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In vitro studies of antimicrobial activity of Gly-His-Lys conjugates as potential and promising candidates for therapeutics in skin and tissue infections

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ABSTRACT

In this Letter, we presented in vitro studies of antimicrobial activity of Gly-His-Lys conjugates that are important point in preliminary biological evaluation of their potential application in skin and tissue therapies. The novel compounds include the conjugation of fatty acids with a modification of the amino acid sequence in the primary structure of Gly-His-Lys (**6i**). All the compounds exhibited strong to moderate activity. Compound **1d** had the most potent antimicrobial activity at MIC ranges 31.3–125.0 μg/mL (against *Escherichia coli* spp. and *Staphylococcus aureus* spp.), 375.0–500.0 μg/mL (against *Pseudomonas aeruginosa* spp.). Conjugate **5b** expressed activity against *Staphylococcus aureus* spp. and *Escherichia coli* spp. at MIC ranges 250.0–500.0 μg/mL and 62.5–125.0 μg/mL, respectively. Both conjugates **1d** and **5b** possessed rapid bactericidal activity against Gram-positive bacteria at 2MIC or 4MIC. Conjugates **1b–c**, **1e**, **2a–b** and **4b** showed noticeable effect against both Gram-positive and Gram-negative bacteria. Compounds **1d**, **1e** and **2e** were the most active against fungus.

The interesting achievement in therapy of skin and tissue infections is the use of endogenous peptidic antibiotics.[1–3] The peptidic compounds are essential due to the natural origin and low incidence of bacterial resistance. They possess a broad spectrum of antimicrobial,[2,3] immunomodulatory[4] and strong repairing activities.[2,4] One of the recently marketed drug is daptomycin (a cycle lipopeptide) which possesses really strong activity and low resistance in treating skin and tissue infections caused by Gram-positive cocci (mainly *Staphylococcus* spp. and *Streptococcus* spp.).[5–7] Many experimental works proved surprisingly high activity of oligopeptides against different microorganisms. Moreover, they are also active against biofilm formation.[8,9] The activity is also potentiated by fatty acid chain linked to the *N*-terminal end of peptide.[10]

Known as lipids, fatty acids are the second group of compounds, that are involved in both physical and immunologic function barriers of the skin. They have been known for nearly several decades for their antimicrobial activity and played a direct role in innate immune defense against epidermal infections.[11–13] Fatty acids also possess antimicrobial activity especially against Gram-positive cocci (*Micrococci* spp., *Staphylococci* spp., *Streptococci* spp.), *Propionibacterium acnes*, and yeast (*Candida albicans*), rather than Gram-negative bacteria such as *Escherichia coli* spp. and *Pseudomonas aeruginosa* spp.[12–17]

Therefore research involving the possibility of their application for therapy of skin infections is still widely discussed. One of the major advantages of this group of compounds is the ability to decrease the development of bacterial resistance in comparison with conventional antibiotics used in treatment of skin lesions and vast array of wounds.[18–20]

Additionally, it was proved that the highest level of biological activity among the saturated fatty acids and their derivatives is represented by lauric acid. It also assuages in in vivo studies the effect of inflammation-related infections[1,21,22] and has a great potential in treatment of acne.[14,15]

In spite of the fact that most fatty acids possess a very strong activity and a low toxicity, especially at higher concentration in comparison with the conventional antibiotics, it makes them very attractive candidates for further modifications.[21] It is also well known that the effective topical therapeutics with antimicrobial activity should selectively target microorganisms, killing bacteria and other microbes, with minimal adverse on healthy cells. Many fatty acid–oligopeptide conjugates exhibit improved biological activity with low cytotoxic activity.[8,23] However, they represent a relatively new group of compounds and the data on their activity are limited to in vitro and in vivo studies. Their amphipathic structure seems to be required for membrane binding. The length of

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fatty acid chain controls the pathogen specificity of the lipopeptides, overall hydrophobicity and oligomeric state of lipopeptides in solution.[24] The unique mechanism of action and relatively safe profile in comparison with native lipo-antibiotics makes them extremely attractive candidates for potential therapeutics in skin and tissue infections.

In our studies, we applied Glycyl-L-Histydyl-L-Lysine as a peptidyl molecule with a variety of important regulatory role in skin inflammation and tissue regeneration. Tripeptide is liberated from extracellular matrix protein, especially α-II chain of human collagen or secreted protein called SPARC in response to even soft tissue damage.[25–30]

The first report on antimicrobial activity of tripeptide was presented by Panlabs and included in vivo strong protection against infection caused by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* spp.[31] Liakopoulou-Kyriakides et al.[32] described in vitro inhibitory activity of tripeptide against *Escherichia coli* spp., which is associated with the inhibition of both protein and DNA synthesis. The activity of tripeptide to overcome bacterial infections is also a result of its ability as a copper complex to interact on metabolism of iron ions. It seems that tripeptide copper complex naturally inhibits the release of iron from ferritin, preventing the microbial growth. Besides peptide induces the gene expression of natural antimicrobial defensins—DEFB1 and DEFB5.[33–35]

We designed a series of peptide conjugates containing Gly-His-Lys analogues modified with *N*-acyl of lauric, palmitic and stearic acid, with *C*-amide or free carboxyl group. The modifications of the amino acid sequence are based on numerous studies reports, that comprise the replacement of histidine to other residue. This modification is a very attractive target in the development of novel analogues due to the improvement of stability and biological activity of the compounds.[36–39] Encouraged by the results obtained earlier, we also replaced His with Hyp, Met, Gly-Hyp, Hyp-Met residues. Some analogues possess additionally D-Lys instead of Lys (Fig. 1).

The modification of Gly-His-Lys analogues with fatty acids increased the affinity to phospholipids membrane in in vitro studies.[27,40–43] We hypothesized that it could also increase the bacterial and fungal membrane binding and insertion, that could also be important point in preliminary studies on their potential application in skin and tissue therapy. In this Letter, we presented in vitro antimicrobial activity of lipopeptides.

All the peptides investigated in our studies were prepared in our laboratory by manual methodology using Fmoc solid-phase technique[44,45] (synthesis details in Supplementary data). Antimicrobial assays for Gly-His-Lys (standard, **6i**) and all the conjugates were performed by determination of MIC and MBC/MFC values (Supplementary data). Minimal inhibitory concentration values against representatives of Gram-positive (*Staphylococcus aureus* spp.), Gram negative (*Escherichia coli* spp., *Pseudomonas aeruginosa* sp.) bacteria and fungus (*Candida albicans* sp.) are presented in Figure 2.

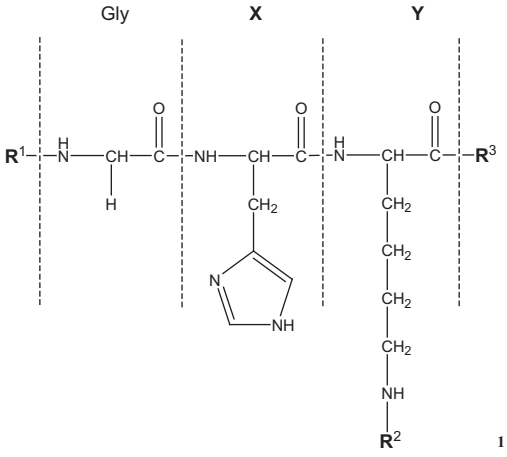
In our previous effort in the development of Gly-His-Lys conjugates (data not shown), we reported that the replacement of natural histidine for hydroxyproline in peptide **1i** gave significantly lower effect of the peptide on Gram-positive bacteria (*Staphylococcus aureus* spp.), whereas it possessed similar activity in comparison with peptide **6i** against representatives of Gram-negative bacteria (*Escherichia coli* spp.).

The compound **4i** showed improved activity against *Staphylococcus aureus* spp. in comparison with peptide **6i**. At the same time, the activity of the compounds **3i** and **4i** against *Escherichia coli* spp. was attenuated, in comparison with more hydrophilic compounds **6i** and **1i**. It could also be associated with the phenotype of genome in *Escherichia coli* spp., that is involved in metabolism of sulfur-containing compounds.[46,47]

The most attractive molecule to further structural modifications was compound **1i**. Firstly, it was found that peptide **1b** exhibited better bacteriostatic activity against *Staphylococcus aureus* spp. and *Pseudomonas aeruginosa* sp. in comparison with peptide **1i** (and **6i**) and conferred also bactericidal activity (Figs. 2B, C and 3A).

Moreover, peptides **5a**, **5b** with the substitution of D-Lys endowed antibacterial activity against *Escherichia coli* spp. with 2- and 4-fold reduction of MIC values, respectively, in comparison with compound **1b**. In spite of the fact that compounds **1b** and **5b** possessed similar inhibitory effect, killing experiments exhibited higher activity of compound **5b** against *Staphylococcus aureus* spp. Whereas the strong bactericidal activity of compound **1b** was observed (at 8MIC) after 18 h, conjugate **5b** presented short-time killing activity at 2-fold lower concentration (4MIC, after 4 h).

Palmitoyl derivative **1c** possessed 2-fold lower MIC value against *Escherichia coli* spp. than peptide **1b**. Moreover, it prevented the growth of *Staphylococcus aureus* spp. in the first time of incubation at 1/2MIC and MIC, whereas at 4MIC it possessed



1 - X=Hyp, Y=Lys
2 - X=Gly-Hyp, Y=Lys
3 - X=Met, Y=Lys
4 - X=Hyp-Met, Y=Lys
5 - X=Hyp, Y=D-Lys
6 - X=His, Y=Lys

i: R^1=H, R^2=H, R^3=OH
a: R^1=CH_3COO, R^2=H, R^3=OH
b: R^1=$CH_3(CH_2)_{10}COO$, R^2=H, R^3=OH
c: R^1=$CH_3(CH_2)_{14}COO$, R^2=H, R^3=OH
d: R^1=$CH_3(CH_2)_{14}COO$, R^2=H, R^3=NH_2
e: R^1=$CH_3(CH_2)_{16}COO$, R^2=H, R^3=OH

Figure 1. The structure of synthesized conjugates of Gly-His-Lys analogues.