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Screening activities of new analogs by single amino acid modifications of 4B8M, c(Pro-Pro-β^3-HoPhe-Phe-), cyclic tetrapeptide sequence

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Abstract: A series of cyclic 4B8M tetrapeptide immunosuppressive analogs were synthesized to evaluate effects of any single amino acid residue of 4B8M sequence, c(Pro-Pro-β^3-HoPhe-Phe-), on therapeutic potential of new analogs and analyze their structure-activity relationship. The compounds were not toxic for human peripheral blood mononuclear cells (PBMC) at 100 µg/mL and were also weakly anti-proliferative at 10 µg/mL. However, some compounds significantly inhibited phytohemagglutinin A (PHA)-induced proliferation of PBMC at 100 µg/mL. Almost all peptides inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF-α) production in human whole blood cultures. A majority of the peptides were significantly inhibitory in the model of carrageenan-induced air poach inflammation as determined by reduction of cell numbers in the exudates. We postulate that among the investigated series of compounds P26 - c(Pro-Oic-β^3-HoPhe-Phe-) and P29 - c(Pro-Tic-β^3-HoPhe-Phe-) exhibited universal immunosuppressive actions by inhibition of cell proliferation, TNF-α production and carrageenan-induced inflammation. P24 - c(Pro-Pro-β^3-HoPhe-Phe[4Cl]-), in turn, presented very selective activity by affecting only carrageenan-induced inflammation, being a laboratory model for clinical rheumatoid arthritis. In conclusion, single amino acid changes in the parent peptide sequence may generate analogs of different immunotropic profiles of potential therapeutic utility.

Keywords: cyclic tetrapeptide, anti-inflammatory activity, carrageenan inflammation, TNF-α

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1. Introduction

Major presently applied classical synthetic therapeutics, including immune suppressants and antibiotics, derive from microorganisms, fungi and plants. Application of immunophilin inhibitors from natural sources, such as cyclosporine A, FK-506 and rapamycin, is associated with risk of undesirable side-effects [Frederics & Holt, 2003; Kovarik & Burtin, 2003]. Cyclic peptides represent a separate important class of immune suppressors [Abdalla, 2016; Wang et al., 2017]. Resistance to proteolysis and increased cell membrane permeability are obvious

advantages of such hydrophobic compounds [Bojarska et al., 2021; Dart et al., 2021]. Cyclolinopeptide A (CLA) was originally found and isolated from linseed oil [Kaufmann & Tobschirbel, 1959] and described as an universal inhibitor of the immune response in vitro and in vivo, graft rejection and graft versus host reaction [Wieczorek et al., 1991]. Gene sequences encoding cyclolinopeptide precursors were established [Reaney, Jia et al., 2013] as well as the distribution and concentration of corresponding cyclopeptides in flaxseed fraction [Reaney, Burnett et al., 2013]. CLA also shows antimalarial properties [Bell et al., 2000] and inhibits cholate uptake in liver cells [Munter et al., 1986; Górski et al., 2001]. Although CLA was shown to bind cyclophilin, similarly as cyclosporin A [Gallo et al., 1995], the affinity of such interaction was weak [Gaymes et al., 1997].

Numerous modifications of the peptide were performed, including linear and cyclic variants, such as chain shortening and amino acid substitutions, as well as search for establishing an essential amino acid sequence [Zimecki & Kaczmarek, 2021]. In a consequence, a cyclic c(Pro-Pro-β^3-HoPhe-Phe-) tetrapeptide (denoted as 4B8M) was synthesized and patented, exhibiting strong anti-inflammatory properties in several in vivo mouse models [Zabrocki et al., 2016; Zimecki et al., 2020]. The mechanism of 4B8M action, associated with prostaglandin metabolism, was established [Zimecki et al., 2020], as well as its antiviral properties [Zaczyńska et al., 2022]. In a search for 4B8M analogs of diverse immunotropic activities a long series of compounds was synthesized differing in single amino acid substitutions. Among these peptides a compound, denoted as P11 c(Pro-Pip-β^3-HoPhe-Phe-), and not investigated in this report, exhibited antitumor properties against melanoma cell lines in vitro [Bojarska et al., 2022].

The aim of this investigation was to screen activities of twenty-seven 4B8M analogs in *in vitro* and *in vivo* experimental models and select peptides of potential therapeutic utility in combat of inflammatory and autoimmune disorders.

2. Materials and methods

2.1. Reagents

Phosphate buffered saline solution (PBS) and Hanks' medium derived from The Institute of Immunology, Wrocław, Poland. RPMI-1640 medium was from Cibi/Life Technologies, UK, fetal calf serum (FCS) from Gibco, dexamethasone (DEX, Dexaven) from Polfa, Poland, dimethyl sulfoxide (DMSO), phytohemagglutinin A (PHA), lipopolysaccharide (LPS) from *Escherichia coli* strain O111:B4, 93-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), N,N-dimethylformamide (DMF) and carrageenan from Sigma-Aldrich, dextran 40 KDa from TdB Consultancy AB, Uppsala, Sweden. All protected amino acids, amino acids attached to the 2-Cl-trityl resin, other peptide reagents and solvents for SPPS were purchased at Iris Biotech GmbH (Marktredwitz Germany). Acetonitrile for HPLC (Regular and Gradient Grade) was purchased from Witko (Łódź, Poland).

2.2. Synthesis of the peptides

All linear tetrapeptide precursors of cyclic peptides were synthesized starting from unprotected amino acids (Phenylalanine and substituted Phenylalanines, Tyr(tBu) and Trp(Boc), Homophenylalanine (HoPhe), 1- and 2-Naphtylalanine (1Nal and 2Nal, respectively, 3-Pirydyloalanine (3Pal) and Tetrahydroisoquino-linecarboxylic acid (Tic) attached to 2-Chlorotrityl resin applying Fmoc/t-Bu strategy on CEMI automatic synthesizer, equipped with a microwave reactor chamber. We used original procedures implemented by Millipore in the software. Addition of each Amino Acid contained the following steps, each of them being microwave assisted:
- soaking of the resin (NMP, 3 x 3 min),
- double coupling [Fmoc-Aaa-OH (4 eq in the first coupling then 2 eq. in the second coupling**)**, HATU/HOAt (3.5 eq in the first coupling then 1.75 eq. in the second coupling**)**, in the presence of equimolar amount of DIPEA (3.5 eq. then 1.75 eq. in N-Methyl Pyrrolidone (NMP), 2 min and 2 min**],**
- capping (Acetic Anhydride/NMP/DIPEA, 2 min)
- washings (NMP, 5 x 1 min)
- deprotection (20% Piperidine in DMF, 3 x 1 min),
- washings (NMP, 5 x 1 min).

Linear precursors were cleaved from the resin with a cocktail composed of DCM, AcOH and TFE (Dichloro-methane/Acetic Acid/2,2,2-Trifluoroethanol,8:1:1). This procedure leaves the side chain protecting groups (Boc and t-Bu) intact. The cleavage cocktail and washings after filtration from the resin were combined, then diluted with n-hexane and evaporated under reduced pressure. A residual crude linear peptide was redissolved in DCM, diluted with n-hexane and evaporated again. This procedure was repeated several times to remove all traces of acetic acid, which could be dangerous during cyclisation. After overnight drying in desiccator over KOH, under reduced pressure, crude linear peptides were checked for purity (HPLC). Crude linear peptides were subjected to cyclisation. All cyclizations were performed in DCM in a very high dilution (0.25 millimole/L DCM) with the aid of EDC hydrochloride (2 equiv.) in the presence of HOAt

(1 equivalent). All cyclizations were verified for completeness by HPLC technique. Sample prepared from 1 mL of the evaporated reaction solution was dissolved in 50% Acetonitrile/water. Disappearance of a starting peptide was followed by concentration of reaction solution to small volume and dilution with 200 mL ethyl acetate. Such solution was extracted with 2 × 50 mL 0.1 M HCl aqueous solution, 1 × 50 mL water, 3 × 50 mL 1M $KHCO_3$ aq. and 1 × 50 mL brine and dried with anhydrous $MgSO_4$. Crude products were obtained after the drying agent was filtered off and the solution was evaporated. They were further subjected to preparative RP-HPLC purification on Kromasil C_8 column (100-5-CB, 21.2 × 150 mm) in a gradient solvent system A+B, composed of part B – azeotrope acetonitrile-water (~81.5%) acidified to pH 2 with 0.38 mL TFA, and part A – 100 water acidified to pH 2 with 0.5 mL TFA. The fractions containing a peptide of correct molecular weight (HPLC-MS, evaluated by independent laboratory) and purity were pooled, concentrated to remove most of acetonitrile, and lyophilized.

For the in vitro experiments the peptides were initially dissolved in DMSO (5 mg/mL) and subsequently in the culture medium to a desired concentration. The amino acid composition of the peptides in presented in Table 1. The chemical character of the substituted, modified amino acids, is described in the table legend.

2.3. Mice

CBA mice 8–12 weeks old, weighing 19–22 g, delivered by the Breeding Centre of Laboratory Animals at the Institute of Occupational Medicine, Łódź, Poland, were used for the study. Mice were housed at 21–22^o C with a 12/12-h light/dark cycle and had free access to commercial laboratory chow and filtered tap water. The local ethics committee at the Institute of Immunology and Experimental Therapy, Wrocław, Poland, approved the study (permission #37/2012).

2.4. Cell viability test

Venous blood from a single donor was withdrawn into heparinized syringes and diluted twice with PBS. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a ficoll-uropoline gradient (density of 1.077 g/mL) and centrifuged at 800 x g for 20 min at 4^o C. Cells from the interphase were then washed 3 x with Hanks' medium, re-suspended in the culture medium at the density of 2 x 10^5 cells/100 µL/well and cultured with the tested peptides for 24 h in a cell culture incubator. Then, the cell viability was evaluated using MTT colorimetric method [Hansen et al., 1989]. The results are presented as the mean OD ± SE from quadruplicate determinations.

2.5. Proliferation test

PBMC were distributed into 96-well flat-bottom plates in 100 µL aliquots (2 × 10^5 cells/well). PHA was added at a concentration of 5 µg/mL. The compounds were tested at doses of 10 and 100 µg/mL. DMSO at appropriate dilutions served as control. Control (-) denotes no addition of PHA and control (PHA) addition of PHA without DMSO. After a four-day incubation in a cell culture incubator, the proliferative response of the cells was determined by the colorimetric MTT method [Hansen et al., 1989]. The results are presented as the mean OD ± SE from quadruplicate determinations.

2.6. Induction of TNF-α activity

Human whole blood was diluted 5 x with RPMI-1640 medium and distributed to 24-well culture plates in 1 mL aliquots. The cultures were stimulated with LPS (1 µg/mL) from *E. coli* and the studied compounds were added at the concentration of 5 and 25 µg/mL. After an overnight incubation the supernatants were harvested and frozen at -20^o C until cytokine determination.

2.7. Determination of TNF-α activity

Tumor necrosis factor alpha (TNF-α) activity was determined by a bioassay [Espevik & Nissen-Meyer, 1986]. In brief, WEHI-164.13 cells were cultured in 96-well plates at a density of 2 x 10^4 cells/well (with addition of actinomycin D (1 µg/mL) with serial dilutions of either culture supernatants or exudates from air poaches. Cell kill was evaluated after 20h using MTT colorimetric method [Hansen et al., 1989]. Optical density (OD) was measured at 550 nm and reference wavelength of 630 nm using a colorimetric reader Dynatech 5000. In that bioassay, one unit of TNF-α (corresponding to 10 pg/mL) is defined as an inverse dilution of the studied samples, at which 50% cells are killed. The results were presented in picograms of TNF-α/mL. Control (-) – no LPS, control (+) addition of LPS, no DMSO.

2.8. Carrageenan inflammation in air poach

The test was performed on mice as described elsewhere with minor modifications [Bottomley et al., 1988]. The experimental groups consisted of 5 mice. Air poaches were formed by a subdermal injection into the dorsal region (in a halothane anesthesia) of 5 mL of air (needle 23G x 11/4, 5 mL syringe). On the next day the air poaches were given an additional 1 mL of air and an inflammatory process was elicited by an injection of 1% of carrageenan in PBS. The peptides were initially dissolved in DMSO (50 mg of the peptide in 0.5 mL of DMSO) and then in PBS to obtain a

concentration of 100 µg/0.2 mL, which was subsequently given intraperitoneally (i.p.) into mice. The compounds were administered at 24h, following formation of the air poach (day 1 of the test). After 90 min carrageenan was injected into the air poach. The mice from the "background" (BG) group were given 0.9% NaCl into the air poaches. Control mice were given carrageenan into the air poaches and appropriately diluted DMSO i.p. On a next day after carrageenan injection (second day of the test) mice were subjected to halothane anesthesia and exudates cells were harvested by injection of 1 mL of 0.9% NaCl into the air poaches and a subsequent aspiration by means of a syringe. In the exudates the cells were diluted with Türk's solution and were counted in a Bürker hemocytometer. The results were presented as the number of the exudates cells (mean values ±SE) per air poach.

2.9. Statistics

The results are presented as mean values ± standard error (SE). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with the Tukey's test to evaluate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal-Wallis' analysis of variance, as indicated in the text. Significance was determined at $P<0.05$. Statistical analysis was performed using STATISTICA 7.0 for Windows.

3. Results

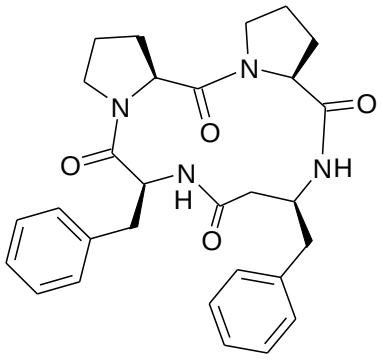
3.1. Characterization of the substitutions in 4B8M sequence

Below we summarize our approach to modify the sequence of the parent 4B8M peptide which structure is shown in Scheme 1.

The character of the substituted amino acids is explained, and peptide labels and sequences are presented in Table 1. The modifications were designed not by theoretical calculations, but simply by switching properties of amino acid side chains at one position at a time (size (bulkiness) and/or hydrophilicity/lipophilicity properties). This matter is described in detail below.

Position 1 or position 2 [both Pro] – the proline ring is replaced with saturated five- and six-membered rings or open-chain side chain with different bulkiness (size) and/or nucleophilic/electrophilic *versus* lipophilic/hydrophilic character.

Scheme 1. The structure of c(Pro-Pro-β^3-HoPhe-Phe-), a sequence of the lead compound (4B8M).



Position 1 – P21 and P21-b [Hydroxyproline and Hyp(tBu)], P22 (dehydroProline), P31 [Octahydroindolecarboxylic acid (Oic)], SP00 and SP00-b [Serine and Ser(tBu)],

Position 2 – P11 [Pipecolic acid *alias* Homoproline (Pip)], P26 (Octahydroindolecarboxylic acid (Oic)[, P29 (Tetrahydroisoquinolinecarboxylic acid, [Tic]), P00S and P00S-b [Serine and Ser(tBu)],

Position 3 [β^3-HoPhe] – β^3-Homologues of other "coded" natural aromatic amino acids: P32 - β^3-HomoTryptophane, (β^3-HoTrp), P33 and P33b - β^3-HomoTyrosine (β^3-HoTyr) and β^3-HoTyr(tBu),

Position 4 [Phe] – Phe phenyl ring is replaced with aromatic rings with different bulkiness (size) and distance between rings [Trp, Tyr(tBu), 1Nal, 2Nal, Phe(4-I),Tic, Bip, HoPhe) and/or nucleophilic/electrophilic *versus* lipophilic/hydrophilic character: P08 (Tryptophane, Trp), P10-b and P10-z (Tyrosine, [Tyr] and [Tyr(tBu)]); P23-1 and P23-2 (1-Naphtylalanine [1Nal] and 2-Naphtylalanine [2Nal]); P24 (4-Chloro-Phenylalanine, [Phe(4-Cl)]); P25 (4- Fluoro-Phenylalanine, [Phe(4-F)]); P25I (4-Iodo-Phenylalanine, [Phe(4-I)]); P27 (4-Methyl-Phenylalanine, [Phe(4-Me)]); P28 (3-Pirydylalanine, [3-Pal]), the most electrophilic and hydrophilic aromatic ring; P30 (tetrahydroisoquinolinecarboxylic acid, [Tic]); P34 (Biphenylalanine, [Bip]); P35 (HomoPhenylalanine, [HoPhe]).

3.1. Evaluation of the peptides on viability of human peripheral blood mononuclear cells (PBMC)

PBMC were incubated with the peptides for 24h at concentration of 10 and 100 µg/mL. The viability of cells was determined by the colorimetric method. The results are presented in Figure 1 ABCD. The compounds were generally not toxic at 10 and 100 µg/mL. Some peptides (P25, P29, P33) showed low toxicity, and P34 even enhanced the cell viability.

Table 1. Peptide labels and sequences.

Peptide	Sequence
4B8M (P00)	**Cyclo-[Pro-Pro-β^3-HoPhe-Phe-]**
P08	**Cyclo-[Pro-Pro-β^3-HoPhe-Trp-]**
P10-b	**Cyclo-[Pro-Pro-β^3-HoPhe-Tyr-]**
P10-z	**Cyclo-[Pro-Pro-β^3-HoPhe-Tyr(t-Bu)-]**
P11	**Cyclo-[Pro-Pip-β^3-HoPhe-Phe-]**
P21	**Cyclo-[Hyp-Pro-β^3-HoPhe-Phe-]**
P21-b	**Cyclo-[Hyp(t-Bu)-Pro-β^3-HoPhe-Phe-]**
P22	**Cyclo-[ΔPro-Pro-β^3-HoPhe-Phe-]**
P23-1	**Cyclo-[Pro-Pro-β^3-HoPhe-1Nal-]**
P23-2	**Cyclo-[Pro-Pro-β^3-HoPhe-2Nal-]**
P24	**Cyclo-[Pro-Pro-β^3-HoPhe-Phe(4Cl)-]**
P25	**Cyclo-[Pro-Pro-β^3-HoPhe-Phe(4F)-]**
P25-i	**Cyclo-[Pro-Pro-β^3-HoPhe-Phe(4I)-]**
P26	**Cyclo-[Pro-Oic-β^3-HoPhe-Phe-]**
P27	**Cyclo-[Pro-Pro-β^3-HoPhe-Phe(4-Me)-]**
P28	**Cyclo-[Pro-Pro-β^3-HoPhe-Tic-]**
P29	**Cyclo-[Pro-Tic-β^3-HoPhe-Phe-]**
P30	**Cyclo-[Pro-Pro-β^3-HoPhe-3-Pal-]**
P31	**Cyclo-[Oic-Pro-β^3-HoPhe-Phe-]**
P32	**Cyclo-[Pro-Pro-β^3-HoTrp-Phe-]**
P33	**Cyclo-[Pro-Pro-β^3-HoTyr-Phe-]**
33-b	**Cyclo-[Pro-Pro-β^3-HoTyr(t-Bu)-Phe-]**
P34	**Cyclo-[Pro-Pro-β^3-HoPhe-Bip-]**
P35	**Cyclo-[Pro-Pro-β^3-HoPhe-HoPhe-]**
P00S	**Cyclo-[Pro-Ser-β^3-HoPhe-Phe-]**
P00S-b	**Cyclo-[Pro-Ser(t-Bu)-β^3-HoPhe-Phe-]**
SP00	**Cyclo-[Ser-Pro-β^3-HoPhe-Phe-]**
SP00-b	**Cyclo-[Ser(t-Bu)-Pro-β^3-HoPhe-Phe-]**

Figure 1A. Effects of P22, P26, P27, P30, P33-b peptides on viability of PBMC. The quadruplicate cultures were incubated with the tested peptides at the concentrations of 10 and 100 μg/mL for 24h and cell viability was evaluated using MTT colorimetric method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: 4B8M NS (P=1.0000); P22 NS (P=0.9753); P26 NS (P=0.9992); P27 NS (P=0.9997); P30 NS (P=0.8178); P33-b NS (P=1.0000); 100 μg/mL: 4B8M NS (P=0.1700); P22 NS (P=1.0000); P26 P=0.0001; P27 P=0.0236; P30 NS (P=0.9882); P33-b P=0.0090 (ANOVA).

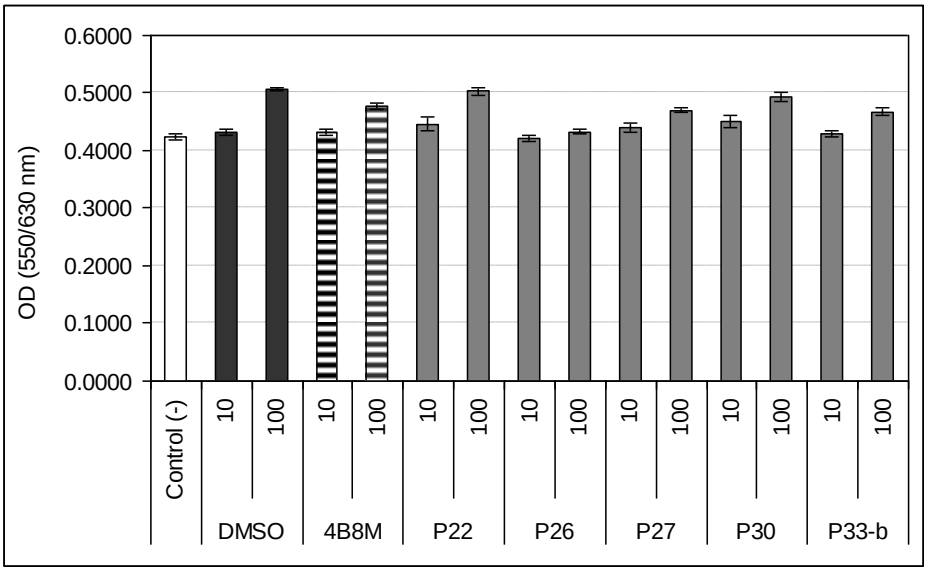


Figure 1B. Effects of P25, P28, P31, P34, P35 peptides on viability of PBMC. The quadruplicate cultures were incubated with the tested peptides at the concentrations of 10 and 100 μg/mL for 24h and cell viability was evaluated using MTT colorimetric method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: P25 NS (P=0.8597); P28 NS (P=0.9997); P31 NS (P=1.0000); P34 P=0.0005; P35 P=0.0222; 100 μg/mL: P25 P=0.0001; P28 NS (P=0.9978); P31 NS (P=0.9531); P34 P=0.0001; P35 NS (P=1.0000) (ANOVA).

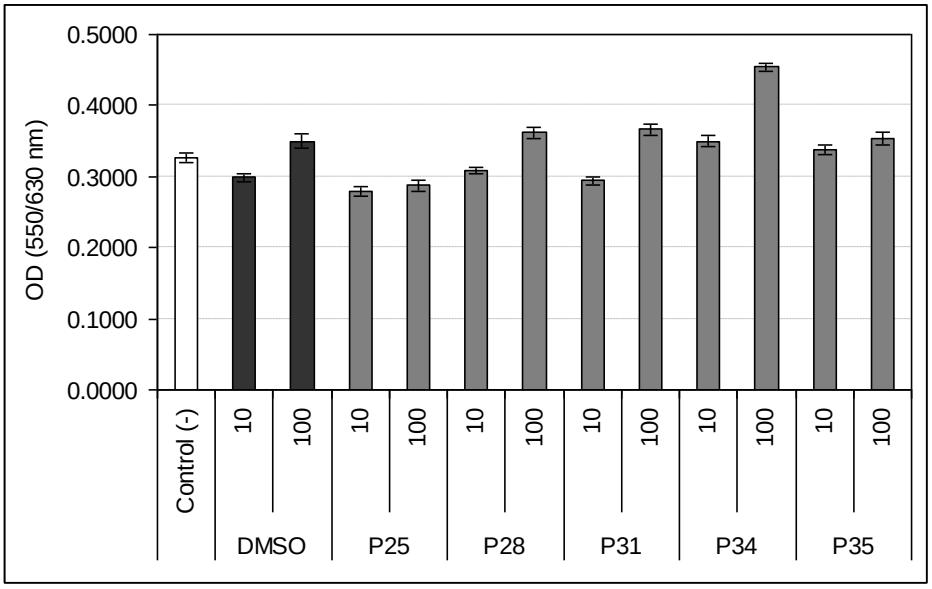


Figure 1C. Effect of P21-b, P25-i, P32, P00S-b, SP00-b peptides on viability of PBMC. The quadruplicate cultures were incubated with the tested peptides at the concentrations of 10 and 100 μg/mL for 24h and cell viability was evaluated using MTT colorimetric method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: P21-b NS (P=0.9976); P25-i NS (P=0.9992); P32 NS (P=0.9954); P00S-b NS (P=0.9962); SP00-b (P=1.0000); 100 μg/mL: P21-b NS (P=0.9185); P25-i NS (P=0.9602); P32 NS (P=0.9984); P00S-b NS (P=0.9250); SP00-b P=0.0466 (ANOVA).

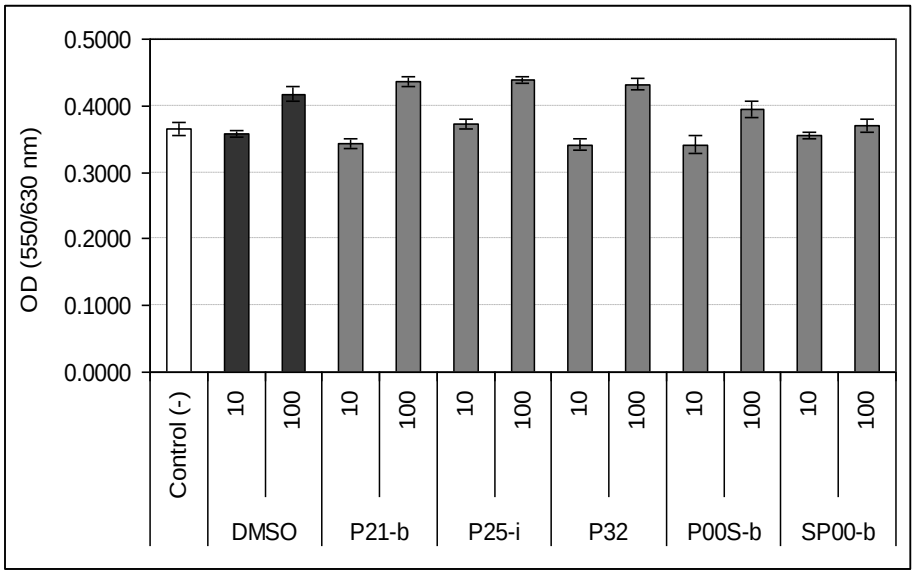


Figure 1D. Effects of P21, P23-2, P24, P29, P33 peptides on viability of PBMC. The quadruplicate cultures were incubated with the tested peptides at the concentrations of 10 and 100 μg/mL for 24h and cell viability was evaluated using MTT colorimetric method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: P21 NS (P=0.6250); P23-2 NS (P=0.9972); P24 NS (P=0.9999); P29 NS (P=0.8876); P33 (P=0.0001); 100 μg/mL, DMSO vs peptides: P21 P=0.0019; P23-2 NS (P=0.4949); P24 NS (P=0.1122); P29 P=0.0001; P33 P=0.0001 (ANOVA).

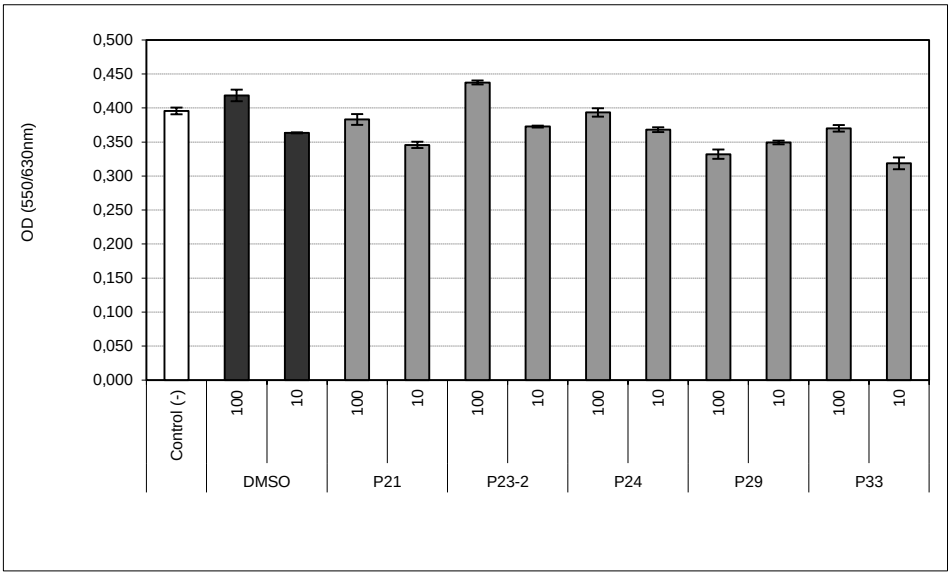
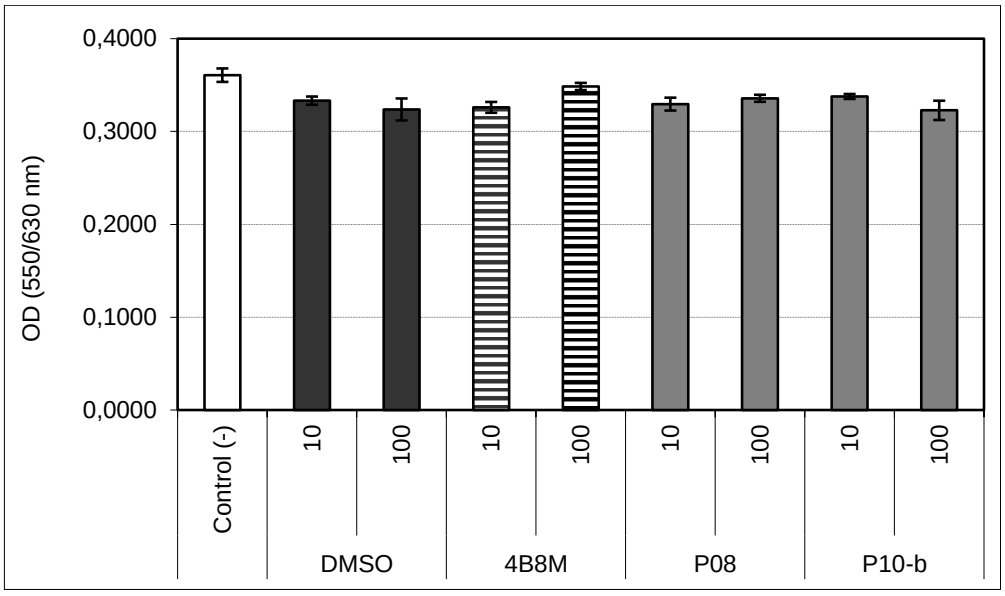


Figure 1E. Effects of 4B8M, P08, P10-b peptides on the viability of PBMC. The quadruplicate cultures were incubated with the tested peptides at the concentrations of 10 and 100 μg/mL for 24h and cell viability was evaluated using MTT colorimetric method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: 4B8M NS (P=0.9999); P08 NS (P=1.0000); P10-b NS (P=1.0000); 1μg/ml: 4B8M NS (P=0.3176); P08 NS (P=0.9999); P10-b NS (P=1.0000); 100 μg/mL: 4B8M NS (P=0.0669); P08 NS (P=0.9957); P10-b NS (P=1.0000) (ANOVA).



3.2. Effects of the peptides on PHA-induced PBMC proliferation

Then, the peptides were tested for their ability to suppress mitogen (PHA)-induced PBMC proliferation at doses of 10 and 100 μg/mL. The peptides were, in general, weakly antiproliferative (lack of inhibition at 10 μg/mL). However, three compounds (P26, P34 and P29) caused significant inhibition of the PHA-induced proliferation at 100 μg/mL. P23-2 and P25-I were less inhibitory (Figure 2 ABCDE). The suppressive action of parent 4B8M was weak, although statistically significant at 100 μg/mL.

Figure 2A. Effects of P22, P26, P27, P30, P33-b peptides on PHA-induced PBMC proliferation. The quadruplicate cultures were stimulated with PHA and the studied compounds were added at the concentrations of 10 and 100 μg/mL. After a four-day incubation the proliferative response of the cells was determined by the colorimetric MTT method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: P22 P=0.0275; P26 P=0.0001; P27 NS (P=0.9121); P30 NS (P=0.9850); P33-b NS (P=0.9850); 100 μg/mL: P22 P=0.0001; P26 P=0.0001; P27 NS (P=0.8195); P30 NS (P=0.9871); P33-b P=0.0010 (ANOVA).

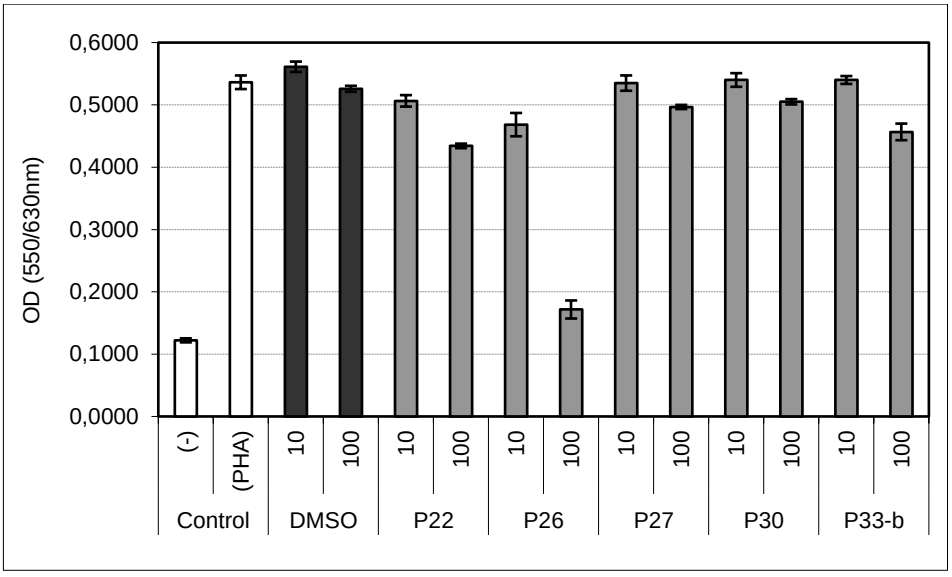


Figure 2B. Effects of P25, P28, P31, P34, P35 peptides on PHA-induced PBMC proliferation. The quadruplicate cultures were stimulated with PHA and the studied compounds were added at the concentrations of 10 and 100 μg/mL. After a four-day incubation the proliferative response of the cells was determined by the colorimetric MTT method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: all comparisons NS (P=1.0000); 100 μg/mL: P25 NS (P=1.0000); P28 NS (P=1.0000); P31 NS (P=1.0000); P34 NS (P=0.1992); P35 NS (P=1.0000) (ANOVA of Kruskal-Wallis).

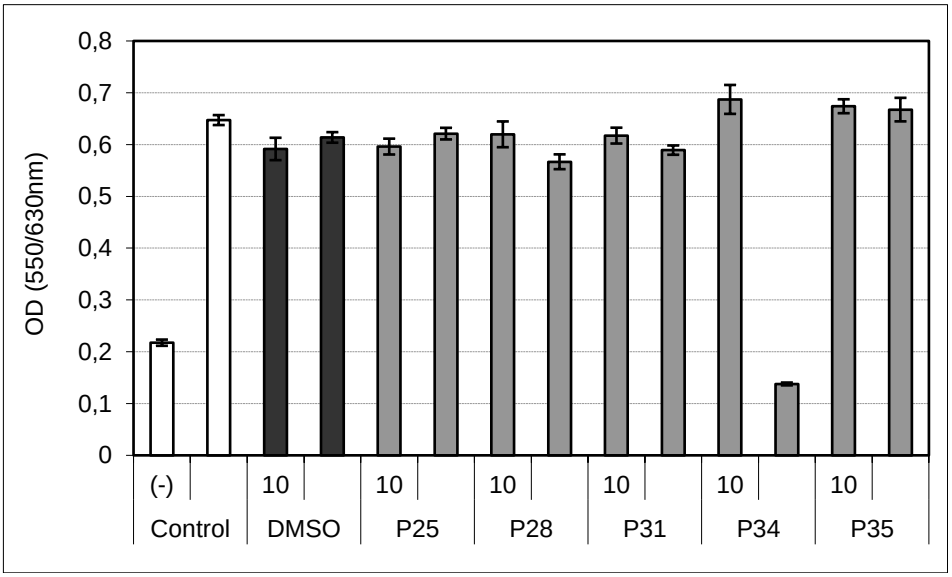


Figure 2C. Effects of 4B8M, P21-b, P25-i, P32, P00S-b, SP00-b peptides on PHA-induced PBMC proliferation. The quadruplicate cultures were stimulated with PHA and the studied compounds were added at the concentrations of 10 and 100 μg/mL. After a four-day incubation the proliferative response of the cells was determined by the colorimetric MTT method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: 4B8M NS (P=0.7530); P21-b P=0.0141; P25-i P=0.0001; P32 P=0.0127; P00S-b NS (P=1.0000); SP00-b NS (P=0.2258); 100 μg/mL: 4B8M P=0.0016; P21-b P=0.0056; P25-i P=0.0001; P32 P=0.0023; P00S-b NS (P=0.7035); SP00-b (P=0.9976) (ANOVA).

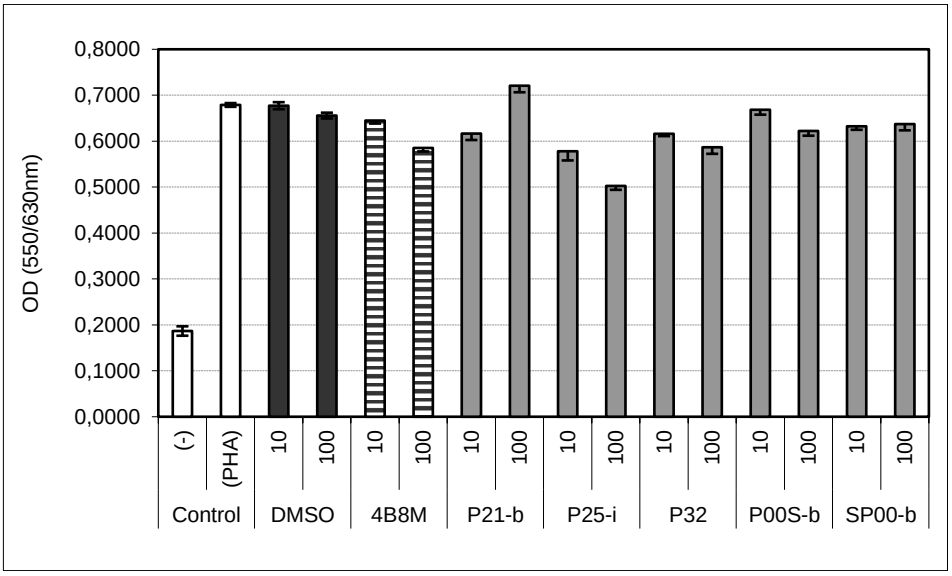


Figure 2D. Effects of P21, P23-2, P24, P29, P33 peptides on PHA-induced PBMC proliferation. The quadruplicate cultures were stimulated with PHA and the studied compounds were added at the concentrations of 10 and 100 μg/mL. After a four-day incubation the proliferative response of the cells was determined by the colorimetric MTT method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 μg/mL: P21 NS (P=0.7122); P23-2 NS (P=1.0000); P24 NS (P=1.0000); P29 NS (P=1.0000); P33 NS (P=1.0000); 100 μg/mL: P21 P=0.0436; P23-2 P=0.0002; P24 NS (P=0.4681); P29 P=0.0001; P33 (P=1.0000) (ANOVA of Kruskal-Wallis).

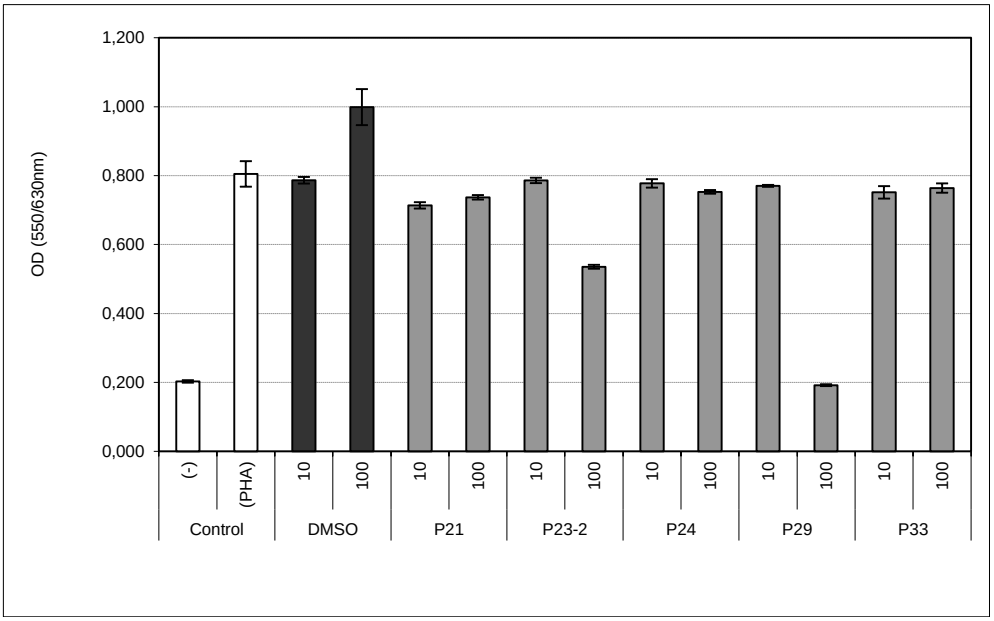
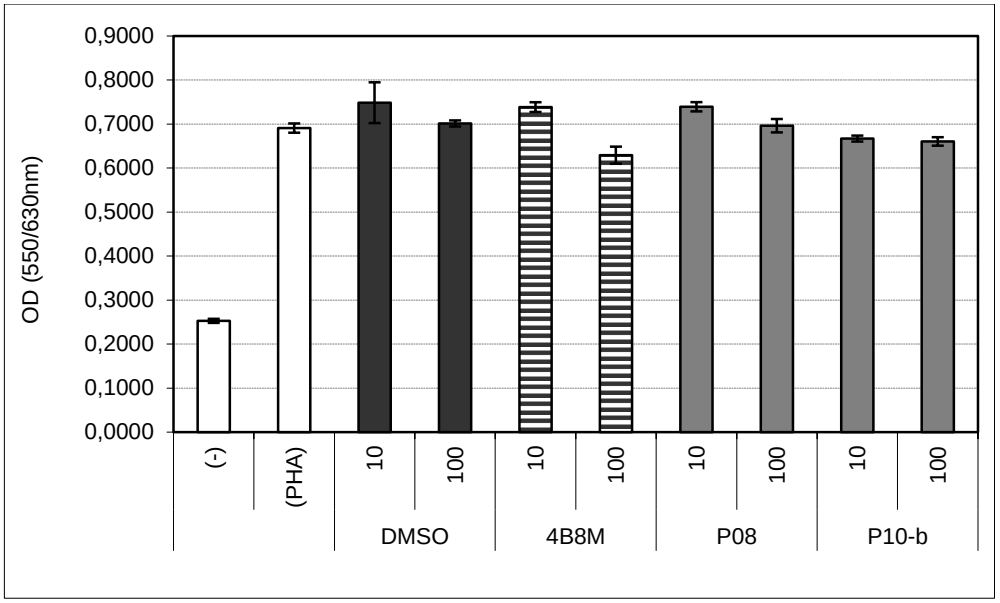


Figure 2E. Effects of 4B8M, P08, P10-b peptides on PHA-induced PBMC proliferation. The quadruplicate cultures were stimulated with PHA and the studied compounds were added at the concentrations of 10 and 100 µg/mL. After a four-day incubation the proliferative response of the cells was determined by the colorimetric MTT method. Statistics (all comparisons vs. DMSO at appropriate dilutions): 10 µg/mL: 4B8M NS (P=1.0000); P08 NS (P=1.0000); P10-b NS (P=0.2763); 100 µg/mL: 4B8M NS (P=0.4941); P08 NS (P=1.0000); P10-b NS (P=0.9933) (ANOVA).



3.3. Effects of the peptides on LPS-induced TNF-α production in whole blood cultures

Almost all peptides inhibited LPS-induced TNF-α production in human whole blood cultures at both studied concentrations (5 µg/mL and 25 µg/mL). Some peptides showed inhibition only at 25µg/mL and SP00-b was stimulatory at 5 µg/mL. Most potent inhibitory peptides were: P22, P26, P27, P23-2, P24 and P29 (Figure 3 ABCD). TNF-α was also inhibited by P08 and P10-b, by 22.7% and 14.0% at 10 µg/mL concentration (data not shown). 4B8M peptide markedly (by about 50%) inhibited TNF-α production at both concentrations.

Figure 3A. Effects of P22, P26, P27, P30, P33-b peptides on LPS-induced TNF-α production in whole human blood cultures. The cultures were stimulated with LPS and the studied compounds were added at the concentration of 5 and 25 µg/mL. After an overnight incubation the supernatants were harvested for cytokine determination.

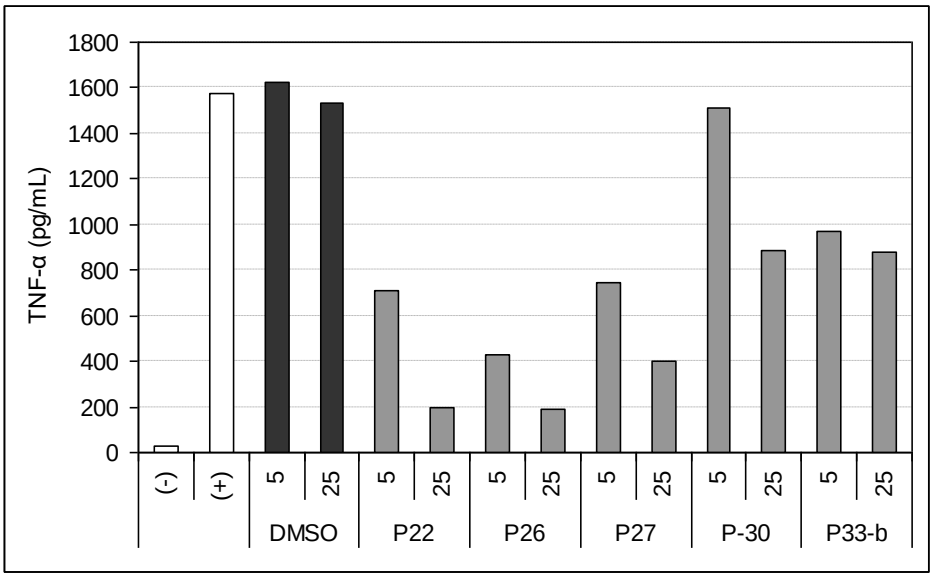


Figure 3B. Effects of P25, P28, P31, P34, P35 peptides on LPS-induced TNF-α production in whole human blood cultures. The cultures were stimulated with LPS and the studied compounds were added at the concentration of 5 and 25 μg/mL. After an overnight incubation the supernatants were harvested for cytokine determination.

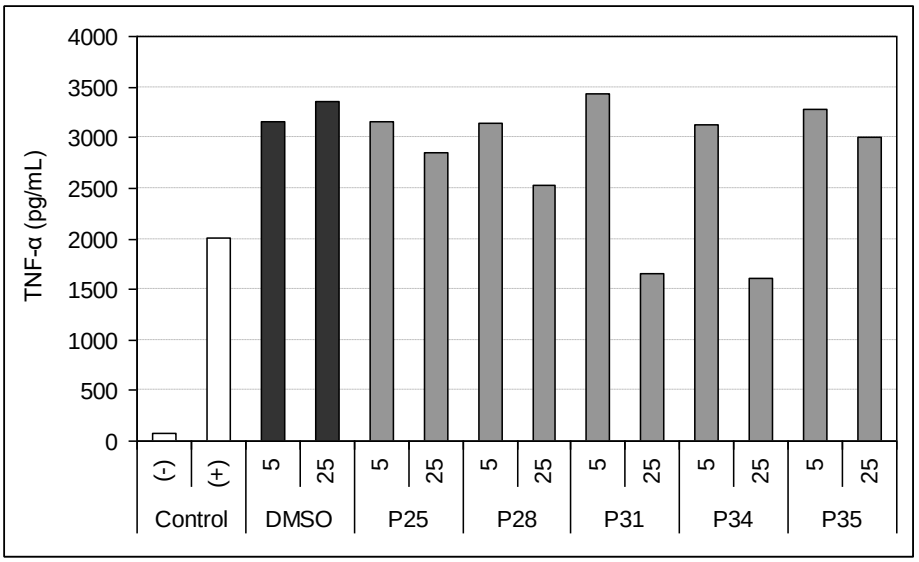


Figure 3C. Effects of P21-b, P25-i, P32, P00S-b, SP00-b peptides on LPS-induced TNF-α production in whole human blood cultures. The cultures were stimulated with LPS and the studied compounds were added at the concentration of 5 and 25 μg/mL. After an overnight incubation the supernatants were harvested for cytokine determination.

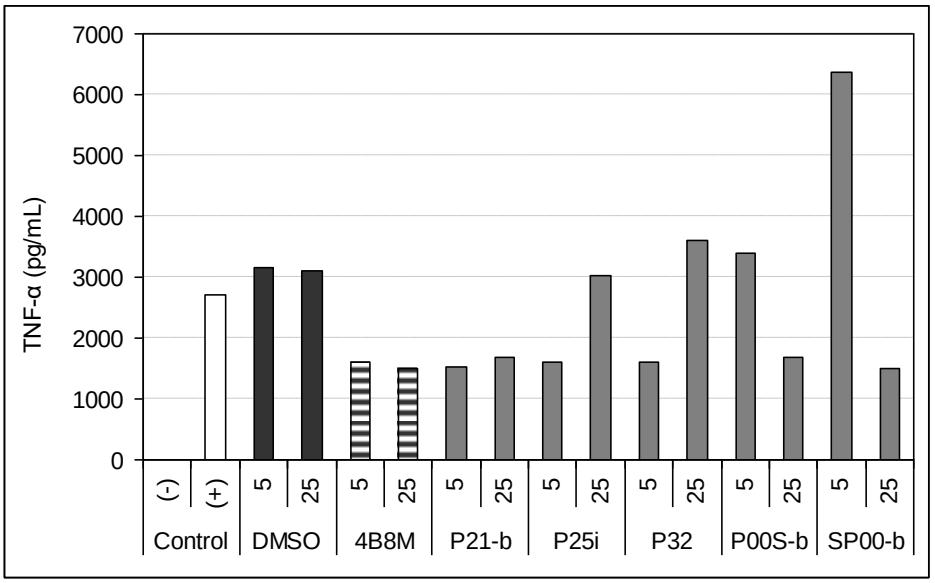
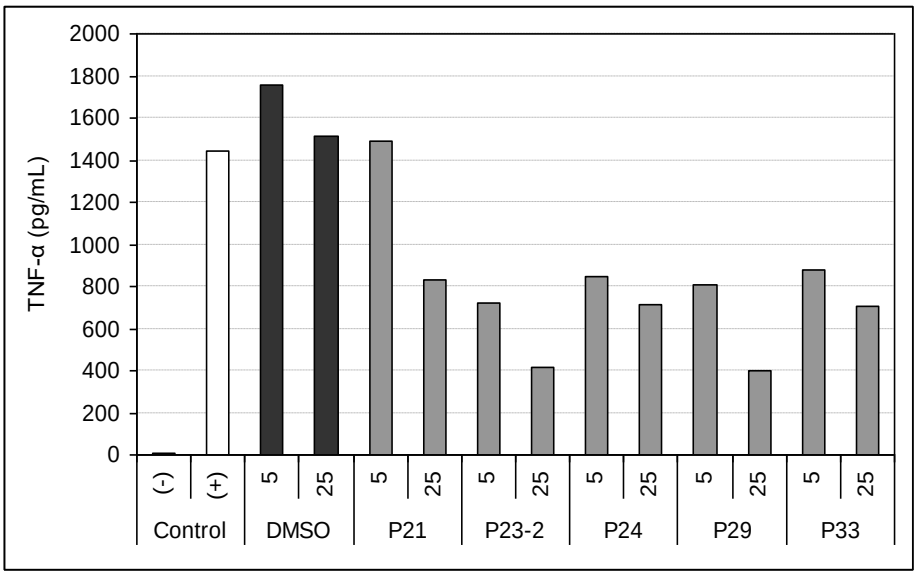


Figure 3D. Effects of P21, P23-2, P24, P29, P33 peptides on LPS-induced TNF-α production in whole blood cultures. The cultures were stimulated with LPS and the studied compounds were added at the concentration of 5 and 25 μg/mL. After an overnight incubation the supernatants were harvested for cytokine determination.



3.4. Effects of the peptides on carrageenan-induced cell infiltration in the air poach model

Effects of the peptides, given i.p., on the number of exudates cells in the air poaches, are shown in Figure 4 AB. The parent 4B8M peptide and dexamethasone were used as reference compounds. Most peptides showed significant inhibitory activities by reducing cell numbers in the air poach exudates. P24, P30 and P23-2 were most potent and the action of P24 exceeded these of dexamethasone and 4B8M.

Figure 4A. Effects of 4B8M, P22, P26, P27, P35 peptides on carrageenan-induced cell infiltration into the air poaches. The peptides were given i.p. into mice at a dose of 100 μg/mouse, at 24h following formation of the air poach. After 90 min carrageenan was injected into the air poach and the exudates were harvested on a next day. Statistics: Control vs: DEX P=0.0034; 4B8M P=0.0005; P22 NS (P=0.7275); P26 P=0.0032; P27 NS (P=0.0659); P35 NS (P=1.0000) (ANOVA of Kruskal-Wallis).

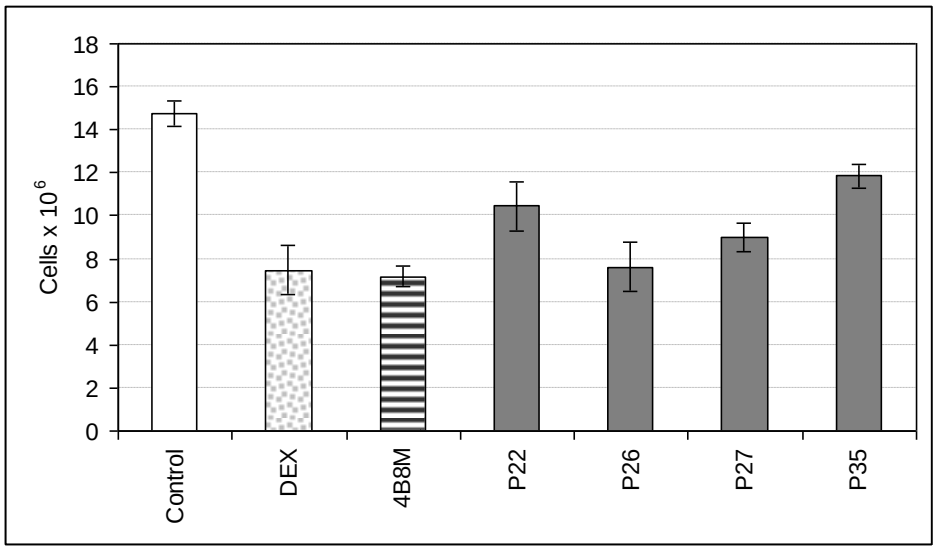
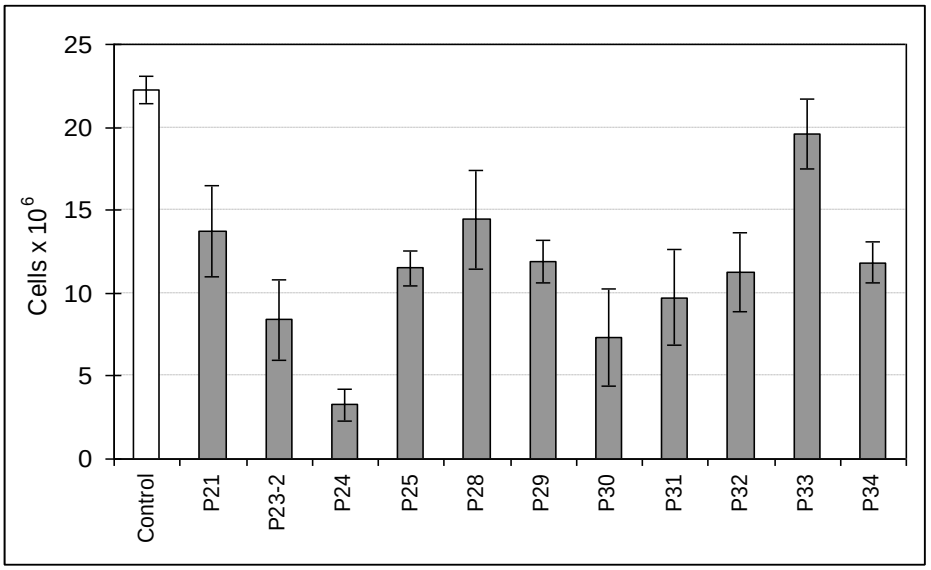


Figure 4B. Effects of P21, P23-2, P24, P25, P28, P29, P30, P31, P32, P33, P34 peptides on carrageenan-induced cell infiltration into the air poaches. The peptides were given i.p. into mice at a dose of 100 μg/mouse, at 24h following formation of the air poach. After 90 min carrageenan was injected into the air poach and the exudates were harvested on the next day. Statistics: Control vs: P21 NS (P=0.322); P23-2 P=0.0020; P24 P=0.0001; P25 P=0.0418; P28 NS (P=0.3502); P29 NS (P=0.0582); P30 P=0.0006; P31 P=0.0078; P32 P=0.0340; P33 NS (P=0.9993); P34 NS (p=0.0566) (ANOVA).



4. Discussion

Modifications of the structures of both linear and cyclolinopeptide A have led to establishment of a core sequence responsible for its generally immunosuppressive activities [Wieczorek et al., 1991; Kaczmarek et al., 2002]. However, further manipulations in the cyclic Pro-Pro-Phe-Phe sequence created a possibility of obtaining analogs of a more directional and selective properties, appropriate to treat certain immune disorders. For example, we recently demonstrated that P11 [Bojarska et al., 2022], belonging to this series of 4B8M analogs (Table 1), displayed moderate antitumor actions against melanoma cell lines. In another investigation, a replacement of L-proline with D proline, in position one or two of the 4B8M sequence, resulted in obtaining two peptides of a different activity (to be published). In this study we have undertaken a laborious attempt to synthesize twenty seven 4B8M analogs by substituting 1-4 positions with commercially available modified amino acids. In the preliminary experiments all compounds were devoid of toxicity in the applied model of peripheral blood mononuclear cells, a desirable feature of potential therapeutics.

Based on the property of inhibiting the proliferative response of PBMC and TNF-α production, we selected most promising compounds to apply in carrageenan-induced inflammation in air poach mouse model. The analysis of the amino acid sequence of the peptides, in parallel with their activity, revealed interesting structure-activity relationships. Below, we focus our discussion on peptides which exhibited more distinctive, directional or intriguing characteristics, and correlate these activities with their anti-inflammatory effects in the air poach model. Of interest, modification of proline residue in position 2, occurring in P26- cyclo-[Pro-Oic-β^3-HoPhe-Phe-], P29-cyclo-[Pro-Tic-β^3-HoPhe-Phe-] and P11- cyclo-[Pro-Pip-β^3-HoPhe-Phe-] [Bojarska et al., 2022] was associated with inhibition of cell proliferation. Such a property may predispose these compounds to act not only as antitumor agents [Popivanova et al., 2008] but have also a significance in suppression of autoimmune diseases [Sadlack et al., 1995; Tago et al., 2008]. In addition, the capability of these peptides to inhibit TNF-α production (Figure 3 AD) may strengthen their antitumor actions taking into account a crucial role of TNF-α in the initiation and progression of inflammation-associated carcinogenesis (e.g. in colitis-associated colon carcinogenesis) [Popivanova et al., 2008; Balkwill, 2009].

Of interest, P29 and P26 were less efficient in inhibition of cell infiltration in the air poach model, a laboratory model of rheumatoid arthritis [Bottomley et al., 1988], where inhibition of TNF-α levels plays a major role. On the other hand, a good correlation between the ability to inhibit LPS-inducible TNF-α and the numbers of the infiltrating cells was found with P24- cyclo-[Pro-Pro-β^3-HoPhe-Phe(4Cl)-], P29- cyclo-[Pro-Tic-β^3-HoPhe-Phe-] and P32- 2-cyclo-[Pro-Pro-β^3-HoPhe-2Nal-], which classifies these peptides to a category of typically anti-inflammatory compounds. The significance of inhibition of TNF-α levels in the carrageenan-induced air poach inflammation was firmly established [Inada & Kamibayashi, 2021; Goncalves et al., 2022]. In addition, inhibition of TNF-α may have clinical significance in prevention of allograft rejection [Azzawi et al., 1999; Brehm et al., 2007].

There were, however, two peptides (P34 and SP00b) with intriguing properties. P34 was found to augment viability of PBMC in 24h culture at high, 100 μg/mL concentration. The peptide also strongly inhibited TNF-α production at 25 μg/mL. Such properties resemble anti-inflammatory properties of DMSO [Huang et al., 2020] routinely serving as a solvent for hydrophobic compounds. In this investigation, DMSO at higher dilutions (equivalent of 100 μg/mL), tends to enhance the cell viability (Figure 1). Since the peptide shows pro survival and anti-inflammatory properties, it is worth further investigations in other models like experimentally induced colitis. In turn, SP00b strongly stimulated TNF-α production at 5 μg/mL, whereas at 25 μg/mL was strongly inhibitory. Its effect on PBMC proliferation were neutral. Such a behavior may suggest,among others, regulation of LPS receptor expression in blood monocytes or elicitation of regulatory signaling pathways.

In summary we showed that modification of single amino acids in the 4B8M sequence is a promising approach to obtain analogs of differential characteristics in terms of their ability to inhibit cell proliferation, TNF-α production and suppressive efficacy in the carrageenan-induced inflammation in the air poach model. Most potent, universal inhibitory peptides were synthesized when Pro in position 2 was modified (P11, P26, P29), and a more selective one upon change in Phe, position 4 (P24). Beside these peptides, other analogs were identified with interesting biological properties, which potential therapeutic value is worth further investigation in experimental models, designed to reflect specific clinical situations and diseases.

The strategy of development of new therapeutics from cyclic peptides contained in plant seeds is very much appreciated [Dahiya et al., 2021]. The diverse activity profiles of the modified core sequence of CLA from linen seeds, described in this report, presents a good example of such approach since newly synthesized peptides acquire other, desirable therapeutic characteristics, although different than the parent peptide.

Author Contributions:

Conceptualization: M.Z., J.Z., K.K., methodology: M.Z., K.K.; validation: J.A.; formal analysis: M.Z.; investigation: J.A., K.K.; data curation: J.A.; writing—original draft preparation: M.Z.; writing—review and editing: J.A., K.K.; visualization and statistical analysis: J.A.; supervision: M.Z., K.K. and J.A.

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Conflicts of interest: M.Z. is a former consultant of Peptaderm and an inventor in the US 09382292 patent. K.K. is a former consultant of Peptaderm and an inventor in the US 09382292 patent. The remaining authors declare no conflict of interest.

The company has no contribution in design, data analysis and interpretation of the results. The company supervisor has approved the content of the manuscript prior to submission to Pharmaceutics.

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