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THE IMMUNOREGULATORY EFFECTS OF EDEINE ANALOGUES IN MICE

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Abstract: The edeines analogs were tested in several *in vitro* and *in vivo* assays using the mouse model, with edeine B (peptide W1) and cyclosporine A as reference compounds. The peptides displayed moderate, stimulatory effects on concanavalin A-induced (ConA-induced) splenocyte proliferation, whereas their effects on pokeweed mitogen-induced (PWM-induced) splenocyte proliferation were inhibitory. The peptides inhibited lipopolysacharide-induced (LPS-induced) tumor necrosis factor alpha production but had little effect on interleukin 6 production. In the model of the humoral immune response *in vitro* to sheep red blood cells, peptide 1 was distinctly stimulatory in the investigated concentrations (1-100 μg/ml), whereas peptides 3 and 4 only stimulated the number of antibody-forming cells at the highest concentration (100 μg/ml). In the model of the delayed type hypersensitivity *in vivo* to ovalbumin, the peptides were moderately suppressive (3 being the most active). The reference peptide W1 stimulated ConA-induced cell proliferation at 1-10 μg/ml but was inhibitory at 100 μg/ml. It also inhibited PWM-induced cell proliferation in a dose-dependent manner. This peptide had no effect on the humoral immune response *in vitro* or on cytokine production, but inhibited DTH reaction *in vivo*. The relationship between structure and activity, and a possible mode of action of the peptides, is discussed in this paper.

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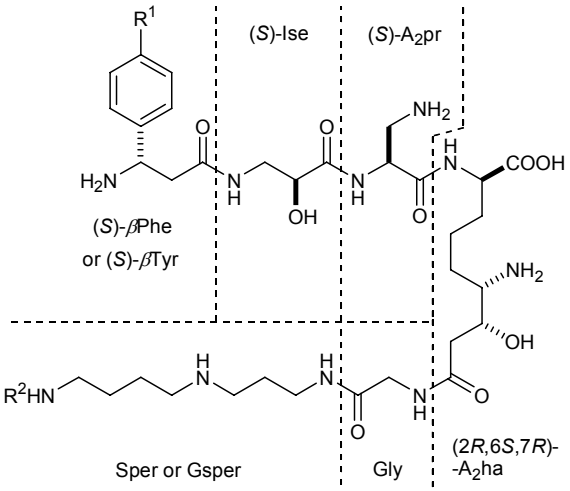
Abbreviations used: AFC – antibody-forming cells; cFa – complete Freund's adjuvant; Con A – concanavalin A; CsA – cyclopsporine; DTH – delayed type hypersensitivity; iFa – incomplete Freund's adjuvant; LPS – lipopolysaccharide; OD – optical density; OVA – ovalbumin; PWM – pokeweed mitogen; SRBC – sheep red blood cells

Key Words: Edeine, Immune response, Mice

INTRODUCTION

Edeines are closely related basic peptide antibiotics produced by the *Bacillus brevis* Vm4 strain [1-3]. These compounds are pentapeptide amides composed of glycine, a polyamine, i.e. spermidine (Sper) or guanylspermidine (Gsper), and four non-protein amino acids: (*S*)-*β*-tyrosine ((*S*)-*β*Tyr) or (*S*)-*β*-phenyl-*β*-alanine ((*S*)-*β*Phe), (*S*)-isoserine ((*S*)-Ise), (*S*)-2,3-diaminopropanoic acid ((*S*)-A_2pr), and (2*R*,6*S*,7*R*)-2,6-diamino-7-hydroxyazelaic acid ((2*R*,6*S*,7*R*)-A_2hp) [4-6]. (2*R*,6*S*,7*R*)-A_2ha is an uncommon amino acid which was detected only in edeines. The method of its chemical synthesis has not yet been elaborated on. The antibiotic complex formed during biosynthesis consists mainly of edeines A, B, D, and F. However, each compound exists as two isomers – the active *α* and the inactive *β*. The linkage between the (*S*)-Ise residue and the (*S*)-A_2pr moiety is via either the *α*- or the *β*-amino group of (*S*)-A_2pr [4-6]. The structures of the edeine *α*-isomers are presented in Fig. 1.



Edeine	R^1	R^2
A	OH	H
B	OH	$C(NH)NH_2$
D	H	H
F	H	$C(NH)NH_2$

Fig. 1. The chemical structures of the *α*-isomers of edeine antibiotics.

Edeine antibiotics reveal a broad spectrum of antimicrobial activity including against Gram-positive and Gram-negative bacteria, fungi [7], and *Mycoplasmas* [8]. These peptides also exhibit considerable immunosuppressive effect, demonstrated in mice [9-11]. Edeines specifically and reversibly inhibit the biosynthesis of DNA [12-14] and possess an ability to differentiate between prokaryotic and eukaryotic microorganisms, based on differences in the function and structure of the DNA replicating systems [15]. Edeine antibiotics are also universal inhibitors of translation [16-19]. The crystal structure of the complex

of the small ribosomal subunit derived from *Thermus thermophilus* was determined with edeine, showing that the binding of this antibiotic might lead to an undesirable stabilization of the subunit conformation, thus contributing to its inhibitory activity [18]. In addition, edeines, as inhibitors of translation in prokaryotic and eukaryotic systems, are useful for studying various aspects of protein synthesis.

At present, there is no available biotechnological source of edeines. Taking into consideration the unique biological properties of these peptides (the immunological activity and the capacity for universal inhibition of protein biosynthesis) and the difficulties in the chemical synthesis of natural antibiotics, we undertook the synthesis of edeine analogues with simplified structures. It was previously demonstrated that the presence of the free ionizable carboxyl group in the (2*R*,6*S*,7*R*)-A_2ha moiety was not essential for the biological activity of edeines [20, 21]. As a continuation of our research project [22], we recently synthesized the following edeine antibiotic analogues (Fig. 2): i) edeine D analogues 1 and 2, in which the (2*R*,6*S*,7*R*)-A_2ha residue was replaced with the (3*R*,4*S*)- or (3*S*,4*S*)-4,5-diamino-3-hydroxypentanoic acid ((3*R*,4*S*)- or (3*S*,4*S*)-A_2hp) moiety, respectively; ii) edeine A analogue 3, with (3*R*,4*S*)-A_2hp instead of (2*R*,6*S*,7*R*)-A_2ha; and iii) edeine D analogue 4, in which the (3*R*,4*S*)-A_2hp moiety was substituted for the (2*R*,6*S*,7*R*)-A_2ha residue and (*S*)-A_2pr was replaced with (*S*)-2-amino-3-(*N*,*N*-dimethylamino) propanoic acid ((*S*)-Me_2A_2pr) to prevent intramolecular isomerization leading to the loss of the biological activity of the α-isomers of edeines in alkaline aqueous solutions. The absolute configuration of the β-hydroxy-γ-amino moiety is unchanged in compounds 1, 3 and 4, containing the (3*R*,4*S*)-A_2hp residue. Peptide 2 has the inverse configuration of the carbon atom bonded with the hydroxyl group relative to the original antibiotics.

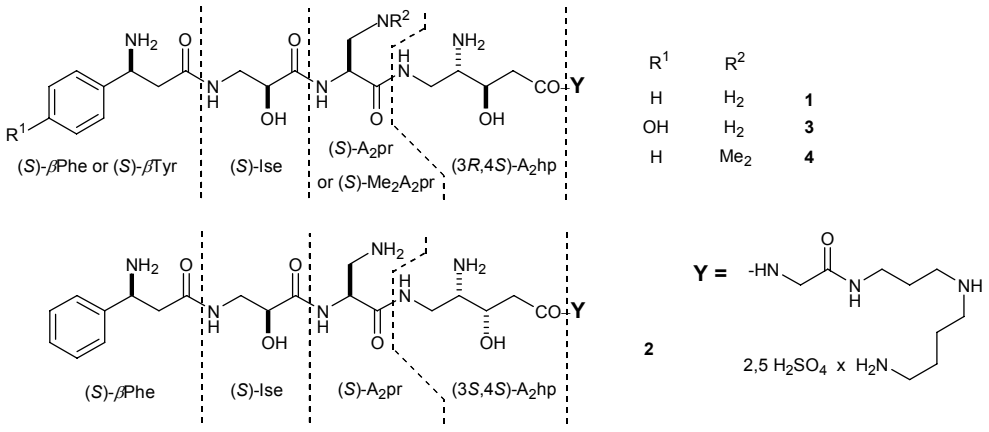


Fig. 2. The chemical structures of analogues of edeines A and D.

Compounds 1-4 were shown to exhibit significantly diminished antibacterial and antifungal activities [22], and they did not display toxic effects towards mammalian cells [23]. Initial studies on the immunological effect of edeine, performed in the mid seventies [9, 11] demonstrated their suppressive actions in the models of the humoral and cellular immune responses of mice *in vivo*. Therefore, it was of interest to determine the immunotropic activities of the newly synthesized edeine analogues in selected immunological assays *in vitro* and *in vivo*. The investigation revealed interesting differential activities of the peptides, with potential therapeutic application.

MATERIALS AND METHODS

Animals and reagents

Twelve-week old CBA mice were used for the experiments. The animals were fed a commercial, pelleted food and filtered tap water *ad libitum*.

Ovalbumin (OVA), complete Freund's adjuvant (cFa), incomplete Freund's adjuvant (iFa), Concanavalin A (ConA), pokeweed mitogen (PWM), lipopolysacharide (LPS) from *E. coli* serotype 0111:B4 ($3x10^6$ E.U. mg^{-1}), and MTT 93-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolim bromide were purchased from Sigma Chemical Company (MO, USA). Cyclosporin A (CsA) was from Sandimmun (Neoral, Sandoz, Basel, Switzerland) in ampoules, edeine B sulphate (peptide W1) from Gdańsk University of Technology (Gdańsk, Poland), RPMI-1640 medium from Cibi/Life Technologies (UK), and FCS-fetal calf serum from Gibco. Peptides 1-4 were synthesized according to the recently described procedure [22].

Proliferation assay

The extracted spleens were pressed against a plastic screen into 0.83% NH_4Cl solution to lyze erythrocytes (5 min incubation at room temperature). The cells were then washed twice with Hanks' medium, passed through a glass wool column to remove debris, and re-suspended in the culture medium, referred to below as the culture medium, consisting of RPMI 1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics. The cells were then distributed into 96-well flat-bottom tissue culture plates (Nunc) at a density of $2x10^5$/100 µl/well. 2.5 µg/ml Concanavalin A (ConA) was added to induce cell proliferation. The compounds were added to the cultures at doses of 1, 10 and 100 µg/ml. After a 3-day incubation, the cell proliferation was determined using a colorimetric MTT assay [24]. The results are presented as the mean optical density (OD) at 550 nm ± standard error (SE) from quadruplicate determinations.

Mitogen-induced cytokine production and determination of cytokine activities

Spleen cells were obtained and prepared as described above. The cell suspension in the culture medium ($5x10^6$ cells/ml) was distributed to 24-well culture plates

in 1 ml aliquots. LPS was added to the cultures at a concentration of 5 μg/ml. The peptides were added at concentrations of 10 and 100 μg/ml at the beginning of the culture. After overnight culture, the supernatants were harvested, aliquoted and kept frozen at -20ºC until needed for cytokine determination. TNF alpha and IL-6 activities were measured by bioassay using WEHI 164.13 and 7TD1 indicator cell lines, respectively [25, 26].

Determination of the secondary immune response *in vitro*

Mice were primed with 0.2 ml of sheep erythrocyte (SRBC) suspension, administered intraperitoneally. After 4 days, the splenocytes were isolated and a single cell suspension was prepared in the culture medium. The cells were incubated in 24-well culture plates at a density of 5 x 10^6 cells/ml with the addition of 50 μl of 0.005% SRBC. The peptides were added to the cultures at the beginning of a 4-day incubation in a cell culture incubator, in doses of 1, 10 and 100 μg/ml. After 4 days, the number of antibody-forming cells (AFC) against SRBC was determined according to Mishell and Dutton [27]. The results are shown as the mean values of AFC number from 4 wells ± SE, calculated per 10^6 viable spleen cells.

Generation of the cellular immune response to ovalbumin

Mice were immunized subcutaneously (s.c.) into the tail base with 5 μg OVA in cFa. The peptides (200 μg doses in 0.2 ml of saline) were given intraperitoneally, 2 h after immunization. After 4 days, the delayed type hypersensitivity (DTH) reaction was elicited by s.c. injection of 50 μg OVA in iFa into the hind feet. The specific DTH reaction was calculated by subtracting the foot pad thickness of naïve mice, given an eliciting dose of the antigen from the DTH reaction of sensitized mice [28].

Statistics

The data is expressed as the means ± SE, except for cytokine determination. Differences between the groups were analyzed using the Student's unpaired *t* test. A P value of 0.05 or less was considered significant.

RESULTS

Synthesized edeines were tested for their ability to affect ConA-induced splenocyte proliferation. The results (Tab. 1) showed that, in general, these peptides exhibited moderate stimulatory properties. The stimulation took place at a concentration of 1 μg/ml, and was higher at 100 μg/ml. Of the peptides studied, compound 4 demonstrated the most uniform and potent stimulatory activities. The reference peptide W1 showed interesting, dose-dependent immunoregulatory properties, i.e. it was stimulatory at 1 μg/ml and inhibitory at 100 μg/ml.

Tab. 1. The effects of the edeines on ConA-induced splenocyte proliferation.

Peptide	Dose [μg/ml]	OD 550/630nm[a]	±SE	*P* (Student's test)
No mitogen		0.052	0.004	
Mitogen, control[b]		0.312	0.022	
	1	0.430	0.007	< 0.01
W 1	10	0.366	0.002	> 0.05
	100	0.230	0.010	< 0.02
	1	0.375	0.016	> 0.05
1	10	0.388	0.006	< 0.02
	100	0.434	0.016	< 0.01
	1	0.409	0.014	< 0.01
2	10	0.399	0.012	< 0.02
	100	0.437	0.023	< 0.01
	1	0.412	0.006	< 0.01
3	10	0.386	0.007	< 0.02
	100	0.466	0.019	< 0.01
	1	0.435	0.005	< 0.01
4	10	0.424	0.008	< 0.01
	100	0.473	0.008	< 0.001
	1	0.079	0.002	< 0.001
CsA	10	0.009	0.001	< 0.001
	100	0.005	0.001	< 0.001

[a]Results are expressed as the mean OD value of quadruplicate determinations ± SE, [b]ConA only (2.5 μg/ml).

The effects of the peptides on PWM-induced splenocyte proliferation are presented in Tab. 2. The effects of the peptides were differential, although generally inhibitory. The reference peptide W1 displayed dose-dependent suppression of cell proliferation, reaching about 70% inhibition at 100 μg/ml. Compound 2 showed moderate inhibitory activity at 10-100 μg/ml doses (44-42% inhibition). 3 and 4 were rather weak inhibitors in the studied dose range.

The peptides were investigated for their ability to alter the LPS-induced production of TNF alpha and interleukin 6 (IL-6) in splenocyte cultures (Tabs 3 and 4). It appeared (Tab. 3) that peptides 1-3 inhibited TNF alpha production to a similar degree at both doses (about 50% inhibition). 4 was only inhibitory at 100 μg/ml, and the reference peptide W1 was devoid of any inhibitory activity. The effects of the peptides on LPS-induced IL-6 production were absent except for compound 4**,** which stimulated IL-6 production (by 2-fold at 100 μg/ml).

The effects of the peptides on the magnitude of the humoral immune response *in vitro*, expressed as the number of antibody-forming cells (AFC) is shown in Tab. 5. The results revealed marked immunostimulatory effects of peptide 1. Peptides 3 and 4 only significantly elevated the AFC numbers at a concentration of 100 μg/ml. W1 and 2 did not change the immune response in that model.

Tab. 2. The effects of the edeines on PWM-induced splenocyte proliferation.

Peptide	Dose [μg/ml]	OD 550/630nm	± SE	*P* (Student's test)
No mitogen		0.124[a]	0.003	
Mitogen, control[b]		0.479	0.009	
	1	0.314	0.009	< 0.001
W 1	10	0.314	0.009	< 0.001
	100	0.150	0.008	< 0.001
	1	0.340	0.021	< 0.001
1	10	0.407	0.012	< 0.01
	100	0.300	0.011	< 0.001
	1	0.440	0.006	< 0.02
2	10	0.270	0.008	< 0.001
	100	0.278	0.007	< 0.001
	1	0.378	0.016	< 0.01
3	10	0.331	0.012	< 0.001
	100	0.396	0.007	< 0.001
	1	0.359	0.011	< 0.001
4	10	0.415	0.007	< 0.01
	100	0.355	0.009	< 0.001
	1	0.096	0.002	< 0.001
CsA	10	0.019	0.001	< 0.001
	100	0.004	0.001	< 0.001

[a]Results are expressed the mean OD value of quadruplicate determinations ± SE, [b]PWM only (2.5 μg/ml).

Tab. 3. The effects of the edeines on LPS-induced TNF alpha production in splenocyte cultures.

Preparation	Dose [μg/ml]	TNF alpha [pg/ml][a]
No LPS		53
LPS, control[b]		206
W1	10	217
	100	194
1	10	105
	100	85
2	10	105
	100	108
3	10	101
	100	109
4	10	198
	100	102
	0.1	59
CsA	1	36
	5	24

[a]TNF alpha activity was determined by bioassay, [b]LPS only (5 μg/ml).

Tab. 4. The effects of the edeines on LPS-induced IL6 production by splenocyte cultures.

Peptide	Dose [μg/ml]	IL-6 [pg/ml][a]
No LPS		0.4
LPS, control[b]		17
W1	10	21
	100	18
1	10	18
	100	17
2	10	14
	100	15
3	10	16
	100	17
4	10	27
	100	35
CsA	0.1	25
	1	23
	5	32

[a]IL-6 activity was determined by bioassay, [b]LPS only (5 μg/ml).

Tab. 5. The effects of the edeines on the humoral immune response *in vitro* to SRBC by mouse splenocytes.

Peptide	Dose [μg/ml]	AFC/10^6	± SE[a]	*P* (Student's test)
Control[b]		975	34.1	
W 1	1	1060	25.9	> 0.05
	10	1030	44.4	> 0.05
	100	865	31.0	> 0.05
1	1	1375	66.5	< 0.01
	10	1355	40.4	< 0.001
	100	1945	153.9	< 0.001
2	1	845	45.8	> 0.05
	10	965	64.0	> 0.05
	100	1005	16.3	> 0.05
3	1	815	49.3	> 0.05
	10	970	59.8	> 0.05
	100	1770	58.0	< 0.001
4	1	845	25.0	> 0.05
	10	1005	85.1	> 0.05
	100	1410	17.3	< 0.001

[a]The results are expressed as the mean of 4 determinations ± SE, [b]Control: RPMI medium.

The results (Tab. 6) showed that most of the peptides exhibited differential inhibitory action in terms of the magnitude of the cellular immune response to OVA (with the exception of compound 1). The strongest suppressive activity was demonstrated by compound 3.

Tab. 6. The effects of the edeines on delayed type hypersensitivity to OVA *in vivo*.

Peptide	Dose [μg/ml]	DTH units[a]	± SE[b]	*P* (Student's test)
Control[b]		13.4	0.61	
W 1	200	8.4	0.78	< 0.001
1	200	11.6	0.41	>0.05
2	200	11.5	0.33	>0.05
3	200	8.2	0.42	< 0.001
4	200	10.6	0.36	< 0.01
CsA	200	8.5	0.47	< 0.001

[a]One unit = 10^{-2} cm, [b]The results are expressed as the mean ± SE of five mice, [c]Control 0.9% NaCl solution.

DISCUSSION

The results described in this article reveal the differential activities of the peptides in several selected immunological models, which should be analyzed individually. The peptides represent various modifications of the W1 precursor inhibitory peptide [9, 11], the suppressive properties of which in some models were also confirmed. W1 peptide significantly suppressed PWM-induced splenocyte proliferation, in particular at 100 μg/ml, while its effect on ConA-induced proliferation was strictly dependent on the dose used; the peptide was stimulatory at a low dose (1 μg/ml) and inhibitory at 100 μg/ml. Such results indicate that W1 is inhibitory for B-cell proliferation and regulatory with respect to T-cell proliferation. The derivatives of W1 retained the inhibitory property of that precursor with respect to B-cell proliferation, but they lost the property to inhibit T-cell proliferation since all of them, to various degrees, were stimulatory in that model. W1 was, in addition, significantly inhibitory in the model of delayed type hypersensitivity, with a potency similar to that of CsA. However, it did not affect the humoral immune response. These results are consistent with early studies on the activity of edeine on the cellular immune response to *Listeria monocytogenes* [11]. Preliminary studies on the effects of edeine on the primary humoral immune response in mice showed that it was significantly suppressive in that model when administered at the time of immunization and on subsequent days after immunization [9]. In this investigation, W1 was not active (Tab. 5). However, the investigated experimental models were different: we used the model of the secondary immune response *in vitro*, which is less susceptible to inhibition because of the existence of antigen-specific T cells.

The immunological activity of peptide 1 is clearly directed to enhance the humoral immune response (Tab. 5), since at all doses used, it was significantly stimulatory. However, it did not significantly affect the cellular immune response (Tab. 6). In addition, it stimulated the proliferation of splenocytes by the T-cell mitogen ConA and inhibited the proliferation of splenocytes by the B-cell mitogen PWM. Compound 1 differs from 2 by the inversion of the absolute configuration of the C-3 carbon atom in the diastereoisomers of 4,5-diamino-3-hydroxypentanoic acid. It is of interest that such a subtle modification of the basic structure of the almost immunologically inactive 2 may lead to such a profound change in activity.

Peptide 2 was not active in the humoral immune response, and demonstrated little activity in the DTH reaction. However, it retained the properties of compound 1 with regard to its influence on mitogen-induced cell proliferation. The marginal effect of peptide 2 on the immune response could be a result of the alteration of the absolute configuration of the β-hydroxy-γ-amino moiety in comparison to natural antibiotics and compound 1.

The immunostimulatory action of peptide 3 in the humoral immune response was strong, but occurred only at a concentration of 100 μg/ml (Tab. 5). On the other hand, this peptide was strongly inhibitory in the DTH model *in vivo* (Tab. 6). Such bidirectional activity was an exception among the peptides studied in this report. Interestingly, it differs from 1 by a substitution of a hydrogen atom in the *para* position of the aromatic ring with the hydroxyl group. So, the addition of that group to the structure of compound 1 led to the formation of a compound exhibiting quite different properties in the immune response.

The analysis of the peptide 4 activity revealed particularly strong and uniform stimulation of T-cell proliferation (Tab. 1) at all doses. The inhibition of B-cell proliferation was rather moderate (Tab. 2). This peptide was also interesting, since it behaved differently in the models of cytokine induction. Whereas none of the other peptides affected IL-6 production, 4 stimulated the production of that cytokine. In addition, it only inhibited TNF alpha production at higher (100 μg/ml) concentrations. It differs from 1 by the replacement of the 3-amino group in (*S*)-2,3-diaminopropanoic acid with a 3-*N*,*N*-dimethylamino group.

The effects of edeine derivatives on T- and B-cell proliferation may, at first glance, contradict their effects on the cellular and humoral immune response. However, mitogen-induced cell proliferation engages all mature lymphocytes, whereas the antigen-specific immune response is mediated by a very small proportion of cells. In addition, recruitment of antigen-specific cells and non-specific polyclonal stimulation of lymphocytes involve different cellular mechanisms and signaling pathways. Consequently, the mechanism of actions of edeine derivatives in the models of specific and non-specific cell stimulation may be different.

These results on the immunotropic activity of W1-derived peptides, although preliminary, demonstrated the interesting relationship between the structure and activity of the peptides. Each of the compounds clearly exhibited different

characteristics. The properties of the described peptides could be altered by quite minor changes (substitutions) in the general structure. Some of the peptides, e.g. compound 2, may be of no further interest because of their lack of effect on the immune response. From the presented model, it is difficult to propose an exact mechanism of action of the peptides on the immune response. Nevertheless, the peptides may affect the induction process of the immune response (DTH model), since they were active when added 2 h after immunization. In turn, their effects on the later stages of the immune response cannot be excluded because they were active (stimulatory) in the secondary humoral immune response *in vitro*. Of interest, most of the peptides inhibited LPS-induced TNF alpha production, this phenomenon suggesting potential anti-inflammatory properties. That effect could be due to the direct binding of LPS by highly basic edeine molecules. It is also evident that the substantial decrease in the antibacterial and antifungal activity [22] correlated with the acquisition of interesting immunomodulatory properties (this article). In conclusion, some of the peptides deserve further studies in other experimental models, which could reveal potential therapeutic benefit.

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