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NEW LEAD STRUCTURES IN THE ISOXAZOLE SYSTEM: RELATIONSHIP BETWEEN QUANTUM CHEMICAL PARAMETERS AND IMMUNOLOGICAL ACTIVITY

MARCIN MĄCZYŃSKI[1]*, STANISŁAW RYNG[1], JOLANTA ARTYM[2], MAJA KOCIĘBA[2], MICHAŁ ZIMECKI[2], KATARZYNA BRUDNIK[3] and JERZY T. JODKOWSKI[3]

[1]Department of Oorganic Chemistry, Faculty of Pharmacy,
Wroclaw Medical University, Borowska 211a, 50-556 Wrocław, Poland
[2]Laboratory of Immunobiology, Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences, Weigla 12, 53-114 Wrocław, Poland
[3]Department of Physical Chemistry, Faculty of Pharmacy, Wroclaw Medical University,
Borowska 211a, 50-556 Wrocław, Poland

Abstract: Potential immunological activities of three compounds: RM54 and its two derivatives RM55 and RM56, were evaluated in several, selected *in vitro* and *in vivo* tests such as: mitogen-induced lymphocyte proliferation, cytokine production, the humoral immune response *in vitro* and carrageenan test. Leflunomide served as a reference drug. The studied compounds showed differential, generally immunosuppressive properties. RM56 exhibited stronger suppressive activities as compared to RM54 and RM55. In particular, RM56 displayed the strongest activity in suppression of the carrageenan inflammation that was correlated with strong suppression of the humoral immune response *in vitro* and lymphocyte proliferation. Density Functional Theory (DFT) was employed to shed a light on molecular properties of the investigated compounds. The geometrical parameters of the studied molecular structures were fully optimized at the B3LYP/6-311G(d,p) level. The atomic charges distribution derived on the base of the Mulliken population analysis was correlated with immunological activity of RM54, RM55 and RM56. The obtained relationships show that the isoxazole ring plays an important role in the observed immunological activities. We also suggest that due to strong anti-inflammatory and anti-proliferative properties of RM-56, potential therapeutic applications of this derivative can be broad.

Keywords: isoxazole, DFT, immune response, TNF-α, carrageenan inflammation, mice

Among drugs targeting the immune system a few categories, both of natural origin and synthetic ones can be mentioned, such as calcineurin inhibitors (cyclosporine, tacrolimus) or isoxazole derivatives (acivicin, leflunomide) of well documented biological activity. Although these therapeutics are commonly used, their side-effects encourage to search new compounds characterized by more beneficial therapeutic properties.

Our efforts to obtain new immunomodulatory compounds resulted in synthesis of new amide derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid (1). The compounds demonstrated strong and differential immunological activities. In the first series, we initially found high antitumor activity of the compounds *in vivo* (2), and subsequently lack of cytotoxicity associated with an immunostimulatory action (3). These findings opened new possibilities in search for new antitumor drugs that act by a different mechanism than cytotoxicity. Therefore, syntheses of three series of amide derivatives (aromatic, heterocyclic and aliphatic) were designed. The syntheses resulted in obtaining a series of amide derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid **I-III** (2-4) (Fig. 1).

After establishing interdependences between types of the amide substituent, the most intriguing question regarding structure-activity relationship was whether the immunostimulatory effect was dependent on the amide structure. The previously conducted studies on quantitative activity relationship indicated special correlations between charge of the carbonyl groups atoms and the biological

* Corresponding author: e-mail: marcin.maczynski@umed.wroc.pl; phone: +48 71 784 03 44, fax: +48 71 784 03 42

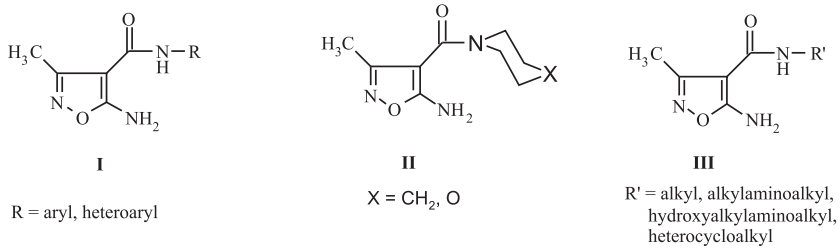


Figure 1. The structures of amide derivatives of 5-amino-3-methyl-4-isoxazolecarboxylic acid

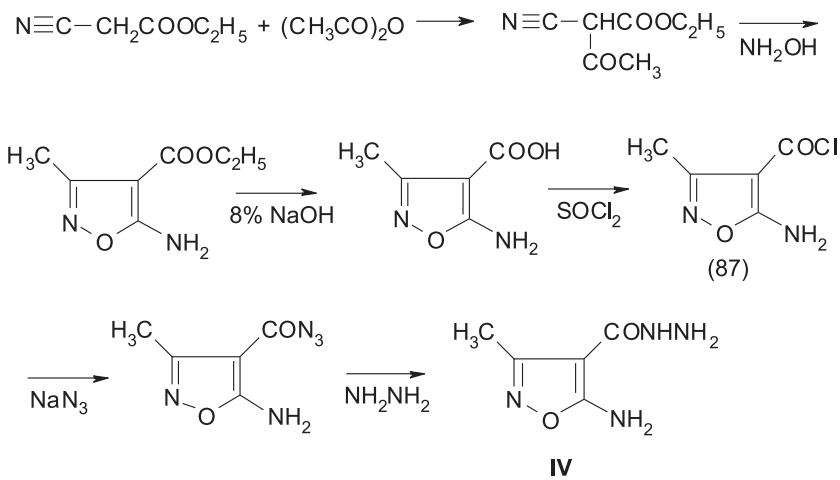


Figure 2. The synthesis and structure of hydrazide of 5-amino-3-methyl-4-isoxazolecarboxylic acid **IV**

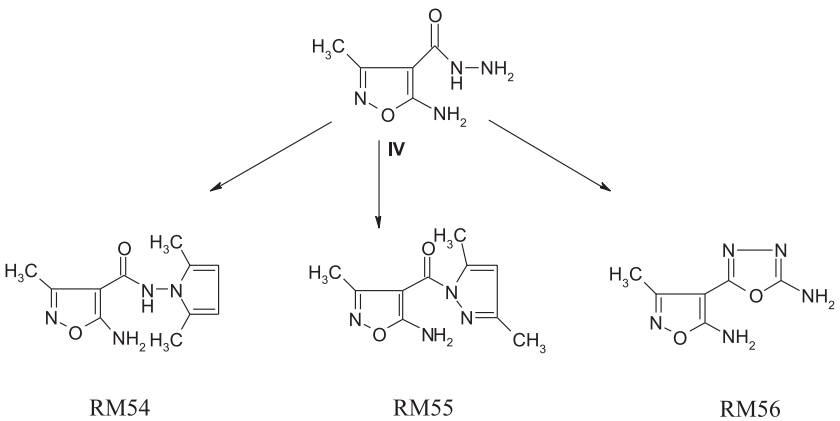
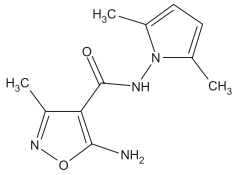
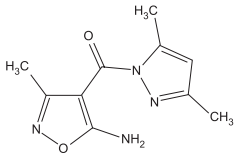
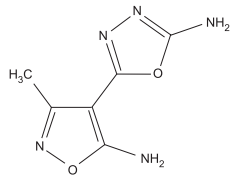


Figure 3. The synthesis and structures: 5-amino-3-methyl-4-(2,5-dimethylpyrrole-aminocarbonyl)-isoxazole RM54, 5-amino-3-methyl-4-(3,5-dimethylpyrazolecarbonyl)-isoxazole RM55 and 5-amino-3-methyl-4-[2-(5-amino-1,3,4-oxadiazole)]-isoxazole RM56

Table 1. The structural and spectroscopic (IR, ^{1}H NMR) data for the obtained compounds.

Compound	Chemical structure	Spectroscopic data
RM54		IR (cm^{-1}): C=O 1712; ^{1}H NMR (DMSO-d_6, δ, ppm): 2.1 (s, 6H, $2CH_3$-pyrrole); 2.4 (s, 3H, CH_3-isoxazole); 5.7 (s, 2H, $-NH_2$); 7.6 (s, 2H, 2CH-pyrrole); 9.7 (s, 1H, NH)
RM55		IR (cm^{-1}): C=O 1708, ^{1}H NMR (DMSO-d_6, δ, ppm): 2.16 (s, 3H, CH_3-pyrazole); 2.21 (s, 3H, CH_3-pyrazole); 2.4 (s, 3H, CH_3-isoxazole); 6.2 (s, 2H, $-NH_2$); 8.2 (s, 1H, pyrazole)
RM56		IR (cm^{-1}): =C-O-C= 1688, ^{1}H NMR (DMSO-d_6, δ, ppm): 2.3 (s, 3H, CH_3); 8.6 (s, 4H, $2NH_2$-isoxazole and oxadiazole)

effect exerted by the amide groups of the derivatives. In the course of the earlier conducted investigations encompassing synthesis and immunological activity we found interdependences between structure and activities of the synthesized compounds. Most active series of compounds were studied in a more detailed manner using quantum-chemical methods and most relevant interdependencies were found by comparing immune activity with distribution of atom charges. These correlations occurred most frequently between activity and atom charges located predominantly on isoxazole heteroatoms and atoms of amide and carbonyl groups (5).

Therefore, it seemed particularly interesting to obtain new derivatives possessing significant differences within these groups and to assess their biologic activities. To address the problem, a new half-product, substrates and appropriate methods were elaborated to enable obtaining such differentiated derivatives.

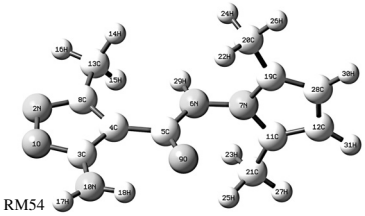
In the reactions of hydrazide of 5-amino-3-methyl-4-isoxazolecarboxylic acid **IV** (Fig. 2) with acetonylacetone (hexanedione-2,5), acetylacetone (pentanedione-2,4) and bromocyanide we obtained new lead structures: 5-amino-3-methyl-4-(2,5-dimethylpyrrole-aminocarbonyl)-isoxazole RM54, 5-amino-3-methyl-4-(3,5-dimethylpyrazolecarbonyl)-isoxazole RM55 and 5-amino-3-methyl-4-[2-(5-amino-1,3,4-oxadiazole)]-isoxazole RM56, respectively (see Fig. 3) (6-8). As a results of these syntheses we obtained a compound with the amide group where the nitrogen atom was bound to the pyrrole nitrogen atom RM54, in compound RM55 the amide nitrogen atom was integrated within the heterocyclic structure of pyrazole, and in compound RM56 atoms: C, N and O of the amide group could be found in the heterocyclic structure of oxadiazole. These combinations enabled evaluation of effects of the amide group on the biologic activity of the new structures. In each of the products, at location 4 of the isoxazole ring, five-element heteroaromatic substituent exists, which significantly improved the bioaccessibility.

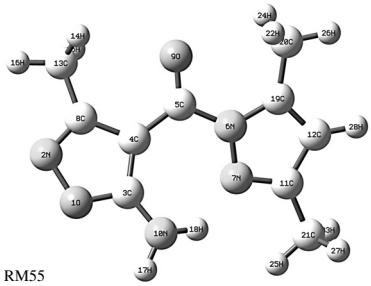
These findings prompted us to synthesize isoxazole derivatives where most essential differences occur within amide structures. As a result, compound RM54 possessing a complete amide group, and compound RM55, where the nitrogen atom in the amide group belongs to the pyrazole structure were obtained. In compound RM56 the whole amide group participates in the oxadiazole structure.

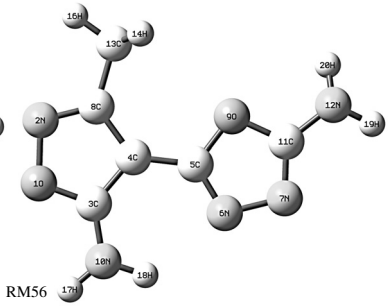
The aim of this study was to evaluate the immunosuppressive activities of the derivatives in relation to quantum chemistry analysis. The calculated atomic charge distributions in RM54, RM55 and RM56 were used to obtain relationships between structure and immunological activity of the studied compounds.

Table 2. The structural parameters of the most stable conformers of RM54, RM55 and RM56 calculated at the B3LYP/6-311G(d,p) in the presence of DMSO.

Atom RM54	Atomic charge (e) RM54	Atom RM55	Atomic charge (e) RM55	Atom RM56	Atomic charge (e) RM56
1 O	**-0.2755**	**1 O**	**-0.2753**	**1 O**	**-0.2750**
2 N	**-0.2130**	**2 N**	**-0.2099**	**2 N**	**-0.2208**
3 C	**0.5743**	**3 C**	**0.5585**	**3 C**	**0.5850**
4 C	-0.4443	4 C	-0.4041	4 C	-0.4349
5 C	0.4886	5 C	0.4620	5 C	0.4000
6 N	-0.3308	6 N	-0.3397	6 N	-0.3430
7 N	-0.3079	7 N	-0.3212	7 N	-0.3087
8 C	**0.2042**	**8 C**	**0.1790**	**8 C**	**0.2087**
9 O	-0.4512	9 O	-0.3825	9 O	-0.2925
10 N	-0.4694	10 N	-0.4546	10 N	-0.4748
11 C	0.1380	11 C	0.1731	11 C	0.4443
12 C	-0.2123	12 C	-0.3261	12 N	-0.4881
13 C	-0.3036	13 C	-0.2256	13 C	-0.2390
14 H	0.1522	14 H	0.1353	14 H	0.1402
15 H	0.1529	15 H	0.1326	15 H	0.1399
16 H	0.1399	16 H	0.1184	16 H	0.1270
17 H	0.2637	17 H	0.2540	17 H	0.2639
18 H	0.2647	18 H	0.2664	18 H	0.2563
19 C	0.1381	19 C	0.2280	19 H	0.2551
20 C	-0.2544	20 C	-0.2138	20 H	0.2566
21 C	-0.2542	21 C	-0.2607		
22 H	0.1252	22 H	0.1315		
23 H	0.1244	23 H	0.1352		
24 H	0.1245	24 H	0.1350		
25 H	0.1251	25 H	0.1264		
26 H	0.1257	26 H	0.1218		
27 H	0.1256	27 H	0.1368		
28 C	-0.2124	28 H	0.1196		
29 H	0.2758				
30 H	0.0931				
31 H	0.0931				


RM54


RM55


RM56

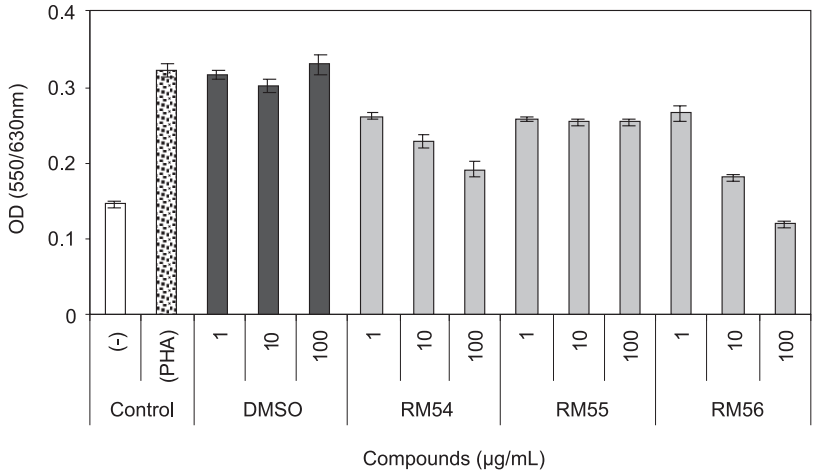


Figure 4. Effects of the compounds on PHA-induced proliferation of human PBMC. RM54, RM55 and RM56 were added to the human PBMC cultures at concentrations: 1, 10 and 100 µg/mL. PHA was added at a concentration of 5 µg/mL. After a four-day incubation, the proliferative response of the cells was determined. Appropriate DMSO dilutions served as control cultures. (-) – no additions. The data are presented as a mean OD value from quadruplicate wells ± SE. Statistics: DMSO *vs*. RM54: 1 µg/mL: p = 0.0133; 10 µg/mL: p = 0.0045; 100 µg/mL: p = 0.0001; DMSO *vs*. RM55: 1 µg/mL: p = 0.0051; 10 µg/mL: NS (p = 0.0901); 100 µg/mL: p = 0.0039; DMSO *vs*. RM56: 1 µg/mL: p = 0.0219; 10 µg/mL: p = 0.0001; 100 µg/mL: p = 0.0001 (ANOVA)

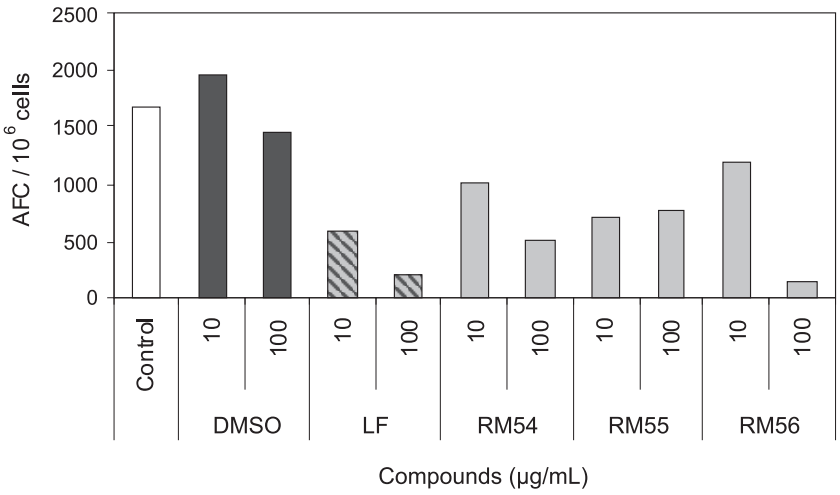


Figure 5. Effect of the compounds on the secondary humoral immune response of mouse splenocytes to sheep erythrocytes. Splenocytes from mice sensitized with SRBC were restimulated with SRBC *in vitro*. The compounds were added to the cell cultures in the beginning of the 4-day incubation at concentration 10 and 100 µg/mL. The results are shown as the mean values of AFC numbers from 4 wells ± SE, calculated per 10^6 viable cells. Statistics: DMSO *vs*. leflunomide (LF): 10 µg/mL: p = 0.0002; 100 µg/mL: p = 0.0003; DMSO *vs*. RM54: 10 µg/mL: p = 0.0067; 100 µg/mL: p = 0.0086; DMSO *vs*. RM55: 10 µg/mL: p = 0.0003; 100 µg/mL: NS (p = 0.1217); DMSO *vs*. RM56: 10 µg/mL: NS (p = 0.0580); 100 µg/mL: p = 0.0002 (ANOVA)

EXPERIMENTAL

Chemistry

Melting points were determined on 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 $CHCl_3$ – CH_3OH 9 : 1 v/v, and detected with UV Fisher Bioblock Scientific 254 nm lamps. IR spectra were recorded with a Specord M-80 spectrophotometer (Carl Zeiss, Jena, Germany) in Nujol mull support-

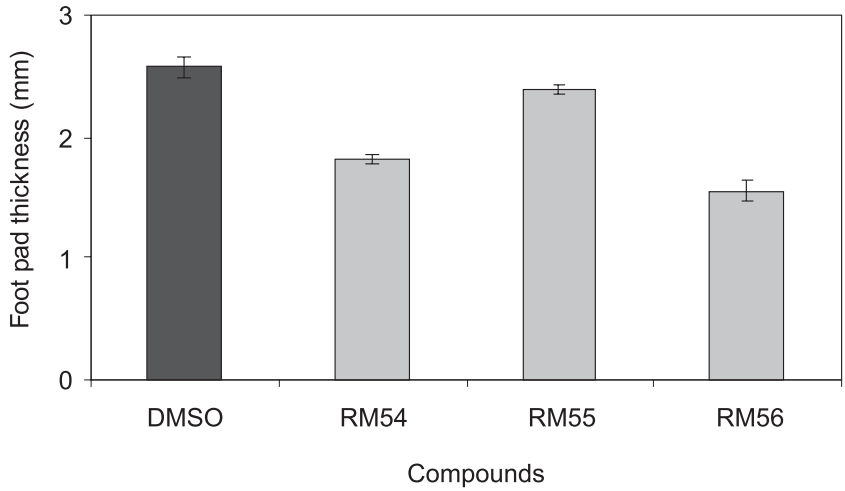


Figure 6. Effect of the compounds on carrageenan reaction in mice. Mice were given 2% carrageenan solution *s.c.* into hind foot pads and after 3 h the foot pad thickness was measured. The compounds (100 µg/mouse) were administered *i.p.* at 48 h and 24 h before carrageenan injection. Control – mice treated with appropriate dilutions of DMSO. The background foot pad thickness (1.5 mm) of naive mice was subtracted from the response of sensitized mice. The results are presented as the mean foot pad thickness from 5 mice/group (10 determinations) and expressed in mm. Statistics: DMSO *vs.* RM54 p = 0.0025; DMSO *vs.* RM55 NS (p = 1.0000); DMSO *vs.* RM56 p = 0.0001 (ANOVA).

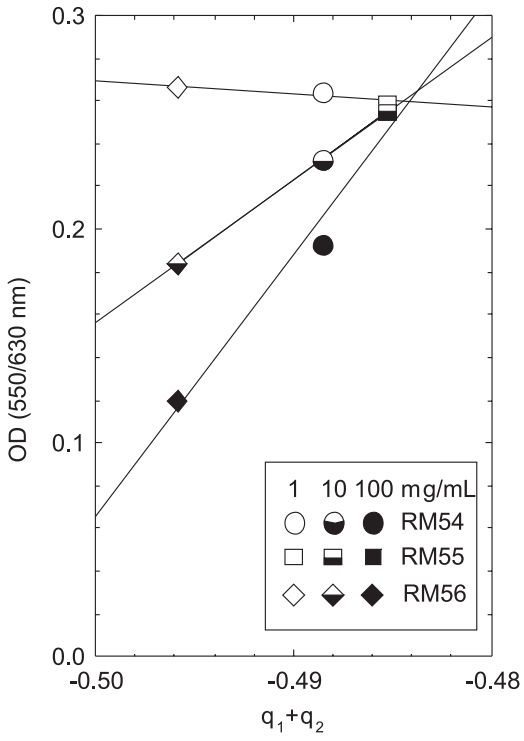


Figure 7. The effects of the studied compounds on PHA-induced proliferation of human PBMC as a function of the sum of the charges on atoms O(1) and N(2) denoted by $q_1 + q_2$

ed on a KBr disk, and ^{1}H NMR spectra were obtained in DMSO-d_6 using a Bruker ARX 300 MHz spectrometer (using TMS as the internal standard). Mass spectrometry was performed on micrOTOF-Q spectrometer (manufactured by Bruker Daltonic) using electrospray ionization (ESI) method, in cation mode with two quadrupole analizators and time of flight (TOF). The spectroscopic data are presented in Table 1. Elemental analyses were performed within ± 0.3% of the theoretical values (Carlo Erba NA, 1500 analyzer).

Compounds preparation

New derivatives of isoxazole were synthesized by using 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide and three different compounds in the presence of isopropanol.

Synthesis of 5-amino-3-methyl-4-(2,5-dimethyl-pyrrole-aminocarbonyl)-isoxazole RM54

The mixture of isopropanol (50 mL) and hexane-2,5-dione (3.3 mmol) was added to the 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide (3 mmol). The solution was heated for 10 min to the boiling temperature with stirring and refluxed for 7 h. Then, the suspension was cooled and filtered. The crude product was recrystallized from ethanol. White crystal product was obtained with 49.3% of the theoretical yield and the melting temperature was 224-225^{o}C.

Synthesis of 5-amino-3-methyl-4-(3,5-dimethyl-pyrazolecarbonyl)-isoxazole RM55

The mixture of isopropanol (50 mL) and pentane-2,4-dione (3.3 mmol) was added to the 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide (3 mmol). The solution was stirred and heated

for 10 min to the boiling temperature and refluxed for 2 h. The solvent was evaporated under pressure to obtain a dry sediment. Then, 50 mL of THF was added. The mixture was heated to the boiling, cooled and finally filtered. The crude product was recrystallized from ethanol. A white crystal product was received with 56% of theoretical yield. The melting temperature was 159-161°C.

Synthesis of 5-amino-3-methyl-4[2-(5-amino-1,3,4-oxadiazole)]-isoxazole RM56

The mixture of isopropanol (50 mL), cyanogen bromide (12 mmol) and anhydrous K_2CO_3 (109 mmol) was added to 5-amino-3-methyl-4-isoxazole-carboxylic acid hydrazide (12 mmol). The solution was stirred and heated for 10 min to the boiling point and refluxed for 2 h. Potassium bromide was separated by filtration, then the solution was evaporated under pressure to obtain a dry sediment. Then, the THF (50 mL) was added and the mixture was heated to the boiling point. Then, the solution was cooled, filtered and recrystallized from ethanol. A white crystal product was obtained with 72% of the theoretical yield. The melting point was 225°C.

Biology

Animals

CBA mice of both sexes, 8-12 week old, delivered by the Institute of Laboratory Medicine, Łódź, Poland, were used in this study. Mice had free access to laboratory chow and filtered tap water. The local ethics committee approved the study.

Reagents

RPMI-1640 medium was purchased from Sigma-Aldrich, fetal calf serum (FCS) from Gibco, lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4, phytohemagglutinin A (PHA), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and leflunomide (LF) were from Sigma-Aldrich. Sheep erythrocytes (SRBC) were delivered by Wroclaw University of Life and Environmental Sciences, Wrocław, Poland. SRBC were stored in Alsever's solution at 4°C until use. The studied peptides were initially dissolved in DMSO and subsequently in a culture medium at concentration of 1 mg/mL (stock solution).

Isolation of the peripheral blood mononuclear cells (PBMC)

Venous blood was withdrawn into heparinized syringes and diluted twice with PBS. PBMC were isolated by centrifugation on Ficoll-uropoline gradient (density 1.077 g/mL) and centrifuged at 800 × g for 20 min at 4°C. The interphase cells, consisting of lymphocytes (20%) and monocytes (80%) 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 pyru-

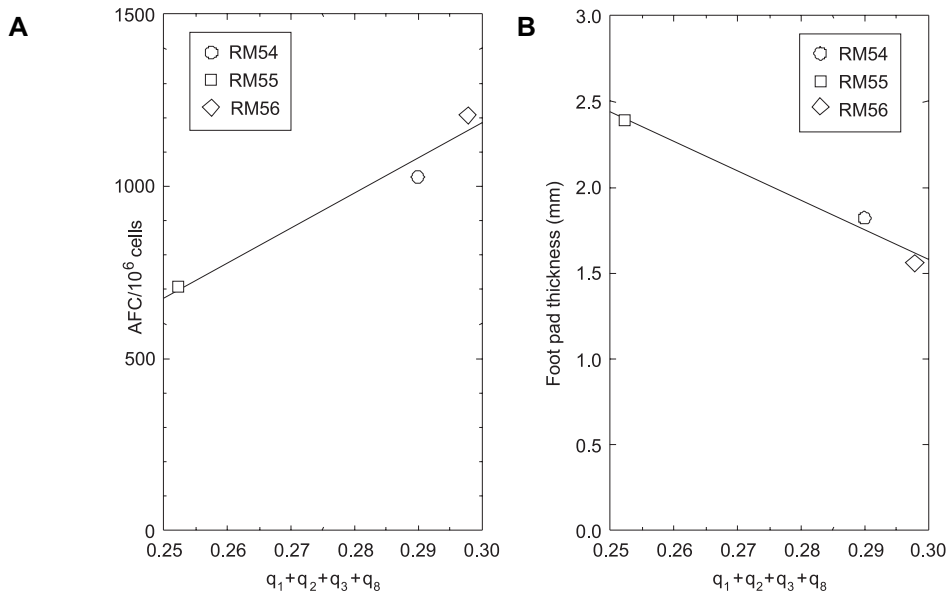


Figure 8. The relationships between the atomic charges on the atoms of isoxazole ring (N, O, C_3 and C_8) denoted by $q_1 + q_2 + q_3 + q_8$ and: (**A**) AFC production at the compounds concentrations of 10 μg/mL, (**B**) carrageenan-induced foot pad thickness *in vivo* at 100 μg dose

Table 3. The structural parameters of the studied compounds calculated at the B3LYP/6-311G(d,p) level in the presence of DMSO as a solvent.

RM54

Molecular mass: 234.11168 amu, total energy at 0 K (ZPE included): -796.982775 a.u., rotational constants: 0.90884, 0.26950, 0.26208 GHz, ionization potential: 5.64 eV, dipole moment: 1.3808 D.

Atom	Orthogonal coordinates (Å) charge (e)			Atomic (cm^{-1})	Harmonic frequencies		
1 O	3.981831	-0.368706	-0.016442	-0.2755	18	30	59
2 N	3.759306	1.052151	0.030687	-0.2130	70	90	123
3 C	2.799897	-0.985774	-0.030625	0.5743	133	150	160
4 C	1.766247	-0.049524	0.004003	-0.4443	173	208	231
5 C	0.368556	-0.458515	-0.013156	0.4886	237	283	283
6 N	-0.583019	0.539861	0.024551	-0.3308	292	305	350
7 N	-1.924977	0.243634	0.009498	-0.3079	360	371	426
8 C	2.460766	1.203609	0.041797	0.2042	512	533	569
9 O	0.041647	-1.646574	-0.059493	-0.4512	589	608	626
10 N	2.766620	-2.316306	-0.060574	-0.4694	631	639	713
11 C	-2.695039	0.019413	1.145843	0.1380	729	761	776
12 C	-3.966762	-0.248993	0.701250	-0.2123	784	788	832
13 C	1.916697	2.597597	0.089391	-0.3036	863	909	981
14 H	1.296997	2.808506	-0.786413	0.1522	986	1024	1034
15 H	1.314898	2.754949	0.988649	0.1529	1041	1055	1057
16 H	2.739652	3.311767	0.102640	0.1399	1062	1064	1106
17 H	3.611751	-2.855924	-0.152389	0.2637	1120	1226	1241
18 H	1.853943	-2.747353	-0.117744	0.2647	1330	1343	1373
19 C	-2.690042	0.110090	-1.144433	0.1381	1377	1410	1413
20 C	-2.107326	0.285834	-2.506495	-0.2544	1415	1440	1468
21 C	-2.118772	0.087974	2.520193	-0.2542	1468	1473	1482
22 H	-1.327472	-0.454016	-2.714334	0.1252	1483	1485	1495
23 H	-1.676331	1.066490	2.733054	0.1244	1520	1539	1562
24 H	-1.661064	1.276711	-2.638822	0.1245	1587	1624	1644
25 H	-1.337427	-0.663741	2.672256	0.1251	1688	3018	3020
26 H	-2.891651	0.167255	-3.255125	0.1257	3036	3062	3062
27 H	-2.906054	-0.091988	3.253329	0.1256	3093	3114	3114
28 C	-3.963695	-0.192125	-0.728042	-0.2124	3129	3220	3234
29 H	-0.333357	1.516571	0.060423	0.2758	3552	3619	3702
30 H	-4.807179	-0.356269	-1.382112	0.0931			
31 H	-4.813219	-0.463618	1.336589	0.0931			

vate, 2-mercaptoethanol and antibiotics, at density of 2×10^6 cells/mL.

Proliferation of PBMC

The isolated PBMC were distributed into 96-well flat-bottom plates in 100 µL aliquots (2×10^5 cells/well). PHA was added at a concentration of 5 µg/mL. The compounds were tested at doses 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 colorimetric MTT method (9). The data are presented as a mean OD value from quadruplicate wells ± SE.

Table 3. cont.
RM55
Molecular mass: 220.09603 amu, total energy at 0 K (ZPE included): -757.702611 a.u., rotational constants: 0.88871, 0.40029, 0.28722 GHz, ionization potential: 6.43 eV, dipole moment: 6.82 D.

Atom	Orthogonal coordinates (Å) charge (e)			Atomic (cm^{-1})	Harmonic frequencies		
1 O	2.845387	-1.703010	-0.267236	-0.2753	30	42	98
2 N	3.609139	-0.549855	0.107309	-0.2099	120	127	158
3 C	1.550209	-1.378849	-0.331433	0.5585	178	191	207
4 C	1.387176	-0.022599	-0.012675	-0.4041	219	248	261
5 C	0.270773	0.911572	-0.029803	0.4620	309	338	354
6 N	-1.074556	0.439062	-0.034944	-0.3397	362	401	437
7 N	-1.394116	-0.858541	0.281460	-0.3212	457	502	585
8 C	2.740466	0.412131	0.238645	0.1790	593	618	620
9 O	0.446643	2.120760	-0.032098	-0.3825	648	659	680
10 N	0.715297	-2.344151	-0.720811	-0.4546	723	768	788
11 C	-2.714284	-0.913661	0.304456	0.1731	794	830	879
12 C	-3.273295	0.356713	0.004356	-0.3261	904	988	1005
13 C	3.239529	1.765111	0.634506	-0.2256	1029	1053	1060
14 H	3.046909	2.491372	-0.155586	0.1353	1064	1064	1067
15 H	2.724235	2.119030	1.529202	0.1326	1101	1159	1184
16 H	4.311020	1.712281	0.830396	0.1184	1248	1313	1335
17 H	1.077534	-3.284399	-0.777091	0.2540	1367	1404	1416
18 H	-0.258813	-2.189481	-0.464052	0.2664	1418	1442	1456
19 C	-2.218289	1.208613	-0.203578	0.2280	1464	1464	1465
20 C	-2.255684	2.658909	-0.555471	-0.2138	1472	1473	1487
21 C	-3.431995	-2.189895	0.607587	-0.2607	1516	1542	1598
22 H	-1.667980	2.871634	-1.450205	0.1315	1613	1647	1695
23 H	-4.133018	-2.052936	1.434950	0.1352	3036	3049	3053
24 H	-1.848439	3.275820	0.246807	0.1350	3088	3109	3118
25 H	-2.723944	-2.974008	0.877874	0.1264	3122	3124	3133
26 H	-3.292096	2.947490	-0.736038	0.1218	3249	3405	3646
27 H	-4.010511	-2.525920	-0.257629	0.1368			
28 H	-4.319133	0.613240	-0.054588	0.1196			

The secondary humoral immune response to SRBC

Mice were sensitized intraperitoneally (*i.p.*) with 0.2 mL of 5% SRBC suspension in 0.9% NaCl. After 4 days, spleens from these mice were isolated, splenocyte single cell suspension was prepared and resuspended in the culture medium at a density of 5 × 10^6/mL. The cells were distributed to 24-well culture plates in 1 mL aliquots and 0.05 mL of 0.005% SRBC suspension was added as antigen. The compounds were added to the cultures at the beginning of the 4-day incubation period at concentration of 10 and 100 μg/mL. The numbers of antibody-forming cells (AFC) in the cultures were determined using a method of local hemolysis in agar gel (10) and presented as the mean values of cell number/10^6 cells ± SE from quadruplicate determinations.

Carrageenan test

Mice were given 2% carrageenan solution, subcutaneously (*s.c.*), into hind foot pads and after 3 h the foot pad thickness was measured by means of

Table 3. cont.
RM56
Molecular mass: 181.05997 amu, total energy at 0 K (ZPE included): -657.045234 a.u., rotational constants: 1.57484, 0.57551, 0.42276 GHz, ionization potential: 5.81 eV, dipole moment: 4.15 D.

Atom	Orthogonal coordinates (Å) charge (e)			Atomic (cm^{-1})	Harmonic frequencies		
1 O	-3.014411	-0.369925	-0.017540	-0.2750	52	99	103
2 N	-2.824848	1.056285	-0.013534	-0.2208	141	159	196
3 C	-1.817519	-0.961016	-0.001509	0.5850	248	261	271
4 C	-0.815050	-0.000141	0.011123	-0.4349	313	360	386
5 C	0.581021	-0.312113	0.013676	0.4000	414	443	512
6 N	1.127191	-1.488103	0.014991	-0.3430	546	558	610
7 N	2.521357	-1.294541	-0.006937	-0.3087	625	695	729
8 C	-1.527439	1.236208	0.004287	0.2087	750	761	783
9 O	1.522133	0.687976	0.005007	-0.2925	858	970	976
10 N	-1.741004	-2.295523	0.026876	-0.4748	1023	1027	1048
11 C	2.694004	-0.010106	-0.005613	0.4443	1063	1097	1119
12 N	3.845707	0.707713	0.058380	-0.4881	1144	1340	1376
13 C	-0.977018	2.626167	0.012604	-0.2390	1414	1435	1463
14 H	-0.363100	2.791374	0.900795	0.1402	1468	1470	1553
15 H	-0.343967	2.794519	-0.861432	0.1399	1589	1604	1635
16 H	-1.792765	3.348875	0.004946	0.1270	1658	1688	3047
17 H	-2.561747	-2.858185	-0.130138	0.2639	3105	3133	3544
18 H	-0.821553	-2.708941	-0.057363	0.2563	3570	3671	3691
19 H	4.678833	0.183717	-0.167939	0.2551			
20 H	3.815719	1.629423	-0.354429	0.2566			

a spring caliper. The compounds were administered *i.p.* at a dose of 100 µg per mouse at 48 h and 24 h before carrageenan injection (11).

Statistics

The results are presented as the mean values ± standard error (SE). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the difference between groups. Significance was determined at $p < 0.05$. Statistical analysis was performed using STATISTICA 7.0 for Windows.

Computational details

The Density Functional Theory (DFT) was used in the calculations of the electronic structure of the compounds under investigation. The DFT technique used in the present calculations utilizes the Becke's three parameter functional (12) together with the local correlation part of Vosko et al. (13) and the nonlocal part of Lee et al. (14) denoted as B3LYP. All molecular structures were fully optimized using the analytical gradients method without any symmetry constraints to locate the lowest-energy isomers of the studied isoxazole derivatives. Vibrational frequency calculations were carried out on each optimized structure to make sure that they are true minima on the respective energy surfaces (absence of any imaginary frequency). All quantum mechanical *ab initio* calculations were carried out using the Gaussian 09 program (15) package. The geometrical parameters of the studied molecular structures were calculated with the 6-311G(d,p) basis set. The atomic charges were derived on the base of the Mulliken population scheme. All calculations were performed in the presence of DMSO as a solvent. However, the results of calculations show that the modification of the structural parameters of the studied molecules in the presence of DMSO is only small.

RESULTS AND DISCUSSION

Effect of the compounds on PHA-induced PBMC proliferation

Figure 4 presents effects of the compounds on PHA-induced proliferation of human PBMC. All compounds had the ability to suppress the proliferation in a dose-dependent manner. The inhibitory potency of the compounds was increasing in the following sequence: RM55, RM54 and RM56.

Effect of the compounds on the secondary humoral immune response of mouse splenocytes to sheep erythrocytes

In the model of secondary, humoral immune response to sheep erythrocytes (SRBC), all the studied compounds at concentrations of 10 and 100 μg/mL, displayed to various degrees suppressive activities (Fig. 5). Leflunomide, the reference drug, was significantly inhibitory (70 and 87%, respectively). The degrees of the inhibitory effects for the studied compounds were the following: RM54 – 50 and 65%, RM55 – 64 and 48%, and RM56 – 39 and 89% (for 10 and 100 mg/mL, respectively).

Effect of the compounds on carrageenan reaction in mice

The compounds were tested in the carrageenan model of foot pad inflammation in mice (Fig. 6). The compounds were administered *i.p.* at 100 μg doses, 48 h and 24 h before elicitation of the carrageenan reaction. The reaction was measured at 3 h after administration of carrageenan. It appeared that only two compounds exhibited strong anti-inflammatory property: RM54 inhibited the reaction by 30% and RM56 by 40%. RM55 was not active (8% inhibition).

The studied compounds demonstrated differential, immunosuppressive properties. RM56 exhibited stronger immunosuppressive properties as compared to RM54.

In particular, its deepest anti-inflammatory property in the carrageenan test is worth noticing, and was, in addition, correlated with the strongest suppression of the humoral immune response *in vitro* and the proliferative response of lymphocytes to PHA. Its mechanism of action probably differs from that of leflunomide (16), since RM54 markedly stimulated IL-6 (data not shown) which controls inflammatory processes by stimulation of acute protein production (17). On the other hand, the immunological activity of RM56 differs from that of another strong immune suppressor RM 33 (18, 19) which was devoid of antiproliferative properties. In conclusion, potential application of RM56 could be broad, such as inhibition of autoimmune diseases progression and other inflammatory processes, tumor growth and prevention of allograft rejection.

Quantum chemistry analysis

The results of the performed *ab initio* calculations including the optimized geometrical parameters, the harmonic vibrational frequencies, the rotational constants, the dipole moments, the vertical ionization potentials, and the total energies at 0 K (ZPE included) of the studied molecular structures are presented in Table 3.

The investigated RM54, RM55 and RM56 compounds are conformationally complicated molecules with many rotational degrees of freedom. At low values of the rotational barriers, the conformers can easy interconvert one to another at room temperature. The molecular arrangements and a key of atom numbering of the most stable rotational isomers of RM54, RM55 and RM56 are given in Table 2. The results of geometry optimization show that the values of bond distances and angles derived for the studied compounds are very close to those observed in the other isoxazole derivatives.

The isoxazole and pyrrole rings are almost perpendicular in the most stable conformer of RM54. The dihedral angle C(5)-N(6)-N(7)-C(11) is 89.5°. The amine group $–NH_2$ is almost co-planar with isoxazole ring. The torsion angles H(18)-N(10)-C(3)-C(4) and H(17)-N(10)-C(3)-O(1) are of 3.7° and 5.3°, respectively. The contact distance O(9)…H(18) of 2.12 Å enables a stabilization of molecule of RM54 by the intramolecular hydrogen bond. The atomic charges of the nitrogen and oxygen atoms, O(1) and N(2) of isoxazole ring are found negative of 0.276e and 0.213e, respectively. The harmonic frequency $\nu_{C=O}$ of 1688 cm^{-1} of RM54 was calculated at the B3LYP/6-311G(d,p) level of theory.

The most stable isomer of RM55 is also not a flat structure. The dihedral angle C(4)-C(5)-N(6)-N(7) between the isoxazole and pyrazole rings of RM55 is found of 19.6°. The amine group is almost co-planar with isoxazole ring, similarly to RM54. The interatomic distances, N(7)…H(18), O(9)…H(14), O(9)…H(15), O(9)…H(22) and O(9)...H(24) are of 1.90 Å, 2.76 Å, 2.62 Å, 2.65 Å and 2.58 Å, respectively. This enables a formation of the intramolecular hydrogen bonds. The oxygen O(1) and nitrogen N(2) atoms of RM55 are negative of 0.2753e and 0.2099e, respectively. The calculated frequency $\nu_{C=O}$ of RM55 is 1695 cm^{-1}.

RM56 is the smallest studied molecule, which consists of 20 atoms. Both isoxazole and oxadiazole rings of RM56 are almost co-planar. In contrast to RM54 and RM55, the most stable conformer of RM56 is a nearly flat molecular structure. The hydrogen bond occurs between the N(6) and H(18) atoms of RM56. The values of the charge on the atoms O(1) and N(2) are similar to those derived for RM54 and RM55.

Theoretical correlations

The differences in the observed immunosuppressive properties of the studied derivatives of isoxazole are a good reason for theoretical investigations. The performed *ab initio* calculations provided useful information on the electron charge distribution in RM54, RM55 and RM56 molecules. The isoxazole ring is common part to any studied compound and can be considered as the reference molecular subunit. The charge distribution of the isoxazole ring should be related with the electronic structure of whole molecule. The O(1) and N(2) atoms are the most characteristic points of the isoxazole ring. The results of calculations show that these atoms are distinctly negative centers of the isoxazole ring capable of forming a stable complex with water or the other polar solvent. One can expect that the atomic charges on the O(1) and N(2) atoms should be correlated with immunological activity of the studied compounds.

Figure 7 shows the relationships between the sum of the charges on atoms O(1) and N(2) denoted by $q_1 + q_2$ and the effects of the studied compounds on PHA-induced proliferation of human PBMC. At any concentration of RM54, RM55 and RM56 the linear correlation retains validity.

The analysis of the atomic charge distribution of RM54, RM55 and RM56 showed that the influence of these compounds on either the secondary humoral immune response of mouse splenocytes to sheep erythrocytes or the carrageenan reaction in mice depends on charges of greater number of atoms of the isoxazole ring. The plots in Figure 8 A,B show that the sum of atomic charges of the O(1), N(2), C(3) and C(8) atoms can be successfully used in description of the influence of RM54, RM55 and RM56 compounds on AFC production and foot pad thickness in the carrageenan test. It seems that a directly proportional correlation exists between the increasing immunological activities of the compounds: RM55 < RM54 < RM56 and the sum of atomic charges on the isoxazole ring (Fig. 8AB). The obtained relationships also show that the isoxazole ring plays an important role in the observed immunological activities and that the flat structure of RM56 determines its highest immunological activity.

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