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Short communication

New conjugates of muramyl dipeptide and nor-muramyl dipeptide linked to tuftsin and retro-tuftsin derivatives significantly influence their biological activity

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Abstract:

The synthesis and biological activity of new conjugates of muramyl dipeptide (MDP) and nor-muramyl dipeptide (nor-MDP) with tuftsin and retro-tuftsin derivatives containing isopeptide bond between ϵ -amino group of lysine and carboxyl group of simple amino acids such as Ala, Gly and Val are presented. We presumed, based on the cytokine profile, that the examined conjugates of tuftsin and MDP were capable of activating antibacterial mechanisms by switching on Th1 immune response. The most active were compounds **11**, **14** and **19–23**.

Key words:

muramyl dipeptide, nor-muramyl dipeptide, tuftsin derivatives, synthesis, immunomodulator

Introduction

Muramyl dipeptide (MurNAc-L-Ala-D-isoGln; MDP) **1** (Fig. 1), a component of peptidoglycan, stimulates various functions of macrophages and increases non-specific resistance of the host against numerous microorganisms [1, 4, 7, 15–17]. Another immunomodulator, tuftsin, is a natural tetrapeptide, H-Thr-Lys-Pro-Arg-OH (TKPR) **2**, present in the peripheral blood of humans and other mammals, where it stimulates monocytes, macrophages, and neutrophils [14,

20]. We have previously reported the synthesis of the conjugates of MDP and nor-muramyl dipeptide (nor-MDP) with tuftsin [2, 3, 6, 18, 19]. The most propitious compounds were then further examined *in vivo*. The elaborated animal model of experimentally induced sepsis allowed us to screen them reliably as potential therapeutic agents [18]. Importantly, tested already compounds were found as efficient stimulators of innate immunity, which resulted in slowing down of experimental sepsis. Hence, the examined already conjugates are reckoned rather as adjuvants than separate therapeutic agents [18].

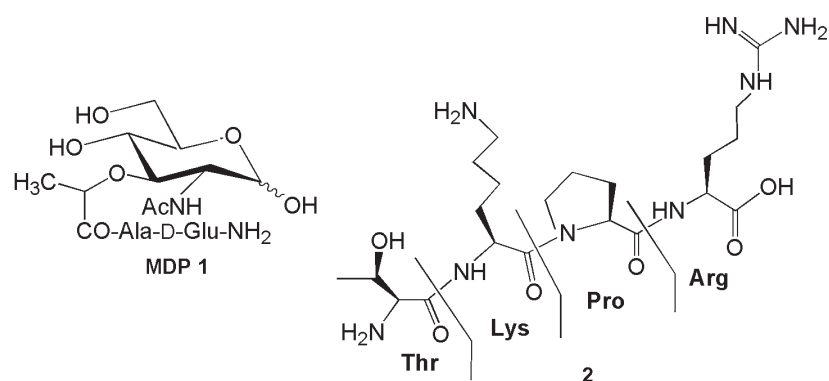
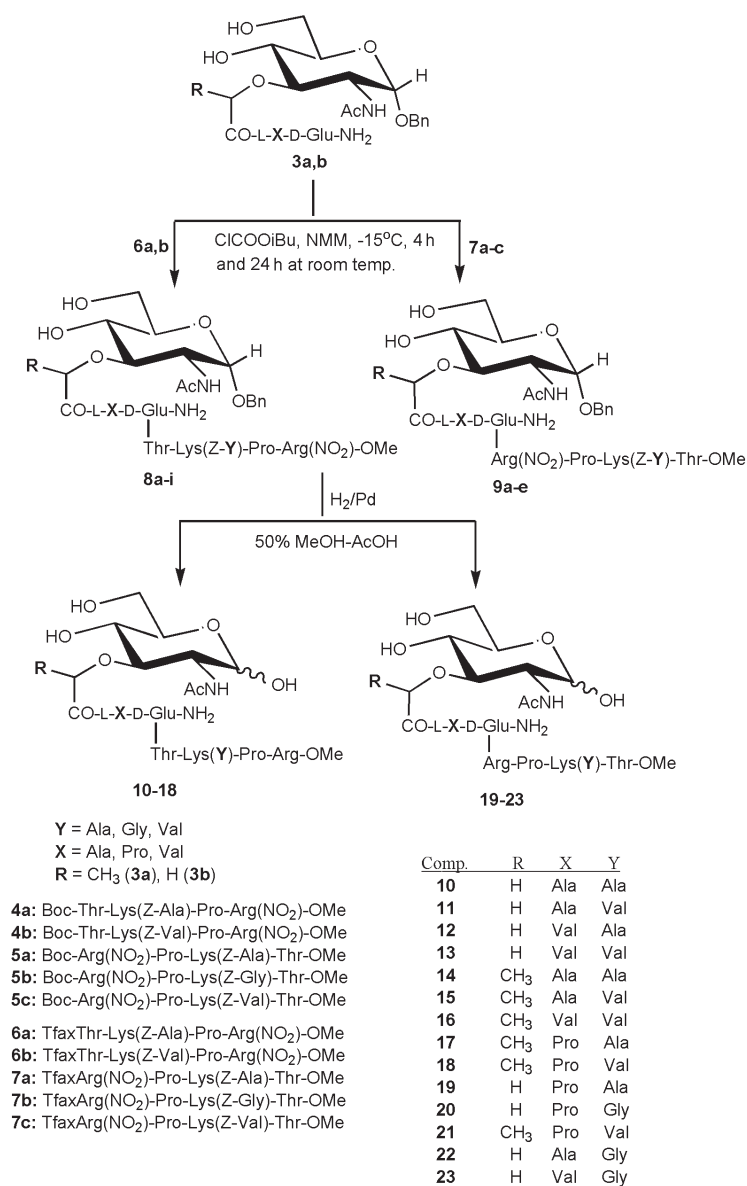


Fig. 1. Structure of muramyl dipeptide (MDP) 1 and tuftsin (TKPR) 2



Scheme 1. Synthesis of MDP or nor-MDP with tuftsin 10–18 and retro-tuftsin 19–23

In this study, we present the synthesis of new set of the conjugates **10–23** (Scheme 1) together with the screening for their immunomodulatory activity. Novel conjugates contain MDP or nor-MDP with tuftsin and retro-tuftsin derivatives with isopeptide bond. Introduction of the additional residue at ϵ -amino group of lysine by -NHCO- formation made the isopeptide bond stronger than peptide bond in central chain. This modification increased chemical resistance and activity of the conjugates as compared to tuftsin [9–11, 13]. We hope that it will also increase the half-time of the compounds and their bioavailability, which need to be verified in structure-activity relationship studies.

Materials and Methods

1-Benzyl-MDP **3a** and 1-benzyl-nor-MDP **3b** described in previous papers [5, 8, 12] were used for the synthesis of conjugates with tuftsin derivatives (H-Thr-Lys(Y)-Pro-Arg-OMe, Y = Ala, Val) and retro-tuftsin derivatives (H-Arg-Pro-Lys(Y)-Thr-OMe, Y = Ala, Gly, Val) (Scheme 1). Acylation of the Thr or Arg amino group of partially protected pentapeptides by MDP or nor-MDP was performed using the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM) in dry dimethylformamide (DMF). The protected conjugates were isolated and purified by preparative TLC. The protected pentapeptides (Boc-Thr-Lys(Z-Y)-Pro-Arg(NO₂)-OMe, Y = Ala, Val) **4a,b** or (Boc-Arg(NO₂)-Pro-Lys(Z-Y)-Thr-OMe, Y = Ala, Gly, Val) **5a–c** were synthesized by the conventional chemical procedure also using mixed anhydride method, isolated by column chromatography and purified with preparative TLC on silica gel. Finally, the Boc group was removed from the peptides by the treatment with trifluoroacetic acid (TFA) to transform them to trifluoroacetates **6a,b** and **7a–c**, which were subsequently used for the synthesis of conjugates with MDP or nor-MDP. The final products **8a–i** and **9a–e** were hydrogenolyzed in 50% methanolic acetic acid containing palladium black, purified with preparative TLC and lyophilized to obtain hygroscopic solids. Qualitative amino acid analyses of the hydrolyzates of the compounds were performed by TLC. Detection by: UV and ninhydrin. The purity of the conjugates was confirmed with ¹H-NMR and MS. The mass spectrometry analysis was carried

out on a MALDI MS (a Biflex III MALDI-TOF spectrometer, Bruker Daltonics, Germany).

General procedure for the preparation of compounds 10–23

To a stirred solution of MDP **3a** or nor-MDP **3b** derivatives (0.1108 mmol) in anhydrous DMF (1 ml) cooled to –15°C, NMM (0.1108 mmol) and isobutyl chloroformate (0.1108 mmol) were added, followed after 15 min by the addition of cooled solution of Thr-Lys(Z-Y)-Pro-Arg(NO₂)-OMe trifluoroacetate **6a,b** or Arg(NO₂)-Pro-Lys(Z-Y)-Thr-OMe trifluoroacetate **7a–c** (0.1293 mmol) and NMM (0.1293 mmol) in anhydrous DMF (0.5 ml) and the stirring was continued for 4 h at –15°C and then for 24 h at room temperature. After evaporation of the solvent, the reaction mixture was purified using radial chromatography and preparative TLC in solvent CHCl₃-MeOH (4:1, v/v) or CHCl₃-MeOH (9:1, v/v) to obtain compounds **8a–i** and **9a–e**. The final products **10–23** were hydrogenolyzed in 50% methanolic acetic acid containing palladium black, purified with preparative TLC in solvent *n*-BuOH-AcOH-H₂O (4:2:2, v/v/v), and lyophilized. Below we present analytical characteristics of the examined compounds:

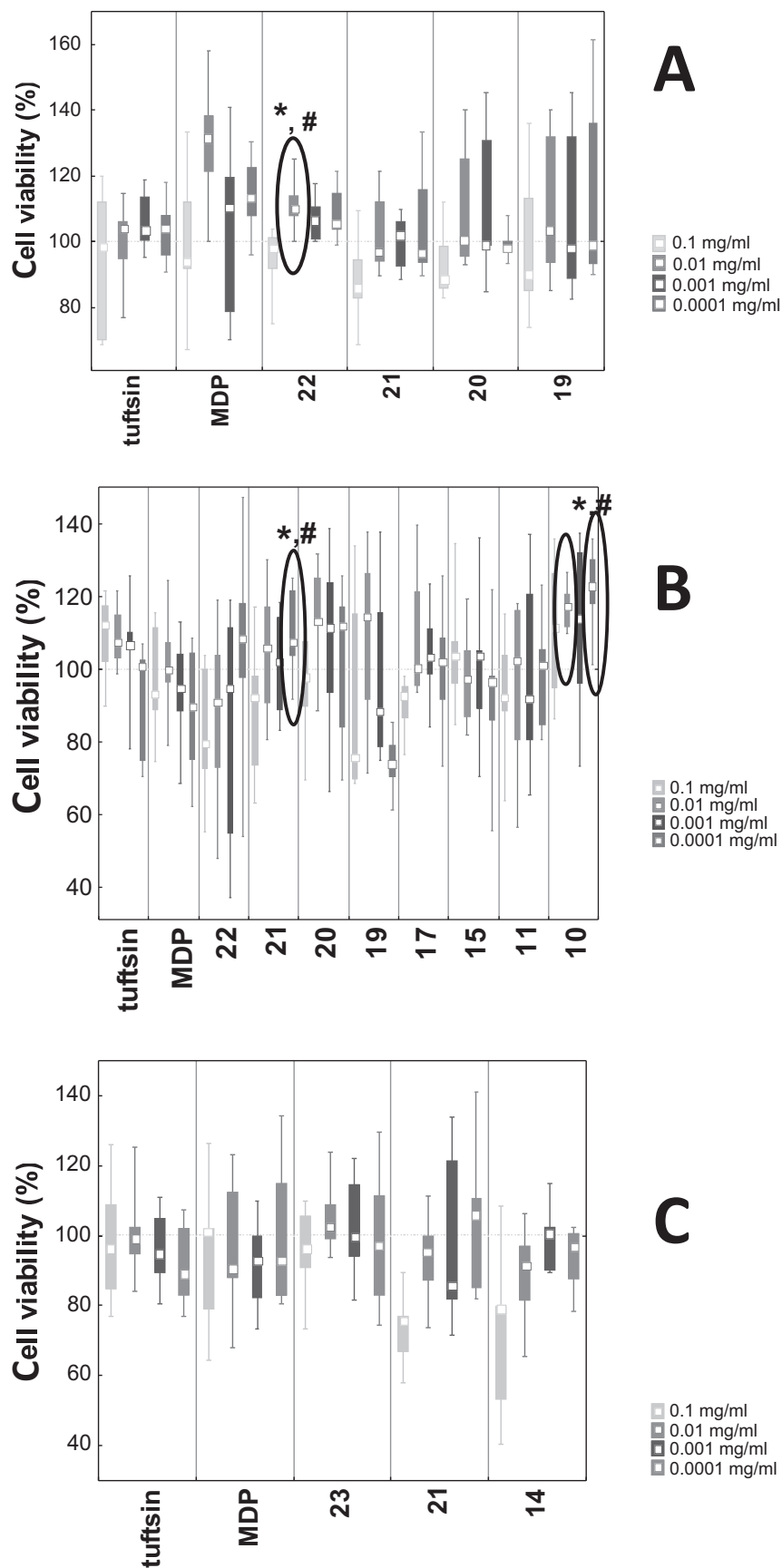
Nor-Mur(NAc)-Ala-D-Glu(Thr-Lys(Ala)-Pro-Arg-Ome)-NH₂ **10**. Yield 42%; amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr; MALDI-TOF calcd. for C₄₃H₇₅N₁₃O₁₇, 1046.1, found 1046.9 (M+H)⁺.

Nor-Mur(NAc)-Pro-D-Glu(Arg-Pro-Lys(Ala)-Thr-OMe)-NH₂ **19**. Yield 30%; amino acid analysis (6 M, 110°C, 20 h): Arg, Glu, Lys, Pro, Thr, Val; MALDI-TOF calcd. for C₄₅H₇₇N₁₃O₁₇, 1072.2, found 1073.1 (M+H)⁺.

General procedure for viability tests

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats by standard ficol gradient centrifugation and separated to peripheral blood leukocytes (PBL) and monocytes. All subsets were then disposed (4 × 10⁴ cells per well) in RPMI 1640 medium containing 10% fetal calf serum and incubated for 24 h in an atmosphere of 5% CO₂ at 37°C in triplicates with the following final concentration of the examined conjugates: 0 (control), 0.0001, 0.001, 0.01, and 0.1 mg/ml. The readout of the test was performed with colorimetric MTT assay. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was added to the wells in the final con-

Fig. 2. The viability tests – the effect of the chosen conjugates is presented. Results of MTT assay are presented as the percentage of the viability obtained in control cultures (without tested compounds, control culture = 100%). **A** – Viability of PBMC. Conjugate '19' is circled as it was significantly different from native compounds. **B** – Viability of PBL. The circled conjugates are those significantly different from native compounds. **C** – Viability of monocytes. Asterisk (*) marks significant difference between the conjugate and tuftsin and the cross (#) marks significant difference between the conjugate and MDP. The results throughout the figure are presented as medians (rectangles inside the boxes), 25–75% percentiles (the boxes) and minimum-maximum (error bars outside the boxes)



centration of 1 mg/ml and the plates were incubated for additional 4 h. Then, the reaction was stopped by addition of 100 μ l of isopropanol. Optical density was read at 570 nm on the automated plate reader (FL600, Bio-Tec, Japan). The results were then adjusted to control cultures (cultures with 0 mg/ml of the conjugates), where the viability of control was treated as 100%.

Cytokine profile

Separate set of the cultures of PBMC, PBL and monocytes was performed in order to assess conjugate-stimulated secretion of the cytokines in culture supernatants. The concentration of the examined compounds used to stimulate blood cells was 0.01 mg/ml. At the end of the culture, 50 μ l of the supernatant from each culture was collected and TNF α , IL-6 and IL-10 were measured using FlexSet kits (BDBioscience, Poland) according to the manufacturer's instructions.

Statistical analysis

The data were evaluated by means of Mann-Whitney U test using Statistica 8.1 (StatSoft, Poland). The p value < 0.05 was considered statistically significant. All the results obtained for the examined compounds were compared to: control group (unstimulated cells) and to native immunomodulators – MDP and tuftsin, in order to estimate whether the new compound surpass their activity.

Results and Discussion

The synthesized conjugates were tested for their biological activity. Viability test is a basic assay for the assessment of immunomodulatory activity of any compound. It reveals biologic activity of the compounds and identifies those potentially toxic. Using such a biological assay, we verified that some of the analyzed chemical bonds between MDP and tuftsin changed the activity of the conjugates towards immunosuppression, while others resulted in immunostimulatory activity of the compounds. Apart from the nature of the chemical bond, also the concentration of particular conjugates and the subset of the examined

immune cells influenced the results. These activities were then further confirmed with cytokine release assay [18]. We have assessed the influence of the synthesized compounds on the viability of white blood cells: either heterogeneous population of PBMC or PBL and monocytes. In the next step, we analyzed the cytokine profile secreted by immune cells when stimulated with the examined compounds. Three cytokines, two proinflammatory (IL-6, TNF α), as well as anti-inflammatory (IL-10), were detected.

Viability tests

PBMC – the viability of those cells was higher after incubation with the following conjugates: **19–21** and **22** (Fig. 2A). PBL – isolated leukocytes were the most viable in the presence of the above mentioned compounds (**19–22**) as well as conjugates **11** and **15** (Fig. 2B). Monocytes – only conjugates **14**, **21** and **23** had promising impact on the viability of these phagocytes (Fig. 2C).

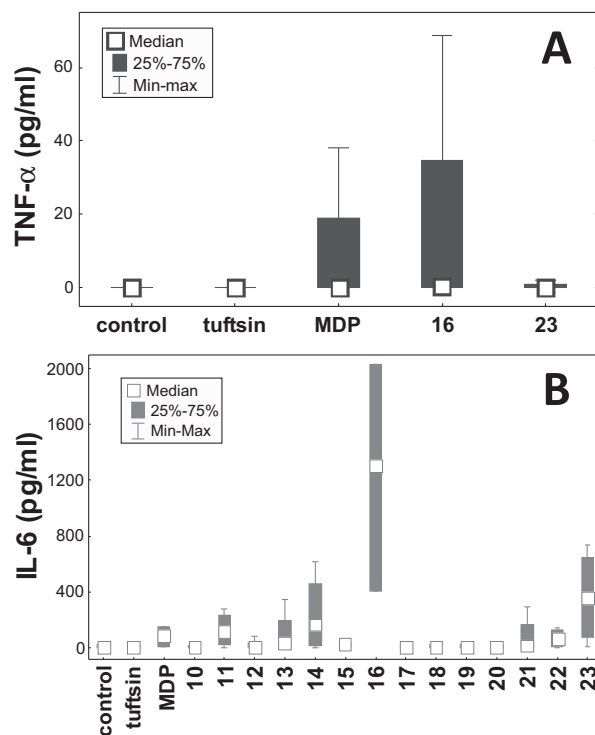


Fig. 3. The cytokine profile – selected results are presented. Results of Flexset assay are presented for reference cultures (control, tuftsin and muramyl dipeptide – MDP) and cultures with detected levels of cytokines. **A** – Secretion of TNF α . **B** – Secretion of IL-6. The results throughout the figure are presented as medians (rectangles inside the boxes), 25–75% percentiles (the boxes) and minimum-maximum (error bars outside the boxes)

Cytokine profile

The examined conjugates had little impact on TNF α production by PBMC, only compounds **16** and **23** slightly induced the secretion of this cytokine (Fig. 3A). The other analyzed proinflammatory cytokine – IL-6 – was secreted in the presence of several conjugates: **11**, **14** and **22**, and the most efficient were **16** and **23** (Fig. 3B). The examined conjugates merely influenced production of IL-10.

In conclusion, we described the synthesis and preliminary biological activity of the new conjugates MDP-containing tuftsin derivatives **10–23** (Scheme 1). We have revealed that the majority of the conjugates decreased the viability of the cells in PBMC cultures. This effect seemed to be time- and dose-dependent. Since PBMC are a heterogeneous population, it was decided to perform the viability tests using separated PBL and monocytes. These tests confirmed the inhibitory effect of the examined compounds on the viability of monocytes. The most efficient compounds were **11**, **14** and **19–23**.

The above mentioned analysis of new immunomodulating molecules is extremely important in a continuing pursuit for potent antibacterial compounds useful in the treatment of severe bacterial infections. At this point, it is difficult to give definitive statement on their usefulness or superiority over previously described conjugates. While preliminary *in vitro* tests with presented conjugates are promising, it is necessary to further verify their activity *in vivo*.

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