin and 70% isopropanol were used as negative and positive controls, respectively.

Fluorescent-dextran diffusion assay

To investigate the diameter of bacterial membrane lesions, the fluorescence-labeled dextrans were used. *E. coli* MG1655 was grown on Luria–Bertani liquid medium at 37°C for 12 h. After cultivation, bacterial cells were precipitated and resuspended in 5 mmol L^{-1} HEPES (pH 7.5) buffer to an optical density (OD_{620}) of 0.15 (corresponding to 10^7 CFU mL^{-1}). Bacterial cells were added to the wells of a 96-well flat-bottom microtiter plate (Eppendorf, Germany) containing serial dilutions of In-58 and fluorescein isothiocyanate-dextran molecules [4.4 and 10 kDa of fluorescien isothiocyanate (FITC)-dextran taken at 0.5 mg ml^{-1}; Sigma-Aldrich, Sydney, Australia]. The intact cells incubated only with 4.4 kDa FITC-dextran served as a negative control. After incubation at 4°C for 1 h, bacterial cells were washed with HEPES buffer and transferred to a glass cuvette for spectroscopy detection of fluorescence (485 nm excitation and 490–550 nm emission).

Molecular Modeling

Modeling of spatial structures of indolicidin and its derivatives was made by PyMOL software (version 0.9.3.) (DeLano Scientific, USA).

Statistical Analysis

All experiments were performed using three independent series. Statistical manipulation of obtained results was realized with STATISTICA 6.0 (StatSoft Inc., USA) software.

Results and Discussion

Structure–Function Relationships between Indolicidins

It was previously found that the residues of tryptophan do not play a determining role in the antibacterial activity of indolicidin [10]. Not only did the replacement of tryptophan with non-natural aromatic amino acid residues not decrease the activity of the analogues obtained, but in some cases, it even led to its increase. And preliminary, it is typically that complete tryptophan mutation (for instance, to L-leucine, L-phenilalanine of L-tyrosine at 4, 6, 8, 9, and 11 positions) could lead to significant decreasing of the antimicrobial activity against Gram-positive bacterial strains only (*Bacillus subtilus* and *Staphylococcus aureus*) but not for Gram-negative (*E. coli*). Interestingly that some single or double substitutions (Trp/Leu, Trp/Lys, Trp/Tyr) were not quite critical for biological activity [15]. [D-Phe (4, 6, 8, 9, 11)]-indolicidin also had a pronounced antibacterial activity [10]. However, to date, analogues of indolicidin contained D-phenylalanine instead of tryptophan has not been studied yet. In order to study the effects of the substitution of amino acid residues in positions of 4, 6, 8, 9, and 11 on the biological activity of the indolicidin molecule, we synthesized analogues containing phenylalanine derivatives carrying electron-donor and electron-acceptor substituents at the aromatic ring (4-nitrophenylalanine, 4-chlorophenylalanine, 4-methoxyphenylalanine, and others) in these positions. Also, [D-Phe (4, 6, 8, 9, 11)]-indolicidin and the analogue whose N-α-amino group was modified by an unsaturated acid fatty acid have been synthesized. Preliminary tests for antimicrobial activity and cytotoxicity revealed the most promising peptide NH_2–$(CH_2)_{10}$–CO–Ile–Leu–Pro–D–Phe–Lys–D–Phe–Pro–D–Phe–D–Phe–Pro–D–Phe–Arg–Arg–NH_2 [12].

The main properties of peptides that determine their antimicrobial activity are known: amphiphilicity (amphipathicity) of molecules; the net charge of a molecule significant for initial electrostatic adsorption on a bacterial surface. The increasing of positive charge is one of the ways to enhance the antimicrobial properties of peptide molecules [16]. However, a strong positive charge usually determines the undesirable toxicity of peptides against the eukaryotic cell [3]. In-58 has the same charge in comparison with the natural molecule (+4 at neutral pH value). This charge value (Table 1) is optimal and sufficient to interact with the

Table 1. Comparative properties of natural indolicidin and its synthetic analogue In-58

	Amino acid sequence	MALDI-TOF MS		Net charge at pH 7.0*	μH [a]
		Obs., da	Calc., da		
Indolicidin	NH_2-ILPWKWPWWPWRR-NH_2	1906.3	1907.6	+ 4	0.485
In-58	NH_2-$(CH_2)_{10}$-CO-ILP(D)FK(D)FP(D)F(D)FP(D)FRR-NH_2	1917	1894.45	+ 4	0.495

[a]μH – the relative hydrophobic moment was calculated online using the web-resource "EMBOSS PROTEIN PROPERTIES" available at http://www.bioinformatics.nl/emboss-explorer

[b]calculated using the web-resource "APD3: Antimicrobial Peptide Calculator and Predictor" available at http://www.aps.unmc.edu

MALDI-TOF MS, Matrix-assisted laser desorption ionization-time of flight mass spectrometry.

surface of a bacterial cell but not with membranes of eukaryotic cells. Reduction of hemolytic properties is also possible because of the replacement of individual amino acid residues by analogues. Thus, it was found that the replacement of tryptophan with other hydrophobic residues (leucine, isoleucine, and lysine) does not reduce the antimicrobial properties of the peptide, but it significantly reduces its cytotoxicity [10,11].

Evaluation of the In-58 hemolytic activity, a significant decrease in cytotoxicity was found in comparison with the natural molecule (Figure 1). Indolicidin hemolyzed 50% of the erythrocytes at a concentration of 100 μg mL^{-1}, while its modified analogue at the same concentrations hemolyzed less than 10%. The decrease in hemolytic activity is mainly determined by the replacement of amino acid residues by D-stereoisomer which is one of the approaches for reducing peptide cytotoxicity [17,18].

The enhancement of the antimicrobial properties of indolicidin can be achieved by conjugating the peptide with a fatty acid at the N-terminus. This approach was used to increase the bactericidal properties of magainin, modified with fatty acid with different chain hydrocarbon lengths; while the dependence of antimicrobial properties on the length of the fatty acid tail was traced [19].

In-58 and indolicidin have antimicrobial activity against some Gram-positive and Gram-negative microorganisms at micromolar concentrations. In general, Gram-negative bacteria were more resistant to the tested peptides than Gram-positive. If comparing both peptides, the MIC of indolicidin was twofold higher (Table 2). It is in need to note what kind of performed substitution of amino acid residues affects the relative hydrophobic moment (μH) reflected amphipathicity of peptides (Table 1). So, Trp/Phe substitutions slightly increase the amphipathicity of In-58, which what increase the antibacterial effect.

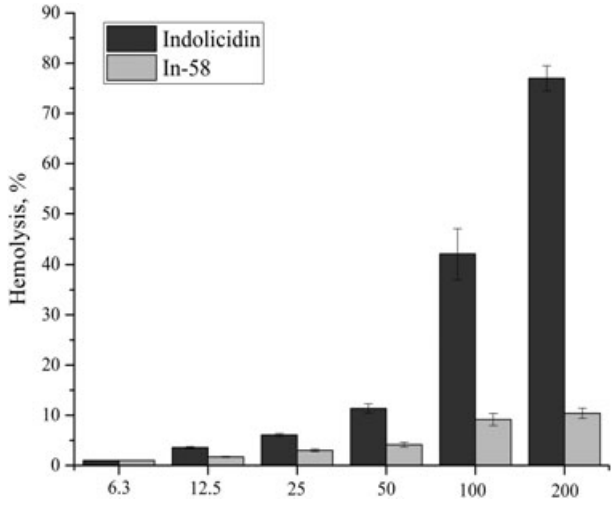

Figure 1. The hemolytic activity of indolicidin and its analogue in-58.

The studied dynamics of antimicrobial action revealed certain features according to *E. coli* MG1655 and *B. cereus* ATCC 14893 which are a Gram-negative and a Gram-positive microorganisms with similar rode-shaped form of the cells. These strains were chosen as the model microorganisms for the experiment. The growth of these bacteria treated with In-58 was slower than the growth of the non-treated ones. It was also possible to identify some differences in the reaction of Gram-negative (Figure 2A) and Gram-positive (Figure 2B) bacteria.

The optical density of the Gram-negative *E. coli* decreased by 25% within 2.5 h of incubation, whereas the optical density of the Gram-positive *B. cereus* decreased by 25% within 1.5 h. This inequality is likely because of differences in the cell walls of these bacteria. Interestingly, the addition of high concentrations of the peptide (>50 μg mL^{-1}) led to a rapid increase in the optical density of the bacteria-peptide suspension, which started immediately followed by a decrease at the end of the observation (Figure S1). A similar effect was noted by Falla *et al.* [9]. However, microscopy imaging within 15 min of peptide addition did not reveal abnormal cell forms. Thus, we assume that this rapid increase in optical density was not associated with the formation of the cell filaments and is likely associated with cell aggregation. Performed atomic-force microscopy revealed a large aggregate of cells treated with 100 μg mL^{-1} indolicidin (Figure S2). Similar results were shown for short buffalo cathelicidins [20] and eosinophilic cationic proteins [21]. The treatment of bacteria by these peptides promoted agglutination of the cells through rearrangement of the proteins that expose the hydrophobic patch of the peptide leading to the disruption of the lipophilic barrier of the cells.

The performed modifications of indolicidin resulted in an improved resistance of In-58 to rapid degradation by proteolytic enzyme. Thus, antimicrobial activity of indolicidin completely disappeared when exposed to proteinase K at a concentration of 2.5 mg mL^{-1}, while the modified analogue was preliminary resistant to this action is about two times (5 mg mL^{-1}). Thus, modification of the indolicidin by the fatty acid increased of antimicrobial properties and resistance to proteinase K because of the fact that hydrophobicity, secondary structure, and self-association of obtained analogue are changed [20].

Table 2. The antibacterial activity of indolicidin and its analogue In-58 against Gram-negative and Gram-positive bacteria

Bacterial cover type	Strains	MIC, μg mL^{-1}			
		Indolicidin	In-58	Vancomycin	Norfloxacin
Gram-negative	*Escherichia coli* MG1655	25	12.5	>200	0.08
	Salmonella enterica ATCC 14028	100	50	>200	1.25
	Pseudomonas aeruginosa ATCC 27853	100	100	>200	2.5
Gram-positive	*Bacillus cereus* ATCC 14893	12.5	6.25	12.5	>28
	Staphylococcus aureus FDA 209 P	12.5	6.25	3.1	0.31
	Listeria monocytogenes EGDe	3.25	0.75	0.38	1.75

MIC, minimum inhibitory concentration.

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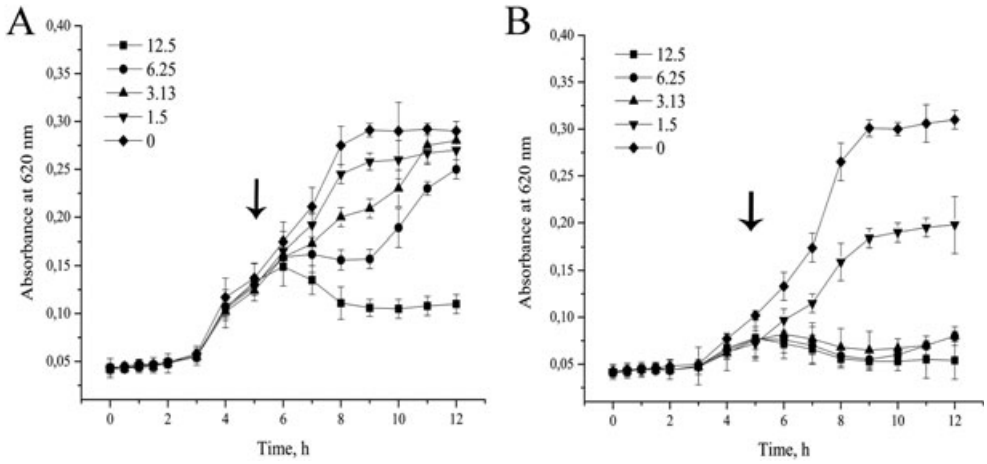

Figure 2. The effect of In-58 on the growth of *Escherichia coli* K12 MG 1655 (A) and *Bacillus cereus* ATCC 14893 (B). Various concentrations of In-58 were added after 4.5 h of culturing (arrows).

Some interesting considerations can be generated when previous data are being analyzed. Turning back to increased protease resistance, a pseudo cyclic indolicidin peptide that generates disulfide bridge between the second and the fourteenth amino acid residues has been obtained and characterized by NMR experiments [22]. This molecule was more stable to proteolysis, but the modifications led to complete loosing of alpha-helical conformation of the mutant peptide, and thereby its three-dimensional structure is observed by random coil only. In another study, it was received indolicidin mutant molecule that contains Pro/Ala (CP10A) substitution that is probably critical for alpha-helix formation and possessed increased antibacterial activity against Gram-positive *S. aureus* and *Staphylococcus epidermidis* [23]. Based on spatial configuration of CP10A (PDB ID: 1HR1), we made a molecular modeling of the polypeptide chains for native indolicidin and In-58 (Figure 3). It was discovered that for the native peptide, there is no any possible surface changes (Figure 3A,B) concerning Pro/Ala mutant (Figure 3C,D), but for Trp/Phe, we are able to find some reorientation of residual side chains (Figure 3E,F) that could be one of the key factors of more significant antibacterial activity based on polypeptide part of the molecule only, excepting alkyl hydrocarbon substituent.

Investigation of Mode of Action at Cellular Level Using a Microscopy and Fluorescence Techniques

Most AMPs disrupt the integrity of bacterial barrier structures. Some studies with indolicidin failed to find membrane-disordered effects [8], whereas the others found these effects [13]. Recently, using the liposomal models of different lipid composition, we demonstrated that indolicidin molecules can be active as organic anion carrier without pore formation in uncharged vesicles [24]. This revealed carrier-type mechanism may be involved in the hemolytic action of indolicidin: however, bacterial cells have a negatively charged surface. In this work, using various fluorescent probes, we traced fluorophore accumulation in bacterial cells and found a permeabilization effect of indolicidin (Figure 4A) and In-58 (Figure 4B). The SYTO 9 fluorophore immediately penetrates bacterial cells, reaching the maximum values within 5 min. The addition of indolicidin or In-58 at the step of SYTO 9 internalization led to instantaneously decreasing (within 30 sec) of its fluorescence.

SYTO 9 is a low molecular weight dye (~ 400 Da) that freely diffuses into the cell and binds to DNA. Whereas PI is unable to penetrate cells with intact membranes. The addition of In-58 causes the immediate formation of lesions, allowing PI to enter the

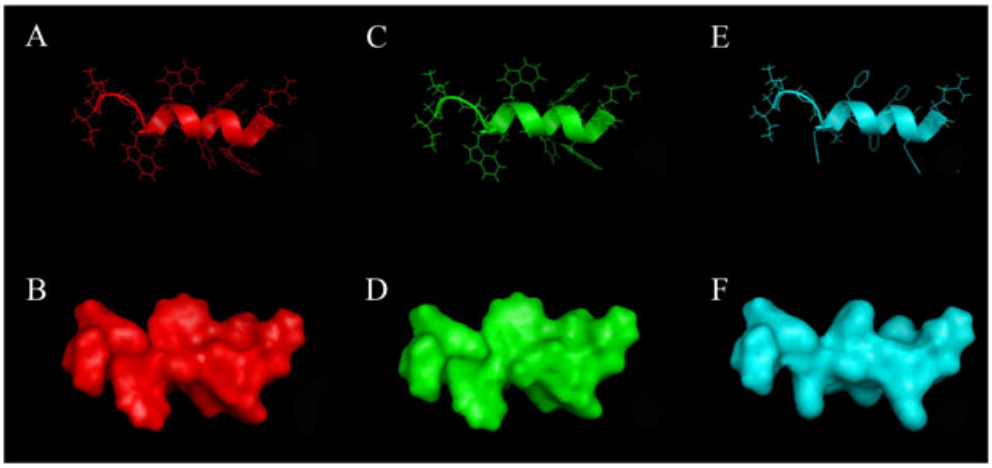

Figure 3. Modeling of 3D structures of indolicidin and analogues based on spatial configuration of C10A (PDB ID: 1HR1). Ribbon (A, C, E) and surface models are presented: native indolicidin (A, B) [7], C10A modified peptide (C, D) [24], and In-58 modified peptide (E, F) (this work).

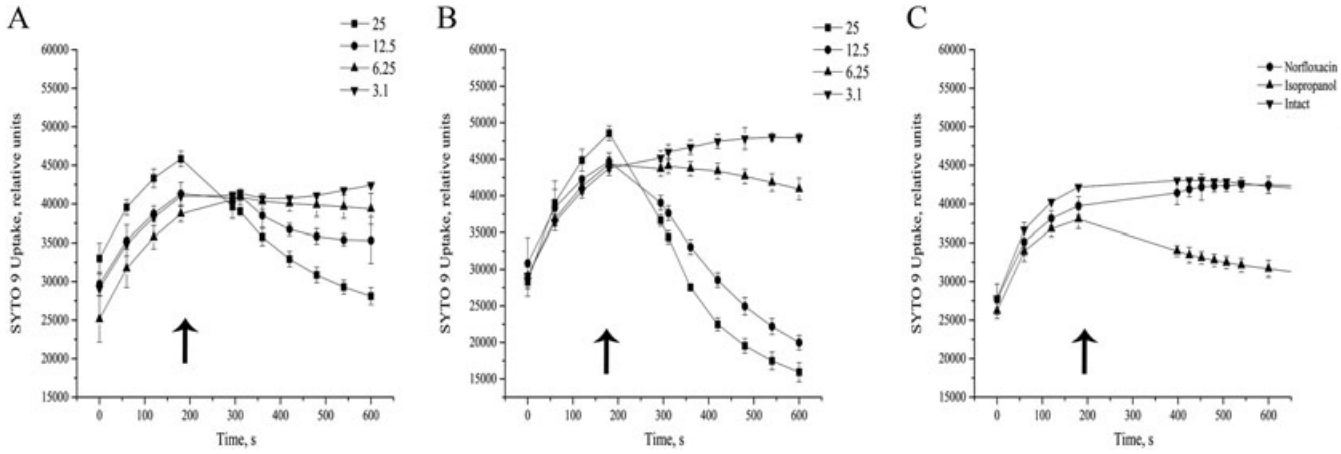

Figure 4. SYTO 9 fluorescence kinetic demonstrating permeation of fluorophore into *Escherichia coli* MG1655 cells treated with indolicidin (A) or In-58 (B). Various controls were used to investigate the relevance of data obtained by measurements of fluorescence kinetic. The negative controls was the norfloxacin that acts on bacteria as a non-permeabilizing agent; 70 % isopropanol served as a positive controls (C). Arrows show the time of test-substance addition. If bacterial membranes are permeabilized, propidium iodide are penetrated into the cell follow displacing the SYTO 9 from DNA, which leads to a decrease in luminescence intensity in a green region of the spectrum.

bacterial cells, displacing SYTO 9, and suppressing its fluorescence (intact cells – green; damaged cells – red) (Figure 5A). Also, we noted rounded formations on green cells treated with either In-58 (Figure 5B) or indolicidin (Figure S3). Given that indolicidin induces local thinning of the bilayer [25,26]; these formations are likely vesicles formed from phospholipids of the cell's membrane and containing part of the intracellular material. Similar images were obtained by confocal microscopy of *E. coli* treated with human beta-defensins-4 modified by introducing disulfide bridges [27].

Fluorescence spectroscopy (Figure 5E) of bacterial populations, treated with increasing concentrations of In-58, showed disrupted the bacterial cell membrane in a dose-dependent manner (Figure 5E). In-58 similarly permeabilized Gram-negative and Gram-positive bacteria.

Disruption of bacterial membranes by peptides has been described by several different models involving the formation of a barrel-stave, toroidal-pores, and carpet mechanism [28–30]. Various models can be described by some parameters among which the dimensional properties can be estimated experimentally quite easily. These are around ~1.8 nm for barrel-stave and ~3.0 nm for toroidal pores, while a carpet model is characterized by significant disruption >50 nm [31 - 33].

We used fluorescence-labeled dextran molecules of various molecular weights to estimate the size of the lesions in the bacterial membrane caused by In-58. This approach is based on the influx of FITC-dextran into the bacterial cytosol through membrane lesions. The accumulation of certain probes will reflect the size of the lesions. This approach has been successfully used to determine actions of various AMPs including temporin L [31], magainin [32], maculatin [33], and hemocidines [34].

The coincubation of bacteria with In-58 leads to the preferential accumulation of 4.4 kDa FITC-dextran that corresponds to a Stokes radius of 1.4 nm. However, conjugates with a mass of 10 kDa (2.3 nm) also infuse but to a lesser degree (Figure 6). These data suggest that In-58 creates lesions in the bacterial membrane,

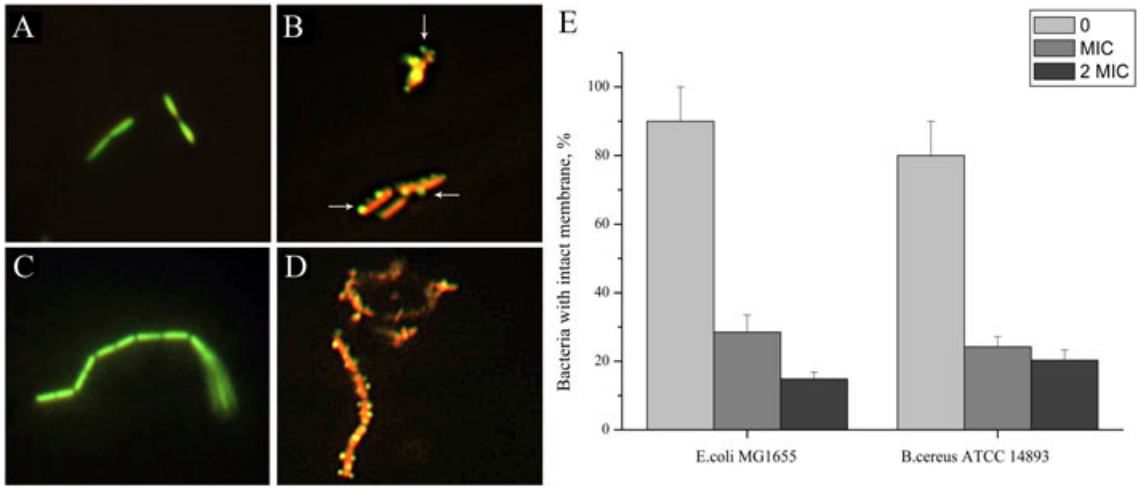

Figure 5. Microscopy investigations of In-58 action on *Escherichia coli* MG1655 (A, B) and *Bacillus cereus* ATCC 14893 (C, D). Fluorescence microscopy images of intact (A, C) and In-58-treated [minimum inhibitory concentration (MIC)] cells (B, D). White arrows indicate lipopolysaccharide protrusions caused by In-58. (E) The effect of In-58 on bacterial cytoplasmic membrane integrity as revealed by fluorescence spectroscopy.

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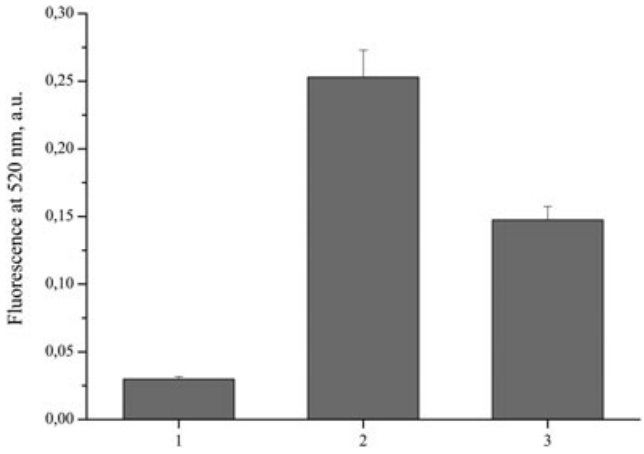

Figure 6. Fluorescence intensity of fluorescien isothiocyanate (FITC)-dextran with various molecular mass in *Escherichia coli* MG 1655 cells. Designations of x-axis: 1) bacteria incubated with 4.4 kDa FITC-dextran without In-58 treatment (negative); 2) bacteria treated with In-58 at minimum inhibitory concentration and incubated with 4.4 kDa FITC-dextran; and 3) In-58-treated bacteria incubated with 10 kDa FITC-dextran.

comparable in size to toroidal pores [28]. Such permeabilization occurs because of the increased lateral pressure in the region of the bilayer, close to the water–lipid interface which might lead to the transient formation of a peptide–lipid toroidal pore [35].

Testing the Antimicrobial Peptide Mode of Action at the Subcellular Level Using a Bioluminescence Assay

The aforementioned data show that indolicidin disrupts bacterial membranes which is in line with the study of Falla *et al.* [13]. However, indolicidin might have multiple targets. For example, some synthetic peptides affect membranes and electrostatically interact with the bacterial nucleic acids [36,37].

The data on the interactions between indolicidin and nucleic acids are based on effects on the nucleic acids themselves [38,39] or, more rarely, on bacteria [8]. Indolicidin forms stable complexes with dsDNA [27]. As it was revealed by spectroscopic investigation, indolicidin binds to the major groove of duplex B-type DNA. This can lead to the subsequent inhibition of DNA replication and transcription.

Using biosensor *E. coli* strain MG1655 (pcolD′::lux), we evaluated the interaction between indolicidin and intracellular DNA. This strain contains hybrid plasmids with promoter of gene *colD* crosslinked with reporter genes *luxCDABE P. luminescens*. Biosensor is characterized by a relatively low background level of luminescence. However, when replication of DNA is disrupted bioluminescence increases. Thus, the light emission of the test strain measurement allows to quantify in real-time the level of activation of the bacterial SOS system. Earlier, some studies were performed using this strain. The *E. coli* MG1655 (pcolD′::lux) lux biosensor exhibited high sensitivity to the detection of antibiotics that form adducts directly with nitrogenous bases of DNA (mitomycin C) and forms interstrand links [40]. Also, such successful approach was applied to detect the antibiotics which action resulting in accumulation of single and double strand breaks (quinolone) leads to impaired replication and cell death [41].

By this approach, we found that subinhibitory concentrations of indolicidin induce the bioluminescence of *E. coli* MG1655 (pcolD′::lux) in a dose-dependent manner (Figure 7A). A similar trend was found for In-58 (Figure 7B). While increasing the active concentration over one half, MIC did not led to increasing of relative light unit-values, but it conversely led to decreasing of bioluminescence. Most likely, this is because of the fact that these AMPs have a membrane's disordering effect on bacteria at MIC. What affects the functions of cell's biosynthetic apparatus, which is involved in bioluminescence.

Earlier *in vitro* studies have shown that the tryptophan residues of the indolicidin molecule have a major role in its interaction with native DNA [38]. Also, it was established that replacing tryptophan with phenylalanine reduces the thermodynamic stability of the peptide-DNA complex [39]. However, here, we found no significant difference in the SOS response of bacteria treated with either indolicidin or its phenylalanine-containing analogue (In-58). It is possible, what violation of the structural integrity of the DNA molecule itself is more significant that the thermodynamic stability of peptide-DNA complex. This may indicate that the results of studies of biological processes obtained in an *in vitro* system cannot always be directly transferred to processes occurring in a living organism.

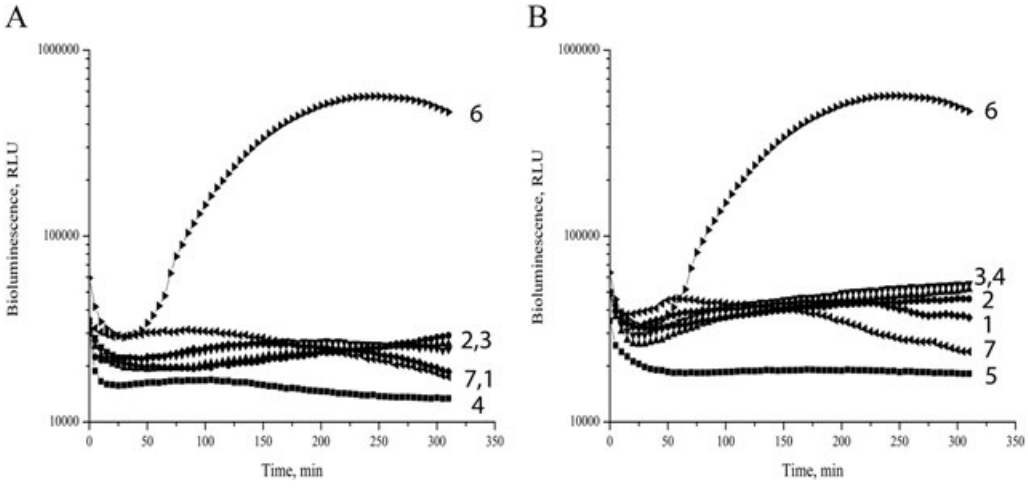

Figure 7. Response of *Escherichia coli* MG1655 (pcolD′::lux), to In-58 (A) and indolicidin (B). Designations: 1) 1.5 μg mL^{-1}; 2) 3.12 μg mL^{-1}; 3) 6.25 μg mL^{-1}; 4) 12.5 μg mL^{-1}; 5) 25 μg mL^{-1}; 6) norfloxacin at 0.5 minimum inhibitory concentration (positive control); 7) pure water.

Conclusion

Natural peptides with antimicrobial activity are extremely diverse. At the same time, they may be improved by modifications, depending on their intended tasks. Here, we investigated the biological properties of indolicidin and its analogue In-58. Compared with indolicidin, In-58 is more bactericidal and less toxic to mammalian cells. We found that indolicidin and In-58 damage bacterial membranes. In addition, for the first time using the luminescent biosensor strains *E. coli* MG1655 (pcolD'::lux), the action of indolicidin and its synthetic analogue In-58 at the subcellular level was shown. It was also shown that peptides taken at subinhibitory concentrations induce an SOS response of bacterial cells. Finally, we demonstrated the killing effect of indolicidin, and its analogue was realized because of the disturbance of the bacterial cell wall, while the interaction with intracellular DNA may lead to other effects; for example, to affecting of expression of some genes [36].

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Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Statement of Human and Animal Rights

This article does not contain studies with human or animal subjects performed by any of the authors that should be approved by Ethics Committee.

Statement of Informed Consent

The article does not contain any studies in patients by any of the authors.

Author Contributions

Conceived and designed the experiments: V. A. S. Performed the experiments: V. A. S., P. T. M., V. A. V., S. M. V., P. T. M., and R. E. A. Analyzed the data: V. A. S., M. I. V., and R. E. A. Contributed reagents/materials/analysis tools: V. A. S., M. I. V., Z. G. B., S. M. P., K. N. I., S. E. A., K. O. L., D. G. K., and S. A. S. Contributed to the writing of the manuscript: V. A. S. and R. E. A.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. The growth of the *E. coli* MG1655 (a) and *B. cereus* ATCC 14893 (b) with addition of In-58 at various concentrations.
Figure S2. The atomic-force microscopy image of *E.coli* MG 1655 treated with high concentration of indolicidin (100 μg mL^{-1}). The observation was obtained with an atomic force microscope SMM-2000 (JSC Proton-MIET Plant, Russia) in contact mode in air. The instrument was equipped with silicon nitride cantilever MSCT-AUNM (Veeco Instruments Inc., USA) with a pyramidal (V-shaped) tip with a typical radius of ~ 10 nm.
Figure S3. The fluorescence microscopy images of intact *E. coli* MG 1655 (a) and *B. cereus* ATCC 14893 (c); *E. coli* MG 1655 (b) and *B. cereus* ATCC 14893 (d) treated with indolicidin taken at MIC.

