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## **Immunosuppressive properties of amino acid and peptide derivatives of mycophenolic acid**

Agnieszka Siebert,<sup>a</sup> Grzegorz Cholewiński,<sup>a\*</sup> Piotr Trzonkowski,<sup>b</sup> Janusz Rachon<sup>a</sup>

<sup>a</sup> Department of Organic Chemistry, Gdansk University of Technology,  
G. Narutowicza 11/12, 80-233 Gdansk, Poland

<sup>b</sup> Department of Clinical Immunology and Transplantology, Medical University of Gdansk,  
St Debinki 7, 80-211 Gdansk, Poland

Corresponding author

E-mail address: grzchole@pg.gda.pl (Grzegorz Cholewiński), tel.: +48 58 347 23 00,  
fax: +48 58 347 26 94.

### **Abstract**

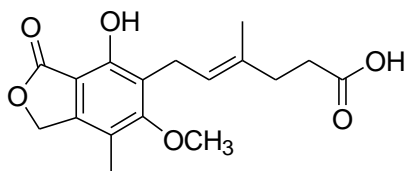
Mycophenolic acid (MPA) was coupled with amino acids and biologically active peptides including derivatives of tuftsin to modify its immunosuppressive properties. Both amino acid unit in the case of simple MPA amides and modifications within peptide moiety of MPA - tuftsin conjugates influenced the observed activity. Antiproliferative potential of the obtained conjugates was investigated *in vitro* and MPA amides with threonine methyl ester and conjugate of MPA with retro-tuftisin occurred to be more selective against PBMC in comparison to parent MPA. Both amino acid and peptide derivatives of MPA acted as inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitors.

### **Keywords**

Mycophenolic acid; amino acids; peptides; tuftsin; IMPDH inhibitors; immunosuppressants.

### **1. Introduction**

Mycophenolic acid (MPA) **1** (Fig. 1) was isolated first time by B. Gosio from *Penicillium brevicompactum*. It possesses antibacterial, antiviral, anticancer and immunosuppressive properties [1,2].



1

**Fig. 1** Structure of mycophenolic acid.

MPA is an uncompetitive and reversible inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH) and its prodrugs are applied in clinic as immunosuppressants. In 1995 morpholine ester of mycophenolic acid – mycophenolate mofetil (MMF, CellCept<sup>®</sup>, Roche AG) was approved by FDA as drug in solid organ transplantation (kidney, liver, heart) for decrease risk of rejection. The second form of the drug is mycophenolic acid sodium salt (MPS, Myfortic<sup>®</sup>, Novartis Farma AG). Both forms are used together with other immunosuppressants, like cyclosporine, tacrolimus in transplantation and autoimmune disorders treatment, e.g. psoriasis [1-4]. MPA inhibits IMPDH *via* blocking binding site of NAD<sup>+</sup> cofactor placed near to active center of the enzyme. The structure of IMPDH enzyme in complex with MPA **1** was reported [5] and the role of functional group of **1** in maintenance of activity was explained, like free phenol or carboxylic groups, which are able to hydrogen bond interactions with the enzyme. Biosynthesis of lymphocytes and DNA depends on IMPDH activity, since it involves nucleotides biosynthesis *de novo*. Other cells use both *de novo* and salvage pathway, when nucleobases are recycled. As a result, MPA selectively inhibits proliferation of lymphocytes B and T [1-4]. Furthermore, IMPDH exists in the two isoforms I and II, where IMPDH I is expressed in normal cells, and IMPDH II is up-regulated in activated lymphocytes and neoplastic cells. MPA inhibits both forms with higher activity against IMPDH II [6]. As a result, numerous IMPDH inhibitors revealed not only immunosuppressive, but also anticancer properties [7-11].

Despite of the progress in immunosuppressive treatment, both the risk of rejection and serious side – effects have been not eliminated so far. As a result, numerous studies were performed to increase efficiency and diminish toxicity of novel mycophenolic acid derivatives [12-19].

In our previous work we designed amino acid MPA derivatives possessing potent immunosuppressive activity [20]. These results were in agreement with literature data, that polar group at the end of side chain in MPA is important for maintenance of anti-proliferative activity, since enables hydrogen bond interactions with Ser 276 of IMPDH [21].

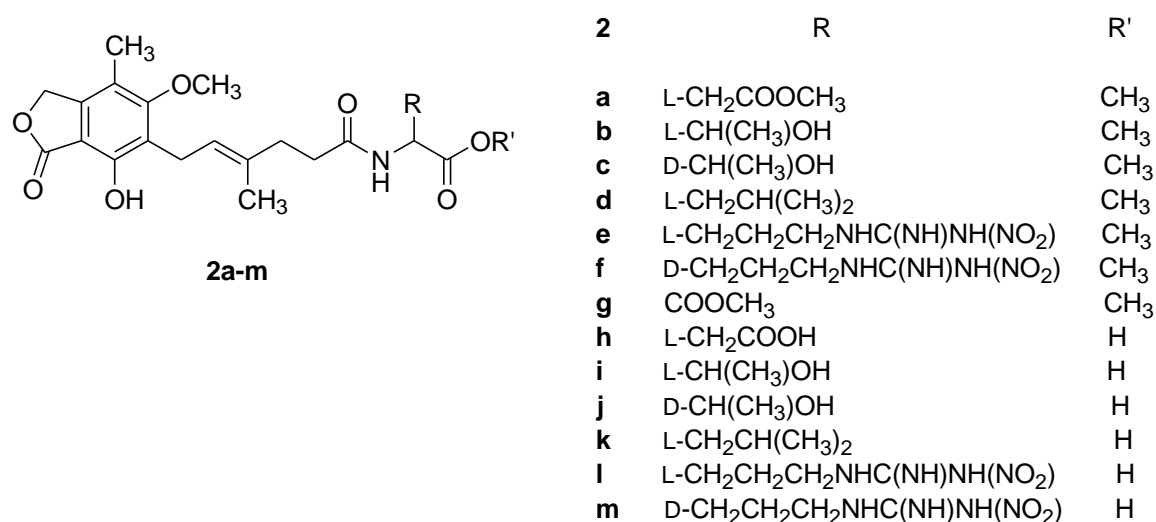
On the other hand, tuftsin is an endogenous tetra-peptide with the sequence Thr-Lys-Pro-Arg, naturally occurring in human blood, which can influence immune properties [22-26]. Moreover, conjugate formation can provide substance with optimized activity, including improved

potency and reduced toxicity [27-32]. Therefore, we decided to obtain and investigate amino acid and peptide derivatives of MPA as promising immunosuppressive agents.

## 2. Results and discussion

### 2.1 Chemistry

Synthesis of amino acid derivatives **2a-g** was performed *via* coupling of amino acid esters with MPA. Then, respective methyl esters were hydrolyzed to free acids **2h-m** according to previously published procedure [20,25].

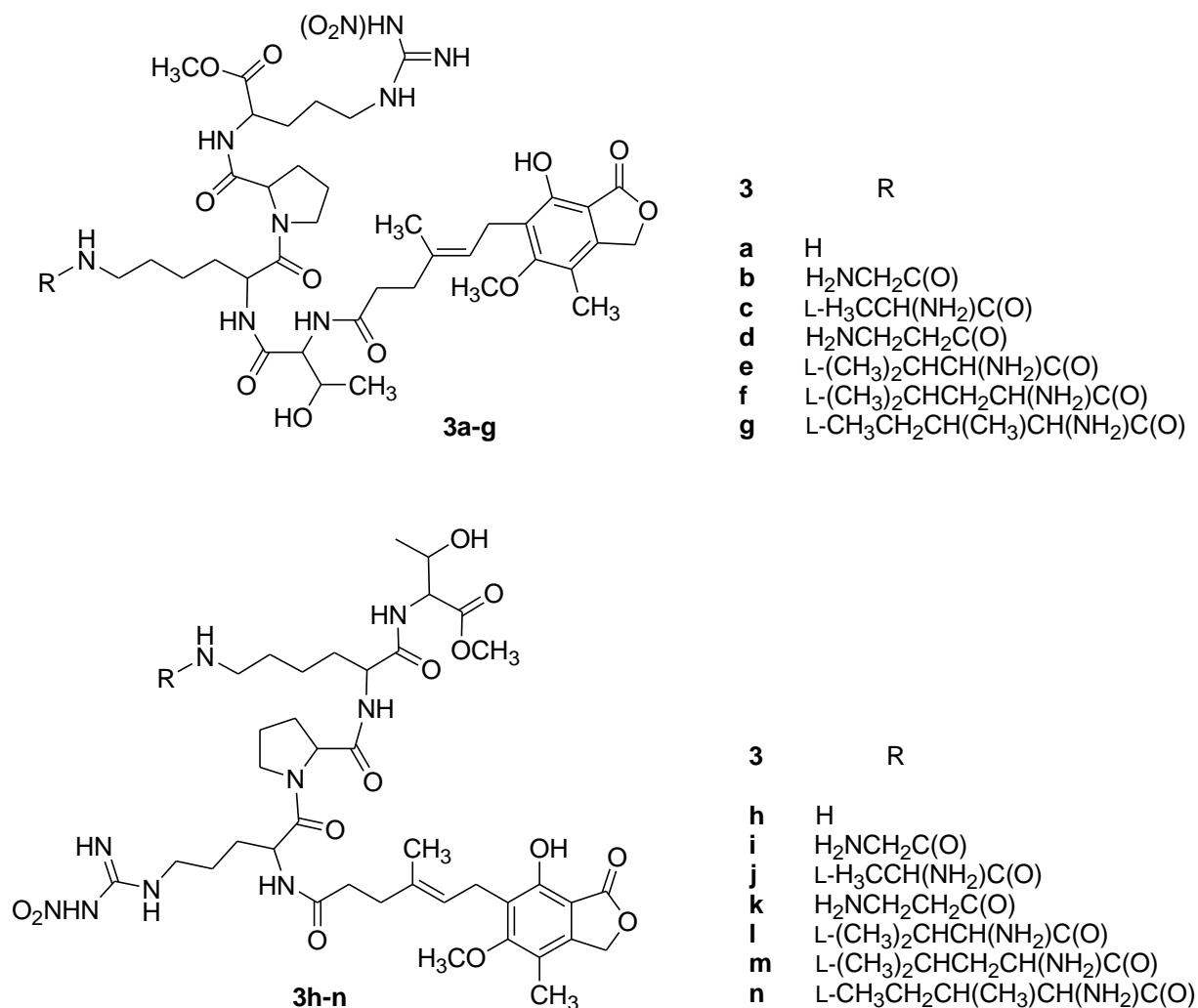


**Figure 2.** Amino acid derivatives of MPA **2a-m**.

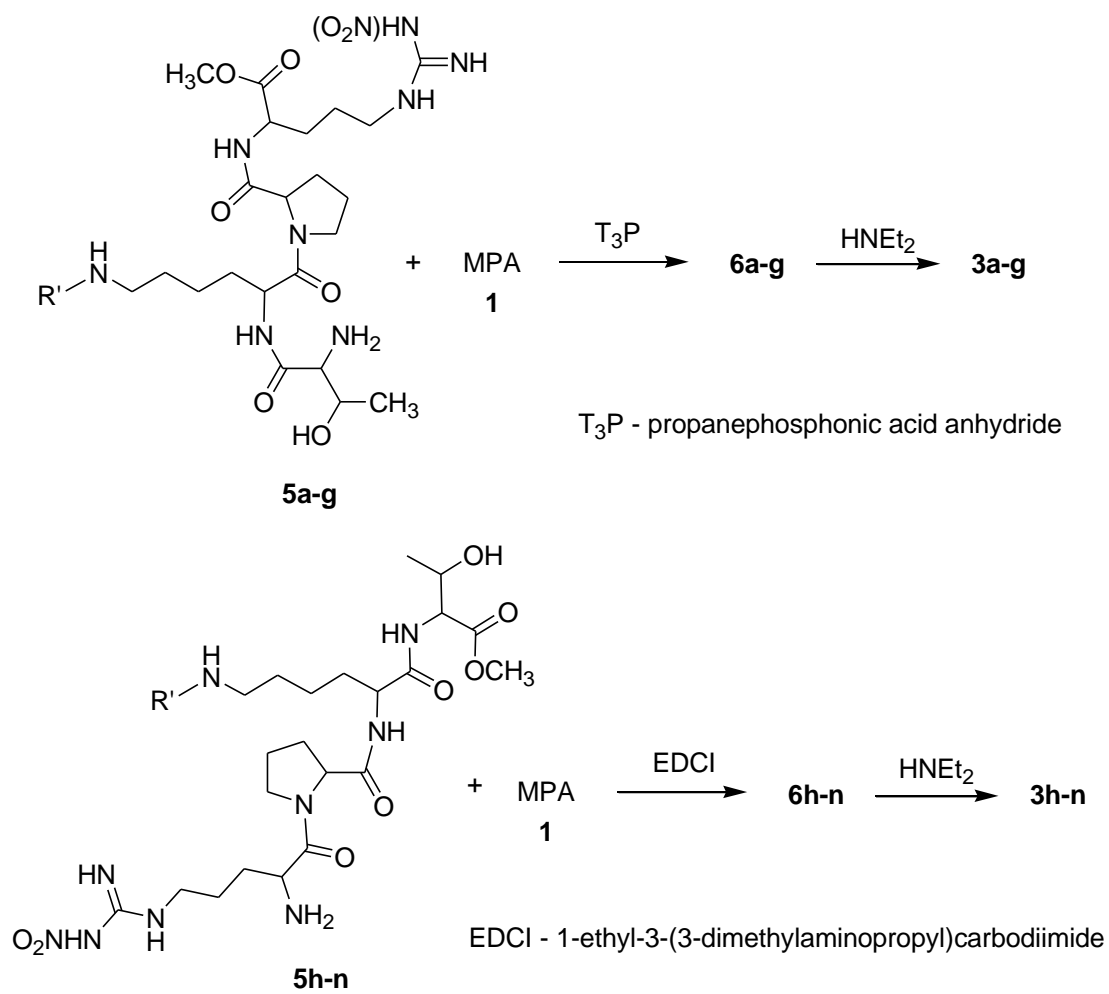
The next series of amide derivatives of MPA consisted peptide analogs, based on tuftsin **3a-g** and retro-tuftsin **3h-n** (Fig. 3). However, tuftsin is not stable and its half – time in plasma is 16 minutes. Therefore, we included also retro tuftsin, a peptide with reversed sequence Arg-Pro-Lys-Thr. Moreover, tuftsin can be stabilized by bonding with biological active compounds or modification at  $\epsilon$ -amine group in lysine moiety, where next amino acid can be attached [22-24]. As a results, we designed pentapeptides **5a-n** (Fig. 4) through acylation of  $\epsilon$ -amine group of lysine with amino acids like  $\alpha$ -alanine,  $\beta$ -alanine, valine, leucine, isoleucine with methods described by Dzierzbicka and co-workers [22-24].

Then, Fmoc-protected peptides **5a-n** were combined with MPA by means of condensing agents such as EDCI, EEDQ or T<sub>3</sub>P. The coupling of Fmoc-protected tuftsin analogs **6a-g** (Fig. 5) with MPA **1** was optimized with T<sub>3</sub>P procedure, whereas the most effective coupling reagent in the case of retro-tuftsin conjugates **6h-n** occurred to be EDCI in the presence of DMAP. The reaction carried out in anhydrous DMF gave the F-moc protected derivatives **6a-n** in 39-65 % yield. In order to produce the final compounds **3a-n**, Fmoc protecting group was removed with 20-30% solution of diethylamine in chloroform in 80-85% yield.

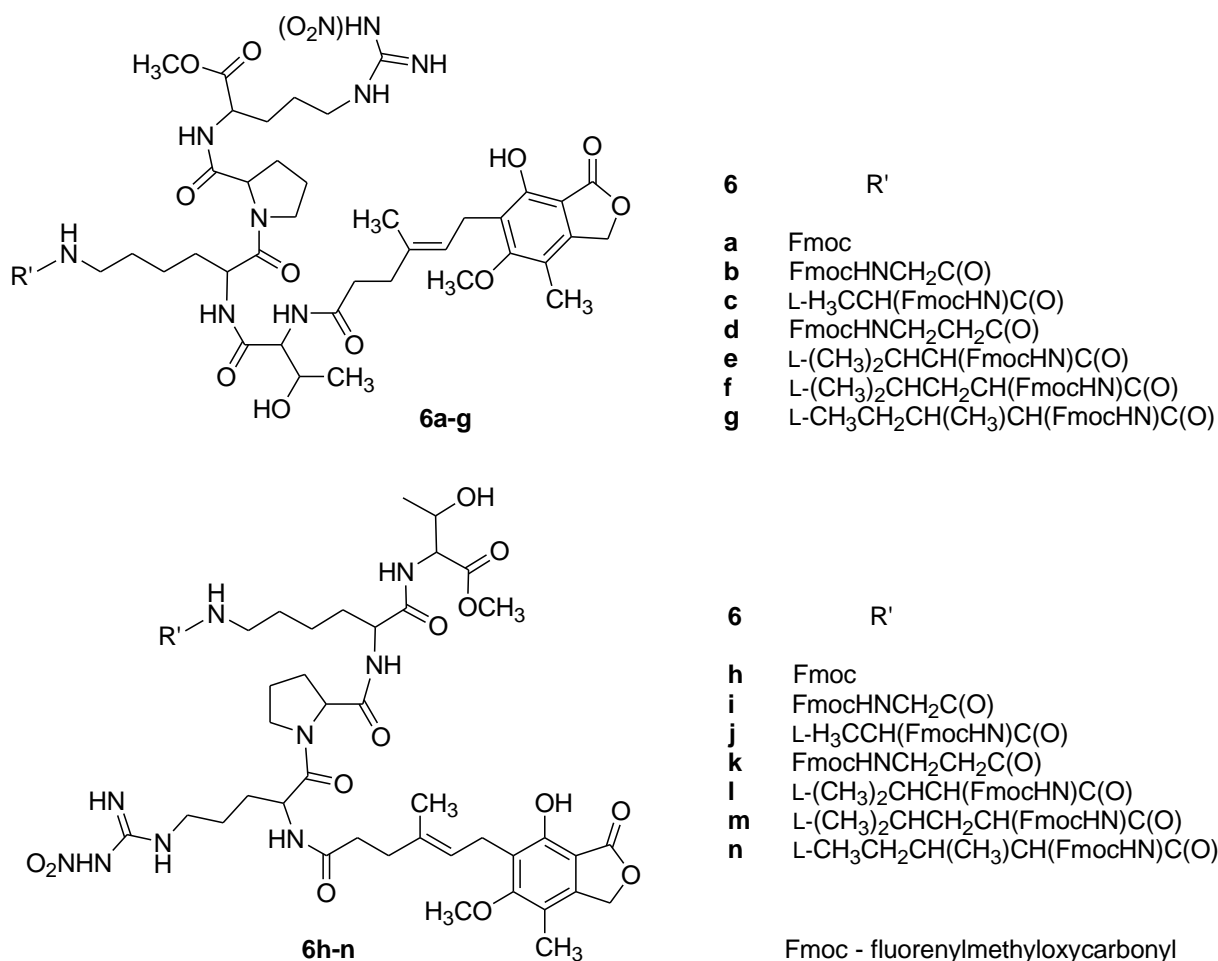
The structures and purities was confirmed with  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, MS, MS-HPLC techniques. Additionally, characteristics were extended with COESY, HMBC, HSQC, ROESY, and TOCSY measurements. Examples of NMR, MS spectra, HPLC chromatograms are available in the supplementary material.



**Figure 3.** Peptides derivatives of MPA **3a-n**.



**Figure 4.** Synthesis of peptide derivatives of MPA **3a-n**.



**Figure 5.** Fmoc – protected conjugates of MPA with tuftsin **6a-g** and retro-tuftsin **6h-n**.

## 2.2. Immunosuppressive evaluation

To assess the immunosuppressive activity, we performed both MTT cytotoxicity assay and a proliferation test using the VPD450 dye on the Jurkat lymphoid cell line and peripheral blood mononuclear cells (PBMCs). Jurkat cells are used as a model line of human lymphocytes, whereas PBMCs are an *in vitro* model of the immune response. These tests were performed in triplicate.

Compounds were dissolved in 20  $\mu$ L of DMSO, then refilled with media to 1 mL, resulting in a starting concentration of 1 mg/mL. Then, further solutions were prepared by serial dilution, obtaining the lowest tested concentration of  $10^{-7}$  mg/mL.

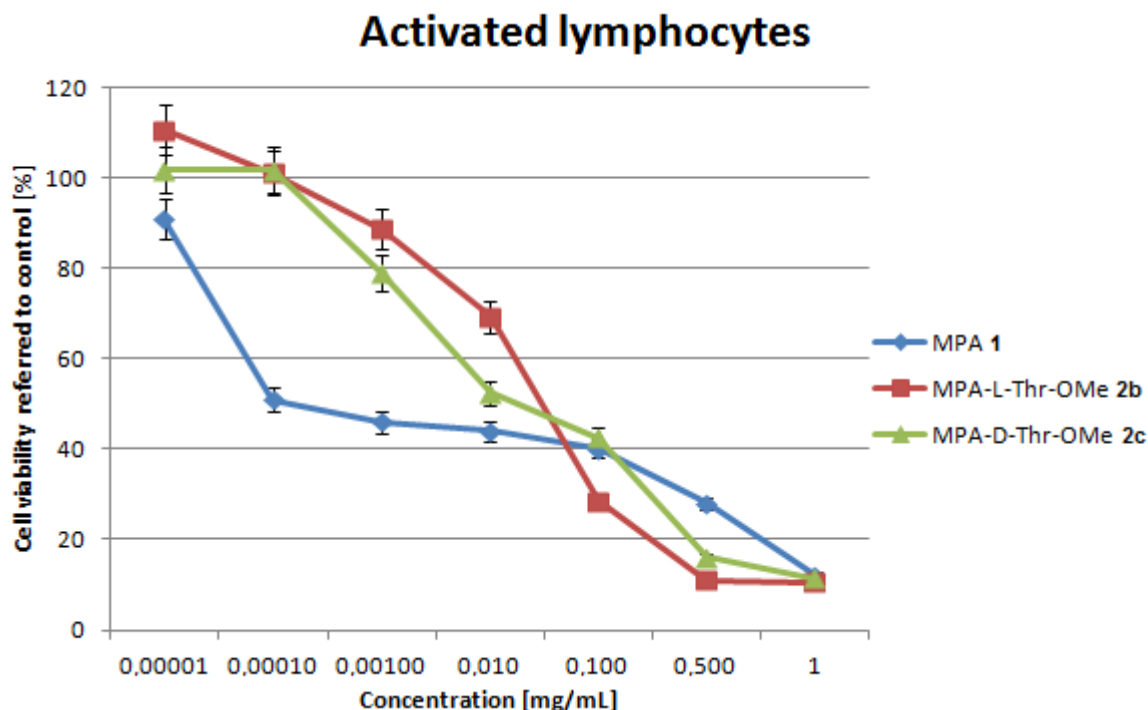
### 2.2.1. Determination of cytotoxic activity of tested compounds against Jurkat and PBMC cell lines using the MTT method

MPA derivatives at various concentrations were incubated with Jurkat or PBMC cells. In addition, the proliferation of human lymphocytes was stimulated with anti-CD3 / anti-CD-28 antibodies. Then, MTT was used and IC<sub>50</sub> established from the obtained data (Tables 1,2).



### 2.2.1.1. Amino acid analogs of MPA

Table 1 shows the results of the MTT cytotoxicity assay, which was performed for the amino acid analogs of MPA **2a-m** on the T-Jurkat cell line and on PBMCs. In the case of PBMCs, all new amino acid analogs of the MPA were found to be less cytotoxic than the parent MPA **1**.



**Figure 6.** Comparison of cytotoxicity of compounds **2b** and **2c** – amino acid derivatives with the best SI (see Table 5) to MPA **1** against PBMC.

In contrast, compounds **2a, b, d, i** gave higher toxicity against T-Jurkat cell line in comparison to **1**. Analyzing the results it can be seen that the amino acid methyl esters **2a-g** were more cytotoxic than their counterparts with the free carboxyl group **2h-m**. This is noticeable in each case for both the T-Jurkat cell line and PBMC cells. The absolute configuration in amino acid moiety also influenced cytotoxicity. Both in the case of methyl esters **2a-g** and derivatives with a free carboxyl group **2h-m**, it can be seen that D enantiomers are less cytotoxic than L enantiomers for both types of tested cells.

MPA derivatives with arginine bearing free carboxyl group, both L **2l** and D **2m** enantiomer, showed the lowest toxicity. In the case of the T-Jurkat cell line for these derivatives, the  $IC_{50}$  values in at used concentrations could not be determined. Thus, the viability at the highest tested concentration (1 mg / mL) was calculated. The cell viability was 59.12% for compound **2l** and 75.14% for **2m**. These analogs indicated also low cytotoxicity to PBMCs, and their  $IC_{50}$  values were 283.35  $\mu$ M for **2l** and 422.2  $\mu$ M in case of **2m**.

The MPA-L-Ile-OMe **2d** was found to be the most cytotoxic to both types of cells, its IC<sub>50</sub> for the Jurkat cell line was 8 μM (approximately 8 times lower than for MPA **1**), whereas against PBMC cells **2d** gave 2.2 μM. Figure 6 shows a comparison of the cytotoxicity of compounds **2b** and **2c** to mycophenolic acid **1** at the tested concentrations against activated lymphocytes. Both threonine esters **2b,c** were less cytotoxic than MPA **1** at lower concentrations, but in the range of 0.01 to 1g/mL observed activity was similar.



**Table 1.** IC<sub>50</sub> [μM] values of amino acid derivatives of MPA **2a-m** based on MTT test.

Compound	No	T-JURKAT			PBMC			
		IC <sub>50</sub> [μM]	Viability [%]*	p	F	IC <sub>50</sub> [μM]	p	F
MPA	<b>1</b>	60.6 ± NAN				0.14 ± 0.06		
<b>MPA-L-Asp(OMe)-OMe</b>	<b>2a</b>	20 ± 3		< 0.05	1.54	<b>18 ± 1.8</b>	< 0.05	33.5
MPA-L-Thr-OMe	<b>2b</b>	17 ± 9		< 0.05	188	<b>57 ± 31.4</b>	< 0.05	30.1
MPA-D-Thr-OMe	<b>2c</b>	<b>115 ± 3.6</b>		< 0.05	266	<b>69 ± 58.2</b>	< 0.05	25.9
MPA-L-Ile-OMe	<b>2d</b>	8 ± 5.7		< 0.05	8775	<b>2.2 ± 2</b>	< 0.05	46.6
MPA-L-Arg-OMe	<b>2e</b>	<b>346.98 ± NAN</b>		0.2	14.1	<b>14 ± 4.8</b>	< 0.05	40.2
MPA-D-Arg-OMe	<b>2f</b>	<b>572 ± 92.9</b>		< 0.05	186	<b>71 ± 63.1</b>	< 0.05	29.5
MPA-Mal-(OMe) <sub>2</sub>	<b>2g</b>	<b>101 ± 20.8</b>		< 0.05	3128	<b>3.5 ± NAN</b>	< 0.05	28.5
<b>MPA-L-Asp(OH)-OH</b>	<b>2h</b>	<b>539.67 ± NAN</b>		< 0.05	236	<b>50 ± 25.7</b>	< 0.05	42.1
MPA-L-Thr-OH	<b>2i</b>	43 ± 5.4		< 0.05	420	<b>82 ± 58.2</b>	< 0.05	25.5
MPA-D-Thr-OH	<b>2j</b>	<b>229 ± 35.9</b>		0.105	50.5	<b>118 ± 114.9</b>	< 0.05	3.99
MPA-L-Ile-OH	<b>2k</b>	<b>73.74 ± NAN</b>		0.083	80.9	<b>20.87 ± NAN</b>	< 0.05	10.0
MPA-L-Arg-OH	<b>2l</b>		59.12%			<b>283.35 ± NAN</b>	< 0.05	25.5
MPA-D-Arg-OH	<b>2m</b>		75.14%			<b>422.2 ± NAN</b>	< 0.05	50.3

IC<sub>50</sub> higher than for MPA, *p* – statistical significance of the difference, *F* – Fisher test to MPA.

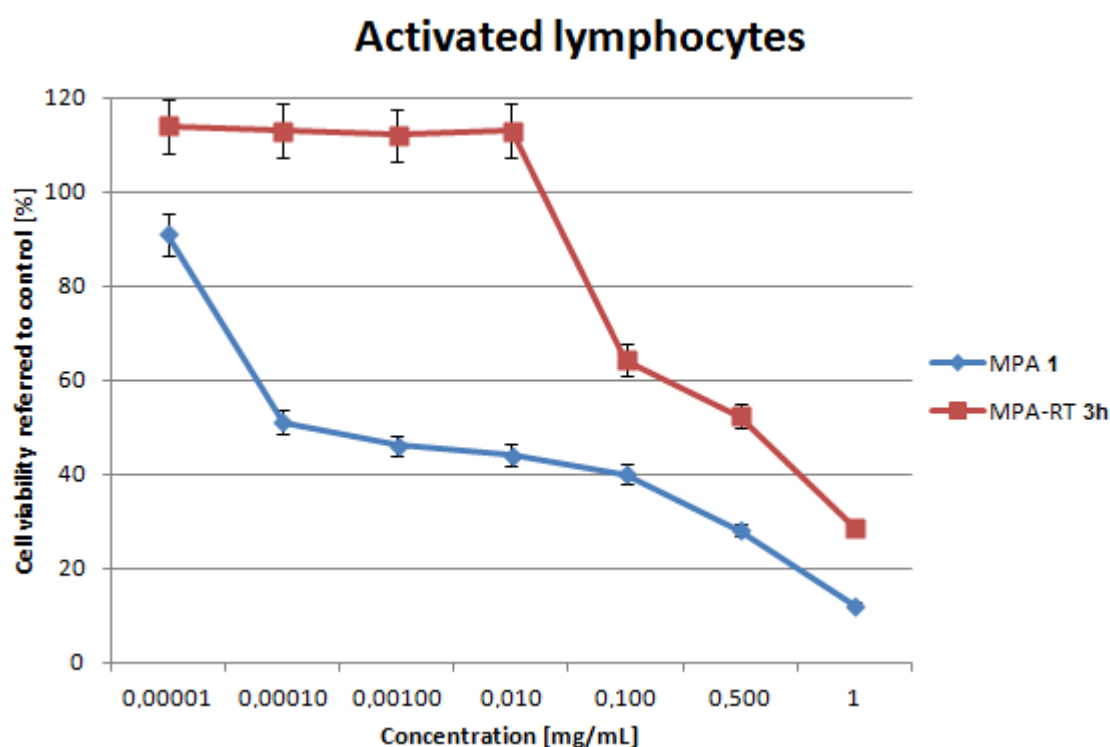
\* cell viability calculated at highest tested concentration 1 mg/mL



### 2.2.1.2. Peptide derivatives of MPA

Table 2 presents the  $IC_{50}$  values obtained for cytotoxicity studies of peptide analogs of MPA **3a-n**. All peptide analogs of MPA are much less toxic than mycophenolic acid **1**.

In the case of more than half of these compounds, determination of  $IC_{50}$  against Jurkat cell line values at the concentration under investigation failed. Therefore, cell viability in the presence of these compounds at the highest concentration of 1 mg / mL was established and gave 52.48% for compound **3n** to 85.82% for **3a**. Peptide derivatives exhibited  $IC_{50}$  values against the Jurkat cell line are over 13 times if compare to MPA **1**. Contrary, in the case of PBMCs,  $IC_{50}$  values are over 3500 times higher than for MPA **1**. This indicates a minimal, in comparison to MPA **1**, cytotoxic activity of peptide analogs against PBMCs.



**Figure 7.** Comparison of cytotoxicity of compound **3h** – peptide derivative with the best SI (see Table 5) to MPA **1** against PBMC.

**Table 2.** IC<sub>50</sub> [μM] values of peptide MPA derivatives **3a-n** based on MTT test.

Compound	No	T-JURKAT			PBMC				
		IC <sub>50</sub> [μM]	Viability [%]*	p	F	IC <sub>50</sub> [μM]	p	F	Viability [%]*
MPA	<b>1</b>	60.56 ± NAN				0.14 ± 0.056			
MPA-T	<b>3a</b>		85.82%				-		99.09%
MPA-T-Gly	<b>3b</b>		53.40%			<b>628 ± 57.5</b>	< 0.05	21.4	
MPA-T-αAla	<b>3c</b>	<b>1001 ± 110</b>		0.099	57.0	<b>560 ± 118.9</b>	< 0.05	42.2	
MPA-T-βAla	<b>3d</b>	<b>1020 ± 51</b>		< 0.05	4674	<b>526 ± 7.9</b>	< 0.05	27.6	
MPA-T-Val	<b>3e</b>		66.56%						56.81%
MPA-T-Leu	<b>3f</b>		67.04%			<b>687 ± 152.8</b>	< 0.05	12.0	
MPA-T-Ile	<b>3g</b>	<b>808.73 ± NAN</b>		30.8	0.134	<b>461 ± 199</b>	< 0.05	11.2	
MPA-RT	<b>3h</b>	<b>971 ± 108.9</b>		0.116	41.0	<b>633.9 ± 0.13</b>	< 0.05	63.6	
MPA-RT-Gly	<b>3i</b>	<b>1096 ± 83.9</b>		< 0.05	284	<b>865 ± 492.7</b>	< 0.05	40.4	
MPA-RT-αAla	<b>3j</b>		53.90%			<b>750 ± 88.6</b>	< 0.05	36.0	
MPA-RT-βAla	<b>3k</b>	<b>786 ± 75.7</b>		0.114	42.9	<b>587.64 ± NAN</b>	< 0.05	42.8	
MPA-RT-Val	<b>3l</b>		63.35%			<b>548 ± 56.5</b>	< 0.05	108.5	
MPA-RT-Leu	<b>3m</b>		61.34%			<b>519 ± 390.6</b>	< 0.05	86.6	
MPA-RT-Ile	<b>3n</b>		52.48%			<b>533 ± 182.6</b>	< 0.05	45.1	

IC<sub>50</sub> higher than for MPA, p – statistical significance of the difference, F – Fisher test to MPA.

\* cell viability calculated at highest tested concentration 1mg/mL



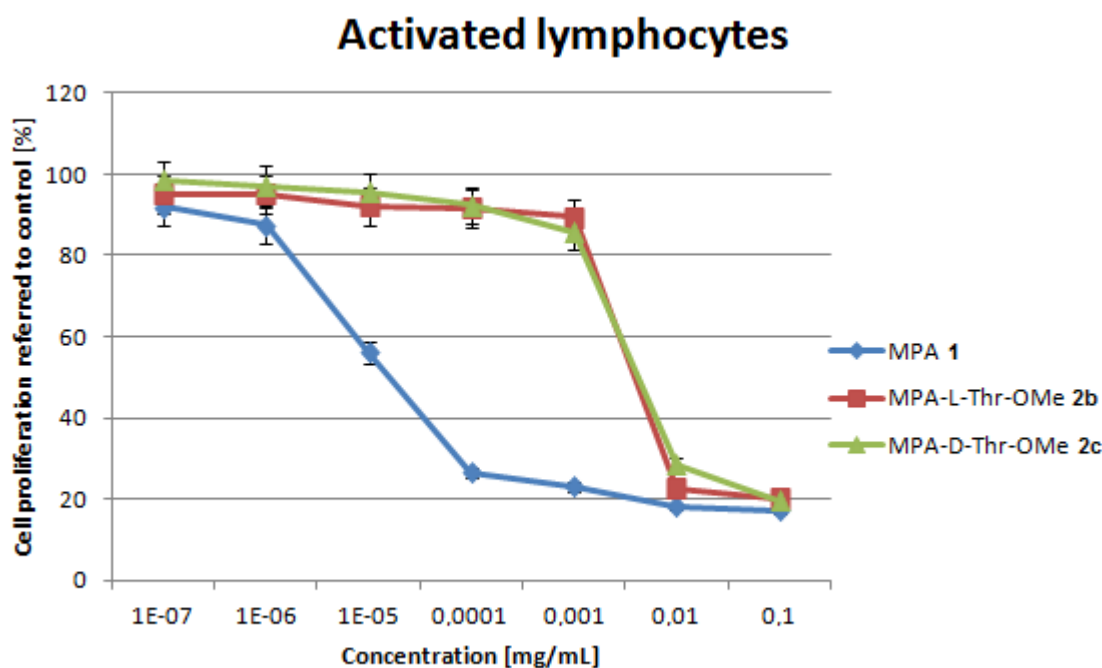
We observed higher toxicity of MPA analogs with retro-tuftsins **3h-n** than compounds bearing tuftsins **3a-g**. An exception constituted two pentapeptide derivatives of MPA possessing  $\alpha$ -alanine and isoleucine units against T-Jurkat cell line and one pentapeptide analog MPA with glycine against PBMCs, where tuftsin conjugates were more toxic. According to these results, we conclude that MPA conjugates holding retro-tuftsins showed slightly higher cytotoxicity than tuftsin counterparts. The MPA-T **3a** derivative was not toxic against both types of cells. Figure 7 shows a comparison of the cytotoxicity of the MPA conjugate having retro-tuftsins **3h** to the parent MPA **1** in the range of considered concentrations against the activated lymphocytes. Conjugate **3h** gave cytotoxicity lower than parent MPA **1**, especially within the range of concentration 0.00001 – 0.1 g/mL.

#### *2.2.2. Determination of antiproliferative activity of tested compounds 2a-m, 3a-g and 3h-n against Jurkat and PBMC cell lines using VPD450.*

In this method VPD450 dye undergoes distribution between child cells uniformly, so that each progeny cell retains about half of the fluorescent intensity of the VPD450 of its parent cell. Fluorescence measurements were then made using flow cytometry to check cell proliferation. After that, the  $EC_{50}$  value was determined from the received data (Tables 3,4).

##### *2.2.2.1. Amino acid MPA derivatives*

In the Table 3 are collected  $EC_{50}$  values of amino acid analogues of MPA. None of these compounds showed such a good anti-proliferative effect as mycophenolic acid **1**. The results of tests carried out on the Jurkat cell line and on PBMCs are consistent. In both cases, the most active compounds occurred to be **2a,b,c,d,k**. The MPA derivative with isoleucine gave the highest activity, where methyl ester **2d** ( $EC_{50} = 5.12 \mu\text{M}$  for PBMC) and free acid **2k** ( $EC_{50} = 9 \mu\text{M}$  for PBMCs) were characterized by the lowest  $EC_{50}$  value. In contrast to that, the least active derivatives of MPA in relation to both types of cells were found to be **2h,i,m**.

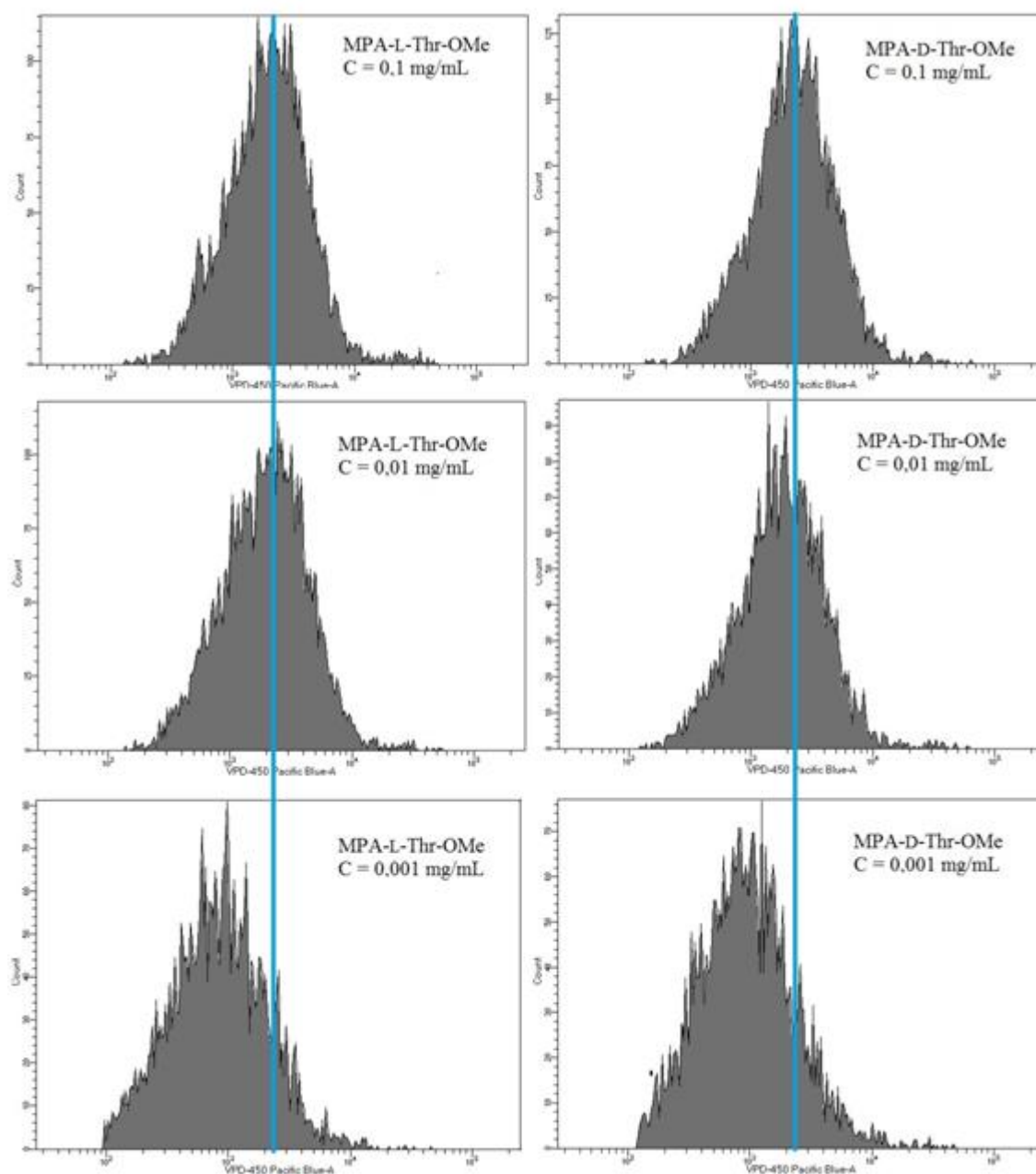


**Figure 8.** Comparison of antiproliferative activity of compounds **2b**, **c** – amino acid derivatives with the best SI (see Table 5) to MPA **1** against PBMC.

Again, the deprotection of methyl esters influenced the observed activity. Methyl esters **2a,b,c** were mostly much more active than their counterparts with a free carboxyl group **2h,i,j**. It suggests the significant role of cell membrane penetration [11]. On the other hand, MPA-L-Ile-OH derivative **2k** ( $EC_{50} = 2.3 \mu\text{M}$ ) was characterized by a lower  $EC_{50}$  value than MPA-L-Ile-OMe **2d** ( $EC_{50} = 9 \mu\text{M}$ ) for the T-Jurkat cell line. According to these results, bulky *sec*-butyl substituent in isoleucine moiety was advantageous for antiproliferative activity and gave together with polar carboxylic group the lowest  $EC_{50}$  within amino acid MPA derivatives **2a-m**.

The effect of configuration in amino acid unit on anti-proliferative activity was lower if compared with the cytotoxicity test. However, only in the case of the MPA-Thr-OH derivative (against Jurkat cell line), the D enantiomer **2j** was more active than L **2i**. According to the other results,  $EC_{50}$  values for L enantiomers are lower or very close to their D counterparts. Figure 8 shows the comparison of antiproliferative activity of compounds **2b** and **2c** to MPA **1** against activated lymphocytes. According to these data, MPA **1** was more active than threonine esters **2b,c** significantly in the range of concentrations 0.000001 – 0.01 mg/mL. Histograms (Figure 9) depict cell divisions containing VPD450 dye. Shifting the median fluorescence peak to the left (increasing number of cell divisions) correlates with decreasing concentration of compounds. The blue line is assigned to the fluorescence peak of cells stained with VPD450 in the presence

of the highest concentration of compound. The peak moves to the left together with decreasing compound concentrations, which indicates more intensive cell proliferation.



**Figure 9.** Histograms showing the division of cells containing dye VPD450 against PBMC in the case of tyrosine analogs **2b** and **2c**.

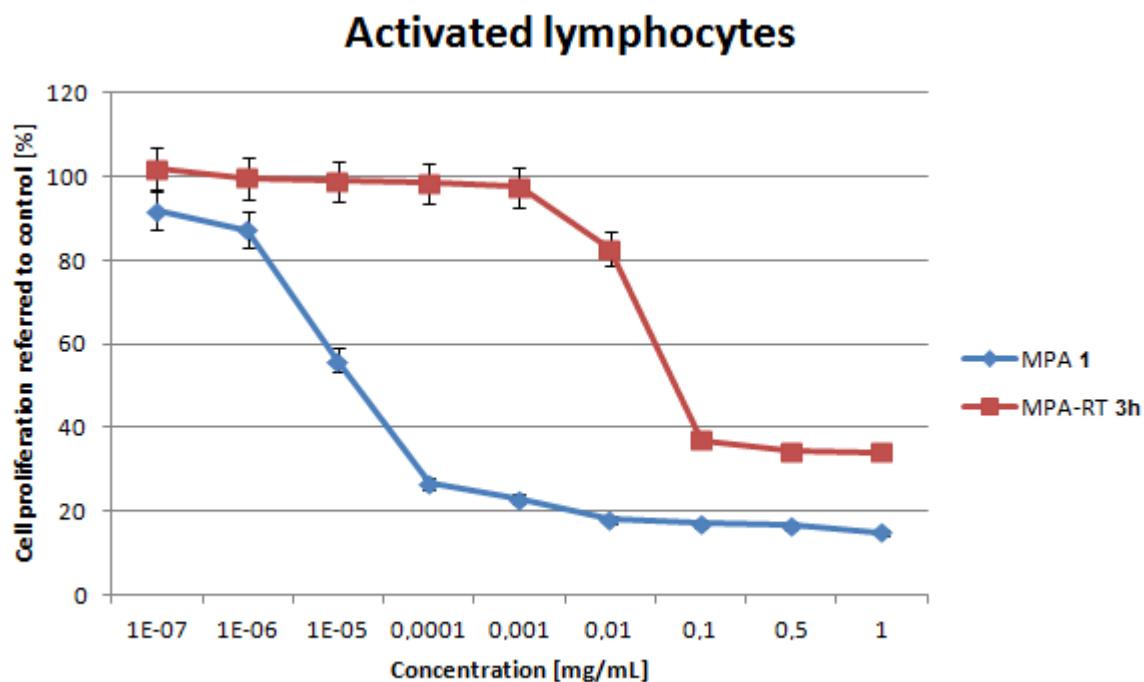
**Table 3.** EC<sub>50</sub> [μM] values of amino acid MPA derivatives **2a-m** based on proliferative test with VPD450.

Compound	No	T-JURKAT			PBMC		
		EC <sub>50</sub> [μM]	p	F	EC <sub>50</sub> [μM]	p	F
MPA	<b>1</b>	0.3 ± 0.2			0.0301 ± 0.0003		
MPA-L-Asp(OMe)-OMe	<b>2a</b>	16.7 ± 0.23	0.738	0.190	8 ± 2.1	0.121	27.0
MPA-L-Thr-OMe	<b>2b</b>	10 ± 7.1	0.293	4.05	10 ± 7.8	0.091	48.0
MPA-D-Thr-OMe	<b>2c</b>	14 ± 5.4	0.379	2.18	9 ± 4.8	0.099	41.0
MPA-L-Ile-OMe	<b>2d</b>	9 ± 5.1	0.105	36.4	5.12 ± NAN	0.667	0.33
MPA-L-Arg-OMe	<b>2e</b>	22.3 ± 0.51	< 0.05	3114	77 ± 2.9	< 0.05	162
MPA-D-Arg-OMe	<b>2f</b>	69 ± 40.9	< 0.05	723	95.57 ± NAN	< 0.05	492
MPA-Mal-(OMe) <sub>2</sub>	<b>2g</b>	65 ± 7.8	0.091	48.1	11 ± 5.5	0.124	25.7
MPA-L-Asp(OH)-OH	<b>2h</b>	396 ± 66.7	< 0.05	1366	211.0166 ± 3.6952	< 0.05	1310
MPA-L-Thr-OH	<b>2i</b>	83 ± 70.5	< 0.05	834	118.63 ± NAN	< 0.05	17638
MPA-D-Thr-OH	<b>2j</b>	21.41 ± NAN	< 0.05	672	121.51 ± NAN	< 0.05	5007
MPA-L-Ile-OH	<b>2k</b>	2.3 ± 0.52	0.182	11.5	9 ± 5.4	0.118	28.3
MPA-L-Arg-OH	<b>2l</b>	1190 ± 474.8	< 0.05	6110	132 ± 2.8	< 0.05	1729
MPA-D-Arg-OH	<b>2m</b>	1037 ± 16.2	< 0.05	3616	588 ± 11.2	< 0.05	7042

*p* – statistical significance of the difference, *F* – Fisher test to MPA.

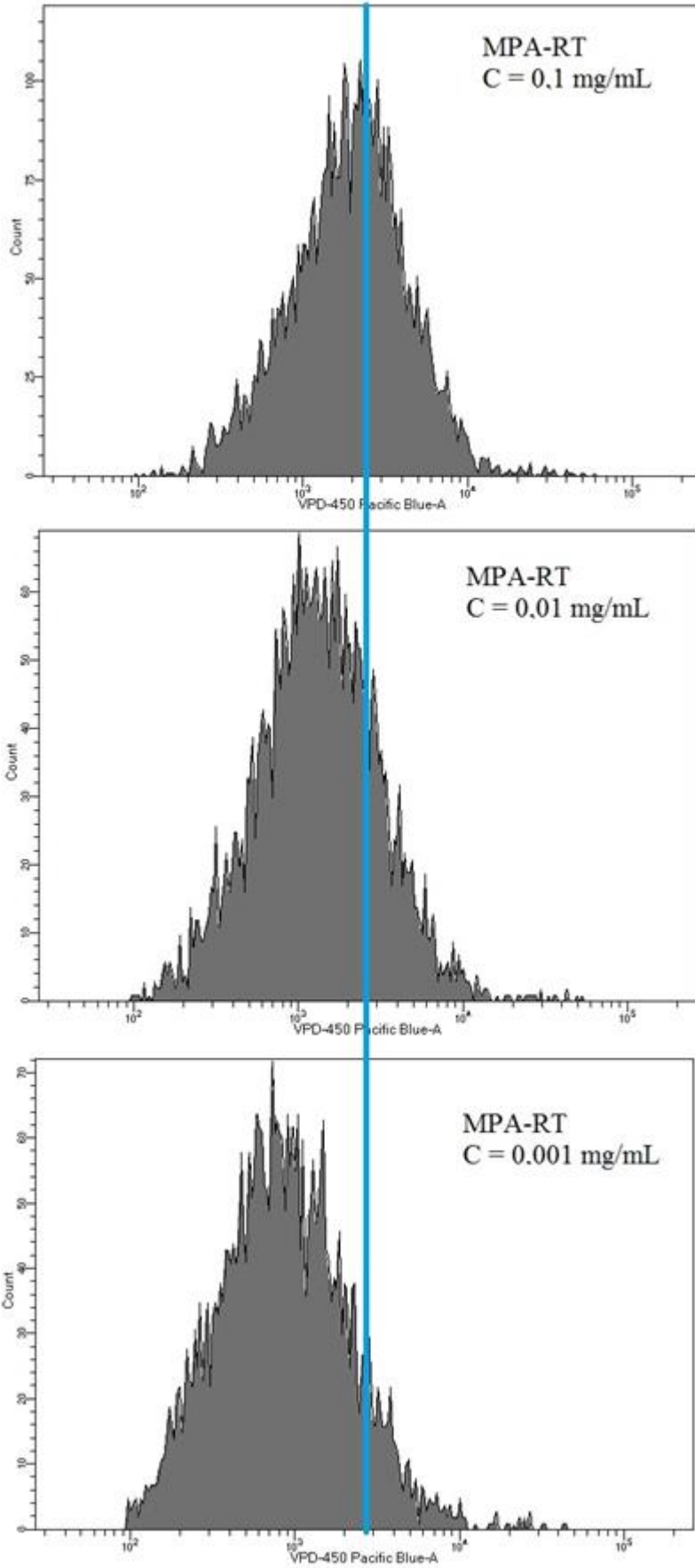
### 2.2.2.2. Peptide derivatives of MPA

Table 4 presents the results of anti-proliferative activity of peptide derivatives of MPA **3a-n**. For some compounds, in the case of the T-Jurkat cell line, it was unable to determine the  $EC_{50}$  value. The most active compounds were **3h** ( $EC_{50} = 663.93 \mu\text{M}$ ) and **3l** ( $EC_{50} = 556 \mu\text{M}$ ). However, even these analogs showed over 2000 times lower activity than MPA **1**.



**Figure 10.** Comparison of antiproliferative activity of compound **3h** – peptide derivative with best SI (see Table 5) do MPA **1** against PBMC.





**Figure 11.** Histograms showing division of cells containing dye VPD450 against PBMC for 3h.

**Table 4.** EC<sub>50</sub> [μM] values of peptide MPA derivatives **3a-n** based on proliferative test with VPD 450.

Compound	No	T-JURKAT			PBMC			
		EC <sub>50</sub> [μM]	Viability [%]*	p	F	EC <sub>50</sub> [μM]	p	F
MPA	<b>1</b>	0.3 ± 0.2				0.0301 ± 0.0003		
MPA-T	<b>3a</b>		60.78%			130 ± 68.4	< 0.05	1204
MPA-T-Gly	<b>3b</b>	1030 ± 50.9		< 0.05	19448	590 ± 266.1	< 0.05	30012
MPA-T-αAla	<b>3c</b>		55.31%			259 ± 54.9	< 0.05	2129
MPA-T-βAla	<b>3d</b>	941 ± 367.5		< 0.05	8494	211 ± 98.2	< 0.05	23935
MPA-T-Val	<b>3e</b>		55.10%			432 ± 108.4	< 0.05	33685
MPA-T-Leu	<b>3f</b>	801 ± 54.3		< 0.05	13790	551 ± 3.2	< 0.05	30012
MPA-T-Ile	<b>3g</b>	924 ± 175.2		< 0.05	12455	357 ± 107.1	< 0.05	19110
MPA-RT	<b>3h</b>	663.93 ± NAN		< 0.05	7500	35.43 ± NAN	< 0.05	29410
MPA-RT-Gly	<b>3i</b>		56.72%			565.92 ± NAN	< 0.05	37509
MPA-RT-αAla	<b>3j</b>	839 ± 85.5		< 0.05	17956	168 ± 75.3	< 0.05	19776
MPA-RT-βAla	<b>3k</b>		75.89%			154 ± 92.9	< 0.05	34031
MPA-RT-Val	<b>3l</b>	556 ± 113		< 0.05	11781	129 ± 77.4	< 0.05	15052
MPA-RT-Leu	<b>3m</b>		63.04%			161 ± 62.4	< 0.05	17072
MPA-RT-Ile	<b>3n</b>		52.77%			152.23 ± NAN	< 0.05	19219

p – statistical significance of the difference, F – Fisher test to MPA.

\* cell viability calculated at highest tested concentration 1mg/mL



Analyzing the results of tests carried out on PBMCs, the most potent was derivative **3h**, which gave  $EC_{50} = 35.43 \mu\text{M}$ . However, it is more than 1000 times lower than for MPA **1** ( $EC_{50} = 0.0301 \mu\text{M}$ ). MPA conjugates from retro-tuftsins **3h-n** indicated higher activity than MPA derivatives with tuftsins **3a-g**, which is clearly visible against PBMCs. Figure 10 shows a comparison of the antiproliferative activity of the MPA conjugate with retro-tuftsins **3h** to mycophenolic acid **1** against PBMCs, and conjugate **3h** increased clearly its activity from concentration of 0.001 g/mL. Figure 11 presents the number of cell divisions depending on the concentration of **3h**. Again, the blue line indicates the fluorescence peak of cells stained with VPD450 in the presence of the highest concentration of compound. In this case, as the concentration of compound decreases, it shifts to the left, which indicates more intensive cell proliferation.

### 2.2.3. Selection of MPA derivatives with the most favorable antiproliferative properties

Determination of the selectivity index (SI), which is the ratio of the cytotoxicity of the compound ( $IC_{50}$ ) to its antiproliferative activity ( $EC_{50}$ ) enables to compare the obtained derivatives with one another and to the parent MPA **1** as well as. On this basis, we selected analogue with the best activity and the lowest toxicity. With the increase of SI, the toxicity of the compound decreases.

Table 5 shows the selectivity coefficients SI for the obtained amino acid and peptide analogs of mycophenolic acid. In the case of the T-Jurkat cell line, none of MPA derivatives was characterized by a higher selectivity index than MPA **1**. In contrast, when analyzing the results of studies carried out on PBMCs, three compounds: **2b,c** and **3h** indicated significant activities and were selected for further studies as potential immunosuppressants. Although the  $EC_{50}$  value of these analogues is higher than in the case of MPA **1** (Table 4), they were characterized by much lower cytotoxicity if compared to mycophenolic acid **1**. The peptide derivatives **3j,l** had a similar selectivity ratio as the MPA **1**, but a high dose of substance would be needed to inhibit cell proliferation (168  $\mu\text{M}$  for **3j** and 129  $\mu\text{M}$  for **3l**).

**Table 5.** Selectivity index (SI) against Jurkat and PBMC cell lines.

Compound	No	SI = IC <sub>50</sub> /EC <sub>50</sub>		Compound	No	SI = IC <sub>50</sub> /EC <sub>50</sub>	
		JURKAT	PBMC			JURKAT	PBMC
MPA	<b>1</b>	228	4.6	MPA	<b>1</b>	228	4.6
<b>MPA-L-Asp(OMe)-OMe</b>	<b>2a</b>	1.2	2.2	MPA-T	<b>3a</b>	-	-
MPA-L-Thr-OMe	<b>2b</b>	1.8	<b>5.7</b>	MPA-T-Gly	<b>3b</b>	-	1.1
MPA-D-Thr-OMe	<b>2c</b>	8.3	<b>7.3</b>	MPA-T-αAla	<b>3c</b>	-	2.2
MPA-L-Ile-OMe	<b>2d</b>	0.9	0.4	MPA-T-βAla	<b>3d</b>	1.1	2.5
MPA-L-Arg-OMe	<b>2e</b>	16	0.2	MPA-T-Val	<b>3e</b>	-	
MPA-D-Arg-OMe	<b>2f</b>	8.2	0.7	MPA-T-Leu	<b>3f</b>	-	1.2
MPA-Mal-(OMe) <sub>2</sub>	<b>2g</b>	1.6	0.3	MPA-T-Ile	<b>3g</b>	0.9	1.3
<b>MPA-L-Asp(OH)-OH</b>	<b>2h</b>	1.4	0.2	MPA-RT	<b>3h</b>	1.5	<b>18</b>
MPA-L-Thr-OH	<b>2i</b>	0.5	0.7	MPA-RT-Gly	<b>3i</b>	-	1.5
MPA-D-Thr-OH	<b>2j</b>	11	1	MPA-RT-aAla	<b>3j</b>	-	4.5
MPA-L-Ile-OH	<b>2k</b>	33	2.3	MPA-RT-bAla	<b>3k</b>	-	3.8
MPA-L-Arg-OH	<b>2l</b>	-	2.2	MPA-RT-Val	<b>3l</b>	-	4.3
MPA-D-Arg-OH	<b>2m</b>	-	0.7	MPA-RT-Leu	<b>3m</b>	-	3.2
				MPA-RT-Ile	<b>3n</b>	-	3.5

SI higher than for MPA

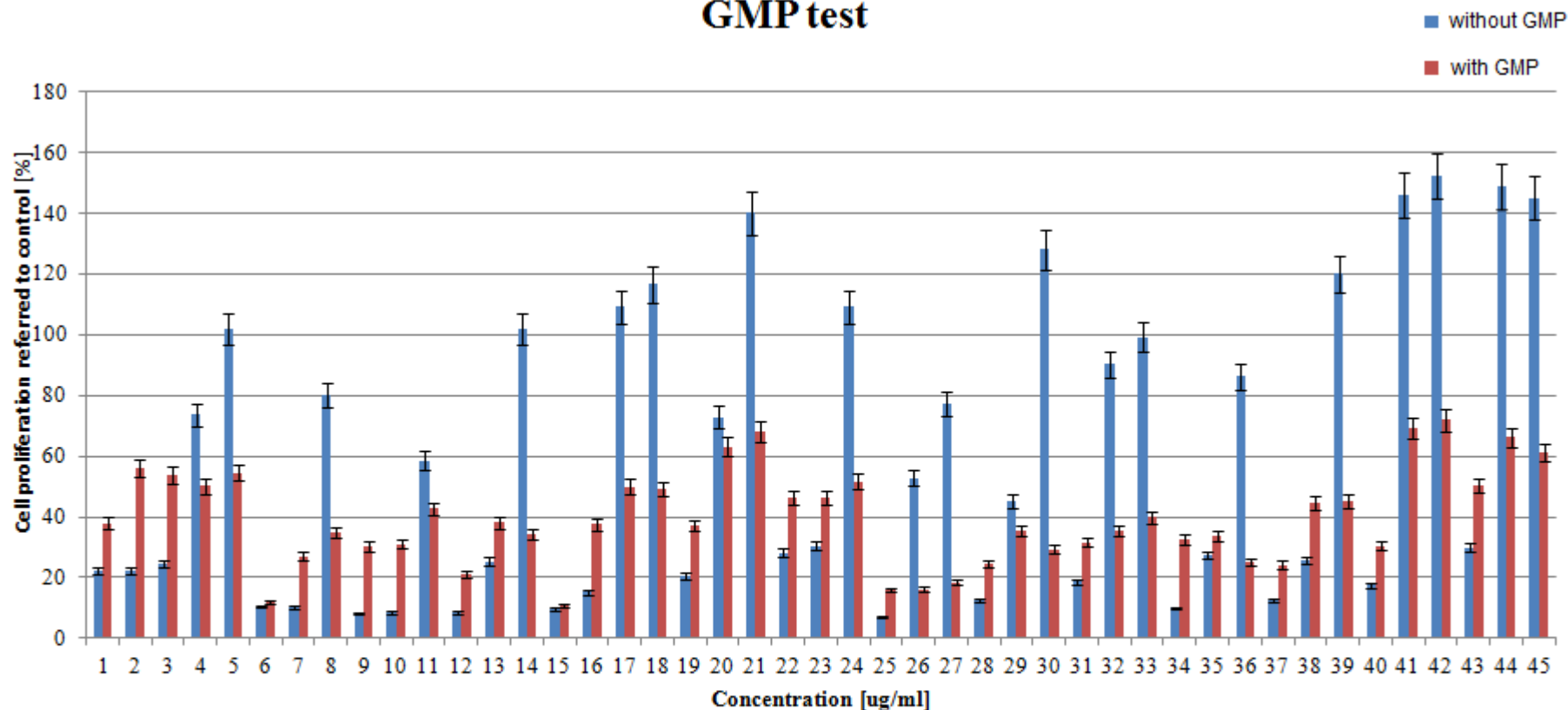


#### 2.2.4. Determination of antiproliferative activity of compounds **2a-m**, **3d**, **e**, **3h**, **j**, **l** on PBMCs using the VPD450 dye in the presence of GMP

The inosine 5'-monophosphate dehydrogenase enzyme, inhibits nucleotide biosynthesis *de novo*. Data in the literature indicate that suppression of cell proliferation under the influence of IMPDH inhibitors is reversible by the addition of guanosine, GMP, GTP or deoxy-GMP [6]. Therefore, antiproliferative tests with the addition of GMP was performed to check whether the antiproliferative activity of MPA derivatives was based on the same mechanism of action as mycophenolic acid **1**. For this purpose, we carried out an antiproliferation test using the VPD450 dye with the addition of 50  $\mu$ M GMP (concentration of GMP in the well).

Figures 12, 13 present the proliferative activity of cells relative to controls at respective concentrations of examined compounds. The mechanism of action of MPA derivatives was consistent with the action of MPA **1**. In the range of concentrations in which inhibition of proliferation occurs without the addition of GMP, it can be seen that after the addition of GMP, proliferation increases definitely. In contrast, at concentrations in which proliferation without GMP was higher than about 50%, it decreased rapidly after GMP addition. In other words, the reversal of proliferation was observed again. At lower concentrations, in which the compounds did not significantly inhibit lymphocyte proliferation, the IMPDH enzyme was likely inhibited by GMP (product), which might be due to substrate-product imbalance caused by an excess of guanosine-5'-monophosphate. At these concentrations, it was also possible the toxic effects of GMP known from the netosis or its effects on other metabolic pathways (as a third transmitter or substrate for other enzymes).

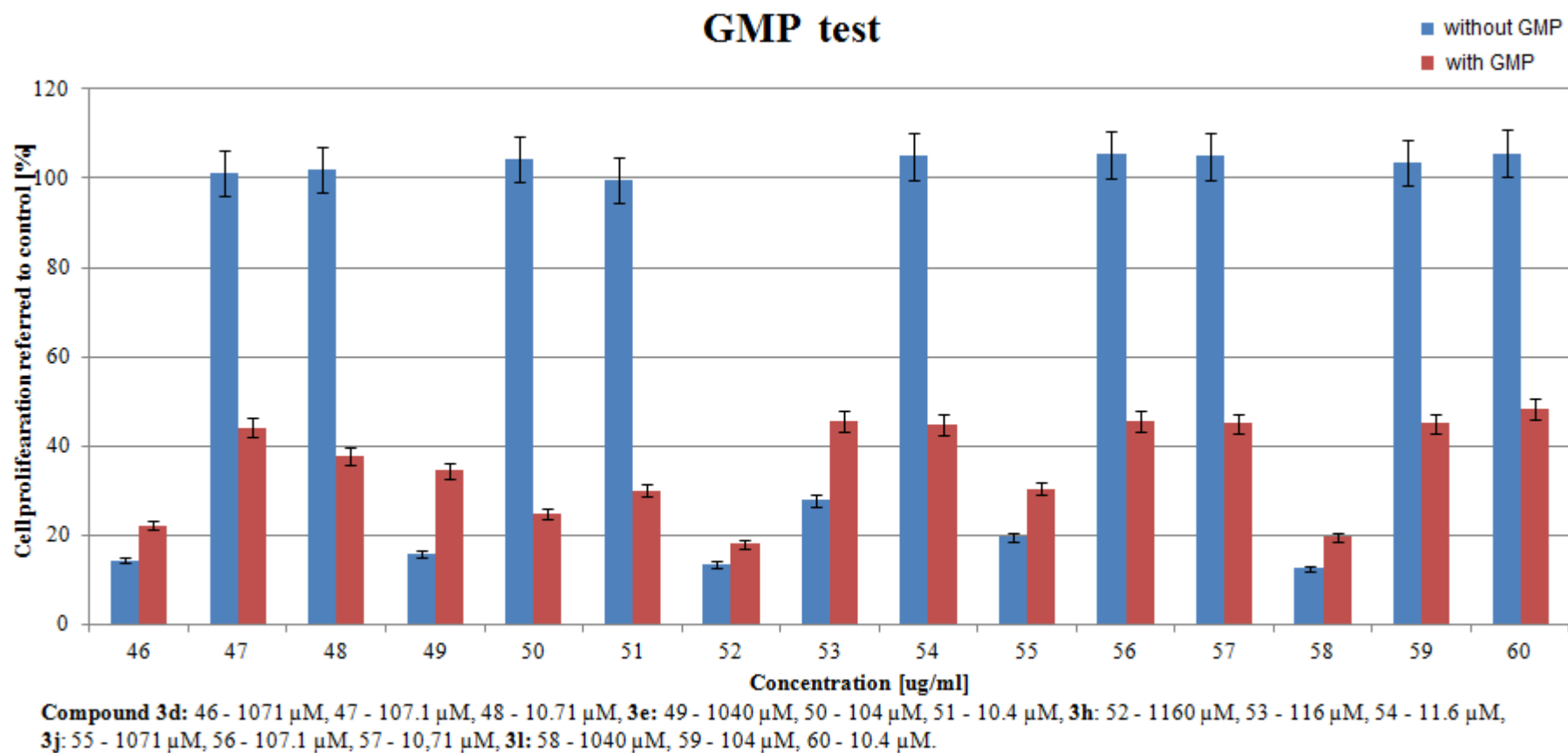
## GMP test



**Compound 1:** 1 - 312.2  $\mu$ M, 2 - 31.22  $\mu$ M, 3 - 3.122  $\mu$ M, 4 - 0.3122  $\mu$ M, 5 - 0.03122  $\mu$ M, **2a:** 6 - 215.8  $\mu$ M, 7 - 21.8  $\mu$ M, 8 - 2.158  $\mu$ M, **2b:** 9 - 229.6  $\mu$ M, 10 - 22.96  $\mu$ M, 11 - 2.296  $\mu$ M, **2c:** 12 - 229.6  $\mu$ M, 13 - 22.96  $\mu$ M, 14 - 2.296  $\mu$ M, **2d:** 15 - 223.4  $\mu$ M, 16 - 22.34  $\mu$ M, 17 - 2.234  $\mu$ M, 18 - 0.2234  $\mu$ M, **2e:** 19 - 186.7  $\mu$ M, 20 - 18.67  $\mu$ M, 21 - 1.867  $\mu$ M, **2f:** 22 - 1867  $\mu$ M, 23 - 186.7  $\mu$ M, 24 - 18.67  $\mu$ M, **2g:** 25 - 222.5  $\mu$ M, 26 - 22.25  $\mu$ M, 27 - 2.225  $\mu$ M, **2h:** 28 - 229.7  $\mu$ M, 29 - 22.97  $\mu$ M, 30 - 2.297  $\mu$ M, **2i:** 31 - 237.3  $\mu$ M, 32 - 23.73  $\mu$ M, 33 - 2.373  $\mu$ M, **2j:** 34 - 237.3  $\mu$ M, 35 - 23.73  $\mu$ M, 36 - 2.373  $\mu$ M, **2k:** 37 - 230.7  $\mu$ M, 38 - 23.07  $\mu$ M, 39 - 2.307  $\mu$ M, **2l:** 40 - 1917  $\mu$ M, 41 - 191.7  $\mu$ M, 42 - 19.17  $\mu$ M, **2m:** 43 - 1917  $\mu$ M, 44 - 191.7  $\mu$ M, 45 - 19.17  $\mu$ M.

**Figure 12.** Antiproliferative activity of amino acid derivatives of MPA **2a-m** in the presence of GMP.



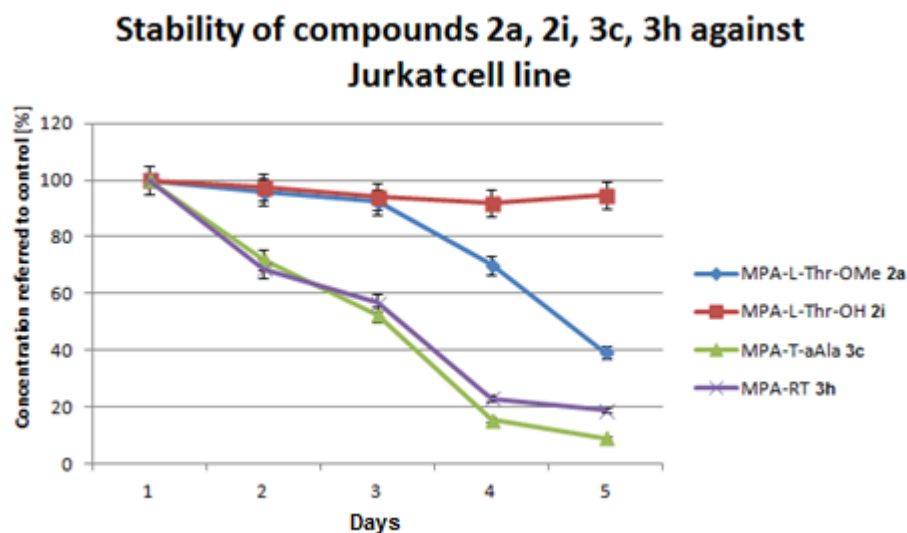


**Figure 13.** Antiproliferative activity of peptide derivatives of MPA **3d, e, h, j, l** in the presence of GMP.



### 2.2.5. Determination of stability of selected compounds against Jurkat cells using HPLC-MS

In order to check the stability of the obtained MPA analogs against cell cultures, an HPLC-MS analysis was carried out to determine the amount of compounds within incubation with Jurkat cells. Compounds **2a**, **i**, **3c**, **h** at EC<sub>50</sub> concentrations were incubated with Jurkat cells for 5 days, a sample was taken each day for HPLC-MS.



**Figure 14.** The rate of consumption of compounds **2a,i**, **3c,h** against Jurkat during a five-days incubation.

Figure 14 presents the stability of compounds **2a**, **i**, **3c**, **h** against Jurkat cells during a five-day incubation. The area of the peak of the first day was marked as 100%. Analyzing the data in the graph can be seen, that the derivative possessing threonine **3i** with a free carboxyl group was characterized by considerable stability, the amount of compound during the five-day incubation did not fall below 90%. The MPA analogue modified with threonine with a carboxyl group protected with methyl ester **2b** also showed good stability towards the tested cell line, however on the fifth day of incubation, the amount of derivative **2b** was about 40%. Therefore, addition of appropriate portion of the analogue **2b** could be advantageous for incubation for longer than 5 days. The peptide derivatives of MPA turned out to be much less stable, which can be explained by their extensive structure. After the third day of incubation, slightly more than 50% of the **3c** and **3h** analogs remained in the well. Thus, addition of another portion of compounds **3c** and **3h** during incubation over 4 days would be necessary.

### 3. Summary

In the article we presented the synthesis and immunosuppressive studies of amino acid and peptide analogs of mycophenolic acid. The most favorable conditions for the synthesis included



EDCI / DMAP and T3P / TEA procedures. We performed cytotoxicity (MTT) and antiproliferation tests (VPD 450, flow cytometry) to select compounds possessing improved parameters to MPA **1**. The tests on the T-Jurkat lymphoidal cell line and peripheral blood mononuclear cells (PBMCs) were carried out. The obtained compounds fulfilled the assumption and proved to be less toxic than mycophenolic acid (against PBMC), whereas higher toxicity showed only four amino acids derivatives **2a**, **2b**, **2d** and **2i** (T-Jurkat). The highest cytotoxicity against both types of cells indicated MPA-L-Ile-OMe **2d**, and the all peptide analogs of MPA were much less toxic than mycophenolic acid.

None of the designed compounds showed such a good antiproliferative effect as MPA **1**. The most active occurred to be derivatives **2a-d** and **2k** (T-Jurkat and PBMC). The MPA derivative with isoleucine both in the form of methyl ester **2d** ( $EC_{50} = 5.12 \mu\text{M}$  for PBMC) and deprotected **2k** ( $EC_{50} = 9 \mu\text{M}$  for PBMC) had the lowest  $EC_{50}$  value making it the most active MPA analog. The lowest activity against both types of cells was found in the case of **2h**, **2l** and **2m**. The highest activity among peptide derivatives indicated compound **3h**, whose  $EC_{50}$  value was  $663.93 \mu\text{M}$  against T-Jurkat cell line and  $35.43 \mu\text{M}$  against PBMCs.

The cytotoxicity and antiproliferative activity of amide MPA derivatives depended both on the presence of methyl ester and the absolute amino acid configuration. The MPA analogs protected with the methyl ester were more active than their counterparts with the free carboxyl group, which can be due to better cell membrane penetration [11]. In addition, L-enantiomers occurred to be more active than the enantiomers with the D-configuration. Most peptide derivatives negatively affected the action of MPA, but MPA conjugates with retro-tuftsins exhibited slightly higher activity than those with tuftsins.

According to the SI selectivity coefficient, we chose three analogs with a higher SI value than MPA ( $SI = 4.6$ ). These compounds include MPA derivatives with threonine of L and D configuration in the form of methyl esters, **2b** and **2c** ( $SI = 5.7$  for **2b** and  $7.3$  for **2c**) and MPA conjugate with retro-tuftsins **3h** ( $SI = 18$ ). The two peptide derivatives are characterized by a selectivity coefficient similar to mycophenolic acid **1**, they are conjugates of MPA modified with  $\alpha$ -alanine **3j** and valine **3l**. Despite the lower antiproliferative activity of selected compounds, they were less toxic. Therefore, it would be necessary to use a higher dose of the drug to achieve the same antiproliferative effect as in case of MPA **1**.

Proliferation studies with the addition of GMP, performed on the obtained MPA derivatives, indicated inhibitory activity against IMPDH similarly to mycophenolic acid.



## Acknowledgments

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## 4. Experimental section

All reactions were performed in inert atmosphere with magnetic stirring. DMF was purified by distillation from benzene/water. The reactions were monitored by TLC on Merck F254 silica gel precoated plates. The following solvent systems (by volume) were used for TLC development: (A), CHCl<sub>3</sub>:MeOH:NH<sub>3</sub> (5:1:0.2, v/v/v) (B). A: CHCl<sub>3</sub>:MeOH (9:1, v/v), B: CHCl<sub>3</sub>:MeOH:NH<sub>3</sub> (9:1:0.2, v/v/v), C; CHCl<sub>3</sub>:MeOH:NH<sub>3</sub> (3:1:0.2, v/v/v).

<sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were taken on the spectrometer Varian Unity 500 Plus in CD<sub>3</sub>OD or DMSO. Mass spectra were performed at the Laboratory of Mass Spectrometry MALDI-TOF on the matrix DHB (BIFLEX III Bruker).

Conditions of chromatographic HPLC separation and detection of examined compounds: column - Poroshell ECC18 (3.0 × 150 mm), 2.7 mm, Agilent Technologies; column temperature - 40°C; injection volume - 2 mL; flow rate - 0.4 mL/min; eluents: (A) 0.1% HCOOH in water, (B) 0.1% HCOOH in ACN/MeOH (1:1, v/v); gradient program:

### Gradient program for HPLC

Time [min]	% A	% B
0	90	10
20	0	100
30	0	100

Post time – 10 min; UV-Vis detection; wavelengths UV: 254 nm; Vis: 580 nm; peak width > 0.1 min (2s); ESI MS detection

### 4.1. General procedure for the preparation of MPA amino acid analogues **2a-m**

The amino acid analogues of the MPA, were obtained according to general procedure for preparation amino acid derivatives described in our last work [20,25].

### 4.2. Preparation of tuftsin/retro-tuftsin derivatives **3a-n**

The procedure for the synthesis of compounds **3a** and **3h** by the mixed anhydride method has been published [22-25]. The Fmoc-protecting groups was removed by treatment with diethylamine in chloroform.

#### 4.2.1. Conjugates of MPA with tuftsin derivatives **3a-h**.

##### 4.2.1.1 Coupling of MPA **1** with peptides **5a-g**

Derivative TFA×Thr-Lys(Fmoc)-Pro-Arg(NO<sub>2</sub>)-OCH<sub>3</sub> **5a** 0.025 g (0.0289 mmol), MPA 0.0068 g (0.0212 mmol), TEA 0.0014 ml (0.0112 mmol) were dissolved in 1 ml anhydrous DMF, under nitrogen. Then, the reaction mixture was cooled to 0°C, followed by addition of T3P 0.030 ml (0.052 mmol, 50 % solution in DMF) and stirred for 2 h. Subsequently, stirring was continued for 48 h at room temperature. The progress of the reaction was monitored with TLC (solvent system A). When the reaction was complete, the solvent was distilled off under vacuum, and the product isolated with preparative thin layer chromatography (PTLC), solvent system A.

##### 4.2.1.1.1. Compound MPA-Thr-Lys(Fmoc)-Pro-Arg(NO<sub>2</sub>)-OMe **6a**:

Product **6a** was obtained with yield 65% as white powder.

MS *m/z* calculated for C<sub>54</sub>H<sub>69</sub>N<sub>9</sub>O<sub>15</sub> 1084.1; found 1084.5 [M+H]<sup>+</sup>

R<sub>f</sub> = 0.512 (solvent system A).

##### 4.2.1.1.2. Compound MPA-Thr-Lys(FmocGly)-Pro-Arg(NO<sub>2</sub>)-OMe **6b**:

Product **6b** was obtained with yield 48 % as white powder.

MS *m/z* calculated for C<sub>56</sub>H<sub>72</sub>N<sub>10</sub>O<sub>16</sub> 1141.2; found 1141.5 M<sup>+</sup>

R<sub>f</sub> = 0.589 (solvent system A).

##### 4.2.1.1.3. Compound MPA-Thr-Lys(FmocαAla)-Pro-Arg(NO<sub>2</sub>)-OMe **6c**:

Product **6c** was obtained with yield 53 % as white powder.

MS *m/z* calculated for C<sub>58</sub>H<sub>76</sub>N<sub>10</sub>O<sub>16</sub> 1155.2; found: 1155.5 M<sup>+</sup>

R<sub>f</sub> = 0.542 (solvent system A).

##### 4.2.1.1.4. Compound MPA-Thr-Lys(FmocβAla)-Pro-Arg(NO<sub>2</sub>)-OMe **6d**:

Product **6d** was obtained with yield 51 % as white powder.

MS *m/z* calculated for C<sub>58</sub>H<sub>76</sub>N<sub>10</sub>O<sub>16</sub> 1155.2; found: 1155.5 M<sup>+</sup>

R<sub>f</sub> = 0.502 (solvent system A).

##### 4.2.1.1.5. Compound MPA-Thr-Lys(FmocVal)-Pro-Arg(NO<sub>2</sub>)-OMe **6e**:

Product **6e** was obtained with yield 49 % as white powder.

MS *m/z* calculated for C<sub>59</sub>H<sub>78</sub>N<sub>10</sub>O<sub>16</sub> 1183.3; found 1183.6 M<sup>+</sup>

R<sub>f</sub> = 0.583 (solvent system A).

4.2.1.1.6. Compound MPA-Thr-Lys(FmocLeu)-Pro-Arg(NO<sub>2</sub>)-OMe **6f**:

Product **6f** was obtained with yield 42 % as white powder.

MS *m/z* calculated for C<sub>60</sub>H<sub>80</sub>N<sub>10</sub>O<sub>16</sub> 1197.3; found 1197.6 M<sup>+</sup>

R<sub>f</sub> = 0.602 (solvent system A).

4.2.1.1.7. Compound MPA-Thr-Lys(FmocIle)-Pro-Arg(NO<sub>2</sub>)-OMe **6g**:

Product **6g** was obtained with yield 45 % as white powder.

MS *m/z* calculated for C<sub>60</sub>H<sub>80</sub>N<sub>10</sub>O<sub>16</sub> 1197.3; found 1197.6 M<sup>+</sup>

R<sub>f</sub> = 0.571 (solvent system A).

4.2.1.2. Deprotection of MPA conjugates with tuftsin to **3a-g**

Derivative MPA-Thr-Lys(Fmoc)-Pro-Arg(NO<sub>2</sub>)-OMe **6a** 0.05 g (0.046 mmol) was dissolved in 1 ml of chloroform and 0.2-0.3 ml of diethylamine was added. Then, the reaction mixture was stirred overnight and controlled with TLC (solvent system A). Subsequently, solvent was evaporated under vacuum, and the product isolated with preparative this layer chromatography (PTLC), solvent systems B and C. The conjugate **3a** was given in yield 84% as a white powder.

4.2.1.2.1. Compound MPA-Thr-Lys-Pro-Arg(NO<sub>2</sub>)-OMe **3a**:

Product **3a** was obtained with yield 84 % as white powder.

**MPA-T 3a**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 0.97 (d, J=6.3 Hz, 3H, γ-T4), 1.36 (m, 2H, γ-K4), 1.53 (m, 5H, δ-K5, β-K3b, γ-R4), 1.63 (m, 2H, β-K3a, β-R3a), 1.74 (s, 3H, f), 1.86 (m, 3H, β-P3b, γ-P4b, γ-P4a), 2.03 (m, 1H, β-P3a), 2.07 (s, 3H, e), 2.14 (m, 2H, g), 2.125 (m, 2H, h), 2.88 (m, 2H, ε-K6), 3.17 (m, 2H, δ-R5), 3.29 (d, J=6,8 Hz, 2H, d), 3.52 (m, 1H, δ-P5b), 3.62 (s, 3H, COOMe), 3.65 (m, 1H, δ-P5a), 3.69 (s, 3H, c), 3.92 (m, 1H, β-T3), 4.17 (m, 1H, α-T2), 4.21 (m, 1H, α-R2), 4.35 (m, 1H, α-P2), 4.50 (m, 1H, α-K2), 5.14 (t, J=6.4 Hz, 1H, a), 5.23 (s, 2H, b), 7.81 (m, 2H, α-KNH, α-TNH), 8.34 (d, J=8.34 Hz, 1H, α-RNH);

<sup>13</sup>CNMR (CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>): 10.02 (e), 14.93 (f), 18.83 (γ-T4), 21.73 (γ-K4), 22.25 (d), 24.79 (γ-P4), 26.7 (γ-R4), 28.19 (β-R3), 29.11 (δ-K5), 30.28 (β-P3), 34.26 (h), 35.13 (g), 39.10 (ε-K6), 40.15 (δ-R5), 42.15 (δ-P5), 48.46 (α-R2), 50.83 (α-K2), 51.46 (OMe), 56.93 (α-T2), 58.87 (α-P2), 61.17 (c), 66.99 (β-T3), 69.39 (b), 106.36 (l), 116.21 (r), 122.31 (o), 123.11 (a), 133.70 (j), 145.27 (m), 153.82 (p), 163.43 (k), 170.83 (T1), 171.31 (K1), 172.12 (n), 172.47 (R1), 173.10 (P1), 174.75 (i);

MS *m/z* calculated for C<sub>39</sub>H<sub>59</sub>N<sub>9</sub>O<sub>13</sub> 861.4; found 861.9 M<sup>+</sup>

R<sub>f</sub> = 0.458 (solvent system B);

Purity HPLC 100%.



#### 4.2.1.2.2. Compound MPA-Thr-Lys(Gly)-Pro-Arg(NO<sub>2</sub>)-OMe **3b**:

Product **3b** was obtained with yield 81 % as white powder.

**MPA-T-Gly 3b**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 0.94 (d, J=6,3 Hz, 3H, γ-T4), 1.24 (m, 2H, γ-K4), 1.33 (m, 2H, δ-K5), 1.46 (m, 5H, β-K3b, γ-R4, β-K3a, β-K3a), 1.67 (m, 4H, f, β-R3a), 1.75 (m, 3H, β-P3b, γ-P4b, γ-P4a), 2.01 (m, 4H, β-P3a, e), 2.10 (m, 2H, g), 2.17 (m, 2H, hb, ha), 3.03 (m, 2H, ε-K6), 3.10 (m, 4H, δ-R5, α-G2), 3.23 (d, J=6.8 Hz, 2H, d), 3.48 (m, 1H, δ-P5b), 3.61 (s, 3H, COOMe), 3.63 (m, 1H, δ-P5a), 3.65 (s, 3H, c), 3.89 (m, 1H, β-T3), 4.14 (m, 1H, α-T2), 4.18 (m, 1H, α-R2), 4.32 (m, 1H, α-P2), 4.44 (m, 1H, α-K2), 5.09 (t, J=6.5 Hz, 1H, a), 5.15 (s, 2H, b), 6.70 (m, 2H, α-KNH, α-TNH), 7.86 (t, J=5.5 Hz, 1H, ε-NHK), 8.22 (d, J=7.4 Hz, 1H, α-RNH);

<sup>13</sup>CNMR (CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>): 11.48 (e), 16.51 (f), 20.19 (γ-T4), 22.52 (γ-K4), 22.95 (d), 24.90 (γ-P4/γ-R4), 28.46 (β-R3), 29.17 (δ-K5), 39.45 (β-P3), 31.24 (β-K3), 34.58 (h), 35.50 (g), 38.54 (ε-K6), 40.63 (δ-P5), 43.71 (α-G2), 50.63 (α-R2), 52.02 (α-K2), 52.29 (COOMe), 58.44 (α-T2), 59.43 (α-P2), 60.99 (c), 67.03 (β-T3), 68.86 (b), 105.90 (l), 107.27 (r), 123.01 (o), 123.23 (a), 134.18 (j), 146.11 (m), 159.78 (p), 162.98 (k), 170.29 (T1), 170.42 (K1), 170.91 (n), 171.93 (G1), 172.24 (P1), 172.56 (R1), 172.80 (i);

MS *m/z* calculated for C<sub>41</sub>H<sub>62</sub>N<sub>10</sub>O<sub>14</sub> 919.0; found 919.4 M<sup>+</sup>

**Rf** = 0.372 (solvent system B)

Purity HPLC 100%.

#### 4.2.1.2.3. Compound MPA-Thr-Lys(αAla)-Pro-Arg(NO<sub>2</sub>)-OMe **3c**:

Product **3c** was obtained with yield 79% as white powder.

**MPA-T-αAla 3c**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 0.97 (d, J=6.3 Hz, 3H, γ-T4), 1.18 (d, J=6.3 Hz, 3H, β-A3), 1.29 (m, 2H, γ-K4), 1.39 (m, 3H, δ-K5, β-K3b), 1.48 (m, 2H, γ-R4) 1.56 (m, 1H, β-R3b), 1.62 (m, 1H, β-K3a), 1.68 (m, 1H, β-R3a), 1.74 (s, 3H, f), 1.79 (m, 2H, β-P3b, γ-P4b), 1.89 (m, 1H, γ-P4a), 2.03 (m, 1H, β-P3a), 2.07 (s, 3H, e), 2.15 (m, 2H, g), 2.27 (m, 2H, h), 3.05 (m, 2H, ε-K6), 3.17 (m, 2H, δ-R5), 3.28 (d, J=6.8 Hz, 2H, d), 3.05 (m, 1H, δ-P5b), 3.41 (dd, J=13.9 Hz, J=6.9 Hz, 1H, α-A2), 3.62 (s, 3H, COOMe), 3.65 (m, 1H, δ-P5a), 3.69 (s, 3H, c), 3.92 (m, 1H, β-T3), 4.17 (m, 1H, α-T2), 4.22 (m, 1H, α-R2), 4.35 (m, 1H, α-P2), 4.47 (m, 1H, α-K2), 5.15 (t, J=6.5 Hz, 1H, a), 5.21 (s, 2H, b), 7.74 (m, 2H, α-KNH, α-TNH), 7.97 (m, 1H, ε-KNH), 8.30 (d, J=7.4 Hz, 1H, α-RNH);

<sup>13</sup>CNMR (CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>) δ ppm: 10.05 (e), 14.98 (f), 18.86 (γ-T4), 18.92 (β-A3), 22.17 (γ-K4), 22.29 (d), 24.81 (γ-P4), 28.22 (γ-R4), 28.50 (β-R3), 29.04 (δ-K5), 30.49 (β-P3), 31.28 (β-K3), 34.32 (g), 35.15 (h), 38.55 (ε-K6), 40.15 (δ-R5), 46.89 (δ-P5), 48.48

( $\alpha$ -R2), 49.81 ( $\alpha$ -A2), 51.1 ( $\alpha$ -K2), 51.48 (OMe), 58.67 ( $\alpha$ -T2), 59.76 ( $\alpha$ -P2), 61.17 (c), 69.03 ( $\beta$ -T3), 69.33 (b), 106.37 (l), 115.91 (r), 122.40 (o), 123.17 (a), 133.65 (j), 145.26 (m), 154.20 (p), 163.40 (k), 171.12 (T1), 171.30 (K1, A1), 172.07 (n), 172.51 (R1), 173.01 (P1), 174.67 (i); MS  $m/z$  calculated for C<sub>42</sub>H<sub>64</sub>N<sub>10</sub>O<sub>14</sub> 933.0; found 933.4 M<sup>+</sup>

Purity HPLC 100%

R<sub>f</sub> = 0.442 (solvent system B).

#### 4.2.1.2.4. Compound MPA-Thr-Lys( $\beta$ Ala)-Pro-Arg(NO<sub>2</sub>)-OMe **3d**:

Product **3d** was obtained with yield 78% as white powder.

MPA-T- $\beta$ Ala **3d**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  ppm: 0.97 (d, J=6.3 Hz, 3H,  $\gamma$ -T4), 1.33 (m, 2H,  $\gamma$ -K4), 1.38 (m, 3H,  $\delta$ -K5,  $\beta$ -K3b), 1.47 (m, 2H,  $\gamma$ -R4), 1.55 (m, 1H,  $\beta$ -R3b), 1.63 (m, 1H,  $\beta$ -K3a), 1.71 (m, 1H,  $\beta$ -R3a), 1.74 (s, 3H, f), 1.78 (m, 1H,  $\beta$ -P3b), 1.88 (m, 2H,  $\gamma$ -P4b,  $\gamma$ -P4a), 2.04 (m, 1H,  $\beta$ -P3a), 2.08 (s, 3H, e), 2.16 (m, 2H, g), 2.27 (m, 2H, h), 2.46 (m, 2H,  $\alpha$ -A2), 2.89 (q, J=7.3 Hz, 2H,  $\beta$ -A3), 3.02 (m, 2H,  $\epsilon$ -K6), 3.15 (m, 2H,  $\delta$ -R5), 3.30 (d, J=7.0 Hz, 2H, d), 3.5 (m, 1H,  $\delta$ -P5b), 3.65 (s, 3H, COOMe), 3.65 (m, 1H,  $\delta$ -P5a), 3.70 (s, 3H, c), 3.91 (m, 1H,  $\beta$ -T3), 4.18 (m, 2H,  $\alpha$ -T2,  $\alpha$ -R2), 4.35 (m, 1H,  $\alpha$ -P2), 4.47 (m, 1H,  $\alpha$ -K2), 5.13 (t, J=6.4 Hz, 1H, a), 5.25 (s, 2H, b), 7.79 (m, 2H,  $\alpha$ -KNH,  $\alpha$ -TNH), 7.81 (m, 1H,  $\epsilon$ -KNH), 8.33 (d, J=7.2 Hz, 1H,  $\alpha$ -RNH);

<sup>13</sup>CNMR CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>)  $\delta$  ppm: 10.07 (e), 14.95 (f), 18.82 ( $\gamma$ -T4), 22.18 ( $\gamma$ -K4), 22.24 (d), 24.81 ( $\gamma$ -P4), 28.21 ( $\gamma$ -R4), 28.45 ( $\beta$ -R3), 29.03 ( $\delta$ -K5), 30.46 ( $\beta$ -P3), 31.39 ( $\beta$ -K3), 34.33 (h), 35.16 (g), 35.88 ( $\alpha$ -A2), 38.54 ( $\beta$ -A3), 40.18 ( $\epsilon$ -K6), 42.15 ( $\delta$ -R5), 46.92 ( $\delta$ -P5), 48.48 ( $\alpha$ -R2,  $\alpha$ -K2), 51.49 (OMe), 58.63 ( $\alpha$ -T2), 60.08 ( $\alpha$ -P2), 60.22 (c), 67.10 ( $\beta$ -T3), 69.47 (b), 106.33 (l), 116.53 (r), 122.24 (o), 123.01 (a), 133.82 (j), 145.28 (m), 153.28 (p), 163.44 (k), 170.77 (T1), 171.13 (K1), 171.25 (A1), 172.15 (n), 172.41 (R1), 173.04 (P1), 174.67 (i);

MS  $m/z$  calculated for C<sub>42</sub>H<sub>64</sub>N<sub>10</sub>O<sub>14</sub> 933.0; found 933.5 M<sup>+</sup>

R<sub>f</sub> = 0.370 (solvent system B)

Purity HPLC 94.8 %.

#### 4.2.1.2.5. Compound MPA-Thr-Lys(Val)-Pro-Arg(NO<sub>2</sub>)-OMe **3e**:

Product **3e** was obtained with yield 82% as white powder.

MPA-T-Val **3e**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  ppm: 0.81 (d, J=6.8 Hz, 3H,  $\gamma$ -V4), 0.86 (d, J=6.9 Hz, 3H,  $\gamma$ -V4), 0.96 (d, J=6.3 Hz, 3H,  $\gamma$ -T4), 1.22 (m, 5H,  $\gamma$ -K4,  $\delta$ -K5,  $\beta$ -K3b), 1.48 (m, 5H,  $\gamma$ -R4,  $\beta$ -K3a,  $\beta$ -R3a,  $\beta$ -R3b), 1.68 (s, 3H, f), 1.77 (m, 4H,  $\beta$ -P3b,  $\beta$ -V2,  $\gamma$ -P4b,  $\gamma$ -P4a), 2.01 (m, 4H,  $\beta$ -P3a, e), 2.10 (m, 4H, g,h), 2.99 (m, 5H,  $\epsilon$ -K6,  $\delta$ -R5,  $\alpha$ -V2), 3.25 (d, J=6.9 Hz,

2H, d), 3.48 (m, 1H,  $\delta$ -P5b), 3.59 (s, 3H, COOMe), 3.64 (m, 1H,  $\delta$ -P5a), 3.66 (s, 3H, c), 3.88 (m, 1H,  $\beta$ -T3), 4.15 (m, 2H,  $\alpha$ -T2,  $\alpha$ -R2), 4.33 (m, 1H,  $\alpha$ -P2), 4.44 (m, 1H,  $\alpha$ -K2), 5.10 (t, J=5.14 Hz, 1H, a), 5.20 (s, 2H, b), 7.72 (m, 2H,  $\alpha$ -KNH,  $\alpha$ -TNH), 7.98 (m, 1H,  $\epsilon$ -NHK), 8.29 (d, J=8.3 Hz, 1H,  $\alpha$ -RNH);

$^{13}\text{C}$ NMR (CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>): 10.03 (e), 14.92 (f), 16.70 ( $\gamma$ -V4), 18.02 ( $\gamma$ -T5), 18.83 ( $\gamma$ -T4), 22.25 (d), 24.81 ( $\gamma$ -R4), 24.87 ( $\gamma$ -P4), 28.22 ( $\beta$ -R3), 28.52 ( $\delta$ -R5), 29.03 ( $\beta$ -P3), 30.48 ( $\beta$ -K3), 31.31 ( $\beta$ -V3), 34.34 ( $\delta$ -K5), 35.17 ( $\beta$ -P3), 38.55 ( $\beta$ -R3), 40.14 (g), 51.11 ( $\alpha$ -K2), 51.46 (COOMe), 58.67 ( $\alpha$ -T2), 59.76 ( $\alpha$ -P2), 60.18 (c), 67.04 ( $\beta$ -T3), 69.40 (b), 106.34 (l), 116.34 (r), 122.29 (o), 123.07 (a), 133.74 (j), 145.25 (m), 159.46 (p), 163.43 (k), 171.12 (K1), 171.30 (T1), 172.05 (n/V1), 172.44 (P1), 173.02 (R1), 174.66 (i);

MS  $m/z$  calculated for C<sub>43</sub>H<sub>66</sub>N<sub>10</sub>O<sub>14</sub> 947.0; found 947.5 M<sup>+</sup>

R<sub>f</sub> = 0.58 (solvent system B)

Purity HPLC 99.3%.

#### 4.2.1.2.6. Compound MPA-Thr-Lys(Leu)-Pro-Arg(NO<sub>2</sub>)-OMe **3f**:

Product **3f** was obtained with yield 83% as white powder.

MPA-T-Leu **3f**:  $^1\text{H}$ NMR (400 MHz, DMSO-d<sub>6</sub>)  $\delta$  ppm: 0.81 (m, 6H,  $\delta$ -L5,  $\epsilon$ -L6), 0.96 (d, J=6.3 Hz, 3H,  $\gamma$ -T4), 1.24 (m, 7H,  $\gamma$ -K4,  $\beta$ -L2,  $\delta$ -K5,  $\beta$ -K3b,  $\beta$ -R3b), 1.53 (m, 4H,  $\gamma$ -R4,  $\beta$ -K3a,  $\beta$ -R3a), 1.74 (s, 3H, f), 1.77 (m, 4H,  $\beta$ -P3b,  $\gamma$ -L4,  $\gamma$ -P4b,  $\gamma$ -P4a), 2.05 (m, 4H,  $\beta$ -P3a, e), 2.13 (m, 4H, g,h), 3.01 (m, 4H,  $\epsilon$ -K6,  $\delta$ -R5), 3.28 (d, J=6.8 Hz, 2H, d), 3.44 (m, 1H,  $\delta$ -P5b), 3.62 (m, 4H, COOMe,  $\delta$ -P5a), 3.69 (s, 3H, c), 3.89 (m, 1H,  $\beta$ -T3), 4.16 (m, 2H,  $\alpha$ -T2,  $\alpha$ -R2), 4.33 (m, 1H,  $\alpha$ -P2), 4.44 (m, 1H,  $\alpha$ -K2), 5.12 (t, J=6.5 Hz, 1H, a), 5.24 (s, 2H, b), 7.73 (m, 2H,  $\alpha$ -KNH,  $\alpha$ -TNH), 8.13 (m, 1H,  $\epsilon$ -NHK), 8.29 (d, J=8.3 Hz, 1H,  $\alpha$ -RNH);

$^{13}\text{C}$ NMR (CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>): 10.60 (e), 14.97 (f), 18.07 ( $\gamma$ -T4), 21.10 (d/  $\gamma$ -K4), 21.68 ( $\epsilon$ -L6) 22.26 ( $\delta$ -L5), 24.34 ( $\gamma$ -L4), 24.82 ( $\gamma$ -R4/ $\gamma$ -P4), 28.24 ( $\beta$ -R3), 28.48 ( $\delta$ -R5), 29.03 ( $\beta$ -P3), 30.48 ( $\beta$ -K3), 34.35 ( $\delta$ -K5), 35.17 ( $\beta$ -P3), 38.55 ( $\beta$ -R3), 40.16 (g), 42.35 ( $\epsilon$ -K6), 48.47 ( $\alpha$ -R2), 51.11 ( $\alpha$ -K2), 51.48 ( $\alpha$ -L2), 52.63 (COOMe), 58.69 ( $\alpha$ -T2), 60.19 (c), 67.05 ( $\beta$ -T3), 69.41 (b), 106.34 (l), 116.31 (r), 122.29 (o), 123.07 (a), 133.75 (j), 145.25 (m), 159.44 (p), 163.44 (k), 171.13 (K1), 171.31 (T1), 172.05 (n/L1), 172.44 (P1), 173.03 (R1), 174.65 (i);

MS  $m/z$  calculated for C<sub>45</sub>H<sub>70</sub>N<sub>10</sub>O<sub>14</sub> 975.1; found 975.3 M<sup>+</sup>

R<sub>f</sub> = 0.548 (solvent system B)

Purity HPLC 98.7%.



#### 4.2.1.2.7. Compound MPA-Thr-Lys(Ile)-Pro-Arg(NO<sub>2</sub>)-OMe **3g**:

Product **3g** was obtained with yield 77.7% as a white powder.

MPA-T-Ile **3g**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 0.81 (m, 6H, δ-I5, ε-I6), 0.96 (d, J=6.3 Hz, 3H, γ-T4), 1.26 (m, 6H, γ-K4, β-I3, δ-K5, β-R3b), 1.51 (m, 5H, γ-R4, β-K3b, β-R3a, β-K3a), 1.70 (s, 4H, f, γ-I4b), 1.77 (m, 4H, β-P3b, γ-I4a, γ-P4b, γ-P4a), 2.00 (m, 1H, β-P3a), 2.08 (s, 3H, e), 2.15 (m, 4H, g, h), 2.99 (m, 4H, ε-K6, δ-R5), 3.22 (d, J=3.2 Hz, 1H, α-I2), 3.28 (d, J=6.8 Hz, 2H, d), 3.48 (m, 1H, δ-P5b), 3.62 (m, 3H, COOMe), 3.64 (m, 1H, δ-P5a), 3.69 (s, 3H, c), 3.90 (m, 1H, β-T3), 4.16 (m, 2H, α-T2, α-R2), 4.33 (m, 1H, α-P2), 4.44 (m, 1H, α-K2), 5.12 (t, J=6.4 Hz, 1H, a), 5.23 (s, 2H, b), 7.73 (m, 2H, α-KNH, α-TNH), 8.13 (m, 1H, ε-NHK), 8.29 (d, J=8.3 Hz, 1H, α-RNH);

<sup>13</sup>CNMR (CD<sub>3</sub>OD, 400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>): 11.52 (e), 11.81 (δ-I5), 15.68 (ε-I6), 16.52 (f), 20.15 (γ-T4), 22.67 (γ-K4), 22.90 (d), 24.48 (γ-L4), 24.90 (γ-P4), 28.44 (β-R3), 29.15 (δ-P5), 29.47 (β-P3), 31.29 (β-K3), 34.56 (δ-K5), 35.52 (β-P3), 37.77 (h), 38.66 (g), 47.24 (α-P2), 49.04 (α-I2), 50.70 (α-K2), 52.61 (COOMe), 58.45 (α-P2), 59.42 (α-T2), 61.06 (c), 67.13 (β-T3), 69.02 (b), 107.35 (l), 115.86 (r), 122.96 (o), 123.05 (a), 134.40 (j), 146.20 (m), 159.88 (p), 163.03 (k), 170.31 (K1), 170.46 (T1), 170.76 (n/II), 172.26 (P1), 172.49 (R1), 172.82 (i);

MS *m/z* calculated for C<sub>45</sub>H<sub>70</sub>N<sub>10</sub>O<sub>14</sub> 975.1 found 975.5 M<sup>+</sup>

R<sub>f</sub> = 0.532 (solvent system B)

Purity HPLC 98.5%.

#### 4.2.2. MPA conjugates with retro-tuftsin **3h-n**

##### 4.2.2.1. Coupling of MPA **1** with peptides **5h-n**

Compound TFA×Arg(NO<sub>2</sub>)-Pro-Lys(Fmoc)-Thr-OCH<sub>3</sub> **5h** 0.01 g (0.011 mmol), MPA 0.0026 g (0.008 mmol), DMAP 0.015 g (0.011 mmol) were dissolved in 1 ml of anhydrous DMF under nitrogen. Then, the reaction mixture was cooled to 0 °C, followed by addition of EDCI 0.0017 g (0.0107 mmol), and after 2 h stirred for 48 h at room temperature. The progress of the reaction was monitored with TLC (solvent system A). When the reaction was complete, solvent was distilled off under vacuum and the product isolated with preparative thin layer chromatography (PTLC), solvent system A.

##### 4.2.2.1.1. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(Fmoc)-Thr-OCH<sub>3</sub> **6h**:

Product **6h** was obtained with yield 64% as a white powder.

MS *m/z* calculated for C<sub>54</sub>H<sub>69</sub>N<sub>9</sub>O<sub>15</sub> 1084.1; found 1084.5 M<sup>+</sup>

R<sub>f</sub> = 0.489 (solvent system A).



4.2.2.1.2 Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(FmocGly)-Thr-OCH<sub>3</sub> **6i**:

Product **6i** was obtained with yield 28% as white powder.

MS *m/z* was calculated for C<sub>56</sub>H<sub>72</sub>N<sub>10</sub>O<sub>16</sub> 1141.2; found 1141.6 M<sup>+</sup>

R<sub>f</sub> = 0.474 (solvent system A).

4.2.2.1.3. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(FmocαAla)-Thr-OCH<sub>3</sub> **6j**:

Product **6j** was obtained with yield 28% as white powder.

MS *m/z* calculated for C<sub>58</sub>H<sub>74</sub>N<sub>10</sub>O<sub>16</sub> 1155.3; found 1155.5 M<sup>+</sup>

R<sub>f</sub> = 0.480 (solvent system B).

4.2.2.1.4. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(FmocβAla)-Thr-OCH<sub>3</sub> **6k**:

Product **6k** was obtained with yield 28% as white powder.

MS *m/z* calculated for C<sub>58</sub>H<sub>74</sub>N<sub>10</sub>O<sub>16</sub> 1155.2; found 1155.6 M<sup>+</sup>

R<sub>f</sub> = 0.420 (solvent system B).

4.2.2.1.5. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(FmocVal)-Thr-OCH<sub>3</sub> **6l**:

Product **6l** was obtained with yield 28% as white powder.

MS *m/z* calculated for C<sub>59</sub>H<sub>78</sub>N<sub>10</sub>O<sub>16</sub> 1183.3; found 1183.6 M<sup>+</sup>

R<sub>f</sub> = 0.465 (solvent system A).

4.2.2.1.6. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(FmocLeu)-Thr-OCH<sub>3</sub> **6m**:

Product **6m** was obtained with yield 28% as white powder.

MS *m/z* calculated for C<sub>60</sub>H<sub>80</sub>N<sub>10</sub>O<sub>16</sub> 1197.3; found 1197.6 M<sup>+</sup>

R<sub>f</sub> = 0.512 (solvent system A).

4.2.2.1.7. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(FmocIle)-Thr-OCH<sub>3</sub> **6n**:

Product **6n** was obtained with yield 28% as white powder.

MS *m/z* calculated for C<sub>60</sub>H<sub>80</sub>N<sub>10</sub>O<sub>16</sub> 1197.3; found 1197.6 M<sup>+</sup>

R<sub>f</sub> = 0.585 (solvent system A).

4.2.2.2. Deprotection of MPA conjugates with retro-tuftsins to **3h-n**

Derivative MPA-Arg(NO<sub>2</sub>)-Pro-Lys(Fmoc)-Thr-OMe **6h** 0.05 g (0.046 mmol) was dissolved in 1 ml of chloroform and 0.2-0.3 ml of diethylamine was added. Then, the reaction mixture was stirred overnight and controlled with TLC (solvent system A). Subsequently, solvent was evaporated under vacuum, and the product isolated with preparative this layer chromatography (PTLC), solvent systems B and C. The conjugate **3h** was given in yield 86% as a white powder.

#### 4.2.2.2.1. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys-Thr-OMe **3h**:

Product **3h** was obtained with yield 86% as white powder.

MPA-RT **3h**: <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 1.057 (m, 3H, γ-T4), 1.348 (m, 2H, γ-K4), 1.487 (m, 3H, δ-K5, β-R3b), 1.532 (m, 3H, β-K3b, γ-R4), 1.640 (m, 2H, β-K3a, β-R3a), 1.728 (s, 3H, f), 1.810 (m, 2H, γ-P4b, β-P3b), 1.908 (m, 1H, γ-P4a), 1.992 (m, 1H, β-P3b), 2.083 (s, 3H, e), 2.132 (m, 2H, g), 2.171 (m, 2H, h), 2.735 (m, 2H, ε-K6), 3.135 (m, 2H, δ-R5), 3.283 (d, J=6.7 Hz, 2H, d), 3.548 (m, 1H, δ-P5b), 3.621 (s, 3H, COOMe), 3.649 (m, 1H, δ-P5a), 3.696 (s, 3H, c), 4.106 (m, 1H, β-T3), 4.237 (m, 1H, α-T2), 4.338 (m, 2H, α-K2, α-P2), 4.424 (m, 1H, α-R2), 5.099 (m, 1H, a), 5.253 (s, 2H, b), 7.972 (d, J=8.2 Hz, 1H, α-TNH), 8.045 (m, 2H, ε-KNH), 8.114 (d, J=7.6 Hz, 1H, α-RNH), 8.162 (d, J=7.8 Hz, 1H, α-KNH);

<sup>13</sup>C NMR (CD<sub>3</sub>OD, 400 MHz) δ ppm: 11.54 (e), 16.49 (f), 19.03 (γ-T4), 20.52 (d), 22.42 (γ-K4), 22.87 (γ-P4), 24.96 (δ-K5), 26.77 (γ-R4), 28.65 (β-R3), 29.55 (β-P3), 31.57 (β-K3), 34.27 (h), 35.46 (g), 38.97 (ε-K6), 41.67 (δ-R5), 47.33 (δ-P5), 52.30 (OMe), 52.50 (α-R2), 56.47 (α-K2), 58.35 (α-T2), 59.76 (α-P2), 61.12 (c), 66.82 (β-T3), 69.10 (b), 107.44 (o), 116.45 (r,l), 122.96 (a), 134.54 (j), 146.27 (m), 153.17 (p), 163.05 (k), 170.61 (n), 170.69 (T1), 171.46 (P1), 171.88 (i), 172.23 (K1), 172.54 (R1);

MS m/z calculated for C<sub>39</sub>H<sub>59</sub>N<sub>9</sub>O<sub>13</sub> 861.4; found 862.4 M<sup>+</sup>

R<sub>f</sub> = 0.583 (solvent system B);

Purity HPLC 100%.

#### 4.2.2.2.2. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(Gly)-Thr-OMe **3i**:

Product **3i** was obtained with yield 81% as white powder.

MPA-RT-Gly **3i**: <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 1.06 (d, J = 6.6 Hz, 3H, γ-T4), 1.29 (m, 2H, γ-K4), 1.38 (m, 3H, δ-K5, β-R3), 1.46 (m, 3H, β-K3, γ-R4), 1.65 (m, 2H, β-K3, β-R3), 1.73 (s, 3H), 1.81 (m, 2H, γ-P4, β-P3), 1.88 (m, 1H, γ-P4), 1.98 (m, 1H, β-P3), 2.06 (s, 3H), 2.13 (m, 2H), 2.19 (m, 2H), 3.07 (m, 2H, ε-K6), 3.11 (m, 2H, δ-R5), 3.26 (d, J = 6.7 Hz, 2H), 3.36 (m, 2H, a-G2), 3.51 (m, 1H, δ-P5), 3.60 (m, 1H, δ-P5), 3.63 (s, 3H, COOCH<sub>3</sub>), 3.68 (s, 3H), 4.10 (m, 1H, β-T3), 4.26 (m, 1H, α-T2), 4.28 (m, 1H, α-K2), 4.34 (m, 2H, α-P2), 4.45 (m, 1H, α-R2), 5.12 (t, J = 6.5 Hz, 1H), 5.21 (s, 2H), 7.82 (d, J = 8.4 Hz, 1H, α-TNH), 7.97 (m, 3H, α-RNH, a-KNH, ε-KNH);

<sup>13</sup>C NMR (400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>) δ ppm: 10.04 (e), 14.99 (f), 18.97 (γ-T4), 22.59 (d), 24.65 (γ-K4), 28.43 (γ-P4), 29.22 (γ-R4), 31.05 (β-R3), 33.94 (δ-K5), 35.05 (β-P3), 38.65 (β-K3), 40.53 (h), 42.17 (g), 42.78 (ε-K6), 48.47 (δ-R5), 50.77 (δ-P5), 51.47 (α-R2), 53.28 (OMe), 56.93 (α-K2), 57.81 (α-T2), 60.11 (α-P2), 60.15 (c), 66.99 (β-T3), 69.33 (b), 106.38 (o), 122.41

(r,l), 123.16 (a), 133.57 (j), 145.29 (m), 159.56 (p), 163.41 (k), 171 (n), 171.13 (T1), 172.56 (P1), 172.97 (i,K1), 173.25 (R1), 174.23 (G1);

MS m/z calculated for C<sub>41</sub>H<sub>62</sub>N<sub>10</sub>O<sub>14</sub> 919.0; found 919.4 M<sup>+</sup>.

R<sub>f</sub> = 0.454 (solvent system B).

Purity HPLC 100%.

#### 4.2.2.2.3. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(αAla)-Thr-OMe **3j**:

Product **3j** was obtained with yield 80% as white powder.

MPA-RT-αAla **3j**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 1.043 (d, J=5.7 Hz, 3H, γ-T4), 1.211 (d, J=6.9 Hz, 3H, β-A3), 1.290 (m, 2H, γ-K4), 1.380 (m, 3H, δ-K5, β-R3b), 1.5483 (m, 3H, β-K3b, γ-R4), 1.653 (m, 2H, β-K3a, β-R3a), 1.727 (s, 3H, f), 1.802 (m, 2H, γ-P4b, β-P3b), 1.894 (m, 1H, γ-P4a), 1.998 (m, 1H, β-P3a), 2.064 (s, 3H, e), 2.128 (m, 2H, g), 2.183 (m, 2H, h), 3.043 (m, 2H, ε-K6), 3.130 (m, 2H, δ-R5), 3.266 (d, J=6.5 Hz, 2H, d), 3.488 (m, 1H, α-A2), 3.532 (m, 1H, δ-P5b), 3.617 (m, 1H, δ-P5a), 3.625 (s, 3H, COOMe), 3.682 (s, 3H, c), 4.103 (m, 1H, β-T3), 4.257 (m, 1H, α-T2), 4.278 (m, 1H, α-K2), 4.341 (m, 2H, α-P2), 4.442 (m, 1H, α-R2), 5.114 (t, J=6.6 Hz, 1H, a), 5.217 (s, 2H, b), 7.827 (d, J=8.4 Hz, 1H, α-TNH), 8.026 (m, 3H, α-RNH, α-KNH, ε-KNH);

<sup>13</sup>CNMR (400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>) δ ppm: 10.06 (e), 14.98 (f), 18.56 (β-A3), 18.98 (γ-T4), 22.30 (d), 24.65 (γ-K4), 28.43 (γ-P4), 29.22 (γ-R4), 31.05 (β-R3), 33.96 (δ-K5), 35.07 (β-P3), 38.74 (β-K3), 40.53 (h), 49.68 (δ-R5), 50.77 (δ-P5, α-A2), 51.48 (α-R2), 53.32 (OMe), 56.94 (α-K2), 57.81 (α-T2), 60.11 (α-P2), 60.18 (c), 67.01 (β-T3), 69.38 (b), 106.38 (o), 116.13 (l), 122.34 (r), 123.1 (a), 133.65 (j), 145.29 (m), 159.55 (p), 163.42 (k), 171.01 (n), 171.14 (T1), 172.48 (P1), 172.96 (i), 173.23 (K1), 173.80 (R1), 174.22 (A1);

MS m/z calculated for C<sub>42</sub>H<sub>64</sub>N<sub>10</sub>O<sub>14</sub> 933.0; found 933.5 M<sup>+</sup>.

R<sub>f</sub> = 0.480 (solvent system B)

Purity HPLC 97%.

#### 4.2.2.2.4. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(βAla)-Thr-OMe **3k**:

Product **3k** was obtained with yield 88% as white powder.

MPA-RT-βAla **3k**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 1.043 (d, J=7 Hz, 3H, γ-T4), 1.270 (m, 2H, γ-K4), 1.385 (m, 3H, δ-K5, β-R3b), 1.5484 (m, 3H, β-K3b, γ-R4), 1.653 (m, 2H, β-K3a, β-R3a), 1.727 (s, 3H, f), 1.817 (m, 2H, γ-P4b, β-P3b), 1.886 (m, 1H, γ-P4a), 2.00 (m, 1H, β-P3a), 2.068 (s, 3H, e), 2.105 (m, 2H, g), 2.162 (m, 2H, h), 2.444 (t, J=6.8 Hz, 2H, α-A2), 2.946 (t, J=5.3 Hz, 2H, β-A3), 3.026 (m, 2H, ε-K6), 3.128 (m, 2H, δ-R5), 3.270 (d, J=6.8 Hz, 2H, d), 3.516 (m, 1H, δ-P5b), 3.615 (m, 1H, δ-P5a), 3.623 (s, 3H, COOMe),

3.686 (s, 3H, c), 4.102 (m, 1H,  $\beta$ -T3), 4.253 (m, 1H,  $\alpha$ -T2), 4.282 (m, 1H,  $\alpha$ -K2), 4.340 (m, 2H,  $\alpha$ -P2), 4.436 (m, 1H,  $\alpha$ -R2), 5.114 (t, J=6.9 Hz, 1H, a), 5.222 (s, 2H, b), 7.847 (d, J=8.4 Hz, 1H,  $\alpha$ -TNH), 8.043 (m, 3H,  $\alpha$ -RNH,  $\alpha$ -KNH,  $\epsilon$ -KNH);

$^{13}\text{C}$ NMR (400 MHz,  $\text{CD}_3\text{OD-d}_4$ )  $\delta$  ppm: 10.06 (e), 14.98 (f), 18.98 ( $\gamma$ -T4), 22.31 (d), 24.66 ( $\gamma$ -K4), 28.30 ( $\gamma$ -P4), 29.22 ( $\gamma$ -R4), 31.00 ( $\beta$ -R3), 31.96 ( $\beta$ -A3), 33.95 ( $\delta$ -K5), 35.06 ( $\beta$ -P3), 36.06 ( $\alpha$ -A2), 38.66 ( $\beta$ -K3), 40.56 (h), 42.151 (g), 46.868 ( $\epsilon$ -K6), 48.332 ( $\delta$ -R5) 50.85 ( $\delta$ -P5), 51.48 ( $\alpha$ -R2), 53.29 (OMe), 56.93 ( $\alpha$ -K2), 57.81 ( $\alpha$ -T2), 60.18 (c), 67.01 ( $\beta$ -T3), 69.37 (b), 106.37 (o), 116.03 (l), 122.37 (r), 123.12 (a), 133.63 (j), 145.31 (m), 159.56 (p), 163.42 (k), 170.96 (n), 170.99 (T1), 172.51 (P1), 172.98 (i) 173.26 (K1), 174.24 (R1,A1);

MS  $m/z$  calculated for  $\text{C}_{42}\text{H}_{64}\text{N}_{10}\text{O}_{14}$  933.0; found 933.5  $\text{M}^+$ .

R<sub>f</sub> = 0.309 (solvent system B).

Purity HPLC 100%.

#### 4.2.2.2.5. Compound MPA-Arg( $\text{NO}_2$ )-Pro-Lys(Val)-Thr-OMe **3l**:

Product **3l** was obtained with yield 79% as white powder.

MPA-RT-Val **3l**:  $^1\text{H}$ NMR (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  ppm: 0.807 (d, J=6.9 Hz, 3H,  $\gamma$ -V4), 0.858 (d, J=6.8 Hz, 3H,  $\delta$ -V5), 1.026 (d, J=4.2 Hz, 3H,  $\gamma$ -T4), 1.279 (m, 2H,  $\gamma$ -K4), 1.369 (m, 3H,  $\delta$ -K5,  $\beta$ -R3b), 1.461 (m, 3H,  $\beta$ -K3b,  $\gamma$ -R4), 1.627 (m, 2H,  $\beta$ -K3a,  $\beta$ -R3a), 1.714 (s, 3H, f), 1.792 (m, 2H,  $\gamma$ -P4b,  $\beta$ -P3b), 1.861 (m, 2H,  $\gamma$ -P4a,  $\beta$ -V3), 1.977 (m, 1H,  $\beta$ -P3a), 2.061 (s, 3H, e), 2.114 (m, 2H, g), 2.183 (m, 2H, h), 3.005 (m, 5H,  $\alpha$ -V2,  $\epsilon$ -K6,  $\delta$ -R5), 3.262 (d, J=6.7 Hz, 2H, d), 3.502 (m, 1H,  $\delta$ -P5b), 3.604 (m, 1H,  $\delta$ -P5a), 3.625 (s, 3H, COOMe), 3.676 (s, 3H, c), 4.096 (m, 1H,  $\beta$ -T3), 4.252 (m, 1H,  $\alpha$ -T2), 4.269 (m, 1H,  $\alpha$ -K2), 4.3 (m, 1H,  $\alpha$ -P2), 4.437 (m, 1H,  $\alpha$ -R2), 5.096 (t, J=6,5 Hz, 1H, a), 5.222 (s, 2H, b), 7.803 (d, J=8.4 Hz, 1H,  $\alpha$ -TNH), 8.022 (m, 3H,  $\alpha$ -RNH,  $\alpha$ -KNH,  $\epsilon$ -KNH);

$^{13}\text{C}$ NMR (400 MHz,  $\text{CD}_3\text{OD-d}_4$ )  $\delta$  ppm: 10.05 (e), 14.96 (f), 16.99 ( $\delta$ -V5), 18.22 ( $\gamma$ -V4), 18.97 ( $\gamma$ -T4), 22.28 (d), 24.65 ( $\gamma$ -K4), 28.49 ( $\gamma$ -P4), 29.22 ( $\gamma$ -R4), 31.05 ( $\beta$ -R3), 31.56 ( $\beta$ -V3), 33.96 ( $\delta$ -K5), 35.07 ( $\beta$ -P3), 38.68 ( $\beta$ -K3), 40.53 (h), 42.16 (g), 46.648 ( $\epsilon$ -K6), 47.904 ( $\delta$ -R5) 50.74 ( $\delta$ -P5), 51.46 ( $\alpha$ -R2), 53.33 (OMe), 56.93 ( $\alpha$ -T2), 57.80 ( $\alpha$ -V2), 60.1 ( $\alpha$ -P2), 60.18 (c), 67.01 ( $\beta$ -T3), 69.39 (b), 106.35 (o), 116.26 (l), 122.3 (r), 123.08 (a), 133.67 (j), 145.28 (m), 159.57 (p), 163.42 (k), 170.98 (n), 171.12 (T1), 172.43 (P1), 172.97 (i) 173.22 (K1), 173.41 (R1), 174.21 (V1);

MS  $m/z$  calculated for  $\text{C}_{43}\text{H}_{66}\text{N}_{10}\text{O}_{14}$  947.0; found 948.4  $[\text{M}+\text{H}]^+$

R<sub>f</sub> = 0.436 (solvent system B)

Purity HPLC 99%.

#### 4.2.2.2.6. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(Leu)-Thr-OMe **3m**:

Product **3m** was obtained with yield 91% as white powder.

MPA-RT-Leu **3m**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 0.77 (m, 6H, δ-L5, ε-L6), 1.014 (m, 3H, γ-T4), 1.22 (m, 10H, γ-K4, β-L3b, β-L3a, δ-K5, β-R3b, β-K3b, γ-R4), 1.59 (m, 3H, β-K3a, β-R3a, γ-L4b), 1.69 (s, 3H, f), 1.79 (m, 3H, γ-P4b, β-P3b, γ-L4a), 1.98 (m, 1H, γ-P4a), 2.05 (s, 3H, e), 2.11 (m, 4H, g, h), 3.03 (m, 2H, ε-K6), 3.11 (m, 2H, δ-R5), 3.25 (d, 2H, J=6.2 Hz, d), 3.32 (m, 1H, α-L2), 3.52 (m, 2H, δ-P5b, δ-P5a), 3.60 (s, 3H, COOMe), 3.67 (s, 3H, c), 4.09 (m, 1H, β-T3), 4.24 (m, 2H, α-T2, α-K2), 4.32 (m, 1H, α-P2), 4.43 (m, 1H, α-R2), 5.10 (t, J=6.6 Hz, 1H, a), 5.21 (s, 2H, b), 7.82 (d, J=8.4 Hz, 1H, α-TNH), 8.08 (m, 2H, α-RNH, α-KNH), 8.12 (m, 1H, ε-KNH);

<sup>13</sup>CNMR (400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>) δ ppm: 11.49 (e), 16.44 (f), 20.48 (γ-T4), 23.39 (d/γ-K4), 23.35 (δ-L5), 24.41 (γ-L4), 24.87 (γ-P4/γ-R4), 28.83 (β-R3), 29.17 (δ-K5), 29.52 (β-P3), 31.81 (β-K3), 34.25 (h), 35.48 (g), 38.81 (ε-K6), 40.54 (δ-R5), 43.17 (β-L3), 47.18 (δ-P5), 50.26 (α-R2), 52.28 (OMe), 52.74 (α-L2), 52.85 (α-K2), 58.13 (α-T2), 59.58 (α-P2), 60.95 (c), 66.76 (β-T3), 68.98 (b), 122.94 (o/a), 146.11 (m), 159.72 (p), 162.98 (k), 170.48 (n), 171.43 (T1), 171.82 (P1), 172.14 (i), 172.59 (K1);

MS *m/z* calculated for C<sub>45</sub>H<sub>70</sub>N<sub>10</sub>O<sub>14</sub> 975.1; found 975.5 M<sup>+</sup>

R<sub>f</sub> = 0.678 (solvent system B)

Purity HPLC 99.5%.

#### 4.2.2.2.7. Compound MPA-Arg(NO<sub>2</sub>)-Pro-Lys(Ile)-Thr-OMe **3n**:

Product **3n** was obtained with yield 84% as white powder.

MPA-RT-Ile **3n**: <sup>1</sup>HNMR (400 MHz, DMSO-d<sub>6</sub>) δ ppm: 0.806 (m, 6H, δ-I5, ε-I6), 1.041 (d, J=6.3 Hz, 3H, γ-T4), 1.078 (m, 2H, γ-I4), 1.273 (m, 2H, γ-K4), 1.393 (m, 3H, δ-K5, β-R3b), 1.459 (m, 3H, β-K3b, γ-R4), 1.634 (m, 3H, β-K3a, β-R3a, β-I3), 1.727 (s, 3H, f), 1.804 (m, 2H, γ-P4b, β-P3b), 1.894 (m, 2H, γ-P4a, β-T3), 1.995 (m, 1H, β-P3a), 2.071 (s, 3H, e), 2.129 (m, 4H, g, h), 3.007 (m, 5H, α-I2, ε-K6, δ-R5), 3.272 (d, J=6.5 Hz, 2H, d), 3.515 (m, 1H, δ-P5b), 3.617 (m, 1H, δ-P5a), 3.625 (s, 3H, COOMe), 3.687 (s, 3H, c), 4.103 (m, 1H, β-T3), 4.25 (m, 2H, α-T2, α-K2), 4.339 (m, 1H, α-P2), 4.444 (m, 1H, α-R2), 5.142 (t, J=6.7 Hz, 1H, a), 5.228 (s, 2H, b), 7.817 (d, J=8.2 Hz, 1H, α-TNH), 8.018 (m, 3H, α-RNH, α-KNH, ε-KNH);

<sup>13</sup>CNMR (400 MHz, CD<sub>3</sub>OD-d<sub>4</sub>) δ ppm: 10.07 (e), 10.45 (δ-I5), 14.45 (ε-I6), 14.93 (f), 18.97 (γ-T4), 22.29 (d), 24.22 (γ-I4), 24.66 (γ-K4), 28.48 (γ-P4), 29.22 (γ-R4), 31.04 (β-R3), 33.97 (δ-K5), 35.08 (β-P3), 38.24 (β-I3), 38.69 (β-K3), 40.53 (h), 42.16 (g), 46.894 (ε-K6), 48.357 (δ-R5), 50.76 (δ-P5), 51.47 (α-R2), 53.35 (OMe), 56.93 (α-T2), 57.81 (α-I2), 59.08 (α-K2),

60.11 ( $\alpha$ -P2), 60.18 (c), 67.02 ( $\beta$ -T3), 69.39 (b), 106.35 (o), 116.2 (l), 122.32 (r), 123.09 (a), 133.66 (j), 145.29 (m), 159.56 (p), 163.42 (k), 170.99 (n), 171.14 (T1), 172.45 (P1), 172.97 (i) 173.23 (K1), 173.42 (R1), 174.21 (II);

MS  $m/z$  calculated for  $C_{45}H_{70}N_{10}O_{14}$  975.1; found 975.5  $M^+$

R<sub>f</sub> = 0.635 (solvent system B)

Purity HPLC 98%.

### *4.3. Investigation of immunosuppressive activity*

#### *4.1. Cell lines*

T-Jurkat cell line, a type of acute lymphoblastic leukemia was obtained from a cell bank.

#### *4.2. Growing media*

The T-Jurkat cell line and PBMCs were suspended in a liquid medium containing RPMI-1640, 10% FBS and P/S. Jurkat cells were grown in sterile culture bottles secured with a sterile filter cap providing free gas exchange

#### *4.3. Jurkat cell line culture*

##### *4.3.1. Passage of the cell line*

Cells were suspended in the culture medium, placed in a incubator at 37 °C, in a 5% CO<sub>2</sub> atmosphere. Every 2-3 days, were passed by pipetting. Then, the used medium was exchanged for a new one and the culture placed in a thermostat.

##### *4.3.2. Thawing the cell line*

Jurkat cells were stored in cryospheres in a cell bank under liquid nitrogen. Cells were thawed and suspension centrifuged. Next, the supernatant was separated and the rest suspended in nutrient solution and pipetted into sterile bottles. Finally, the culture bottles were filled up to 10 mL with culture medium containing RPMI-1640, 10% FBS and P/S.

##### *4.3.3. Freezing the cell line*

The cells suspended in the culture medium were centrifuged and the supernatant decanted. The pellet was suspended in medium with the addition (10% final volume) of FBS and 10% (10% final volume) of DMSO. Then, a cell suspension was placed in cryoamphlets and frozen at -80 °C.

#### *4.4. Isolation of PBMCs*

Peripheral blood mononuclear cells (PBMCs) were obtained from a buffy coat, which is treated as waste in the process of producing erythrocyte mass. A buffy coat was taken from the Regional Center for Blood Donation and Blood Treatment in Gdańsk, Poland (RCKiK). The

obtained test material came from anonymous and healthy donors. PBMC cells were obtained by centrifugation in a gradient of venous blood density. The blood was diluted with PBS in a 1: 1 ratio. Then, the diluted blood was layered in 15 mL vortex tubes containing 4 mL of Ficoll and Uropoline (1:1), followed by spinning for 18 min. at 1800 rpm. After this time, it a separation to individual fractions in test tubes was observed. Lymphocytes with monocytes formed a "leukocytic" coat at the border of Ficoll and plasma. A buffy coat was carefully collected and rinsed twice with PBS buffer, spinning at 1500 rpm for 5 min. After pouring the buffer, 2 mL of lysis buffer was added to the PBMCs (pH 7.4-7.5) and left at room temperature for 5 min. Subsequently, erythrocytes lysis occurred, and the lysis buffer was diluted with PBS 1: 1 and centrifuged (1500 rpm, 5 min). Depending on the test, PBMC cells were suspended the in the culture medium to obtain  $10^6$  cells in 1 ml, or left in PBS.

#### *4.5. Preparation of dilutions of MPA analogs*

To prepare the appropriate concentration in eppendorf tube, 1 mg of the compound was dissolved in 20  $\mu$ L of DMSO, and subsequently diluted with medium to 1 mL, resulting in a concentration of 1 mg/mL. Subsequent concentrations were prepared by serial dilution, obtaining the lowest test concentration of 10<sup>-8</sup> mg/mL in the case of MPA and 10<sup>-5</sup> mg/mL in the case of MPA derivatives. When performing the repetitions, a respective compound (1 mg) was dissolved each time to avoid the storage of samples in the form of solutions.

#### *4.6. Testing used in biological investigations*

##### *4.6.1. Colorimetric MTT test*

##### *4.6.1.1. Jurkat cell line*

Jurkat cells were transferred from the culture bottle to the centrifuge tube, then swirled (1500 rpm, 5 min) and the spent medium removed. Cells were suspended in culture medium (50  $\mu$ L) and placed in 96-well, flat bottom plates at  $50 \times 10^3$  cells per well. Subsequently, the appropriate dilutions of compounds in triplicate were added to each well, and the plates was kept in an incubator (37 °C, 5% CO<sub>2</sub>). After 48 hours of incubation, 20  $\mu$ L of MTT solution (5mg / ml H<sub>2</sub>O) was placed in each well. Then the plate was incubated for a further 3 hours. After the next incubation 100  $\mu$ L of acidified 0.4 N HCl of isopropanol was added to each well, followed by shaking for 15 minutes to dissolve the precipitated formazan crystals. Then, the intensity of the color at 570 nm using a spectrophotometer was measured.



#### 4.6.1.2. PBMC

The were isolated according to the procedure described in section 4.6.1.1., followed by suspension in an appropriate amount of culture medium (50  $\mu\text{L}$ ) and placed in a 96-well flat-bottomed plate at  $50 \times 10^3$  cells per well. In addition, 1  $\mu\text{L}$  of monoclonal antibodies anti-CD3 / anti-CD28 were added to each well. Then, 50  $\mu\text{L}$  of the compounds were added to the plate in appropriately prepared dilutions in triplicate. The plates were incubated for 72 hours in a heating oven (37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$ ). Next, 20  $\mu\text{L}$  of MTT solution was added to each well and incubated for 3 hours, followed by acidification (0.4N HCl) with isopropanol. The intensity of the color was read using a wavelength of 570 nm. The test on PBMC cells was performed for three different patients, and the result was averaged.

#### 4.6.2. Proliferation test using VPD450

##### 4.6.2.1. Preparation of the dye solution

Solution 1 mM of VPD450 dye in DMSO was prepared in centrifuge tube DMSO. Then one-off portions of the dye solution (2  $\mu\text{L}$ ) were prepared before use. VPD450 dye solutions were stored at -80  $^{\circ}\text{C}$  without light.

##### 4.6.2.2. Cell staining

The cells  $10\text{-}30 \times 10^6$  were suspended in 1 mL of PBS and 2  $\mu\text{L}$  of the previously prepared dye solution (1 mM) was added. The cells were incubated for 15 min in a water bath at 37  $^{\circ}\text{C}$ . Subsequently, the cells were rinsed with PBS and spun. The supernatant was poured off and the cell pellet was rinsed with 10 mL of culture medium and spun. The liquid was separated after rinsing. The color of the cells was checked with flow cytometer before applying.

##### 4.6.2.2.1. Jurkat cell line

Jurkat cells were transferred from the culture bottle to the centrifuge tube (1500 rpm, 5 min) and the spent medium was removed. Jurkat cells were rinsed twice with PBS and stained according to 4.6.2.2. procedure. Next, cells were suspended in culture medium (50  $\mu\text{L}$ ) and placed in 96-well, round-bottom plates (  $50 \times 10^3$  cells per well). Subsequently, the appropriate dilutions of compounds in triplicate was added to each well, followed by incubation the plates in an incubator (37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , 48h). Then, cells with compounds were transferred to 5 mL tubes with rinsing the wells with cold PBS. Subsequently, the cells were centrifuged and the supernatant separated. The pellet was suspended in 300  $\mu\text{L}$  of PBS, stirred and analyzed by flow cytometry.





#### 4.6.2.2.2. PBMC

The cells isolated according to the procedure described in section 2.2.4. were suspended in PBS and stained as described in 4.6.2.2. Stained cells were suspended in an appropriate amount of culture medium (50  $\mu$ L) and placed in a 96-well round-bottom plate ( $50 \times 10^3$  cells per well). Next, 1  $\mu$ L of anti-CD3 / anti-CD28 monoclonal antibodies to each well were added. Then, 50  $\mu$ L of the compounds were added to the plate in appropriately prepared dilutions in triplicate. The plates were incubated for 72 hours in a heating oven (37 °C, 5% CO<sub>2</sub>). After this time, PBMC cells and compounds were transferred from wells to 5 mL tubes and the wells rinsed with cold PBS. Then the cells were centrifuged and the supernatant was separated. The residue was suspended in 300  $\mu$ L of PBS, thoroughly stirred and analyzed by flow cytometry.

#### 4.6.3. Data analysis

The results from both tests were then adjusted with SigmaPlot Software in order to obtain IC<sub>50</sub> or EC<sub>50</sub> values for each compound.

#### 4.6.4. PBMC in the presence of GMP

The test for the determination of antiproliferative activity using flow cytometry against PBMC cell line in the presence of GMP was carried out analogously as described in 4.6.2. GMP solution (50  $\mu$ M) was added to cell culture with respective compound and antibodies.

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