

Synthesis and Immunomodulation of Human Lymphocyte Proliferation and Cytokine (Interferon- γ) Production of Four Novel Malonitridamides

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Leflunomide is an immunomodulator drug with applications in the management of arthritis rheumatoid. In this study, four novel analogs (4a–d) of A771726, the active metabolite of leflunomide were synthesized and examined *in vitro* for their immunomodulation activity by examining human lymphocyte proliferation and determination of the cytokine interferon- γ concentrations in human lymphocyte cell culture. For this purpose, 5×10^4 human lymphocyte cells were incubated at 37 °C in 5% CO₂ with phytohemagglutinin and one of the analogs (concentrations 1–100 mM), negative controls or cyclosporine (0.1 mM). Effects of the compounds on lymphocyte proliferation and interferon- γ production were determined by MTT assay and enzyme-linked immunosorbent assay, respectively. Our results showed that all four compounds dose-dependently suppressed lymphocyte proliferation. Moreover, these compounds at some concentrations reduced interferon- γ production which is an indicator of the immune response. Generally, the most potent analog was 4b with an amide linkage (X=NH) and the weakest analog was 4a with an ester linkage (X=O). Compound 4a has little similarity with the leflunomide active metabolite which has an amide linkage. In this study, four novel compounds were synthesized that showed considerable immunosuppressive effects that deserve further investigations.

Key words: immunomodulation, interferon- γ , leflunomide, malonitridamides, MTT assay, synthesis

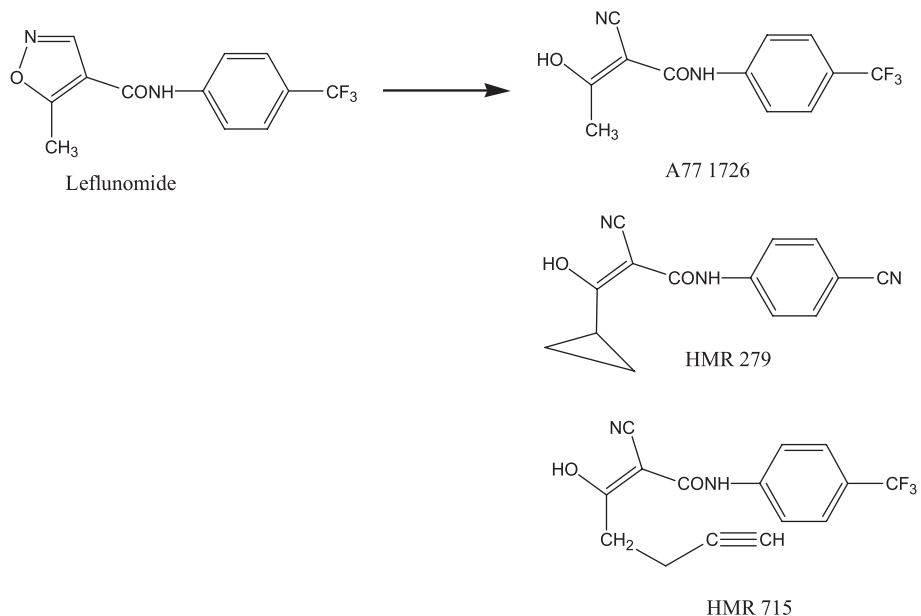
Received 21 December 2008, revised 3 March 2009 and accepted for publication 19 March 2009

Search for new ways to modulate the immune system is a vital and ongoing venture with important potential applications in the pharmacology and pharmacotherapy of several diseases. Malonitridamides are a novel class of immunosuppressive drugs effective in various experimental models of autoimmune disease and allo- or xenotransplantation. Leflunomide (LFM), the lead compound of this new group of therapeutic agents is an immunomodulatory drug-inhibiting dihydroorotate dehydrogenase which is an enzyme involved in *de novo* pyrimidine synthesis (1,2). Genuine antiproliferative activity of LFM has been strongly documented (2). Additionally, several experimental models (both *in vivo* and *in vitro*) have demonstrated an anti-inflammatory effect (3). This double action is supposed to slow progression of the disease and to cause remission/relief of symptoms of rheumatoid arthritis and psoriatic arthritis such as joint tenderness and decreased joint and general mobility in human patients. Leflunomide has been assigned orphan drug status for the prevention of solid organ rejection after allograft transplantations when co-administered with commonly used first line agents (4).

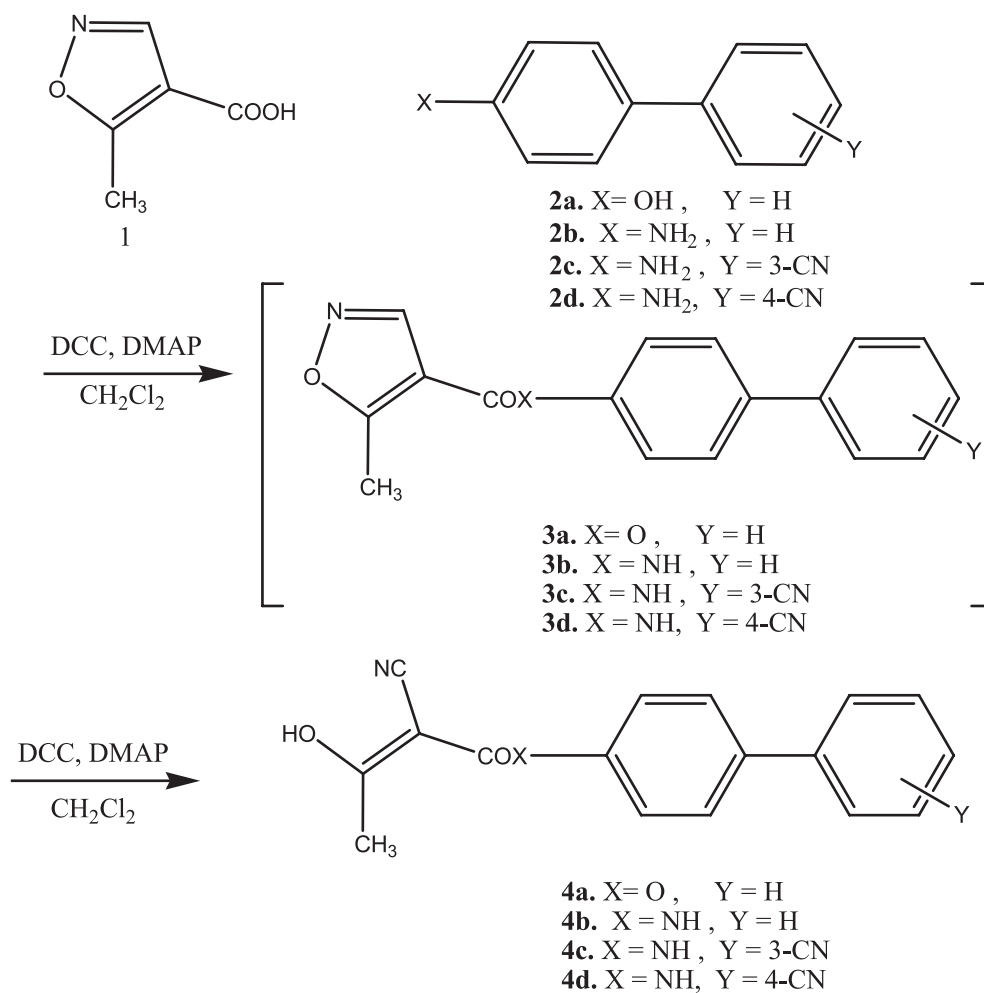
The immunomodulatory activity of LFM is attributed to its primary active metabolite, A771726 (5). Malonitridamide analogs of A771726, HMR279 (6) and HMR715 (7), have previously been synthesized and are currently in development for the pharmacotherapy of organ transplantation (8,9).

In this work, four novel analogs of A771726 (4a–d) in which 4-trifluoromethylphenyl has been replaced with the bisphenyl moiety in A771726 were synthesized and examined for their immunosuppressant activity through lymphocyte proliferation inhibition assay and determination of interferon gamma (IFN- γ) concentrations in human lymphocyte cell culture.

5-Methylisoxazole-4-carboxylic acid (**1**) was reacted with bisphenyl (2a–d) in dichloromethane (10) in the presence of dicyclohexyl carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to give intermediate **3a–d** which interestingly were converted *in situ* to *N* or



Structure of leflunomide and its active metabolite A771726 and its analogues HMR279 and HMR715



Scheme 1: Synthetic method of compounds 4a–d.

O-(biphenyl-4-yl)-2-cyano-3-hydroxycrotonate (**4a–d**). At a similar reaction in basic medium, LFM is converted to A771726 (11). Compounds **4a–d** are structurally similar to A771726. All the compounds were identified by spectroscopic methods including NMR and IR (Scheme 1).

Material and Methods

Chemical synthesis

Melting points were determined on Electrothermal Capillary apparatus and are uncorrected. The IR spectra were obtained using a Perkin-Elmer Model 1000 (Perkin-Elmer, Waltham, MA, USA). ¹H NMR was obtained on Bruker Ac-80 spectrophotometer (Bruker, Ettlingen, Germany) and chemical shifts (δ) are in ppm relative to internal tetramethylsilane. Errors of elemental analyses were within $\pm 0.4\%$ of theoretical values. Compound **1** was prepared as described previously (12).

Preparation of *O*-(biphenyl-4-yl)-2-cyano-3-hydroxycrotonate (**4a**)

Under a nitrogen atmosphere, a 250 mL, oven-dried, round-bottomed flask containing anhydrous dichloromethane (50 mL) is charged with 5-methylisoxazole-4-carboxylic acid (**1**, 0.51 g, 4 mm), bisphenol (**2a**, 0.68 g, 4 mm), DCC (1.1g, 5.6 mm) and DMAP (0.097 g, 0.8 mm). After the mixture is stirred for 12 h at room temperature, the white precipitate that formed is discarded by filtration through a Buchner funnel. From the clear filtrate, the solvent is removed by rotary evaporation to give a yellow-orange solid. Filtration through a short silica gel column (5 \times 40 cm column, silica gel 0.06 mm, 150 g; eluent: chloroform/ethanol 5:3) delivers 0.56 g (50%) of white-colored *O*-(biphenyl-4-yl)-2-cyano-3-hydroxycrotonate **4a**, m.p. 115–8 °C (decomp). IR (KBr): 1750 (C=O), 2100 cm^{-1} (CN); ¹H-NMR (CDCl₃): δ 8.62 (s, 1H, OH enolic), 7.8–7.0 (m, 9H, arom) 2.77 ppm (s, 3H, CH₃).

Preparation of *N*-(biphenyl-4-yl)-2-cyano-3-hydroxycrotonate (**4b**)

It was prepared as described for **4a** with 37.8% yield, m.p. 270–5 °C (decomp). IR (KBr): 1625 (C=O), 2100 cm^{-1} (CN); ¹H-NMR (CD₃OD): δ 7.9–7.1 (m, 11H, arom, NH, OH enolic) 2.14 ppm (s, 3H, CH₃).

Preparation of *N*-(3'-cyano-biphenyl-4-yl)-2-cyano-3-hydroxycrotonate (**4c**)

It was prepared as described for **4a** with 30% yield, m.p. 260–2 °C (decomp); IR (KBr): 1625 (C=O), 2125 cm^{-1} (CN); ¹H-NMR (CD₃OD): δ 8.0–7.4 (m, 10H, arom, NH, OH enolic) 2.16 ppm (s, 3H, CH₃).

Preparation of *N*-(4'-cyano-biphenyl-4-yl)-2-cyano-3-hydroxycrotonate (**4d**)

It was prepared as described for **4a** with 37.5% yield, m.p. 275–6 °C (decomp); IR (KBr): 1625 (C=O), 2125 cm^{-1} (CN); ¹H-NMR (CD₃OD): δ 7.9–7.1 (m, 10H, arom, NH, OH enolic) 2.03 ppm (s, 3H, CH₃).

In vitro immunosuppressive activity

Measuring the ability of the synthesized compounds to suppress lymphocyte proliferation by the MTT assay is used in this study as an indication of their immunosuppressive activities (13,14). For this purpose, heparinized whole blood from volunteers (5 mL) was mixed with an equal volume of normal saline. This mixture was slowly poured over 5 mL of Ficoll solution, and tubes were centrifuged at 750 $\times g$ (Pars Azma, Iran) for 20 min at room temperature. Then, the middle layer containing the mononuclear cells was removed and placed in a clean tube, washed with normal saline and centrifuged at 250 $\times g$ for 10 min. The washing step was repeated twice.

The remaining pellet of cells was resuspended in 2 mL of growth medium (RPMI 1640; fetal calf serum, 10% v/v; L-glutamine, 5 mM; penicillin, 50 IU/mL; and streptomycin, 50 $\mu\text{g}/\text{mL}$; Biosera, East Sussex, UK). Viability of cells was tested by trypan blue dye exclusion method. Viability of more than 95% was considered acceptable for further experimentation. Cells were counted with a hemocytometer. An aliquot of cell suspension equal to 50 000 cells was seeded in a 96-well flat bottom tissue culture plate ($n = 6$) and 20 μL of phytohemagglutinin (PHA) (Gibco, Carlsbad, CA, USA) solution (5 $\mu\text{g}/\text{mL}$) was added to each well for the induction of T-cell proliferation (15,16). Then, 50 μL of one of the four synthesized compounds **4a–d** solution in DMSO was added to each well. The final compounds concentrations in the wells were adjusted at 1, 5, 10, 50 and 100 mM. The total amount of DMSO in each well did not exceed 1%. The control wells consisted of T cells in the presence of vehicle control DMSO 1%, T cells without PHA and wells containing only growth media. A cyclosporine positive control with concentration of 0.1 mM was also employed. The plates were incubated at 37 °C in 5% CO₂/95% O₂ for 4 days. After the incubation period, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye) (5 mg/mL in PBS) was added to each well, and they were incubated in the dark for 4 h. The plates were centrifuged at 750 $\times g$ for 15 min, the growth medium was removed and 200 μL of DMSO and 20 μL of glycine buffer were added to each well. Then, the absorbance of each well was measured by an enzyme-linked immunosorbent assay (ELISA) reader (Statfax-2100; Awareness Technology, Palm City, FL, USA) at 570 nm. The percent inhibition of T-cell proliferation was calculated for each compound (**4a–d**). In all cases, percentage of T-cell proliferation inhibition was expressed in comparison with the cells treated only with DMSO and PHA, which was taken as 100%.

Cytokine (IFN- γ) assay

Measuring IFN- γ is another indicator of the immune response, particularly Th-1 maturation, NK and macrophage activity and cellular immunity (17,18). For this purpose, heparinized whole blood from volunteers (5 mL) was mixed with an equal volume of normal saline. This mixture was slowly poured over 5 mL of Ficoll solution, and tubes were centrifuged at 750 $\times g$ (Pars Azma, Iran) for 20 min at room temperature. Then, the middle layer containing the mononuclear cells was removed and placed in a clean tube, washed with normal saline and centrifuged at 250 $\times g$ for 10 min. The washing step was repeated twice.

The remaining pellet of cells was resuspended in 2 mL of growth medium (RPMI 1640; fetal calf serum, 10% v/v; L-glutamine, 5 mM;

penicillin, 50 IU/mL; and streptomycin, 50 μ g/mL; Biosera). Viability of cells was tested by trypan blue dye exclusion method. Viability of more than 95% was considered acceptable for further experimentation. Cells were counted with a hemocytometer. An aliquot of cell suspension equal to 50 000 cells was seeded in a 96-well flat bottom tissue culture plate ($n = 3$) and 20 μ L of PHA (Gibco) solution (5 μ g/mL) was added to each well for the induction of T-cell proliferation (15). Then, 50 μ L of one of the four synthesized compounds **4a–d** solution in DMSO was added to each well. The final compounds concentrations in the wells were adjusted at 1, 5, 10, 50 and 100 mM. The total amount of DMSO in each well did not exceed 1%. The control wells consisted of T cells in the presence of vehicle control DMSO 1%, T cells without PHA and wells containing only growth media. A positive cyclosporine positive control with concentration of 0.1 mM was also employed. Cells were cultured for 48 h (16). Supernatants (0.8–0.9 mL) were collected from separate wells after 48 h of culture. All supernatants were stored at -80 °C for cytokine analysis. Cytokine levels were analyzed in supernatants by sandwich ELISA. A human IFN- γ ELISA kit (catalog no. BMS228) to determine the level of IFN- γ was used (Bender MedSystems, Vienna, Austria). In this kit, an anti-human IFN- γ coating antibody is adsorbed onto microwells. Human IFN- γ present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human IFN- γ antibody is added and binds to human IFN- γ captured by the first antibody. Following incubation unbound biotin-conjugated anti-human IFN- γ antibody is removed during a wash step. Streptavidin–horseradish peroxidase (HRP) is added and binds to the biotin-conjugated anti-human IFN- γ antibody.

Following incubation, unbound Streptavidin–HRP is removed during a wash step and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of human IFN- γ present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human IFN- γ standard dilutions and human IFN- γ sample concentration determined. The detection limit for the assays was 0.99 pg/mL for IFN- γ .

Results and Discussion

Effects of compounds **4a–d** on lymphocyte proliferation

As seen in Figures 1–4, all compounds with concentrations 5–100 mM inhibited lymphocyte proliferation dose-dependently ($p < 0.05$). Compound **4a** with ester linkage ($X=O$) was the weakest one. Compounds **4b–d** with amide linkage ($X=NH$) reduced lymphocytes proliferation similarly ($p < 0.05$). These results are similar to the inhibitory effect of A771726 on lymphocyte proliferation (19).

Effects of compounds **4a–d** on cytokine IFN- γ production

As seen in Figures 5–8, all compounds **4a–d** at some concentrations show significant differences with the negative control. This

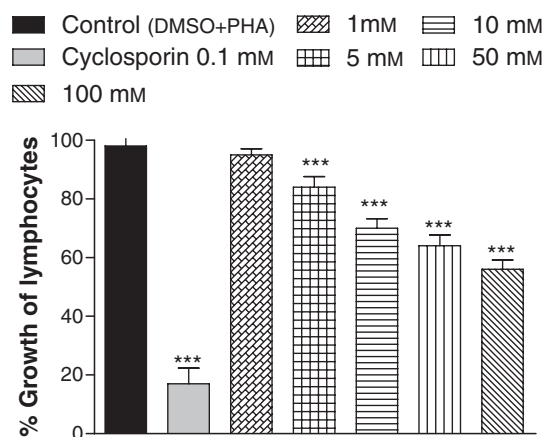


Figure 1: Effects of **4a** on lymphocyte proliferation. Each bar represents mean \pm SEM ($n = 6$), *** $p < 0.001$.

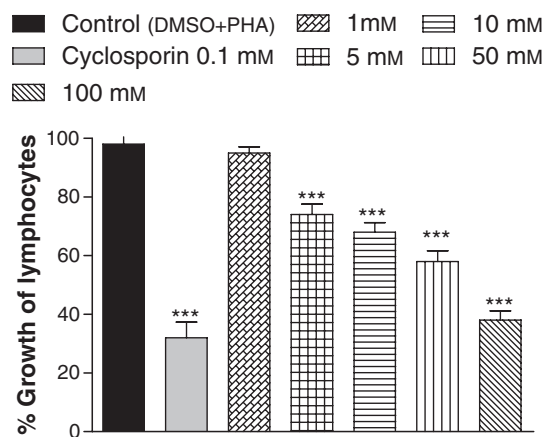


Figure 2: Effects of **4b** on lymphocyte proliferation. Each bar represents mean \pm SEM ($n = 6$), *** $p < 0.001$.

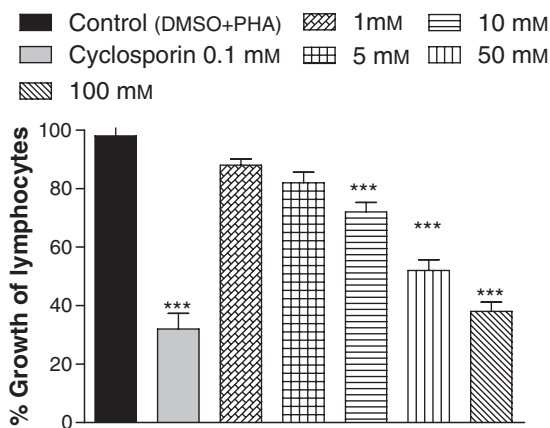


Figure 3: Effects of **4c** on lymphocyte proliferation. Each bar represents mean \pm SEM ($n = 6$), *** $p < 0.001$.

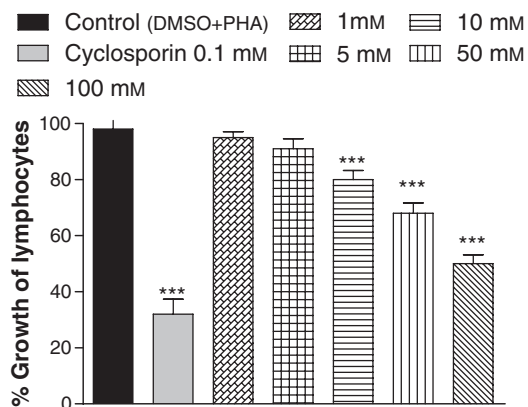


Figure 4: Effects of **4d** on lymphocyte proliferation. Each bar represents mean \pm SEM ($n = 6$), *** $p < 0.001$.

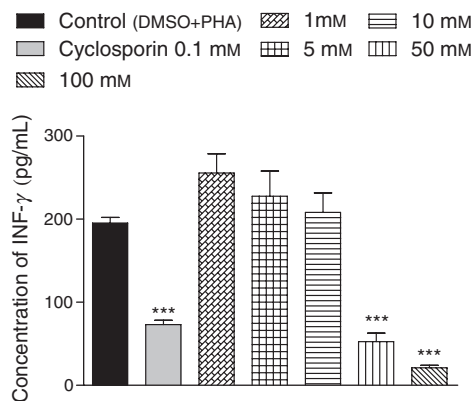


Figure 7: Effects of **4c** on IFN- γ production in cultured lymphocytes. Each bar represents mean \pm SEM ($n = 3$), *** $p < 0.001$.

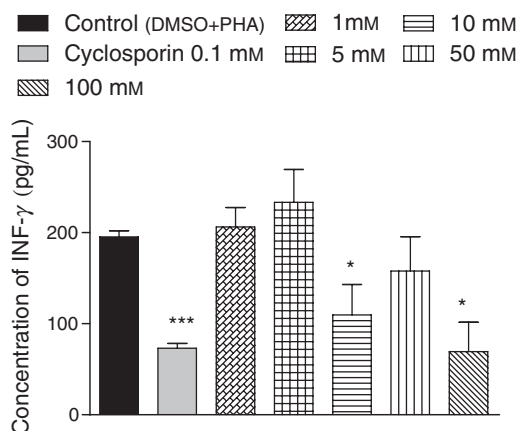


Figure 5: Effects of **4a** on IFN- γ production in cultured lymphocytes. Each bar represents mean \pm SEM ($n = 3$), * $p < 0.05$, *** $p < 0.001$.

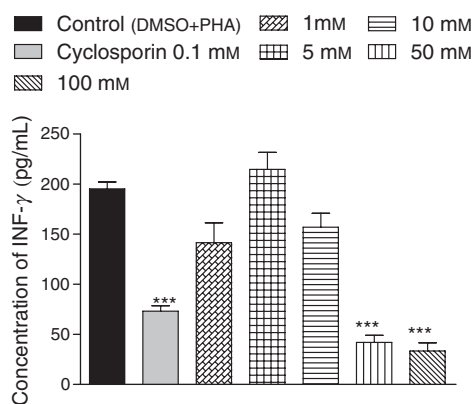


Figure 8: Effects of **4d** on IFN- γ production in cultured lymphocytes. Each bar represents mean \pm SEM of ($n = 3$), *** $p < 0.001$.

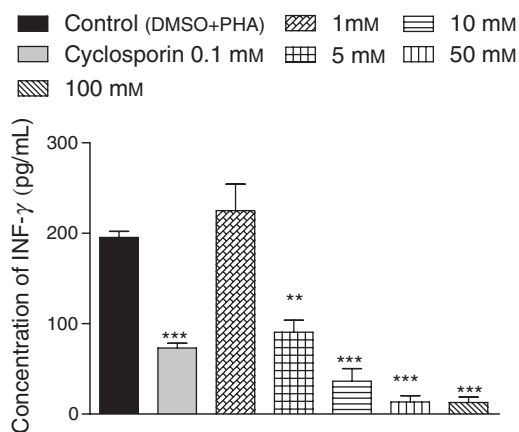


Figure 6: Effects of **4b** on IFN- γ production in cultured lymphocytes. Each bar represents mean \pm SEM ($n = 3$), ** $p < 0.01$, *** $p < 0.001$.

implies that at these concentrations, compounds **4a–d** suppressed the cytokine production by lymphocytes.

Compound **4b** with amide linkage ($X=NH$) and without any substituent on the bisphenyl ring ($Y=H$) was the most potent one and demonstrated a clear concentration-dependent effect. It was effective at concentrations 5–100 mM ($p < 0.05$). On the other hand, compound **4a** with ester linkage ($X=O$) was found to be the weakest one. No concentration-dependent effects were observed. It was effective only at 10 and 100 mM ($p < 0.05$). Compounds **4c** and **4d** with amide linkage ($X=NH$) and a substituent on bisphenyl ring ($Y=CN$) behaved similarly. They were effective at concentrations 50 and 100 mM ($p < 0.05$). It seems that substitution on bisphenyl ring reduces activity of compounds on cytokine production.

In conclusion, because of concerns about the prolonged half-life and potential for hepatotoxicity of LFM that have tempered the enthusiasm for its use and the fact that most synthesized analogs are shorter acting agents that are preferable to LFM, synthesizing

Table 1. Summary of biological activities of compounds **4a–d**

	4a	4b	4c	4d
Lymphocyte production	5–100 mM p < 0.001	5–100 mM p < 0.001	10–100 mM p < 0.001	10–100mM p < 0.001
IFN- γ production	10 and 100 mM p < 0.05	5–100 mM p < 0.001, very effective	50 and 100 mM p < 0.001	50 and 100 mM p < 0.001

and studying the effects of such analogs are important in discovering novel immunosuppressive agents (20).

As shown in Table 1, compound **4b** suppresses IFN- γ production even more significantly than cyclosporine. Also, this compound is relatively effective in inhibiting lymphocyte proliferation. Other compounds have less and varying degrees of activities on lymphocyte proliferation and IFN- γ production.

Conclusion

In this study, four analogs of A771726, the active metabolite of LFM showed some promising activities as inhibitors of lymphocyte proliferation and IFN- γ production. The value of these effects in clinical settings deserves further investigation of these four compounds. Therefore, it is suggested that further studies, particularly *in vivo* work, to be pursued to clarify their effects.

Acknowledgment

The authors are thankful to the financial support of the Research Council of Mashhad University of Medical Sciences.

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