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(71) Applicant (for all designated States except US): **CELL-TECH R & D LIMITED** [GB/GB]; 208 Bath Road, Slough, Berkshire SL1 3WE (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BIRD, John** [GB/GB]; Celltech R & D Limited, 208 Bath Road, Slough, Berkshire SL1 3WE (GB). **MCCLOUGHLIN, Sheila, Bridget** [GB/GB]; Celltech R & D Limited, 208 Bath Road, Slough, Berkshire SL1 3WE (GB). **BARNES, Matthew, Joseph** [GB/GB]; Celltech R & D Limited, 208 Bath Road, Slough, Berkshire SL1 3WE (GB).

(74) Agent: **THOMPSON, John**; Celltech R & D Ltd, 208 Bath Road, Slough, Berkshire SL1 3WE (GB).

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(54) Title: PHARMACEUTICAL USE OF AN ADAM 10 INHIBITORY SUBSTANCE

(57) Abstract: The present invention provides the use of a substance capable of inhibiting ADAM 10, an enzyme involved in the shedding of ectodomain CD23, for the manufacture of a medicament for the treatment of allergic diseases, including asthma, atopic dermatitis and allergic rhinitis.



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## **PHARMACEUTICAL USE OF AN ADAM 10 INHIBITORY SUBSTANCE**

### **FIELD OF THE INVENTION**

5 The invention relates to the use of a substance capable of inhibiting ADAM 10 for the manufacture of a medicament for the treatment of allergic disease. Such substances are capable of inhibiting ADAM 10-mediated cleavage of CD23. The invention also relates to a method of identifying substances capable of binding ADAM 10.

10

### **BACKGROUND TO THE INVENTION**

CD23 is the low affinity receptor for IgE (FcεRII) with homology to the c-type lectin family of proteins and consists of a transmembrane domain, a stalk  
15 region and a lectin domain which is responsible for the binding of IgE. To date, two isoforms of CD23 have been identified which differ only marginally at the intracellular N-terminus of the protein although the expression of the isoforms is notably differential (Yokota *et al.*, Proc. Natl. Acad. Sci. USA, 1;89 (11):50, pp30-4, 1992).

20

CD23 is not only an IgE receptor, but also a membrane-bound precursor of soluble molecules that still bind IgE (sCD23 or IgE-binding factors) (Sarfati. M. *et al.*, Immunol. Res., 1992, 11, pp. 260-272; Letellier *et al.*, Mol Immunol, 26(12), 1105-12, 1989). These soluble fragments of CD23 are thought to be  
25 involved in the regulation of IgE by inducing the differentiation of B-cells into IgE secreting plasma cells via a receptor-ligand interaction with CD21 (Aubry *et al.*, Nature, 358(6386), 505-7, 1992).

Soluble fragments are generated via multiple cleavage events, for example  
30 sCD23 of molecular weights 37, 33, 29, 25 and 17kDa arise by an autocatalytic cleavage process postulated to involve a metalloprotease cleavage of membrane-bound CD23 (Marolewski *et al.*, Biochem J, 333 (Pt 3), 573-9, 1998) in a process known as ectodomain shedding, although the

identity of the precise metalloproteinase enzyme involved is currently unknown. However, the mechanistic role of this metalloproteinase in the regulation of IgE via inhibition of CD23 shedding has been previously demonstrated by the use of hydroxamic-based compounds, which attenuated the secretion of IgE *in vitro* (Christie *et al.*, Eur J Immunol, 27(12), 3228-35, 1997; Mayer *et al.*, Clin Exp Allergy, 30(5), 719-27, 2000).

There is increasing evidence that sCD23 fragments may exert several effects, either alone or in conjunction with other cytokines, on a large variety of haematopoietic cells. These effects include the regulation of IgE synthesis, promotion of B- and T-cell proliferation, inhibition of monocyte migration and in synergy with interleukin 1 (IL1) it may be implicated in the differentiation of early thymocytes, myeloid cell precursors and some germinal centre B cells.

In particular the three higher molecular weight sCD23 fragments (37, 33 and 29 kDa) have multifunctional cytokine properties which appear to play a major role in IgE production. The excessive formation of sCD23 has been implicated in the overproduction of IgE, which is the hallmark of allergic diseases such as extrinsic asthma, rhinitis, allergic conjunctivitis, eczema, atopic dermatitis and anaphylaxis (Sutton and Gould, Nature, 1993, 366, pp421-428). Elevated levels of sCD23 have also been observed in the synovial fluids of patients with rheumatoid arthritis (Chomarat P *et al.*, Arthritis and Rheumatism, 1993, 36, pp. 234-242).

Metalloproteinases represent a class of the proteinase enzyme family with zinc-dependent catalytic activity. They can be broadly divided into matrix metalloproteinases, which catalyse the degradation of protein constituents of basement membrane and the extracellular matrix, and non-matrix metalloproteinases. The ADAM (a disintegrin and metalloproteinase) family represent a sub-class of non-matrix metalloproteinase enzymes of which 33 have been identified to date that are involved in various biological processes including ectodomain shedding, cell-cell interactions and development (Primakoff and Myles, Trends Genet, 16(2), 83-7, 2000). Several members of the ADAM family have demonstrated the zinc-dependent catalytic activity

typical of metalloenzymes and have been shown to exist in a membrane-associated form both of which are consistent with their inherent structure containing a pro-domain, catalytic domain, disintegrin domain, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains  
5 (Wolfsberg *et al.*, Dev Biol, 169(1), 378-83, 1995). More specifically, ADAM 10 has been identified as a catalytically active ADAM through the cleavage of a 12-amino acid peptide based around the pro-tumour necrosis factor (TNF) cleavage site (Rosendahl *et al.*, J Biol Chem, 272(39), 24588-93, 1997) and also been implicated in the ectodomain shedding of protein (Qi *et al.*, Science,  
10 283, 91-94, 1999). In addition, ADAM 9 has also been shown as catalytically active through the cleavage of peptides, myelin basic protein (Amour *et al.*, FEBS Letters, 524, 154-158, 2002) and has also been proposed to have a role in the ectodomain shedding of CD23 (European Patent Application EP1142910).

15 International Patent Application WO-A-98/37092 discloses a disintegrin protein and its use in detecting and testing drugs for ailments such as osteoarthritis.

20 We have now found that the metalloprotease ADAM 10, a member of the ADAM family, is the much sought after enzyme involved in the shedding of ectodomain CD23, as shown by the experimental observations as described hereinafter. These observations suggest that modulation of ADAM 10 activity results in the inhibition of CD23 shedding and thus the subsequent generation  
25 of IgE, thus providing a possible target for drug therapy in allergic diseases.

The invention provides the means to identify compounds for use in the treatment of allergic disease.

30 Thus one aspect of the invention encompasses the use of a substance capable of inhibiting ADAM 10 for the manufacture of a medicament for the treatment of allergic disease.

A further aspect of the invention encompasses the use of a substance capable of inhibiting ADAM 10 for the manufacture of a medicament for the treatment of allergic disease, characterised in that said substance is obtained by an assay comprising the steps of:

- 5           i)       incubating a test substance with ADAM 10 and a substrate
- ii)       determining whether the test substance inhibits the activity of ADAM 10 mediated cleavage of said substrate.

Such substances may be of use in the treatment or prevention of conditions in  
10   which IgE up-regulation is implicated, in particular allergic diseases.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows that ADAM 10 protein is expressed in the RPMI8866 cell line  
15   and primary human tonsillar B-cells

Figure 2 shows the cleavage site for ADAM 10-mediated cleavage of a peptide based on the N-terminus of the 33 kDa cleavage site CD23 (Substrate 1)  
20

Figure 3 shows ADAM 10 mediated cleavage of a recombinant CD23 protein (Substrate 4) in the presence and absence of various metalloproteinase inhibitors

#### 25   DETAILED DESCRIPTION OF THE INVENTION

The phrase "ADAM 10" refers to a metal dependent proteinase (Rosendahl *et al.*, J Biol Chem, 272(39), 24588-93, 1997), a fragment, a mutant or homologue thereof, which still maintains its catalytic function. The invention is  
30   intended to encompass ADAM 10 from any source, including the native protein from any species, and those prepared by recombinant methods, *in vitro* methods, or standard peptide synthesis. Preferably the proteinase is human or a mutant thereof. In particular, a recombinant ADAM 10, especially

human (e.g. R+D Systems 936-AD-020), produced in, for example, an insect cell line or a mammalian cell line may be used.

Any suitable substrate known to those skilled in the art may be employed in the assay. As used herein, the phrase "substrate" refers to any peptide or protein, comprising one or more amino acids, that possesses a cleavage site that is capable of being cleaved by ADAM 10. Preferably the substrate is "CD23-based", such that it includes one or more cleavage sites of membrane-bound CD23 (see for example Letellier M *et al.*, Mol. Immunology, Vol 26, pp 1105-1112, 1989). In particular, the substrate is based on the 37, 33, 29, 25 or 17 kDa cleavage sites, especially the 37 or 33 kDa cleavage sites, most especially the 33 kDa cleavage site, of membrane-bound CD23.

In one particular aspect of the invention the said CD23-based substrate is a peptide of greater than 1 amino acid. One particular example of a peptide of this type is the 12-amino acid Ac-RAEQQRLKSQDL-NH<sub>2</sub> (Substrate 1).

In another aspect of the invention the CD23-based substrate is a protein, containing the amino acid sequence of CD23 or a fragment, mutant or homologue thereof, such that it includes one or more cleavage sites of membrane-bound CD23. The invention is intended to encompass CD23 protein from any source, including the native protein from any species, and those prepared by recombinant methods, *in vitro* methods, or standard peptide synthesis. Preferably the protein is human or a mutant thereof. One particular example of a protein of this type is the ectodomain of CD23, which comprises the amino acid residues 48-321.

The cleavage of said substrates may be followed by conventional methods, either directly with such methods as high-performance liquid chromatography (HPLC) or mass spectrometry (MS), or indirectly with such methods as immunoblotting.

The substrate may, in a further aspect of the invention, contain a suitable label in order to provide a more facile means of detecting the cleavage of said substrate.

- 5 The term "label" or "tag" refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can, for example, be attached to the substrate to help detect cleavage of said substrate. Suitable labels include, but are not limited to, fluorophores, chromophores, radioactive isotopes, or chemical groups, peptides or proteins
- 10 which act as affinity ligands through which cleavage products can be identified. For example, useful labels include fluorophores such as 7-methoxycoumarin-4-acetic acid (Mca), chromophores such as *p*-nitroanilide, <sup>14</sup>C labelled amino acids, affinity ligands such as biotin, poly-histidine, immunoreactive peptide sequences e.g. c-myc (EQKLISEEDLGGC),
- 15 glutathione-S transferase (GST), or the FC domain of IgG. These labels can be detected using appropriate methods including fluorescence, absorbance, radioactivity, or by highly specific interaction with another molecule, such as streptavidin, metal ions or an antibody.
- 20 If a fluorometric substrate is used, the course of the assay may be followed by measuring the change in fluorescence intensity of the assay medium directly due to the accumulation of the label, e.g. Mca, comprising product as a result of cleavage, at an appropriate wavelength, e.g. 328/396 nm, or by using any convenient filter pair, such as 340/405 nm. As the assay can be used as a
- 25 discontinuous assay in the visible spectrum it has the advantage that cheap plastic microtitre plates can be used to detect the effects of test substances on ADAM 10 activity.

- In another example, a fluorometric method based on Fluorescence
- 30 Resonance Energy Transfer (FRET) utilizing a synthetic peptide substrate for measuring protease activity may be used to generate a spectroscopic response to protease cleavage. In this type of substrate, both an acceptor molecule and a fluorescent molecule are attached to the peptide or protein. The acceptor molecule is carefully chosen so that its absorbance overlaps

with the fluorophore's excited-state fluorescence, thus ensuring that the fluorescence is quenched through resonance energy transfer. Enzyme hydrolysis of the substrate results in spatial separation of the fluorophore and the acceptor molecule, thereby restoring the fluorophore's fluorescence. One particular example of a suitable substrate of this type is the quenched fluorescent peptide Mca-RAEQQLKSQDLE-Dpa (Substrate 2). Substrate 2 is a peptide that includes a CD23 protease cleavage site for ADAM 10, along with two covalently modified amino acid residues: Mca (7-methoxycoumarin-4-acetic acid), which acts as the fluorophore; and Dpa (3-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid), which acts as the quenching group. ADAM 10 cleavage of Substrate 2 releases a fragment containing only the Mca fluorophore, thus liberating it from the quenching effect of the nearby Dpa. ADAM 10 protease activity can be measured by exciting the sample at 328 nm and measuring the fluorescence emission at 396 nm. This fluorometric peptide substrate has been demonstrated to be cleaved quantitatively by ADAM 10. Another particular example of a suitable fluorometric substrate of this type is the commercially available generic metalloprotease peptide Mca-PLAQAVDap(Dnp)RSSSR (Substrate 3, (Bachem (UK) Ltd) M2255) [(7-methoxycoumarin-4-yl) acetyl-Pro-Leu-Ala-Gln-Ala-Val-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Arg-Ser-Ser-Ser-Arg-NH<sub>2</sub> (R&D Systems' Catalog # ES003)].

Alternatively the substrate, in particular a protein substrate, may contain labels that can be detected using, amongst other methods, immunoblotting techniques. Appropriate labels may include, but are not limited to, short sequences of amino acids or proteins, which have been co-engineered with the substrate at either the N-terminal or the C-terminal. For example, suitable labels include a Flag, c-myc, poly-histidine, V5, glutathione-S-transferase, green fluorescent protein, maltose binding protein (MBP), influenza A virus haemagglutinin (HA),  $\beta$ -galactosidase, and GAL4, which are capable of interacting with highly specific antibodies to the engineered label. Suitable examples may include tagged recombinant ectodomain CD23 protein, which can also contain one or more tags. One particular example of a suitable

substrate of this type is a dual tagged protein, H<sub>2</sub>N-c-myc-[human CD23 residues 48-321]-V5-[hexa-histidine]-COOH (Substrate 4). Substrate 4 is a recombinant protein that includes the extracellular portion of the CD23 protein containing all the known cleavage sites of CD23, along with one peptide tag at the N-terminus and two separate tags at the C-terminus.

Any suitable format, known to those skilled in the art, may be used for the assay for identifying inhibitors of ADAM 10. Thus, for example, a typical enzyme reaction may be commenced by the addition of a substrate for ADAM 10 and if assaying for ADAM 10 inhibitors, addition of the substrate may be preceded by addition of a medium, typically a buffered medium, containing a test substance and ADAM 10. The assay may, for example, be followed by depletion of the substrate or by increase of the cleaved product, using the detection methods described herein, depending on substrate.

The assay may be carried out at any temperature or pH at which ADAM 10, in the absence of any inhibitor, is active, and is typically carried out in the range from 25-37°C, pH7-8, in any suitable buffer compatible for use in the assay. A detergent may also be employed in the assay. The buffer may, for example, be a Hepes buffer, preferably 50 mM Hepes, pH7.2 and 0.0015% Brij 35. Final assay conditions may therefore consist of an assay mixture containing 50 mM Hepes, pH7.2, 0.0015% Brij 35, 0.15 µg/well recombinant human ADAM 10 and 10 µM substrate which is incubated at 37°C for 2 hrs. Cleavage may be measured by comparing with controls. Appropriate controls may include wells monitoring the progress of the assay in the absence of the test substance; and wells including substrate and buffer in the absence of ADAM 10.

The assay is suitable for adaptation to 96 well and 384 well plate technologies and can be automated using liquid handling robots, allowing modern high throughput screening techniques to be applied. The invention therefore permits high throughput, flexible and inexpensive screening for substances which inhibit ADAM 10. This will increase the likelihood of potent bioavailable drugs being identified.

A substance which inhibits the activity of ADAM 10 may be a reversible, irreversible, competitive, non-competitive or mixed inhibitor, or may inhibit by binding to the substrate thus preventing enzyme-substrate binding.

5

Suitable test substances may include defined chemical entities, peptides or peptide mimetics, oligonucleotides, and antibodies. Particular antibodies include anti-ADAM 10 antibodies or fragments thereof, for example monoclonal or polyclonal antibodies, any antigen binding fragment such as scFv, Fv, Fab, Fab' or F(ab)<sub>2</sub>, or recombinant antibodies such as chimeric, CDR-grafted or other engineered antibodies. Further, the assay of the invention may be used to screen libraries, such as combinatorial libraries, defined chemical entity libraries, peptide or peptide mimetic libraries, oligonucleotide libraries or natural product libraries.

15

Test substances, including libraries, may be obtained using conventional chemical or biological means, for example by simple chemical synthesis, peptide synthesis or recombinant DNA technology, or from naturally occurring sources, for example a microorganism.

20

A substance capable of inhibiting ADAM 10 reduces the ability of ADAM 10 to catalyse the cleavage of membrane-bound CD23 or a suitable substrate thereof. Preferably such substances inhibit ADAM 10 with a high affinity, having an IC<sub>50</sub> of less than  $20 \times 10^{-6}$  M, preferably less than  $1 \times 10^{-6}$  M, most especially less than  $1 \times 10^{-7}$  M, or even lower.

25

The present invention enables a substance to be identified that is capable of inhibiting the activity of ADAM 10 and consequently the shedding of soluble CD23 fragments. Thus, in particular, such a substance may be used in a method of treating or modulating allergic or IgE-mediated diseases. Typical diseases include allergic diseases such as asthma, atopic dermatitis and other atopic diseases, allergic rhinitis, gastrointestinal allergies such as food allergies, eosinophilia, conjunctivitis, glomerular nephritis, graft-v-host disease, systemic anaphylaxis or hypersensitivity responses, urticaria, shock,

30

drug allergies, insect sting allergies and parasite infections. Particular conditions include asthma, atopic dermatitis and allergic rhinitis .

5 The formulation of a substance identified according to the invention will depend upon the nature of the identified substance. Typically the pharmaceutical composition would comprise the identified substance together with one or more pharmaceutically acceptable carriers, excipients or diluents.

10 Alternative compositions may comprise the identified substance or a salt thereof; an additional agent selected from an immunosuppressant or an anti-inflammatory agent; and any pharmaceutically acceptable carrier, adjuvant or vehicle.

For example, pharmaceutical compositions may take a form suitable for oral, 15 buccal, parenteral, nasal, topical, vaginal or rectal administration, or a form suitable for administration by inhalation or insufflation.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets, lozenges or capsules prepared by conventional 20 means with pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium glycolate); or wetting agents (e.g. 25 sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with 30 pharmaceutically acceptable additives such as suspending agents, emulsifying agents, non-aqueous vehicles and preservatives. The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

5 For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

The identified substance may be formulated for parenteral administration by injection, e.g. by bolus injection or infusion. Formulations for injection may be presented in unit dosage form, e.g. in glass ampoules, or multi-dose  
10 containers, e.g. glass vials. The compositions for injection may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising, preserving and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free  
15 water, before use. For particle-mediated administration the substance may be coated on particles such as microscopic gold particles.

In addition to the formulations described above, the identified substance may also be formulated as a depot preparation. Such long-acting formulations  
20 may be administered by implantation or by intramuscular injection.

For nasal administration or administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation for pressurised packs or a nebuliser, with the  
25 use of suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas or mixture of gases.

For vaginal or rectal administration the identified substance may be formulated  
30 as a suppository. These formulations may be prepared by mixing the active ingredient with a suitable non-irritating excipient which is a solid at room temperature but liquid at the body temperature. Such materials include, for example, cocoa butter and polyethylene glycols.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack or dispensing device may be accompanied by instructions for administration.

5

The quantity of an identified substance required for the prophylaxis or treatment of a particular condition will vary depending on the identified substance, and the condition of the patient to be treated. In general, however, daily dosages may range from around 100 ng/kg to 100 mg/kg, e.g. around  
10 0.01 mg/kg to 40 mg/kg body weight for oral or buccal administration, from around 10 ng/kg to 50 mg/kg body weight for parenteral administration, and around 0.05 mg to around 1000 mg, e.g. around 0.5 mg to around 1000 mg, for nasal administration or administration by inhalation or insufflation.

15 The following examples illustrate the series of events that led to the invention and the practical use of the invention for identifying inhibitors of ADAM 10-mediated cleavage of CD23.

These examples demonstrate that ADAM 10 is co-expressed with CD23 in  
20 physiologically relevant human cellular systems and that ADAM 10 can mediate the cleavage of a recombinant ectodomain CD23 protein *in vitro*. To further define this cleavage event, ADAM 10 was also incubated with a peptide (Substrate 1) based on a region flanking the previously identified N-terminus of the 33 kDa soluble CD23 fragment and found to cleave at the  
25 same site exhibited during the shedding of soluble CD23, suggesting a relationship between ADAM 10 and the unknown CD23 sheddase. In addition, an array of hydroxamic-based inhibitors plus endogenous inhibitors of metalloproteinases (TIMPs) ranging in potency against CD23 shedding were also profiled for activity in a recombinant ADAM 10 enzyme assay using  
30 both Substrate 2 and Substrate 3 and IC<sub>50</sub> values determined. Results generated indicate a significant correlation between IC<sub>50</sub> values generated in each assay, suggesting a strong relationship between ADAM 10 activity and CD23 shedding and thus strengthening the relationship between ADAM 10 and the CD23 sheddase. In addition, these data demonstrate that inhibition of

ADAM 10 activity by a chemical entity in the assay is readily detectable and readable, which thus illustrates the practical application of the invention.

Abbreviations:

- 5 LC-MS - liquid chromatography-mass spectroscopy  
TFA - trifluoroacetic acid                      DMSO - dimethylsulfoxide  
MeCN - acetonitrile                              EDTA - ethylenediaminetetraacetic acid

**Example 1**

10 **ADAM 10 is expressed in physiologically relevant cellular systems**

Previous studies have shown that both the RPMI8866 cell line and isolated primary human B-cells express CD23 (Bujanowski-Weber *et al.*, Immunology, 66(4), 505-11, 1989) and under certain conditions they have also been shown  
15 to shed the protein from the cell surface (Christie *et al.*, Eur J Immunol, 27(12), 3228-35, 1997; Marolewski *et al.*, Biochem J, 333 (Pt 3), 573-9, 1998), suggesting that these cellular systems are also likely to express the metalloproteinase responsible for the shedding of the substrate, CD23.

20 To analyse whether ADAM 10 was expressed in physiologically relevant cellular systems (RPMI8866 cell line and isolated primary human B-cells) immunoblotting experiments were carried out using a commercially available purified rabbit polyclonal antibody (Chemicon-AB19026), which had been raised against a peptide corresponding to amino acids 732-748 of human  
25 ADAM 10.

**Preparation of RPMI8866 cells:**

Cells of the human B lymphoid RPMI8866 cell line were maintained in RPMI1640 medium (Sigma, Catalogue Number R0883) supplemented with 2  
30 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% foetal bovine serum (FBS) at 37°C with 5% CO<sub>2</sub> until they reached a density of  $8 \times 10^5$  cells/ml. The cells were resuspended in unsupplemented RPMI1640 medium at a density of  $1 \times 10^7$  cells/ml and maintained for 1 hour

at 37°C with 5% CO<sub>2</sub>. The cells were then washed with Dulbecco's phosphate buffered saline (DPBS) (0.2 g/l KCl, 0.2 g/l anhydrous KH<sub>2</sub>PO<sub>4</sub>, 8.0 g/l NaCl, 1.15 g/l anhydrous Na<sub>2</sub>HPO<sub>4</sub>; Sigma, Catalogue Number D8537) (1 ml/2.72 x 10<sup>7</sup> cells), snap frozen and stored at -80°C until use.

5

Preparation of primary human tonsillar B-cells:

A pair of human tonsils were roughly chopped, mixed with 30 ml DPBS and forced through a 40 micron sieve. The resulting cell suspension was then layered onto 20 ml Ficoll-Plaque Plus (Amersham Biosciences) and  
10 centrifuged at 1180 x g for 30 minutes. The tonsillar mononuclear cells were then recovered from the DPBS/Ficoll-Plaque Plus boundary, washed with 50 ml DPBS and resuspended in FBS/EDTA (0.99% (v/v) FBS prepared using 0.02% EDTA in DPBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>; Sigma, Catalogue Number E8008) (1.6 ml/2 x 10<sup>8</sup> cells). The resulting cell suspension was then mixed  
15 with MACS CD19 Microbeads (Miltenyi Biotec, Catalogue Number 503-01) (0.4 ml/2 x 10<sup>8</sup> cells) and incubated for 15 minutes at 4°C. Next, the cell suspension was diluted to 50 ml with FBS/EDTA, the cells recovered by centrifugation and resuspended in FBS/EDTA (1 ml/2 x 10<sup>8</sup> cells). An autoMACS machine (Miltenyi Biotec) was then used to separate the tonsillar  
20 B-cells, which express CD19, from other mononuclear cells, which lack CD19. The isolated tonsillar B-cells were diluted to 50 ml with DPBS, recovered by centrifugation and resuspended at a density of 1 x 10<sup>7</sup> cells/ml in RPMI1640/2% FBS (RPMI1640 medium (Sigma, Catalogue Number R0883) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml  
25 streptomycin and 2% (v/v) FBS). Next, 2 ml aliquots of the resulting cell suspension were mixed with 2 ml RPMI1640/0% FBS (RPMI1640 medium with 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin) that was either unsupplemented or contained 200 ng/ml recombinant human interleukin-4 (IL-4; R&D Systems, Catalogue Number 204-IL-005). The  
30 resulting cell suspensions, which contained 5 x 10<sup>6</sup> cells/ml in RPMI1640 medium with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1% (v/v) FBS ± 100 ng/ml IL-4, were then maintained for 24

hours at 37°C with 5% CO<sub>2</sub>. The cells were then washed with DPBS (1 ml/2 x 10<sup>7</sup> cells), snap frozen and stored at -80°C until use.

Preparation of cell lysates for immunoblotting studies:

- 5 Cell samples were thawed to 4°C and resuspended (using 10 ml/g of cells) in DPBS (4°C) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets from Roche used according to the manufacturer's instructions). They were then briefly sonicated at 4°C and the resulting lysates dispensed into aliquots and stored at -20°C. Finally, the protein
- 10 concentration of each cell lysate was determined by BCA protein assay (Pierce, Catalogue Number 23225X, Microtiter Plate Protocol) using bovine serum albumin as a standard.

SDS-PAGE and Western Blotting:

- 15 Protein samples were subjected to SDS-PAGE and then transferred onto nitrocellulose using the NuPAGE Bis-Tris Electrophoresis System (Invitrogen) according the manufacturer's instructions. Samples of cell lysate containing 20 µg total protein were mixed with 6.25 µl NuPAGE LDS Sample Buffer and 2.5 µl NuPAGE Sample Reducing Agent and enough ultrapure water to give a
- 20 final volume of 25 µl. After incubation for 10 minutes at 70°C, samples were loaded onto a NuPAGE Novex 4-12% Bis-Tris Gel (1.0 mm thick with 15 wells) and subjected to SDS-PAGE under reducing conditions using NuPAGE MOPS SDS Running Buffer. After electrophoresis, proteins were then transferred onto Hybond ECL nitrocellulose (Amersham Biosciences) by
- 25 Western blotting.

Immunoblotting:

- The nitrocellulose Western blot was blocked overnight at 4°C in 50 ml Milk Mix (PBS (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4 at
- 30 25°C; Sigma, Catalogue Number P4417) containing 0.05% (v/v) Tween 20 (Sigma) and 5% (w/v) 'Marvel' non-fat dried milk) and then incubated for 3.5 hours at 20°C with 3 ml Milk Mix containing 1 µg/ml of anti-ADAM 10 antibody (Chemicon International). The blot was maintained at 20°C, washed 5 times

(each 6 minutes) with 20 ml Milk Mix, then incubated for 45 minutes with 3 ml of Milk Mix containing a 1:1000 dilution of protein A-horseradish peroxidase (Amersham Biosciences). Finally, blots were washed 5 times (each 6 minutes) with 20 ml PBS/0.05% (v/v) Tween 20, then washed 5 times (each 6 minutes) with 20 ml PBS and antigen-antibody complexes located using enhanced chemiluminescence (ECL). This was performed using ECL Western Blotting Detection Reagents and Hyperfilm ECL (both from Amersham Biosciences). The results shown in Figure 1 were obtained during a 10 minute period of ECL.

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ADAM 10 was found to be expressed (60 kDa and 85 kDa) in both the RPMI8866 cell line and primary human B-cells isolated from tonsillar tissue both in the presence and absence of IL-4 stimulation (Figure 1). Expression in the Jurkat cell line was included as a positive control for expression of ADAM 10 (Wu *et al.*, Biochem Biophys Research Comm, 235(2), 437-442, 1997).

15

## **Example 2**

### **ADAM 10 mediated cleavage of a peptide based on 33KDa cleavage site of CD 23**

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To demonstrate recombinant ADAM 10 (R+D Systems 936-AD-020) could potentially cleave the 33 kDa cleavage site of CD23, the enzyme was incubated with Ac-RAEQQLKSDNL-NH<sub>2</sub> (Substrate 1) based on the 33 kDa cleavage site of CD23 (Figure 2) and cleavage products were analysed by mass spectroscopy.

25

#### **Exposing Substrate 1 to ADAM 10:**

Substrate 1 was supplied lyophilized and reconstituted to 1 mM using sterile ultrapure H<sub>2</sub>O. Recombinant human ADAM 10 (amino acids 214-672 of human ADAM 10 with a C-terminal 10X His tag) was obtained from R&D Systems; this was supplied lyophilized and reconstituted to 100 µg/ml using sterile ultrapure H<sub>2</sub>O.

30

Assays were carried out in a final volume of 200  $\mu$ l, containing 50 mM Hepes, pH 7.2, 0.0015% Brij 35, recombinant human ADAM 10 (200 ng), Substrate 1 (20  $\mu$ l of a 1 mM stock solution) and either DMSO (20  $\mu$ l of 20%) or one of the  
5 test compounds, SB257702, Marimastat or Trocade (20  $\mu$ l of 30  $\mu$ M stock solution in 20% DMSO). The resulting reaction mixtures were then incubated for 18 h at 37°C, and then stored at -80°C prior to analysis.

#### LC-MS:

10 Gradient LC was performed using a Vydac 300Å Protein C18 column (250 x 4.6 mm) with a mobile phase of 0.1% TFA (aqueous) and 0.1% TFA (MeCN). Mass spectra were determined under electrospray ionisation conditions (cone voltage 25V), using a Micromass LC-Z mass spectrometer.

### 15 Example 3

#### Inhibitors of ADAM 10 as modulators of CD23 shedding

Following the above experiments, Substrate 2 (Mca-RAEQQRLKSQDLE-Dpa, a fluorogenic version of Substrate 1) was prepared and used subsequently to  
20 develop a method for identifying inhibitors of ADAM 10-mediated cleavage of CD23. ADAM 10 was incubated with Substrate 2 and inhibition ( $IC_{50}$  values) determined in the presence of a panel of metalloproteinase inhibitors.

In addition, ADAM 10 activity was also demonstrated following incubation with  
25 a commercially available fluorescent metalloproteinase peptide substrate, Mca-PLAQAVDap(Dnp)RSSSR (Bachem (UK) Ltd) (Substrate 3), which has previously been shown to be cleaved by human recombinant ADAM 10 (Rosendahl *et al.*, J Biol Chem, 272(39), 24588-93, 1997). ADAM 10 was incubated with Substrate 3 and inhibition ( $IC_{50}$  values) determined in the  
30 presence of a panel of metalloproteinase inhibitors.

Assays were carried out in a 96-well plate format, in a final volume of 100  $\mu$ l, containing 50 mM Hepes, pH 7.2, 0.0015% Brij 35, recombinant human ADAM 10 (150 ng), Substrate 2 or Substrate 3 (10  $\mu$ l of 100  $\mu$ M) and either

DMSO (10  $\mu$ l of 20%) or one of the test compounds, SB257702, SB435466, Marimastat or Trocade (10  $\mu$ l of 30  $\mu$ M stock solution, or dilution thereof, in 20% DMSO), or one of the Tissue Inhibitors of Metalloproteinases (TIMP1, 2 & 3; obtained from R&D Systems) (10  $\mu$ l of 2.5  $\mu$ M stock solution, or a dilution thereof, in assay buffer). After incubation at 37°C for 1-2 h, protease activities were determined by measuring the change in fluorescence intensity of the assay medium in a fluorescence plate reader (Tecan Ultra) using 340/405 nm filter pair.

IC<sub>50</sub> values generated in either the Substrate 2- or Substrate 3-based assay were compared to those obtained from a CD23 shedding membrane preparation assay which was based on a previously published method (Bailey *et al.*, Bioorg Med Chem Lett, 8(1), 23-8, 1998; Marolewski *et al.*, Biochem J, 333 (Pt 3), 573-9, 1998). IC<sub>50</sub> values were defined as the concentration of drug required to inhibit the enzyme or shedding activity by 50%. The enzyme or shedding activity for a given concentration of drug was divided by the mean value obtained from the control (without drug) and multiplied by 100 to give values equivalent to the percentage of the control count. Non-linear least squares regression was then used to fit the Hill equation to the data. The results are shown in the Table of Biological Results below.

Results generated suggest a significant relationship between IC<sub>50</sub> values of inhibitors against CD23 shedding and ADAM 10 activity, as shown in the Table below. More specifically, both selective inhibitors of CD23 shedding (SB257702; SB435466 (RSC-SCI Medicinal Chemistry - 11th Symposium (Part I), Cambridge, UK, 2001)) and more broad-spectrum metalloproteinase inhibitors that also inhibit CD23 shedding (Marimastat; TIMP1 and TIMP3 (Tissue Inhibitors of Metalloproteinases)) were found to have similar activity in the aforementioned recombinant ADAM 10 assay whereas relatively poor inhibitors of CD23 shedding (Trocade and TIMP2) were correspondingly found to be relatively poor inhibitors of ADAM 10 activity.

**Example 4****ADAM 10 mediated cleavage of recombinant CD23 protein**

Having demonstrated that ADAM 10 could cleave a peptide based on 33 kDa  
5 cleavage site of CD23 at an identical position to the CD23 sheddase and that  
there was a relationship between the activity of metalloproteinase inhibitors in  
CD23 sheddase and ADAM 10 assays, subsequent experiments attempted to  
confirm these observations on the protein level (Figure 3).

10 **Exposing Substrate 4 to ADAM 10:**

Medium containing the dual-tagged CD23 ectodomain (Substrate 4) was  
thawed to 4°C. Aliquots (70 µl) were left untreated or supplemented with  
ADAM 10 (10 µl of 100 µg/ml) and either DMSO (5 µl of 8.5%) or one of the  
test compounds, SB257702, Marimastat or Trocade (5 µl of 17 µM in 8.5%  
15 DMSO). The resulting reaction mixtures were then incubated overnight at  
37°C.

**SDS-PAGE and Western Blotting:**

Reaction mixtures were subjected to SDS-PAGE and then transferred onto  
20 nitrocellulose using the NuPAGE Bis-Tris Electrophoresis System (Invitrogen)  
according the manufacturer's instructions. Samples of reaction mixture  
containing 7 µl of medium containing Substrate 4 were mixed with enough  
NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent to give a  
4-fold dilution of the former and a 10-fold dilution of the latter. After incubation  
25 for 10 minutes at 70°C, samples were loaded onto a NuPAGE Novex 10%  
Bis-Tris Gel (1.0 mm thick with 10 wells) and subjected to SDS-PAGE under  
reducing conditions using NuPAGE MOPS SDS Running Buffer. After  
electrophoresis, proteins were then transferred onto Hybond ECL  
nitrocellulose (Amersham Biosciences) by Western blotting.

30

**Immunoblotting:**

The nitrocellulose Western blot was blocked by incubating for 1 hour at 20°C  
in 50 ml Milk Mix (PBS (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl,

pH 7.4 at 25°C; Sigma, Catalogue Number P4417) containing 0.05% (v/v) Tween 20 (Sigma) and 5% (w/v) 'Marvel' non-fat dried milk) and then incubated for 1 hour at 20°C with 10 ml PBS/0.05% (v/v) Tween 20 containing a 1:5000 dilution of anti-V5-horseradish peroxidase (Invitrogen, Catalogue Number R961-25). The blot was maintained at 20°C and rinsed twice with 100 ml of PBS/0.05% (v/v) Tween 20, then washed twice (each 10 minutes) with 200 ml PBS/0.05% (v/v) Tween 20. Antigen-antibody complexes were then located using enhanced chemiluminescence (ECL). This was performed using ECL Western Blotting Detection Reagents and Hyperfilm ECL (both from Amersham Biosciences). The results shown in Figure 3 were obtained during a 15 second period of ECL.

Dual tagged ectodomain CD23 (Substrate 4) was found to be cleaved into lower molecular weight fragments following co-incubation with recombinant ADAM 10 (Lane 2) but found to be stable when incubated alone (Lane 1). This apparent cleavage into lower molecular weight fragments was inhibited by 1  $\mu$ M SB257702 (a specific CD23 sheddase inhibitor) and 1  $\mu$ M Marimastat (a broad spectrum metalloproteinase inhibitor) but not by 1  $\mu$ M Trocade (a specific matrix metalloproteinase inhibitor), a profile consistent with inhibition of CD23 shedding (see Table of Biological Results below).

Table of Biological Results

IC<sub>50</sub> values of CD23 shedding and ADAM 10 following exposure to various  
5 metalloproteinase inhibitors.

| <b>Compound</b> | <b>Inhibits</b>              | <b>CD23<br/>shedding<br/>(IC<sub>50</sub>)</b> | <b>ADAM 10<br/>(Substrate 3)<br/>(IC<sub>50</sub>)</b> | <b>ADAM 10<br/>(Substrate 2)<br/>(IC<sub>50</sub>)</b> |
|-----------------|------------------------------|------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|
| SB257702        | CD23<br>Shedding             | 23 nM                                          | 27 nM                                                  | 36 nM                                                  |
| SB435466        | CD23<br>Shedding             | 39 nM                                          | 10 nM                                                  | 56 nM                                                  |
| Marimastat      | Metalloproteinases           | 92 nM                                          | 78 nM                                                  | 99 nM                                                  |
| Trocade         | Matrix<br>Metalloproteinases | >30 µM                                         | >5.0 µM                                                | >5.0 µM                                                |
| TIMP1           | Metalloproteinases           | 10 nM                                          | 10 nM                                                  | 9 nM                                                   |
| TIMP2           | Matrix<br>Metalloproteinases | >250 nM                                        | >250 nM                                                | >250 nM                                                |
| TIMP3           | Metalloproteinases           | 0.4 nM                                         | 33 nM                                                  | 24 nM                                                  |

Substrate 2 - Mca-RAEQQLKSDLE-Dpa

Substrate 3 - Mca-PLAQVDap(Dnp)RSSSR (M-2255; Bachem (UK) Ltd.)

**CLAIMS**

1. The use of a substance capable of inhibiting ADAM 10 for the manufacture of a medicament for the treatment of allergic disease.
- 5 2. The use according to Claim 1, characterised in that said substance is obtained by an assay comprising the steps of:
  - i) incubating a test substance with ADAM 10 and a substrate
  - 10 ii) determining whether the test substance inhibits the activity of ADAM 10 mediated cleavage of said substrate.
3. The use according to Claim 2 wherein said substrate is CD23-based.
4. The use according to Claim 2 or Claim 3 wherein said substrate is  
15 labelled.
5. The use according to any one of Claims 2 to 4 wherein said substrate is a peptide.
- 20 6. The use according to Claim 5 wherein said substrate is Ac-RAEQQLKSDLE-NH<sub>2</sub>.
7. The use according to Claim 5 wherein said substrate is Mca-RAEQQLKSDLE-Dpa, wherein Mca is a fluorophore and Dpa  
25 is a group capable of quenching Mca fluorescence.
8. The use according to Claim 5 wherein said substrate is Mca-PLAQAVDap(Dnp)RSSSR, wherein Mca is a fluorophore and Dap(Dnp) is a group capable of quenching Mca fluorescence.  
30
9. The use according to any one of Claims 2 to 4 wherein said substrate is a protein.
10. The use according to Claim 9 wherein the protein is recombinant.

11. The use according to Claim 10 wherein said substrate is  
H<sub>2</sub>N-c-myc-[human CD23 residues 48-321]-V5-[hexa-histidine]-COOH.

5

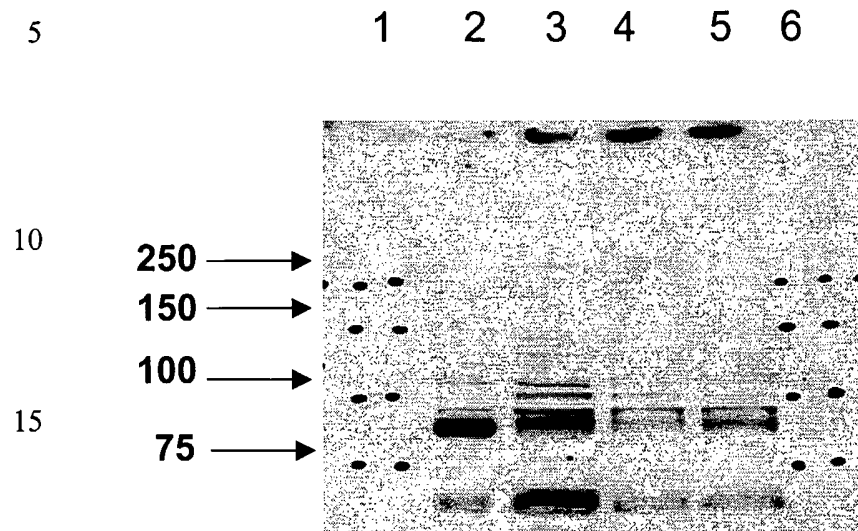
12. The use according to Claim 1 or 2 wherein the substance is an anti-  
ADAM 10 antibody or a fragment thereof.

13. The use according to Claim 1 or 2 wherein the substance is a defined  
chemical entity.

10

**Figure 1**

**ADAM 10 protein is expressed in the RPMI8866 cell line and primary human tonsillar B-cells**



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Lane 1= prestained precision protein standards (Bio-Rad Laboratories) (3.25  $\mu$ g)

Lane 2= Jurkat cells (25  $\mu$ g protein) (+ve control)

30 Lane 3= RPMI8866 cells (20  $\mu$ g protein)

Lane 4= IL-4 stimulated primary human tonsillar B-cells (20  $\mu$ g protein)

Lane 5= primary human tonsillar B-cells (20  $\mu$ g protein)

Lane 6= prestained precision protein standards (3.25  $\mu$ g protein)

**Figure 2**

**ADAM 10-mediated cleavage of a peptide based on the N-terminus of the 33 kDa cleavage site CD23 (Substrate 1)**

