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Dimeric analogs of immunosuppressive decapeptide fragment of ubiquitin‡

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Our previous studies revealed that ubiquitin and its decapeptide fragment with the LEDGRTLSDY sequence, located on the exposed molecule loop, strongly suppressed the immune response. This suggested that the loop may serve as a functional epitope of ubiquitin molecule and that a possible mechanism of biological action of the synthesized peptides is associated with interfering in interactions of ubiquitin with other molecules. Ubiquitin is known to exist in oligomeric forms, which can interact with various oligomeric receptors. We designed and synthesized new dimeric analogs of the ubiquitin fragment, to probe whether dimeric peptides may have higher affinity towards the ubiquitin receptors responsible for immunosuppression, which are believed to form oligomeric structures. Three dimerization strategies, N-terminus to N-terminus, C-terminus to C-terminus, and N-terminus to C-terminus (head-to-tail) via PEG derivatives were used to synthesize the dimeric peptides on solid support. In the course of our research, we developed a new and straightforward procedure of dimerization where α-amino groups of the C-terminal lysine residues of two peptide fragments were linked by PEG spacer directly on solid support.

The effect of dimeric analogs on the immunological response was tested in the AFC *in vitro* experiment. The immunological tests showed that the head-to-tail dimerization caused a more profound increase in the biological activity than other tested dimerization methods. Our results suggest that such orientation of peptide components may correspond to orientation of the hypothetic ubiquitin receptors responsible for the immunomodulatory activity. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dimerization strategies; dimerization on solid support; PEG linker; linker length; ubiquitin fragments

Introduction

Dimerization or oligomerization of receptors is an essential step in various cellular signal transduction processes. Understanding of the multivalent character of some bioreceptors may be helpful in designing new compounds with high affinity and enhanced selectivity for a given receptor. Substances that are able to modulate the receptor dimerization may control such a process and therefore affect the biological outcome.

Dimerization of an active biomodulator often results in enhanced binding and improved pharmacological properties. Compounds consisting of two ligands connected by a proper spacer have a potential for bridging vicinal receptors. Therefore, the synthesis and biological evaluation of dimeric analogs of biologically active compounds is of great interest in biomedical applications [1]. Many biological ligands are also composed of clustered binding epitopes [2]. Properly designed dimeric analogs of bioligands should enhance interactions between neighboring receptors, possibly by reducing unfavorable entropic effects. The dimerization strategy was successfully applied to antagonists of chemokine receptor CXCR4, responsible for regulation of inflammation and immune response, with linkers composed of oligoglycine and dicarboxylic acids [3]. Multimeric forms of cyclic RGD analogs attached to rigid scaffolds presented increased binding avidity towards αvβ3 integrin, although addition of oligo(ethylene glycol) spacers reduced the effect, presumably by enthropic effect [4]. On the other hand, the ^{18}F-labeled PEG_4-E[PEG_4-c(RGDfK)]$_2$ construct showed increased *in vitro* receptor binding affinity and significantly enhanced tumor uptake *in vivo* compared with PEG_4-E[c(RGDfK)]$_2$ without the two PEG_4 spacers between the two RGD motifs [5]. The influence of dimerization and linker length on the activity of Smac fragments, binding to XIAP, an important regulator of apoptosis, was analyzed by biological and physicochemical methods [6,7].

A combinatorial approach based on dipeptide linkers attached to a central scaffold (bis-3,5-aminomethyl benzoic acid) was applied to the design of bivalent glutathione *S*-transferase inhibitors, resulting in compounds with increased affinity and selectivity, and better solubility [8]. Two peptide fragments of discontinuous HLA-DQ epitope were linked by γ-Abu residue to increase their immunosuppressory

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Dedicated to Prof. Ignacy Z. Siemion on the occasion of his 80th birthday.

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Abbreviations used: *HLA, human leukocyte antigen; AFC, antibody-forming cells; SRBC, sheep red blood cells*

Journal of PeptideScience

activity [9]. Several short linkers, ranging from single Asp residue to PEG diacid, were used in the synthesis of N-to-N and N-to-C dimers in search for more efficient antagonists of the human IgG–human FcRn interaction [10].

It may be expected that properly designed dimeric analogs of biologically active bioligands are more selective in their affinity towards dimeric receptors. Therefore, the dimeric ligands may serve as a tool to examine the phenomenon of receptor dimerization.

Ubiquitin is a conservative 76-amino acid polypeptide present in all eukaryotic cells. Apart from its key function in proteasomal degradation [11], this polypeptide is involved in many intracellular signal pathways [12] and also plays an important role as the extracellular immunomodulator and antimicrobial agent [13]. It has been postulated that cryptides originating from ubiquitin could interfere in the ubiquitination process, by disruption of some ubiquitin activities. Moreover, ubiquitin-derived peptides were shown to express various important biological activities [14]. We pointed out some topological similarities between 50–59 loop of ubiquitin and β164–172 loop of HLA-DQ molecule with TPQRGDVYT sequence, known for suppression of the humoral and cellular immune response and inhibition of integrins activity [15,16]. We found that the 50–59 ubiquitin fragment LEDGRTLSDY, containing the retro-RGD sequence, exhibited strong immunosuppressive effects on the cellular and humoral immune responses, comparable with that of cyclosporine [17]. This finding suggested that ubiquitin fragment with LEDGRTLSDY sequence could interact with similar receptor type as the immunosuppressive fragments of HLA. The dimerization of the nonapeptide VPRSGEVYT, designed to mimic the discontinuous immunosuppressive epitopes of the HLA-DR superdimers [18], resulted in the enhancement of immunosuppressive potency [19,20] and prompted us to use such approach to ubiquitin fragments.

Ubiquitin is known to exist in an oligomeric form that can interact with various oligomeric receptors [21,22]. It was established that the ubiquitin system is involved in the regulation of immune response by the activation of the NF-κB pathway [23], with linear polyubiquitination and linear ubiquitin chain assembly complex playing a role in B cell activation and differentiation, regulation of type I interferon production, genotoxic stress response, and osteogenesis [24]. However, the biological significance of some interactions of the oligomeric ubiquitin still remains unknown. It is feasible that the dimeric analogs of ubiquitin fragments (Figure 1) are able to interact with the dimeric receptors, which may cause the modulation of the interaction of oligoubiquitin with the oligomeric receptors. We decided to synthesize dimeric analogs of the immunosuppressive decapeptide fragment of ubiquitin, utilizing three dimerization strategies N-terminus to N-terminus, C-terminus to C-terminus, and N-terminus to C-terminus (head-to-tail) (Figure 1) to investigate a possibility of multivalency of the receptors.

Materials and Methods

Reagents

The derivatives of amino acids for peptide synthesis, the coupling reagent 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate *N*-oxide (TBTU) and *N*-hydroxybenzotriazole (HOBt) were purchased from IrisBiotech (Marktredwitz, Germany) and NovaBiochem (as Merck, Darmstadt, Germany). The side chain protecting groups for Fmoc-amino acids were *t*-butyl for Glu, Asp, Thr,

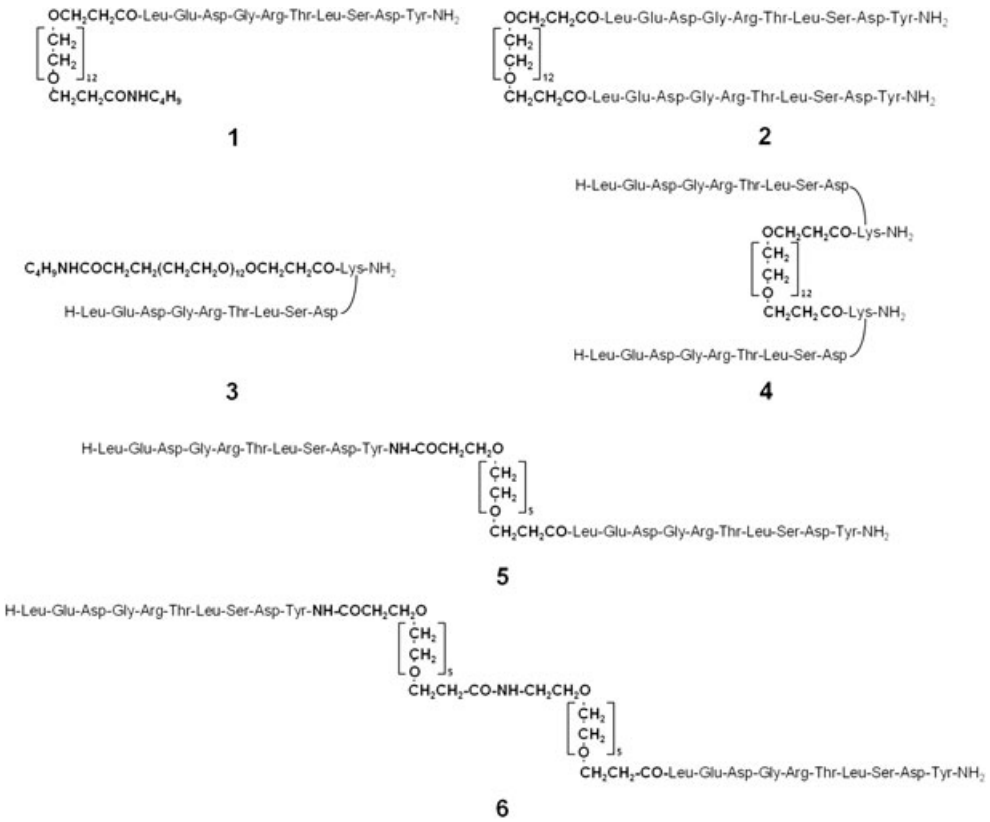

Figure 1. The synthetic analogs of the immunosuppressive ubiquitin fragment.

Ser, and Tyr; Pmc for Arg; and Mtt for Lys. The MBHA Rink amide resin (0.69 mmol/g) was purchased from NovaBiochem. The HOOC-$CH_2CH_2O(CH_2CH_2O)_{12}CH_2CH_2$-COOH and Fmoc-NH-$CH_2CH_2O(CH_2CH_2O)_5CH_2CH_2$-COOH were obtained from Aldrich (as Sigma-Aldrich, St. Louis, MO, USA) and NovaBiochem, respectively. TFA was from IrisBiotech. Thioanisole, pentafluorophenol, and triisopropylsilane (TIS) were purchased from Fluka (as Sigma-Aldrich, St. Louis, MO, USA); phenol from Aldrich; piperidine from Merck (Darmstadt, Germany); and dicyclohexylcarbodiimide (DCC) from Riedel-de Haën (as Sigma-Aldrich, St. Louis, MO, USA). The solvents for peptide synthesis were obtained from Aldrich (DMF and DCM) and IrisBiotech (DIPEA). Other chemicals were purchased from POCH (Gliwice, Poland).

General Methods

Mass spectrometry

MS and MS/MS experiments were carried out on micrOTOF-Q and Apex-Qe Ultra 7T FT-ICR instruments (Bruker Daltonic, Bremen, Germany) equipped with an electrospray source. Spectra were recorded using 50:50 acetonitrile–water mixture containing 0.1% HCOOH at the peptide concentration 0.5 μM. The potential between the spray needle and the orifice was set to 4.5 kV. In the MS/MS mode, the quadrupole was used to select the precursor ions, which were fragmented in the hexapole collision cell generating product ions that were subsequently mass analyzed by the mass analyzer. Argon was used as the collision gas. In the mass spectrometric (MS/MS) experiments, the collision energy was optimized for the best fragmentation.

Purification

Analytical HPLC was performed on Vydac C18 column (4.6 mm × 250 mm) (Grace, Deerfield, IL, USA). Solvent system: S_1, 0.1% aqueous TFA; S_2, 80% acetonitrile + 0.1% TFA, linear gradient from 0% to 100% of S_2 for 60 min, flow rate 1.0 ml/min, UV detection at 220 nm. For compounds **1**, **2**, **4**, **5**, and **6**, additional HPLC analyses were performed, using YMC-Pack ODS-AQ column (4.6 mm × 250 mm) (YMC, Kyoto, Japan) and Varian Microsorb-MV 100-5 CN (4.6 mm × 250 mm) (Varian, Palo Alto, CA, USA) in linear gradient from 0% to 80% of S_2 for 40 min, flow rate 1.0 ml/min (retention times for analyses are given as R_{t1}, R_{t2}, and R_{t3}, respectively). Preparative reversed-phase HPLC was performed on Tosoh TSKgel ODS-120T column (21.5 mm × 300 mm) (Tosoh, Tokyo, Japan) using the same solvent system (gradient 0.5%/min, flow rate 7 ml/min). Purified compounds (purity determined by HPLC >98%) were transformed into acetate salts by dissolving in 50% acetic acid, dilution, and lyophilization [25].

CD spectroscopy

CD spectra were recorded on a Jasco J-600 spectropolarimeter (Easton, MD, USA). Peptides were dissolved at concentrations of approximately 70 μg/ml, and the spectra were recorded in water, 10% aqueous sodium chloride, TFE, and aqueous solution of TFE (20% and 50%) at room temperature, at pH 6. The spectrum of the solvent was recorded under identical conditions and subtracted during data analysis. A rectangular quartz cuvette of 1 mm path length was used. Each spectrum represents the average of eight scans. Data are presented as molar ellipticity (Θ) per amino acid residue.

Synthesis

General peptide synthesis

Peptides were prepared by manual solid-phase technique by using the standard Fmoc (9-fluorenylmethoxycarbonyl) synthesis procedure. The MBHA Rink amide resin was functionalized with Fmoc-Tyr(Bu^t)-OH (for compounds **1**, **2**, **5**, and **6**) and Fmoc-Lys(Mtt)-OH (for compounds **3** and **4**). Fmoc-protecting groups were removed in the presence of 25% piperidine in DMF. The ε-amino groups of lysine were deprotected using 1% TFA in DCM (TFA/TIS/DCM 1/4/95 v/v). Single coupling by TBTU was performed in DMF. The reaction was monitored by Kaiser test [26]. Lithium chloride was used during synthesis of dimeric compounds as chaotropic agent. All synthesized compounds were cleaved from the resin and deprotected using modified reagent K (TFA/water/phenol/thioanisole/TIS 82.5/5/5/5/2.5 v/v) at room temperature for 2 h, the resin was rinsed with TFA, and the products were precipitated with cold diethyl ether (Et_2O). The yield of synthesis is presented according to the participation of the peak representing the synthesized compound in HPLC profile of crude product at 220 nm.

*PfpOOC-$CH_2CH_2O(CH_2CH_2O)_{12}CH_2CH_2$-COOPfp (reagent **A**).* HOOC-$CH_2CH_2O(CH_2CH_2O)_{12}CH_2CH_2$-COOH (3 g, 4.34 mmol) and pentafluorophenol (1.6 g, 8.69 mmol) were dissolved in 20 ml of ethyl acetate at 0 °C, and DCC (1.8 g, 8.69 mmol) was added. The reaction mixture was stirred for 1.5 h at 0 °C. Dicyclohexylurea was filtered off, and the filtrate was evaporated *in vacuo*. The obtained product was used for synthesis of dimeric compounds **2** and **4** without further purification.

ESI-MS: *m/z* 1023.345, calculated for $[M+H]^+$ 1023.343 (monoisotopic mass); *m/z* 1045.327, calculated for $[M+Na]^+$ 1045.325 (monoisotopic mass); *m/z* 1061.301, calculated for $[M+K]^+$ 1061.298 (monoisotopic mass).

*PfpOOC-$CH_2CH_2O(CH_2CH_2O)_{12}CH_2CH_2$-CONHC$_4H_9$ (reagent **B**).* Reagent **A** (3 g, 3 mmol) was dissolved in DMF (10 ml). Then, *n*-butylamine (148 μl, 1.5 mmol) was added to the solution in three portions at 15-min intervals. The reaction was completed after 1.5 h as judged by the ninhydrin test. The obtained product was used for synthesis of monomeric compounds **1** and **3** without further purification.

ESI-MS: *m/z* 912.430, calculated for $[M+H]^+$ 912.437 (monoisotopic mass).

Peptide 1. α-Amino group of protected peptide H-Leu-Glu(OBu^t)-Asp(OBu^t)-Gly-Arg(Pmc)-Thr(Bu^t)-Leu-Ser(Bu^t)-Asp(OBu^t)-Tyr(Bu^t) attached to the resin (200 mg, 0.14 mmol) was acylated by Reagent **B** (1.3 g, 1.4 mmol) in the presence of HOBt (190 mg, 1.4 mmol) dissolved in DMF. The reaction was performed for 24 h. The peptide was cleaved from the resin and purified by reversed-phase HPLC. The fractions were collected, lyophilized, and transformed into acetate forms.

Yield 52%, R_{t1} 20.8 min, R_{t2} 25.6 min, R_{t3} 27.7 min.

ESI-MS: *m/z* 948.002, calculated for $[M+2H]^{2+}$ 948.003 (monoisotopic mass); *m/z* 632.339, calculated for $[M+3H]^{3+}$ 632.338 (monoisotopic mass).

MS/MS on parent ion 632.34 ($[M+3H]^{3+}$) (*m/z* value for the most abundant peaks): 857.96^{+2} (calc. for b_9 857.96^{+2}); 421.27^{+2} (calc. for b_1 421.27^{+2}); 1054.48^{+1} (calc. for y_9 1054.48^{+1}); 383.1^{+1} (calc. for y_3 383.1^{+1}).

Peptide 2. The strategy of the synthesis of the dimeric compound **2** (Figure 2) was similar to the one described by us previously [20]. Two adjacent resin-bound peptide chains (H-Leu-Glu(OBu^t)-Asp(OBu^t)-Gly-Arg(Pmc)-Thr(Bu^t)-Leu-Ser(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-) were linked through their N-termini by reagent **A** in DMF at room temperature in the presence of HOBt. A solution of reagent **A** (51 mg, 0.05 mmol) and HOBt (33.8 mg, 0.25 mmol) in 1 ml DMF

Journal of PeptideScience

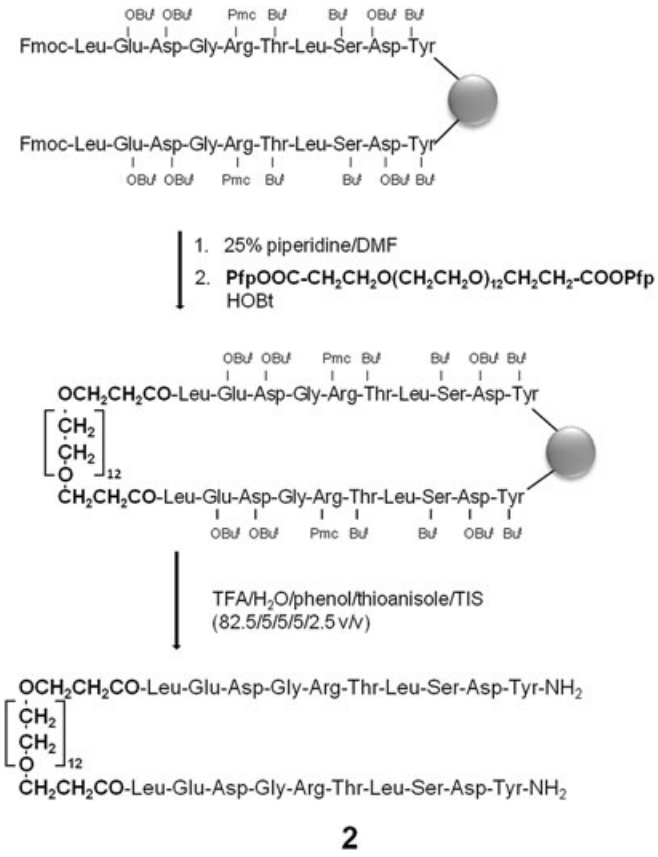

Figure 2. The synthesis strategy of compound **2**. In synthesis of monomeric analog **1**, reagent **B** was used instead of di-pentafluorophenyl ester of PEG diacid (reagent **A**). Grey balls indicate MBHA Rink amide resin bead.

was added to the peptidyl resin (150 mg, 0.1 mmol) suspended in DMF in a few portions during 24 h. The peptide was cleaved from the resin and purified by reversed-phase HPLC. The fractions were collected, lyophilized, and transformed into acetate forms.

Yield 61%, R_{t1} 17.6 min, R_{t2} 23.7 min, R_{t3} 22.6 min.

ESI-MS: *m/z* 1495.720, calculated for $[M+2H]^{2+}$ 1495.722 (monoisotopic mass); *m/z* 997.481, calculated for $[M+3H]^{3+}$ 997.484 (monoisotopic mass); *m/z* 748.365, calculated for $[M+4H]^{4+}$ 748.365 (monoisotopic mass).

MS/MS on parent ion 997.48 ($[M+3H]^{3+}$) (*m/z* values for the most abundant peaks): 1304.15^{+2} (calc. for b_7 1304.15^{+2}); 968.49^{+2} (calc. for b_1 968.49^{+2}); 528.24^{+2} (calc. for y_9 528.24^{+2}); 384.14^{+1} (calc. for y_3 384.14^{+1}); 297.11^{+1} (calc. for y_2 297.11^{+1}).

Peptide 3. Fmoc-Lys(Mtt) was attached to the MBHA Rink amide resin. Then Fmoc-protecting group was removed, and the mixture of reagent **B** (1.3 g, 1.4 mmol) and HOBt (190 mg, 1.4 mmol) dissolved in DMF was added to the resin with attached Lys(Mtt) residue (200 mg, 0.14 mmol) suspended in DMF. The reaction was performed for 24 h. Next, the Mtt-protecting group from the ε-amine group of Lys was removed with 1% TFA in DCM (TFA/TIS/DCM 1/4/95 v/v), and the synthesis of peptide chain was continued. The peptide was cleaved from the resin, and the crude product was characterized and purified by reversed-phase HPLC. The fractions were collected, lyophilized, and transformed into acetate forms.

Yield 64%, R_{t1} 23.5 min.

ESI-MS: *m/z* 930.515, calculated for $[M+2H]^{2+}$ 930.518 (monoisotopic mass); *m/z* 620.681, calculated for $[M+3H]^{3+}$ 620.682 (monoisotopic mass). MS/MS on parent ion 620.68 ($[M+3H]^{3+}$) (*m/z* value for the most abundant peaks): 494.24^{+2} (calc. for b_9 494.24^{+2}); 393.21^{+2} (calc. for b_7 393.21^{+2}); 437.29^{+2} (calc. for y_1 437.29^{+2}).

Peptide 4. Fmoc-Lys(Mtt) was attached directly to the MBHA Rink amide resin (200 mg, 0.14 mmol). The Fmoc-protecting group was removed, and the dimerization was performed by linking the α-amine groups of resin-bound lysine residues by reagent **A**. The mixture of reagent **A** (71 mg, 0.07 mmol) and HOBt (47 mg, 0.35 mmol), dissolved in 1 ml DMF, was added to the resin suspended in DMF in several portions during 1 h, and the reaction mixture was stirred for 24 h. Next, the Mtt-protecting group from the ε-amine group of Lys was removed with 1% TFA in DCM (TFA/TIS/DCM 1/4/95 v/v). Further synthesis of peptide chain, attached to the ε-amine group of Lys, was continued according to the Fmoc synthesis procedure. The peptide was cleaved from the resin, and the crude product was characterized and purified by reversed-phase HPLC. The fractions were collected, lyophilized, and transformed into acetate forms.

Yield 70%, R_{t1} 19.1 min, R_{t2} 19.2 min, R_{t3} 17.3 min.

ESI-MS: *m/z* 1459.779, calculated for $[M+2H]^{2+}$ 1459.769 (monoisotopic mass); *m/z* 973.512, calculated for $[M+3H]^{3+}$ 973.515 (monoisotopic mass); *m/z* 730.386, calculated for $[M+4H]^{4+}$ 730.388 (monoisotopic mass). MS/MS on parent ion 730.39 ($[M+4H]^{4+}$) (*m/z* value for the most abundant peaks): 494.24^{+2} (calc. for b_9 494.24^{+2}); 436.73^{+2} (calc. for b_8 436.73^{+2}); 393.21^{+2} (calc. for b_7 393.21^{+2}); 336.67^{+2} (calc. for b_6 336.67^{+2}); 745.35^{+1} (calc. for y_8b_9 745.35^{+1}); 630.32^{+1} (calc. for y_8b_8 630.32^{+1}).

Peptide 5. The Fmoc-NH-$CH_2CH_2O(CH_2CH_2O)_5CH_2CH_2$-COOH (79 mg, 0.14 mmol) was added to the synthesized decapeptide fragment attached to the resin (100 mg, 0.07 mmol) suspended in DMF. The reaction was performed for 5 h in the presence of DIPEA (51 µl, 0.28 mmol) and TBTU (44 mg, 0.14 mmol). After removing the Fmoc-protecting group, the synthesis of the next decapeptide fragment was continued according to the Fmoc synthesis procedure, each coupling was repeated twice, and lithium chloride was used as chaotropic agent. The peptide was cleaved from the resin, and the crude product was characterized and purified by reversed-phase HPLC. The fractions were collected, lyophilized, and transformed into acetate forms.

Yield 76%, R_{t1} 19.3 min, R_{t2} 21.6 min, R_{t3} 20.7 min.

ESI-MS: *m/z* 1326.647, calculated for $[M+2H]^{2+}$ 1326.648 (monoisotopic mass); *m/z* 884.765, calculated for $[M+3H]^{3+}$ 884.768. MS/MS on parent ion 884.77 ($[M+3H]^{3+}$) (*m/z* value for the most abundant peaks): 824.73^{+3} (calc. for b_{20} 824.73^{+3}); 799.91^{+2} (calc. for b_{12} 799.91^{+2}); 751.88^{+2} (calc. for y_{11} 751.88^{+2}).

Peptide 6. The compound was synthesized by the method described for peptide **5**. In order to increase the linker length, coupling of a second Fmoc-NH-$CH_2CH_2O(CH_2CH_2O)_5CH_2CH_2$-COOH molecule was executed in the presence of DIPEA and TBTU after the removal of the Fmoc-protecting group of the immobilized peptide already containing one residue of PEG-amino acid.

Yield 82%, R_{t1} 20.4 min, R_{t2} 22.0 min, R_{t3} 20.9 min.

ESI-MS: *m/z* 1494.251, calculated for $[M+2H]^{2+}$ 1494.245 (monoisotopic mass); *m/z* 996.497, calculated for $[M+3H]^{3+}$ 996.499 (monoisotopic mass); *m/z* 747.625, calculated for $[M+4H]^{4+}$ 747.626 (monoisotopic mass). MS/MS on parent ion 747.63 ($[M+4H]^{4+}$) (*m/z* value for the most abundant peaks): 936.48^{+3} (calc. for b_{21} 936.48^{+3}); 869.12^{+3} (calc. for b_{19} 869.12^{+3}); 636.02^{+3} (calc. for a_{12} 636.02^{+3}).

Biological Tests

Animals

Twelve-week-old male CBA mice were used for the experiments. The mice were kept at the Animal Facility of the Institute of Immunology and Experimental Therapy, Wroclaw, and fed commercial granulated food and water *ad libitum*. The local ethics committee approved the study.

Reagents

SRBC used for priming mice and *in vitro* culture were provided by the Wroclaw University of Life and Environmental Sciences and were stored in Alsever's solution until use.

Determination of the AFC numbers in the cell culture

Mice were sensitized with 0.2 ml of a 1% SRBC suspension in 0.9% NaCl, intravenously. Four days later, the spleens were used for generating the secondary immune response *in vitro*. The spleens were isolated aseptically and pressed through a plastic screen into Hanks' medium. The cells were then centrifuged and treated for 5 min at room temperature with 0.83% ammonium chloride to lyze erythrocytes. Subsequently, the cells were washed twice with Hanks' medium and passed through cotton wool columns to remove cell debris and dead cells. Finally, the splenocytes were resuspended in a culture medium (RPMI 1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, 2-mercaptoethanol, and antibiotics) and distributed to 24-well culture plates (5×10^6 per well). The cell cultures were immunized with 50 μl of 0.001% SRBC, and the peptides were added at the dose range of 1–100 μg/ml. To control cultures, 0.1 ml of the culture medium was added. After 4 days of incubation in a cell culture incubator, the AFC numbers were determined according to Mishell and Dutton [27]. The results are presented as mean AFC numbers from four wells, calculated per 10^6 viable splenocytes ± standard deviation.

Statistics

The results were presented as mean values ± standard deviation (SD). The Levene's test was used to determine the homogeneity of variance between groups. Analysis of variance was applied to estimate the significance of the difference between groups. Significance was determined at $p \leq 0.05$. The statistical analysis was performed using STATISTICA for Windows statistical package.

Results and Discussion

We synthesized four new dimeric analogs consisting of two immunosuppressive peptide fragments of ubiquitin connected by selected derivatives of PEG as linkers (compounds **2**, **4**, **5**, and **6**), as well as two monomeric analogs with PEG attached to N-terminus or C-terminus (compounds **1** and **3**).

The immunosuppressive peptide sequences were connected in various combinations: N-terminus to N-terminus (compound **2**), C-terminus to C-terminus (compound **4**), and N-terminus to C-terminus (compounds **5** and **6**). Synthesis of all analogs was performed directly on solid support.

Previously, we used the α and ε di-oligoglycinated lysine residue ($-(Gly_{4\text{-}6})_2$–Lys-NH_2) as an effective spacer in preparation of dimeric analogs of immunosuppressive HLA fragments, utilizing C-terminus to C-terminus strategy [19]. The synthesis of the linker was performed simultaneously on the α-amino and ε-amino groups of lysine residue attached to solid support. Such dimers with oligoglycyl bridges evoked high immunosuppressive activity; however, poor solubility in physiological fluids excluded their further applicability. To overcome this problem, we selected PEG derivative to serve as a flexible linker in the C-terminus to C-terminus strategy.

The formation of dimeric analogs, where two peptide sequences were covalently linked through their N-termini (Figure 2), was performed according to procedure described by us previously [28]. The dimerization was achieved by cross-linking the N-terminal amino group of the immobilized peptides with the dicarboxylic derivative of PEG, activated by esterification with pentafluorophenol. Development of such derivative of PEG in the synthesis of peptide dimers was presented by us previously [20]. It is worth noting that gradual addition of reagent (0.5 equivalent in respect to resin loading) is required for efficient dimerization.

The second strategy of formation of dimeric analogs directly on solid support was based on linking the α-amino groups of two C-terminal lysine residues by PEG derivative (reagent **A**) and continuing the peptide synthesis on the ε-amino groups. Previously, we found that the Tyr residue in the ubiquitin$^{50\text{–}59}$ immunosuppressive LEDGRTLSDY sequence was not crucial for its immunomodulatory activity [17]. Therefore, for dimerization purposes, we replaced tyrosine by lysine residue, using orthogonally protected Fmoc-Lys(Mtt)-OH derivative.

After the removal of the Fmoc-protecting groups, the two lysine residues were linked through their α-amino groups using the di-pentafluorophenyl ester derivative of PEG (reagent **A**) (Figure 3). This dimerization procedure was similar to that presented for the N-terminus to N-terminus dimer (compound **2**) but was performed on the C-terminal amino acid residues directly attached to the resin. Next, the Mtt-protecting group from the ε-amine group of the dimerized lysine residues was removed, and the synthesis of the immunosuppressive peptide fragment of ubiquitin was continued on resin directly at the ε-amine groups of cross-linked lysine residues.

Relatively high yield of the synthesis of dimeric compounds **2** and **4** indicates that cross-linking of two peptide fragments attached to solid support can be easily performed not only on their N-terminal groups, where the amino group is located far away from the solid support, but also on α-amino groups of the residues attached directly to the resin, despite the sterical interference of the support. To the best of our knowledge, this paper describes the first cross-linking reaction between two α-amino groups of amino acids directly attached to a resin, to produce the desired dimeric products. We propose that this method could be general and may be applied for synthesis of various peptide conjugates.

To evaluate the influence of the dimerization process on the structural and biological properties of ubiquitin fragment, we synthesized two conjugates, in which the PEG derivative was attached to the N-terminal amino group of the decapeptide, to compare with N-terminal dimer **2** (compound **1**), or the α-amino group of C-terminal Lys residue, to compare with dimer **4** (compound **3**) (Figure 1). Reagent **B**, developed for this procedure, was obtained by reaction of pentafluorophenyl diester of PEG diacid with butylamine. By optimizing the reaction conditions during the synthesis of reagent **B** (see Materials and Methods), a product containing monoester–monoamide derivative (the reactive form of reagent **B**) and diamide (non-reactive form) was obtained. For synthesis of monomeric compounds, a significant excess (ten equivalents) of reagent **B** could be used, as the non-reactive component is removed during resin washing.

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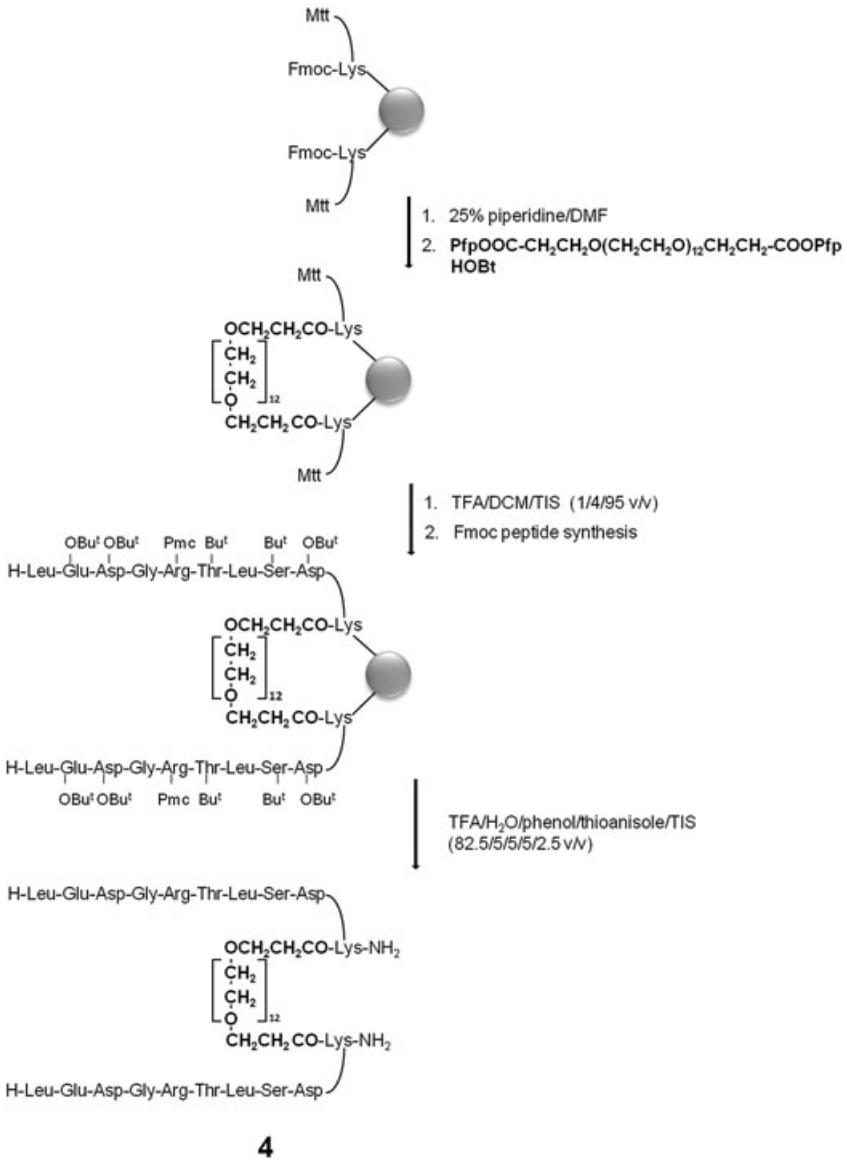

Figure 3. The synthesis procedure of compound **4**. In synthesis of monomeric analog **3**, reagent **B** was used instead of di-pentafluorophenyl ester of PEG diacid (reagent **A**). Grey balls indicate MBHA Rink Amide resin beads.

The synthesis of head-to-tail dimers (compounds **5** and **6**) was performed using commercially available Fmoc-protected PEG-amino acid residue (Fmoc-NH-$CH_2CH_2O(CH_2CH_2O)_5CH_2CH_2$-COOH), according to the standard Fmoc synthesis procedure. Two dimeric analogs were obtained by incorporation of one (compound **5**) or two (compound **6**) Fmoc-PEG-amino acid residues between two peptide chains (Figure 4).

All of the obtained dimeric analogs of the immunosuppressive peptide fragment of ubiquitin were purified by preparative RP-HPLC. The presence of Asp-Gly motif in the investigated peptides did not cause significant synthetic problems. In crude compounds **5** and **6** (21 and 22 synthetic steps, respectively, as compared with other dimers requiring only ten Fmoc protection removal steps), the presence of piperidide by-products, which could indicate the level of succinimide formation and opening during removal of Fmoc protection [29], was practically negligible according to MS analysis, and by-products containing two piperidide modifications were not detected (data not shown). The structure of the decapeptide LEDGRTLSDY was also confirmed by NMR [30]. The molecular weights and structures of the synthesized compounds were confirmed by ESI-MS and ESI-MS/MS analysis, respectively. The ESI-MS/MS spectra (one example is presented in Figure 5) contain peaks derived from typical a_n, b_n, and y_n series and some internal fragments. We found that the fragmentation occurs on peptide bonds only, but not in the PEG linker, which facilitates the interpretation of the spectrum.

The effects of the peptides on the secondary humoral immune response were analyzed by determining the number of AFC in the culture of mouse splenocytes (Figure 6). There are two effects that may be anticipated as a result of the dimerization. First, the modification may have a potential for bridging vicinal receptors, which in turn may affect the binding potency. Second, the introduction of the PEG linker may alter the chemical profile of the peptide. To separate the contributions of these two effects, we synthesized the peptide monomers pegylated on their N- or

Figure 4. The synthesis strategy of compound **5**. Compound **6** was synthesized using two PEG-amino acid linkers. Grey balls indicate MBHA Rink Amide resin.

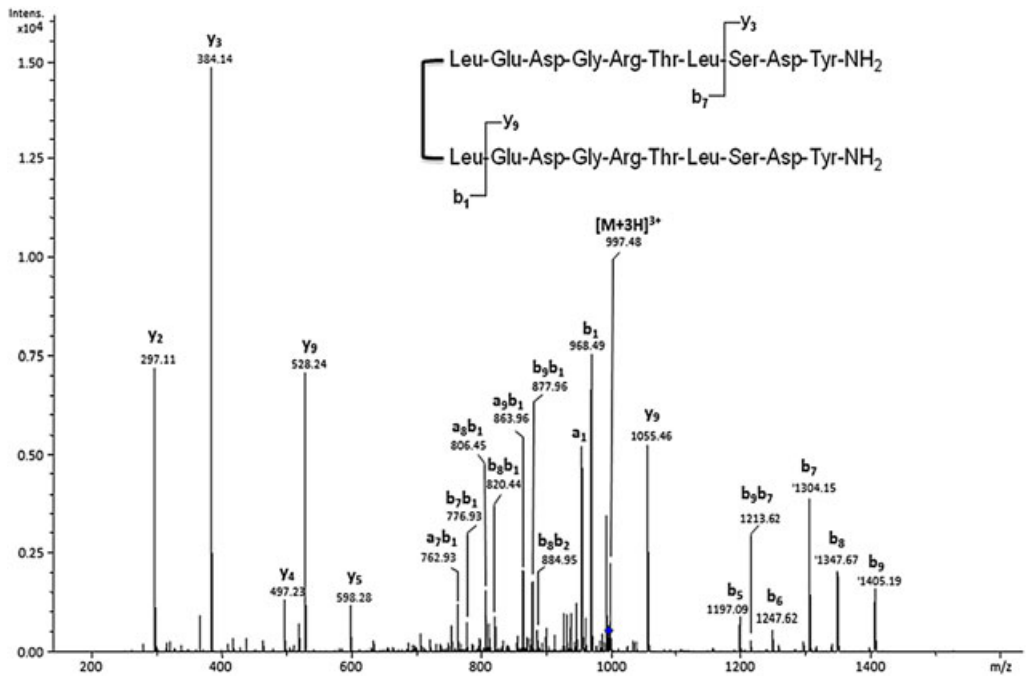

Figure 5. MS/MS spectrum of triply charged peptide **2** (parent ion *m/z* 997.43).

C-terminal amino acid residue (compounds **1** and **3**, respectively). The comparison of the immunomodulatory potencies of the ubiquitin fragment with LEDGRTLSDY-NH_2 sequence with its analogs **1** and **3** suggests that the PEG-containing chain has nearly no effect on the immunosuppressive effect of the ubiquitin fragment. However, the dimeric compounds **2**, **4**, **5**, and **6** generally exhibited higher immunosuppressive potencies than their monomeric counterparts, **1** and **3**. A significant increase in

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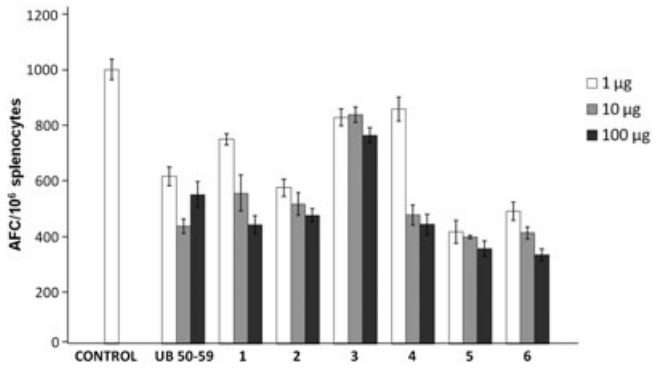

Figure 6. Effect of the synthesized peptides **1–6** on the humoral immune response. AFC numbers in the mouse spleen cell cultures of CBA/liw mice immunized with SRBC and treated *in vitro* with the peptides. The results are expressed as a mean ± SD of six wells. The results were elaborated by the analysis of variance: control *versus* all concentrations of **UB50-59**, **2**, **5**, **and 6** ($p = 0.0001$); control *versus* **1**: 1 μg/ml ($p = 0.0008$), 10 μg/ml ($p = 0.0001$), 100 μg/ml ($p = 0.0001$); control *versus* **3**: 1 μg/ml (NS), 10 μg/ml (NS), 100 μg/ml ($p = 0.0021$); control *versus* **4**: 1 μg/ml (NS), 10 μg/ml ($p = 0.0001$), 100 μg/ml ($p = 0.0001$); NS – not significant.

immunological activity in the case of the head-to-tail dimers (**5** and **6**) was observed, particularly for small concentration (1 μg/ml) of peptide **5**. When examined on molar basis, the immunological activity of dimeric compounds seems actually much higher than that of monomeric compounds **1** and **3**, as nearly the same effect is evoked by only 60% of molecules (the average formula weight of dimers is approximately 3 kDa as compared with 2 kDa of compounds **1** and **3**). However, the examined compounds in general show small changes in activity with increasing dose, and a ten times increase in concentration of the dimer does not result in a respective change of activity. The suppressive effect of ubiquitin-derived peptides *in vitro* at 100 μg/ml may be not spectacular; however, we recently showed that a ubiquitin-derived peptide prolonged allogeneic skin transplant survival in mice almost as potently as FK-506 [31].

Our earlier studies revealed that the immunomodulatory properties of the ubiquitin fragments were similar to that of intact ubiquitin [30,31]. Although mechanism of the immunosuppressive action of the ubiquitin fragments remains unknown, it may interrupt the interactions of ubiquitin with its hypothetical receptors. Because ubiquitin is known to exist in the oligomeric form, a possibility of interaction of oligoubiquitin with some oligomeric receptors cannot be excluded.

Ubiquitin has been reported to interact with numerous proteins. These interactions regulate various processes, including DNA repair, endocytosis, and vesicular trafficking [32]. Recognition is mediated by binding to different ubiquitin binding domains (reviewed by Dikic *et al.* [33]). Although the interactions of ubiquitin with ubiquitin binding domains are rather weak, the affinity is usually increased by polymerization of ubiquitin and multimerization of the ubiquitin receptor [34]. There are reports that tandem repeats of ubiquitin binding domains may also interact with variously linked ubiquitin chains [35,36].

It is known that a linker length may dramatically affect the biological potencies of bivalent bioligands [37]. The bivalent analog should therefore consist of two monomers – ubiquitin fragments – covalently linked by a spacer with a sufficient length, able to position them in the same orientation as in the ubiquitin oligomers. The polyubiquitin chains formed *in vivo* can adopt different topologies that are defined by the lysine residue involved in the formation of the isopeptide bond between ubiquitin subunits. The arrangement of the polyubiquitin chain influences its interaction with ubiquitin receptors and consequently determines its signaling role [38].

To specify the potential molecular target for the most active dimeric analog of fragment LEDGRTLSDY (compound **5**), we compared structures of diubiquitin units formed by linking specific Lys residues (Figure 7). Crystal structures are available for Lys-6-linked and Lys-48-linked dimers [21,39], whereas Lys-11-linked diubiquitin is the dimeric fragment of the ubiquitin oligomer crystal structure [39]. For Lys-63-linkage, the NMR solution structure of ubiquitin dimer interacting with yUIMs receptor was described [40].

The PEG linker utilized in the synthesis of compound **5** is 28 Å long, assuming its fully stretched conformation, which could be rarely adopted in solution. It is very unlikely that this dimeric compound could mimic the interaction of ubiquitin loops located in farther distance. Therefore, the only orientation of two LEDGRTLSDY fragments that can be mimicked by compound **5** is that of diubiquitin connected via Lys-48. Interestingly, similarity to the Lys-48 dimer can be achieved in two ways, as the distances between C- and N-termini of two fragments are 21.0 and 22.3 Å, respectively (Figure 7). Together, this suggests that dimeric compound **5**, which possesses the highest immunosuppressory activity in this study (Figure 6), may act through a signaling pathway connected with recognition of Lys-48 polyubiquitin chains, which are mainly used as signals for targeting proteins to the proteasome [41]. Compound **6** (with linker of 56 Å) also expresses high immunosuppressive effect, which can be explained by the flexibility of the PEG linker. Still, there is a possibility that Lys-6-linked, Lys-11-linked, or Lys-63-linked ubiquitin chains can adopt bent conformation, different from the observed in crystal form, in order to interact with the receptor.

We performed conformational investigations of the synthesized peptides by CD in water, to examine the possible effect of the PEG linker and dimerization on overall conformation of the studied peptides. CD spectra of investigated peptides are presented in Figure 8 as molar ellipticity per amino acid residue to compensate for different lengths of peptides.

CD spectra obtained for nonpegylated peptide LEDGRTLSDY-NH_2 and its pegylated analogs **1–6** dissolved in water (at neutral pH) were characterized by a negative band at 195 nm and a weak negative shoulder at 220 nm (Figure 8). The ellipticity of investigated peptides has the same order of magnitude; however, two conjugates – compounds **2** and **5** – were characterized by significantly lower dichroic intensity of the negative band. The spectra were typical for open conformation [42]. The similarity of the tested compound spectra in water implies that the dimerization has little effect on the stabilization of their structure in water. The unstructured conformation of the peptides in water may also suggest that the PEG linkers may easily adopt extended conformation, which enables them to situate the immunosuppressory peptides in a desired position.

We also found that addition of sodium salt does not significantly affect the CD spectra (results not shown), which indicates that possible interaction of sodium cations with polyoxyethylene chain has little or no effect on conformation of PEG-linked peptide segments.

The enhancement in the immunosuppressory activity of our dimeric analogs could be explained by microaggregation of the

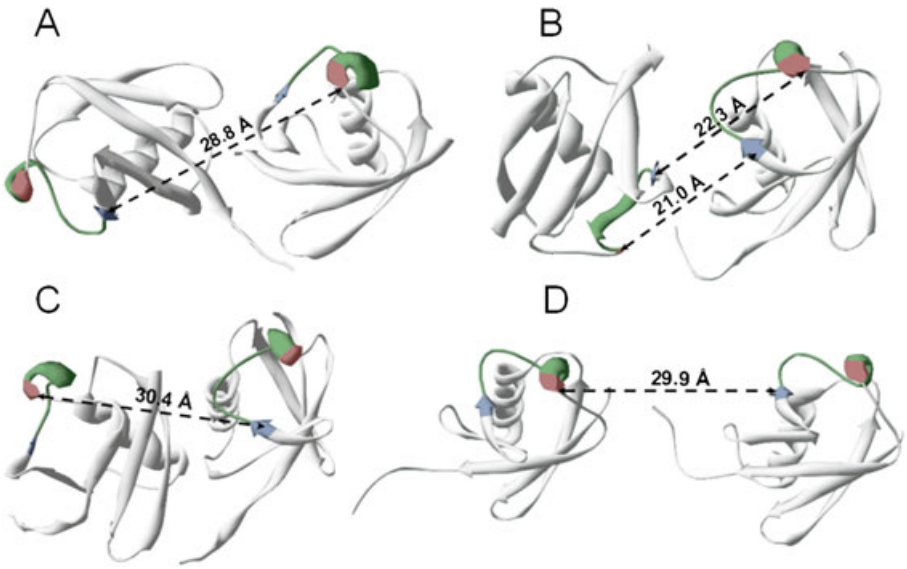

Figure 7. Orientation of immunosuppressory fragment LEDGRTLSDY in the ubiquitin dimers connected by different lysine residues. X-ray structures (Lys-6-linked (A) [39], Lys-48 (B) [21], and Lys-11 (C) [39]) and NMR solution structure (Lys-63 [40] (D)) are presented. The distances between amide nitrogen atom of the N-terminal residue (blue) of the one 50–59 fragment and carbonyl carbon atom of the C-terminal residue (red) of the second 50–59 fragment were shown.

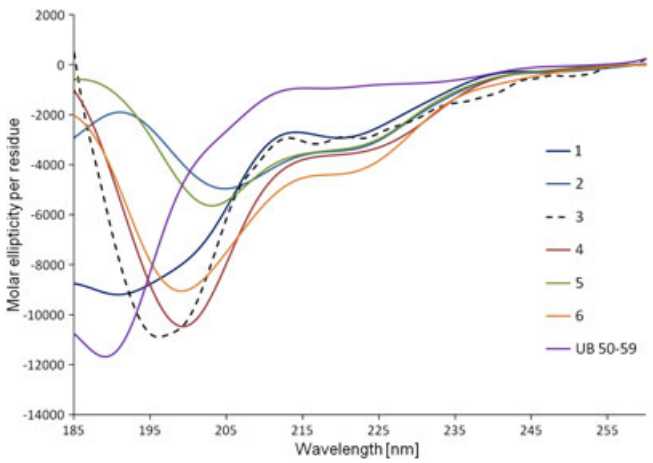

Figure 8. CD spectra of compounds **1–6** and the ubiquitin 50–59 fragment dissolved in water.

receptors at the cell surface induced by the dimeric antagonists, which would prevent the access of the proper ligands to the receptors. Nevertheless, it is known that increased affinity of dimeric analogs of the ligands may be also independent of receptor density because bivalent ligands are known to produce thermodynamically more favorable binding interaction with their receptor than the monovalent ligands [43]. Their potencies may be attributed to a higher concentration of pharmacophores in the proximity of their recognition sites by a simple statistical advantage. However, this effect may not depend on the orientation of the ligands in their dimer analog, nor the linker length. The observed dependence of the immunosuppressory potency on the dimer structure (orientation and length) supports rather a possibility that dimeric structures may be capable of bridging independent recognition sites of the receptor.

It has been previously found that N-terminal dimerizations of the immunosuppressive HLA class II fragments significantly increase the immunomodulatory effect [20]. The dimerization presented in this paper causes limited increase in immunosuppressory effect, which could be interpreted by different molecular targets for ubiquitin and HLA class II fragments.

Conclusions

We synthesized new analogs of the ubiquitin fragment, exploring all dimerization strategies: N-terminus to N-terminus, C-terminus to C-terminus, and N-terminus to C-terminus via PEG derivatives. In the course of our research, we developed a new and straightforward procedure for the direct dimerization of the C-terminal residues of peptide on a solid support using a PEG spacer. Results of the performed immunological tests indicate that the N-terminus to C-terminus dimerization enhances the immunosuppressive activity of the conjugated decapeptide fragments of ubiquitin. Taking into account that differentially linked polyubiquitin chains may be regarded as independent posttranslational modifications evoking various biological effects [44], dimeric fragments of ubiquitin could interact with the hypothetic ubiquitin receptors responsible for the immunomodulatory activity.

Acknowledgement

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