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NEW DERIVATIVES OF 5-AMINO-3-METHYL-4-ISOTHIAZOLECARBOXYLIC ACID AND THEIR IMMUNOLOGICAL ACTIVITY

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Abstract: Several new compounds – 4 and 5-substituted derivatives of 3-methyl-4-isothiazolecarboxylic acid were synthesized. These compounds have aminoacylamino groups in position 5 of the isothiazole ring. In position 4, the carboxylic group was replaced by ethyl ester. The biological activites of the obtained compounds were analyzed in the humoral immune response and delayed type hypersensitivity reaction to sheep red blood cells (SRBC) in the mouse model, as well as in the proliferative response of splenocytes to T-cell and B-cell mitogens *in vitro*.

Keywords: 5-amino-3-methylisothiazolecarboxylic acid derivatives, immunological activity.

Among biologically active isothiazole derivatives synthesized in our laboratory, some of them exhibited antiviral, anti-inflammatory and immunosuppressive activities (1, 2) like, for example, Vratizolin, 5-benzoylamino-3-methyl-4-N-(4-chlorophenyl)-isothiazolecarboxamide. Some compounds displayed antitumor activity (4). Recently, we demonstrated that activity was exhibited by isothiazole derivatives, containing, at position 4 amino acid ester residues and at position 5 the benzoyl residue. A particularly strong activity was expressed by an amide possessing at position 4 the methyl ester of alanine residue and at position 5 the aminoacylamine group (3).

Machoń et al. (4) described antitumor action of the isothiazole derivatives containing at position 4 the carboxylic group and at position 5 the aminoacylamine group. It was, therefore, of interest, to synthesize derivatives containing at position 4 of the isothiazole ring the ester group and the aminoacylamine moiety at position 5. Such substitution should increase lipophilicity of the compound. We have already demonstrated that the biological activity required at position 5 of the isothiazole structure the benzoyl or p-chlorobenzoyl substituent, and at position 4 an aromatic amine residue. Replacement of the aroyl group for the acetyl or propionyl group led to a loss of pharmacological activity (5). On the other hand, the compound was active when position 4 was occupied by the aminoester residue, in paricular by alanine (3).

In this investigation we demonstrated that introduction of the aminoacylamine group in position 5 and a lipophilic ester group in position 4, led to acquirement of the immunological activity. Compound **3e**, possessing p-phenetidine residue, was particularly immunosuppressive.

EXPERIMENTAL

Chemistry

The melting points of all new compounds were measured on a Boetius hot-stage apparatus (VEB Kombinat Negema Wagetechnik Rapido Radebeul, Germany) and were uncorrected. Elemental analysis: carried out in the Microlaboratory of the Pharmacy Department, Wrocław Medical University, showed that all the analytical results were according to the calculated ones within ± 0,5%.

The following equipment was employed: for IR spectra (in KBr) – Specord M80 (Zeiss) apparatus; ^{1}H-NMR spectra: Tesla BS 587 A apparatus at 80 MHz (Tesla), TMS was used as internal reference. TLC was run on Polygram SIL G/UV_{254} (D-52313 Duren, Germany) plates using toluene-methanol (9:1, v/v) as elutent. Detection was performed under UV light and or by treatment with iodine vapor.

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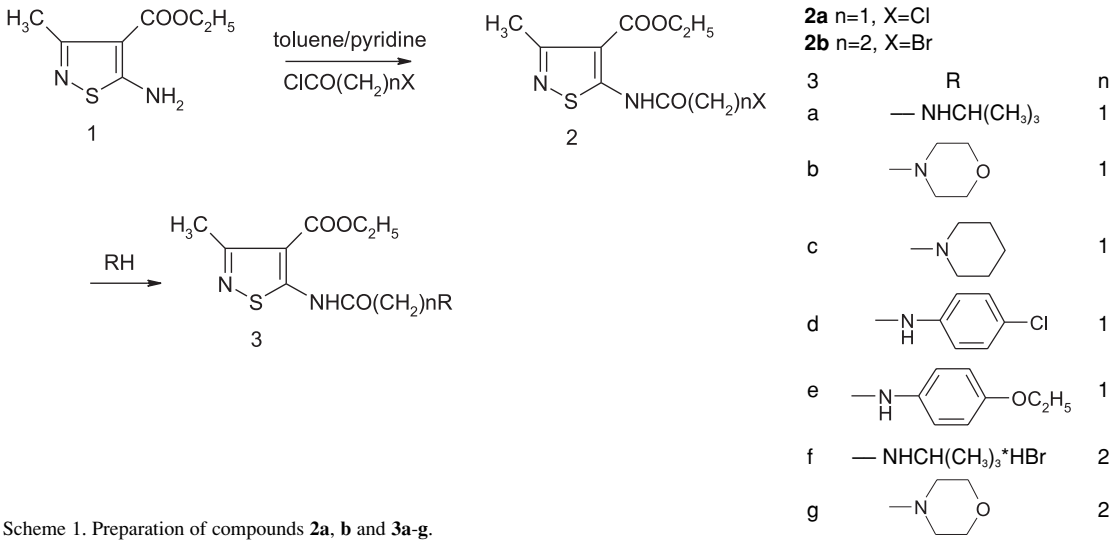

Scheme 1. Preparation of compounds **2a**, **b** and **3a**-**g**.

General method of ethyl 5-halogen acyloamino-3-methylisothiazole-4-carboxylate synthesis

0.01 mole of aminoester **1** was dissolved in 50 mL of anhydrous toluene and 1 mL of pyridine, the solution was cooled and 0.015 mole of (bromo- or chloro-) halogenoacid chloride was added dropwise. Then, the mixture was refluxed for 4 h. The solvent was distilled off under vacuum and the residue was crystallized from methanol. The following compounds were obtained: ethyl 5-(2-chloroacetamido)-3-methylisothiazole-4-carboxylate (**2a**), m.p. 108-109°C, yield 53.4 % and ethyl 5-(3-bromopropanamido)-3-methylisothiazole-4-carboxylate (**2b**), m.p. 98-99°C, yield 65.2 %.

*General method for ethyl 5-(aminoacyl)amino-3-methylisothiazole-4-carboxylates (**3a-3e**)*

0.005 mole of compound **2a** or **2b** in 75 mL of anhydrous toluene and 0.01 mole of respective amine was warmed up for 12-15 h. The course of the reaction was controlled by TLC. After distillation off the solvent under vacuum the residue was crystallized from ethanol. The following compounds were obtained: ethyl 5-(2-isopropylaminoacetamido)-3-methylisothiazole-4-carboxylate (**3a**), m.p. 137-138°C, yield 49.3 %; ethyl 5-(2-morpholinoacetamido)-3-methylisothiazole-4-carboxylate (**3b**), m.p. 119-120°C, yield 64.1 %; ethyl 5-(2-piperidinoacetamido)-3-methylisothiazole-4-carboxylate (**3c**), m.p. 88-90°C, yield 82.5 %; ethyl 5-[2-(4-chlorophenylamino)acetamido]-3-methylisothiazole-4-carboxylate (**3d**), m.p. 126-128°C, yield 42.3 %; ethyl 5-[2-(4-ethoxyphenylamino)acetamido]-3-methylisothiazole-4-carboxylate (**3e**), m.p. 80-82°C, yield 63.2 %; ethyl 5-(3-isopropylaminopropanamido)-3-methylisothiazole-4-carboxylate hydrobromide (**3f**), m.p. 208-210°C, yield 67.6 % and ethyl 5-(3-morpholinopropanamido)-3-methylisothiazole-4-carboxylate (**3g**), m.p. 121-122°C, yield 63.3 %. The properties of the obtained compounds are presented in Table 1.

IMMUNOLOGY

Materials and Methods

Animals: 12-week-old female BALB/c mice were used for the experiments. Mice were kept in the Animal Facility of the Institute of Immunology and Experimental Therapy, Wrocław, fed a commercial, granulated food and water *ad libitum*.

Reagents: Sheep red blood cells (SRBC) were provided by the Wrocław Agriculture Academy. SRBC were kept in Alsever's solution until use. Cyclosporine A (CsA) was from Sandoz, Concanavalin A (ConA), pokeweed mitogen (PWM), DMSO and Cremophor were from Sigma. RPMI 1640 medium and fetal calf serum (FCS) were from Gibco.

Preparation of the compounds for the immunological tests: The compounds were initially dissolved in DMSO and then in RPMI 1640 medium. The compound **3f** (hydrophilic) was dissolved in RPMI 1640 medium only. The compounds were added to the cell culture at doses indicated in Tables 2-5 legends. CsA was used as a reference compound. Appropriate concentrations of the solvent – DMSO, coresponding to the doses of the compounds, were added to the cultures as adequate controls.

Table 1. Physical and analytical data for the obtained compounds.

Compound	Formula Molecular weight	Elemental analysis Calculated/Found			IR (KBr) cm^{-1}	^{1}H-NMR δ [ppm]
		C[%]	H[%]	N[%]		
2a	$C_9H_{11}ClN_2O_3S$ 262.45	41.14 41.21	4.19 4.18	10.67 10.55	1180-1240 CO ester 1665-CONH 1725-$COOC_2H_5$ 3220 CONH	1.39-1.58 (CH_3-CH_2 3H t), 2.63 (CH_3 -3H s), 4.31-4.63 (-CH_2-CH_3 2H q), 4.35 (-CH_2-2H s); 11.0 (NH 1H s)
2b	$C_{10}H_{13}BrN_2O_3S$ 321.18	37.39 37.56	4.05 4.19	8.72 8.51	1220 C-O ester 1670 CONH 1720 $COOC_2H_5$ 2900-2980 (-CH_2-CH_2) 3250 NH-CO	1.35-1.53 (CH_3-CH_2 3H t), 2.62 (CH_3-3H s); 3.67-4.01 (-CH_2-CH_2 –4H m), 4.31-4.57 (-CH_2-CH_3 2H q), 11.2 (NH 1H s)
3a	$C_{12}H_{19}N_3O_3S$ 285.0	50.53 50.25	6.67 6.71	14.74 14.44	1210 (C-O ester) 1680 CONH 1720 $COOC_2H_5$ 2920-2990 isopropyl 3300 (CONH)	1.12-1.21 (-$CH(CH_3)_2$ 6H d), 1.37-1.56 (CH_3-CH_2 3 H t), 2.64 (CH_3-3H s); 2.75-3.08, ($CH(CH_3)_2$ 1H m), 3.6 (-CH_2- 2H s), 4.33-4.61 (CH_2-CH_3 2H q)
3b	$C_{13}H_{19}N_3O_4S$ 313.0	49.84 49.47	6.07 6.17	13.42 13.67	1217 (C-O ester) 1685 CONH 1715 $COOC_2H_5$ 3300-3330 NH-CO	1.35-1.54 (CH_3-CH_2 3H t), 2.62 (CH_3; -CH_2-N-CH_2-7H), 3.33 (-CH_2-2H s); 3.86 (-CH_2-O- CH_2-4H s), 4.3-4.57 (CH_2 -CH_3 2H q), 11.2 (NH 1H s)
3c	$C_{14}H_{21}N_3O_3S$ 311.0	54.02 53.97	6.75 6.52	13.5 13.7	1180 C-O ester 1680 CONH 1720 $COOC_2H_5$ 3290 CONH	1.33-1.36 (-CH_2-CH_3 3H t), 1.63-1.68 (piperid. CH_2 4H m), 2.3-2.53 (piperid. CH_2 6H m), 2.94 (CH_3 – 1H s), 3.3 CH_2 2H s), 4.3-4.4 (CH_2-CH_3 2H q), 10.82 (NH 1 H s)
3d	$C_{15}H_{16}ClN_3O_3S$ 353.5	50.92 51.02	4.52 4.32	11.88 12.00	1180 C-O ester 1690 CONH 1720 $COOC_2H_5$ 1600 –Ar 3320, 3420 CO-NH	1.29-1.32 (-CH_2-CH_3 3H t), 2.9 (CH_3 3H s), 4.15-4.20(-CH_2 2H d), 4.29-4.32 (CH_2-CH_3 2H q), (6.6-6.72 2H d Ar – and 7.13-7.26 2H d Ar), 10.66 (NHCO- 1H s)
3e	$C_{17}H_{21}N_3O_4S$ 363.0 5	6.20 56.52	5.78 5.57	11.57 11.85	1180 C-O ester 1240 C-O ester 1680 CONH 1710 $COOC_2H_5$	1.17-1.29 (-CH_2-CH_3 6H t), 3.1 (CH_3 3H s), 3.8-3.89-CH_2-CH_3 2H q) (eter), 3.98-4.0 (-CH_2-2H d), 4.16-4.23(-CH_2 -CH_3 2H q), 6.53-6.56 (Ar 2H d), 6.72-6.75 (Ar 2H d), 10.52 (NH-CO 1H s)
3f	$C_{13}H_{22}BrN_3O_3S$ 379.0	41.06 40.70	5.75 6.10	11.06 11.17	1230 (C-O ester) 1550 (NH_2^+); 1670 (CONH) 2450, 2720 (NH_2^+) 2920-2980 (isopropyl) 3260 (NHCO)	1.4-1.47 (CH_2-CH_3 and $CH(CH_3)_2$ 9H m), 2.62(CH_3 3H s), 3.21-3.40(CH-CH_2 -CH_2-5H m), 4.31-4.56(-CH_2-CH_3 2H q), 10.5 (NH 1H s)
3g	$C_{14}H_{21}N_3O_4S$ 327.0	51.38 51.29	6.42 6.49	12.84 12.81	1170 (C-O ester) 1670 (NHCO) 1715 $COOC_2H_5$ 2820-2920 (CH_2-CH_2) 3200 (NHCO)	1.35-1.5(-CH_3 3H s), 2.6-2.75 CH_3; -CH_2-N-CH_2-7H d), 3.8-3.98 (-CH_2-O -CH_2-4H m), 4.27-4.54(CH_3 -CH_2-2H q), 11.5 (NH 1H s)

Determination of the antibody-forming cell numbers (AFC) in the cell culture: Mice were sensitized with 0.2 mL of 1 % SRBC suspension in 0.9 % NaCl, intraperitoneally. Four days later the spleens were used for generation of the secondary, humoral immune response *in vitro*. A single cell suspension was prepared by pressing the spleens through a plastic screen into Hanks' medium. The cells were then centrifuged and treated with 0.83% ammonium chloride for 5 min at room temperature to lyze erythrocytes. Subsequently, the cells were washed twice with Hanks' medium and passed through cotton wool to remove cell debris. Finally, splenocytes were resuspended in a culture medium (RMPI 1640, supplemented with 10% fetal calf serum (FCS), glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics) and distributed in 24-well culture plates (5×10^{-6}/well). The cell cultures were immunized with 50 µL of 0.001% SRBC and the peptides were added at 1 – 10 µg/mL doses. After four days of incubation in a cell culture incubator the AFC numbers were determined using Mishell and Dutton assay (13). The results are presented as mean AFC numbers from 4 wells, calculated per 10^6 viable splenocytes ± standard error (SE).

Proliferative response of splenocytes to concanavalin A: Spleen cell suspensions were prepared as described above from naive mice. The cells, resuspended in the culture medium, were distributed into 100 µL aliquots ($2 \times 10/5$/ cells) in 96-flat bottom well plates. Con A was used at concentration of 2.5 µg/mL and the preparations at doses 0.1 – 10 µg/mL. Control cultures contained the solvent at appropriate concentrations. After 3-day culture, cell proliferation was determined using the MTT colorimetric method (14). Cell proliferation was expressed as mean optical density (OD) values from quadruplicate wells ± SE.

Determination of cytotoxicity**:** For determination of cell toxicity a plasmocytoma cell line, 7TD1, dependent on the presence of IL-6 (15) was used.

Proliferative response of plenocytes to pekeweed mitogen: The assay was performed similarly as above using PWM at concentration of 2.5 µg/mL. The cell density was 4×10^5 per well. After 3-day culture, cell proliferation was determined using MTT colorimetric method (14).

Statistics: For the evaluation of data the Student's t-test was applied. The results are presented as mean values from quadruplicate wells (four determinations) ± standard error (SE). The results are regarded as significant when $p < 0.05$. NS = not significant.

Table 2. Cytotoxicity of the compounds against 7TD1 cell line

Compound	Dose (µg/mL)	% viable cells versus control
3c	0.01	117
	0.1	88
	1	77
	10	41
	100	10
3e	0.01	40
	0.1	36
	1	24
	10	26
	100	9
3g	0.01	155
	0.1	130
	1	96
	10	49
	100	0
CsA	0.01	103
	0.1	73
	1	83
	10	6
	100	0

The compounds were incubated at the dose range of 0.01-100 µg/mL with 7TD1 indicator cells stimulate with ConA-conditioned medium, for 48 h. Appropriate solvent concentrations (DMSO for the compounds and Cremophor for CsA) served as respective controls. The cell surivial was determined using MTT colorimetric method. The results are presented as the mean percentage of viable cells as compared to respective control (solvent containing) cultures.

RESULTS

Chemistry

The substrate – ethyl 5-amino-3-methylisothiazole-4-carboxylate 1 was synthesized according to a method described earlier (6). Based on available literature the acylation reaction was carried out using chloride halogenoacids, namely: chloroacetyl chloride and β-bromopropionyl chloride. The reaction was carried out in the presence of pyridine in toluene solution (Scheme 1). According to literature, the reaction of chloroacylation of amines is carried out with application of chloroacid chloride or bromoacid chloride in CH_3CN solution (7), in THF (8), benzene solution (9) or as a biphasic reaction in ether-aqueous solution containing equimolar amount of $NaHCO_3$, amine and chloroacetyl chloride. In our case the reaction yield was 53 and 65%. In the next step the nucleophilic exchange of the chloride or bromide atoms for the amine residue was performed. That type of reactions is usually carried out in: THF solution with a five-fold excess of

Table 3. Effects of the compounds on the secondary humoral immune response *in vitro*

Compound	Dose µg/mL	AFC per 10^6 cells	% inhibition	± SE	p, Student test
Control		650		46.83	
DMSO Control	1 10	719 681	0 0	57.24 34.46	NS NS
3a	1 10	500 437	31 36	17.70 16.6	< 0.05 < 0.001
3b	1 10	250 162	65 76	14.45 29.79	< 0.001 < 0.001
3c	1 10	275 75	62 89	14.45 10.22	< 0.001 < 0.001
3d	1 10	362 112	50 84	38.91 7.23	< 0.01 < 0.001
3e	1 10	44 31	94 96	6.49 7.96	< 0.001 < 0.001
3f	1 10	400 287	39 56	20.44 31.49	< 0.01 < 0.001
3g	1 10	275 119	62 83	27.00 15.75	< 0.001 < 0.001
CsA	1 10	187 94	74 86	16.16 11.98	< 0.001 < 0.001

The compounds were added to the cell cultures at doses of 1 and 10 µg/mL. The results are presented as the mean number of antibody-forming cell in cell cultures, calculated per 10^6 of viable cells, from four wells. The AFC numbers were compared with appropriate DMSO (solvent) concentrations except of **3f** (water-soluble).

amine (7), in acetone in the presence of K_2CO_3 and catalytic concentration of KJ (11), in DMF in catalytic concentration of NaJ (12), in butan-1-ole (8) or by warming up the halogen compound with an excess of amine. In our case a toluene solution of the respective compound **2a** or **2b** was applied, treated with an excess of amine. After completion of the reaction and purification, the structure of the synthesized compounds was determined by means of elemental and spectral analyses (Table 1). When compound **2b** was applied, together with isopropylamine, hydrobromide **3f** was obtained and not the free amine. That compound was very well soluble in water; the elemental and spectral analysis supported such a structure of the synthesized derivatives.

Immunology

In this investigation we evaluated the immunosuppressory properties of a series of compounds in the model of the humoral immune response *in vitro* and in the proliferative response of splenocytes to T and B-cell mitogens. Several compounds appeared to be very active in both tests, in particular **3c**, **3e** and **3g.** Interestingly, **3c** inhibited B-cell proliferative response much stronger than T-cell induced proliferation. At the present stage of investigation, it is difficult to propose an exact mechanism of suppressory action of the compounds studied. It is, however, possible that their inhibitory actions in the humoral immune response are directly associated with the suppression of the proliferative response of T and B cells, as in the case of CsA. Such an association is not always obvious since, as we showed recently (13, 16), an isoxazole derivative RM33 was a very strong suppressor of both the humoral and cellular immune response, while it had no effect on cell proliferation. The ability of the compounds to suppress the proliferative response of lymphocytes, exhibiting at the same time low toxicity, makes them attractive, potential therapeutics for prevention of graft rejection, as cyclosporine and leflunomide. The strong immunosuppressive activites of compound **3e** may be directly associated with its cytotoxicity and not with active suppression without significant cell killing, as in the case of compound **3g**. That compound, at the dose of 10 µg/mL, completely blocked T cell proliferation and very significantly lowered the AFC number but caused only 50% cell kill. At the same dose, CsA, which also blocks T-cell proliferation, causes 94% cell death. What is

Table 4. Effects of the compounds on Concanavalin A-induced splenocyte proliferation

Compound	Dose (μg/mL)	OD 550/630 nm	% suppression	± Set	p, Student test
No Con-A		0.097		0.003	
Con-A only	2.5	0.647		0.014	
DMSO control	0.1	0.595	8	0.013	
	1	0.581	10	0.011	
	10	0.625	3	0.013	
3a	0.1	0.638	0	0.015	NS
	1	0.590	0	0.018	NS
	10	0.546	12	0.007	<0.02
3b	0.1	0.657	0	0.014	<0.02
	1	0.541	7	0.003	<0.05
	10	0.410	34	0.007	<0.001
3c	0.1	0.598	0	0.015	NS
	1	0.505	13	0.013	<0.01
	10	0.128	80	0.003	<0.001
3d	0.1	0.624	0	0.015	NS
	1	0.495	15	0.016	<0.01
	10	0.215	66	0.007	<0.001
3e	0.1	0.245	59	0.003	<0.001
	1	0.157	73	0.005	<0.001
	10	0.045	93	0.002	<0.001
3f	0.1	0.568	12	0.013	<0.01
	1	0.539	17	0.007	<0.05
	10	0.566	13	0.009	<0.01
3g	0.1	0.550	8	0.005	<0.05
	1	0.621	0	0.010	NS
	10	0.001	100	0.000	<0.001
CsA	0.1	0.091	86	0.005	<0.001
	1	0.074	89	0.002	<0.001
	10	0.002	100	0.001	<0.001

The compounds were added to the cell cultures at a dose range 0.1-10 μg/mL. The results are presented as a mean OD value from quadruplicate wells. The OD values were compared with appropriate DMSO (solvent) concetration except of **3f** which is water-soluble.

more interesting, **3g**, at lower doses, increased the cell survival (Table 2).

DISCUSSION AND CONCLUSION

Effects of the compounds on the secondary humoral immune response *in vitro*

The compounds demonstrated differential inhibitory effects on the magnitude of the humoral immune response *in vitro*, expressed as the number of antibody-forming cells in cell culture (Table 3). The suppressive activities of the compounds were dose-dependent and the strongest activities were exhibited by compound **3e**, although other compounds, such as: **3b**, **3d** and **3g**, were also inhibitory, particularly at the dose of 10 μg/mL. Interestingly, the compound **3e** was more potent inhibitor than the reference drug CsA.

Effects of the compounds on the mitogen-induced lymphocyte proliferation

The effects of the compounds on the mitogen-induced splenocyte proliferation are presented in

Tables 4 and 5. In the ConA-induced splenocyte proliferation (Table 4) most potent inhibitory activites were exhibited by compounds **3c**. The activities of **3c** was also significant at the dose of 10 μg/mL.

In the proliferative response of splenocytes to PWM, a B-cell mitogen, best suppressive activities were demonstrated by compounds **3c**, **3e** and **3g**.

Determination of cell toxicity of the compounds

The toxicity of the compounds was tested with respect to 7TD1 plasmocytoma cells (13). We selected the compounds of the strongest immunosuppressive activity, comparing their toxicity to that

Table 5. Effects of the compounds on pokeweed mitogen-induced splenocyte proliferation.

Compound	Dose (μg/mL)	OD 550/630 nm	% suppression	± Set	p, Student test
PWM only	2.5	0.312		0.006	
No PWM		0.055		0.002	
DMSO control	0.1	0.313	0	0.006	NS
	1	0.327	0	0.010	NS
	10	0.308	1	0.004	NS
3a	0.1	0.311	1	0.011	NS
	1	0.275	16	0.006	<0.013a3a
	10	0.260	16	0.015	<0.02
3b	0.1	0.282	10	0.007	<0.02
	1	0.254	22	0.005	<0.001
	10	0.151	51	0.004	<0.001
3c	0.1	0.172	45	0.008	<0.001
	1	0.210	36	0.008	<0.001
	10	0.039	87	0.001	<0.001
3d	0.1	0.316	0	0.008	NS
	1	0.287	12	0.003	<0.02
	10	0.113	6	0.001	<0.001
3e	0.1	0.152	52	0.004	<0.001
	1	0.096	71	0.006	<0.001
	10	0.031	90	0.001	<0.001
3f	0.1	0.316	0	0.007	NS
	1	0.282	10	0.011	<0.05
	10	0.257	18	0.016	<0.05
3g	0.1	0.307	2	0.013	NS
	1	0.284	13	0.008	<0.02
	10	0.099	68	0.010	<0.001
CsA	0.1	0.091	71	0.004	<0.001
	1	0.078	75	0.002	<0.001
	10	0.034	89	0.000	<0.001

The compounds were added to the cell cultures at a dose range 0.1-10 μg/mL. The results are presented as a mean OD value from quadruplicate wells. The OD values were compared with appropriate DMSO (solvent) concetration except of **3f** which is water-soluble.

of CsA. Among the studied compounds (Table 5) **3g** exhibited the lowest cytotoxicity at the dose range 1-10 μg/mL. The cytotoxicity of **3c** was also moderate and lower as compared to CsA. **3e** was highly toxic, more that CsA, at the studied concentration range.

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