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IL FARMACO 59 (2004) 345–352
IL FARMACO
www.elsevier.com/locate/farmac

Modulation of cytokine production by some phthalimido-desmuramyl dipeptides and their cytotoxicity

Stanislav Gobec [a,*], Marija Sollner-Dolenc [a], Uroš Urleb [a,b], Branka Wraber [c], Saša Simčič [c], Metka Filipič [d]

[a] *Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia*
[b] *Lek d.d., Verovškova 57, 1000 Ljubljana, Slovenia*
[c] *Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia*
[d] *Department of Genetic Toxicology and Tumor Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia*

Received 12 December 2003; accepted 12 January 2004

Abstract

Muramyl dipeptide (MDP) is the smallest bacterial cell wall peptidoglycan component having immunomodulatory activity. In an attempt to obtain MDP derivatives with improved and better defined pharmacological profiles we synthesized a new lipophilic phthalimido-desmuramyl dipeptide, LK 508. This novel MDP analogue and three structurally related phthalimido-desmuramyl dipeptides (LK 413, LK 511 and LK 512) were evaluated immunologically. Their ability to modulate the production of cytokines was measured *in vitro* by their inclusion in cultures of human peripheral blood mononuclear cells (PBMC) activated by ionomycin and phorbol-12-myristate-13-acetate (PMA). The results were compared with the analogous activity of MDP. All compounds tested are strong up-regulators of IL-12 synthesis. All compounds except LK 512 also stimulated IFNγ synthesis. LK 508, LK 511 and LK 512 are effective in up-regulating IL-2 production. LK 508 and LK 512 considerably up-regulate the synthesis of IL-4 and IL-10. LK 413 and MDP stimulated the production of Th1 promoting and Th1 (IFNγ and IL-12) cytokines, while LK 508, LK 511 and LK 512 non-selectively up-regulated the production of both Th1 and Th2-types of (IL-4 and IL-10) cytokines. None of the phthalimido-desmuramyl dipeptides was cytotoxic *in vitro* against the normal cell line HUVEC (human endothelial cells) thereby indicating their potential for use *in vivo*.

Keywords: Muramyl dipeptide analogues; Phthalimido-desmuramyldipeptides; Synthesis; Cytokines; Cytotoxic activity

1. Introduction

N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP, Figure 1) has been known since 1974 as the minimal fragment of the *Mycobacterium* cell wall which still retains most of the biological activity, in particular the immunoadjuvant activity [1]. MDP also induces side effects, such as pyrogenic and somnogenic activity, induction of autoimmune responses, and inflammatory reactions [2-4]. In order to obtain molecules with improved and more specific pharmacological profiles, many MDP derivatives and analogues have been designed, synthesized and evaluated biologically [5]. Recently, a lipophilic MDP derivative N^2-[*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-stearoyl-L-lysine (romurtide) was marketed for the treatment of radiotherapy-induced leukopenia [6,7]. Most of the MDP analogues known so far possess an intact dipeptide moiety, L-Ala-D-Glu-NH_2 or L-Ala-D-Glu. On the other hand, it has been generally accepted that the *N*-acetyl-D-glucosamine fragment is not essential for the immunomodulating activity of this class of compounds [5,8-10]. Its derivatization or replacement with various acyl groups is thus an important approach in the design and synthesis of new immunologically active MDP analogues, as demonstrated by FK-156 [11], pimelautide [12], 7-(oxoacyl)-L-alanyl-D-isoglutamines [13], some carbocyclic MDP analogues [5,14], and by the adamantyl-desmuramyl dipeptides [15].

In the search for a suitable *N*-acetylmuramyl surrogate we focused our attention on phthalimido substituted alkanoic

* Corresponding author.
E-mail address: gobecs@ffa.uni-lj.si (S. Gobec).

doi:10.1016/j.farmac.2004.01.003

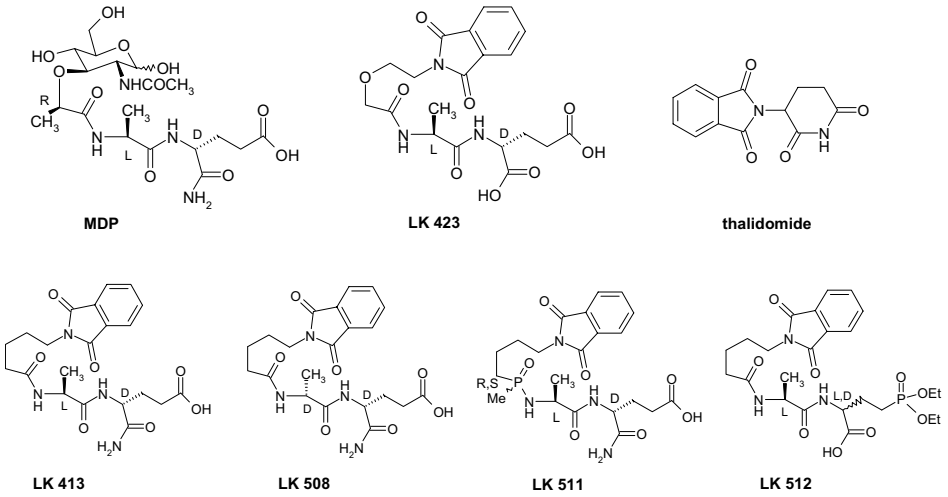


Fig. 1.

acids. The phthalimide containing sedative thalidomide (*N*-phthaloglutarimide, Figure 1) was shown to have immunosuppressive activity [16,17]. It also reduces agonist-induced tumour necrosis factor (TNF) production by accelerating the decay of TNF mRNA [18]. Further investigations have revealed that thalidomide can both enhance and inhibit TNF production, depending on the cell type and the TNF production inducer [19,20]. Certain other compounds bearing a substituted phthalimide fragment can also influence the immune system. Immunosupressive activities were observed for the *N*-phthaloylated derivative of 2-amino-4-chlorophenylthialzole-5-acetic acid [21] and phthalimido substituted 1-aziridinecarboxylic acid derivatives [22]. We postulated that connecting the phthalimide ring to the essential dipeptide part of MDP (L-Ala-D-Glu-NH_2 or its bioisosteres) could produce new immunomodulators with improved pharmacological properties [23]. The most promising compound in the first series of phthalimido-desmuramyl dipeptides was LK 423 (Figure 1), which exhibited very interesting activities in different immunological models. It was found to augment the capacity to produce interleukin-10 in the spleen cells of cyclophosphamide-treated mice [24], and it alleviated dextran sulfate sodium-induced colitis in rodents [25]. LK 423 is thus a candidate substance for development as an anti-inflammatory pharmaceutical agent [25]. The compound was also able to stimulate the production of TNF in *in vitro* phorbol 12-myristate 13-acetate (PMA) and ionomycin-stimulated cultures of human peripheral blood mononuclear cells [26]. To obtain more information about structure-activity relationships, we recently synthesized some phthalimido-desmuramyl dipeptides related to LK 423, where the lead compound was modified bioisosterically at various positions (side chain, peptide bond, terminal carboxyl group) [27-30].

Immunological activities of muramyl dipeptides and related compounds appear to depend on the stimulation or inhibition of the biosynthesis of many cytokines [31]. The production of cytokines is a key event in the activation and effector phases of innate and specific immunity and serves to mediate and regulate these responses. So far, several experimental models have addressed the influence of MDP and some analogues on the production of cytokines [26,32-34]. In an effort to define further the effect of compounds of the MDP series, we have determined the ability of four phthalimido-desmuramyldipeptides, LK 413, LK 508, LK 511, and LK 512 (Figure 1), to modulate cytokine production. IL-12, TNFα, IFNγ, IL-2, IL-4, and IL-10 were assayed in *in vitro* ionomycin and PMA polyclonally activated human peripheral blood mononuclear cell (PBMC) cultures. Taking into account its profound immunomodulatory potential, MDP was used as a comparative substance.

Although MDP has low anticancer activity, some MDP derivatives have proved to be interesting as potential anticancer agents. Acylation of MDP with mycolic acids [35], hydroxy fatty acids [36], and quinonylalkanoic acids [37] resulted in compounds with antitumor activity. Dzierzbicka and coworkers have recently synthesized conjugates of MDP and nor-MDP with the strong anticancer agents acridine, hydroxyacridine, and acridone and shown them to be both immunologically active, and to exhibit potent *in vitro* cytotoxic activity against a panel of human tumour cell lines [38,39]. Conjugates of MDP and nor-MDP with batracylin were not cytotoxic, but some of them reduced proliferation of Ab melanoma cells *in vitro* [40]. Recently, we demonstrated weak cytotoxicity of a series of adamantyl-desmuramyl-dipeptides [41]. In addition, many compounds with the phthalimide motif were either cytotoxic against the growth of human cancer cultured cell lines [42-45] or active in *in vivo* antitumor assays [46].

In this paper we present the results of both *in vitro* immunological evaluation (modulation of cytokine production) and determination of cytotoxicity of a series of phthalimido-desmuramyl dipeptides (Figure 1). The synthesis of one new MDP analogue (LK 508) is also presented.

2. Experimental

2.1. Chemistry

LK 413, LK 511 and LK 512 were synthesized as described previously [23,28,29]. MDP was supplied by Sigma. All reagents and solvents were of commercial grade and used as such. Melting points were determined using a Reichert hot stage microscope and are uncorrected. Optical rotation was measured on a Perkin-Elmer 1241 MC polarimeter. Elemental C, H, N analyses were performed at the Faculty of Chemistry and Chemical Engineering, University of Ljubljana, on a Perkin-Elmer elemental analyzer 240 C. IR spectra were measured by a Perkin-Elmer FTIR 1600 instrument on KBr pelleted samples. Mass spectra were obtained by a Micromass AutospecQ mass spectrometer using FAB ionization. NMR spectra were obtained on a Bruker Avance DPX 300 instrument. ^{1}H NMR was done at 300.13 MHz with tetramethylsilane as an internal standard.

2.2. Synthesis of LK 508

2.2.1. N-(5-Phthalimidopentanoyl)-D-alanine benzyl ester (*1*)

To a stirred solution of 0.741 g (3 mmol) 5-phthalimidopentanoic acid [47] and 1,05 g (3 mmol) D-alanine benzyl ester p-toluene sulfonate in 10 mL DMF, 1 g (3,6 mmol) diphenyphosphoryl azide (DPPA) and 0,83 mL (6 mmol) triethylamine were added at 0° C. The reaction mixture was stirred at 0° C for 3 h and then at room temperature for 48 h. After the usual work-up procedure (addition of 80 mL ethyl acetate and extraction with 5 % citric acid (3 x 15 mL), saturated solution of $NaHCO_3$ (3 x 15 mL) and water (3 x 15 mL)), recrystallization from acetone yielded 0.82 g (67 %) of pure compound **1**. M.p. 111° C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ= 1.26 (d, 3H, J= 7.5 Hz, CH_3), 1.40-1.65 (m, 4H, 2 CH_2), 2.13 (t, 2H, J= 6.8 Hz, $\underline{CH_2}$CONH), 3.55 (t, 2H, J=6.8 Hz, CH_2N), 4.20-4.35 (m, 1H, CH), 5.09 (s, 2H, $\underline{CH_2}$Ph), 7.25-7.45 (m, 5H, Ph), 7.80-7.95 (m, 4H, phthaloyl), 8.24 (d, 1H, J= 7.15 Hz, NH) ppm. MS(FAB): 409 $(M+H)^+$. $[a]_D^{20}$ = 24.9° (0.39; methanol). $C_{23}H_{24}N_2O_5$ (408.4) Calcd. C 67.63 H 5.92 N 6.86 Found C 67.93 H 6.06 N 6.85.

2.2.2. N-(5-Phthalimidopentanoyl)-D-alanine (*2*)

0.6 g (1.47 mmol) compound **1** was dissolved in 25 mL glacial acid. The solution was then treated with 60 mg 10% palladium/carbon and hydrogen for 3 h. The catalyst was removed and the filtrate evaporated. The yield was 406 mg (87 %). M.p. 142° C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ= 1.26 (d, 3H, J= 7.2 Hz, CH_3), 1.45-1.70 (m, 4H, 2 CH_2), 2.13 (t, 2H, J= 6.8 Hz, $\underline{CH_2}$CONH), 3.56 (t, 2H, J=6.8 Hz, CH_2N), 4.10-4.25 (m, 1H, CH), 5.09 (s, 2H, $\underline{CH_2}$Ph), 7.75-7.95 (m, 4H, phthaloyl), 8.06 (d, 1H, J= 7.54 Hz, NH) ppm. MS(FAB): 319 $(M+H)^+$. $[\alpha]_D^{20}$ =14.3° (0.37; methanol). $C_{16}H_{18}N_2O_5$ (318) Calcd. C 60.37 H 5.70 N 8.80 Found C 59.96 H 5.69 N 8.70.

2.2.3. Benzyl N-(5-phthalimidopentanoyl)-D-alanyl-D-isoglutaminate (*3*)

To a stirred solution of 477 mg (1.5 mmol) compound **2** and 409 mg (1.5 mmol) D-isoglutamine benzyl ester hydrochloride in 10 mL DMF, 495 mg (1,8 mmol) diphenyphosphoryl azide (DPPA) and 0,42 mL (3 mmol) triethylamine were added at 0° C. The reaction mixture was stirred at 0° C for 3 h and then at room temperature for 48 h. After the usual work-up procedure (addition of 80 mL ethyl acetate and extraction with 5 % citric acid (3 x 10 mL), saturated solution of $NaHCO_3$ (3 x 10 mL) and water (3 x 10 mL)), recrystallization from DMF/ether yielded 0.6 g (75 %) of pure compound **3**, m.p. 194-196° C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ= 1.17 (d, 3H, J= 7.14 Hz, CH_3), 1.40-1.60 (m, 4H, 2 CH_2), 1.70-1.85 in 1.90-2.05 (2m, 1 H each, β- CH_2-iGln), 2.14 (t, 2H, J= 6.8 Hz, $\underline{CH_2}$CONH), 2.34 (t, 2H, J= 7.5 Hz, CH_2) 3.55 (t, 2H, J=7.5 Hz, CH_2N), 4.15-4.25 (m, 2H, CH-Ala, CH-iGln), 5.06 (s, 2H, $\underline{CH_2}$Ph), 7.04 and 7.27 (2s, 1H each, NH_2), 7.30-7.40 (m, 5H, Ph), 7.80-7.90 (m, 5H, phthaloyl, NH), 8.03 (d, 1H, J= 6.8 Hz, NH) ppm. MS(FAB): 537 $(M+H)^+$. $[\alpha]_D^{20}$ = 15.6° (0.05; methanol). $C_{28}H_{32}N_4O_7$ (536.6) Calcd. C 62.68 H 6.01 N 10.44 Found C 62.25 H 6.07 N 10.57.

2.2.4. N-(5-phthalimidopentanoyl)-D-alanyl-D-isoglutamine (*LK-508*)

90 mg (0.17 mmol) compound **3** was dissolved in 20 mL glacial acid. The solution was then treated with 50 mg 10% palladium/carbon and hydrogen for 45 min. The catalyst was removed and the filtrate evaporated. The yield was 60 mg (79 %). M.p. 200-203° C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ= 1.18 (d, 3H, J= 7.16 Hz, CH_3), 1.40-1.65 (m, 4H, 2 CH_2), 1.65-1.85 in 1.85-2.00 (2m, 1H each, β-CH_2-iGln), 2.10-2.23 (m, 4H, 2 CH_2) 3.58 (t, 2H, J= 6.8 Hz, CH_2N), 4.05-4.30 (m, 2H, CH-Ala, CH-iGln), 7.00 and 7.23 (2s, 1H each, NH_2), 7.80-8.00 (m, 5H, phthaloyl, NH), 8.04 (d, 1H, J= 6.8 Hz, NH) ppm. MS(FAB): 447 $(M+H)^+$. $[\alpha]_D^{20}$ = 33.3° (0.3; methanol). $C_{21}H_{26}N_4O_7$ (446) Calcd. C 56.50 H 5.83 N 12.56 Found C 56.68 H 6.05 N 12.60.

2.3. Biological evaluation

2.3.1. In vitro cytotoxicity

Cytotoxicity of tested compounds was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to Mosmann [48], with minor modifications. This assay measures the conversion of MTT to insoluble formazan by mitochondrial dehydrogenase enzymes of living cells. The *in vitro* cytotoxicity was evaluated against three human tumour cell lines- MDA-MB231 (breast cancer), 103H (large cell lung cancer) and HepG2 (human hepatoma) and normal cell line HUVEC (human endothelial cells).

MDA-MB-231 cells were cultivated in L-15 (Sigma, St. Louis, USA) medium, 103H cells in RPMI 1640 (Sigma, St. Louis, USA), HepG2 cells in William's medium E (Sigma, St. Louis, USA) and HUVEC in EMEM with nonessential

amino acids (Sigma, St. Louis, USA). All the media were supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. The cells were incubated 37°C in humidified atmosphere with 5% CO_2.

Cells were seeded in 200 μL of growth media in Corning 96-well microtiter plates at 25,000 cells per well (MDA-MB231), 15,000 cells per well (103H) or 20,000 cells per well (HepG2 and HUVEC). On the next day the medium was replaced by complete growth medium containing 10^{-4} M test compound and the cells incubated further for 24 hours (control cells were incubated in complete medium containing 0.1% DMSO). The cells were then washed twice with PBS and the medium was replaced by fresh complete growth medium containing MTT (final concentration 0.5 mg/ml). After 3 h the medium was removed and the resulting formazan crystals dissolved in DMSO. The absorbance (A) of each well was measured at 570 nm (reference filter 690 nm) using an ELISA microplate reader. Survival (viability) was determined by comparing absorbance in wells containing treated cells with that of untreated cells. Six replicates were measured for each concentration.

2.3.2. Modulation of cytokine production

The desmuramyldipeptide compounds, LK 413, LK 508, LK 511 and LK 512, and MDP used for *in vitro* stimulation of human PBMC were dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) and further diluted in RPMI 1640 (Sigma, USA) supplemented as described below, so that the final concentration of DMSO did not exceed 0,1 %.

Human PBMC from buffy coat of two healthy blood donors were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden). Cells were cultured in RPMI 1640 supplemented with 100 U/mL penicillin (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA), 2 mM L-glutamine (Sigma, USA), 20 mM Hepes (Sigma, USA) and 10 % heat-inactivated AB normal human serum (Sigma, USA) for TNFα production or 5% heat-inactivated foetal calf serum (Sigma, USA) for the production of other cytokines. 1 x 10^6 cells (final culture volume 1,5 mL) were plated in 24-well culture plates (Nunc, Denmark) with either a combination of a test compound (final concentration in cell cultures was 11 μM) with ionomycin (500 nM) and PMA (3,33 ng/mL) or medium alone, at 37°C, and cell-free supernatants were collected at 40 hours and stored at –70°C before being evaluated for 6 cytokines.

The concentration of cytokines (pg/mL) was measured by commercially available ELISA kits, TNFα from Innogenetics (Belgium), and IL-2, IL-4, IL-10, IL-12 and IFNγ from Endogen (USA). The ability to modulate cytokine synthesis by a test substance was evaluated as an index according to the ionomycin and PMA induced cytokine production (Stimulation/suppression index = [cytokine concentration induced by ionomycin + PMA)/ (cytokine concentration induced by ionomycin + PMA + test substance]). Cytokine concentration indices below 0.8 and above 1.2 are considered as suppression and stimulation indices respectively.) [26].

3. Results and discussion

3.1. Synthesis

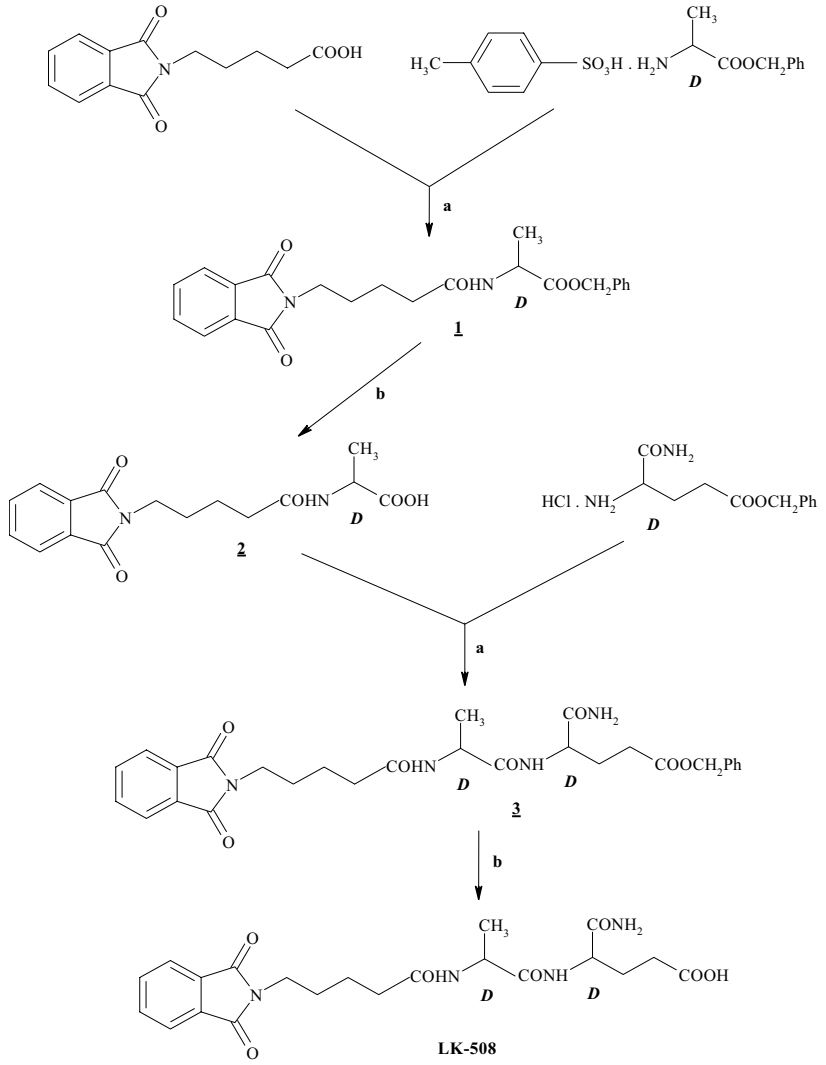
The synthesis of LK 508 is presented in Scheme 1. Starting from carboxyl-protected D-alanine and 5-phthalimidopentanoic acid, *N*-(5-phthalimidopentanoyl)-D-alanine benzyl ester (**1**) was obtained after activation of the carboxylic group with diphenylphosphoryl azide (DPPA). Hydrogenolysis of compound **1** gave *N*-(5-phthalimidopentanoyl)-D-alanine (**2**) which was then coupled with D-isoglutamine benzyl ester hydrochloride. The DPPA-coupling method was very useful in both coupling reactions, giving satisfactory yields and no undesired side products. Finally, the benzyl protecting group of compound **3** was removed by catalytic hydrogenation over Pd/C to give the target compound **LK 508**.

3.2. Cytotoxicity

The *in vitro* cytotoxicity of phthalimido-desmuramyl-dipeptides (LK 413, LK 508, LK 511 and LK 512) was evaluated against three human tumour cell lines, MDA-MB 231 (breast cancer), 103H (large cell lung cancer) and Hep G2 (hepatoma). 100 μM LK 413 reduced the viability of 103H cells by 21% and 100 μM LK 511 reduced the viability of MDA-MB 231 and HepG2 cells by 19 and 15%, respectively. To further evaluate the therapeutic potential and safety of a series of phthalimido-desmuramyl dipeptides, the cytotoxicity against normal cell line HUVEC (human endothelial cells) was determined. None of the compounds at concentration 100 μM reduced the viability of HUVEC cells, indicating that the compounds are not cytotoxic to non-tumour cells. The modest selective cytotoxicity of LK 413 against tumour cells 103H, and of LK 511 against MDA-MB 231 and HepG2 does not qualify this compound for consideration for antitumour therapeutic use.

3.3. Modulation of cytokine production

The immunomodulating activity of test substances LK 413, LK 508, LK 511 and LK 512 was evaluated in terms of their ability to modify IL-12, TNFα, IFNγ, IL-2, IL-4, and IL-10 synthesis, induced in human PBMC cultures by ionomycin and PMA. PBMC were isolated from buffy coat (donor 1). Following ionomycin and PMA stimulation with the test substance added, supernatants were screened for the content of the 6 different cytokines. The levels of each cytokine in turn obtained on incubating PBMC with ionomycin and PMA in the presence and absence of the test substances were compared. In addition, the response to MDP, a well established immunomodulating substance, in the same system, was measured with PBMC from another buffy coat (donor 2) (data published previously [15]). In both experiments the baseline production of cytokines was measured in



Scheme 1. a) DPPA, Et_3N, DMF; b) H_2, Pd/C(10 %), CH_3COOH

non-stimulated PBMC cultures, with added RPMI 1640 only. The cytokine concentrations are displayed in Table 1 and 2. Stimulation/suppression indices were calculated (Table 3) to evaluate the immunomodulating activity of the substances, as described previously [26], and to take into account the different cytokine concentrations produced in PBMC cultures from healthy blood donors [49].

All substances tested are strong up-regulators of IL-12 synthesis (stimulation indices 1.74 to 1.98, Table 3). It is well known that the production of IL-12 by macrophages is induced by various bacteria, bacterial products, intracellular pathogens, and viruses. A major biological effect of IL-12 is regulation of adaptive immunity, especially a strong promotion of Th1 responses [50,51]. IL-12 induces the production of IFNγ, which stimulates the microbial killing activity of macrophages. IFNγ also stimulates macrophages, NK cells and CD8+ T cells to produce higher levels of IL-12, constituting a powerful positive feedback mechanism [50,51]. In our experiment the up-regulation of IL-12 is followed by increased IFNγ synthesis when PBMC are incubated with LK 413, LK 508, LK 511 and MDP. This is not the case with LK 512, a phthalimido-desmuramyl dipeptide where MDP's *N*-acetylmuramic acid has been replaced by the more lipophilic 5-phthalimidopentanoyl fragment and D-isoglutamine has been replaced by racemic 2-amino-4-(diethoxyphosphoryl)butanoic acid. Here the up-regulation of IL-12 is not followed by increased IFNγ production.

IFNγ is a well-defined inducer of TNFα synthesis. In the presence of LK 508, LK 511 and MDP, the synthesis of TNFα was also stimulated. However, LK 413 shows a tendency to downregulate TNFα secretion, without altering the ionomycin/PMA-induced augmentation of IL-12 and IFNγ production. LK 413 is a lipophilic MDP analogue where the L-Ala-D-Glu-NH_2 dipeptide part is *N*-acylated with 5-phthalimidopentanoic acid. We observed similar cytokine inducing properties in the case of the adamantane substituted desmuramyl dipeptide LK 517, which also caused TNFα suppression despite augmented IL-12 and IFNγ production [15].

LK 511 is a lipophilic phthalimido desmuramyldipeptide derived from LK 413, where the amide bond between the dipeptide part and the 5-phthalimidopentanoyl side chain has

Table 1
Concentrations of cytokines (pg/ml) in culture supernatants of human PBMC activated by ionomycin and PMA in the presence and absence of LK 413, LK 508, LK 511 and LK 512, together with the baseline production of cytokines in non-stimulated culture (donor 1)

Cytokine	Non-stimulated culture (RPMI 1640)	Ionomycin/PMA	Ionomycin/PMA + LK 413	Ionomycin/PMA + LK 508	Ionomycin/PMA + LK 511	Ionomycin/PMA + LK 512
IL-12	10.7	93.9	159	161	177	155
TNFα[a]	<3.00	3220	1865	2554	4042	1908
IFNγ	<1.00	2116	3456	3389	3406	2418
IL-2	<1.00	1449	1705	2162	2270	1911
IL-4	<1.00	15.7	18.4	21.6	22.3	17.3
IL-10	3.34	39.6	47.0	60.2	65.7	45.6

[a] From reference [26].

Table 2
Concentrations of cytokines (pg/ml) in culture supernatants of human PBMC activated by ionomycin and PMA in the presence and absence of MDP, together with the baseline production of cytokines in non-stimulated culture (donor 2) [15]

Cytokine	Non-stimulated culture (RPMI 1640)	Ionomycin/PMA	Ionomycin/PMA + MDP
IL-12	1.16	117	207
TNFα	5.90	2116	2838
IFNγ	<1.00	10940	15255
IL-2	<1.00	10480	9965
IL-4	5.56	16.4	14.4
IL-10	7.00	62.0	59.9

Table 3
Immunomodulating effects of LK 413, LK 508, LK 511 and LK 512 compared to MDP on cytokine production in ionomycin and PMA activated human PBMC cultures. Results are expressed in stimulation/suppression indices[a]

Cytokine	Stimulation/supression indices				
	Ionomycin/PMA + LK 413	Ionomycin/PMA + LK 508	Ionomycin/PMA+ LK 511	Ionomycin/PMA+ LK 512	Ionomycin/PMA+ MDP
IL-12	1.78	1.80	1.98	1.74	1.77
TNFα	0.90	1.24	1.96	0.93	1.34
IFNγ	1.63	1.60	1.60	1.14	1.39
IL-2	1.18	1.49	1.57	1.32	0.95
IL-4	1.17	1.38	1.42	1.10	0.85
IL-10	1.19	1.52	1.66	1.15	0.97

[a] Stimulation/suppression index = (cytokine concentration induced by ionomycin + PMA)/ (cytokine concentration induced by ionomycin + PMA + test substance). Cytokine concentration indices below 0.8 and above 1.2 are considered as suppression and stimulation indices respectively [26].

been replaced by phosphinamide isostere. In order to obtain more data about structure-activity relationships we also prepared LK 508, a diastereomer of LK 413, in which L-alanine is replaced by D-alanine. Both LK 508 and LK 511 were highly active in stimulating IL-4 and IL-10 production in T-cells. The main physiological function of Th2 cytokine IL-4 is as a regulator of IgE- and mast cell/eosinophil-mediated immune reactions [51]. IL-10 is an inhibitor of both innate and T cell-mediated specific immune inflammation due to inhibition of cytokine production by macrophages and the inhibition of the accessory function of macrophages in T cell activation [51-53]. Although IL-10 is a well known inhibitor of TNFα and IL-12 production, in the case of compounds LK 508 and LK 511 high concentrations of IL-10 did not inhibit the synthesis of TNFα and IL-12.

IL-2 is the major autocrine and paracrine growth factor for T lymphocytes, which also stimulates NK cells and B cells [51,53]. We found that the synthesis of IL-2 is not significantly affected when PMBC are incubated with LK 413 or MDP. On the other hand, other phthalimido-desmuramyldipeptides tested were highly active in this compartment with LK 511 being the most active (stimulation index 1.57). The introduction of the phosphinamide moiety into phthalimido-desmuramyldipeptide analogues thus resulted in compound with the greatest stimulation of IL-2 production in T-cells.

Cytokines play a critical role in regulating the development of naïve $CD4^+$ T cells into either T helper 1 (Th1) cells or Th2 cells. The former produce IFNγ exclusively and favour cell-mediated responses, macrophage activation, and the production of opsonizing antibodies. The latter produce IL-4, IL-5, IL-13 exclusively, together with a more pronounced production of IL-10, and favour humoral immunity and allergic responses [51-53]. From the cytokine stimulation/suppression pattern exhibited by the compounds investigated here, we can assume that LK 413 and MDP could shift the immune system towards the production of Th1 and Th1 promoting cytokines (strong stimulation of IL-12 and IFNγ production, weak stimulation or even no stimulation of IL-4 and IL-10 production). Other phthalimido-desmuramyldipeptides (LK 508, LK 511 and LK 512) non-

selectively stimulated the production of both Th1 and Th2-types of cytokines.

4. Conclusion

The structurally related phthalimido-desmuramyl dipeptides investigated here were able to modulate the production of cytokines *in vitro*. Small variations in chemical structure of the compounds resulted in different stimulation/supression patterns for cytokine synthesis. LK 413 and MDP could shift the immune system towards the production of Th1 promoting and Th1 cytokines. LK 508, LK 511 and LK 512 non-selectively stimulated the production of both Th1 and Th2-types of cytokines. None of the phthalimido-desmuramyl dipeptides was cytotoxic *in vitro* against the normal cell line HUVEC (human endothelial cells) thereby indicating their potential for use *in vivo*.

Acknowledgements

The authors thank Dr. Roger Pain (Jožef Stefan Institute, Ljubljana, Slovenia) for critical reading of the manuscript. Financial support of this work by the Ministry of Education, Science and Sport of the Republic of Slovenia is gratefully acknowledged.

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