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Investigations on the Immunosuppressive Activity of Derivatives of Mycophenolic Acid in Immature Dendritic Cells

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ABSTRACT

The main activity of mycophenolic acid **1** (MPA) and its analogs is inhibition of proliferation of T cells which exerts immunosuppressive effect. There are also reports that MPA inhibits the activity of antigen-presenting cells (APC) including dendritic cells (DCs). We tested the effect of novel amino acid derivatives of MPA and conjugates of MPA with acridines/acridones on DCs by flow cytometry, ELISA and MLR assay. Both acridines/acridone derivatives could inhibit the maturation of DC, as shown by the decreased expression of B7 family receptors. It was confirmed in the mixed leucocyte reaction (MLR), in which the T cells challenged with DCs pretreated with the analogues showed decreased proliferation and reduced cytokine secretion. The most interesting activity in this series of studies (influence on DCs maturation, cytokine production and mixed leucocyte reaction) exhibited (mycophenoyl-*N*-3-propyl)-9-acridone-4-carboxamide ester **5a** and (mycophenoyl-*N*-5-pentyl)-9-acridone-4-carboxamide ester **5b**. They caused also reduction in both concentration of IL-2 and IL-15, which are pro-inflammatory cytokines. In addition, they caused increase secretion of suppressive IL-10. Similarly, promising results were obtained for the *N*-mycophenoyl-D-glutamic acid **4b**, which previously gave the highest value of selectivity. Acridone derivatives of MPA are therefore good immunosuppressive drug candidates for further testing.

1. INTRODUCTION

The first successful renal transplantation was performed between identical twins and hence there was no need for immunosuppression [1]. Since that time solid organ transplantation has been rapidly evolving as a life-saving therapeutic intervention that greatly contributes to a better quality of life in organ recipients. Genetic donor-recipient

match is still the best guarantee of uneventful post-transplant follow up while appropriate immunosuppression is the most common strategy to keep the transplanted organ in good condition [2]. Unfortunately allograft rejection is still a major cause of graft loss in the first year [3].

The inhibition of lymphocyte proliferation is one of the best way used in immunosuppression therapies. Mycophenolic acid (MPA) **1** is an uncompetitive inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH), the crucial enzyme in *de novo* purine nucleotide biosynthesis. There are two clinically approved derivatives of mycophenolic acid **1** (MPA, Figure 1) – mycophenolate mofetil **2** (2-morpholinoethyl, MMF, CellCept (Roche), Figure 1) and mycophenolate sodium **3** (MPS, Myfortic (Novartis), Figure 1). MMF **2** is a prodrug metabolised to active form (MPA **1**) and first was used in early 1990s as an immunosuppressive drug. Both compounds, MMF **2** and MPS **3**, are used in the prevention of allograft rejection and treatment of autoimmune diseases [3-6].

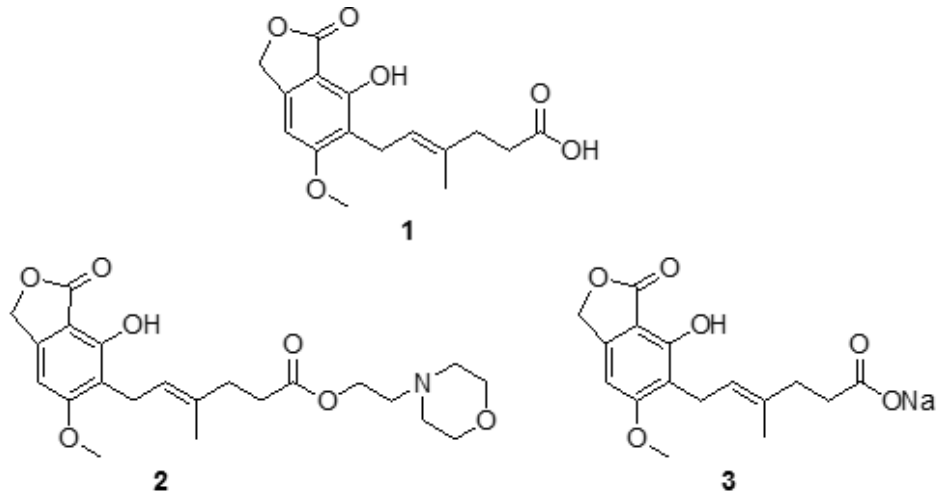

Figure 1. Structure of MPA **1**, MMF **2** and MPS **3**.

The main activity of MPA and its analogs is the inhibition of proliferation of T cells, which stops these cells from allograft rejection [7]. Nevertheless, there are reports proved the inhibition of the activity of antigen-presenting cells (APC) by MPA [8]. It may be a special importance in transplantation as alloantigens presented by antigen-presenting cells (APC) trigger T cell alloresponse and graft rejection. The most important APC in the induction of such an immune response are dendritic cells (DCs) and myeloid subset of DC (moDC), which is mainly considered as involved in organ rejection [9,10]. If it is true, MPA might be an agent that not only stops the proliferation of allosensitised lymphocytes, but also inhibit the allosensitization itself. Some changes to the particle structure might further enhance the latter activity without deteriorating the former one.

Hence, there were designed MPA immunosuppressive derivatives combining antiproliferative and anti-antigen-presentation activities, including amino acids derivatives of MPA and acridone/acridine analogs of MPA [11,12]. We evaluated their cytotoxic (IC_{50} - half maximal inhibitory concentration) in colorimetric test MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) and antiproliferative activity (EC_{50} - half maximal effective concentration) in proliferation test with ^{3}H-thymidine incorporation (3H-TdR) (for an example of the diagram see Figure S-1 in the electronic supplementary information), influence on inosine-5'-monophosphate dehydrogenase (IMPDH) performed in absence or presence of guanosine monophosphate (GMP) and accounted selectivity index (SI). According to these studies, there were selected the most promising compounds for consecutive tests. Methyl ester *N*-mycophenoyl-L-phenyloalanine **4a**, *N*-mycophenoyl-D-glutamic acid **4b**, *N*-mycophenoyl-L-leucine **4c**, (mycophenoyl-*N*-3-propyl)-9-acridone-4-carboxamide ester **5a**, (mycophenoyl-*N*-5-pentyl)-9-acridone-4-carboxamide ester **5b** and (mycophenoyl-*N*-3-propyl)-acridine-4-carboxamide ester **6a**, (mycophenoyl-*N*-5-pentyl)-acridine-4-carboxamide ester **6b**, (mycophenoyl-*N*-6-hexyl)-acridine-4-carboxamide ester **6c**

(Figure 2) were considered for *in vitro* examinations as potential immunosuppressive agents inhibiting antigen-presentation by myeloid DCs.

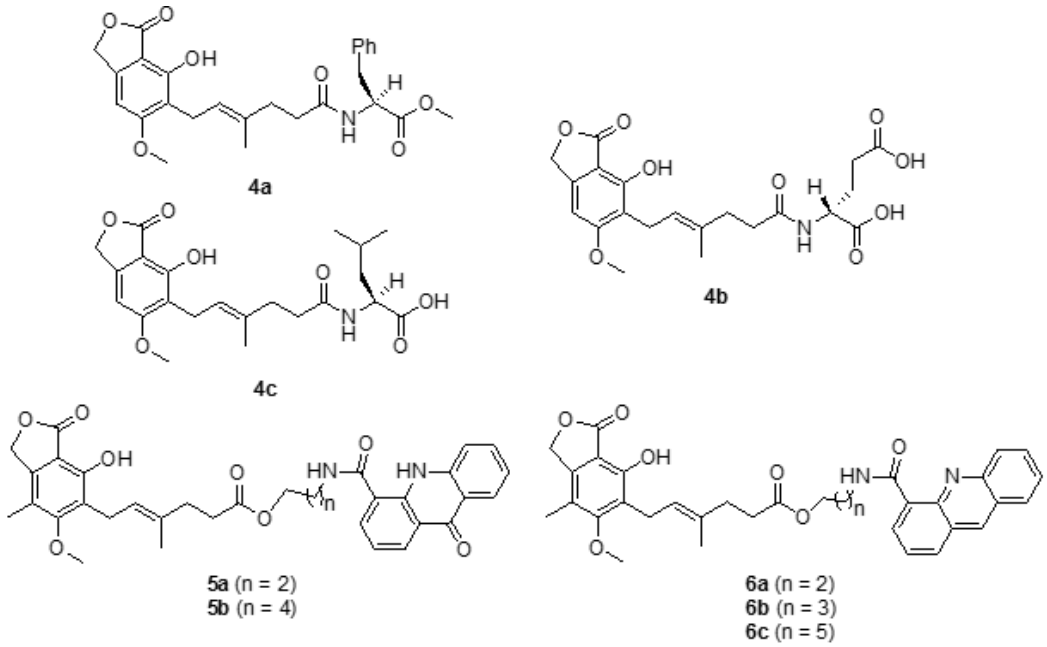

Figure 2. Structures of selected analogs of MPA **4a-c**, **5a,b** and **6a-c**.

2. MATERIALS AND METHODS

2.1. MPA analogs

Tested analogs of MPA **4a-c**, **5a,b** and **6a-c** (Figure 2) were obtained in accordance with general procedure for the preparation amino acid derivatives of MPA **4a-c** described by Iwaszkiewicz-Grzes et al. [11] and ester derivatrives of MPA and acridines **5a,b** or acridones **6a-c** described by Cholewinski *et al*. [12]. Both publications include complete identification of compounds and their characteristic data [11,12]. For clarity, the most promising analogs, such a **4b**, **5a**, and **5b**, are presented in main figures, while results from all analogs are shown in electronic supplementary information.

2.2. Human blood samples

Human buffy coats were obtained from anonymous healthy donors from the Regional Blood Bank in Gdańsk (RCKiK).

2.3. Isolation of peripheral blood mononuclear cells (PBMC)

The heparinized blood was diluted with sterile phosphate-buffered saline (PBS pH7.4 W/O CAMQ USA PLASTIC, Life Technologies) (1.5/1 v/v). PBMC were separated by density-gradient centrifugation (2500 rpm by 25 minutes) in Ficolle-Paque (Gradisol L-Aqua-med). PBMC, as a dense white band above Ficolle-Paque, were collected in 50 ml tube (BD Falcon Tubes PP, Diag-Med). The cells were washed twice (1500 rpm by 10 minutes) with sterile PBS and then resuspended with RPMI-1640 medium (Immuniq, PAA) (10^8 cells/10ml).

2.4. Isolation of monocytes from PBMC

Monocytes were prepared from PBMC by a 2 h adherence step at 37 ˚C in RPMI-1640 medium (Immuniq, PAA) supplemented with 10% FBS (Life Technologies) and P/S (Penicillin-Streptomycin Sterile-Filtered, Sigma-Aldrich). Non-adherent cells were removed by extensive washing with sterile PBS and the remaining adherent cells were immediately subjected to the moDCs.

2.5. Generation of dendritic cells

DCs were generated by previously described protocols, with some modifications [13]. Adherent monocytes were cultured for 7 days in GMP-DC medium (GMP Serum-free Dendritic Cell Medium (DC), CellGenix) containing 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), (R&D systems, Biokom) and 100 ng/ml interleukin-4 (IL-4), (R&D systems, Biokom) in polystyrene 24 well plate (Nunc, Diag-Med) at the concentration of $1x10^6$/ml.

After 7 days immature DCs were stimulated with 100 ng/ml LPS (lipopolysaccharide, Sigma-Aldrich) and MPA analogs (Table 1) by 72h. We have used three conditions: negative control – culture of not stimulated cells, positive control – culture of stimulated cells with LPS, tested analogs of MPA - cells with compounds (concentration as in Table 1) and DMSO - cells with DMSO (the highest concentration we used for dissolve tested analogs [11,12]. Experiment has been performed three times.

Table 1. Final concentrations of compounds. EC_{50} [μM] values of **1**, **4a-c**, **5a,b**, **6a-c** for activated PBMC obtained in antiproliferation test [11,12].

Compound	Concentration [μM]	Ref.
1 (MPA)	0.00006	[11]
4a	0.004*	[11]
4b	0.003*	[11]
4c	0.004*	[11]
5a	5.7*	[12]
5b	1.2*	[12]
6a	0.003*	[12]
6b	0.0016*	[12]
6c	0.0017*	[12]

* statistical significant $p<0.05$

2.6. Flow cytometry

Maturity of DCs was assessed continuously in the culture by staining (lineage cocktail 1; HLA-DR PerCP-Cy5.5; CD11c APC; CD123 PE-CY7; CD80 PE; CD83 PE and CD86 PE) and examined by flow cytometry. DCs were harvested after incubation of plates on ice for 30 minutes. Then, the cells were stained with the following cocktail of antibodies: lineage cocktail 1 (lin 1 - CD3, CD14, CD16, CD19, CD20); HLA-DR PerCP-Cy5.5; CD11c APC; CD123 PE-CY7; CD80 PE; CD83 PE (not shown); CD86 PE (all antibodies from BDBioscience, Belgium) for 25-30 minutes, washed and examined by flow cytometry (BD FACSAria^{TM} Cell Sorter). Maturity of DCs was measured as MFI of CD80, CD83 (not shown) and CD86 receptors on HLA-DR^{+}/lin^{-}CD123^{-}CD11c^{+} cells. Experiment has been performed three times. An example of flow cytometry analysis has been shown on Figure S-2 (see electronic supplementary information).

2.7. Assessment of cytokines levels in DCs cultures

Supernatants from the cultures of DCs stimulated with MPA derivatives and unstimulated DCs were harvested after 10 days and kept at −80°C until analysis. The following ELISA kits were used in this study: human IFN-γ (not shown), IL-2, IL-4, IL-5 (not shown), IL-6, IL-10, IL-12, IL-15, IL-17 (not shown), IL-18 (not shown), TGF-β1 (not shown) (R&D Systems, France). ELISA was performed according to the manufacturer's instructions. Experiment has been performed three times.

2.8. Mixed leucocyte reaction (MLR) assay

To evaluate the effect of DCs generated by MPA derivatives on the proliferation of allogeneic lymphocytes, we carried out mixed leucocyte reaction assay. Experiment has been performed five times. Immature DCs prepared as above was treated for 72 h with LPS (100ng/ml) (Sigma-Aldrich) for extortion maturation of DCs and the compounds were tested in accordance with the previously calculated EC_{50} concentrations for PBMC (Table 1) (values calculated and described by Iwaszkiewicz-Grzes et al. [11] and Cholewinski et al. [12]. Immature DCs prepared with LPS were used as a reference. Then, DCs from all the conditions were irradiated with at least 2518 cGy by 10 minutes. Irradiated cells were used as stimulators and co-cultured with allogenic lymphocytes ($1x10^5$ cells/well) treated as responders in the following DCs:lymhocyte ratios: 2:1, 1:1, 0.5:1, 0.25:1, 0.125:1. Incubation was conducted for further 72h. After this time [^{3}H] thymidine (0.5μCi/well) (MPBiomedicals) was added to each culture and left until the next day (18h). Reading was performed by liquid scintillation counter (LSC-Beckman).

As a control we prepared allogenic lymphocytes treated with antibodies anti-CD3/anti-CD-28 (beads) (Millipore) and DCs treated with DMSO (solvent for the derivatives) under the same conditions as above.

2.9. Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) test

All tested analogs of MPA were subjected to LAL test to detect the presence of endotoxins. LAL was performed according to the manufacturer's instructions (Lonza). LAL test confirmed that compounds do not contain detectable level of endotoxins, which could cause DCs maturation and falsify the effects of tested analogs of MPA (**4a-c**, **5a,b**, **6a-c**).

2.10. Statistics

EC_{50} values were determined using SigmaPlot 11. F (Fisher test) and p (statistical significance) values were determined using two-way ANOVA (STATISTICA 10.0). All results for MPA analogs (**4a-c**, **5a,b** and **6a-c)** were compared to MPA **1**.

3. RESULTS

3.1. Determination of the effect of synthesized compounds (1, 4a-c, 5a,b, 6a-c) on the maturation of dendritic cells

Amino acid derivatives of MPA (**4a-c**) did not inhibit the maturation of DCs from monocytic precursors (Figure S-3, see electronic supplementary information) as strong as MPA **1**. However, there was a relationship between the day when compound was added to the cultures and the level of maturation measured as MFI (Median Fluorescence Intensities) of CD80, CD83 (not shown) and CD86 receptors (Figure S-2, see electronic supplementary information). Addition of MPA (**1**) during the first day of culture resulted in a reduced amount of $CD86^+$ cells compared to seventh day. In addition, MPA (**1**), when added on the first day, was the only compound which decreased the percentage of $CD80^+$ cells compared to the controls containing LPS. Amino acid derivatives exhibited stronger inhibition of the expansion of DCs in the case when compound was added on seventh day. At the same time adding the analog **4c** (0.004 μM; p=0.036, F=78.95945) resulted in a decrease in the percentage of $CD86^+$ cells compared with the control containing only LPS. Hence, we decided to introduce the test compounds during the seventh day with LPS.

Acridone derivatives of MPA (**5a,b**) (Figure S-4, see electronic supplementary information) decreased the expression of CD86 receptor on treated DCs as compared to LPS-

treated DCs (**5a**: 5.7 μM; p=0.033, F=93.02331; **5b**: 1.2 μM; p=0.0355, F=79.75919). There was no effect on the other two receptors. Acridine derivatives (**6a-c**) did not inhibit the expression of the receptors on DCs.

3.2. Influence of dendritic cells generated in the presence of examined compounds on the proliferative activity of lymphocytes in MLR

In the MLR (Figure 3, and Figure S-5, S-6 see electronic supplementary information), which was applied as a functional assay testing capability of DCs to present antigens/stimulate proliferation of allogeneic lymphocytes, compounds **5a** (5.7 μM; p=0.043, F=67.22879), **5b** (1.2 μM; p=0.0399, F=78.12554) showed the strongest inhibition of DCs maturation. It was showed as the lowest proliferation of allogeneic lymphocytes mixed with DCs treated with these compounds (Figure 3). The effect was significant as compared with MPA (**1**). DCs treated with other compounds: amino acids derivatives (Figure S-5, see electronic supplementary information) **4a** (0.004 μM; p=0.0296, F=141.6614), **4b** (0.003 μM; p=0.0067, F=2701.465), **4c** (0.004 μM; p=0.053, F=43.35213) and two acridone derivatives **6a** (0.003 μM; p=0.014, F=636.5057) and **6c** (0.0017 μM; p=0.0043, F=6639.743) also stimulated proliferation of lymphocytes less than MPA (**1**), however only in the cocultures with low ratios of DCs to lymphocytes (0.25:1 and 0.125:1). Noteworthy, in low DC/lymphocytes conditions (0.25:1 and 0.125:1) DC treated with all tested compounds stimulated proliferation of allogeneic lymphocytes less than immature DCs (DCs without LPS).

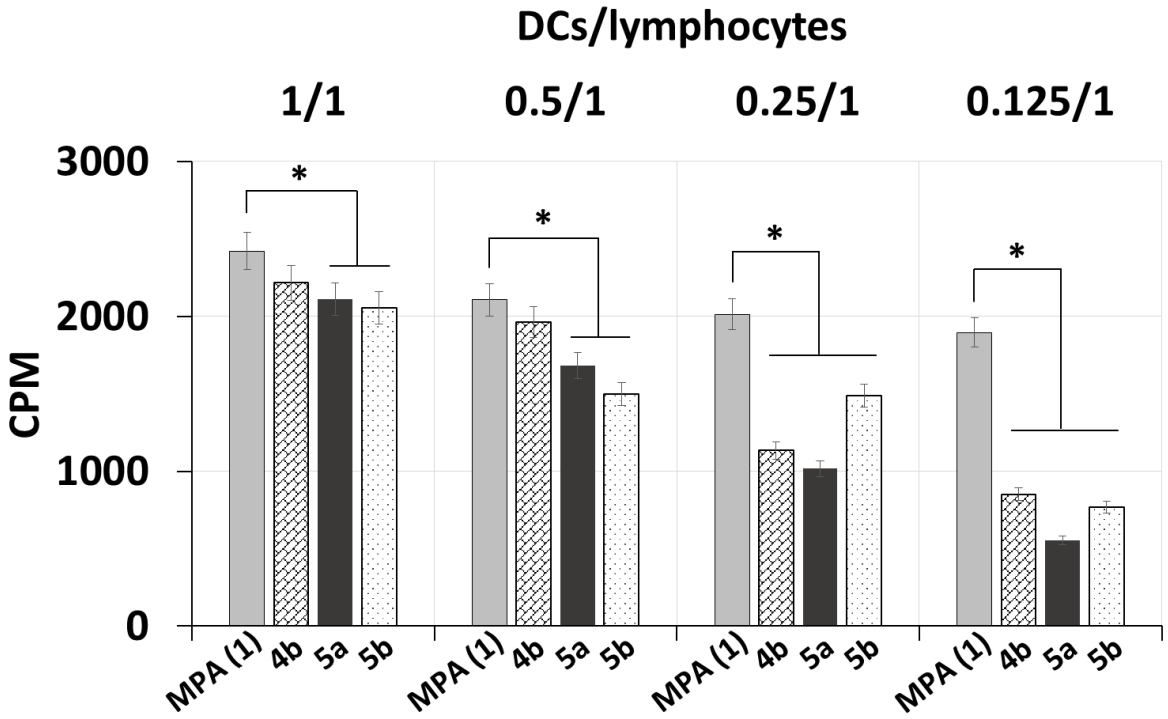

Figure 3. Effect of DCs treated with the most promising derivatives of MPA **4b**, **5a** and **5b** on the proliferation of lymphocytes in MLR test. DCs treated with native MPA (**1**) were used as a reference. "Y" axis shows CPM - Counts Per Minute as units of proliferation. Ratios 1/1, 0.5/1, 0.25/1, 0.125/1 shows the proportions of DCs to lymphocytes in particular conditions. The data present the mean ±SD of triplicate test.

* - statistically significant results

For DCs treated with LPS only, without LPS, with DMSO and cultures of lymphocytes treated with beads instead of DCs and DCs treated by other tested derivatives of MPA see electronic supplementary information.

3.3. ELISA

Samples of supernatants from DCs cultures were tested for the presence of mainly pro-inflammatory and anti-inflammatory cytokines (Table 2, Figure 4 and Figure S-7, S-8, see electronic supplementary information). Cytokines involved in the lymphocyte activity, Th1/Th2 balance and therefore mainly in transplant rejection. IFN-γ, IL-2 (pro-inflammatory cytokines) and IL-4 (anti-inflammatory cytokine) were affected to various extents [9,10].

Table 2. Concentration of interleukins [pg/ml] in the tested samples.

	IL-2	IL-4	IL-6	IL-10	IL-12p70	IL-15
DCs with LPS	6.6±0.3	13.8±0.7	19.1±3.5	2.1± 0.1	13.5±0.7	4.0±1.0
DCs without LPS	4.9±0.2	12.1±0.7	15.6±2.4	2.3±0.1	1.5±0.3	2.6±0.1
1 (MPA)	4.2±0.2	14.0±0.9	19.1±2.8	1.2±0.1	13.4±1.5	6.9±1.0
DMSO	4.7±0.2	14.4±0.9	19.3±2.8	1.7±0.1	11.9±0.4	6.1±0.8
4a	4.0±0.2	13.5±1.1	19.5±3.3	1.3±0.1	12.5±1.4	3.1±0.6*
4b	3.2±0.2*	14.3±1.0	19.7±1.2	1.9±0.1*	15.5±2.6	3.1±0.4*
4c	6.05±0.3	14.6±1.2	18.8±2.2	1.8±0.2*	14.3±1.4	2.3±0.8*
5a	1.6±0.1*	14.0±0.9	18.2±1.6	1.5±0.4*	14.1±1.3	2.8±0.5*
5b	3.4±0.2*	15.7±1.5	19.4±3.5	1.5±0.6*	15.1±2.0	6.4±1.0
6a	4.9±0.2	15.7±1.4	19.4±1.0	2.1±0.5*	18.2±2.6	4.0±0.8*
6b	2.1±0.1*	14.3±1.0	19.4±1.3	1.9±0.1*	14.6±2.0	4.1±0.4*
6c	1.9±0.1*	14.7±2.2	19.1±2.0	2.7±1.0*	13.9±1.3	2.1±0.3*

* statistically significant $p<0,05$

Tested analogs of MPA did not affect the production of IFN-γ or IL-4. However, we observed that three compounds: mainly **5a**, and to a lesser extent **6b** and **6c** significantly reduced the amount of IL-2 as compared to native MPA. Similarly good results were obtained for the *N*-mycophenoyl-D-glutamic acid **4b**. Inflammatory properties of the compounds were analyzed by using IL-6, IL-12 and IL-15. The levels of IL6 and IL-12 were not affected while the production of IL-15 was suppressed by almost all derivatives as compared to the cultures with native MPA (**1**). Importantly, this anti-inflammatory effect of the compounds was additionally

enhanced as the compounds suppressed the production of anti-inflammatory IL-10 significantly less than native MPA (**1**).

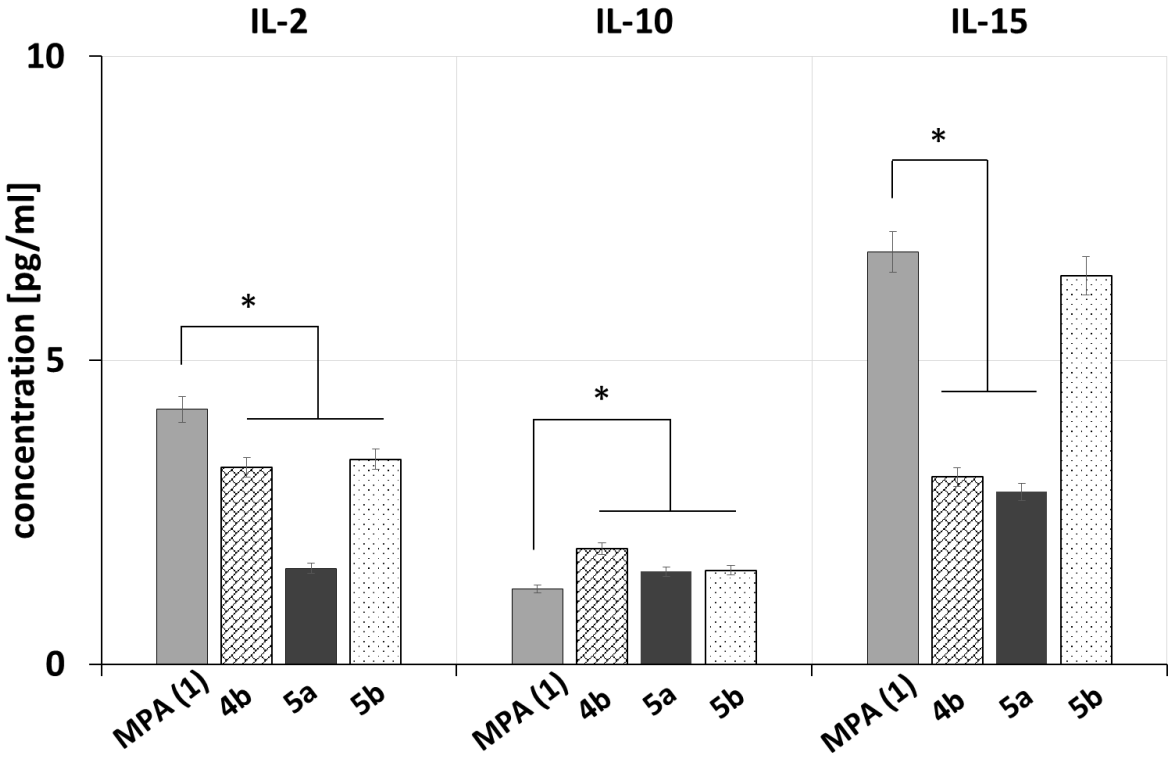

Figure 4. Concentration of pro-inflammatory (IL-2, IL-15) and anti-inflammatory (IL-10) cytokines [pg/ml] from the cultures of DCs treated by the most promising derivatives of MPA (**4b**, **5a** and **5b**). DCs treated with native MPA (**1**) were used as a reference.
* - statistically significant results
For DCs treated with LPS only, without LPS, with DMSO as a reference and DCs treated by other tested derivatives of MPA see electronic supplementary information.

4. DISCUSSION

The study proved that, apart from anti-proliferative activity, agents based on MPA may exert suppressive activity on DCs maturation. These compounds were able to decrease the expression of maturity receptors on treated DCs, suppress function of DCs as stimulators of lymphocyte proliferation in MLR and inhibit the production of cytokines which are

important in organ rejection and inflammation. Acridone derivatives of MPA (**5a,b)** were found as the most promising agents in the study.

The understanding of the activity of current immunosuppressive drugs is usually based on a single mechanism of action. Nevertheless, thorough analysis revealed that many routinely used immunosuppresants can exert other beneficial activities. For example, rapamycin does not only inhibit mTOR protein but also induces T regulatory cells [14]. Depletion of antibodies does not only kill mature cells, but influences the regeneration of the immune system. It is therefore possible that MPA also can be pleiotropic. Development of immunosuppressive drugs and understanding of their action has traditionally been focused on lymphocytes, but recent evidence indicates that these agents interfere with immune responses at the earliest stage, targeting key functions of DCs. Pharmacological inhibition of DCs differentiation can be achieved by immunosuppressive agents, for example corticosteroids, MMF, cyclosporine A etc [14]. Our interest in other activities of MPA focused on DCs as these cells which are exposed to the action of drugs similarly to lymphocytes. DCs are important in the survival of allotransplants. Human dendritic cells derive *in vivo* from $CD34^+$ progenitor cells of the bone marrow. It occurs under the influence of Flt3 ligand, stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF-α) and IL-4. Then DCs traffic into the peripheral blood and further to the tissues [15,16]. It has been shown that DCs arise from two different lines of development: myeloid cells (CMP - common myeloid progenitors) and lymphoid (CLP - common lymphoid progenitors) [15,17]. These cells are also characterized by the secretion of many substances, including cytokines and chemokines, which modulate immune response [18-22]. DCs express a bunch of receptors involved in the processes of adhesion and T cell activation endocytosis and chemotaxis [15-17,20-26]. DCs are involved in immune response to allograft from very early phase. So called direct and indirect recognition of the graft antigens, which lead to

allosensitization, is mediated mainly *via* DCs from donor and recipient, respectively. Phenotypic features of DCs described above can be used to detect activity of DCs during these reactions. Primary function of the immature DCs is to capture antigens. In case of inflammation, DCs are attracted to the area of inflammation and stimulated to capture and internally process antigens. For MHC class II presentation to stimulate CD4+ Th cells, antigen is taken up by phagocytosis or receptor-mediated endocytosis to endosomes where antigen preparation occurs. A key component on DCs in addition to antigen capture, processing and presentation is the presence of costimulatory molecules [27].

The activity of DCs during immune response can be mimicked *in vitro* and it is therefore very easy to analyze the effects of the drugs on these cells, like in this study. Our results showed that derivatives of MPA were able to actively modulate these activities of DCs. As mentioned above, the expression of one or both of the costimulatory molecules CD80 and CD86 on the DCs are essential for the effective activation of T lymphocytes. As we can see only analog **4c** indicated the strongest inhibition of DCs maturation from all amino acids derivatives as well as MPA (Figure S-3, see electronic supplementary information). For acridone and acridine derivatives of MPA (Figure S-4, see electronic supplementary information), two compounds **5a** and **5b** indicated comparable quantity of CD80 in comparison with pure MPA. **5a** also inhibited expression of molecule CD86 as well as MPA. Only **5b** has shown stronger influence on inhibition CD86 over MPA and from all the tested compounds. Upon receipt of satisfying results we performed MLR test. Dendritic cells are uniquely potent at stimulating primary immune responses and the stimulation of allogeneic T cells in the mixed leukocyte reaction (MLR) which is used as a functional test to identify DCs. The indirect presentation of allogeneic MHC class II to responding T cells by recipient DCs is now considered to be the major pathway for initiating graft rejection [28]. In previous tests, only compounds **5a** and **5b** (Figure 3) have shown the strongest inhibition of DCs

maturation in all ratios. Also for compound **4c** we obtained satisfying results. We observed that when DCs (treated by selected compounds) were added in proportions similar to physiology (0.25:1 and 0.125:1) stimulated lymphocytes, proliferation was less than MPA. This is undoubtedly an advantage of the tested compounds. If this compound is used for treatment, we strongly believe that it would lead to a more efficient inhibition of mediated reactions of rejection by lymphocytes in comparison with MPA.

Cytokines are important mediators of the immune response intermediate in allograft rejection. DCs secrete a number of chemokines and express some chemokine receptors, and it seems likely that these properties could also contribute to the capacity of DCs to cause the aggregation and stimulation of naive T cells [28]. The effect on graft survivability, except the risk factors such as age, time of warm and cold ischemia, lack of immunosuppressive regimen etc. can caused level of cytokines before transplantation. It is assumed that increased levels of Th1-type cytokines, particularly IFN-γ, IL-2 and IL-12, may favor the later episodes of acute rejection [26,29,30]. Expression of one or both of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on the DCs are essential for the effective activation of T lymphocytes and for IL-2 production. We tested 11 different cytokines. IL-2 is a factor activating regulatory T cells and can stimulate proliferation of cytotoxic T lymphocytes. We observed that compounds **5a**, **6b** and **6c** reduced the amount of IL-2 the most. Its noteworthy to mention that other compounds **4a**, **4b** and **5b** also showed better activity than MPA in reducing the level of IL-2.

Similarly good results were obtained for the D-glutamine derivative **4b**, which in previous stages of the research showed the highest value of selectivity.

The compounds that showed the most promising activities in this series of studies are **5a** and **5b**. It caused a reduction in both concentration of IL-2 and IL-15 as well as increased the concentration of IL-10, which is responsible for anti-inflammatory properties. IL-10 has an

important regulatory role on monocyte function and on DCs maturation. Furthermore, IL-10 has been documented to have a significant inhibitory effect on several aspects of APC function, for example, the expression of co-stimulatory molecules and the ability to synthesize IL-12. Importantly, IL-10 treated-DCs can be tolerogenic. Dendritic cells secreting IL-10 exhibit minimal or no stimulatory properties in primary MLRs and are markedly inhibitory to T cell proliferation induced by polyclonal activators. Thus, IL-10 producing DCs are functionally and phenotypically inhibitory accessory cells and putatively tolerogenic [28].

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

[1] J.E. Murray, The first successful organ transplants in man, J. Am. Coll. Surgeons 200 (2005) 5-9.

[2] N.J. Sarma, V. Tiriveedhi, T. Angaswamy, N. Mohanakumar, Role of antibodies to self-antigens in chronic allograft rejection: Potential mechanism and therapeutic implications. Hum. Immunol. 73 (2012) 1275-1281.

[3] P. Halloran, T. Mathew, S. Tomlanovich, C. Groth, L. Hooftman, C. Barker, Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection, The international Mycophenolate Mofetil Renal Transplant Study Groups, Transplantation 63 (1997) 39-47.

[4] M. Sandor, S. Csaba, A. Kovaesne-Mezei, T. Tivadar, J. Hajko, J. Aronhime, Patent EP 1908756 (2008).

[5] M. Abdurrazzaque, K.Rudolf Patent US 2008300404 (2008).

[6] P. De, R. Mattheus, V.B. De Erik, T. Neeraj, G.B. Nana, Patent WO 2009003878 (2009).

[7] A. Prémaud, A. Rousseau, G. Johnson, C. Canivet, P. Gandia, F. Muscari, J.M. Peron, L. Rostaing, P. Marquet, N. Kamar, Inhibition of T-cell activation and proliferation by mycophenolic acid in patients awaiting liver transplantation: PK/PD relationships, Pharmacol. Res. 63 (2011) 432-438.

[8] V.R. Cicinnati, J. Hou, M. Lindemann, P.A. Horn, G.C. Sotiropoulos, A. Paul, G. Gerken, S. Beckebaum, Mycophenolic acid impedes the antigen presenting and lymph node homing capacities of human blood myeloid dendritic cells, Transplantation 88 (2009) 504-513.

[9] X. He, R.L. Smeets, H.J. Koenen, P.M. Vink, J. Wagenaars, A.M. Boots, I. Joosten, Mycophenolic Acid-Mediated Suppression of Human CD4+ T Cells: More Than Mere Guanine Nucleotide Deprivation, Am. J. Transplant. 11 (2011) 439-449.

[10] A.E. Morelli, Dendritic cells of myeloid lineage: the masterminds behind acute allograft rejection, Curr. Opin. Organ Transplant. 19 (2014) 20-27.

[11] D. Iwaszkiewicz-Grześ, G. Cholewinski, A. Kot-Wasik, P. Trzonkowski, K. Dzierzbicka, Synthesis and biological activity of mycophenolic acid-amino acid derivative, Eur. J. Med. Chem. 69 (2013) 863-871.

[12] G. Cholewinski, D. Iwaszkiewicz-Grzes, P. Trzonkowski, K. Dzierzbicka, Synthesis and biological activity of ester derivatives of mycophenolic acid and acridines/acridones as potential immunosuppressive agents, J. Enzyme Inhib. Med. Chem. (2016) accepted, DOI: 10.3109/14756366.2015.1077821.

[13] E. Wiesemann, D. Sonmez, F. Heidenreich, A. Windhagen, Interferon-beta increases the stimulatory capacity of monocytederived dendritic cells to induce IL-13, IL-5 and IL-10 in autologous T-cells, J. Neuroimmunol. 123 (2002) 160–169.

[14] H. Hackstein, A.W. Thomson, Dendritic cells: emerging pharmacological targets of immunosuppressive drugs, Nat. Rev. Immunol. 4 (2004) 24-34.

[15] J. Pogorzelska−Dyrbuś, A. Pogorzelska−Antkowiak, E. Hadas, Rola komórek Langerhansa w układzie immunologicznym skóry, Przegl. Derm. 2 (2004) 147–152.

[16] M. Colonna, G. Trinchieri, Y.L. Liu, Plasmacytoid dendritic cells in immunity, Nat. Immunol. 5 (2004) 1219–1226.

[17] A. O'Garra, G. Trinchieri, Are dendritic cells afraid of commitment?, Nat. Immunol. 5 (2004) 1206–1209.

[18] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, K. Palucka, Immunobiology of dendritic cells, Annu. Rev. Immunol. 18 (2000) 767–811.

[19] M. Sochacka, Z. Błach−Olszewska, Mechanizmy wrodzonej odporności, Postepy Hig. Med. Dosw. 59 (2005) 250–258.

[20] J. Węcławek−Tompol, A. Chybicka, B. Rybka, D. Noworolska-Sauren, R. Ryczan, Rola komórek dendrytycznych w układzie odpornościowym, Adv. Clin. Exp. Med. 13 (2004) 651–662.

[21] J. Aliberti, C. Reis e Sousa, M. Schito, S. Hieny, T. Wells, G.B. Huffnagle, A. Sher, CCR5 provides a signal for microbial induced production of IL−12 by CD8α+ dendritic cells, Nat. Immunol. 1 (2000) 83–87.

[22] M. Ato, S. Stäger, C.R. Engwerda, P.M. Kaye, Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis, Nat. Immunol. 3 (2002) 1185–1191.

[23] M.A. Degli−Esposti, M.J. Smyth, Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat. Rev. Immunol. 5 (2005) 112–124.

[24] G.J. Clark, N. Angel, M. Kato, J.A. López, K. MacDonald, S. Vuckovic, D.N. Hart, The role of dendritic cells in the innate immune system, Microbes Infect. 2 (2000) 257–272.

[25] B. Kelsall, C.A. Biron, O. Sharma, P.M. Kaye, Dendritic cells at the host−pathogen interface, Nat. Immunol. 3 (2002) 699–702.

[26] F. Santiago−Schwarz, Positive regulation of myeloid dendritic cells lineage, J. Leu. Biol. 66 (1999) 209–216.

[27] M.F. Lipscomb, B.J. Masten, Dendritic cells: Immune regulators in health and disease, Physiol. Rev. 82 (2002) 97-130.

[28] S. Satthaporn, O. Eremin, Dendritic cells (I): biological functions, J. R. Coll. Surg. Edinb. 46 (2001) 9-20.

[29] M. Sadeghi, V. Daniel, W. Weimar, M. Wiesel, O. Hergesell, G. Opelz, Pre-transplant Th1 and post-transplant Th2 cytokine patterns are associated with early acute rejection in renal transplant recipients, Clin. Transplant. 17 (2003) 151-157.

[30] A. Amirzargar, M. Lessanpezeshki, A. Fathi, M. Amirzargar, F. Khosravi, B. Ansaripour, B. Nikbin, Th1/Th2 cytokine analysis in Iranian renal transplants recipients, Transplant. Proc. 37 (2005) 2985-2987.