

Received Date : 07-Aug-2015

Revised Date : 03-Nov-2015

Accepted Date : 05-Nov-2015

Article type : Research Article

***In vitro* evaluation of the allergic potential of antibacterial peptides: camel and citropin**

Running title: Allergic potential of antibacterial peptides

Keywords: peptides; allergy; basophils; flow cytometry

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/cbdd.12688

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Abstract

Peptide-based drugs are promising group of compounds which are characterized by specificity to their in vivo targets and high potency of action (antineoplastic, immunoregulatory, antibacterial). The peptides, however, involve a relatively high risk of allergic reactions that are not predictable on the basis of their sequence and chemical properties. In this study peripheral blood was obtained from 53 patients including 38 hypersensitive patients and 15 control patients. Basophil activation stimulated by two antibacterial peptides (camel, citropin 1.1) and acetylsalicylic acid was assessed by means of BAT (Basophils Activation Test). Basophil activation stimulated by camel occurred in 7 out of 38 patients with hypersensitivity (18.42%) as well as in 2 out of 15 control patients (13.33%). Basophils were activated by citropin 1.1 in 7 out of 38 hypersensitive patients (18.42%) and in none of the control patients. Using the Structural Database of Allergenic Proteins we confirmed that the examined peptides share some structural similarities with common environmental allergens. Therefore, the cross-reactivity between potentially present anti-allergen IgE with examined peptides cannot be excluded. Our study proved that BAT,

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together with other biological tests and specific databases of allergenic compounds, may serve as an initial selection of new active peptides and proteins.

Introduction

Peptides are extremely promising group of potential drugs due to their high specificity and biological activity. They can mimic naturally occurring compounds such as growth factors, peptides and hormones. Additionally, they can be easily modified, therefore changes in their physicochemical properties, pharmacodynamic and therapeutic applications are feasible (1-5). There is a wide range of possible usage of peptides in therapy, i.e. as antineoplastic agents, immunoregulatory, antibacterial etc. (6-10). Currently, there is an increase in the role of peptides and protein drugs in the medical market. There is also a visible surge in a number of preclinical and clinical studies of new peptide-structured compounds (5,6). Antimicrobial peptides (AMPs) are a broad class of compounds exhibiting potent antimicrobial activity often accompanied by additional biological properties (i.e. antifungal, antineoplastic, anti-inflammatory and others) (11-13). AMPs are the focus of scientists mainly due to the emergence of growing numbers of drug-resistant bacteria strains. This is particularly vital in the management of wounds, as they are often infected with multidrug-resistant strains including *Staphylococcus aureus*. Wound infections lead to delayed healing, and they pose a threat of a systemic infection (sepsis) (14-18). The design of synthetically produced AMPs is generally based on chemical structures of compounds naturally occurring in nature, i.e. in amphibians and insects. Mechanisms of action presented by peptides are completely different from those of conventional antibiotics, therefore development of bacterial resistance to these compounds is rarely observed (19-21). One of the major drawbacks of peptides and proteins is their relatively high immunogenicity and high allergenic potential. The risk of allergy is very difficult to predict based on the sequence and chemical structure of the peptide (5,22).

Therefore, it is extremely important to determine this risk before new compounds are introduced into a very expensive phase of clinical trials (in volunteers and patients) or routine clinical practice (6,23). Currently, the sensitive screening tests assessing allergy risk are being developed in order to utilize them in a preclinical phase. Most of these studies make use of animal models (mice, rabbits), which do not reflect human immune response, despite genetic homology and similar immunological and physiological mechanisms (5,23). In allergic reaction the major role is played by tissue mast cells and their functional equivalents at the periphery - basophils. The latter are easily accessible, that is why they become widely used in allergy tests (24,25). In the presented study we used Basophil Activation Test (BAT) to determine the degree of basophil activation, after the stimulation with synthetically produced peptides citropin 1.1 and camel as well as acetylic acid. In this study the expression of three specific markers was analyzed: CCR3 (marker of basophil population), CD63 and CD203c (basophil activation markers). This method has been described in numerous publications and applied in many studies assessing its performance in food allergies, drug-induced allergies, venom-induced anaphylactic reactions (26,27). The peptide camel is a 15 amino acid hybrid peptide derived from the combination of two insect peptides: cecropin A and melittin. Besides its antibacterial properties, camel also displays antitumor activity (8,27). Citropin 1.1 is one of the antimicrobial peptides with the simplest structure but displaying a very broad spectrum of antibacterial activity. In nature, citropin can be found in green tree frog – *Litoria citropa* (28,29). The objective of this study was to evaluate the allergic potential of camel and citropin in vitro. In addition, we aimed to verify whether people with a variety of hypersensitivities, including those against acetylsalicylic acid, might induce a stronger immunological reaction to the AMPs.

Materials and methods

Peptides

Camel and citropin 1.1 (Table 1) were obtained by Fmoc chemistry on polystyrene AM-RAM resin using Liberty Blue microwave peptide synthesizer (CEM Corporation, USA). The compounds were purified (Luna C18, 30x100mm, 20-60% ACN, 10 ml/min, 60 min) and analyzed (Luna C18, 4.6x150mm, 10-90% ACN, 2ml/min, 10 min.) by reversed-phase high performance liquid chromatography (RP-HPLC). The confirmed purity of both compounds was over 97 %. Identity was confirmed by ESI MS with Waters ACQUITY QDa mass detector (Camel $m/z = 1770,34$ Da; Citropin1.1 $m/z = 1614,98$ Da) (30).

Patients

A total number of 53 participants were included in the study. The first group consisted of 38 patients, including 26 women and 12 men, with suspected aspirin (ASA) hypersensitivity. The diagnosis was based on clinical history with positive basophil activation test and/or provocation test. Single-blinded placebo-controlled aspirin provocation tests were performed in the Department of Allergology, Medical University of Gdańsk, according to two-day protocol. On the first day, 4 doses of placebo were administered every two hours; on the second day – increasing doses of aspirin up to cumulative dose of 1000 mg. Provocation was assessed to be positive in case of fall of FEV_1 of 20% or more, reduction of nasal inspiratory flow of at least 40% or typical clinical signs and symptoms of hypersensitivity reaction (urticaria, angioedema, flush, dyspnea, wheezing, fall in blood pressure etc.). Characteristics of patients and concomitant allergic diseases have been shown in Table 2. Exclusion criteria were: immunologic disorders, immunosuppressive therapy, renal or hepatic insufficiency, cancer and current infection. The participants were recruited among patients hospitalized in the Department of Allergology, Medical University of Gdańsk. Control group comprised 15

healthy volunteers, including 12 women and 3 men, with no history of allergic diseases, immunologic disorders or any clinical conditions that could influence on results of BAT. The study protocol was approved by the Ethics Committee of the Medical University of Gdańsk and the samples of blood were only taken when informed consent from the patients was received.

Basophil Activation Test (BAT)

The reagent kit Flow CAST® highsens (Bühlmann Laboratories, Switzerland) was used to perform basophil activation test. Peripheral blood was collected in EDTA tubes and the test was performed within 24 hours of blood sampling according to manufacturer's instructions. Briefly, 100 µL of blood was incubated separately with 100 µL ASA allergen (provided by Bühlmann Laboratories, Switzerland), citropin 1.1, camel and PBS (the final concentration for citropin 1.1 and Camel was 10 µg/mL, that is 6.19 µM and 5.65 µM respectively). Samples were stained using 40 µL of anti-CD63, anti-CD203c antibodies mixture conjugated with the tandem dye PE-DY647 (PerCP Cy5.5 channel) and anti-CCR3 antibodies conjugated with phycoerythrin (PE channel). The samples were incubated for 15 minutes in water bath in 37°C. Followed incubation step, lysis and wash procedure was performed and the samples were analyzed within two hours using BD FACSCanto II flow cytometer. For each patient sample, negative and positive (anti-FcεRI mAb and tripeptide fMLP) stimulation controls were prepared. For each sample 100 000 events were analyzed according to gating strategy shown in figure 1S. The cut-off values for analyzed allergen were estimated as 2.0 – 2.5 % of negative stimulation control sample. According to the manufacturer recommendation the cut-off value for activation of basophils was estimated as 10%.

Results

Reaction to anti-FcεRI antibodies and fMLP is similar in both groups – hypersensitivity patients and control

Antibodies activating FcεRI and unspecific cell activator fMLP were used as a positive controls in BAT assay. The cut-off value of basophils activation was estimated as 10%, according to the manufacturer recommendation. The obtained results show that the basophil activation presented as the percentage of basophils activated by anti-FcεRI mAb and fMLP do not statistically differ between two analyzed groups (1 - patients with hypersensitivities and 2 - control group). The positive reaction to anti-FcεRI mAb was observed in 35 out of 38 (92.1%) in group 1 and 13 out of 15 (86.6%) in control group (fig. 1A). Whereas fMLP induced positive reaction in 32 out of 38 hypersensitive patients (84.2%) and in 10 out of 15 of healthy controls (66.5%) (fig. 1B).

Activation upon citropin 1.1 and camel differs in hypersensitive patients and healthy donors and correlates with ASA activation.

We also assessed the basophil reaction to citropin 1.1 in patients with hypersensitivity (n=38) and healthy donors (n=15). In the first group, the activation of basophils induced by citropin 1.1 was characterized by more intensive reaction in comparison to control group ($p<0.01$). There were 7 out of 38 patients from the first group, who were characterized by a reaction exceeding the 10% threshold. In the control group the observed activation did not reach the threshold value (fig. 2A). After stimulation with camel, the differences in stimulation between both groups were also noted ($p<0.05$). The activation in the first group was observed in 7 out of 38 analyzed patients, in the second group – 2 out of 15 healthy patients (fig. 2B).

The obtained data show that the whole analyzed population of patients, both hypersensitive and healthy (n=53) was characterized with a positive correlation between the level of basophil activation stimulated with citropin 1.1 peptide and the activation of these cells with acetylsalicylic acid (fig. 3A; $p<0.05$, $r=0.338$). Also the positive correlation for the whole analyzed group was observed for camel and acetylsalicylic acid (fig. 3B; $p<0.05$, $r=0.333$).

Citropin 1.1 and camel share similarities with common environmental allergens

Based on the Structural Database of Allergenic Proteins (31), it was confirmed that both citropin 1.1 and camel peptide share some chemical structure similarities with common environmental allergens. Table 3 and 4 present the examples of allergens (i.e. grass, mites) with the highest similarities to citropin 1.1 and camel peptide (the lowest index PD – property distance similarity index). According to available literature data, a significance cut-off value between 7.5 and 9 would be most appropriate for determining peptides with similar properties (32). In our study, we used both extreme values to find potential similarities between our peptides and common allergens. With the cut-off value $PD=7.5$, no available environmental allergens showed suitable similarity to camel peptide or citropin 1.1. When we analyzed the second PD cut-off value 9.0, we found out that only 12 allergens for citropin 1.1 and 32 for camel peptide shared similarity with examined compounds.

Discussion

Peptides can constitute a very important group of drugs with a variety of therapeutic possibilities. However, there are still many challenges in the fast and reliable assessment of the safety and effectiveness of these compounds (5,6,33). In this paper we present the results of allergenic potential of two antibacterial peptides (citropin 1.1 and camel). The obtained data indicate that the analyzed peptides display the allergenic potential mainly in

hypersensitive patients. This seems vital as patients with hypersensitive reactions, including allergies, become constantly expanding group, thereby posing a significant medical problem (6,34). Therefore, our research may be a valuable clue to preclinical and clinical trials which are mainly based on animal models and patients with no allergies. It should be noted that more than 80% of potential drugs, which have passed the preclinical testing phase, is discarded in clinical trials due to their side effects including toxicity, immunogenicity and induction of allergies (5,23). An allergic reaction is mediated mainly by the tissue mast cells and their equivalents in the peripheral blood, basophils. The BAT assay, used in the present study, is routinely used test allowing for clinical assessment of allergies to various compounds including medicines, insect venom, synthetic dyes, etc. It was shown by other authors that BAT is more sensitive for the evaluation of drug allergies and insect venom than skin prick testing (SPT) or fluorescent enzymeimmunoassay (FEIA). The FEIA measure quantity of allergen-specific IgE antibodies in the serum and diagnoses an allergy to different kind of allergens (e.g. animal, plant, latex) (34). It should be noted that BAT can be also performed with patients undergoing anti-allergic treatment in contrast to SPT (25,35,36). In the present work, we decided to use a concentration of 10 µg/mL for both peptides (camel – 5.65 µM; citropin 1.1 – 6.19 µM). Our previous studies have shown that camel and citropin in certain concentrations exert strong antimicrobial properties, even against clinically isolated strains of *S. aureus*. The MIC50 value for camel was 4 µg/mL (2.26 µM), and for citropin 1.1 8 µg/mL (4.95 µM). The compounds did not exhibit toxicity toward human keratinocytes once applied at concentrations of 10 µg/mL (5.65 µM) (camel) and 25 µg/mL (15.47 µM) (citropin 1.1) (14). In present study the camel peptide proved to be a more effective activator of basophils than citropin 1.1, as it caused activation of these cells in a higher number of healthy patients (13.33% vs 0%). This may be a result of differences in the amino acid sequence and origin of both compounds. The camel peptide presents hybrid structure

composed of fragments two insect-derived peptides, while citropin 1.1 is naturally found in amphibians (27). It is vital to add, that due to various molar mass of the examined peptides, the molar concentration of citropin 1.1 peptide was higher than camel. Furthermore, we observed a positive correlation between basophil activation stimulated by each peptide and acetylsalicylic acid. This observation indicates particular caution while administering new peptide/ protein drugs to patients with hypersensitivity to other available drugs.

In our study basophil activation was obtained due to the stimulation with fMLP (tripeptide causing basophil activation in a non-immunologic and immunologic way) or FcεRI antibodies. The fMLP mode of action utilizes specific receptors on basophils, either formyl peptide receptor (FPR) or formyl peptide receptor-like 1 (FPRL1). Both types of receptors activate intracellular signaling pathways in basophils, leading to increased chemotaxis of cells as well as release of leukotriene and histamine (37,38). Interestingly, basophil activation with either fMLP or anti-FcεRI mAb (positive controls) did not differ statistically between hypersensitivity patients and control patients.

The available database – SDAP and PD index, allowed to find and confirm that the examined peptides share some structural similarities with common environmental allergens (31). PD values or similar indices may allow us to predict the allergenic potential of a protein and to design hypo-allergenic proteins. The PD index is calculated on the basis of the comparison of the interested sequence and a list of sequences available in the database. Therefore, the cross-reactivity between potentially present anti-allergen IgE with examined peptides cannot be excluded (32). The reaction observed in our study could be mediated by both non-IgE dependent mechanisms and classic IgE dependent reaction. The first mechanism may result from the activation of peptide receptors expressing on the surface of basophils (24,38).

Whereas the second reaction with specific IgE, due to previous sensitization with environmental allergens (31,39). In order to assess a full risk of the occurrence of hypersensitivity reaction in patients the possibility of the appearance of IgE after another contact with the drug must be taken into consideration (40,41). It brings us to conclusion, that creating new peptide drugs we need to take into account both mathematical calculations of allergens similarities and data acquired in in vitro tests. In summary, due to growing number of drug-resistant strains of bacteria and the increasing percentage of people with allergies, data and conclusions obtained in this study can be an important clue in a new molecular design and analysis as well as potential peptide drugs screening based on BAT test and specific databases of allergenic compounds.

Acknowledgments

This work was supported by Innovative Economy Operational Programme WND-POIG.01.04.00-22-052/11.

Conflict of interest

The authors state no conflict of interest.

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Table 1. Sequence and experimental data for camel and citropin 1.1 (*measured using mass spectroscopy).

Peptide	Sequence	Mass (Da)		Concentration used in BAT (μM)
		Calculated	Observed*	
Camel	KWKLFKKIGAVLKVL-NH ₂	1770.28	1770.34	5.65
Citropin 1.1	GLFDVIKKVASVIGGL-NH ₂	1614.95	1614.98	6.19

Table 2. Characteristics of study and control groups.

		Hypersensitivity group (n= 38)	Control group (n=15)
Age		47.54 ± 14.09	42.20 ± 14.54
Gender: women/ men		26/12	12/3
% of basophils (mean ± SD)		0.50 ± 0.25	0.65 ± 0.21
Positive BAT test Citropin 1.1		7 (18.42%)	0 (0.0%)
Positive BAT test CAMEL		7 (18.42%)	2 (13.33%)
History of allergy	Food allergy	46.1%	0%
	ASA hypersensitivity	63.6% (positive BAT test and provocation history)	0%
	Asthma	15.2%	0%

Table 3. The examples of allergens sharing similarities with citropin 1.1 peptide.

No	Allergen	Systematic Name (type/common name)	<i>PD</i> Sequence Similarity Index	Start Residue	Matching region to citropin 1.1	End Residue
1	Dau c 5.0101	<i>Daucus carota</i> (carrot)	7.80	67	SLVKAIKQVDVVISTL	82
2	Pyr c 5	<i>Pyrus communis</i> (pear)	8.35	69	SLVKAIKQVDVVISTV	84
3	Bet v 6.0101	<i>Betula verrucosa</i> (white birch)	8.82	69	SLVKAFKQVDVVISTV	84
4	Asp f 17	<i>Aspergillus fumigatus</i> (fungi)	9.02	74	PVQDLTKQVEGVDDL	89
5	Pha v 3.0101	<i>Phaseolus vulgaris</i> (kidney bean)	9.11	70	GICNCLKTAAGAVRGL	85

Table 4. The examples of allergens sharing similarities with camel peptide.

No	Allergen	Systematic Name (type)	<i>PD</i> Sequence Similarity Index	Start Residue	Matching region to Camel peptide	End Residue
1	Der f mag29	<i>Dermatophagoides farinae</i> (mites)	8.23	56	RKKILDKVDEVLKWL	70
2	Phl p 5.0204	<i>Phleum pratense</i> (grass)	8.23	135	ELQIIDKIDAAFKA	149
3	Poa p 5.0101	<i>Poa pratensis</i> (grass)	8.60	166	EQQIIDKIDAAFKA	180
4	Lol p 5	<i>Lolium perenne</i> (grass)	8.87	171	ELQIVDKIDAAFKA	185
5	Pha a 5	<i>Phalaris aquatica</i> (grass)	8.87	42	ELQIVDKIDAAFKA	56

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Figure Legends:

Figure 1. Effect of stimulating antibodies anti-FcεRI (A) and fMLP (B) on basophil activation in hypersensitivity patients (n=38) and control group (n=15). The results are presented as a median (25th and 75th percentiles) and raw data (triangles). There was no statistical difference between groups (NS). Cut-off value was estimated as 10% of activated basophils (according to manufacturer's instructions).

Figure 2. Effect of citropin 1.1 (A) and camel (B) on basophil activation in hypersensitivity patients – first group (n=38) and control group – second group (n=15). Statistical differences were observed between the first group and the second group after citropin and camel stimulation ($p<0.05$ and $p<0.01$ respectively).

Figure 3. Correlation between the level of basophil activation stimulated with citropin 1.1 (A) or camel (B) and the activation of these cells with acetylsalicylic acid (linear regression line and its 95% confidence interval). Statistically significant positive correlation for the whole analyzed group (n=53) was observed in the case of citropin 1.1 (fig. 3A; $p<0.05$, $r=0.338$) and camel (fig. 3B; $p<0.05$, $r=0.333$).

Supplementary data

Figure 1S. Representative dotplots showing basophils gating strategy based on low SSC-A values and CCR-3 expression. Eosinophils located at high SSC position (upper right side) were excluded (A). Basophils were displayed in a graph with CCR3-PE on the x-axis and CD63+CD203cPE-DY647 on the y-axis; intact cells – negative control (B), cells activated with stimulating mAbs (C) – first positive control and fMLP – second positive control (D).



