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Synthesis and biological activity of tuftsin, its analogue and conjugates containing muramyl dipeptides or nor-muramyl dipeptides

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Abstract: Several conjugates of muramyl dipeptide (MDP) or nor-muramyl dipeptide (nor-MDP) with tuftsin were synthesized. Conjugates **8a**–**f** were prepared by acylation of protected tuftsin with the isoglutamine carboxyl group of MDP or nor-MDP **2a**–**f**. Also tuftsin analogue **6** (H-Thr-Lys-Pro-Arg(NO_2)-OH) was obtained. All synthesized compounds were investigated at the Medical University of Gdansk. The biological activity of the examined compounds was estimated using *in vitro* cultures of human monocytes and lymphocytes. The substances displayed cytotoxic effects, as was revealed in the viability tests performed. The effects were most probably mediated by the induction of an oxidative burst in monocytes and the stimulation of redox enzymes in lymphocytes. In addition, the analogues turned out to be efficient stimulators of TNFα and IL6 secretion by monocytes and lymphocytes. Nevertheless, the secretion of cytokines did not affect the viability of the leukocyte population used in the experiments.

The beneficial properties of the compounds examined (mainly **6**, **3**, **8a** and **8c**), which implies their usefulness as potential therapeutic agents, are connected with their rapid start of action and more efficient effects compared with tuftsin alone. An *in vivo* assay on animal models will be performed.

Keywords: muramyl dipeptide conjugates; tuftsin analogues; synthesis; biological activity

INTRODUCTION

Tuftsin, a natural peptide (H-Thr-Lys-Pro-Arg-OH) occurring in the blood of humans and other mammals, is capable of stimulating certain white blood cells (monocytes, macrophages and neutrophils), and was isolated at Tufts University in 1970 [1]. Since then, a good deal of research has been conducted to discover its properties and to detect the mechanism of its tetrapeptide action. Tuftsin has been found to display several types of potentially beneficial activity [2,3]: activation of macrophages toward fighting bacterial infections; improvement of communication among the immune systems (macrophages, T-cells and antibody-producing B-cells) resulting in increased antibody production; enhancement of the immune response against tumours and retardation of the growth of tumours (in animal experiments). Tuftsin is useful in the control of yeast infections and is able to stimulate the production of blood cells by the bone marrow. The susceptibility of tuftsin to biodegradation and the antagonistic properties of its decomposition products inspired scientists to make efforts to find analogues that were more stable, more selective, easier to purify and of longer lasting activity. The other component of the conjugates — muramyl dipeptide (MDP) a minimal structure of bacterial cell wall peptidoglycan — possesses immune potentiating activity [4–6]. Similar to tuftsin (a bioactive peptide of animal origin), MDP (a synthetic bioactive glycopeptide of microbiological origin) stimulates various functions of macrophages (such as phagocytosis, pinocytosis, motility, chemotaxis, bactericidal and antitumour activity) and increases the non-specific resistance of the host against numerous microorganisms. However, its action does not last long and is not very effective. Numerous derivatives of MDP were designed and synthesized with the aim of obtaining molecules of improved and more defined immunological profiles. The efforts gave good results and some of the compounds were introduced to therapy or are under clinical trials [7–9]. A synergistic effect of MDP on many therapeutic agents is well known and its synergy with tuftsin was also checked. Preliminary information on the activity of a mixture of MDP and tuftsin was published in 1993 [10]. Tests performed on macrophage bovine teat revealed that the application of a mixture of tuftsin and MDP intensified the macrophage inhibition effect on intracellular replication of *Brucella abortus type 2308*. Titov *et al.* [11] synthesized conjugates of tuftsin with *N*-acetyl-glucosaminyl muramyl dipeptide (GMDP) in which tuftsin (H-Thr-Lys-Pro-Arg-OH) was attached to the γ-carboxylic group of D-Gln either through the

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α-amino group of terminal Thr residue or through the ε-amino group of Lys. The conjugates of GMDP and tuftsin were prepared from unprotected GMDP by a mixed anhydride procedure. It was found that GMDP facilitated the macrophage-stimulating activity of tuftsin. Immunological tests indicated the high adjuvant activity of GMDP derivatives containing tuftsin in the stimulation of antibody production against ovalbumin, delayed-type hypersensitivity (DTH) and enhancement of phagocytosis. Administered in a saline mixture of GMDP with tuftsin, as well as GMDP coupled covalently to this tetrapeptide possessed activity comparable to that of incomplete Freund's adjuvant (IFA).

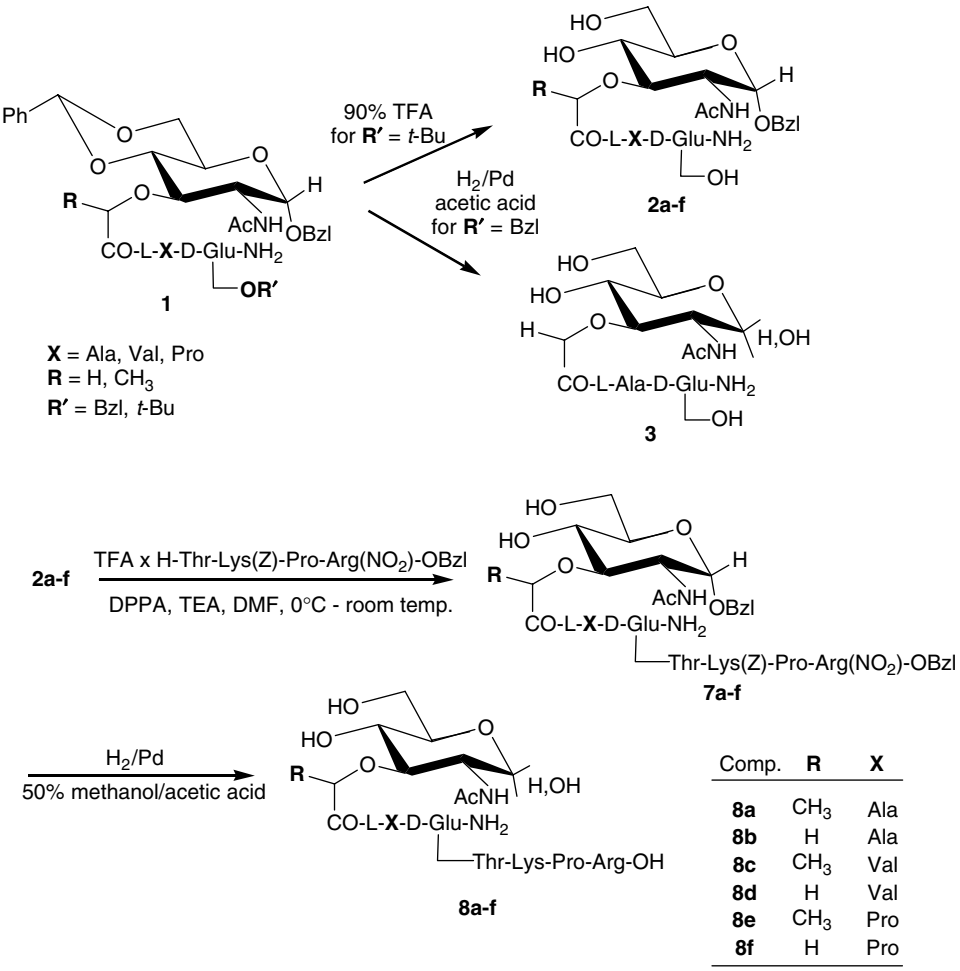
The design of the examined conjugates was based on the assumption that their activity would combine in an enhanced form the biological activities of both tuftsin and MDP, due to the synergistic properties of MDP. The activity of tuftsin is generally directed toward the activation of non-specific elements of the immune system. It mainly stimulates the synthesis of free radicals by granulocytes and, to a lesser extent, monocytes. Another action of tuftsin is connected with the secretion of cytokines by monocytes [2,3]. On the other hand MDP is much more deeply engaged in the stimulation of monocytes. It is believed that MDP, an analogue of the bacterial wall fragment, acts via lipopolysaccharide receptor CD14 and via Toll-like receptors. It is a commonly known fact that the activation of the above-mentioned receptors stimulates a wide range of immune system elements [12]. So, it was our intention to combine the effects of both these immunostimulants together. It seems reasonable to suppose that activation of the non-specific part of the immune system by a strong immunostimulant could be a very valuable type of the therapy for severe infections, either as an alternative to antibiotic treatment or to support it. Another application of the examined conjugates might be related to neoplasms. It is a well-known phenomenon that tumours cause immunosuppression. Very often suppression concerns the acquired immunity. The application of strong immunostimulators during tumour treatment is able to stimulate the nonspecific resistance of the treated organism. Such a reaction is especially helpful for the activation of dendritic cells, the role of which is unquestionable in cancer growth reduction. These cells are very often investigated in the search for new antitumour vaccines.

This paper reports the synthesis of MDP or nor-MDP analogues **8a**–**f** (Scheme 1) which were modified by the incorporation of tuftsin to the *C*-terminal of the muramyl peptides, forming a covalent bond with the isoglutamine carboxylic group. The biological activity of the synthesized compounds was tested at the Medical University of Gdansk, Poland.

CHEMISTRY

The synthesis of the conjugates was carried out according to Scheme 1. The protected MDP or nor-MDP **1** and **2a**–**f** were prepared in a multistep procedure as described previously [13–18]. Tuftsin as a short peptide should be easily obtained according to the standard rules of peptide chemistry. In fact its synthesis is rather complicated because of the presence of amino acids such as Thr, Lys and Arg. These multifunction amino acids required particular protections and special treatment during peptide bond formation, deprotection and purification. The synthesis of tuftsin and its analogues is usually performed in the classical way using EEDQ or DCC as condensing agents; however, the crude product requires careful purification [19–25]. After the removal of the protecting groups from the obtained tetrapeptide, the crude product is purified chromatographically on a column filled with AG 50W-X2 gel, Sephadex G-25 or an ion exchange type Amberlite IR-4B, followed by lyophilization.

This paper describes a modified preparation of protected tuftsins: Boc-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl **4** and Z-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl **5**. They were synthesized using DPPA activation in the presence of TEA or NMM [26]. The yields and melting points of the protected dipeptide, tripeptide and tetrapeptide are shown in Table 1. The method diminishes the formation of impurities and ensures a high chiral purity of the product and a good yield, about 75%–80% at each step. It is worth noting that the yields of the protected tuftsin obtained according to DPPA activation were higher than in the cases referred to in the literature [2,24,25]. Acylation of the Thr amino group of protected tuftsin TFA x H-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl **4a** by MDP or nor-MDP **2** was performed also using the DPPA coupling procedure (Scheme 1). The compounds **2a**–**f** represent the starting substrates for the synthesis of **8a**–**f**. The protected conjugates **7a**–**f** were isolated by radial chromatography on plates covered with Kieselgel 60 PF_{254} and purified with a preparative TLC. The identities of the protected products were confirmed by high resolution ^{1}H NMR (500 MHz, COSY, TOCSY, ROESY) spectroscopy. The final products were hydrogenolysed with H_2/Pd in 50% methanol–acetic acid and purified with preparative TLC with *n*-BuOH–AcOH–H_2O (4 : 2 : 2) as a solvent system. The identities of the final products were confirmed by TLC qualitative amino acid analysis, and elemental analyses. Compound **5** (protected tuftsin) was selectively deblocked with H_2/Pd in 50% methanol–acetic acid for 18 h to give compound **6**, H-Thr-Lys-Pro-Arg(NO_2)-OH. Catalytic hydrogenation of compound **5** for 30 h gave the completely deprotected compound without the NO_2 group. The structure of compound **6** was confirmed by high resolution ^{1}H NMR (500 MHz, COSY, TOCSY, ROESY) spectroscopy and MS.



Comp.	R	X
8a	CH_3	Ala
8b	H	Ala
8c	CH_3	Val
8d	H	Val
8e	CH_3	Pro
8f	H	Pro

Scheme 1

Table 1 Synthesis of Tuftsin Derivatives **4**, **5** and **6**

Compound[a]	Prepared from	Molecular formula	Yield (%)	Mp (°C)	$[\alpha]_{546}^{25}$ MeOH
Boc-Pro-Arg(NO_2)-OBzl	H-Arg(NO_2)-OBzl, Boc-Pro-OH	$C_{23}H_{34}N_6O_7$ (506.56)	69	65–70	−54° (*c* 1.0)
Boc-Lys(Z)-Pro-Arg(NO_2)-OBzl	TFAxH-Pro-Arg(NO_2)-OBzl, Boc-Lys(Z)-OH	$C_{37}H_{52}N_8O_{10}$ (769.23)	73	70–73	−48° (*c* 1.0)
Boc-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl; **4**	TFAxH-Lys(Z)-Pro-Arg(NO_2)-OBzl, Boc-Thr-OH	$C_{41}H_{59}N_9O_{12}$ (869.96)	74	80–81	−43.5° (*c* 0.92)
H-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl trifluoroacetate; **4a**	Boc-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl; **4**	$C_{38}H_{52}N_9O_{12}F_3$ (883.82)	94	oil	—
Z-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl; **5**	TFAxH-Lys(Z)-Pro-Arg(NO_2)-OBzl, Z-Thr-OH	$C_{44}H_{57}N_9O_{12}$ (903.99)	80	88–90	−38.7° (*c* 1.0)
H-Thr-Lys-Pro-Arg(NO_2)-OH; **6**	Z-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl; **5**, H_2/Pd	$C_{25}H_{49}N_9O_{10}$ (683.71)[b]	75	83–87	−32° (*c* 1.0)

[a] ^{1}H NMR spectra of all compounds were as expected.
[b] Anal. calc. for $C_{21}H_{39}N_9O_8 \times 2CH_3COOH \times H_2O$.

RESULTS AND DISCUSSION

All the synthesized conjugates **8a**–**f** and compounds **2b**,**c**,**f**, **3** (nor-MDP) and **6** were tested at the Department of Histology and Immunology, Medical University of Gdansk, Poland. The results of these investigations are described below.

Viability Tests in Peripheral Blood Mononuclear Cells (PBMC) Cultures (Table 2)

The addition of tuftsin to 4 h cultures of PBMC increased the viability of PBMC, while derivatives **6**, **3** and high concentrations of **8a**, **8c**, **8d** and **8f** decreased it significantly. A 12 h incubation revealed

Table 2 IC_{50}/EC_{50} (±SD) of the Examined Substances (mg/ml) Evaluated from 4, 12 and 24 h Cultures of Monocytes, PBL and PBMC Treated with Serial Dilution of the Examined Compounds[a]

Compound	Time of incubation and effect					
	4 h	Effect	12 h	Effect	24 h	Effect
Monocytes						
Tuftsin	0.001 ± 0.02	⇑	0.001 ± 0.01	⇓	0.6 ± 0.2	⇓
6	0.1 ± 0.2	↓	0.001 ± 0.01	↓*	0.05 ± 0.04	↓*
2b	—	—	0.07 ± 0.04	↓	—	—
3	0.3 ± 0.1	↓	0.1 ± 0.06	↓	0.1 ± 0.1	↓*
8b	—	—	0.05 ± 0.05	↓	—	—
8a	—	—	0.2 ± 0.4	↓*	0.1 ± 0.03	↓*
8c	0.09 ± 0.02	↓	0.1 ± 0.03	↓*	0.07 ± 0.06	↓*
8d	0.2 ± 0.1	↓	0.6 ± 0.2	↓*	0.1 ± 0.02	↓*
8e	0.02 ± 0.01	↓	0.8 ± 0.1	↓*	0.7 ± 0.3	↓*
2f	—	—	0.09 ± 0.05	↓	—	—
8f	0.2 ± 0.09	↓	0.7 ± 0.3	↓	0.5 ± 0.02	↓*
2c	0.4 ± 0.1	↓	0.3 ± 0.04	↓	—	—
PBL						
Tuftsin	0.005 ± 0.07	⇑	—	—	—	—
6	0.09 ± 0.2	↓	0.07 ± 0.03	↓*	0.0001 ± 0.001	↓*
2b	0.03 ± 0.02	↑	0.0001 ± 0.05	↑	—	—
3	0.02 ± 0.2	↓	0.06 ± 0.02	↓*	0.0001 ± 0.001	↓*
8b	—	—	—	—	—	—
8a	0.2 ± 0.03	↓	0.009 ± 0.06	↓*	0.01 ± 0.01	↓*
8c	0.1 ± 0.04	↓	0.08 ± 0.01	↓*	0.02 ± 0.05	↓*
8d	0.0001 ± 0.001	↑	0.07 ± 0.005	↓*	0.08 ± 0.05	↓*
8e	0.01 ± 0.02	↓	0.1 ± 0.03	↓*	—	—
2f	0.06 ± 0.05	↓	—	—	0.05 ± 0.04	↓*
8f	—	—	0.08 ± 0.06	↓*	0.08 ± 0.07	↓*
2c	—	—	—	—	—	—
PBMC						
Tuftsin	0.0001 ± 0.001	⇑	0.02 ± 0.01	⇓	0.02 ± 0.09	⇓
6	0.007 ± 0.005	↓	0.01 ± 0.04	↓*	0.0005 ± 0.004	↓
2b	—	—	—	—	0.0001 ± 0.001	↓
3	0.03 ± 0.003	↓	0.0006 ± 0.004	↓*	0.0001 ± 0.001	↓
8b	—	—	0.0004 ± 0.02	↓*	0.0001 ± 0.001	↓
8a	—	—	0.008 ± 0.05	↓	—	—
8c	0.01 ± 0.06	↓	0.07 ± 0.01	↓	0.03 ± 0.02	↓
8d	0.09 ± 0.04	↓	0.09 ± 0.01	↓	0.04 ± 0.04	↓
8e	—	—	0.08 ± 0.02	↓	—	—
2f	—	—	—	—	0.09 ± 0.06	↓
8f	0.03 ± 0.06	↓	0.07 ± 0.03	↓	—	—
2c	—	—	—	—	—	—

[a] Arrows indicate significant decrease (↓) or increase (↑) in the viability of PBMC in the presence of the compounds in comparison with DMSO (solvent), dual-line arrows (⇓ and ⇑) are related to the comparison between tuftsin and DMSO. Asterisks (*) indicate significant difference between the particular conjugate and tuftsin. Hyphen (—) indicates no significant difference. Values are presented only if the whole course of the viability with the serial dilutions of the compound differed significantly from the whole course of the viability in the presence of the serial dilutions of the solvent (DMSO) in the ANOVA test. Whenever the value of IC_{50} or EC_{50} was <0.0001 it was made equal to 0.0001 (0.001 in monocytes) and SD < 0.001 was made equal to 0.001 (0.01 in monocytes).

that both tuftsin and most of its analogues (**8b**, **8a**, **8c**, **8d**, **8e**, **8f**) and compounds **6**, **3** were able to decrease the viability of PBMC compared with DMSO. In addition, higher doses of **6**, **3** and **8b** decreased the viability more efficiently than tuftsin. After 24 h, the cultures incubated with all substances including pure solvent were characterized by a lower viability than at the beginning of the experiment. Nevertheless, the viability of PBMC from cultures incubated with tuftsin and compounds **6**, **2b**, **3**, **8b**, **8c**, **8d** and **2f** was significantly lower than in the case of PBMC incubated with DMSO. Interestingly, low concentrations of compounds **6**, **2b**, **3** and **8b** were more suppressive than tuftsin.

Viability Tests in Cultures of Monocytes (Table 2)

The 4 h incubation with a low concentration of tuftsin increased the viability of monocytes in the treated cultures while it was decreased in cultures incubated with compounds **6**, **3**, **8a**, **8c**, **8d**, **8e**, **8f** and **2c**. The 12 h cultures revealed a decreased viability in cultures stimulated with tuftsin and all examined compounds compared with the pure solvent. The most efficient agents seemed to be analogues **8a**, **8c**, **8d**, **8e** and derivative **6** used in high concentrations. They inhibited the viability of monocytes more efficiently than tuftsin. The viability of monocytes in the cultures incubated for 24 h was decreased in the presence of tuftsin and compounds **6**, **3**, **8a**, **8c**, **8d**, **8e**, **8f** and **2f** compared with the pure solvent. This effect was significantly stronger in the case of high concentrations of compounds **6**, **3**, **8a**, **8c**, **8d**, **8e** and **8f** than in the case of tuftsin.

Viability Tests in Peripheral Blood Lymphocyte (PBL) Cultures (Table 2)

The incubation with tuftsin and compounds **2b**, **8d** for 4 h increased the viability in the PBL cultures. On the other hand, high concentrations of compounds **6**, **3**, **8a**, **8c**, **8e** and **2f** caused a decrease in the viability of PBL. After 12 h, an increased viability of PBL was observed only in the cultures treated with derivative **2b**, while an inhibition of PBL viability was noted in the cultures stimulated with high concentrations of the following compounds: **6**, **3**, **8a**, **8c**, **8d**, **8e** and **8f**. Tuftsin seemed to affect PBL only after a 24 h incubation. The cultures of PBL stimulated with tuftsin or any of the following compounds: **6**, **3**, **8a**, **8c**, **8d**, **8f** and **2f**, for 24 h revealed a decreased viability, and all these compounds exerted a significantly stronger influence in comparison with the solvent or tuftsin.

Generation of Oxidative Burst by Examined Compounds (Figure 1)

The generation of the oxidative burst by the examined compounds in monocytes from PBMC cultures (Figure 1, panel 'monocytes') started just after the addition of the examined substances into the cultures of H_2DCF-loaded PBMC. This effect was most visible in

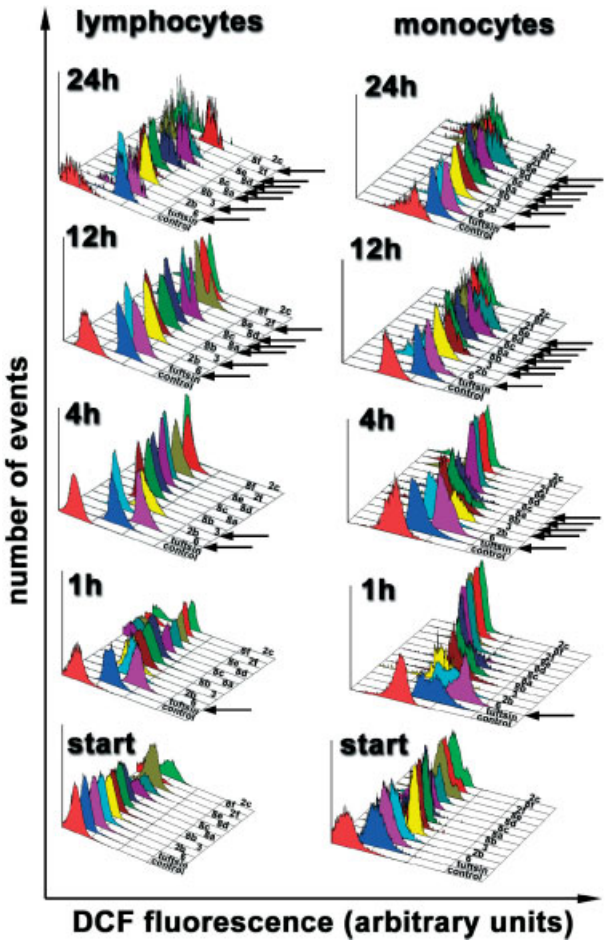


Figure 1 DCF fluorescence shift after stimulation of PBMC with the examined compounds. Monocytes and lymphocytes stimulated with the examined compounds caused a DCF fluorescence shift in cultures stimulated with both 0.001 or 0.1 mg/ml of the examined substances. Since the results of the experiments performed with these two concentrations of the compounds were similar, only those obtained from the cultures stimulated with 0.1 mg/ml are presented. The presented panels 'lymphocytes' and 'monocytes' were obtained from lymphocyte and monocyte gates, respectively. The examples of overlaid histograms obtained from cultures are presented on the charts in the following order: start (just after the addition of the examined substances), 1 h, 4 h, 12 h and 24 h of incubation. Since the results for untreated cultures were identical to those for cultures treated with DMSO alone, only histograms from the former (marked 'control') are shown. Arrows on the charts indicate the beginning of a significant difference in the peak fluorescence signal between the cultures stimulated with the particular compounds and those untreated.

the cultures treated with derivative **6**, in which significant differences became apparent as early as 1 h after the addition of derivative **6** and were stronger than in other examined cultures, including those stimulated by tuftsin. Other compounds which efficiently stimulated the oxidative burst were **3**, **8b**, **8a** and **8c**. Differences in the peak fluorescence signal in the cultures stimulated with these derivatives in comparison with the pure solvent were revealed in 4 h cultures. However, the mean fluorescence shift was comparable between the cultures treated with these derivatives and tuftsin. The above changes were also noted in the cultures stimulated with the derivatives for 12 and 24 h. Under such conditions also conjugates **8d** and **8e** revealed the ability to produce an oxidative burst.

The examined substances also induced an oxidation of 2′,7′-dichloro-dihydrofluorescein (H_2DCF) in lymphocytes (Figure 1, panel 'lymphocytes'), but the 2′,7′-dichlorofluorescein (DCF) fluorescence shift was much weaker than in the case of monocytes. As in the monocytes gate, the most efficient derivative was compound **6** which induced the strongest signal as early as 1 h after the start of the experiment. The derivative **3** was also an efficient stimulator of lymphocytes, however, a significant difference between the level of fluorescence obtained from cultures stimulated with **3** and other derivatives or tuftsin was not visible until after 4 h of stimulation. These effects were also noted during the rest of the experiment, with **6** and **3** after 12 and 24 h of incubation. A longer incubation also revealed a fluorescence shift in the lymphocyte gate in the cultures stimulated with **8a**, **8c**, **8d**, **8e**, **8f** and **2f** but it was significant only in the case of conjugates **8a** and **8c**.

The Compound-induced Synthesis of Cytokines (Table 3)

Not surprisingly, the most effective induction of TNFα was observed in cultures of isolated monocytes. The derivatives **6**, **2b**, **3** and **8b** induced secretion of TNFα from monocytes, while tuftsin and other derivatives were not able to induce such an effect. To a lesser extent, this phenomenon was also noted in cultures of PBMC, where the secretion of TNFα was induced by compounds **6**, **8c**, **8d**, **2f** and **8f**. This effect seemed to be dose-dependent only in the case of **6** and **8b**. On the other hand, the activity of the other derivatives was observed only in a particular concentration of these substances. None of the examined derivatives stimulated secretion of TNFα in the cultures of PBL.

The secretion of IL6 was visible in all the analysed types of cultures. However, not all the derivatives were able to induce it. A significant increase in the amount of IL6 in cultures of PBMC was revealed under stimulation with compounds **2b** and **8b**. The isolated monocytes were efficiently stimulated with **3**, **2f**, and **8b**, **8d**, **8f** analogues. Secretion of IL6 in cultures of PBL was induced by various concentrations of tuftsin and the majority of its conjugates **8a**, **8d**, **8e**, **8f** and derivatives **6**, **2b**, **2c**. It is worth noting that the stimulation of PBL with high concentrations of all the examined substances was less efficient than with low concentrations.

Since some of the examined analogues were able to induce secretion of TNFα or IL6, the impact of this secretion on the results obtained in the viability tests was assessed. Nevertheless, addition of antibodies against TNFα or IL6 to the cultures of PBMC, separated monocytes, and PBL incubated with the examined substances did not change results of the viability test.

Natural Killer (NK) Activity after Stimulation with the Examined Compounds (Table 4)

There was a trend towards an increase in the cytotoxic activity of NK cells treated with some of the examined substances. However, it was significant only in the case of the following analogues: **8d** (0.1 mg/ml), **2f** (0.01–1.0 mg/ml), and **8f** (0.1–1.0 mg/ml), compared with the non-stimulated cultures.

CONCLUSIONS

The current study revealed a suppressive effect on the viability of cells from PBMC cultures incubated in the presence of the majority of the compounds examined. This effect seemed to be time- and dose-dependent. Since PBMC are a heterogeneous population, it was decided to perform the viability tests using separated PBL and monocytes. These tests confirmed the inhibitory effect of the examined compounds on the viability of both lymphocytes and monocytes. Interestingly, low concentrations of some analogues were more suppressive than tuftsin. The most efficient compounds were **6**, **3**, **8a**, **8c** and **8d**.

The majority of the examined tuftsin analogues revealed biological activity similar to tuftsin and MDP, which are parts of their structure. The decrease in the viability of cells treated with the compounds was most probably related to the generation of free radicals by monocytes and an increased activity of redox enzymes in lymphocytes. The action of particular substances differed depending on the time of incubation or cells used in the test. Nevertheless, compounds **6**, **3**, **8a** and **8c** seemed to be characterized by the highest activity, sometimes even stronger than tuftsin. The main difference between tuftsin and its analogues was related to an increased viability of leukocytes from short-lasting cultures treated with tuftsin, which was not revealed in the presence of the majority of the examined substances. Tuftsin probably stimulates mitochondria in the production of enzymes and metabolites that belong to the most important targets used in the viability tests and flow cytometry

Table 3 Secretion of TNFα and Interleukin 6. Secretion of Tumour Necrosis Factor α and Interleukin 6 were Assessed in the Cultures of PBMC, Monocytes, and PBL Stimulated with the Examined Substances. Values are mean ± SD

	Monocytes				**PBL**				**PBMC**			
	Concentration of the compounds in cultures (mg/ml)				Concentration of the compounds in cultures (mg/ml)				Concentration of the compounds in cultures (mg/ml)			
Tumour Necrosis Factor α (pg/ml)												
	0	0.001	0.01	0.1	0	0.001	0.01	0.1	0	0.001	0.01	0.1
DMSO	0.6 ± 0.3	0.8 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.01	0.8 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.7 ± 0.01	0.6 ± 0.1	0.4 ± 0.2
Tuftsin	0.5 ± 0.1	0.9 ± 0.3	0.8 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.3	0.2 ± 0.1	0.5 ± 0.2	0.8 ± 0.2	0.2 ± 0.01
6	0.5 ± 0.1	2.6 ± 0.6	1.6 ± 0.1	1.4 ± 0.1	0.3 ± 0.01	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.2 ± 0.01	0.2 ± 0.1	0.5 ± 0.2	1.1 ± 0.2
2b	0.5 ± 0.2	2.3 ± 0.3	1.7 ± 0.3	0.6 ± 0	0.6 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.01	0.2 ± 0.1	0.3 ± 0.01	0.5 ± 0.1	0.8 ± 0.5
3	0.5 ± 0.2	2.3 ± 0.3	1.7 ± 0.3	0.6 ± 0	0.3 ± 0.2	0.4 ± 0.01	0.5 ± 0.2	0.7 ± 0.2	0.4 ± 0.01	0.2 ± 0.01	0.8 ± 0.5	0.6 ± 0.1
8b	0.9 ± 0.1	3.0 ± 0.7	0.8 ± 0.1	1.0 ± 0.2	0.2 ± 0.01	0.5 ± 0.3	0.8 ± 0.1	0.5 ± 0.3	0.3 ± 0.1	0.1 ± 0.01	0.2 ± 0.1	0.8 ± 0.1
8a	0.5 ± 02	2.7 ± 0.7	1.9 ± 0.7	2.2 ± 0.7	0.6 ± 0.01	0.3 ± 0.01	0.1 ± 0.01	0	0.2 ± 0.01	0.2 ± 0.01	0	0
8c	0	0	0.4 ± 0	0	0.1 ± 0.01	0.4 ± 0.01	0.3 ± 0.01	0	0.2 ± 0.1	1.3 ± 0.01	0	0
8d	0	0.4 ± 0.1	0	0	0.2 ± 0.01	0.4 ± 0.01	0.3 ± 0.01	0	0.2 ± 0.01	0.9 ± 0.01	1.6 ± 0.01	2.3 ± 0.2
8e	0	4.2 ± 0.1	0.1 ± 0	0	0.2 ± 0.01	0.7 ± 0.01	0.3 ± 0.01	0	0.6 ± 0.01	0.8 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
2f	0	0	0	0	0.3 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0	0	0	1.6 ± 0.01	0.2 ± 0.01
8f	0	0	0	0	0	0	0.1 ± 0.01	0	0	1.0 ± 0.01	0.7 ± 0.01	0.3 ± 0.01
2c	0	0	0	0	0.5 ± 0	0.4 ± 0.01	0.3 ± 0.01	0	0	0.2 ± 0.01	0.3 ± 0.01	0.2 ± 0.01
Interleukin 6 (ng/ml)												
DMSO	30.5 ± 19.5	23.6 ± 5.4	36.1 ± 1.1	30 ± 13.9	35.0 ± 5.0	23.6 ± 5.4	36.1 ± 1.1	30 ± 13.9	62.0 ± 16.0	59.8 ± 17.3	49.1 ± 3.8	66.5 ± 10.7
Tuftsin	73.6 ± 13.6	67.8 ± 0.3	35.9 ± 0.9	32.3 ± 0.2	84.1 ± 3.1	373.2 ± 75.8	245.1 ± 44.6	130.9 ± 30.1	72.9 ± 48.9	59.2 ± 14.8	32.8 ± 9.7	21.9 ± 0.6
6	86.5 ± 16.5	98.1 ± 1.1	66.3 ± 19.4	63.1 ± 18.0	49.0 ± 28.3	409.6 ± 69.6	357.7 ± 91.3	61.0 ± 19.8	75.6 ± 26.8	20.4 ± 3.1	53.9 ± 32.9	14.7 ± 0.6
2b	75.4 ± 14.6	78.5 ± 1.9	69.4 ± 5.9	56.1 ± 11.4	71.2 ± 5.9	124.9 ± 26.7	188.2 ± 7.3	43.8 ± 12.8	79.7 ± 10.2	161.9 ± 2.4	254.4 ± 53.5	261.3 ± 60.9
3	57.0 ± 3.0	260 ± 10	52.3 ± 5.5	13.5 ± 3.5	69.1 ± 29.1	65.5 ± 1.4	32.6 ± 11.6	43.9 ± 1.7	70.2 ± 15.9	106.4 ± 31.2	15.8 ± 3.3	15.6 ± 0.1
8b	61.7 ± 19.3	265.0 ± 65.0	254.2 ± 46.8	15.5 ± 5.5	63.0 ± 33.0	90.5 ± 14.5	51.2 ± 13.5	41.7 ± 4.8	58.3 ± 1.8	169.4 ± 3.8	156.5 ± 50.3	158.4 ± 11.5
8a	28.6 ± 6.6	27.5 ± 4.8	16.0 ± 4.0	0	159.1 ± 8.9	231.2 ± 20	104.1 ± 5.9	10.1 ± 0.9	16.1 ± 16.0	37.3 ± 4.7	27.0 ± 5.5	0
8c	17.5 ± 4.0	37.5 ± 12.5	47.5 ± 12.5	0	23.1 ± 7.0	94.9 ± 65.2	0	0	0 ± 0	29.3 ± 3.3	41.9 ± 4.4	0
8d	44.1 ± 4.2	169.5 ± 28.5	35.5 ± 22.5	0	110.8 ± 11.8	218.1 ± 16.1	228.1 ± 7.9	0	22.2 ± 7.1	19.6 ± 9.4	55.8 ± 4.4	45.6 ± 25.5
8e	46.5 ± 9.5	71.5 ± 27.5	39.5 ± 2.5	0	128.3 ± 5.1	314.2 ± 36.0	143.2 ± 8.0	60.5 ± 10.5	23.0 ± 11.6	118.7 ± 7.5	17.1 ± 3.9	50.5 ± 0.1
2f	118.0 ± 19.0	256.0 ± 12.0	296.5 ± 13.5	0	29.5 ± 12.8	201.2 ± 5.0	28.2 ± 2.0	0	56.4 ± 5.7	50.5 ± 0.1	23.3 ± 1.9	32.0 ± 11.0
8f	40.1 ± 13.9	154.5 ± 32.5	39.8 ± 7.2	38.5 ± 8.5	50.9 ± 10.8	166.0 ± 10.1	59.8 ± 44.2	0	28.4 ± 12.9	37.2 ± 0.2	50 ± 2.2	45.9 ± 4.7
2c	53.4 ± 11.6	58.6 ± 12.5	129.0 ± 7.0	0	36.6 ± 21.2	28.6 ± 1.6	141.5 ± 9.4	0	12.4 ± 9.2	50.5 ± 7.6	61.6 ± 25.6	38.8 ± 18,3

Table 4 NK Cytotoxicity. NK Cytotoxicity was Evaluated in the Cultures of PBMC Stimulated with the Compounds. Results are Presented as the Mean Percentage of Cytotoxicity ± SD

Compound	Concentration of the compounds in cultures (mg/ml)				
	0	0.001	0.01	0.1	1.0
DMSO	44.6 ± 2.3	55.1 ± 1.1	54.3 ± 1.7	60.4 ± 1.3	61.5 ± 0.8
Tuftsin	45.9 ± 0.8	43.0 ± 1.3	44.4 ± 6.0	41.8 ± 5.6	47.0 ± 6.2
6	46.9 ± 0.6	48.1 ± 1.7	49.2 ± 4.8	48.0 ± 0.6	45.7 ± 1.4
2b	45.5 ± 1.4	46.6 ± 0.7	48.5 ± 0.7	48.4 ± 0.4	45.8 ± 7.8
3	41.8 ± 0.8	42.7 ± 0.8	38.2 ± 2.3	45.8 ± 2.1	50.2 ± 0.9
8b	44.5 ± 3.6	48.2 ± 1.2	47.9 ± 0.4	54.5 ± 0.9	52.4 ± 0.6
8a	64.1 ± 16.8	21.1 ± 2.5	29.8 ± 1.2	78.1 ± 19.6	59.5 ± 12.0
8c	59.5 ± 15.8	35.4 ± 1.0	36.5 ± 5.6	59.4 ± 21.8	49.0 ± 9.9
8d	61.0 ± 12.7	55.1 ± 0.1	66.1 ± 5.8	93.0 ± 8.8	77.5 ± 10.6
8e	57.1 ± 10	57.2 ± 8.8	16.8 ± 9.7	43.3 ± 4.7	40 ± 14.1
2f	59.1 ± 2.1	50.8 ± 4.3	94.4 ± 5.1	81.7 ± 12.3	78.0 ± 8.5
8f	46.0 ± 8.5	70.6 ± 14.1	63.2 ± 30.6	84.9 ± 21.1	63.5 ± 6.4
2c	53.2 ± 24.4	10.7 ± 1.0	89.9 ± 14.0	85.8 ± 19.2	71.0 ± 4.2

analysis. In the case of the examined compounds, a shorter time from stimulation to reaction, visible in the activity of some compounds compared with tuftsin, might mask the activating effect of the tuftsin part of the analogues. A high concentration of free radicals generated in response to stimulation with the compounds might damage cells, causing a leakage of enzymes and metabolites which are the substrates of the viability tests. Since the viability test measures the activity of live cells, the oxidative burst analysis assesses the redox changes in the whole suspension. Thus, despite a low viability, the compounds revealed a high fluorescence signal of DCF, which was even higher than in the case of tuftsin in short-lasting cultures. Nevertheless, not all the compounds were characterized by the above-mentioned quick response.

Surprisingly, the majority of the examined derivatives did not increase the cytotoxic activity of NK cells, which is in contrast to the MDP derivatives examined previously [27].

The observed biological effects of the compounds also could be dependent on unknown differences in signal transduction inside the cells and in cell-to-cell interactions. This hypothesis may be supported by the fact that leukocytes responded in a slightly different way in cultures of PBMC than in isolation lymphocytes or monocytes, which suggests their cooperation. Another premise pointing to such a conclusion is that the cultures treated with the derivatives secreted cytokines known to carry information between cells and to improve their contact. It is worth noting that the secretion of cytokines did not influence the viability of cells incubated with the compound. Thus, the cytokines seemed to play only an accessory role in our model. Further experiments should explain the differences noted in the viability tests or in the level of secreted cytokines between cultures of PBMC and isolated PBL or monocytes. Models including different kinds of cells are much more similar to a real human body. The estimation of interactions between these cells is of great importance in considering further applications of those substances as drugs in medicine.

The advantages of the examined compounds (especially **6**, **3**, **8a** and **8c**), and their usefulness in future therapies, are connected with their rapid start of action and more efficient effects compared with tuftsin. It could be of great importance for the limitation and eradication of infections, both sepsis and several local infections, in which the rapid start of an intensive innate response mediated by monocytes and the engagement of other parts of the immune system by induced pro-inflammatory cytokines is pivotal. Thus, further *in vivo* tests on animal models should be performed in order to confirm the usefulness of these conjugates as potential drugs.

EXPERIMENTAL SECTION

Melting points (uncorrected) were determined on the Kofler-block apparatus. ^{1}H-NMR spectra were measured in DMSO or $CDCl_3$ solutions with a Varian 500 and 200 NMR spectrometers. Preparative column chromatography and radial chromatography were performed on silica gel (Kieselgel 60, 100–200 mesh) in solvent systems specified in the text. All chemicals and solvents were of reagent grade and were used without further purification. The reactions were monitored by TLC on Merck F_{254} silica gel precoated plates. The following solvent systems (by vol.) were used for TLC and radial chromatography development: (A) *n*-BuOH–pyridine–AcOH–H_2O (60 : 45 : 4 : 30), (B) *n*-BuOH–AcOH–H_2O (4 : 2 : 2), (C) $CHCl_3$–MeOH–AcOH (90 : 10 : 5), (D) $CHCl_3$–MeOH (4 : 1), (E) $CHCl_3$–MeOH (9 : 1),

(F) $CHCl_3$–MeOH (2 : 1). All synthesized protected peptides were homogeneous on TLC in solvent A, C, D or E. Elemental analyses were performed by the Laboratory of Elemental Analysis, University of Gdansk. Qualitative amino acid analyses of the hydrolysates of the compounds were accomplished on TLC in solvent A, and were detected by UV and ninhydrin. The optical rotation values were measured on a Rudolph Research automatic polarimeter Autopol II. HPLC/MS analysis of compound **6** was accomplished by electrospray ionization with positive ion detection mode performed on a HPLC Agilent series 1100 mass spectrometer (HPLC-ES-MS). MS $[M + H]^+$ for $C_{21}H_{39}N_9O_8$ (compound **6**) m/z 547.

Benzyl or *tert*-butyl 1-*O*-benzyl-*N*-acetyl-muramyl (or nor-muramyl)-L-amino acid-D-isoglutaminate **1**, 1-*O*-benzyl-*N*-acetyl-muramyl-L-alanyl-D-isoglutamine **2a**, 1-*O*-benzyl-*N*-acetyl-nor-muramyl-L-alanyl-D-isoglutamine **2b**, 1-*O*-benzyl-*N*-acetyl-muramyl-L-valyl-D-isoglutamine **2c**, 1-*O*-benzyl-*N*-acetyl-normuramyl-L-valyl-D-isoglutamine **2d**, 1-*O*-benzyl-*N*-acetyl-muramyl-L-prolyl-D-isoglutamine **2e**, 1-*O*-benzyl-*N*-acetyl-nor-muramyl-L-prolyl-D-isoglutamine **2f**, *N*-acetyl-normuramyl-L-alanyl-D-isoglutamine **3** [13–18] and tuftsin derivative **6** were prepared in our laboratory. Tuftsin (lot no. 0 537 826) — H-Thr-Lys-Pro-Arg-OH acetate salt was obtained from Bachem AG (Bubendorf-Switzerland).

The peptides: dipeptide [Boc-Pro-Arg(NO_2)-OBzl], tripeptide [Boc-Lys(Z)-Pro-Arg(NO_2)-OBzl] and tetrapeptides [Boc-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl, Z-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl] were synthesized by a DPPA procedure. The analytical data of the intermediate products of the syntheses are shown in Table 1.

General Procedures for the Syntheses of Compounds 8a–f

To a stirred solution of compound **2** (1 mmol) and H-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl trifluoroacetate (1.1 mmol) in anhydrous DMF (4 ml), a solution of DPPA (0.24 ml, 1.1 mmol) was added in anhydrous DMF (1 ml) at 0 °C, followed by addition of TEA (2.2 mmol). The mixture was stirred at 0 °C for 3 h and after that at room temperature for 48 h. After evaporation of the solvent, the reaction mixture was purified using radial chromatography and preparative TLC in solvent D, E or F to obtain compounds **7a**–**f**.

All the protecting groups were removed from the analogues by catalytic hydrogenolysis. Compounds **7a**–**f** were hydrogenated in 50% methanol-acetic acid containing palladium black for 40 h. The supernatant was decanted, the catalyst washed with water, all solutions were combined, filtered and purified using preparative TLC in solvent B and lyophilized.

1-O-Bzl-Mur(NAc)-Ala-D-Glu(Thr-Lys(Z)-Pro-Arg-(NO_2)-OBzl)-NH_2 (7a). Yield 50%; mp 94°–97 °C; ^{1}H-NMR (DMSO) δ 0.99 (d, $J = 6.3$ Hz, 3H, T4), 1.21 (d, $J = 6.9$ Hz, 3H, CH_3-A3), 1.24 (d, $J = 6.9$ Hz, 3H, H3-PrA), 1.29 (m, 2H, K4), 1.36 (m, 2H, K5), 1.53 (m, 2H, R4), 1.61 (m, 1H, R3), 1.69 (m, 1H, P3), 1.70 (m, 1H, Q3), 1.76 (m, 1H, R3),1.78 (s, 3H, AcN), 1.80 (m, 2H, P4), 1.92 (m, 1H, Q3), 1.96 (m, 1H, P3), 2.19 (m, 2H, Q4), 2.94 (q, $J = 6.0$ Hz, K6), 3.13 (m, R5), 3.30 (m, 1H, G4), 3.48 (m, 1H, P5), 3.49 (m, 2H, G3, G5), 3.51 (m, 1H, G6), 3.80 (ddd, $J = 3.4$ Hz, $J = 8.3$ Hz, $J = 10.5$ Hz, G2), 3.62 (m, 1H, P5), 3.91 (m, T3), 3.64 (m, 1H, G6), 4.12 (dt, $J = 5.2$ Hz, $J = 7.9$ Hz, 1H, Q2), 4.18 (dd, $J = 4.3$ Hz, $J = 8.4$ Hz, 1H, T2), 4.26 (m, m, R2, H2), 4.29 (m, 1H, A2), 4.31 (m, 1H, P2), 4.43 (d, $J = 12.4$ Hz, 1H, Ph-CH_2-GlcN), 4.44 (m, 1H, K2), 4.60 (t, $J = 5.9$ Hz, 1H, G6-OH), 4.66 (d, $J = 12.4$ Hz, 1H, Ph-CH_2-GlcN), 4.72 (d, $J = 3.4$ Hz, 1H, G1), 4.76 (d, $J = 5.5$ Hz, 1H, T3-OH), 4.98 (s, 2H, Ph-CH_2-), 5.05 (d, $J = 12.5$ Hz, 1H, Ph-CH_2-), 5.11 (d, $J = 12.5$ Hz, 1H, Ph-CH_2-), 5.30 (d, $J = 6.9$ Hz, 1H, G4-OH), 7.06 and 7.34 (s, s, 2H, Q1-NH2), 7.20 (d, $J = 5.3$ Hz, 1H, K6-NH), 7.25–7.40 (m, 5H, Ph), 7.55 (d, $J = 6.9$ Hz, 1H, A2-NH), 7.67 (d, $J = 8.4$ Hz, 1H, T2-NH), 7.84 (d, $J = 7.6$ Hz, K2-NH), 8.10 (d, $J = 8.3$ Hz, 1H, G2-NH), 8.15 (d, $J = 7.9$ Hz, 1H, Q2-NH), 8.29 (d, $J = 7.5$ Hz, 1H, R2-NH), 8.50 (bs, 1H, R5-NH).

1-O-Bzl-nor-Mur(NAc)-Ala-D-Glu(Thr-Lys(Z)-Pro-Arg-(NO_2)-OBzl)-NH_2 (7b). Yield 51%; mp 56°–58 °C; ^{1}H-NMR (DMSO) δ 0.99 (d, $J = 6.2$ Hz, 3H, T4), 1.22 (d, $J = 6.8$ Hz, 3H, CH_3-A3), 1.29 (m, 2H, K4), 1.36 (m, 2H, K5), 1.53 (m, 2H, R4), 1.62 (m, 1H, R3), 1.68 (m, 1H, P3), 1.72 (m, 1H, Q3), 1.74 (m, 1H, R3), 1.80 (m, P4), 1.82 (s, 3H, AcN), 1.92 (m, 1H, Q3), 1.95 (m, 1H, P3), 2.18 (m, 2H, Q4), 2.94 (q, $J = 5.9$ Hz, K6), 3.13 (m, 2H, R5), 3.30 (m, 1H, G4), 3.48 (m, 1H, P5), 3.49 (m, G3, G5), 3.51 (m, 1H, G6), 3.62 (m, 1H, P5), 3.64 (m, 1H, G6), 3.84 (ddd, $J = 3.4$ Hz, $J = 7.8$ Hz, $J = 10.5$ Hz, G2), 3.91 (m, T3), 4.13 (dt, $J = 5.3$ Hz, $J = 7.9$ Hz, Q2), 4.18 (dd, $J = 4.3$ Hz, $J = 8.4$ Hz, T2), 4.25 (m, 1H, R2), 4.29 (m, 1H, A2), 4.31 (m, 1H, P2), 4.43 (d, $J = 12.2$ Hz, 1H, Ph-CH_2-GlcN), 4.44 (m, 1H, K2), 4.62 (t, $J = 5.9$ Hz, 1H, G6-OH), 4.66 (d, $J = 12.2$ Hz, 1H, Ph-CH_2-GlcN), 4.72 (d, $J = 3.4$ Hz, 1H, G1), 4.75 (d, $J = 5.4$ Hz, 1H, T3-OH), 4.98 (s, 2H, Ph-CH_2-), 5.06 (d, $J = 12.5$ Hz, 1H, Ph-CH_2-), 5.12 (d, $J = 12.5$ Hz, 1H, Ph-CH_2-), 5.40 (d, $J = 5.9$ Hz, 1H, G4-OH), 7.08 and 7.38 (s,s, 2H, Q1-NH2), 7.20 (d, $J = 5.0$ Hz, 1H, K6-NH), 7.25–7.40 (m, 5H, Ph), 7.55 (d, $J = 6.9$ Hz, 1H, A2-NH), 7.68 (d, $J = 8.4$ Hz, 1H, T2-NH), 7.84 (d, $J = 7.6$ Hz, K2-NH), 8.14 (d, $J = 8.3$ Hz, 1H, G2-NH), 8.18 (d, $J = 7.9$ Hz, 1H, Q2-NH), 8.28 (d, $J = 7.5$ Hz, 1H, R2-NH), 8.52 (bs, 1H, R5-NH).

1-O-Bzl-Mur(NAc)-Val-D-Glu(Thr-Lys(Z)-Pro-Arg-(NO_2)-OBzl)-NH_2 (7c). Yield 48%; mp 110°–113 °C; ^{1}H-NMR (DMSO) δ 0.80 and 0.82 (d,d, $J = 6.3$ Hz, 6H, CH_3-V), 0.99 (d, $J = 6.2$ Hz, 3H, T4), 1.24 (d, $J = 6.4$ Hz, 3H, H3-PrA), 1.29 (m, 2H, K4), 1.36 (m, 2H, K5), 1.53 (m, 2H, R4), 1.62 (m, 1H, R3), 1.69 (m, 1H, P3), 1.70 (m, 1H, Q3), 1.74 (m, 1H, R3), 1.78 (s, 3H, AcN), 1.80 (m, 2H, P4), 1.92 (m, 1H, Q3), 1.96 (m, 1H, P3), 1.98 (m, 1H, -CH-$(CH_3)_2$ V), 2.19 (m, 2H, Q4), 2.94 (q, $J = 5.9$ Hz, K6), 3.10 (m, 2H, R5), 3.30 (m, 1H, G4), 3.48 (m, 1H, P5), 3.49 (m, 2H, G3, G5), 3.51 (m, 1H, G6), 3.62 (m, 1H, P5), 3.64 (m, 1H, G6), 3.80 (d,d,d, $J = 3.5$ Hz, $J = 8.2$ Hz, $J = 11.2$ Hz, G2), 3.91 (m, T3), 4.02 (t, $J = 7.6$ Hz, 1H, NHCHCO-V), 4.12 (dt, $J = 5.2$ Hz, $J = 7.9$ Hz, Q2), 4.18 (dd, $J = 4.4$ Hz, J = 8.4 Hz, 1H, T2), 4.26 (m, R2), 4.29 (m, A2), 4.32 (m, P2), 4.42 (d, $J = 12.2$ Hz, 1H, Ph-CH_2-GlcN), 4.44 (m, K2), 4.60 (t, $J = 5.9$ Hz, G6-OH), 4.65 (d, $J = 12.2$ Hz, 1H, Ph-CH_2-GlcN), 4.72 (d, $J = 3.4$ Hz, 1H, G1), 4.76 (d, $J = 5.5$ Hz, 1H, T3-OH), 4.98 (s, 2H, Ph-CH_2-), 5.06 (d, $J = 12.5$ Hz, 1H, Ph-CH_2-), 5.12 (d, $J = 12.5$ Hz, 1H, Ph-CH_2-), 5.34 (d, $J = 6.9$ Hz, G4-OH), 7.06 and 7.34 (s,s, Q1-NH2), 7.20 (d, $J = 5.3$ Hz, K6-NH), 7.25–7.40 (m, 5H, Ph), 7.66 (d, $J = 8.3$ Hz, 1H, T2-NH), 7.84 (d, $J = 7.3$ Hz, 1H, K2-NH), 8.10 (d, $J = 8.3$ Hz, G2-NH), 8.25 (d, $J = 7.9$ Hz, 1H, Q2-NH), 8.29 (d, $J = 7.5$ Hz, 1H, R2-NH), 8.30 (d, $J = 8.3$ Hz, 1H, V-NH), 8.50 (bs, 1H, R5-NH).

1-O-Bzl-nor-Mur(NAc)-Val-D-Glu(Thr-Lys(Z)-Pro-Arg-(NO$_2$)-OBzl)-NH$_2$ (7d). Yield 54%; mp 69°–72 °C; ^{1}H-NMR (DMSO) δ 0.78 and 0.81 (d,d, J = 6.3 Hz, 6H, CH_3-V), 0.99 (d, J = 6.2 Hz, 3H, T4), 1.28 (m, 2H, K4), 1.36 (m, 2H, K5), 1.53 (m, 2H, R4), 1.60 (m, 1H, R3), 1.69 (m, 1H, P3), 1.70 (m, 1H, Q3), 1.73 (m, 1H, R3), 1.80 (s, 3H, AcN), 1.82 (m, 2H, P4), 1.92 (m, 1H, Q3), 1.95 (m, 1H, -CH-(CH_3)$_2$-V), 1.97 (m, 1H, P3), 2.19 (m, 2H, Q4), 2.94 (q, J = 5.9 Hz, K6), 3.12 (m, R5), 3.30 (m, 1H, G4), 3.48 (m, 1H, P5), 3.50 (m, G3, G5), 3.51 and 3.64 (m, m, 1H, G6), 3.63 (m, 1H, P5), 3.80 (d,d,d, J = 3.2 Hz, J = 7.9 Hz, J = 10.2 Hz, G2), 3.91 (m, T3), 4.00 (t, J = 7.6 Hz, 1H, -NHCHCO-V), 4.12 (dt, J = 5.2 Hz, J = 7.9 Hz, Q2), 4.18 (dd, J = 4.4 Hz, J = 8.4 Hz, T2), 4.25 (m, 1H, R2), 4.29 (m, 1H, A2), 4.33 (m, 1H, P2), 4.42 (d, J = 12.6 Hz, 1H, Ph-CH_2-GlcN), 4.65 (d, J = 12.6 Hz, 1H, Ph-CH_2-GlcN), 4.44 (m, 1H, K2), 4.60 (t, J = 5.9 Hz, 1H, G6-OH), 4.72 (d, J = 3.4 Hz, 1H, G1), 4.76 (d, J = 5.5 Hz, 1H, T3-OH), 4.98 (s, 2H, Ph-CH_2-), 5.04 (d, J = 12.5 Hz, 1H, Ph-CH_2-), 5.10 (d, J = 12.5 Hz, 1H, Ph-CH_2-), 5.34 (d, J = 6.9 Hz, 1H, G4-OH), 7.06 and 7.34 (s,s, Q1-NH2), 7.20 (d, J = 5.2 Hz, 1H, K6-NH), 7.25–7.40 (m, 5H, Ph), 7.63 (d, J = 8.3 Hz, 1H, T2-NH), 7.82 (d, J = 7.3 Hz, 1H, K2-NH), 8.10 (d, J = 8.3 Hz, 1H, G2-NH), 8.25 (d, J = 7.9 Hz, 1H, Q2-NH), 8.29 (d, J = 7.5 Hz, 1H, R2-NH), 8.30 (d, J = 8.3 Hz, 1H, V-NH), 8.50 (bs, 1H, R5-NH).

1-O-Bzl-Mur(NAc)-Pro-D-Glu(Thr-Lys(Z)-Pro-Arg-(NO$_2$)-OBzl)-NH$_2$ (7e). Yield 50%; mp 105°–108 °C; ^{1}H-NMR (DMSO) δ 1.0 (d, J = 6.3 Hz, 3H, T4), 1.23 (d, J = 6.4 Hz, 3H, H3-PrA), 1.30 (m, 2H, K4), 1.38 (m, 2H, K5), 1.54 (m, 2H, R4), 1.63 (m, 1H, R3), 1.67–2.07 (m, PP′3, PP′4), 1.73 (m, 1H, Q3), 1.75 (m, 1H, R3), 1.79 (s, 3H, AcN), 2.01 (m, 1H, Q3), 2.21 (m, 2H, Q4), 2.95 (q, J = 5.8 Hz, K6), 3.14 (m, 2H, R5), 3.20 (m, 1H, G4), 3.52 and 3.64 (m, PP′5), 3.41 and 3.51 (m, G5, G3), 3.50 and 3.62 (m, m, 2H, G6), 3.61 (d,d,d, J = 3.1 Hz, J = 7.7 Hz, J = 11.0 Hz, G2), 3.92 (m, T3), 4.19 (dt, J = 5.2 Hz, J = 7.8 Hz, Q2), 4.22 (dd, J = 4.4 Hz, J = 8.4 Hz, T2), 4.27 (m, R2), 4.32 (m, PP′2), 4.34 (d, J = 12.2 Hz, 1H, Ph-CH_2-GlcN), 4.45 (m, K2), 4.58 (t, J = 5.9 Hz, 1H, G6-OH), 4.83 (d, J = 3.4 Hz, 1H, G1), 4.65 (d, J = 12.2 Hz, 1H, Ph-CH_2-GlcN), 4.78 (d, J = 5.5 Hz, 1H, T3-OH), 4.98 (s, 2H, Ph-CH_2), 5.07 (d, J = 12.5 Hz, 1H, Ph-CH_2-), 5.12 (d, J = 12.5 Hz, 1H, Ph-CH_2-R), 5.38 (d, J = 6.9 Hz, 1H, G4-OH), 7.03 and 7.17 (s,s, 2H, Q1-NH2), 7.22 (d, J = 5.2 Hz, K6-NH), 7.24–7.38 (m, 5H, Ph), 7.70 (d, J = 8.3 Hz, 1H, T2-NH), 7.88 (d, J = 7.3 Hz, 1H, K2-NH), 7.93 (d, J = 8.3 Hz, 1H, G2-NH), 8.31 (d, J = 7.5 Hz, 1H, R2-NH), 8.32 (d, J = 7.9 Hz, 1H, Q2-NH), 8.53 (bs, 1H, R6-NH).

1-O-Bzl-nor-Mur(NAc)-Pro-D-Glu(Thr-Lys(Z)-Pro-Arg-(NO$_2$)-OBzl)-NH$_2$ (7f). Yield 46%; mp 92°–95 °C; ^{1}H-NMR (DMSO) δ 0.98 (d, J = 6.3 Hz, 3H, T4), 1.32 (m, 2H, K4), 1.37 (m, 2H, K5), 1.54 (m, 2H, R4), 1.62 (m, 1H, R3), 1.67–2.07 (m, 4H, PP′3, PP′4), 1.71 (m, 1H, Q3), 1.74 (m, 1H, R3), 1.86 (s, 3H, AcN), 2.0 (m, 1H, Q3), 2.21 (m, Q4), 2.94 (q, J = 5.7 Hz, K6), 3.16 (m, R5), 3.20 (m, 1H, G4), 3.52 and 3.64 (m, 4H, PP′5), 3.42 and 3.53 (m, G5, G3), 3.50 and 3.62 (m, m, G6), 3.60 (d,d, J = 8.3 Hz, J = 10.5 Hz, G2), 3.92 (m, T3), 4.19 (dt, J = 5.2 Hz, J = 7.8 Hz, Q2), 4.22 (dd, J = 4.4 Hz, J = 8.4 Hz, T2), 4.27 (m, R2), 4.32 (m, PP′2), 4.34 (d, J = 12.2 Hz, 1H, Ph-CH_2-GlcN), 4.65 (d, J = 12.2 Hz, 1H, Ph-CH_2-GlcN), 4.45 (m, K2), 4.58 (t, J = 5.8 Hz, 1H, G6-OH), 4.83 (d, J = 3.4 Hz, 1H, G1), 4.78 (d, J = 5.5 Hz, 1H, T3-OH), 4.98 (s, 2H, Ph-CH_2-), 5.07 (d, J = 12.4 Hz, 1H, Ph-CH_2-), 5.12 (d, J = 12.4 Hz, 1H, Ph-CH_2-R), 5.38 (d, J = 6.9 Hz, 1H, G4-OH), 7.03 and 7.17 (s,s, 2H, Q1-NH2), 7.22 (d, J = 5.2 Hz, 1H, K6-NH), 7.24–7.38 (m, 5H, Ph), 7.70 (d, J = 8.3 Hz, 1H, T2-NH), 7.86 (d, J = 7.3 Hz, 1H, K2-NH), 7.98 (d, J = 8.2 Hz, 1H, G2-NH), 8.32 (d, J = 7.8 Hz, 1H, Q2-NH), 8.33 (d, J = 7.5 Hz, 1H, R2-NH), 8.53 (bs, 1H, R6-NH).

Mur(NAc)-Ala-D-Glu(Thr-Lys-Pro-Arg-OH)-NH$_2$ (8a). Yield 0.19 g (45%); amino acid analysis (6 M HCl, 110 °C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Found: C, 47.39; H, 7.29; N, 15.13; calcd. for $C_{40}H_{70}N_{12}O_{16} \times 2CH_3COOH \times H_2O$: C, 47.48; H, 7.24; N, 15.099.

nor-Mur(NAc)-Ala-D-Glu(Thr-Lys-Pro-Arg-OH)-NH$_2$ (8b). Yield 0.20 g (47%); amino acid analysis (6 M HCl, 110 °C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Found: C, 46.76; H, 7.34; N, 14.99; calcd. for $C_{39}H_{68}N_{12}O_{16} \times 2CH_3COOH \times H_2O$: C, 46.99; H, 7.15; N, 15.29.

Mur(NAc)-Val-D-Glu(Thr-Lys-Pro-Arg-OH)-NH$_2$ (8c). Yield 0.23 g (40%); amino acid analysis (6 M HCl, 110 °C, 20 h): Arg, Glu, Lys, Pro, Thr, Val. Found: C, 48.52; H, 7.53; N, 14.98; calcd. for $C_{42}H_{74}N_{12}O_{16} \times 2CH_3COOH \times H_2O$: C, 48.41; H, 7.42; N, 14.73.

nor-Mur(NAc)-Val-D-Glu(Thr-Lys-Pro-Arg-OH)-NH$_2$ (8d). Yield 0.17 g (42%); amino acid analysis (6 M HCl, 110 °C, 20 h): Arg, Glu, Lys, Pro, Thr, Val. Found: C, 48.09; H, 7.42; N, 15.12; calcd. for $C_{41}H_{72}N_{12}O_{16} \times 2CH_3COOH \times H_2O$: C, 47.95; H, 7.33; N, 14.91.

Mur(NAc)-Pro-D-Glu(Thr-Lys-Pro-Arg-OH)-NH$_2$ (8e). Yield 0.15 g (38%); amino acid analysis (6 M HCl, 110 °C, 20 h): Arg, Glu, Lys, Pro, Thr. Found: C, 48.63; H, 7.37; N, 14.92; calcd. for $C_{42}H_{72}N_{12}O_{16} \times 2CH_3COOH \times H_2O$: C, 48.49; H, 7.25; N, 14.75.

nor-Mur(NAc)-Pro-D-Glu(Thr-Lys-Pro-Arg-OH)-NH$_2$ (8f). Yield 0.17 g (42%); amino acid analysis (6 M HCl, 110 °C, 20 h): Arg, Glu, Lys, Pro, Thr. Found: C, 48.32; H, 7.26; N, 15.12; calcd. for $C_{41}H_{70}N_{12}O_{16} \times 2CH_3COOH \times H_2O$: C, 48.04; H, 7.17; N, 14.94.

BIOLOGICAL TESTS

Acquisition of Leukocytes

Venous blood samples were obtained from volunteers recruited during routine medical consultation in one of the nursing homes in Gdansk. Only fully healthy individuals according to criteria of physical examination and laboratory tests were included in the study. People suffering from chronic illnesses, chronically taking drugs, suffering from acute illnesses or vaccinated for 6 weeks before venopuncture were excluded. All subjects were fully informed about the study and signed written consent approved by the Ethical Committee of the Medical University of Gdansk. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of whole blood in a Ficoll-Hypaque gradient and subsequently cultured. Separation of

monocytes and peripheral blood lymphocytes (PBL) started from PBMC which were cultured for 1 h on adhesive Petri dishes in RPMI containing 5% heat-inactivated fetal calf serum (FCS). After this time, the monocytes adhered to the bottom of the Petri dish, while PBL did not. Then, medium containing PBL was gently pipetted and transferred into another dish. The dish containing the monocytes was completed with medium and the monocytes were harvested for further tests using a scraper. Each set of experiments described below requiring different concentrations of the particular derivative was performed with blood samples from the same donor. It allowed more accurate results to be obtained, however, in some cases this limited the number of possible combinations in the experiments.

Proliferation/Viability Tests

In all tests the cells were incubated for 4, 12 and 24 h in an atmosphere of 5% CO_2 at 37 °C on plastic 96-well flat-bottomed plates (Corning, Science Products, USA) in triplicate. PBMC, PBL and monocytes obtained from volunteers were dispensed at 1×10^5 cells per well in 100 µl of RPMI medium with 5% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamate (Gibco, Life Technologies Inc., USA). The cultures were incubated with the following final concentrations of tuftsin and its derivatives: 0, 0.0001, 0.001, 0.01, 0.1 and 1.0 mg/ml. In the case of monocytes, the cultures incubated with 0.01 mg/ml of the derivatives could not be performed because of the low amount of monocytes yielded from the blood sample of a single individual. Since the examined substances were dissolved with dimethyl sulphoxide (DMSO), additional control cultures were performed only with serial dilutions of the solvent. Since the observed effects of the derivatives were suspected to be dependent on the secreted cytokines another set of viability tests was performed with addition of anti-cytokine antibodies. Rabbit anti-human IL6 antibody (Genzyme, USA) or rabbit anti-human TNFα antibody (Genzyme, USA) were added in a final concentration of 1 µg/ml to the examined wells and incubated as described above. After particular periods of incubation, plates from all performed viability tests were prepared using the colorimetric MTT assay described below.

Stimulation of PBMC, PBL and Monocytes for Bioassays

The PBMC, PBL and monocytes obtained were subsequently cultured on the plastic 24-well plates (Nunc, Denmark) at 1×10^6 cells per well, in 1 ml of RPMI containing 5% FCS. The cultures were incubated with the following serial dilution of tuftsin and its derivatives: 0, 0.001, 0.01 and 0.1 mg/ml. Incubation was carried out for 24 h in a humidified atmosphere in 5% CO_2 at 37 °C. Then, supernatants were aspirated, immediately frozen and stored at −80 °C.

Bioassay for Interleukin 6 (IL6) and Tumour Necrosis Factor α (TNFα)

The IL6-dependent murine hybridoma cell line B9 cells (kindly provided by Dr L. Aarden, Netherland Red Cross, Amsterdam, Netherlanden) were cultured for 48 h on 96-well plastic plates at a concentration of 10×10^3 per well in RPMI medium containing 10% FCS, 2 mM L-glutamate and gentamycin. Five µl of the supernatants was added in triplicate to each well. The optical densities obtained from the experimental wells were fitted with titration standards of recombinant IL6. Rabbit anti-IL6 antibody was added to the wells in order to confirm the specificity of the test. The anti-IL6 antibody completely blocked the proliferative effect of the sera or supernatants on B9 cells. The level of TNFα was assessed similarly to the above-mentioned method for IL6 with the exception that the cell line used was TNFα-sensitive WEHI 164 (from Dr T. Espevick, Institute of Cancer Research, University of Trodheim, Trodheim, Norway) cultured for 24 h in medium containing in addition 1.0 mg/ml actinomycin-D (Sigma Chemical Co., USA). Five µl of the supernatants was added, in triplicate, to each well of the plate. The optical densities obtained from the experimental wells were fitted with titration standards of recombinant TNFα. Confirmation of the specificity of the test was made using anti-TNFα antibody. After incubation, the plates were prepared using the colorimetric MTT assay described below.

Colorimetric MTT Assay

Cells in the viability tests or bioassays after particular periods of incubation were treated in the following way. After incubation, (3–4.5 dimethylthiazol-2-yl)-2.5 diphenyl-tetrazolium bromide (MTT) (Sigma Chemical Co., USA) was added to all wells in a final concentration of 1 mg/ml and the plates were incubated at 37 °C in an atmosphere of 5% CO_2 at 37 °C for an additional 4 h. Then, the reaction was stopped by the addition of 100 µl of isopropanol. The optical density was read at 570 nm on an automated plate reader (FL600, Bio-tec, USA). In the case of proliferation/viability tests, the results were analysed in the absorbance mode using the computer program Kineticalc 4 (Bio-Tec, USA). The absorbance of the control samples (without the examined substances, containing only cells in medium with 5% FCS) was taken to be 100%. The absorbance of the other samples was calculated as a percentage of the control. In the case of bioassays, the values of optical density were fitted against titration standards of IL6 or TNFα.

Cytometric Evaluation of Oxidative Burst in Subpopulations of PBMC

Evaluation of the increase in the oxidative activity induced by tuftsin derivatives in monocytes and PBL was based on oxidation of 2′,7′-dichlorodihydrofluorescein (H_2DCF) to the parent fluorescent dye 2′,7′-dichloro-fluorescein (DCF) [28]. Cells in PBMC cultures were loaded with H_2DCF for 1 h. Then, the examined substances were added to the cultures at a final concentration 0.001 or 0.1 mg/ml. The fluorescence of the generated DCF was measured using flow cytometry just after the addition and at 1 h, 4 h, 12 h and 24 h after incubation with the examined substances.

The gates containing lymphocytes and monocytes in flow cytometry analysis were established using forward angle scatter (size) and side angle scatter (granularity) light characteristics of these populations. Dead cells were excluded under the same conditions. The appropriateness of the established gates was additionally confirmed using the CD14/CD45 leucogate protocol [29]. Then, histograms visualizing DCF fluorescence were generated from established gates. Mean and peak fluorescence signals of DCF from the examined cultures were measured and compared in arbitrary channel units.

Assessment of Natural Killer (NK) Cells Cytotoxic Activity

The cytotoxic activity of NK cells was assessed from PBMC cultured with the examined derivatives for 12 or 24 h in a colorimetric lactate dehydrogenase (LDH) release assay. K562 cells (NK-sensitive erythromyeloid cell line from ATCC collection) were used as the target. A total of 2×10^4 K562 cells was harvested during the log phase with effector cells (PBMC containing NK cells) in a ratio of 1 : 10. Mixed cells were incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2 in 96-well round-bottomed plates (Nunc, Denmark). After incubation the samples were centrifuged and 100 µl of the supernatants was transferred into 96-well flat-bottomed optical plates (Nunc, Denmark). The activity of LDH released from K562 was assessed after 30 min of incubation of the supernatants with 100 µl of LDH substrate (Boehringer Mannheim, Germany). The absorbance of the coloured product was measured at 492 nm using an automated plate reader (FL600, Bio-tec, USA). The following formula was used for the calculations: % of cytotoxicity = (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100%.

Assessment of IC_{50}/EC_{50}

The values of the serial dilutions of the conjugates versus the arbitrary absorbance units obtained in the plate reader were used to assess IC_{50} or EC_{50} in the computer program Prism 3.0 (GraphPad, USA). IC_{50} were given when the conjugate inhibited viability compared with the solvent (DMSO) and ED_{50} were given when the conjugate increased the viability compared with the solvent. Whenever a value of IC_{50} or EC_{50} was <0.0001 it was made equal to 0.0001 and SD < 0.001 was made equal to 0.001. Values were given only if the differences between the viability in the presence of the conjugate and the solvent were statistically significant (Table 2).

Statistical Analysis

Data were computed using program Statistica 5.5 (Statsoft, Poland). Analysis was based on non-parametric statistics: Kruskal-Wallis and Friedmann ANOVA or, when the date achieved normal distribution, on parametric ANOVA and *post hoc* tests.

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