SYNTHESIS AND IMMUNOREGULATORY PROPERTIES OF SELECTED 5-AMINO-3-METHYL-4-ISOXAZOLECARBOXYLIC ACID BENZYLAMIDES

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Abstract: The aim of the study was to characterize a series of isoxazole derivatives in several immunological tests in vitro and in vivo, in mouse and human models. The human model included measurement of: viability of peripheral blood mononuclear cells (PBMC), phytohemagglutinin A (PHA)-induced proliferation of PBMC, production of tumor necrosis factor α (TNF α) in whole blood cultures stimulated with lipopolysaccharide (LPS) and growth of SW-948 and L1210 tumor cell lines. Experiments in mice encompassed the following tests: secondary, humoral immune response splenocytes to sheep erythrocytes (SRBC) in vitro, delayed type hypersensitivity (DTH) to ovalbumin (OVA) and carrageenan-induced foot edema. All compounds were non-toxic against PBMC and displayed differential, dose-dependent suppressive properties in the model of PHA-induced PMBC proliferation. They also exhibited differential, mostly inhibitory effects on TNF α production. The inhibitory actions on growth of tumor cell lines were moderate. MO5 (5-amino-3-methyl-N-(4-methyl-benzyl)-4-isoxazolecarboxamide) was most suppressive in the proliferation and TNF α production tests, it was, therefore, selected for in vitro and in vivo studies in the mouse models. The compound inhibited the humoral immune response in vitro, stimulated the inductive phase of DTH in vivo, although it inhibited the eliciting phase of that response. The compound also inhibited the carrageenan skin reaction. MO5 combines strong anti-proliferative and anti-inflammatory activities, it is therefore attractive for further studies in more advanced animal models as a potential therapeutic.

Keywords: carrageenan; immune response; isoxazoles; PBMC; PHA; TNF α

The hitherto efforts, aimed to supply for the pharmaceutical market new therapeutics that would prove effective in treatment of diseases resulting from overstimulation of suppression of the immune system, have been insufficient. Therefore, extensive search for new compounds from various classes of chemicals is continued. The isoxazole system has been a source of valuable drugs. Isoxazoles belong to the most important azoles (1). A number of pharmaceuticals as well as bioactive natural products integrate azoles, and specifically isoxazoles, as key pharmacophores pivotal for biological activities of drugs such as: valdecoxib (COX-2 inhibitor), sulfamethoxazole (PABA antagonist), oxacillin (β-lactam antibiotic), isoxacilutole (4-hydroxyphenylpyruvate dioxygenase inhibitor) or leflunomide (anti-rheumatic drug) (2). In these molecules, isoxazole acts as an indispensable pharmacophore enforcing the desired pharmacological activity due to a unique positioning in a 1,2-relationship of two electronegative heteroatoms that are capable of engaging in hydrogen bond donor/acceptor interactions with a variety of enzymes, receptors and messengers, unavailable for other rings systems and potential therapeutics (3, 4).

Isoxazole derivatives were reported to possess a variety of biological activities including antidepressant (5), dopamine D 4 receptor activity (6), anticonvulsant (7), antiviral (8), and antifungal (9). In our studies, (10-12) isoxazole derivatives with immunostimulatory and immunosuppressive properties were found. We obtained the mono-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides (Fig. 1) with the immunostimulatory activity equal to or higher than levamisole (a reference drug).

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Further characterization of these compounds showed their very low toxicity (10-12).

Among these compounds, 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides, substituted in position 4 with a strong electrophilic group, were most active. 4-Chlorophenylamide of 5-amino-3-methyl-4-isoxazolecarboxylic acid (Fig. 2) proved strong stimulating effect on the humoral and cellular immune responses (10-12).

In contrast, di- and tri-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides showed immunosuppressive activity more effective than cyclosporine A (a reference drug). Dichloro- and trimethoxy- derivatives were inhibitors of the humoral immune response (11-13).

Also, N’-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide (Fig. 3) showed immunosuppressive activity in mouse and human experimental models (14).

An interesting immunomodulatory activity of the compounds described above led us to synthesize a new series of 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides substituted on the phenyl ring (Fig. 4).

Then, biological and quantum-chemical studies for obtained compounds were performed. The

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**Figure 1.** Mono-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides

**Figure 2.** 4-Chlorophenylamide of 5-amino-3-methyl-4-isoxazolecarboxylic acid

**Figure 3.** N’-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide

**Figure 4.** 5-Amino-3-methyl-4-isoxazolecarboxylic acid benzylamides substituted on the phenyl ring

**Figure 5.** Toxicity of the compounds against human PBMC. The compounds were used at concentrations of 1, 10 and 100 µg/mL. The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations. The cell toxicity was compared with toxicity of DMSO used as solvent.
results led to evaluation of the immunological activities and the structure-activity relationships of this class of compounds.

The aim of this study was to synthesize substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides, evaluate their immunological properties in preliminary models in vitro, select a most active compound and study its activity in the mouse models on nonspecific and specific immune responses in vivo.

EXPERIMENTAL

Biology

Animals

CBA mice of both sexes, 8-12 week old, derived from a breeding colony in Ilkowice, Poland were used. The mice were kept in standard conditions with free access to granulated food and filtered water. The local ethics committee for animal experiments approved the study (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław, 20/01/2010, 8/2010).

Reagents

Hanks’ medium, RPMI-1640 medium, Eagle’s medium and LSM (lymphocyte separation medium; 1.077 g/mL) were from Cytogen, Sinn, Germany; MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), L-glutamine, sodium pyruvate, 2-mercaptoethanol, 0.83% NH₄Cl solution, antibiotics (penicillin and streptomycin), lipopolysaccharide (LPS) from Escherichia coli, serotype O:111:B4, concanavalin A (ConA), cyclosporine A (CsA), cisplatin and carrageenan were from Sigma, Saint Louis, U.S.A. Freund’s complete adjuvant (cFa), Freund’s incomplete adjuvant (iFa) and fetal calf serum (FCS) were from BD Biosciences, U.S.A.. Dexamethasone (Dexaven®) was from Jelfa, Poland. Sheep red blood cells (SRBC) were supplied by Wrocław University of Life and Environmental Sciences, Poland. TNF α was measured by enzyme-linked immunosassay (ELISA Ready-SET-Go Affymetrix eBioscience). Cyclooxygenase (COX) Inhibitor Screening Assay Kit was supplied by Cayman Chemical, Ann Arbor, U.S.A.

Preparation of the compounds for biological assays

The compounds were dissolved in DMSO (5 mg/200 µL) and subsequently diluted to 1 mL of RPMI-1640 medium for in vitro studies. As a control, appropriate dilutions of DMSO in RMPI-1640 medium were used. For in vivo administration the compound was further diluted in 0.9% saline.

Isolation of the human peripheral blood mononuclear cells (PBMC)

Venous peripheral blood from a single donor (healthy adult volunteer) was withdrawn into heparinized syringes and diluted twice with PBS. PBMC were isolated by centrifugation on lymphocyte separation medium and centrifuged at 400 × g for 20 min at 4°C. The interphase cells were then washed three times with Hanks’ medium and resuspended in a culture medium, referred to below as the culture medium, consisting of RPMI-1640, supplemented with 10% FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, at density of 2 × 10⁶ cells/mL.

Cytotoxicity of the compounds against human PBMC

PBMC at density of 3 × 10⁶/100 µL/well, resuspended in the culture medium, were cultured for 24 h in a cell culture incubator with the compounds at 1, 10 and 100 µg/mL concentrations. Cell survival was determined by MTT colorimetric method (15). The data are presented as a mean optical density (OD) values at 550 nm ± standard error (SE) from quadruplicate determinations.

Phytohemagglutinin A (PHA)-induced proliferation of human blood mononuclear cells

PBMC were distributed into 96-well flat-bottom plates in 100 µL aliquots (2 × 10⁵ cells/well). PHA was added at a concentration of 5 µg/mL. The compounds were tested at doses of 1, 10 and 100 µg/mL. DMSO at appropriate dilutions served as control. After a four-day incubation in a cell culture incubator, the proliferative response of the cells was determined by the MTT colorimetric method (15). The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations.

Lipopolysaccharide (LPS)-induced TNF α production in whole blood cell culture

Venous blood from a single donor (healthy adult volunteer) was diluted 10 × with RPMI-1640 medium and distributed in 1 mL aliquots in 24-well culture plates. The cultures were stimulated by addition of 1 µg/mL of LPS. The compounds were added to the cultures at concentrations of 1-100 µg/mL. Appropriate dilutions of DMSO served as controls. After overnight incubation in a cell culture incubator, the supernatants were harvested and frozen at -20°C until cytokine determination by ELISA kit
according to manufacturer’s instructions. The results are presented in pg/mL.

**Tumor cell lines**

L-1210 lymphoma and SW-948 colon tumor cell lines derived from the Collection of Cell Lines of The Institute of Immunology and Experimental Therapy, Wrocław, Poland. The lines were resuspended in the culture medium and distributed into 96-well flat bottom plates. L-1210 was present at 1.5 \( \times 10^4 \) cells/100 µL/well while SW-948 at 2.5 \( \times 10^4 \) cells/100 µL/well. Cisplatin served as a reference drug. The studied preparations were added to the wells at the indicated concentrations. After 3-day incubation in a cell culture incubator, the proliferation was determined using MTT colorimetric method (15). The data are presented as mean OD values at 550 nm ± SE from quadruplicate determinations.

**Secondary humoral immune response in vitro**

Mice (n = 5) were sensitized intraperitoneally (i.p.) with 0.2 mL of 5% sheep red blood cells (SRBC) suspension. After 4 days, the spleens were isolated, a single cell suspension prepared by pressing the organs against a plastic screen into a cold Hanks’ medium, washed 2× with Hanks’ medium and resuspended in the culture medium at a density of 5 \( \times 10^6 \)/mL. The cells were distributed to 24-well culture plates in 1 mL aliquots and 0.05 mL of 0.005% SRBC was added as antigen. MO5 compound was used at 1, 10 and 100 µg/mL. The number of antibody forming cells (AFC) in the cultures was determined using the MTT colorimetric method.

Table 1. The analytical data of the obtained compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting point (°C)</th>
<th>Yield (%)</th>
<th>Formula</th>
<th>Molecular weight (g/mol)</th>
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<tr>
<td>MO1</td>
<td>126.5-127.5</td>
<td>45</td>
<td>C₇H₁₃N₃O₂</td>
<td>231.25</td>
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<td>MO2</td>
<td>146-147</td>
<td>52</td>
<td>C₇H₁₂ClN₃O₂</td>
<td>265.70</td>
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<tr>
<td>MO3</td>
<td>167-168</td>
<td>54</td>
<td>C₇H₁₁Cl₂N₃O₂</td>
<td>300.15</td>
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<tr>
<td>MO4</td>
<td>133-134</td>
<td>48</td>
<td>C₇H₁₁ClN₃O₂</td>
<td>265.70</td>
</tr>
<tr>
<td>MO5</td>
<td>152-153</td>
<td>63</td>
<td>C₇H₁₂N₂O₂</td>
<td>245.28</td>
</tr>
<tr>
<td>MO6</td>
<td>145-146</td>
<td>58</td>
<td>C₇H₁₂N₂O₂</td>
<td>261.28</td>
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<tr>
<td>MO7</td>
<td>176-177</td>
<td>49</td>
<td>C₇H₁₃FN₃O₂</td>
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<tr>
<td>MO8</td>
<td>167-168</td>
<td>64</td>
<td>C₇H₁₃N₂O₂</td>
<td>261.28</td>
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<tr>
<td>MO9</td>
<td>162-163</td>
<td>57</td>
<td>C₇H₁₄N₃O₄</td>
<td>291.30</td>
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<tr>
<td>MO10</td>
<td>187-188</td>
<td>52</td>
<td>C₇H₁₃F₂N₂O₂</td>
<td>299.25</td>
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were determined using a method of local hemolysis in agar gel (16). The results are presented as a mean numbers of AFC per 10^6 splenocytes ± SE.

**Delayed type hypersensitivity to ovalbumin (OVA)**

Mice (n = 5) were sensitized subcutaneously (s.c.) with 5 µg ovalbumin (OVA) emulsified in Freund’s complete adjuvant (cFa) in the tail base. After 4 days, the mice were challenged s.c. with 50 µg OVA in incomplete Freund’s antigen (iFa) in the hind footpads. Following next 24 h, the footpad thickness was measured using a spring caliper with 0.05 mm accuracy. MO5 compound was administered to mice i.p., at 100 µg dose, 30 min before sen-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical structure</th>
<th>Spectroscopic data</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO1</td>
<td><img src="MO1.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.288 (s, 3H, CH3), 4.381-4.401 (d, 2H, CH2), 7.221-7.300 (m, 5H, CH-aromat), 7.415 (t, 1H, NH), 7.453 (s, 2H, NH2). IR (cm^-1): 1659 C=O, 3324 NH, 3428-3441 NH2.</td>
</tr>
<tr>
<td>MO2</td>
<td><img src="MO2.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.282 (s, 3H, CH3), 4.350-4.370 (d, 2H, CH2), 7.292-7.320 (d, 2H, CH-aromat), 7.343-7.372 (d, 2H, CH-aromat), 7.468 (t, 1H, NH), 7.478 (s, 2H, NH2). IR (cm^-1): 1652 C=O, 3324 NH, 3424-3446 NH2.</td>
</tr>
<tr>
<td>MO3</td>
<td><img src="MO3.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.310 (s, 3H, CH3), 4.404-4.423 (d, 2H, CH2), 7.322-7.408 (m, 2H, CH-aromat), 7.415 (s, 1H, CH-aromat), 7.444 (t, 1H, NH), 7.478 (s, 2H, NH2). IR (cm^-1): 1658 C=O, 3316, 3417-3442 NH2.</td>
</tr>
<tr>
<td>MO4</td>
<td><img src="MO4.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.290 (s, 3H, CH3), 4.36-4.368 (d, 2H, CH2), 7.270-7.338 (m, 3H, CH-aromat), 7.363 (s, 1H, CH-aromat), 7.444 (t, 1H, NH), 7.478 (s, 2H, NH2). IR (cm^-1): 1660 C=O, 3322 NH, 3420-3445 NH2.</td>
</tr>
<tr>
<td>MO5</td>
<td><img src="MO5.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.317 (s, 3H, CH3), 2.345 (s, 3H, CH3), 4.522-4.541 (d, 2H, CH2), 7.085-7.112 (d, 2H, CH-aromat), 7.16-7.188 (d, 2H, CH-aromat), 7.304-7.376 (t, 1H, NH), 7.452 (s, 2H, NH2). IR (cm^-1): 1657 C=O, 3319 NH, 3421-3443 NH2.</td>
</tr>
<tr>
<td>MO6</td>
<td><img src="MO6.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.268 (s, 3H, CH3), 3.705 (s, 3H, OCH3), 4.301-4.321 (d, 2H, CH2), 6.843-6.872 (d, 2H, CH-aromat), 7.202-7.231 (d, 2H, CH-aromat), 7.319-7.359 (t, 1H, NH), 7.445 (s, 2H, NH2). IR (cm^-1): 1655 C=O, 3315 NH, 3416-3439 NH2.</td>
</tr>
<tr>
<td>MO7</td>
<td><img src="MO7.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.280 (s, 3H, CH3), 4.350-4.369 (d, 2H, CH2), 7.117-7.146 (d, 2H, CH-aromat), 7.302-7.321 (d, 2H, CH-aromat), 7.349-7.413 (t, 1H, NH), 7.455 (s, 2H, NH2). IR (cm^-1): 1661 C=O, 3322 NH, 3423-3447 NH2.</td>
</tr>
<tr>
<td>MO8</td>
<td><img src="MO8.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.299 (s, 3H, CH3), 3.811 (s, 3H, OCH3), 4.359-4.379 (d, 2H, CH2), 6.866-7.166 (m, 4H, CH-aromat), 7.183-7.235 (t, 1H, NH), 7.448 (s, 2H, NH2). IR (cm^-1): 1657 C=O, 3323 NH, 3418-3443 NH2.</td>
</tr>
<tr>
<td>MO9</td>
<td><img src="MO9.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.278 (s, 3H, CH3), 3.722 (s, 3H, OCH3), 3.794 (s, 3H, OCH3), 4.285-4.304 (d, 2H, CH2), 6.446-6.541 (m, 2H, CH-aromat), 7.055 (s, 1H, CH-aromat), 7.083-7.105 (t, 1H, NH), 7.437 (s, 2H, NH2). IR (cm^-1): 1655 C=O, 3314 NH, 3423-3447 NH2.</td>
</tr>
<tr>
<td>MO10</td>
<td><img src="MO10.png" alt="Image" /></td>
<td>1H NMR (DMSO-d6, δ ppm): 2.296 (s, 3H, CH3), 4.452-4.471 (d, 2H, CH2), 7.539 (s, 1H, CH-aromat), 7.489-7.518 (m, 3H, CH-aromat), 7.563-7.601 (t, 1H, NH), 7.642 (s, 2H, NH2). IR (cm^-1): 1659 C=O, 3324 NH, 3417-3438 NH2.</td>
</tr>
</tbody>
</table>
sitization of mice or 30 min before elicitation of the response. Only DMSO (solvent for compound) was administered to control DMSO mice. The background, non-specific inflammatory response was inducted by administration of an eliciting dose of OVA to naive mice and was subtracted from the response of sensitized mice. The results were presented as a mean value of antigen-specific increase of footpad thickness measured in 5 mice (10 measurements) and expressed in DTH units (one DTH unit = 10^-2 cm) ± SE.

Carrageenan-induced foot pad edema
The compound MO5 was given i.p. at a dose of 100 µg per mouse at 24 h and 1 h before carrageenan injection. Dexamethasone (Dex) as reference drug, was used at a dose of 40 µg, i.p., at 1 h before carrageenan injection. Mice (n = 5) were given 2% carrageenan solution (100 µg in 50 µL 0.9% saline) s.c. into hind foot pads and after 4 h the foot pad thickness was measured by means of a spring caliper with 0.05 mm accuracy. The background foot pads thickness was induced by s.c. administration of 0.9% NaCl into hind foot pads of naive mice and was subtracted from the response of carrageenan injected mice. The results were presented as a mean value of antigen-specific increase of footpad thickness measured in 5 mice (10 measurements) and expressed in mm ± SE.

Figure 6. Effect of the compounds on PHA-induced human PBMC proliferation. The compounds were used at concentrations of 1, 10 and 100 µg/mL. The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations. The proliferative response was compared with DMSO used as solvent. *p < 0.05, when compared with DMSO cultures

Figure 7. Effect of the compounds on LPS-induced TNF-α production in human whole blood cell culture. The compounds were used at concentrations of 1, 10 and 100 µg/mL. The results are presented in pg/mL. The results were compared with DMSO used as solvent
Data analysis and statistical procedures

The results are presented as mean values ± SE; n represents the number of mice in each of experimental group. Brown-Forsyth’s test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with the Tukey’s test to estimate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal-Wallis analysis of variance, as indicated in the text. Significance was determined at *p < 0.05. Statistical analysis was performed using STATISTICA 7 for Windows.

Chemistry

General techniques

Melting points were determined in a Büchi apparatus (Laboratoriums-Technik AG, Flawil, Switzerland), heated table Kofler system (Wagner&Munz) and were uncorrected. Thin layer chromatography (TLC) was carried out on Polygram SIL G/UV 254 nm glass silica gel plates (Macherey-Nagel), using the developing system CHCl₃-CH₃OH 9 : 1, v/v and detected with UV Fisher Bioblock Scientific 254 nm lamps. IR spectra were recorded with Perkin Elmer Spectrum Version 10.03.08, and ¹H NMR spectra were obtained in DMSO-d₆, using a Bruker ARX 300 MHz spectrometer (using TMS as the internal standard).

Elemental analyses were performed within ± 0.3% of the theoretical values (Carlo Erba NA, 1500 equipment).

General procedure for preparation of the compounds MO1-M10

New, not described, derivatives 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides...
were synthesized from 5-amino-3-methyl-4-isoxazolecarboxylic acid azide in reaction with relevant substituted benzylamines (Scheme 1).

Five mL of propanol-2 and 5 mmol of relevant substituted benzylamines were added to 2.5 mmol of 5-amino-3-methyl-4-isoxazolecarboxylic acid azide, obtained according to a previously described method (17). The solution was stirred and heated for 4 h in temp. 82-83°C, then the reaction mixture was stirred for 24 h at room temperature. At the end of the reaction (controlled by TLC), the solid, which separated out, was filtered and washed with propanol-2. When the solid was not formed, propanol-2 was evaporated in vacuo from the mixture. The crude product was recrystallized from propan-2-ol. The analytical data of the obtained compounds are presented in Table 1 and the structure and spectroscopic data of the obtained compounds are presented in Table 2.

RESULTS AND DISCUSSION

In the first phase of the investigation we monitored the compounds for their possible cytotoxic effects against human PBMC. The results (Fig. 5) indicated that the studied compounds, at the concentration range of 1-100 µg/mL, did not decrease cell viability of human PBMC in 24 h cultures in comparison with respective DMSO controls. Moreover, at 100 µg/mL, the viability of cells increased. This effect was, in a way, correlated with the cell protective effect of DMSO at 100 µg/mL.

Next, the compounds were screened for their potential inhibitory activities in the model of human PBMC stimulated in culture with PHA – the T-cell mitogen. The results shown in Figure 6 revealed differential and dose-dependent actions of the compounds on PHA-induced PBMC proliferation. The suppressive effects were already evident for the concentration of 10 µg/mL. The actions of some compounds at 100 µg/mL resulted in a total inhibition of mitogen-induced proliferation. MO1, MO7 and MO10 compounds were weakly suppressive whereas other compounds belonged to moderately acting inhibitors.

The compounds were also checked for their ability to inhibit LPS-induced TNF α production in human whole blood cell cultures (Fig. 7). The compounds displayed differential, dose-dependent, mostly suppressive (at concentration of 100 µg/mL) effects on the cytokine production. MO6 and MO9 compounds showed stimulatory actions at low doses. MO5 particularly strongly suppressed TNF α production.

Taking into account relatively strong anti-proliferative properties of some compounds we wished to check effects of MO5 on growth of two tumor cell lines (colon cancer SW-948 and lymphocytic leukemia L-1210). Cisplatin was chosen as a reference drug. The results are presented in Figure 8ab. A growth inhibitory effect (at 25 µg/mL) was relatively quickly achieved with SW-948 cell line, further increase of concentration did not, however, increase the inhibitory effect of the compound (Fig. 8a). In
the case of L-1210 cells (Fig. 8b) the growth suppressive effect was distinctly weaker, although dose-dependent.

Subsequently, we decided to investigate the effects of MO5 on the antigen-specific immune response. First, the effect of MO5 compound was checked in the model of the secondary, humoral immune response of mouse splenocytes to SRBC in vitro. The results presented in Figure 9 show that the suppression of the numbers of cells producing anti-SRBC antibodies by MO5 occurred only at concentration of 10 µg/mL. No further suppression was observed at 100 µg/mL. The suppressive effect by cyclosporine A (CsA) – the reference drug – was significantly stronger (till the background levels at 10 µg/mL).

The mouse model of the delayed type hypersensitivity (DTH) in vivo allows to dissect effects of a given compound on induction or elicitation phases of this type of the immune response (Fig. 10ab). MO5 was administered to mice 30 min before sensitization of animals with OVA (Fig. 10a) or 30 min before elicitation of the DTH reaction with the antigen (Fig. 10b). MO5 enhanced the response to OVA when given before immunization (Fig. 10a). However, the compound given before the sensitizing dose of antigen strongly inhibited that type of immune response (Fig. 10b).

Lastly, we wished to investigate the effect of the compound on a nonspecific, carrageenan-induced foot pad inflammation. The mice were
given MO5 compound (100 µg) i.p., at 24 h and 1 h before injection of carrageenan. The compound significantly reduced the foot pad edema when measured 4 h after carrageenan administration (Fig. 11). Dexamethasone, the reference drug, given in a single 50 µg dose 1 h before carrageenan, was more potent.

DISCUSSION

In this investigation we presented anti-proliferative and anti-inflammatory properties of the studied compounds with regard to human PBMC with no apparent toxicity against these cells. MO5, selected as most interesting compound, inhibited the secondary humoral immune response in vitro, suppressed the effector phase of the DTH reaction and the carrageenan reaction. Its suppressive effect may chiefly depend on inhibition of activity of inflammation mediators accompanying both antigen-specific immune response (DTH) as well as the early, nonspecific inflammatory response to carrageenan. In the latter test, MO5 behaved similarly as RM33 (3,5,7-trimetyloizoxazolo[5,4-e]triazepin-4-on) in carrageenan-induced foot pad edema in rats (18). The inhibition of foot pad edema by MO5 was also correlated with its ability to strongly suppress LPS-induced TNF α in the whole blood cell model. It is, therefore, likely that inhibition of pro-inflammatory cytokine production and induction of mastocyte apoptosis, as in the case of leflunomide (19), could account for a possible mechanism of action in this case. On the other hand, MO5 stimulated the inductive phase of the cellular immune response. That interesting phenomenon could involve stimulation of antigen presentation process and/or preferential recruitment of antigen-specific T cells of Th1 type. Thus, the immunologic characteristic of the compound appears to be very intriguing. The ability to stimulate the cellular immune response by the compound administered prior to antigen may also indicate its potential adjuvant value. The structure/activity analysis of MO5 suggests that the CH3 group at position 4 of phenyl ring may be critical for its particularly strong activity.

At this stage of investigation, it is difficult to propose a mechanism of action for MO compounds. We excluded a possibility that MO5 could be a cyclooxygenase 1 or 2 inhibitor (data not shown) as, for example another oxazolone derivative (20). Other mechanisms of its action, possibly involving arrest in a definite cell cycle, apoptosis or inhibition of NFκB expression (21-24) are likely and will be investigated in forthcoming investigations.

CONCLUSIONS

The activity screening of the series of selected 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides and subsequent studies in the mouse
models revealed a potential therapeutic value of 5-amino-3-methyl-N-(4-methylbenzyl)-4-isoxazole-carboxamide (MO5). The compound exhibited strong anti-proliferative and anti-inflammatory properties. On the other hand, MO5 had the ability to enhance manifestation of the cellular immune response. Its mechanism of action and potential therapeutic utility in more advanced experimental models remain to be established.

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