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Anticancer properties of amino acid and peptide derivatives of mycophenolic acid

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Structured abstract

Background

Although mycophenolic acid (MPA) is applied as prodrugs in clinic as immunosuppressant, it possesses also anticancer activity. MPA acts as inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitor, where carboxylic group at the end of the side chain interacts with Ser 276 of the enzyme *via* hydrogen bonds. Therefore, MPA derivatives with other polar groups indicated high inhibition too. On the other hand, potent anticancer agents like dacarbazine and cisplatin give numerous side-effects.

Objective

Based on the literature data, MPA derivatives should be explored towards anticancer properties. Conversion of carboxylic group of MPA to amide could maintain antiproliferative

activity. Therefore, we decided to investigate several amino acid and peptide derivatives of MPA against chosen cancer cell lines *in vitro*.

Methods

Amides of MPA hold threonine and arginine amino acid unit. These amino acid derivatives were tested as L and D enantiomers and both in free acid and methyl esters forms.

Additionally, MPA was modified with tuftsin or retro-tuftsin as biologically active peptides, which could act as a drug carrier.

Results

Amino acid and peptide derivatives of MPA were investigated *in vitro* as potential anticancer agents on cell lines: Ab melanoma, A375 melanoma and SHSY5Y neuroblastoma. The activity of the tested compounds was compared to parent MPA and known chemotherapeutics: dacarbazine and cisplatin.

Conclusion

Amino acid moiety and sequence of amino acids in peptide part influenced observed activity. The most active amino acid MPA analogues occurred to be D and L-threonine derivatives as methyl esters, probably due to better cell membrane penetration.

1. Introduction

Mycophenolic acid (MPA) **1** (Fig. 1) was isolated for the first time in 1893 as a natural metabolite of *Penicillium* fungi [1-3]. MPA has a broad spectrum of biological activity. This compound is characterized by antibacterial, antifungal, antiviral, anticancer and is used as an immunosuppressive drug, inhibiting T cell proliferation [4]. It is applied during both acute and chronic transplant rejection and belongs to the group of the most frequently prescribed immunosuppressants [4-7].

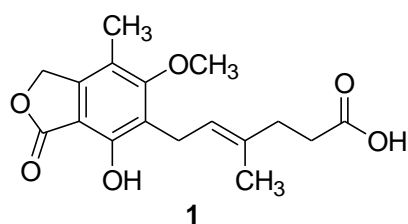


Figure 1. Structure of mycophenolic acid (MPA) **1** [4]

Mycophenolic acid is a non-competitive and reversible inhibitor of the inosine 5'-monophosphate dehydrogenase (IMPDH). The enzyme exists in the two isoforms, where IMPDH I is expressed in normal cells, and IMPDH II is a predominant form in neoplastic cells. MPA indicated activity against both isoforms, but with selectivity towards IMPDH II. Therefore, IMPDH inhibitors based on MPA derivatives revealed also anticancer potential [8-13]. However mycophenolic acid is metabolized in the liver (to a lesser extent in the kidneys and digestive tract) where it is inactivated during glucuronidation which significantly limits the use of its anti-cancer properties [14]. As a result of this process, mainly two metabolites are formed: pharmacologically inactive 7-*O*-glucuronide MPA (MPAG) and acylated glucuronide (AcMPAG) [15-16]. This unfavorable MPA metabolism forces the use of larger amounts of pharmaceutical to obtain the required therapeutic concentration, resulting in a huge number of side effects such as: ailments of the digestive, urogenital, blood or nervous systems. This situation led to the design of new derivatives of MPA, which will be characterized by greater efficiency, stability, selectivity and diminished toxicity [4].

According to literature data, hydroxyl and methyl groups in the aromatic ring, and the *trans* bond in side the chain are necessary to maintain the activity of mycophenolic acid [17-19]. These groups interact with the amino acids belonging to IMPDH, and the *trans* bond results in the proper arrangement of the carboxyl group. Currently, most of the MPA derivatives are based on modifications in the six-carbon side chain, leaving the phthalide moiety intact [4]. Hydrogen bonding between the carboxyl group of MPA and Ser 276 of IMPDH is one of the most important interactions in the MPA-IMPDH complex [18]. Studies confirmed that modifications of this polar group at the end of the chain are promising within designing of active analogs of mycophenolic acid [19]. On the other hand, conjugate formation can improve potential therapeutic properties including both activity and diminish toxicity [20].

In connection with the above, we modified the carboxylic group of MPA, by amide bond formation with an amino group belonging to selected amino acids and peptides. We assumed that the combination of MPA with natural substances could reduce the toxicity of the discussed pharmaceutical together with maintenance of biological activity. In the previous work we described the synthesis of amino acids (containing in their structure L, D-threonine, L, D-arginine, aspartic acid, isoleucine and dimethyl malonate) and peptide (containing in the structure peptide such as: tuftsin modified by- β -Ala, retro-tuftsin modified by Gly and β -Ala) derivatives of MPA followed by their microbiological activity [21,22]. In this work we focused on anticancer properties of selected MPA amino acid derivatives and theirs



conjugates with peptides. The research was carried out on three cell lines: Ab hamster melanoma, A375 human melanoma and SHSY5Y human neuroblastoma.

2. Materials and Methods

Amino acid MPA analogues **2-9** (Fig. 2) were obtained according to general procedure for preparation amino acid derivatives described in our recent work [21,22].

Peptide MPA derivative **10** and **11** (Fig. 3) were synthesized with the mixed anhydride method, followed by condensation of peptide moiety with MPA [21,22].

2.1. Anticancer activity evaluation

For the biological evaluation we used cells of the Ab amelanotic cell line, created as a result of spontaneous transformation from the melanotic form (Ma) of a transplantable hamster melanoma known as the Bomirski melanoma model. Both melanoma lines differ in many biological features. The most important of which characterizes the Ab line is inhibition of melanogenesis (amelanotic cells), faster tumor growth rate compared to the Ma melanotic line and a number of changes in cell structure [23].

The melanoma cells used in the study were obtained from the tumor according to the previously developed non-enzymatic method of obtaining a single cell suspension [24]. For this purpose, cells were mechanically dispersed from tumor mass, isolated on Histopague 1077 (Sigma Aldrich). Melanoma cells of the amelanotic line were isolated after 10-12 days of tumor growth. The viability of such isolated cells was determined in each experiment with the trypan blue assay and it was 80-95%. The experiments' procedures were approved by the Animal Ethics Committee at Medical University of Gdansk (Poland) and conducted in accordance with National Health and Medical Research Council's guide for the care and use of laboratory.

Melanoma cells were incubated in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% Fetal Bovine Serum (Sigma Aldrich) and antibiotics: 100 µg/mL streptomycin, 100 units/mL penicillin (Sigma Aldrich) at 37°C, in a 5% CO₂ atmosphere.

2.1.1. Melanoma, human A375 cell line

Human A375 amelanotic melanoma cells were established from a skin biopsy of 54-year old female malignant melanoma. Cells were obtained from the ATCC collection. They

were grown in DMEM HG medium (Sigma Aldrich) supplemented with 10% Fetal Bovine Serum and antibiotics: 100 µg/mL streptomycin, 100 units/mL penicillin at 37°C in a 5% CO₂ atmosphere.

2.1.2. Neuroblastoma SHSY5Y

Human SH-SY5Y neuroblastoma was originally established from bone marrow biopsy of a neuroblastoma patient in the early 1970s. It was derived from the ATCC collection. Cells between the 10th and 25th passages were used in experiments. The cells were grown in DMEM HG medium (Sigma Aldrich) containing 10% Fetal Bovine Serum and antibiotics: 50 µg/mL streptomycin, 50 µg/mL penicillin at 37°C, in a 5% CO₂ atmosphere.

2.2. Reference compounds

Dacarbazine (Sigma Aldrich) and cisplatin (Sigma Aldrich) as chemotherapeutics used in the treatment of melanoma and neuroblastoma have been used as reference compounds. Dacarbazine and cisplatin were dissolved in 1M HCl and 0,9 % NaCl respectively and diluted in appropriate medium for testing.

2.3. Compounds' samples for testing

The samples of investigated compounds were prepared in concentrations: 0.1; 1; 10; 20; 40; 50; 100; 150 µM. All dilutions of the tested compounds were prepared in the medium and the starting concentration in sterile distilled water with 5% DMSO (Sigma-Aldrich)

2.4. Determination of inhibitory effect (IC₅₀) in the XTT cytotoxicity assay

5x10³ cells per well were plated on 96 well plates in medium for the given cell line (composition above). After 24 hours, the medium was changed and tested compounds were added at concentrations: 0.1; 1; 10; 20; 40; 50; 100; 150 µM. Incubation was carried out for 48 and 72 hours. Cell viability was assessed by XTT test (Sigma-Aldrich). This test assesses the activity of mitochondria that reduce the tetrazolium salt XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) to the water soluble formazan form. The absorbance of the orange product was measured using a microplate reader (Multiscan FC, ThermoScientific) at 450 nm. The measurement of the cytotoxic activity of the tested compounds was based on the IC₅₀ concentration (inhibitory concentration) in which the proliferation/viability, was established by the mitochondrial cell activity, inhibited in 50% relative to the control (100%). When it was not possible to determine the IC₅₀ value, the

percentage of living cells after treatment with the highest tested concentration (150 μ M) was calculated.

3. Results

For the evaluation of anticancer properties, we chose methyl esters of amino acid derivatives of MPA (MPA-Thr-OMe **2**, MPA-Arg(NO₂)-OMe **6**), which in recent studies indicated higher antimicrobial activity [22] in comparison to analogs with free carboxylic groups, probably due to better cell permeability. It was also found that derivatives of D configuration were much less active than those of L configuration [22]. In order to check this effect on the antitumor properties we selected to *in vitro* investigations MPA-D-Thr-OMe **3**, MPA-Thr-OH **4**, MPA-D-Thr-OH **5**, MPA-D-Arg(NO₂)-OMe **7**, MPA-Arg(NO₂)-OH **8** and MPA-D-Arg(NO₂)-OH **9** (Fig. 2).

Amino acid MPA derivatives **2,3,6,7** were obtained by means of a condensation reagent 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI) in the presence of 4-(dimethylamino)pyridine (DMAP) acting as a base in anhydrous *N,N*-dimethylformamide (DMF). Next, methyl esters were hydrolyzed with lithium hydroxide monohydrate to produce derivatives with free carboxylic group **4,5,8,9**. A detailed synthesis was presented in our work [21,22].

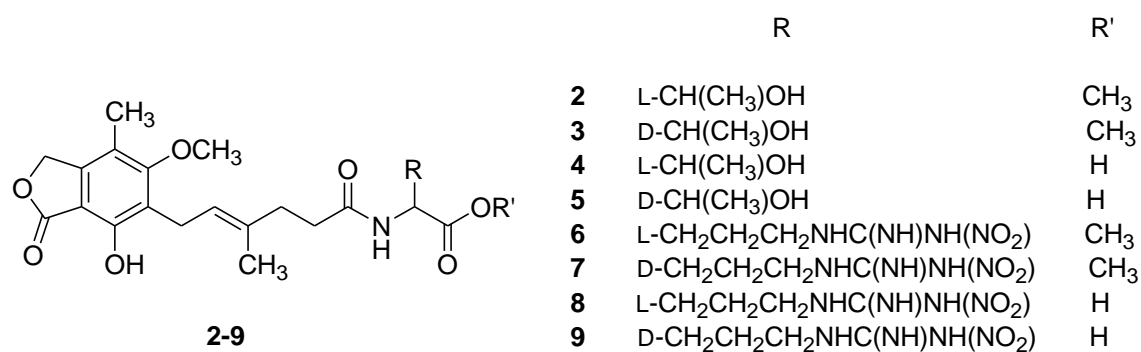


Figure 2 Structures of amino acid derivatives of MPA **2-9**

Subsequently, we focused on MPA conjugates with tuftsin, a natural, produced by the spleen, peptide (Fig. 3). It possesses both immunomodulatory and anticancer properties [25]. Tuftsin is a tetrapeptide (Thr-Lys-Pro-Arg) that specifically binds monocytes, macrophages, and polymorphonuclear leukocytes and potentiates their natural killer (NK) activity against tumors and pathogens [26]. Moreover, conversion of active substance to conjugate with component possessing other biological activity can improve potential therapeutic properties including toxicity [8, 13, 14, 27]. Therefore, we decided to investigate the combination of

mycophenolic acid covalently bonded with tuftsin or retro-tuftsin (reversed amino acids sequence) as conjugates **10** and **11** [22, 27-29], which structures were depicted in Figure 3.

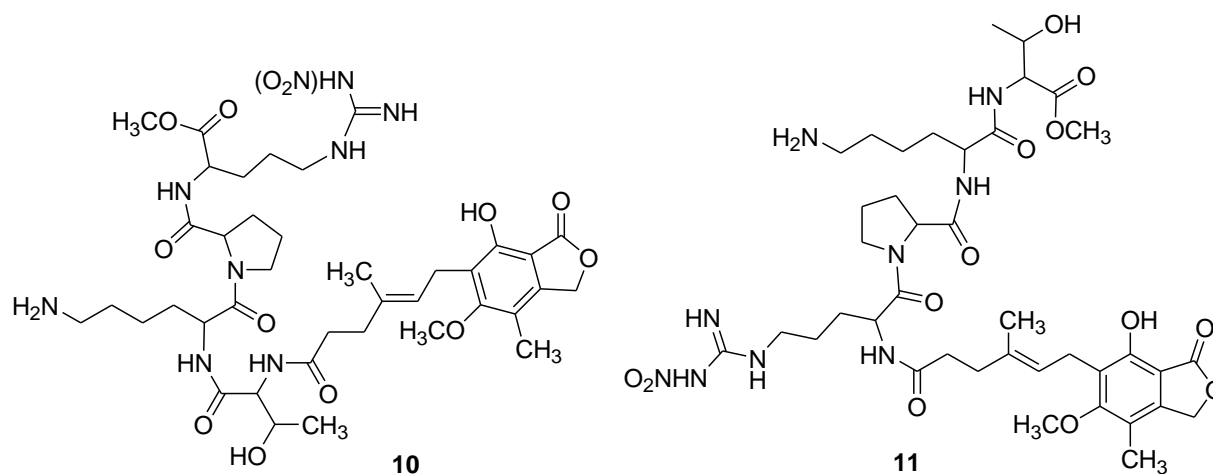


Figure 3. Conjugates of MPA with tuftsin **10** and retro-tuftsin **11**.

3.1. Anticancer evaluation

Melanomas are tumors of melanocyte origin that could be defined as melanotic or amelanotic. Amelanotic melanomas are often difficult to diagnose owing to the lack of melanin production in cells that presence is one of the diagnostic factors [30, 31]. Malignant melanomas are highly aggressive tumors and have an unpredictable course with poor prognosis. Radical excision of the primary tumor is the main stay of treatment. Malignant melanoma is relatively chemoresistant and radioresistant tumor [32-35]. Neuroblastoma (NB) is a neuroendocrine tumor that most commonly originates in the adrenal glands, but can also develop anywhere along the sympathetic nervous system in the neck, chest, abdomen or pelvis [36]. The mainstay of neuroblastoma chemotherapy is combination therapy involving dose-intensive cycles of cisplatin and etoposide alternating with vincristine, doxorubicin, and cyclophosphamide [37]. NB shows a wide range of illnesses. Some neuroblastomas may simply disappear in infants without treatment (spontaneous regression). Other NB can survive a very intense multi-drug therapy, which is why NB is known as one of the most aggressive and difficult to treat cancers in children. Taking into account the fact that neuroblastoma is a pediatric cancer, it is extremely important to look for new drugs that will completely cure cancer without the possibility of its return.

The biological activity of MPA **1** and synthesized derivatives **2-11** were evaluated against cells of two lines of amelanotic melanoma of various origin: the Bomirski hamster Ab melanoma cell line, human A375 melanoma and human SHSY5Y neuroblastoma cells. The



results of preliminary studies on the antitumor activity of MPA and its derivatives are summarized in Table 1.

As reference compounds, we used two standard chemotherapeutics: dacarbazine and cisplatin. As of mid-2006, dacarbazine is used as a single agent in the treatment of metastatic melanoma [38, 39] and as a part of the ABVD chemotherapy regimen for Hodgkin's lymphoma treatment [40] and in the MAID regimen for sarcoma [41, 42]. Cisplatin is a chemotherapy medication used to treat a number of cancers [43]. This includes testicular cancer, ovarian cancer, cervical cancer, breast cancer, bladder cancer, head and neck cancer, esophageal cancer, lung cancer, mesothelioma, brain tumors and neuroblastoma [43].

Table 1. Cytotoxicity of MPA and its derivatives evaluated by XTT assay for melanoma cells (Bomirski hamster Ab melanoma cell line, human A735 melanoma cell line) and human SHSY5Y neuroblastoma cells. Values are arithmetic means \pm standard deviation of at least 3 experiments. IC₅₀ = inhibition dose; concentration at which cell viability (inhibition of mitochondrial activity) is inhibited by 50% in relation to control (100%).

Compound	Ab melanoma				A735 melanoma				SHSY5Y neuroblastoma	
	IC ₅₀ [uM]		% viability at concentration 150 μ M		IC ₅₀ uM		% viability at concentration 150 μ M		IC ₅₀ [uM]	% viability at concentration 150 μ M
	48h	72h	48h	72h	48h	72h	48h	72h	72h	72h
MPA 1	0.2\pm0.1	-	-	-	2.3\pm1.7	2\pm0.9			4.4\pm8.1	
MPA-Thr-OMe 2	15.9 \pm 0.4	18.9\pm6.2	-	-			51.4 \pm 8.6	59.5 \pm 11.3	139.2\pm7.7	

MPA-D-Thr-OMe 3	24.8±7.3	23.6±8.2	-	-	103.8±31.7			54.7±12.5		76.6±8.8
MPA-Thr-OH 4	NA	NA	NA	NA	NO	NO	NO	NO	NO	NO
MPA-D-Thr-OH 5	NA	NA	NA	NA	NO	NO	NO	NO	NO	NO
MPA-Arg(NO ₂)- OMe 6	-	-	50.7±12.6	61.9±27.5	NO	NO	NO	NO	NO	NO
MPA-Arg(NO ₂)- OH 8	85.9±36.4	62.3±12.1	-	-	NO	NO	NO	NO	NO	NO
MPA-D- Arg(NO ₂)-OH 9	NA	-	NA	48.4±29.4	NO	NO	NO	NO	NO	NO
MPA-T 10	NA	-	NA	75.1±12.6	NO	NO	NO	NO	NO	67.9±15.5
MPA-RT 11	NA	-	NA	68.9±15.4	NO	NO	NO	NO	NO	58.5± 6.7
Dacarbazine	-	68.5±20.9	47.5±9.7	-	-	35.2±6.2	48.6±10.7	-	NO	NO
Cisplatin	NO	NO	NO	NO	NO	NO	NO	NO	3.5±3.5	-

NA - inactive
NO - not marked

For all tested cell lines, IC₅₀ doses for MPA **1** were determined. They were 0.2 μM, 2 μM, 4.4 μM for Ab, A375 melanomas and SHSY5Y neuroblastoma, respectively. It should be added here that the IC₅₀ doses for standard chemotherapeutics used in the treatment of both cancers: dacarbazine (melanoma) and cisplatin (neuroblastoma) on the tested cells were 69 μM, 35 μM, 3.5 μM for Ab, A375 melanomas and SHSY5Y neuroblastoma cells respectively. Noteworthy, is the lower dose of MPA **1** required to obtain the IC₅₀ effect on cells of both amelanotic melanoma lines compared to dacarbazine used in the systemic treatment of this cancer. What is more IC₅₀ concentration was calculated after 48 hour stimulation, when for dacarbazine it was not possible to determine IC₅₀ value after this time. A comparable dose of MPA **1** was necessary to obtain the IC₅₀ effect in SHSY5Y cells with respect to cisplatin used in the treatment of neuroblastoma. The results show that mycophenolic acid was more effective on melanoma cells and on neuroblastoma cells almost as effective as commonly used anticancer drugs. MPA worked also faster than dacarbazine on both melanoma cell lines.

The MPA derivatives **2** and **3** showed definitely lower cytotoxic activity than MPA **1**. Both compounds, MPA-Thr-OMe **2** and MPA-D-Thr-OMe **3** inhibited mitochondrial activity in 50% of Ab-melanoma cells at a dose of about 20 μM. This is a dose many times higher than for MPA (0.2 μM) but still three times lower than dacarbazine (69 μM). Worth mentioning is also the fact that IC₅₀ values for these two derivatives were calculated after 48-



hour treatment, which suggest that on this cell line they, like MPA, work faster than dacarbazine. Due to better activity of compounds **2** and **3** than dacarbazine on the Bomirski hamster Ab melanoma cell line of, we checked the effect of derivatives **2** and **3** on the human A375 melanoma cell line. Unfortunately, these cells were less sensitive because under 150 μM dose over 55% of cells were still alive. Decarbazine is characterized by greater cytotoxicity against A375 cell line. Human melanoma cells were slightly more sensitive to the MPA-D-Thr-OMe **3** derivative for which the IC_{50} was 104 μM . The analog MPA-Thr-OMe **2** at 150 μM resulted in inhibition of mitochondria in approximately 40% of A375 cells. The performed tests on the SHSY5Y neuroblastoma cell line revealed the similar situation. NB cell line was slightly more sensitive to the MPA-Thr-OMe **2** derivative for which the IC_{50} was 139.2 μM . The compound MPA-D-Thr-OMe **3** at a dose of 150 μM caused the inhibition of mitochondria in just over 20% on SHSY5Y cells. Both compounds showed significantly worse activity than cisplatin. Compounds containing threonine with a free carboxylic group **4** and **5** did not show any activity on any of the tested cancer cell lines.

Analog of MPA-Arg(NO_2)-OMe **6** at a concentration of 150 μM inhibited 40-50% of mitochondrial activity of Ab melanoma, therefore no studies were carried out on subsequent cell lines. Unfortunately, the MPA-D-Arg(NO_2)-OMe **7** derivative proved to be insoluble in medium and was not tested.

Evaluation of the activity of compounds with free carboxylic group **8** and **9** on Ab melanoma cells showed that analog MPA-Arg(NO_2)-OH **8** inhibited mitochondrial activity in 50% of cells with IC_{50} results of 62 μM . This value is very similar to the IC_{50} determined for dacarbazine, which is 68.5 μM . However, the derivative MPA-D-Arg(NO_2)-OH **9** caused a similar effect at a dose slightly over twice higher (150 μM).

Peptide analogs of MPA containing in their structure: tuftsin **10** and retro-tuftsin **11** in the highest tested concentration (150 μM) exhibited a similar effect on melanoma Ab and neuroblastoma SHSY5Y cells lines, resulting in inhibition of mitochondrial activity in about 30% of cells.

4. Discussion

Parent MPA **1** indicated the high activity to all tested cell lines. Analyzing the results, it can be concluded that the free, unmodified carboxyl group is responsible for such good activity.

In the case of MPA derivatives with threonine **2-5** we noted the decisive advantage of compounds with a carboxyl group protected with a methyl ester of **2,3** compared to those with



a free carboxyl group **4,5** which showed no activity. The superiority of derivatives covered with methyl ester was also found during previous studies [19]. Analyzing the effect of configuration, we note that the MPA-Thr-OMe **2** analog better inhibited mitochondrial activity in two cell lines: Ab melanoma and SHSYBY neuroblastoma than a derivative in configuration D **3**. This fact gives a slight advantage of L configuration over D.

All three tested MPA derivatives with arginine **6,8** and **9** occurred to be active. However, the IC₅₀ value was only attributable to the compound of the L configuration with the free carboxylic group **8**. The presence of the nitro group, which has a protective function for the guanidinium group, may affect the activity of all three compounds. Analyzing the effect of the amino acid configuration, we can conclude that compounds **6** and **8** with the L configuration indicated better activity compared to the D **9** isomer.

The combination of MPA with peptides such as tuftsin **10** and retro-tuftsin **11** allowed for the inhibition of proliferation of Ab melanoma cells and SHSYBY neuroblastoma in approximately 30%. We did not notice a significant difference in the biological activities between this two peptide derivatives. The relatively low inhibition of cell activity may be due to the rapid disintegration of compounds **10** and **11** by proteases.

5. Conclusion

MPA amide derivatives **2-11** were evaluated in biological tests on three cancer cell lines: hamster Ab melanoma, human A375 melanoma and SHSY5Y neuroblastoma. As reference compounds, we chose the drugs used in anticancer therapy: dacarbazine and cisplatin. We found that the parent compound MPA **1** exhibited better activity than dacarbazine on cells of both amelanotic melanoma lines and cytotoxicity comparable to cisplatin on neuroblastoma cell line.

Among the tested novel amino acid: analogs of MPA, considerable properties showed: MPA-Thr-OMe **2**, MPA-D-Thr-OMe **3** and MPA-Arg(NO₂)-OH **8**. Analogs L **2** and D Thr **3** indicated three times better activity against the Ab cell line than dacarbazine. Unfortunately, in case of the human melanoma cell line, their activity was definitely weaker in relation to the pharmaceutical used in the clinics. The same situation occurred for the SHSY5Y neuroblastoma.

The MPA modification method presented by us may lead to a reduction of the toxicity of the parent compound itself while maintaining biological activity. This fact may in the future contribute to the introduction of mycophenolic acid analogues as chemotherapeutics.



Obtained data also suggest that MPA and its derivatives can potentially be useful in treatment of amelanotic melanoma and neuroblastoma.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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