



Anti-allergic properties of a new all-D synthetic immunoglobulin-binding peptide

Maria Rossi, Menotti Ruvo¹, Daniela Marasco¹,
Maurizio Colombo, Giovanni Cassani, Antonio Verdoliva*

Tecnogen S.p.A., Località La Fagianeria, 81015 Piana di Monte Verna, CE, Italy

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Abstract

Using a combinatorial chemistry approach, we identified a tetrameric tripeptide, denoted Protein A Mimetic (PAM) or TG19318, able to bind to immunoglobulins of different classes and species. The inverso variant, with the tripeptide in the all-D configuration (D-PAM or TG19320), is described as retaining binding properties to Ig. This peptide has now been assayed as a binder for E class immunoglobulins, in linear and competitive ELISA experiments, dot-blot and surface plasmon resonance (SPR) analyses. We show that D-PAM binds IgE with high specificity and selectivity, the interaction being sufficient to inhibit anaphylactic release of β -hexosaminidase from RBL 2H3 cells, with an IC_{50} value of 10 μ g/mL. Intradermal administration of D-PAM suppresses PCA in the rat, with an IC_{50} of 1.25 μ g/kg dose of peptide, while its intraperitoneal injection inhibits mouse PCA with an IC_{50} of about 7 mg/kg and an efficacy comparable to that of ketotifen. Similarly, D-PAM inhibits ACA in the mouse, with 50% suppression at 10 mg/kg.

The results presented here show that the peptide is active on the studied models, with effective doses below toxicity level, hence the molecule is a promising candidate for development of a new class of anti-allergic drugs.

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1. Introduction

Allergic diseases represent the most common disorders of the immune system, affecting about 20% of the world's population (Meltzer and Grant, 1999). Due to the risk of severe anaphylactic reactions and the ineffectiveness in patients responsive to various allergens, the use of classical immunotherapy, based on subcutaneous injections of small but gradually increasing amounts of allergen (Till et al., 2004), has greatly decreased in recent years. In addition, pharmacological treatment of allergic disorders allows their control at a lower risk-to-benefit ratio than immunotherapy (Adkinson et al., 1997). Most of the current therapies for treatment of atopic diseases are based on drugs that inhibit or suppress components of the allergic inflammatory response. Antihistamines, bronchodilators, anti-allergic

drugs and corticosteroids remain by far the most effective therapeutic response to allergic diseases in reducing symptoms and concomitant inflammatory reactions (Inagaki and Nagai, 2001; Barnes, 1999; Barnes et al., 1998; Marshall, 2000). Nevertheless, corticosteroids sometimes induce deleterious side effects, including liver damage and osteoporosis (Baylink, 1983; Piper et al., 1991), while patients given antihistaminic compounds need constant, lifelong therapeutic treatment (Meltzer and Grant, 1999).

Current research is aimed at finding medications targeting specific steps in the allergic cascade. A promising therapy concerns the block of IgE activation by administration of anti-IgE monoclonal antibodies with very high binding specificity (Presta et al., 1993; Bousquet et al., 2005). In addition to the neutralization of circulating IgEs and inhibition of their synthesis by B cells, these immunoglobulins show several indirect effects, including accumulation of IgE/anti-IgE immune complexes and downregulation of the high-affinity $Fc\epsilon RI$, which represents a further, obvious target for atopy treatment. In fact, IgE/ $Fc\epsilon RI$ interaction was the focus of several studies aimed at designing

* Corresponding author. Tel.: +39 0823 612 215; fax: +39 0823 612 230.

E-mail address: verdo@tecnogen.it (A. Verdoliva).

¹ Present address: Istituto di Biostrutture e Bioimmagini del CNR, Sezione Biostrutture, via Mezzocannone 16, 80134, Naples, Italy.

inhibitor compounds, including synthetic peptides derived from IgE or Fc ϵ RI structures and high-affinity oligonucleotide ligands, useful in allergy treatment (Wiegand et al., 1996; Kinet et al., 1991; Nakamura et al., 2001, 2002; Buku et al., 2005; Rigby et al., 2000).

We previously described a synthetic peptide, denoted Protein A Mimetic (PAM) or TG19318, able to specifically and selectively bind immunoglobulins of different classes and species, including IgE (Fassina et al., 1996; Palombo et al., 1998a,b,c; Verdoliva et al., 2000). PAM/IgE interaction has been extensively investigated and a number of peptide-derivatized matrices for antibody purification are now commercially available (Verdoliva et al., 2002; Michaelsen et al., 2003; Yan et al., 2002). The peptide in its inverso form (D-PAM or TG19320) was evaluated in *in vitro* and *in vivo* models of LES as a potential anti-lupus agent, showing promise for utilization in autoimmune disorders (Marino et al., 2000, 2002). In this work, we extended the characterization of D-PAM/IgE binding by investigating the anti-allergic properties of the peptide in several *in vitro* and *in vivo* experiments. We thus evaluated its ability to inhibit mast cell degranulation by measuring the release of β -hexosaminidase from RBL 2H3 cells and the peptide inhibitory effects on atopic reactions in different experimental animal models of allergic disorders, such as passive cutaneous anaphylaxis (PCA) in rats and mice, and active cutaneous anaphylaxis (ACA) in mice.

2. Materials and methods

2.1. Peptide synthesis

All peptides were produced by solid phase peptide synthesis following Fmoc methodology, using a fully automated 431A peptide synthesizer (Perkin-Elmer, Boston, MA, USA) as previously described (Fassina et al., 1996). After resin cleavage, peptides were purified by reverse phase chromatography (RP-HPLC) and their identity confirmed by amino acid analysis and ESI mass spectrometry, which provided molecular weights identical to the expected values, within experimental error. The 16-D-PAM, a highly branched version of the original peptide containing 16 copies of the monomeric tripeptide (R)Arg-(R,S)Thr-(R)(Tyr), was prepared following standard procedures of solid phase peptide synthesis on an Fmoc-Gly-WANG resin, by four subsequent couplings with Fmoc-L-Lys(Fmoc)-OH. To achieve the tripeptide sequence, protected amino acid derivatives were coupled to obtain the desired final compound. The crude polypeptide was purified by two subsequent steps of dialysis and RP-HPLC and characterized by size exclusion chromatography and amino acid analysis. The 16-D-PAM was used only for the ELISA assay in order to improve peptide coating to the microtiter plates. All other experiments were performed with the original tetrameric peptide.

2.2. IgE and DNP biotinylation

Mouse anti-DNP IgE, human IgE and 1 mg of DNP were dissolved in 1 mL of 50 mM sodium phosphate, pH 7.5, and treated

with 10 μ L of biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma–Aldrich, Milan, Italy) at a concentration of 10 mg/mL in dimethylsulphoxide (DMSO), under agitation at room temperature. After 4 h of incubation, 120 μ L of a 1 M lysine solution was added to deactivate residual active groups. The reaction was left under stirring for 2 h, and the biotinylated DNP and IgEs were extensively dialyzed against 50 mM sodium phosphate, pH 7.5.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter plates (Falcon) were incubated overnight at 4 °C with 100 μ L/well of a 16-D-PAM solution in PBS, pH 7.2. After coating, the plates were washed with PBS, 0.1% Tween 20 (PBS-T, washing buffer) and blocked with 200 μ L of PBS containing 3% BSA, for 2 h at 37 °C. For the immunoreactivity analysis, the plates were filled with biotinylated anti-DNP IgE at varying concentrations (0.01–50 μ g/mL), previously diluted with PBS containing 0.5% BSA (PBS-B) and incubated at 37 °C for 2 h. Each dilution was performed in duplicate. Plates were washed again with PBS-T and filled with 100 μ L of streptavidin peroxidase diluted 1:1000 with PBS-B. After 1.5 h of incubation at 37 °C, 100 μ L of a chromogenic substrate solution consisting of 0.2 mg/mL 3-ethyl benzothiazoline-6-sulphonic acid (ABTS, Sigma–Aldrich) in 0.1 M sodium citrate buffer, pH 5.0, containing 5 mM hydrogen peroxidase, were added. The absorbance at 405 nm of each sample was measured with a Model 2250 EIA Reader (Bio-Rad, Milan, Italy). For competitive ELISA, 2 μ g/mL of biotinylated IgE were mixed with increasing concentrations of peptides as competitors (0.1–500 μ g/mL) and 100 μ L/well of this mixture were added to the 16-D-PAM-coated plates (20 μ g/mL). Optimal concentrations of IgE and 16-D-PAM were determined in preliminary experiments. Incubation with streptavidin peroxidase and chromogenic development were performed as described above.

2.4. SPR analysis

Monoclonal anti-DNP IgEs were immobilized on a CM5 dextran-coated sensor chip by a standard amine coupling procedure, according to manufacturer's instructions (Biosense, Milan, Italy). Briefly, the carboxymethylated dextran surface was activated by a 7 min injection of a solution containing 200 mM EDC and 50 mM NHS, followed by an IgE injection (50 μ g/mL solution in 10 mM sodium acetate, pH 5.5, at a flow rate of 20 μ L/min for 3 min.). A continuous flow of HBS (Hepes 10 mM, NaCl 150 mM, EDTA 3.4 mM) at 20 μ L/min was maintained, with capping of unreacted sites achieved by injecting 1 M ethanolamine, pH 8.5, for 7 min. Blank surfaces were obtained in the same manner but without injection of proteins. Sensorgrams for kinetic measurements were generated by injection of D-PAM (at concentrations ranging from 1.5 to 150 μ M) in HBS at a flow rate of 30 μ L/min. Kinetic data were collected in duplicate for each D-PAM concentration. The peptide was allowed to bind for 2 min and dissociate for another two before final washing with HBS. Biosensor data were prepared, modelled and fitted

by means of BIAevaluation 4.1 software (Biacore AB, Uppsala, Sweden) using the equation:

$$RU_{eq} = \frac{K_A C R_{max}}{[K_A C (n + 1)]}$$

where K_A is the association constant ($K_A = 1/K_D$), n is the binding factor, C is the peptide concentration, R_{max} is the response maximum value and K_D is the dissociation constant.

2.5. Dot-blot assay

Solutions of 10 μ L PBS (50 mM phosphate, 150 mM sodium chloride, pH 7.5), containing varying amounts of D-PAM peptide (0.01–1 μ g), were immobilized on a nitrocellulose filter and incubated, under mild stirring, for 2 h at room temperature in PBS containing 5% dried milk (Sigma–Aldrich) as blocking buffer. The filter was then washed twice with PBS and incubated with 10 μ g/mL of biotinylated IgE in PBS with 0.5% milk (PBS-M). After 1 h of incubation at room temperature, the filter was washed twice with PBS containing 0.05% Tween (PBS-T) and then incubated for 1 h at room temperature with streptavidin peroxidase diluted 1:1000 with PBS-M. The membrane was washed with PBS-T and developed with a chromogenic substrate solution containing 0.7 mg/mL 3,3'-diaminobenzidine and 0.17 mg/mL urea hydrogen peroxide in 60 mM Tris (Sigma–Aldrich). This substrate produces an intense brown-black precipitate at the enzyme binding site.

2.6. Binding of IgE to Fc ϵ RI and specific antigen

2.6.1. Cell culture

RBL 2H3 cells (ATCC, Manassas, VA, USA) were maintained in MEM (Sigma–Aldrich) supplemented with 10% heat-inactivated FCS, 4 mM L-glutamate, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were harvested by treatment with 0.25% trypsin (w/w) and 0.1 mM EDTA and recovered by centrifugation (250 $\times$ g for 5 min at room temperature).

2.6.2. CELISA assay

The plates (Nunc, Milan, Italy) were saturated for 2 h at room temperature with 2% BSA in PBS and then washed three times with PBS (250 μ L/well). The RBL 2H3 cells were adjusted at 6 $\times$ 10⁶/mL in 1% BSA in PBS (PBS-B) and the biotinylated anti-DNP IgE monoclonal antibody (Sigma–Aldrich) was diluted in the same buffer (PBS-B) to obtain concentrations ranging from 0.01 to 20 μ g/mL. Fifty microliters of RBL 2H3 suspension and 50 μ L of the IgE dilutions were mixed in each well and the plates incubated for 1 h at 37 °C.

For the Fc ϵ RI/IgE interaction analysis, plates were washed three times with 250 μ L PBS/well by centrifugation for 5 min at 450 $\times$ g, 50 μ L of streptavidin peroxidase, diluted 1:1000 in PBS-B, were added to each well and the plates incubated again for 1 h at 37 °C. After three final washes with PBS, plates were developed with ABTS and the absorbance at 405 nm of each sample was measured with a model 2250 EIA Reader (Bio-

Rad). In the competition assays, D-PAM and IgE solutions, at concentrations ranging from 0.01 to 500 μ g/mL in PBS-B, were added to a 1 μ g/mL of biotinylated IgE solution and the mixture dispensed into the plate wells containing the cells. As control reaction, the addition of competitor or biotinylated IgE was omitted (maximum rate and background rate, respectively).

To investigate the effect of D-PAM on the binding of DNP and anti-DNP IgE, 50 μ L of PBS-B containing the biotinylated antigen at concentrations ranging from 0.005 to 25 μ g/mL were added to 0.5 μ g/mL of anti-DNP IgE complexed with the Fc ϵ RI. After three washes with PBS, plates were incubated with streptavidin peroxidase and developed with ABTS as previously described. In the competition assays, D-PAM and DNP solutions, at concentrations ranging from 0.01 to 500 μ g/mL in PBS-B, were added to a 0.05 μ g/mL of biotinylated DNP solution and the mixture dispensed into the plate wells containing the cells. As control reaction, the addition of competitor or biotinylated DNP was omitted (maximum rate and background rate, respectively).

2.7. IgE-mediated β -hexosaminidase release assay

The activity of β -hexosaminidase, released from stimulated RBL 2H3 cells, was determined by the method described by Yamanishi et al. (1995), with appropriate modifications. In brief, from confluent cultures, 1 $\times$ 10⁵ trypsinized RBL 2H3 cells, dispersed in 80 μ L of the medium, were inoculated into each well of a 96-well microtiter plate (Corning-Costar, Milan, Italy). After 24 h, 20 μ L of the medium containing mouse anti-DNP IgE monoclonal antibody (Sigma–Aldrich) were added. After 1 h of incubation at 37 °C, the microplate was washed three times with 100 μ L of HEPES buffered saline (HBS, 10 mM HEPES buffer at pH 7.4 containing 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂ and 0.5 mM MgCl₂) and incubated with 100 μ L of HBS containing 2,4-dinitrophenol- (DNP) conjugated human serum albumin (Sigma–Aldrich). Total cellular enzyme release was obtained by adding 100 μ L of HBS containing 1% Triton X-100 to wells with an equal number of cells, whereas spontaneous release was determined with an equal number of unstimulated cells in HBS.

After incubating at 37 °C for 2 h, 30 μ L of the conditioned medium and cell lysate were transferred to the wells of another microplate filled with 50 μ L of a sodium citrate buffer at pH 4.5 containing 4 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma–Aldrich), a specific substrate for β -hexosaminidase. Two hours later, the reaction was stopped by adding 100 μ L of 0.2 M glycine solution at pH 10.7, with enzyme activity determined by measuring absorbance at 405 nm with a model 3550 EIA Microplate Reader.

In preliminary experiments, IgE and antigen were added to each well at different concentrations to determine the optimal concentrations of reagents to produce release with acceptable OD signal (0.8 AU were obtained with 0.5 μ g/mL of IgE and 0.05 μ g/mL DNP) under nonsaturating conditions.

In competitive experiments, D-PAM solutions, at peptide concentrations ranging from 0.1 to 500 μ g/mL, were mixed with the antigen. As control reactions, the addition of peptide or IgE

was omitted to obtain the maximum rate and background rate, respectively.

2.8. Animals

CD1 rats (180–200 g) and Balb/c mice (20–22 g) were purchased from Charles River (Calco, Italy). They were fed a standard diet and kept in controlled climatic conditions. Food and drinking water were available ad libitum.

2.9. Rat passive cutaneous anaphylaxis (PCA)

PCA was induced following the procedure originally described by Ovary (1958). In brief, male CD1 rats were anesthetized by ether and 50 μ L of a diluted ascitic fluid containing anti-ovalbumin IgE antibodies (kindly provided by IBB labs, Naples, Italy) were intradermally injected into six/seven sites on the shaved back. As a negative control reaction, the same rats received an injection of 50 μ L of PBS. Twenty-four hours after sensitization, a PCA reaction was elicited by an intravenous injection of 1 mg of ovalbumin (OVA, Sigma–Aldrich, Milan, Italy) in 1 mL PBS containing 2% (w/v) Evans blue dye (Sigma–Aldrich). Thirty minutes after the antigenic challenge, the blue spot wheels appearing on the back skin were measured to evaluate allergic reaction. Pictures of these reactions were collected and rats sacrificed 1 h later. The dimensions and intensity of the blue lesions were measured by a quantitative densitometric analysis of the scanned pictures.

In competition assays, ascitic fluid containing IgE (diluted 200-fold) and D-PAM (from 0.125 μ g/kg to 12.5 mg/kg of peptide) were mixed and simultaneously injected intradermally. As control reaction, 50 μ L of ascitic fluid without peptide and 50 μ L of PBS were injected to obtain the maximum rate and background rate, respectively.

2.10. Mouse ear passive cutaneous anaphylaxis (PCA)

Mouse ear PCA was induced following the procedure originally described by Inagaki et al. (1985). In brief, female BALB/c mice were anesthetized by ether and 10 μ L of a diluted ascitic fluid containing anti-ovalbumin IgE antibodies, at different concentrations, were intradermally injected into the ear lobes. A group of mice received an injection of a similar volume of PBS.

Forty-eight hours after sensitization, a PCA reaction was elicited by an intravenous injection of 0.25 mg of OVA dissolved in 70 μ L of PBS containing 1.8% (w/v) Evans blue. Thirty minutes after the antigenic challenge, blue spot wheels appeared on the lobes, the mice were sacrificed and their ears removed for measurement of extravasated dye.

Dye extraction and measurement were performed as follows: each ear was dissolved with 0.5 mL of 1 M KOH solution at 37 °C overnight, and 2 mL of a mixture of 0.6 M phosphoric acid and acetone (5:13) were added. After vigorous shaking, precipitates were filtered off and the amount of dye was determined spectrophotometrically at 620 nm.

In preliminary experiments, ascitic fluid containing IgE was injected into the ears at different dilutions in order to deter-

mine the optimal concentrations of reagents to produce blueing reactions confined within lobes (ascitic fluid diluted 20-fold).

In competition assays, D-PAM, dissolved in PBS, was given intraperitoneally at doses of 5, 50 and 100 mg/kg to groups of mice sensitized by injection of ascitic fluid diluted 20-fold, 30 min prior to eliciting PCA by antigenic challenge.

A group of sensitized animals, used as positive control, and a group of nonsensitized animals, used as negative control, received an intraperitoneal injection of 100 μ L of PBS. In addition, another group of sensitized mice received an i.p. injection of 10 mg/kg ketotifen in 100 μ L PBS.

2.11. Mouse ear active cutaneous anaphylaxis (ACA)

Mouse ear ACA was induced following the procedure originally described by Inagaki et al. (1992). In brief, female BALB/c mice were immunized by a 100 μ L intraperitoneal injection of PBS containing 25 μ g of ovalbumin adsorbed on 1 mg of aluminum hydroxide gel (alum, Sigma–Aldrich) as adjuvant. Another group of mice received an intraperitoneal injection of 1 mg of alum without the antigen (negative control).

Two weeks later, ACA was elicited in mice ears by intradermally injecting the antigen in 10 μ L of PBS, followed by an intravenous injection of 70 μ L of PBS containing 1.8% (w/v) Evans blue. The control group of nonimmunized mice received the same treatment. Thirty minutes after the antigenic challenge, blue spot wheels appeared on the lobes, mice were sacrificed and ears removed for measurement of extravasated dye. During these operations the animals were kept under ether anesthesia. Extraction and measurement of extravasated Evans blue dye were performed as previously described.

In preliminary experiments, 10 μ L of antigen were injected into the ears of immunized mice at different concentrations (0.001–0.01–0.1 μ g/ μ L) to determine the optimal concentrations of reagents needed to produce blueing reactions limited within lobes (dose of 0.01 μ g/ μ L).

For competition assays, D-PAM dissolved in 100 μ L PBS was given intraperitoneally to groups of immunized mice at doses of 5, 50 and 100 mg/kg, 30 min prior to eliciting ACA by injection of 0.1 μ g of ovalbumin. A group of immunized animals, used as positive control, and a group of nonimmunized mice, used as negative control, received an intraperitoneal injection of 100 μ L of PBS.

3. Results

3.1. In vitro experiments

D-PAM (TG19320) represents the inverso derivative of the original PAM (TG19318) with all amino acids in D configuration. This peptide is not degraded by treatment with proteolytic enzymes, such as trypsin and α -chymotrypsin (Verdoliva et al., 2002; Marino et al., 2002), and, consequently, represents the more suitable form for in vivo studies. Replacement of amino acids with the D equivalents also preserved IgE recognition properties. Fig. 1A shows that the peptide, synthesized as multibranched 16-D-PAM to increase its coating capability to

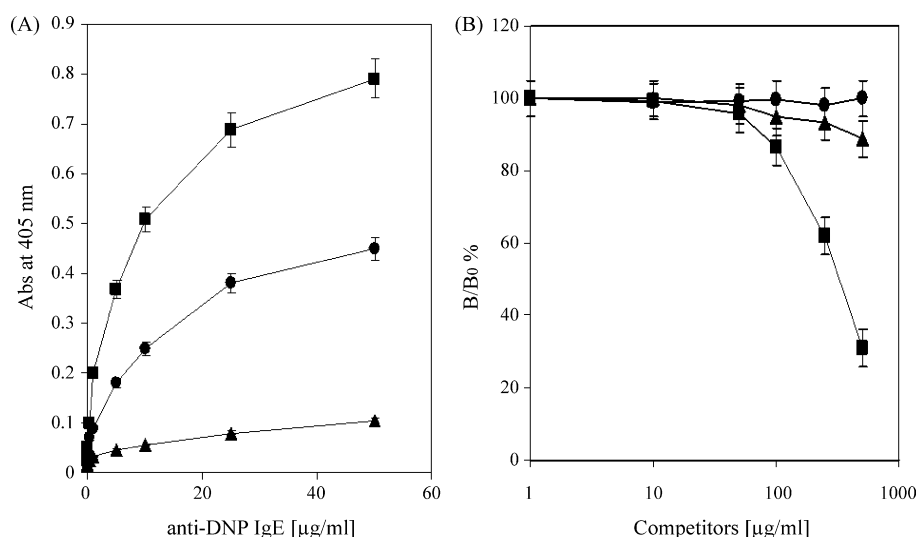


Fig. 1. Panel A: Binding of progressive amounts of biotinylated anti-DNP IgE (0.01–50 µg/mL) to BSA (▲) and 16-D-PAM coated on microtiter plates at 5 (●) and 20 (■) µg/mL. Panel B: Inhibition of interaction between biotinylated anti-DNP IgE (2 µg/mL) and coated 16-D-PAM (20 µg/mL), performed with increasing amounts of D-PAM (■), monomeric peptide rty (▲) and scrambled peptide (●) (1–500 µg/mL). Results represent the means of three independent experiments. B/B_0 is calculated as $[(\text{absorbance with competitor} - \text{background rate})/(\text{absorbance without competitor} - \text{background rate})] \times 100$.

microtiter plates, was able to bind biotinylated mouse anti-DNP IgE in a dose-dependent manner. Binding to the E immunoglobulins was specific, since it was inhibited in a dose-dependent manner by the peptide itself, while control peptides, such as scrambled D-PAM (tyr) K_2 KG and monomeric D-PAM (rty), did not inhibit D-PAM/IgE binding (Fig. 1B), thus suggesting that the interaction is sequence-specific and that the entire tetravalent structure is required for recognition. In strict agreement with these results, the peptide, immobilized on nitrocellulose filters

and incubated with biotinylated mouse anti-DNP and human IgE, was able to bind both mouse and human E immunoglobulins in a dose-dependent manner (data not shown). SPR sensorgrams obtained by injection of D-PAM on coated IgE (Fig. 2A), show that the association and dissociation kinetics of the peptide/IgE interaction were too fast to allow calculation of kinetic rate constants, while the equilibrium dissociation constant (K_D), calculated by a steady-state model for the fitting of experimental data (Fig. 2B), was 2.2×10^{-4} M.

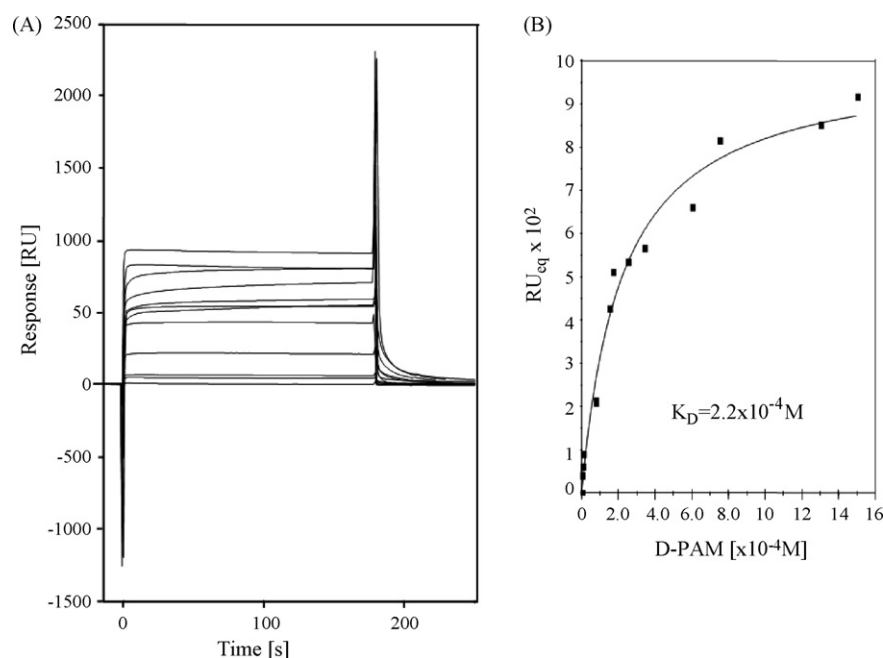


Fig. 2. Panel A: overlay plot of BiaCore SPR sensorgrams showing interaction between D-PAM and anti-DNP IgE immobilized on a CM5 dextran-coated sensor chip. Peptide was injected at concentrations ranging from 1.5 to 150 µM and a flow rate of 30 µL/min. Two-min. association and 2-min. dissociation times were allowed. Panel B: Response maximum values of each binding curve are reported against D-PAM concentrations (1.5–150 µM). The best fitting, obtained using BIAevaluation 4.1 software, gives a K_D of 2.2×10^{-4} M.

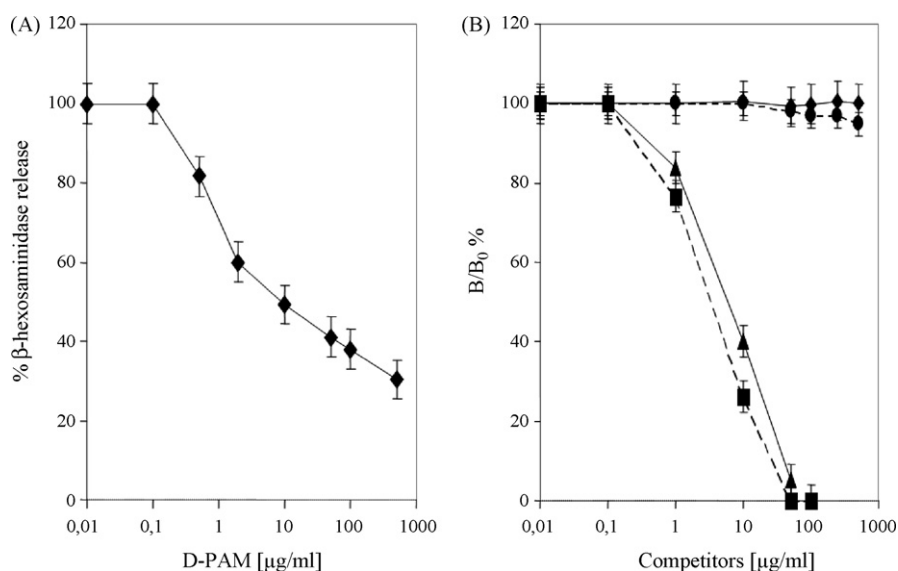


Fig. 3. Panel A: Inhibition of IgE-mediated β -hexosaminidase release by D-PAM. DNP antigen was incubated with or without increasing amounts of peptide (0.1–500 $\mu\text{g/ml}$), and the mixture used to sensitize RBL 2H3 cells, expressing Fc ϵ RI receptors. Net β -hexosaminidase release was determined and plotted against the concentration of IgE ligand. The range of six replicate samples was less than 5% of mean values. Panel B, solid lines: inhibition of the interaction between biotinylated anti-DNP IgE (1 $\mu\text{g/ml}$) and Fc ϵ RI receptors, performed with increasing amounts of D-PAM (●) (0.01–500 $\mu\text{g/ml}$) and anti-DNP IgE (▲) (0.01–50 $\mu\text{g/ml}$). Panel B, dotted lines: Inhibition of binding between anti-DNP IgE complexed with Fc ϵ RI receptors and biotinylated DNP antigen (0.05 $\mu\text{g/ml}$), performed with increasing amounts of D-PAM (◆) (0.01–500 $\mu\text{g/ml}$) and DNP (■) (0.01–100 $\mu\text{g/ml}$). Results represent the means of three independent experiments. B/B_0 is calculated as [(absorbance with competitor – background rate)/(absorbance without competitor – background rate)] $\times$ 100.

To verify whether the D-PAM/IgE interaction was sufficient to prevent IgE-dependent mast cell mediator release, specific experiments were performed using RBL 2H3 cells that release chemical mediators of inflammation in response to the cross-linking of IgE by a multivalent antigen. The β -hexosaminidase enzyme, which coexists with histamine in the secretory granules of the mast cell and basophils, represents a marker of cell degranulation; hence, quantification of enzyme activity released into the conditioned medium can be employed to evaluate the inhibitory effect of the peptide on the degranulation event *in vitro* (Lee et al., 1999; Hoffmann et al., 1999). Fig. 3A shows that the anaphylactic release of β -hexosaminidase from RBL 2H3 cells was significantly inhibited by D-PAM in a dose-dependent manner with an IC_{50} value of 10 $\mu\text{g/ml}$ (about 4.6 μM), thus demonstrating its effectiveness as an interfering agent in mast cell degranulation.

The ability of this compound to interfere with the binding of IgE to Fc ϵ RI and its specific DNP antigen, was evaluated by a tissue culture cell-based assay on the RBL 2H3 cell line, which expresses a high affinity receptor for IgE on the surface. Nevertheless, the peptide, incubated at different concentrations with biotinylated IgE or biotinylated DNP just before the mixture was added to the cells, did not induce significant binding reduction of IgE to Fc ϵ RI and corresponding DNP antigen, while IgE/Fc ϵ RI and IgE/DNP interactions were completely inhibited using, respectively, nonbiotinylated IgE and DNP (Fig. 3B).

3.2. *In vivo* experiments

The *in vivo* activity of the D-PAM peptide as an anti-allergic agent was evaluated using different animal models of aller-

gic disorders, such as passive cutaneous anaphylaxis (PCA) in the rat and mouse and active cutaneous anaphylaxis (ACA) in the mouse. PCA in rat was elicited by intradermal injection of mouse ascitic fluid containing anti-ovalbumin IgE, and, after 24 h, by intravenous injection of ovalbumin treated with Evans blue dye to induce an allergic response. Since the PCA reaction involves mast cell activation and a subsequent increase in vascular permeability, the cutaneous reaction is visualized by extravasation of the dye (Fig. 4). The dimension of blue lesions, measured by densitometric analysis and summarized in Table 1, demonstrated that D-PAM was able to inhibit rat PCA in a dose-dependent fashion, with 50% suppression produced by a dose of 1.25 $\mu\text{g/kg}$, corresponding to about 0.58 nmol/kg of peptide.

The anti-allergic properties of D-PAM were confirmed by eliciting PCA in the mouse, which represents the established experimental animal, due to the availability of many strains of mice with unique immunological characteristics (van der Kleij et al., 2004; Alter et al., 1993). Intraperitoneal administration of D-

Table 1
Effects of D-PAM on 24 h rat PCA reaction (mean $\pm$ S.E.M., $n = 3$)

Drug substance	Dose		Extension of blueing reaction (%)
	($\mu\text{g/kg}$)	(nmol/kg)	
–	0	0	100 $\pm$ 5.0
D-PAM	0.125	0.058	70 $\pm$ 5.0
D-PAM	1.25	0.58	50 $\pm$ 8.0
D-PAM	12.5	5.8	35 $\pm$ 10.0
D-PAM	125	58	15 $\pm$ 10.0
D-PAM	1250	580	5 $\pm$ 15.0
D-PAM	12500	5800	0 $\pm$ 15.0

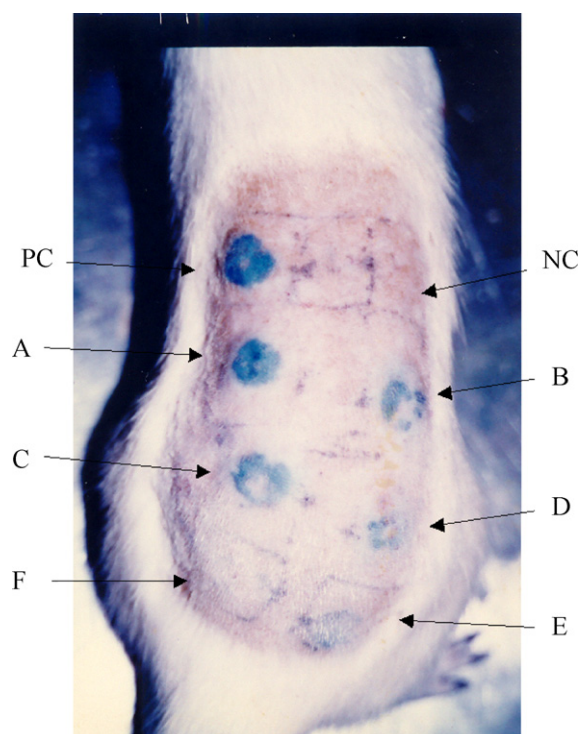


Fig. 4. Rat passive cutaneous anaphylaxis inhibition by D-PAM. Increasing doses of peptide, from A (0.125 μ g/kg) to F (12.5 mg/kg), were administered intradermally together with IgE antibodies, 24 h prior to the antigenic challenge. Positive (PC) and negative (NC) controls were obtained by administering IgE without peptide and PBS, respectively.

PAM 30 min prior to the antigenic challenge resulted in a strong inhibition of PCA reaction in comparison to the positive control group; Table 2 shows that suppression was dose-dependent. The smallest dose of 5 mg/kg (2.3 μ mol/kg) of D-PAM inhibited the blueing reaction to 45%, whereas the highest dose of drug, corresponding to 100 mg/kg (46 μ mol/kg), caused almost complete inhibition of the allergic reaction. To verify the effectiveness of these results, the antiallergic properties of ketotifen, a prophylactic anti-allergic drug which acts by stabilizing mast cell membranes, consequently decreasing the release of histamine (Joly et al., 1987; Russo et al., 2005), were also comparatively evaluated in PCA mouse ear experiments. Table 2 shows that ketotifen and D-PAM were found to be essentially equipotent: at a dose of 23 μ mol/kg, suppression of an immediate hypersensitivity reaction by ketotifen was comparable or lower to

Table 2
Effects of D-PAM on 48 h mouse PCA reaction (mean $\pm$ S.E.M., $n = 5$)

Drug substance	Dose		Inhibition (%) ^a
	(mg/kg)	(μ mol/kg)	
–	0	0	0 $\pm$ 2.0
D-PAM	5	2.3	45 $\pm$ 6.0
D-PAM	50	23.0	75 $\pm$ 10.0
D-PAM	100	46.0	94 $\pm$ 10.0
Ketotifen	10	23.0	65 $\pm$ 3.0

^a Inhibition percent: $1 - \{[\text{mean of experimental group} - \text{mean of negative group}]/[\text{mean of positive control group} - \text{mean of negative group}]\} \times 100$.

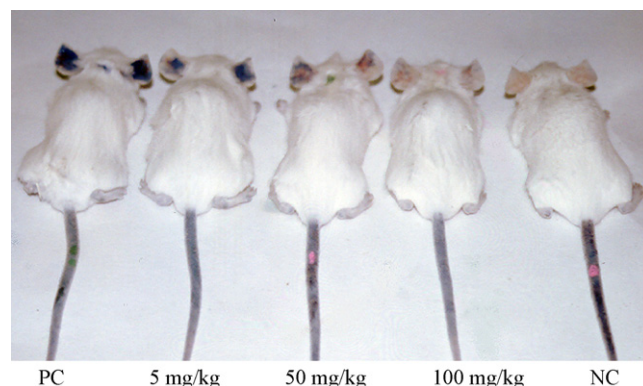


Fig. 5. Mouse ear active cutaneous anaphylaxis inhibition by D-PAM. Peptide was administered intraperitoneally 30 min prior to the antigenic challenge at doses of 5, 50, 100 mg/kg. Positive (PC) and negative (NC) controls were obtained by administering IgE without peptide and PBS, respectively.

Table 3
Effects of D-PAM on mouse ACA reaction (mean $\pm$ S.E.M., $n = 5$)

Drug substance	Dose		Inhibition (%) ^a
	(mg/kg)	(μ mol/kg)	
–	0	0	0 $\pm$ 3.5
D-PAM	5	2.3	30 $\pm$ 4.0
D-PAM	50	23.0	72 $\pm$ 6.0
D-PAM	100	46.0	92 $\pm$ 5.5

^a Inhibition percent: $1 - \{[\text{mean of experimental group} - \text{mean of negative group}]/[\text{mean of positive control group} - \text{mean of negative group}]\} \times 100$.

that induced by D-PAM (65% and 75% of the positive control, respectively).

Although passive sensitization provides a simple and useful experimental model for studying drugs for allergy, active sensitization is also important to investigate the mechanisms involved in both allergic reactions and drug action, since allergic disorders are caused by active sensitization. Therefore, we extended the PCA technique in the mouse ear to the active cutaneous anaphylaxis (ACA) elicited in female Balb/c mice. Animals were immunized with ovalbumin in the presence of aluminum hydroxide gel, and, after two weeks, ACA was elicited in the mouse ear by intradermal injection of the antigen followed by an intravenous injection of Evans blue solution. Fig. 5 shows that administration of D-PAM, injected intraperitoneally 30 min prior to the antigenic challenge, inhibits ACA response in a dose-dependent manner, in comparison to the positive control group. Measurements of extravasated dye, collected in Table 3, indicate that the suppression caused at doses of 5 and 50 mg/kg of D-PAM was 30 and 75%, respectively. The highest drug dose, corresponding to 100 mg/kg, caused inhibition above 90%.

4. Discussion

D-PAM has been extensively described as a binder for different classes of immunoglobulins. Structurally, the molecule (Marino et al., 2000) is a tetrameric tripeptide where four copies of the peptide (R)Arg-(R-S)Thr-(R)Tyr are inserted into a core composed of three (S) lysines and a glycine. In the present study,

we show that the peptide binds, with high affinity and specificity, both mouse anti-DNP and human IgE, as determined by ELISA and dot-blot analyses, and that the D-PAM/IgE interaction is sufficient to induce a dose-dependent reduction of IgE-promoted β -hexosaminidase release from cultured RBL 2H3 cells. As IgE-dependent cellular degranulation is a basic step involved in the pathogenesis of allergic and inflammatory disorders, the inhibitory effect of D-PAM is clearly of therapeutic interest, as confirmed by several in vivo experiments using different animal models of allergic disorders. Intradermal administration of D-PAM resulted in remarkable suppression of passive cutaneous anaphylaxis (PCA) in the rat, with an IC_{50} produced by a dose of 1.25 μ g/kg (0.58 nmol/kg) of peptide. Consistent with these results, the intraperitoneal injection of D-PAM was able to inhibit mouse PCA with an efficacy comparable to that of ketotifen, a potent mast cell membrane stabilizer, with suppression of allergic reaction, at a dose of 5 mg/kg (23 μ mol/kg), of 75 and 65%, respectively. Similarly, D-PAM inhibited active cutaneous anaphylaxis (ACA) in the mouse, with 50% suppression produced by a dose of about 10 mg/kg (4.6 μ mol/kg).

Since the compound inhibited the release of histamine and other mediators, but was unable to block the binding of IgE to Fc ϵ RI, as well as the binding of antigen to IgE, we hypothesized that D-PAM may act by inhibiting the cross-linking of IgE complexed with Fc ϵ Rs, located on the surface of mast cells and basophils, and that this effect could be explained by the polycationic nature of the molecule, given the presence of four positively charged arginine residues. The binding of the peptide to IgE, fixed to Fc ϵ receptors, adds a broad positive charge to immunoglobulins that could induce strong repulsion effects and structural modifications sufficient to block the cross-linking of IgE following re-exposure to the antigen, and thus the pathological reaction.

Despite a relatively high molecular weight (2141.46 amu) and high hydrophilicity, this compound shows low immunogenicity in mice and rabbits, and is rapidly adsorbed and cleared (Marino et al., 2002). Furthermore, long-term treatment with repeated peptide administration did not induce evident signs of toxicity in mice, as determined by postmortem autopsies, and intraperitoneal LD₅₀ was around 300 mg/kg (Marino et al., 2002). Collectively, the pharmacological properties of D-PAM appear very promising and the compound could be a valid candidate for development of a new therapeutic agent in the treatment of allergic diseases, with a different and innovative mechanism, compared to the anti-allergic compounds currently in use.

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