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SYNTHESIS, IMMUNOLOGICAL ACTIVITY AND COMPUTATIONAL STUDY OF 5-AMINO-3-METHYL-4-ISOXAZOLECARBOXYLIC ACID SEMICARBAZIDES AND THIOSEMICARBAZIDES

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Abstract: A series of 5-amino-3-methyl-4-isoxazolecarboxylic acid semicarbazides and thiosemicarbazides (**M1**-**M9**) were obtained by reacting 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide with isocyanate and isothiocyanate. In order to determine structure-activity relationships for the series of nine compounds (**M1**-**M9**) geometry optimization was carried out using quantum-chemical DFT calculations at B3LYP/6-31g* level. The compounds, administered to mice at 1µg or 10 µg doses, four hours prior to immunization with sheep erythrocytes (SRBC), significantly suppressed the primary, humoral immune response in mice as measured by the number of antibody-forming cells (AFC) in the spleens. Structure/activity relationships, regarding the studied compounds, were discussed.

Keywords: AFC, 5-amino-3-methyl-4-isoxazolecarboxylic acid semicarbazides and thiosemicarbazides, computational study

Intensive studies on new, biologically active isoxazole derivatives, have been conducted for the last few decades. The significance of any progress in a search for new, potential, immunomodulatory compounds can not be underestimated. Our hitherto investigations have demonstrated high immunomodulatory activity of both mono- and bicyclic isoxazole derivatives. Among bicyclic derivatives we studied 5-substituted 3-methylisoxazolo(5,4-*d*)-pyrimidin-4-one derivatives (1) and 5-substituted 3-methylisoxazole(5,4-*d*)-1,2,3-triazin-4-one derivatives (2, 3).

In the group of monocyclic compounds, the pharmacophore structure was a fragment of 5-amino-3-methyl-4-isoxazolecarboxylic acid. From this structure, via the azide of that acid, and, subsequently the hydrazide (4), we obtained N'-substituted hydrazides (5) of high immunosuppressive activity. The importance of the pharmacophore structure of 5-amino-3-methyl-4-isoxazolecarboxylic acid, contained in isoxazoleoxadiazole (6), isoxazolepyrazole (7) and isoxazolepyrrole (8), for high immunomodulatory activity, was confirmed in several patents.

In reaction of 5-amino-3-methylo-4-isoxazolecarboxyl acid hydrazide with isocyanates or isothiocyanates we obtained the described series of semicarbazides and thiosemicarbazides of that acid. A part of the studies, regarding the immunosuppressive activity of one compound from that series, 1-(5-amino-3-methyl-4-isoxazolyl)-4-phenylthiosemicarbazide (**M1**), was presented in (9).

EXPERIMENTAL

Chemistry

Melting points were determined on a Büchi apparatus (Laboratoriums-Technik AG, Flawil, Switzerland) and hot stage Kofler system (Wagner & Munz) and were uncorrected. Thin layer chro-

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matography (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 KBr disks, and ^1H NMR spectra were obtained in DMSO-d_6 using a Bruker ARX 300 MHz spectrometer (using TMS as an internal standard). Elemental analyses (C, H, N) were obtained within ± 0.3% of the theoretical values (Carlo Erba NA, 1500 equipment).

General procedure for preparation of compounds M1-M9

25 mL of anhydrous tetrahydrofurane (THF) and 6.4 mmol of appropriate isocyanate or isothiocyanate were added to 6.4 mmol of 5-amino-3-methylisoxazole-4-carboxylic acid hydrazide obtained according to a method described in (10). The solution was stirred and heated for 2 h. At the end of reaction (controlled by TLC), the solid, which separated out, was filtered and washed with ethylhexane. The crude product was recrystalized from THF: hexane (1:1, v/v).

Biological activity

Animals: 12-week-old CBA mice were delivered by the animal breeding center in Ilkowice/Krakow and kept in the Animal Facility of the Institute of Immunology and Experimental Therapy, Wrocław. The animals were fed with granulated food and water *ad libitum*.

Antigens: Sheep red blood cells (SRBC) were delivered by the Wrocław Agriculture Academy and kept in Alsever's solution at 4°C until use.

Reagents: Cyclosporin A (CsA) in ampoules (in Cremophor as solvent) was purchased from Sandimun, Sandoz (Switzerland).

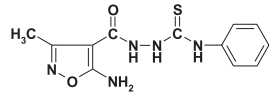
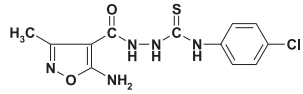
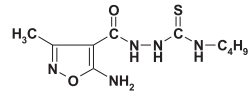
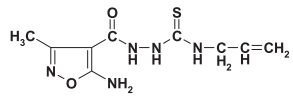
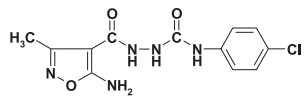
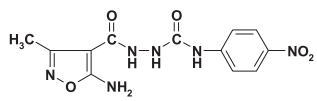
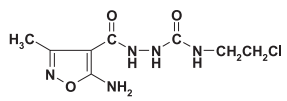
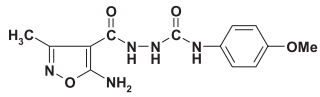
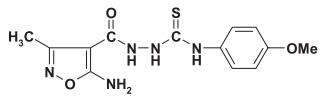
Administration of the preparations: The compounds were initially dissolved in DMSO and then diluted by 0.9% saline. The compounds were given to mice intraperitoneally (1 µg and 10 µg in 0.2 mL), 4 h before intraperitoneal immunization of mice with 0.2 mL of 5% SRBC suspension.

Determination of the humoral immune response: Four days after immunization the number of antibody-forming cells (AFC) in the splenocytes was determined according to Mishell and Dutton (11). The magnitude of the response was expressed as the AFC number per 10^6 splenocytes.

Table 1. Physical data and elemental analyses for the obtained compounds (**M1**-**M9**)

Compound	Melting point (°C) /THF:hexane (1:1)	Yield (%)	Formula Molecular weight (g/mol)	Elemental analysis Calculated/Found		
				%C	%H	%N
M1	190-191	82	$C_{12}H_{13}N_5O_2S$ 291,33	49,47 49,93	4,50 4,71	24,04 24,29
M2	198-199	76	$C_{12}H_{12}ClN_5O_2S$ 325,78	44,24 44,18	3,71 3,84	21,50 21,39
M3	197-199	64	$C_7H_{11}N_5O_2S$ 229,26	36,67 36,90	4,84 4,89	30,55 30,63
M4	198-200	75	$C_9H_{13}N_5O_2S$ 255,30	42,34 42,23	5,13 5,02	27,43 27,51
M5	196-197	68	$C_{12}H_{12}$ Cl N_5O_3 309,71	46,54 46,69	3,91 4,01	22,61 22,72
M6	238-239	58	$C_{12}H_{12}N_6O_5$ 320,27	45,00 45,11	3,78 3,84	26,24 26,29
M7	148-149	72	$C_7H_{11}N_5O_3$ 213,20	39,44 39,32	5,20 5,08	32,85 32,77
M8	200-202	81	$C_{13}H_{15}N_5O_4$ 305,30	51,15 51,04	4,95 4,90	22,94 22,86
M9	197-199	75	$C_{13}H_{15}N_5O_3S$ 321,36	48,59 48,63	4,70 4,62	21,79 21,62

Table 2. The structural and spectroscopic data for the obtained compounds (**M1**-**M9**)

Compound	Chemical structure	Spectroscopic data
M1		IR (cm^{-1}): C=O 1657, C=S 1405, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.30 (s, 3H, CH_3), 7.50 (m, 5H, aromat), 4.35 (br. s, 2H, NH_2), 8.20 (s, 1H, NH), 8.50 (s, 1H, NH), 9.10 (s, 1H, NH).
M2		IR (cm^{-1}): C=O 1660, C=S 1400, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.33 (s, 3H, CH_3), 4.25 (br. s, 2H, NH_2), 7.60 (dd, 4H, aromat) J = 8.9 Hz, 7.90 (s, 1H, NH), 8.40 (s, 1H, NH), 9.10 (s, 1H, NH).
M3		IR (cm^{-1}): C=O 1652, C=S 1415, ^{1}H NMR (DMSO-d_6) (δ, ppm) 0.90-1.00 (t, 2H, CH_2), 1.35-1.45 (m, 2H, CH_2), 1.65-1.70 (m, 2H, CH_2), 2.35 (s, 3H, CH_3), 3.20-3.25 (t, 3H, CH_3), 7.50 (s, 2H, NH_2), 7.95 (t, 1H, NH), 8.70 (s, 1H, NH), 8.90 (s, 1H, NH).
M4		IR (cm^{-1}): C=O 1668, C=S 1420, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.30 (s, 3H, CH_3), 4.1 (t, 2H, CH_2), 5.1-5.16 (dd, 2H, CH_2) J = 10,8 Hz, 5.76-5.90 (m, 1H, CH), 7.50 (s, 2H, NH_2), 8.15 (t, 1H, NH), 8.80 (s, 1H, NH), 9.20 (s, 1H, NH).
M5		IR (cm^{-1}): C=O 1665, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.25 (s, 3H, CH_3), 4.30 (br. s, 2H, NH_2), 7.65 (dd, 4H, aromat) J = 8.9 Hz, 7.85 (s, 1H, NH), 8.35 (s, 1H, NH), 9.35 (s, 1H, NH).
M6		IR (cm^{-1}): C=O 1655, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.20 (s, 3H, CH_3), 4.20 (br.. s, 2H, NH_2), 7.60 (dd, 4H, aromat) J = 8.8 Hz, 7.80 (s, 1H, NH), 8.55 (s, 1H, NH), 9.15 (s, 1H, NH).
M7		IR (cm^{-1}): C=O 1668, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.30 (s, 3H, CH_3), 3.3-3.6 (m, 4H, CH_2 -CH_2), 7.5 (s, 2H, NH_2), 7.90 (t, 1H, NH), 8.60 (s, 1H, NH), 8.80 (s, 1H, NH).
M8		IR (cm^{-1}): C=O 1660, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.20 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.15 (br. s, 2H, NH_2), 7.60 (dd, 4H, aromat) J = 8.7 Hz, 7.90 (s, 1H, NH), 8.40 (s, 1H, NH), 9.10 (s, 1H, NH).
M9		IR (cm^{-1}): C=O 1645, ^{1}H NMR (DMSO-d_6) (δ, ppm): 2.20 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.15 (br. s, 2H, NH_2), 7.60 (dd, 4H, aromat) J = 8.7 Hz, 7.90 (s, 1H, NH), 8.40 (s, 1H, NH), 9.10 (s, 1H, NH).

Statistics: The experimental groups consisted of 5 mice each. The results are presented as the mean values ± standard error (SE). The Student's *t*-test was applied to determine significance of the data. The differences were regarded significant when $p \leq 0.05$

RESULTS AND DISCUSSION

Chemistry

The description of the synthesis and structure of 5-amino-3-methyl-4-isoxazolecarboxylic acid semicarbazides and thiosemicarbazides (**M1**-**M9**) was written in our previous work (12). The physical data and elemental analyses are presented in Table 1, the spectroscopic data and the structure of the compounds (**M1**-**M9**) in Table 2.

Immunology

The effects of administration of the compounds on the humoral immune response in mice are shown in Table 3 as the number of AFC and the percentage of inhibition with respect to the control response. The actions of the compounds were generally

strongly inhibitory in a dose-dependent manner with exception of compound **M1**. Compound **M9** was active only at 10 μg dose and compound **M3** may be regarded as a relatively weak suppressor. Compound **M5**, in turn, appeared to exhibit the strongest inhibitory property, comparable to that of Cyclosporin A (CsA), the reference drug. At the dose of 10 μg/mouse compounds **M2** and **M8** were also strongly suppressive. The strong suppressive actions of the compounds, administered 4 h before immunization, indicate that they affect the early phase of the immune response like antigen uptake, processing and/or presentation. These strong inhibitory actions, even at low doses, suggest also very good bioaccessibility and high affinity to extra- or intracellular receptors.

Structure activity relationships

The analysis of the structure/activity relationships leads to the following conclusions. Compound **M1**, containing a non-substituted phenyl ring and thiosemicarbazide group does not show significant immunosuppressive activity. Compound **M2**, containing electronegative chlorine atom in position 4 of the phenyl ring and the thiosemicarbazide group, was a stronger suppressor than CsA at concentration of 10 μg/mouse. Compound **M5**, having electronegative chlorine atom in position 4 of the phenyl ring and the semicarbazide group, appeared to be a very strong suppressor (similar in potency to CsA in both doses). So, the change of the thiosemicarbazide group (**M2**) to semicarbazide one (**M5**) with preservation of the 4-chlorophenyl group was associated with increased immunosuppressive activity. Compound **M6**, bearing the semicarbazide group and a strongly electronegative nitro group, exceeded the activity of CsA but only at 10 μg/mouse. Compound **M7**, in turn, possessing the semicarbazide and aliphatic β-chloroethyl group, demonstrated the same suppressive property as CsA at the 1 μg/mouse dose. Lastly, compound **M8**, containing the semicarbazide group and a metoxyphenyl fragment, demonstrated a stronger suppressive action than CsA, but only at 10 μg/mouse. The change of the semicarbazide group (**M8**) to the thiosemicarbazide one (**M9**) with preservation of the 4-metoxyphenyl fragment, abolished the activity of compound **M8** at the lower dose and weakened the suppressive action at the higher dose.

Table 3. Effects of the compounds on the primary humoral immune response of mice to sheep erythrocytes.

Compound	Mouse μg/mL	AFC/10^6 splenocytes	% inhibition	± SE	Student *t*-test
Control	775		38.6		
M1	1 10	675 406	13 48	60.6 44.5	NS NS
M2	1 10	331 112	57 86	27.1 6.3	< 0.01 < 0.001
M3	1 10	344 469	56 40	29.8 27.1	< 0.01 < 0.001
M4	1 10	362 244	54 69	14.0 33.5	< 0.01 < 0.001
M5	1 10	212 162	73 80	37.0 18.8	< 0.01 < 0.001
M6	1 10	406 125	48 84	35.8 8.8	< 0.02 < 0.001
M7	1 10	175 387	77 50	8.8 10.8	< 0.001 < 0.001
M8	1 10	363 119	53 85	20.6 10.4	< 0.01 < 0.001
M9	1 10	794 250	 68	58.9 15.3	NS < 0.001
CsA	1 10	175 131	77 83	8.8 13.6	< 0.001 < 0.001

Table 4. Descriptions of the coded descriptors

Code	Description	Class
SlogP_VSA3	Sum of ν_I such that L_i is in (0, 0.1). (Denotes contribution to logP(o/w) for atom i as calculated in the SlogP descriptor.)	2D
TPSA	Polar surface area calculated using group contributions to approximate the polar surface area from connection table information only.	2D
SMR_VSA3	Sum of ν_I such that R_i is in (0.35, 0.39). (Denotes contribution to molar refractivity for atom i as calculated in the SMR descriptor.)	2D
BCUT_PEOE_1	The BCUT descriptors are calculated from the eigenvalues of a modified adjacency matrix. The diagonal takes the value of the PEOE partial charge.	2D
b_1rotR	Fraction of rotatable single bonds: b_1rotN (Number of rotatable single bonds. Conjugated single bonds are not included) divided by b_heavy (Number of bonds between heavy atoms).	2D

Table 5. Correlation matrix for equations developed for dose1 mg/mL.

	AFC/10^6	SlogP_VSA3	TPSA	SMR_VSA3
PFC/10^6	100	-63	80	57
SlogP_VSA3	-63	100	-74	-55
TPSA	80	-74	100	32
SMR_VSA3	57	-55	32	100

Table 6. Correlation matrix for equations developed for dose10 mg/mL.

	AFC/10^6	BCUT_PEOE_1	B_1rotR
AFC/10^6	100	69	67
BCUT_PEOE_1	69	100	91
B_1rotR	67	91	100

Computational study

Dataset consisted of nine compounds, which display immunological activity. The value of bioactivity was expressed as AFC – the effect of the compounds on the humoral immune response *in vivo*. One compound (**M1**) was rejected from dataset because of decreasing the quality of the statistical descriptions. In reference to the results of humoral immune response test *in vivo*, the compound exhibited the activity distinctly below the activity values for the reference compound – CsA (Table 3). Because of a low number of compounds in the dataset, the maximum number of descriptors is two if one wants to obtain a reliable model. It was necessary for each dose to reject one compound from dataset because of its low relevance average value in the population. The molecular structures were optimized using quantum-chemical density functional theory (DFT) calculations (13, 14) with the B3LYP hybrid exchange-correlation energy functional and 6-31g* basis set. Geometry optimization of nine compounds was performed using the Gaussian 03 software package (15). The second part of theoretical study was an execution of the linear QSAR. The tasks were performed in the following sequence: geometry optimization by quantum chemical DFT calculations at B3LYP/6-31g* level, calculations of descriptors and their selection, and finding quantitative correlations between biological activity and molecular structure by linear QSAR. In an attempt to find quantitative structure-activity relationship, the studies were performed in Molecular Operating Environment software package (MOE). All the 181 2D descriptors only use the atoms and connections information of the molecule for the calculation. 3D coordinates and individual conformation are not considered and internal 3D descriptors use 3D coordinate information about each molecule; however, they are invariant to rotations and translations of the conformation descriptors available in MOE, were

calculated for each molecule (16). After correlation analysis of the descriptors and their discrimination, the linear model using Partial Least Squares method (PLS) was generated. Model performance was measured as the square of correlation coefficient (R^2) and cross-validated R^2. The predictive power of equations was validated by leave one out (LOO) cross-validation method (R^2_{CV}). The calculations were carried out independently for each dose because an analysis of the performed experiments showed drastic dose-dependent changes of activity in all the experiments. The names of the descriptors were coded and this coding was used in the equations and is presenting in Table 4. Also in Tables 5 and 6 correlations of descriptors and their correlation with biological activity are presented.

Below there are presented linear models for each dose, statistical quality of the developed equations and correlation matrix for models.

dose1 μg/mL

1) $AFC/10^6 = -742.23902+9,51199\times TPSA$

$R^2 = 0.91$ $R^2cv = 0.73$

2) $AFC/10^6 = -750.62533+8.58539\times TPSA+17.89289\times SMR\_VSA3$

$R^2 = 0.97$ $R^2cv = 0.93$

3) $AFC/10^6 = -329.52638+6.92331\times TPSA-4.22282\times SlogP\_VSA3$

$R^2 = 0.96$ $R^2cv = 0.83$

4) $AFC/10^6 = -540.49860+7.51429\times TPSA-2.12835\times SlogP\_VSA3+13.38144\times SMR\_VSA3$

$R^2 = 0.98$ $R^2cv = 0.86$

dose10 μg/mL

1) $AFC/10^6 = 899.36206+1294.46145\times BCUT\_PEOE\_1$

$R^2 = 0.82$ $R^2cv = 0.72$

2) $AFC/10^6 = -162.18498+2118.90940\times b\_1rotR$

$R^2 = 0.80$ $R^2cv = 0.66$

The dose of 10 mg/mL allows to obtain only single parameter model. Any additional parameter didn't contribute relevantly to the description of relationship.

In case of 1 mg/mL dose it was possible to generate single-, two- and three parameters equation. Thus we can conclude, that biological response is dependent on more than only one physicochemical property.

Three parameters model may be unreliable due to the number of descriptors corresponding to the number of variable parameters.

From above equations the crucial descriptors for this dataset clearly emerged. They are as follows: TPSA (dose 1 μg/mL) and BCUT_PEOE_1 (dose10 μg/mL). Both descriptors are generally connected with charge distribution within the molecule. Descriptors giving less contribution to models confirm that electronic properties of that compounds have main impact on humoral immune response.

QSAR study revealed quite high correlations with biological activity and structure. The obtained QSAR models may be considered as starting points towards modifications of the investigated compounds, that would be the most advantageous regarding their biological activity. The obtained results may be the basis for predictions of biological activity of yet non-synthesized or non-investigated compounds.

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