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(54) Title: METHOD FOR AMELIORATING AN INFLAMMATORY SKIN CONDITION

(57) Abstract: The present invention relates to the use of thioredoxin in the manufacture of a medicament suitable for application to a skin surface for ameliorating an inflammatory skin condition. The present invention further relates to a method of ameliorating an inflammatory skin condition comprising applying to a skin surface an effective amount of a composition comprising thioredoxin. The invention further relates to a pharmaceutical composition suitable for ameliorating an inflammatory skin condition comprising from 0.0001 to 0.5 w/v thioredoxin.

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Method for ameliorating an inflammatory skin condition

The present invention relates, *inter alia*, to a method of ameliorating an inflammatory skin condition.

Inflammatory skin conditions are known to be associated with chemokines and cytokines, and in particular the activities of pro-inflammatory cytokines such as IL-1α, IL-1β and tumour necrosis factor α (TNF-α). These same cytokines are known also to play pivotal roles in the initiation of skin immune responses, and in fact provide mandatory signals for the migration of epidermal Langerhans cells (LC) from the skin. The movement of LC from the skin, and their subsequent accumulation in skin-draining lymph nodes provides a mechanism for the transport of antigen to the sites (regional lymph nodes) where immune responses are induced.

Our understanding that the migration of LC from the epidermis is dependent upon the provision of signals by IL-1α, IL-1β and TNF-α provides an experimental system for investigating the availability and functional activity of these cytokines in skin tissues. Experience has shown that factors that are known to inhibit the availability or function of IL-1α, IL-1β or TNF-α are associated with a significant inhibition of induced LC migration.

In addition to being required for the stimulation of LC mobilisation, IL-1β is known to cause skin inflammation and has been implicated, directly or indirectly, in the pathogenesis of several cutaneous inflammatory disorders. IL-1β is synthesised as an inactive intracellular precursor protein, which is cleaved and secreted to yield mature carboxy-terminal fragments that are biologically active and exert their effect by binding to specific cell surface receptors found on almost all cell types and triggering a range of responses.

The present invention is based on the surprising discovery that certain molecules are able, when applied topically to the skin, to inhibit the production and/or availability of bioactive IL-1α and/or IL-1β. As such these molecules are suitable, *inter alia*, for

the treatment of inflammatory skin conditions where IL-1α and/or IL-1β are implicated in the pathogenesis. Suitable molecules include thioredoxin (TRX), a 12-kDa protein with a Cys-Gly-Pro-Cys active site, and additionally "redox-inactive" TRX molecules, wherein the cysteines at the active site are replaced by amino acids other than cysteine. Whilst it has been shown that these molecules are likely to exert their therapeutic effect by inhibiting the production or activity of IL-1α or IL-1β – it is also possible that they exert an associated or additional beneficial effect by stimulating the production of anti-inflammatory cytokine(s), such as interleukin-10 (IL-10).

According to the present invention there is provided a polypeptide capable of ameliorating an inflammatory skin condition wherein said polypeptide is a modified thioredoxin, the modification comprising:-

a. substituting Cys_1 and Cys_2 in the motif Cys_1-Gly-Pro-Cys_2 present in the unmodified thioredoxin with an amino acid other than cysteine with the proviso that if one Cys is substituted with Ser the other Cys is not substituted with Ser; or
b. substituting either of Cys_1 and Cys_2 in the motif Cys_1-Gly-Pro-Cys_2 present in the unmodified thioredoxin with an amino acid other than cysteine and deleting the non-substituted cysteine.

Preferably, the modification consists of independently substituting both Cys_1 and Cys_2 with an amino acid other than Ser. The modification of the active site renders the active site redox-inactive and, surprisingly, it has been found that such redox-inactive molecules are capable of ameliorating an inflammatory skin condition.

The present invention further provides a modified thioredoxin wherein if the unmodified thioredoxin contains one or more cysteines in addition to Cys_1 and Cys_2, then the modification further comprises substituting and/or deleting one or more of the additional cysteines.

Both Cys_1 and Cys_2 may be independently substituted. For example, one embodiment of the polypeptide of the present invention could comprise Ser-Gly-Pro-Ala, another

Ala-Gly-Pro-Ser. In a preferred embodiment of the invention Cys_1 and Cys_2 are both substituted by Ala to give Ala-Gly-Pro-Ala. More preferred is a polypeptide wherein the unmodified TRX is human TRX, and more preferred still is the polypeptide selected from the group consisting of SEQ ID NO. 3, SEQ ID NO. 9 and SEQ ID NO. 10.

A further embodiment of the present invention is a DNA sequence that encodes a polypeptide of the present invention. The exact nature of the DNA sequence would, of course, depend on the specific nature of the polypeptide and the intended use of the DNA sequence. For example, codon-optimisation of the DNA sequence may be required for expression of the DNA sequence in a recombinant expression system (an example of a codon-optimised sequence is provided as SEQ ID NO. 6). The techniques required to provide such DNA sequences are well within the knowledge of the skilled man. A preferred DNA sequence of the present invention is depicted in SEQ ID NO. 4.

The present invention also relates to the use of the polypeptides of the present invention as a pharmaceutical – and a pharmaceutical composition/medicament - suitable for treating inflammatory skin conditions preferably comprising the polypeptide(s) of the present invention. For therapeutic purposes the polypeptide(s) of the present invention may be administered by any conventional means, either as an individual therapeutic agent or in combination with other therapeutic agents. The pharmaceutical compositions of the present invention can be adapted, using methods well known to those skilled in the pharmaceutical art, depending on the exact route of administration desired. Compositions of the present invention include, but are not limited to, those suitable for application to the skin via, for example, topical application and subcutaneous application. For the treatment of psoriasis, topical application is sufficient to give a therapeutic effect.

The present invention further relates to methods of producing the polypeptide of the present invention. Such methods would include recombinant expression of said polypeptide and in particular transforming an organism with a vector comprising a DNA sequence encoding the polypeptide, wherein said vector is capable of

expressing said DNA sequence in said organism and growing said organism in conditions which allow the expression of said DNA sequence to produce said polypeptide. By growing it is meant increasing biomass, for example where the organism is a unicellular organism growing means increasing cell number. The term "organism" includes any organism that is suitable for the recombinant expression of the polypeptides of the present invention. Suitable recombinant expression systems include, but are not limited to, mammalian cell cultures, yeast and bacteria. Particularly preferred is *E.coli*. Vectors suitable for expression in host cell such as these would be readily apparent to the skilled man and include, for example vectors that harbour the T7 promoter, such as pET vectors, for expression in *E. coli* and other vectors suitable for expression in the yeast *Pichia pastoris*. The method of producing the polypeptide may also include the purification of the polypeptide. By purification it is meant obtaining the recombinant polypeptide from the production materials. Methods such as these could be employed during the Good Manufacturing Practice (GMP) production of these polypeptides.

The present invention further relates to a method of ameliorating an inflammatory skin condition comprising applying to a skin surface an effective amount of a composition comprising a molecule selected from the group consisting of:

a. a protein comprising a thioredoxin active site (Cys_1-Gly-Pro-Cys_2);
b. a thioredoxin (TRX);
c. a modified thioredoxin wherein said modification comprising substituting and/or deleting at least one of the cysteines present in the unmodified thioredoxin with an amino acid other than cysteine;
d. a polypeptide according to the present invention; and
e. a molecule that comprises a region of three dimesional similarity to a region present within the three dimensional structure of the protein depicted in SEQ ID NO. 1, and which is capable of ameliorating an inflammatory skin condition.

The term "inflammatory skin condition" includes, for example, a human inflammatory skin condition and an animal inflammatory skin condition. In a preferred embodiment the inflammatory skin condition is selected from the group

consisting of psoriasis, lichen planus, atopic eczema, irritant or allergic contact dermatitis, contact urticaria, infantile eczema and acne. The methods of the present invention are also useful in assisting wound healing, and in the treatment of burns, especially sunburn. Psoriasis is a chronic inflammatory skin condition characterised by the appearance of discrete psoriatic plaques. Psoriasis is associated with a number of changes in skin morphology. There is increasing evidence that pro-inflammatory cytokines play important roles in the pathogenesis of psoriasis. Current treatments include local topical administration of anti-inflammatory agents – typically a corticosteroid. Such treatments are not fully effective and are associated with unwanted side effects. Another therapeutic strategy is disruption of TNF-α function, but this also has been found to cause adverse reactions. There is a need therefore to provide further molecules that are effective in the treatment of inflammatory skin disorders, but which exhibit little or no adverse side effects and which ideally can be delivered by a non-invasive method, for example by application directly onto the inflammation.

Preferred for use in the method of the present invention is a molecule capable of inhibiting production and/or activity of IL-1α and/or IL-1β and/or capable of stimulating or enhancing the production and/or activity of IL-10.

TRX is a small (10-14 kDa), ubiquitous protein that is an important component of the cellular redox regulation system. Suitable TRX for use in the method of the present invention include TRX from (1) a prokaryote (e.g *E. coli* – SEQ ID NO.7), (2) a plant (e.g *Arabidopsis* – SEQ ID NO.8) and (3) an animal (e.g human – SEQ ID NO. 1). TRX can exist in a reduced state (wherein the two cysteines at the active site (Cys_1-Gly-Pro-Cys_2) provide a dithiol) and an oxidised state (wherein there is a disulphide bridge formed between the two cysteines at the active site). Under physiological conditions both redox states can exist – and both forms can be utilised in respect of the present invention. Furthermore, it is known that certain thioredoxins can exist in multimeric forms. For example, it is known that human TRX (hTRX) can form dimers wherein a disulphide bridge exists between Cys-73. These multimeric forms of the molecules may also be utilised, in addition to the monomeric form, within the methods of the present invention. It is however, preferred, that the molecule, for

example thioredoxin, is in a substantially reduced state. By substantially reduced it is meant that >80%, preferably >90%, more preferably >95% of the molecules present are in a reduced state. A preferred molecule for use in the method is the recombinant human thioredoxin depicted in SEQ ID NO. 1 – since this protein is an endogenous human protein, and is therefore unlikely to cause either adverse effects, or an immune response when administered to patients. Other molecules suitable for use in the method of the present invention include a protein that comprises a thioredoxin active site in which one or both of the cysteines at the active site are replaced by an amino acid other than cysteine. Examples wherein one or other of the cysteines is replaced include Cys_1-Gly-Pro-Ala and Ala-Gly-Pro-Cys_2. Surprisingly, it has been discovered that redox-inactive TRX molecules, in which both Cys_1 and Cys_2 are replaced, can also be successfully used in the present inventive methods. Furthermore, it has been shown that where the TRX molecule comprises additional cysteines other than at the active site these additional cysteines can also be replaced without any loss in activity. For example, in respect of human thioredoxin, which contains five cysteines (C_{32}, C_{35}, C_{62}, C_{69} and C_{73}) it has been shown that modified human thioredoxins comprising (1) C73A, (2) C32A, C35A and C73A; and (3) C32A, C35A, C62A, C69A and C73A retain biological activity. It has also been shown that activity is retained if cysteines present in the unmodified thioredoxin other than the active site cysteines are substituted and/or deleted. For example, the protein depicted in SEQ ID NO. 11 (C73A) has been shown to be active. It has also been found that the active molecules can be rendered inactive by heat treatment at 95°C for 30 min, or 56°C for 30 min, indicating that there is a structural feature associated with these molecules that is responsible for the observed activity. Thus the present invention further relates a molecule which comprises a region of three dimensional homology to a region present within the three dimensional structure of the active molecules disclosed in the present application, for example SEQ ID NO. 1, that are capable of ameliorating an inflammatory skin condition. Particularly preferred for use in the method are the polypeptides of the present invention, including the polypeptide sequences depicted in SEQ ID NO. 3, SEQ ID NO. 9 SEQ ID NO. 10 and SEQ ID NO. 11.

It has been shown that the molecule(s) described can be used to treat inflammatory skin conditions at extremely low application rates. Accordingly, the present invention

further provides a pharmaceutical composition wherein the concentration of the active molecule within the pharmaceutical composition is preferably from 0.0001 to 0.5 w/v (1 μg/ml to 5 mg/ml) more preferably 0.0001 to 0.1% w/v, more preferably 0.0001% to 0.01% w/v, and still more preferably 0.0001% to 0.001% w/v. If the composition is a cream then it is particularly preferred that the active molecule is present at a concentration from 0.0001% to 0.02% w/v. Compositions comprising recombinant human thioredoxin in a substantially reduced, monomeric state are particularly preferred.

The application rate of the molecules described above to the skin surface is preferably 0.05 to 10 μg / cm^2, more preferably 0.05 to 5 μg / cm^2, and more preferably 0.1 to 1 μg / cm^2. It is preferred that human thioredoxin in a substantially reduced, monomeric state is applied to the skin surface.

The present invention further relates to a method of treating inflammatory skin conditions comprising applying to a skin surface an effective amount of a composition comprising a molecule described above and an additional active ingredient. By additional active it is meant an ingredient that also has a pharmaceutical effect – which could be either additive or synergistic to the said molecule. Examples of additional active ingredients include lactoferrin (e.g. as depicted in SEQ ID NO. 5) and/or corticosteroids. The present invention also relates to a pharmaceutical composition comprising a molecule described above and an additional active ingredient. Preferred additional active ingredients include lactoferrin (e.g. as depicted in SEQ ID NO. 5), and/or corticosteroids and/or other topical medicaments suitable for the treatment of inflammatory skin conditions. A preferred composition is wherein the molecule is human thioredoxin depicted in SEQ ID NO. 1 and/or the modified TRX depicted in SEQ ID NO.3 and wherein the additional active ingredient is lactoferrin, depicted in SEQ ID NO. 5. The compositions of the present invention may also comprise further ingredients, for example anti-oxidants such as glutathione, vitamin A, vitamin C, vitamin E, or indeed extracts from plants such as, for example, *Aloe vera*. The pharmaceutical compositions of the present invention can also be used in a combination therapy for the treatment of severe inflammatory skin conditions.

It is preferred that composition of the present invention is suitable for application to the skin. Accordingly the composition will typically be formulated as a solution, gel, lotion, ointment, cream, suspension, paste, liniment, powder, tincture, aerosol, transdermal drug delivery system, or similar in a pharmaceutically acceptable form by methods well known in the art. Substances that enhance the penetration of the active ingredients through the skin may also be added including, for example, dimethylsulfoxide, dimethylacetamide, dimethylformamide, surfactants, azone, alcohol, acetone, propylene glycol and polyethylene glycol. The compositions may be applied directly to the skin or via various transdermal drug delivery systems, such as patches.

The present invention further relates to the use of a polypeptide capable of ameliorating an inflammatory skin condition wherein said polypeptide is a modified thioredoxin, the modification comprising:-

a. substituting Cys_1 and Cys_2 in the motif Cys_1-Gly-Pro-Cys_2 present in the unmodified thioredoxin with an amino acid other than cysteine; or

b. substituting either of Cys_1 and Cys_2 in the motif Cys_1-Gly-Pro-Cys_2 present in the unmodified thioredoxin with an amino acid other than cysteine and deleting the non-substituted cysteine;

as a pharmaceutical.

The present invention further relates to the use of the polypeptide depicted in SEQ ID NO.s 11 and 17 as a pharmaceutical.

The present invention further relates to the use of a polypeptide capable of ameliorating an inflammatory skin condition wherein said polypeptide is a modified thioredoxin, the modification comprising:-

a. substituting Cys_1 and Cys_2 in the motif Cys_1-Gly-Pro-Cys_2 present in the unmodified thioredoxin with an amino acid other than cysteine; or

b. substituting either of Cys_1 and Cys_2 in the motif Cys_1-Gly-Pro-Cys_2 present in the unmodified thioredoxin with an amino acid other than cysteine and deleting the non-substituted cysteine.

in the manufacture of a medicament suitable for application to a skin surface for ameliorating an inflammatory skin condition; the use of thioredoxin in the manufacture of a medicament suitable for application to a skin surface for ameliorating an inflammatory skin condition; and the use of human thioredoxin depicted in SEQ ID NO. 1 in the manufacture suitable for application to a skin surface for ameliorating an inflammatory skin condition.

The present invention further relates to the use of the polypeptide depicted in SEQ ID NO.s 11 and 17 in the manufacture of a medicament suitable for application to a skin surface for ameliorating an inflammatory skin condition.

List of Sequences

All sequences are provided herewith with an N-terminal methionine. For the avoidance of doubt, it should be understood that the present invention also includes sequences wherein the N-terminal methionine is absent.

SEQ ID NO. 1 Human TRX (protein)

SEQ ID NO. 2 Human TRX (DNA)

SEQ ID NO. 3 Modified Human TRX (protein)

SEQ ID NO. 4 Modified Human TRX (DNA)

SEQ ID NO. 5 Human Lactoferrin (protein)

SEQ ID NO. 6 DNA sequence encoding human TRX optimised for expression in *E. coli*.

SEQ ID NO. 7 *E.coli* thioredoxin.

SEQ ID NO. 8 *Arabidopsis* thioredoxin

SEQ ID NO. 9 Triple modified human thioredoxin (C32A, C35A, C73A).

SEQ ID NO. 10 Cysteine free human thioredoxin (C32A, C35A, C62A, C69A, C73A).

SEQ ID NO. 11 Modified Human TRX (C73A).

SEQ ID NO. 12 Modified Human TRX (C32S).

SEQ ID NO. 13 Modified Human TRX (C35S).

SEQ ID NO. 14 Modified Human TRX (C32S C35S).

SEQ ID NO. 15 Modified Human TRX (C32S C69S).

SEQ ID NO. 16 Modified Human TRX (C35S C69S).

SEQ ID NO. 17 Modified Human TRX (C73S)

List of Figures

The terms "TRX" and "Thio" are both used interchangeably as abbreviations for thioredoxin.

Figure 1. Groups of mice (n=3) received 30 µl of aqueous cream (cr) or 30 µl of native human TRX (0.5 µg; TRX – SEQ ID NO. 1) on the dorsum of both ears. Two hours later, mice were exposed topically on the dorsum of both ears to 0.5% oxazolone (Ox) or to vehicle alone (acetone:olive oil; AOO). Control mice were untreated (naïve; -). Epidermal sheets were prepared for analysis of major histocompatibility complex (MHC) class II (Ia)$^+$ LC frequencies 4 h later. LC numbers (mean ± SE) are derived from analysis of n=6 epidermal sheets/treatment group.

Figure 2. Groups of mice (n=3) received 30 µl of aqueous cream (cr) or 30 µl of native human TRX (0.5 µg; Thio) on the dorsum of both ears. Two hours later, mice received 50ng of murine TNFα or IL-1β by intradermal injection into ear pinnae. Control mice were untreated (naïve; -). Epidermal sheets were prepared for analysis of MHC class II (Ia)$^+$ LC frequencies 4 h (IL-1b) or 30 min (TNFα) later. LC numbers (mean ± SE) are derived from analysis of n=6 epidermal sheets/treatment group.

Figure 3. Groups of mice (n=10) received 30 µl of aqueous cream (cr) or 30 µl of native human TRX (0.5 µg; Thio) on the dorsum of both ears. Two hours later, mice received 50ng of murine TNFα or IL-1β by intradermal injection into ear pinnae. Control mice were untreated (naïve). Draining auricular lymph nodes were excised 17h (IL-1β) or 4 h (TNFα) later, pooled for each experimental group and a single cell suspension of LNC prepared. DC were enriched by density gradient centrifugation. DC numbers were assessed following direct morphological examination of DC-enriched fractions and are expressed as number of DC per node.

Figure 4. Groups of mice (n=3) received 30 µl of aqueous cream (cr), 30 µl of native human TRX (0.5 µg; hTRX) or 30 µl of modified human TRX (0.5 µg; C32AC35A – SEQ ID NO.3) on the dorsum of both ears. Two hours later, mice were exposed topically on the dorsum of both ears to 0.5% oxazolone (Ox). Control mice were untreated (naïve). Epidermal sheets were prepared for analysis of MHC class II $(Ia)^+$ LC frequencies 4 h later. LC numbers (mean ± SE) are derived from analysis of n=6 epidermal sheets/treatment group.

Figure 5. Groups of mice (n=3) received 30 µl of aqueous cream (cr), 30 µl of native human TRX (0.5 µg; hTRX) or 30 ml of various amounts of modified human TRX (0.5, 0.1 or 0.05 µg; C32AC35A – SEQ ID NO. 3) on the dorsum of both ears. Two hours later, mice were exposed topically on the dorsum of both ears to 0.5% oxazolone (Ox). Control mice were untreated (naïve). Epidermal sheets were prepared for analysis of MHC class II $(Ia)^+$ LC frequencies 4 h later. LC numbers (mean ± SE) are derived from analysis of n=6 epidermal sheets/treatment group.

Figure 6. Groups of mice (n=3) received 30 µl of aqueous cream (cr), 30 µl of modified human TRX (0.5 µg; C32AC35A – SEQ ID NO. 3) or 30 µl of various amounts native human TRX (0.5, 0.1 or 0.05 µg; hTRX) on the dorsum of both ears. Two hours later, mice were exposed topically on the dorsum of both ears to 0.5% oxazolone (Ox). Control mice were untreated (naïve). Epidermal sheets were prepared for analysis of MHC class II $(Ia)^+$ LC frequencies 4 h later. LC numbers (mean ± SE) are derived from analysis of n=6 epidermal sheets/treatment group.

Figure 7. Healthy volunteers (a and b) were exposed topically at two sites to native human TRX (Trx; 0.5µg in 50µl) and at a further two sites to an equivalent volume of aqueous cream alone. Two hours later, human recombinant TNF-α (500U) or an equal volume of saline was injected intradermally into paired sites (one pre-treated with Trx and one with cream) and biopsies taken 2 h later. $CD1a^+$ LC densities were assessed following

indirect immunofluorescence staining of epidermal sheets. Results are expressed as the mean ± SD number of cells/mm2 derived from examination of 50 fields/sample.

Figure 8. Graph indicating that the modified human TRX (C32A/C35A – SEQ ID NO.3) is redox-inactive.

Figure 9. Groups of mice (n=5) received 30 µl of aqueous cream (cr), 30 µl of native human thioredoxin (0.5 µg; hTRX) of on the dorsum of both ears. Two hours later, mice were exposed topically on the dorsum of both ears to 0.5% oxazolone (Ox). Control mice received an equal volume of vehicle (AOO) alone. Two hours later, ears were excised and explants prepared and cultured for 16 h at 37°C. IL-10 content was analyzed by Bioplex cytokine array and results are expressed as pg/ml IL-10 produced per mouse.

Figure 10. Inhibition of oxazolone-induced LC migration by the monomeric hTRX mutant C73A in mice. Groups of mice (n=3) were exposed topically on the dorsum of both ears to 30µl aqueous cream BP containing 0.5µg oligomeric hTRX, 0.5µg C73A or cream alone, 2h prior to application at the same site of 0.5% oxazolone (Ox) suspended in vehicle (4:1 acetone:olive oil). Control mice were untreated (naïve). After 4h, ears were removed and epidermal sheets were prepared from dorsal ear halves for indirect immunofluorescence staining for MHC class II (Ia) expression. Results are displayed as the mean number (± SE) of Ia^+ LC/mm^2 of epidermis following examination of 10 fields/ear for each of 6 ears.

Figure 11. Inhibition of oxazolone-induced LC migration by the cysteine-free hTRX mutant (C32AC35AC62AC69AC73A) in mice. Groups of mice (n=3) were exposed topically on the dorsum of both ears to 30µl aqueous cream BP containing 0.5µg oligomeric hTRX, 0.5µg C32AC35AC62AC69AC73A (Cys-free) or cream alone, 2h prior to application at the same site of 0.5% oxazolone (Ox) suspended in vehicle (4:1 acetone:olive oil). Control mice were untreated (naïve). After 4h, ears were removed and epidermal sheets were prepared from dorsal ear halves for indirect immunofluorescence staining for MHC class II (Ia) expression. Results are displayed

as the mean number (± SE) of Ia^+ LC/mm^2 of epidermis following examination of 10 fields/ear for each of 6 ears.

Figure 12. Inhibition of oxazolone-induced LC migration by the triple mutant C32AC35AC73A in mice. Groups of mice (n=3) were exposed topically on the dorsum of both ears to 30µl aqueous cream BP containing 0.5µg oligomeric hTRX, 0.5µg C32AC35AC73A or cream alone, 2h prior to application at the same site of 0.5% oxazolone (Ox) suspended in vehicle (4:1 acetone:olive oil). Control mice were untreated (naïve). After 4h, ears were removed and epidermal sheets were prepared from dorsal ear halves for indirect immunofluorescence staining for MHC class II (Ia) expression. Results are displayed as the mean number (± SE) of Ia^+ LC/mm^2 of epidermis following examination of 10 fields/ear for each of 6 ears.

Figure 13. Influence of heat treatment (95°C for 30min) on inhibition of oxazolone-induced LC migration by oligomeric hTRX in mice. Groups of mice (n=3) were exposed topically on the dorsum of both ears to 30µl aqueous cream BP containing 0.5µg oligomeric hTRX, 0.5µg heat treated (95°C for 30min) oligomeric hTRX (hTRX-HT) or cream alone, 2h prior to application at the same site of 0.5% oxazolone (Ox) suspended in vehicle (4:1 acetone:olive oil). Control mice were untreated (naïve). After 4h, ears were removed and epidermal sheets were prepared from dorsal ear halves for indirect immunofluorescence staining for MHC class II (Ia) expression. Results are displayed as the mean number (± SE) of Ia^+ LC/mm^2 of epidermis following examination of 10 fields/ear for each of 6 ears.

Figure 14. Influence of heat treatment (56°C for 30min) on inhibition of oxazolone-induced LC migration by oligomeric hTRX in mice. Groups of mice (n=3) were exposed topically on the dorsum of both ears to 30µl aqueous cream BP containing 0.5µg oligomeric hTRX, 0.5µg heat treated (56°C for 30min) oligomeric hTRX (hTRX-HT) or cream alone, 2h prior to application at the same site of 0.5% oxazolone (Ox) suspended in vehicle (4:1 acetone:olive oil). Control mice were untreated (naïve). After 4h, ears were removed and epidermal sheets were prepared from dorsal ear halves for indirect immunofluorescence staining for MHC class II

(Ia) expression. Results are displayed as the mean number (± SE) of Ia^+ LC/mm^2 of epidermis following examination of 10 fields/ear for each of 6 ears.

Figure 15. Inhibition of oxazolone-induced LC migration in mice by reduced monomeric hTRX. Groups of mice (n=3) were exposed topically on the dorsum of both ears to 30µl aqueous cream BP containing various concentrations of hTRXrm (0.5µg, 4µg, 20µg), or cream alone, 2h prior to application at the same site of 0.5% oxazolone (Ox) suspended in vehicle (4:1 acetone:olive oil). Control mice were untreated (naïve). After 4h, ears were removed and epidermal sheets were prepared from dorsal ear halves for indirect immunofluorescence staining for MHC class II (Ia) expression. Results are displayed as the mean number (± SE) of Ia^+ LC/mm^2 of epidermis following examination of 10 fields/ear for each of 6 ears.

EXPERIMENTS

Mouse studies

Mice

Young adult (6- to 8-week old) male BALB/c strain mice obtained from the Specific Pathogen Free Breeding Unit (Alderley Park, Cheshire, UK) were used throughout these investigations.

Thioredoxin

Recombinant native human TRX (hTRX – SEQ ID NO.1) or modified human TRX (SEQ ID NO. 3) were diluted to 16.7µg/ml in aqueous cream BP and 30µl (0.5µg TRX) applied topically to the dorsum of both ears 2 hours prior to exposure at the same site to chemical or cytokine. Control mice received an equivalent volume of cream alone. In some experiments, animals received 0.5, 0.1 and 0.05 µg of TRX.

Chemicals and exposure

The skin sensitising chemical 4-ethoxy-2-phenyloxazol-5-one (oxazolone; Sigma Chemical Co., St Louis, MO) was dissolved in 4:1 acetone:olive oil (AOO). Groups of mice received 25µl of 0.5% oxazolone, or vehicle (AOO) alone, on the dorsum of both ears. Other control animals were untreated (naïve).

Cytokines

Recombinant murine TNF-α (specific activity $2x10^8$U/mg by L929 cytotoxicity assay; endotoxin level: 0.009ng/μg) was obtained from Genzyme (West Malling, Kent, UK). Recombinant murine IL-1β (specific activity $1\text{-}2x10^8$U/mg; endotoxin level: <0.1ng/μg) was purchased from R&D Systems (Oxon, UK). Cytokines were either supplied as, or reconstituted in, sterile solutions of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) as carrier protein. Cytokines were diluted with sterile PBS containing 0.1% BSA and were administered using 1ml syringes with 30-gauge stainless steel needles. Mice received 30μl intradermal injections into both ear pinnae.

Preparation and analysis of epidermal sheets

Ears were removed either 4 h following exposure to chemical or IL-1β, or 30 min after treatment with TNF-α. Samples were split with the aid of forceps into dorsal and ventral ear halves. The dorsal halves were incubated for 90 min at 37°C with 0.02M ethylenediamine tetra-acetic acid (EDTA; Sigma) dissolved in PBS. The epidermis was separated from the dermis using forceps and washed in PBS. Epidermal sheets were fixed in acetone for 20 min at -20°C. Following fixation, sheets were washed in PBS and then incubated at room temperature for 30 min with anti-mouse MHC ($I\text{-}A^d/I\text{-}E^d$) monoclonal antibody diluted to 5μg/ml in 0.1%BSA/PBS. Sheets were then washed prior to incubation for a further 30 min with FITC-conjugated $F(ab)_2$ goat anti-rat IgG, diluted 1:100 in 0.1%BSA/PBS. Finally, sheets were washed in PBS and mounted on microscope slides in Citifluor (Citifluor Ltd., London, UK) and sealed with nail varnish. Samples were examined in a blinded fashion by fluorescence microscopy and the frequency of stained cells assessed using an eyepiece with a calibrated grid (0.32 x 0.213 at x40 magnification). For each sample 10 consecutive fields in the central portion of the ear were examined.

Measurement of epidermal cytokine production

Ears were removed 2 h following exposure to 0.5% oxazolone and prepared for explant culture under aseptic conditions. Ears were washed immediately in 70%

ethanol, rinsed in PBS and were split with the aid of forceps into dorsal and ventral halves. Dorsal halves were floated on 250 µl RPMI-1640 medium in 24-well tissue culture plates (1 dorsal ear half/well). Supernatants were collected after 16 h of culture, pooled for each mouse and centrifuged at 150 g for 5 min prior to storage at -70°C. The IL-10 content was measured in supernatants using the Bio-Plex™ cytokine array system according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Preparation and analysis of Dendritic Cells (DC)

Draining auricular lymph nodes were excised 18 h following treatment with chemical, or 4 h and 17 h following administration of the cytokines TNF-α and IL-1β, respectively. Nodes were pooled for each experimental group. A single cell suspension of lymph node cells (LNC) was prepared under aseptic conditions by mechanical disaggregation through sterile 200-mesh stainless steel gauze and resuspended in RPMI-1640 growth medium (Gibco, Renfrewshire, UK) supplemented with 25 mM HEPES, 400 µg/ml streptomycin, 400 µg/ml ampicillin and 10% heat-inactivated fetal calf serum (RPMI-FCS). Viable cell counts were performed by exclusion of trypan blue dye and the total cellularity per lymph node recorded. The cell concentration was adjusted to $5x10^6$ cells/ml in RPMI-FCS and DC-enriched populations were prepared by discontinuous gradient centrifugation on Metrizamide (Sigma Chemical Co.; 14.5% in RPMI-FCS). The frequency of DC in such low buoyant density fractions was assessed routinely by direct morphological examination using phase contrast microscopy.

Human Studies

TRX and exposure

TRX in aqueous cream (0.5µg in 50µl) was applied topically to two skin sites (each $2cm^2$ area) identified on non-sun-exposed buttock or hip. A further two sites on the contralateral buttock or hip received 50µl of aqueous cream alone. Two hours later, volunteers received two 50µl intradermal injections of 500U of homologous recombinant TNF-α diluted in sterile normal saline and two control injections of 50µl of sterile saline alone to paired sites (one exposed previously to TRX and one

exposed to cream alone). Punch biopsies (6mm) were taken under local anaesthesia (1% lignocaine) from each of the treated sites 2 h later.

Preparation and analysis of epidermal sheets

Epidermal Langerhans cells (LC) were identified on the basis of their expression of CD1a; a membrane determinant that characterises LC in human epidermis. To stain for LC, biopsies were placed immediately in 0.02M ethylenediamine tetraacetic acid (Sigma, St Louis, MO, USA) dissolved in phosphate buffered saline (PBS) and incubated for 2 h at 37°C. The epidermis was separated from the dermis using forceps, washed in PBS and fixed in acetone at –20°C. After washing in PBS, epidermal sheets were incubated at room temperature for 30 min with monoclonal antibodies specific for CD1a [clone NA1/34 (mouse IgG2a); DAKO Ltd, Cambridge, UK] diluted to 10μg/ml in PBS containing 0.1% bovine serum albumin (BSA). Sheets were washed prior to incubation for a further 30 min with fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse immunoglobulins (DAKO) diluted 1:100 in 0.1% BSA/PBS. Finally, sheets were washed in PBS and mounted on microscope slides in Citifluor (Citifluor Ltd., London, UK) and sealed with nail varnish. The identity of each slide was then masked using tape.

Samples were examined by fluorescence microscopy and the frequency of stained cells assessed in a blinded fashion using an eyepiece with a calibrated grid (0.32 x 0.213mm at x40 magnification). For each sample, 50 consecutive fields were examined. The identity of each slide was revealed after all samples have been counted. Results are expressed as the mean ± SD number of cells/mm^2.

Experiment 1

The purpose this experiment was to determine whether topical application of native hTRX to mouse skin was able to influence the integrity of LC migration induced by subsequent exposure at the same site to oxazolone, a potent contact allergen. The results of a representative experiment are illustrated in Figure 1. The results reveal that prior exposure to hTRX causes a complete inhibition of allergen-induced LC migration. The conclusion drawn is that topically applied hTRX is able to reach the

viable epidermis of mouse skin at concentrations sufficient to inhibit one or more biological processes required for the effective mobilisation and migration of LC in response to a stimulus, in this instance a contact allergen.

Experiment 2

Previous studies have provided clear evidence that the migration of epidermal LC, in both mouse and man, is dependent upon the availability of certain cytokines and chemokines, two of those known to be of particular importance being interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α). There is a precedent for perturbation of cytokine function resulting in compromised LC migration. In the next experiments we therefore investigated whether hTRX could affect LC migration induced by either IL-1β or TNF-α. The results of a representative experiment are displayed in Figure 2. These data reveal that prior topical exposure of mice to hTRX was able to cause an almost complete inhibition of LC migration induced by the intradermal (id) injection of homologous TNF-α. In contrast, hTRX applied in the same way was without influence on the integrity of LC migration provoked by id administration of homologous IL-1β. The interpretation is that topical administration of hTRX is associated with a perturbation of IL-1β function. Thus, hTRX was able to inhibit very effectively LC mobilisation in response to either allergen (oxazolone) (Figure 1), or TNF-α (Figure 2) in both of which circumstances there is a requirement for the availability of bioactive IL-1β. However, the inhibitory effects of hTRX can be overcome by the addition of an exogenous source of IL-1β in which case the effectiveness of migration is unimpaired.

Experiment 3

In a parallel series of experiments the same question as addressed in Experiment 2 was explored, but using a supplementary endpoint. In this case the endpoint used was the accumulation of dendritic cells (DC) in skin-draining regional lymph nodes. The relevance of this measurement is that the epidermal LC that are provoked to migrate from the skin traffic via afferent lymphatics to draining lymph nodes (in order to interact with the adaptive immune system). The effectiveness of LC mobilisation can

therefore be measured either as a function of the loss of LC from the epidermis, or as a function of their subsequent accumulation in skin-draining lymph nodes. A representative experiment is illustrated in Figure 3 where the impact of hTRX on DC accumulation in lymph nodes following id administration of either IL-1β or TNF-α has been examined. The results are consistent with those shown in Figure 2. That is, hTRX was found to inhibit DC accumulation in response to TNF-α, but not in response to IL-1β

Experiment 4

Most biological properties of TRX are considered to be a function of the redox activity of this protein. There are available redox-inactive mutant variants of the protein that have discrete amino substitutions that render the protein redox-inactive. One such mutant is C32A/C35A, as depicted in SEQ ID NO. 3. In another series of experiments the ability of C32A/C35A to inhibit LC migration was investigated and compared with the activity of native hTRX (SEQ ID NO. 1). A representative experiment is shown in Figure 4. In these experiments LC mobilisation was stimulated with the chemical allergen oxazolone and the ability of either hTRX or C32A/C35A to inhibit this response was measured. The results summarised in Figure 4 demonstrate clearly that both native hTRX and the redox-inactive mutant C32A/C35A are able to inhibit very substantially the integrity of LC migration. The conclusion drawn is that the effects of TRX on LC migration (and the integrity of IL-1β signalling) are independent of active redox function.

Experiment 5

In subsequent experiments the relative potency of native hTRX (SEQ ID NO. 1) and of C32A/C35A (SEQ ID NO. 3) were compared with respect to inhibition of LC migration. In one experimental design various concentrations of the redox-inactive mutant protein were compared with a single concentration of the native hTRX. The results of a representative experiment are summarised in Figure 5. The data available reveal a dose-dependent inhibition of LC migration. Exposure of mice to 0.5μg of C32A/C35A (or to 0.5μg of native hTRX) was characterised by a complete inhibition

of allergen-induced LC migration. Although lower concentrations of C32A/C35A (0.1μg or 0.05μg) were able to inhibit allergen-induced LC migration their effects were less complete than that seen with the higher dose of protein.

Experiment 6

In parallel investigations the same experimental design was employed with the reverse orientation. That is, a dose response was performed with the native hTRX and the results compared with the effects of a single dose of the redox-inactive mutant. A representative experiment is illustrated in Figure 6. Again, a clear dose response relationship was observed. Treatment of mice with 0.5μg of hTRX (or with 0.5μg of C32A/C35A) caused a complete inhibition of allergen-induced LC migration. Lower doses of hTRX (0.1μg or 0.05μg) although having some effect, caused a less complete inhibition of migration than did 0.5μg. Taken together these data confirm that hTRX and C32A/C35A both cause an inhibition of LC migration, and do so with comparable potency.

Experiment 7

In the next series of experiments the impact of hTRX on the integrity of LC migration in humans was investigated using healthy adult volunteers. The results obtained using two such volunteers are illustrated in Figure 7. In common with previous studies conducted in mice (see Figure 2 above), it was observed in each of the two volunteers that prior topical exposure to hTRX caused a significant inhibition of LC migration stimulated subsequently by the id administration of homologous recombinant TNF-α. These data confirm that hTRX effects changes in human skin comparable to those observed initially in mouse skin.

Experiment 8

This experiment was designed to show that the C32A/C35A modified human TRX was redox inactive. The assay was run at room temperature for 15 min and the reduction of dithionitrobenzoic acid (DTNB) followed at 412 nm overtime with a

spectrophotometer. The reaction mixture contains an excessive concentration of NADPH that is consumed by the TRX reductase to reduce TRX. After that, TRX reduces preferentially DTNB and TRX is recycled in its reduced form by the reductase and NADPH. Figure 8 indicates the results obtained in this experiment, which confirms that the C32A/C35A modified human TRX is redox-inactive.

Experiment 9

In a separate series of experiments the influence in mice of topical treatment withTRX on the elaboration by skin cells of IL-10 was measured. A representative experiment is shown in Figure 9. Skin tissue isolated from control animals, that were exposed to vehicle (AOO) alone, but were not sensitised with the contact allergen oxazolone, produced very low levels of IL-10 and hTRX was without impact on IL-10 production. In contrast, however, hTRX was able to enhance the production of IL-10 in response to sensitisation with oxazolone. The implication is that an additional property of TRX is to augment production by skin cells of IL-10; a cytokine that is known to have anti-inflammatory effects in the skin and other tissues.

SEQUENCE LISTING

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C

SEQ ID NO.3
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SEQ ID NO. 4 Modified Human Thioredoxin (DNA)
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SEQ ID NO. 5 Lactoferrin (protein)
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SEQ ID NO. 9. (Triple mutant)
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SEQ ID NO. 11. (C73A)
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What is claimed is:

1. Use of thioredoxin in the manufacture of a medicament suitable for application to a skin surface for ameliorating an inflammatory skin condition.

2. Use according to claim 1, wherein the thioredoxin is human thioredoxin.

3. Use according to either claim 1 or claim 2, wherein the thioredoxin has the sequence depicted in SEQ ID NO. 1.

4. Use according to any one of the previous claims, wherein the thioredoxin is in a substantially reduced state.

5. Use according to any one of the previous claims, wherein the thioredoxin is in a multimeric form.

6. Use according to claim 1, wherein the thioredoxin is capable of inhibiting the production and/or activity of interleukin 1α and /or interleukin 1β.

7. Use according to claim 1, wherein the thioredoxin is capable of stimulating and/or enhancing the production and/or activity of interleukin 10.

8. Use according to claim 1, wherein the medicament is selected from the group consisting of a solution, a gel, a lotion, an ointment, a cream and a paste.

9. Use according to claim any one of the previous claims, wherein the inflammatory skin condition is selected from the group consisting of psoriasis, lichen planus, atopic eczema, irritant or allergic contact dermatitis, contact urticaria, infantile eczema and acne vulgaris.

10. Use according to claim 9, wherein the skin condition is psoriasis.

11. Use according to any one of claims 1 to 10, wherein the medicament further comprises an additional active ingredient.

12. Use according to claim 11, wherein the additional active ingredient is a corticosteroid and/or lactoferrin and/or any other topical medicament effective in the treatment of cutaneous inflammatory diseases.

13. Use according to claim 12, wherein the additional active ingredient is lactoferrin.

14. A method of ameliorating an inflammatory skin condition comprising applying to a skin surface an effective amount of a composition comprising thioredoxin.

15. A method according to claim 14, wherein said thioredoxin is human thioredoxin depicted in SEQ ID NO.1 in a substantially reduced state.

16. A method according to claim 14 or claim 15, wherein the thioredoxin is applied to the skin surface at a concentration of 0.05 to 5 $\mu g / cm^2$.

17. A pharmaceutical composition suitable for ameliorating an inflammatory skin condition comprising from 0.0001 to 0.5% w/v thioredoxin.

18. A pharmaceutical composition according to claim 17, wherein the thioredoxin is human thioredoxin depicted in SEQ ID NO. 1 in a substantially reduced state.

Figure 1

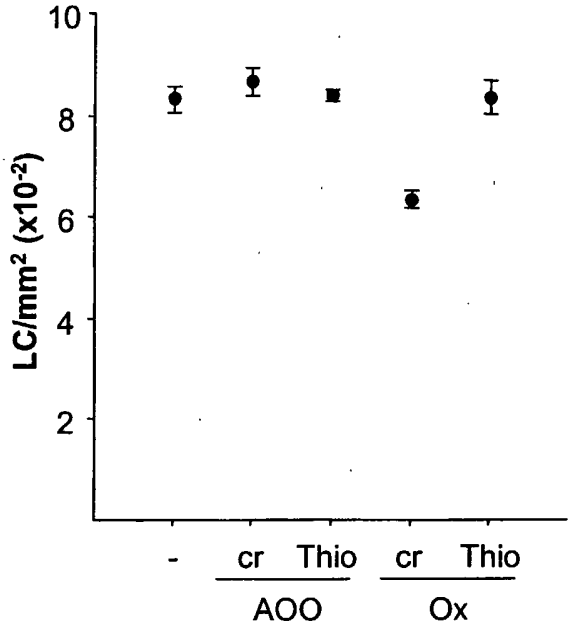


Figure 2

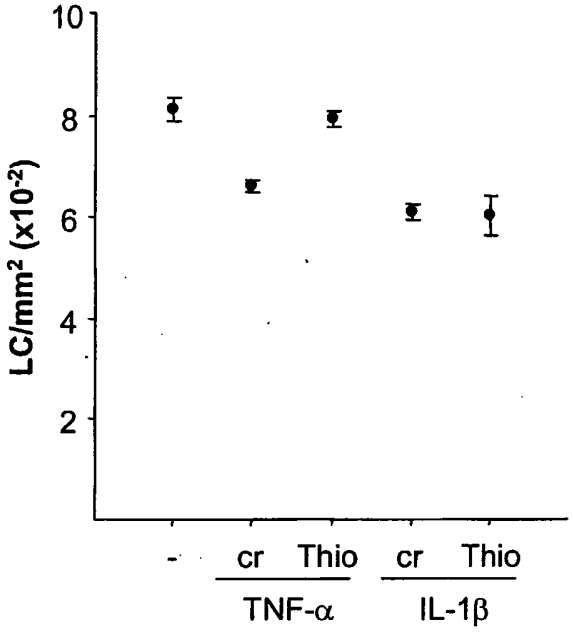


Figure 3

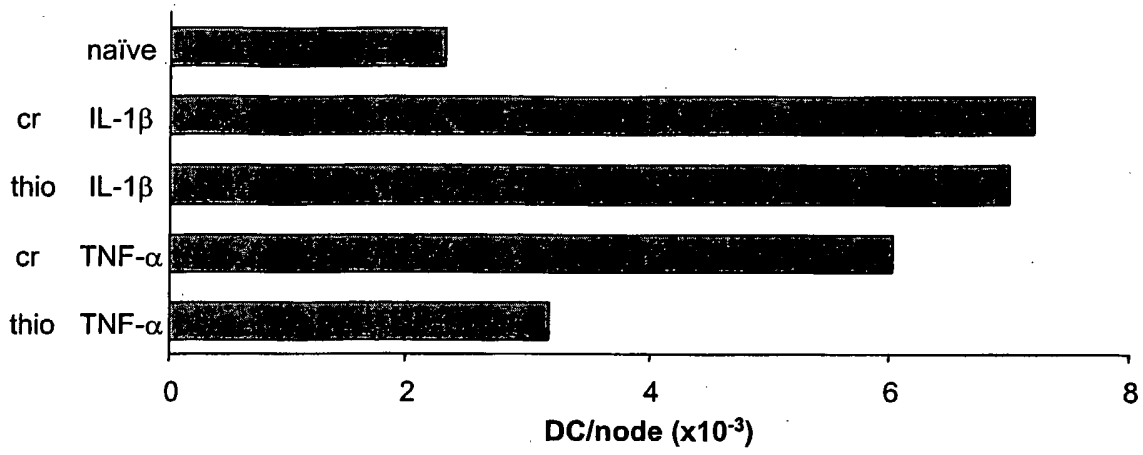


Figure 4

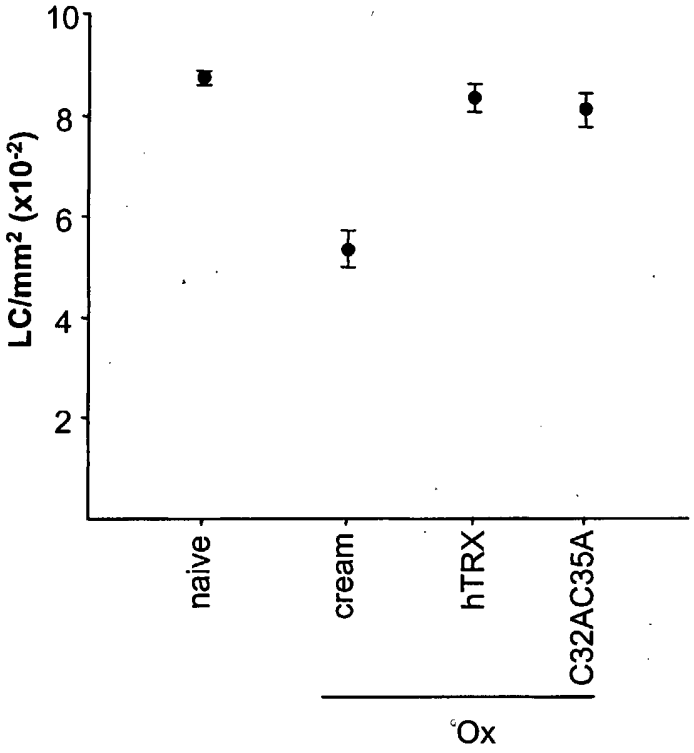


Figure 5

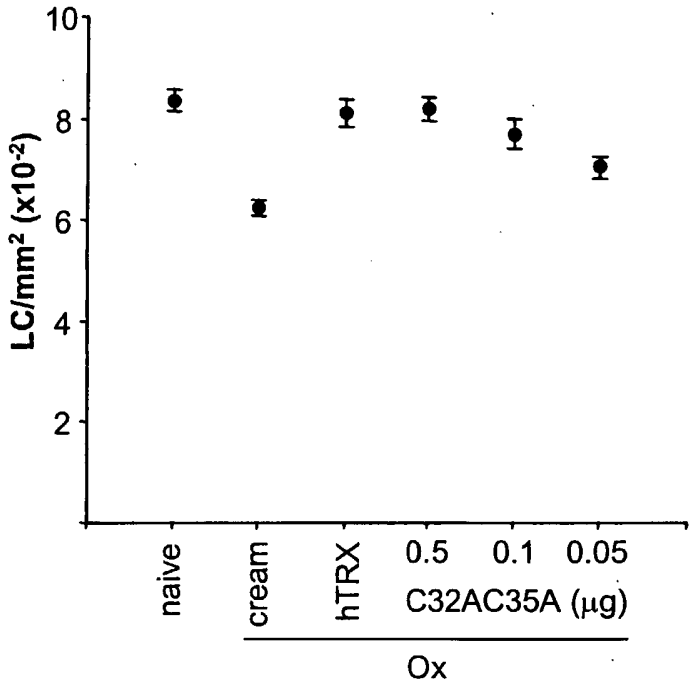


Figure 6

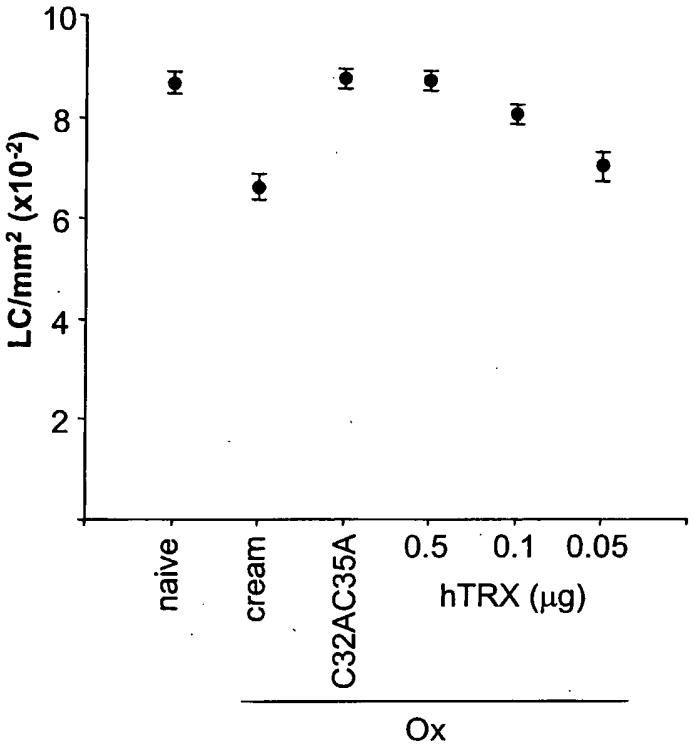
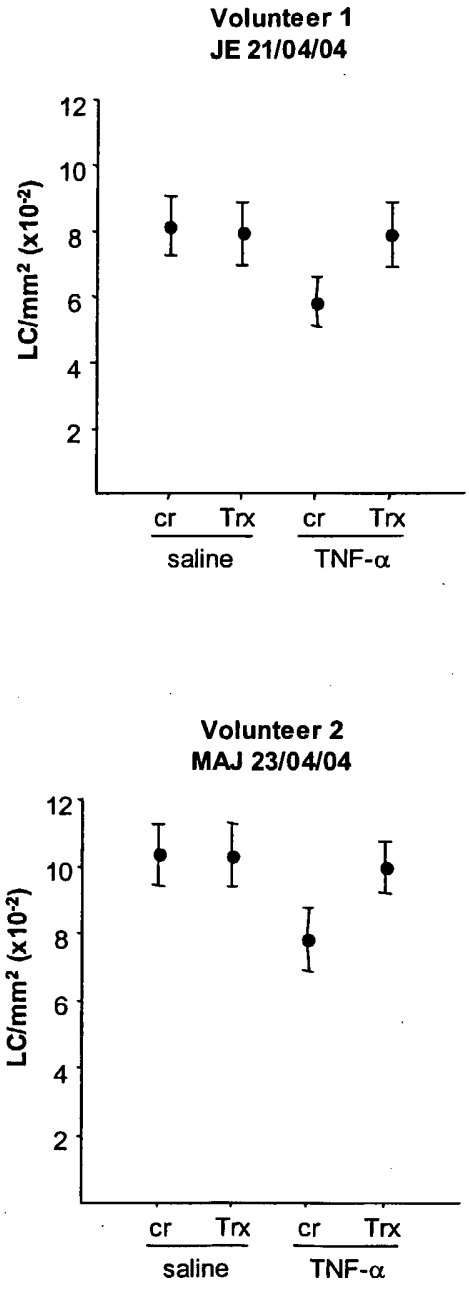


Figure 7


Volunteer 1
JE 21/04/04
LC/mm² (x10⁻²)
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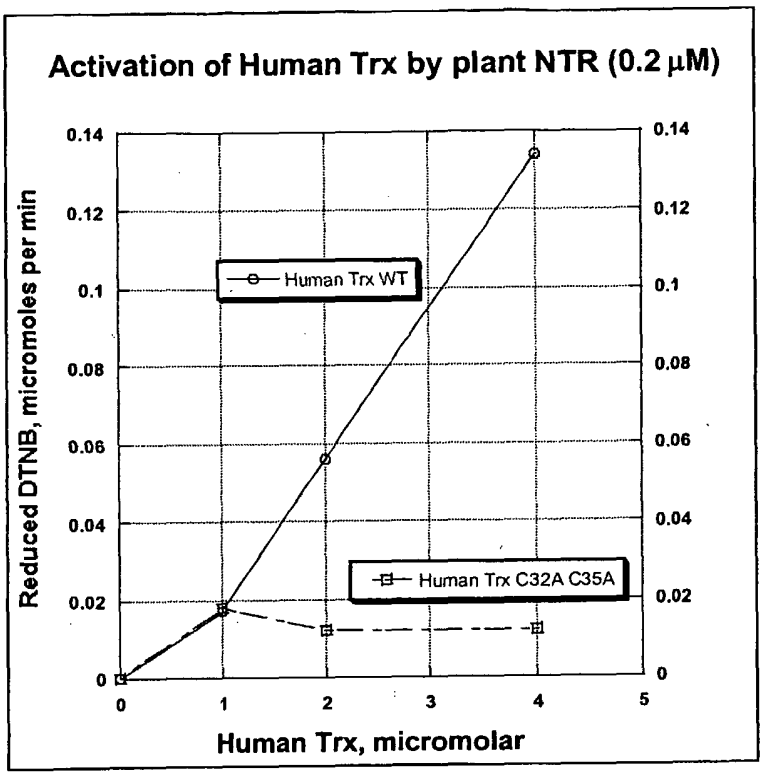
10
8
6
4
2
cr
Trx
cr
Trx
saline
TNF-α
Volunteer 2
MAJ 23/04/04
LC/mm² (x10⁻²)
12
10
8
6
4
2
cr
Trx
cr
Trx
saline
TNF-α

Figure 8


Activation of Human Trx by plant NTR (0.2 μM)
Reduced DTNB, micromoles per min
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0.12
0.1
0.08
0.06
0.04
0.02
0
Human Trx WT
Human Trx C32A C35A
0
1
2
3
4
5
Human Trx, micromolar

Figure 9

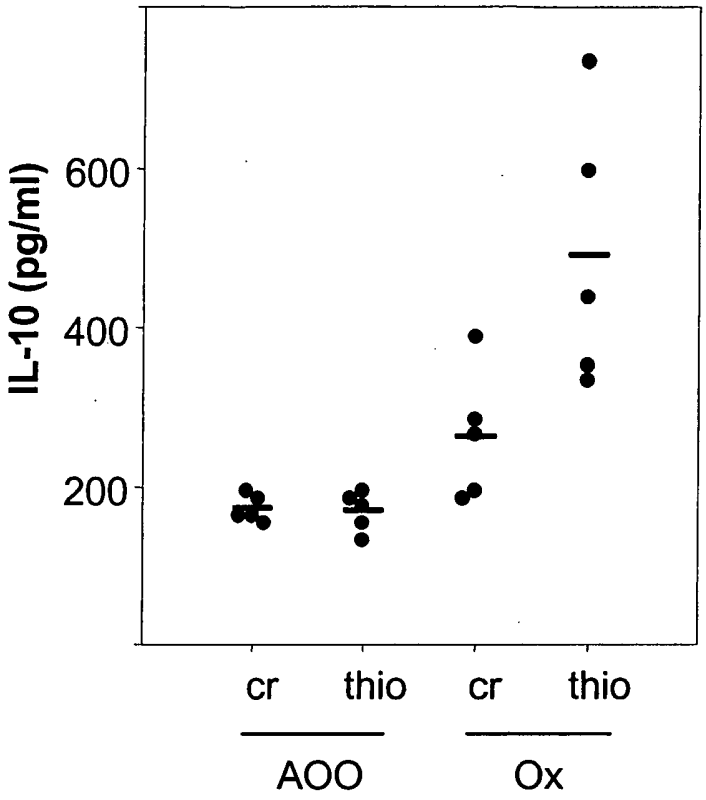


Figure 10

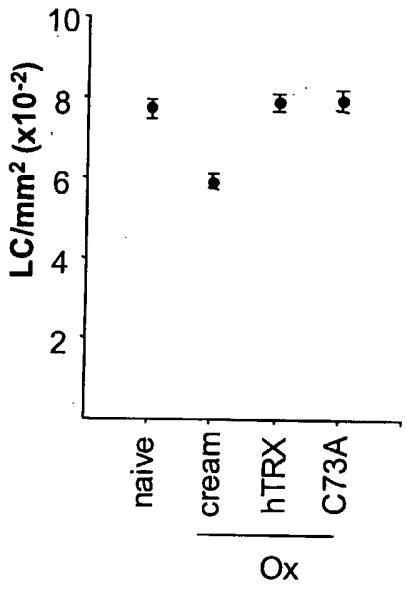


Figure 11

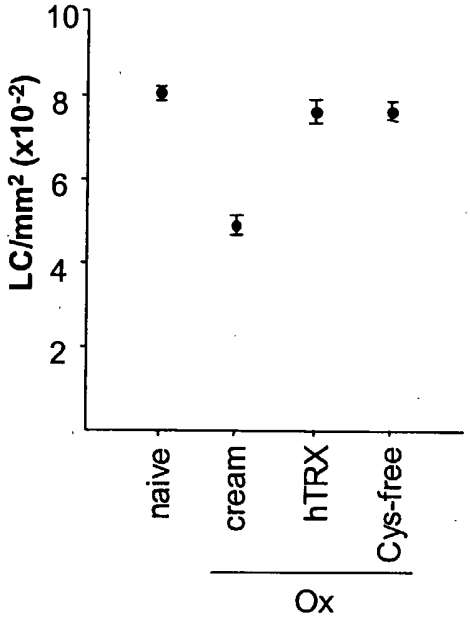


Figure 12

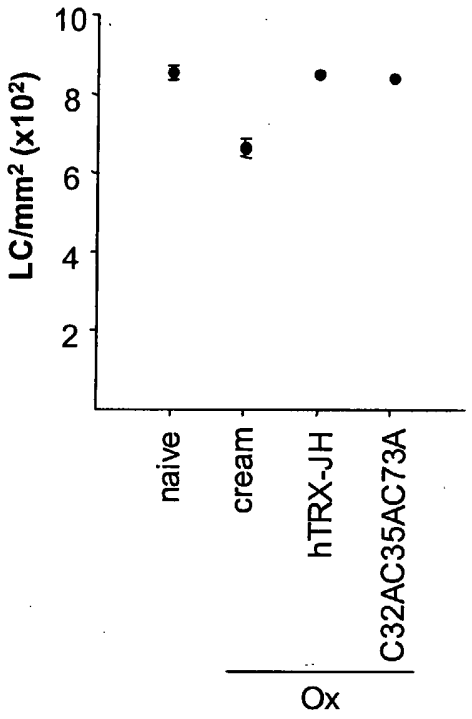


Figure 13

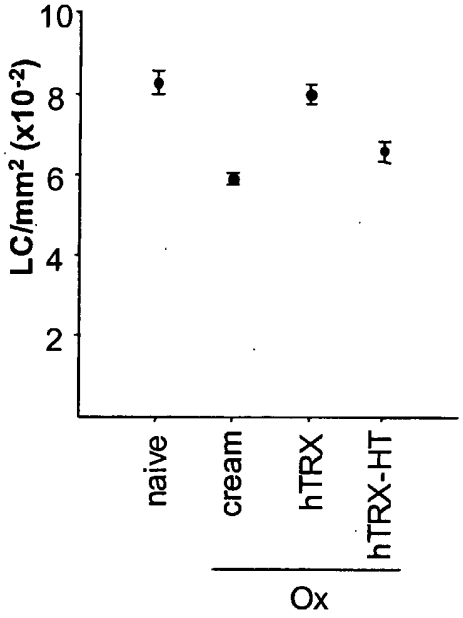


Figure 14

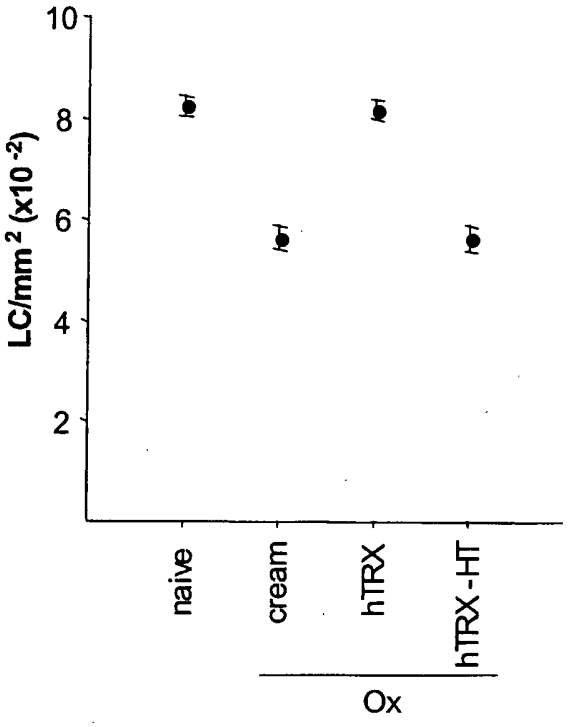
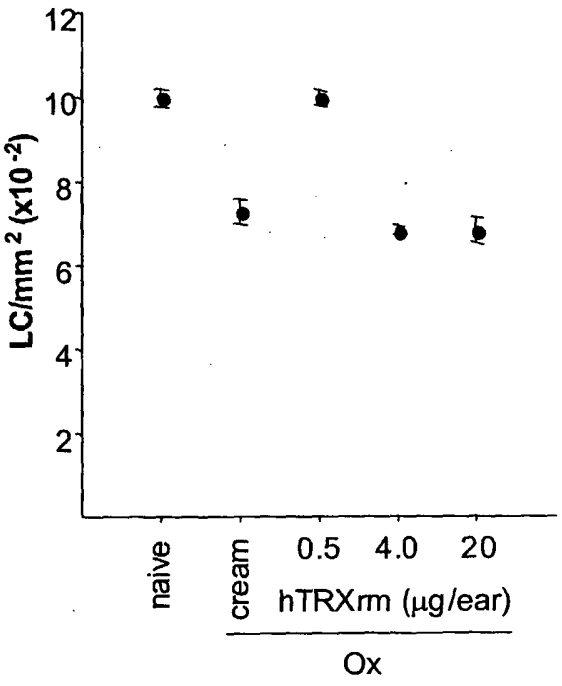


Figure 15



SEQUENCE LISTING

<110> Syngenta Limited

<120> Method for ameliorating an inflammatory skin condition

<130> 70479

<160> 17

<170> PatentIn version 3.1

<210> 1

<211> 105

<212> PRT

<213> Homo sapiens

<400> 1

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1               5                  10                  15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Cys
            20                  25                  30

Gly Pro Cys Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
        35                  40                  45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
    50                  55                  60

Val Ala Ser Glu Cys Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65                  70                  75                  80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
                85                  90                  95
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Leu Glu Ala Thr Ile Asn Glu Leu Val
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<211> 315

<212> DNA

<213> Homo sapiens

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aaacttgtag tagttgactt ctcagccacg tggtgtgggc cttgcaaaat gatcaagcct
120

ttctttcatt ccctctctga aaagtattcc aacgtgatat tccttgaagt agatgtggat
180

gactgtcagg atgttgcttc agagtgtgaa gtcaaatgca tgccaacatt ccagtttttt
240

aagaagggac aaaaggtggg tgaattttct ggagccaata aggaaaagct tgaagccacc
300

attaatgaat tagtc
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<210> 3

<211> 105

<212> PRT

<213> Artificial Sequence

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<223> Artificial

<400> 3

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Ala
20 25 30

Gly Pro Ala Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 4

<211> 315

<212> DNA

<213> Artificial Sequence

<220>

<223> Artificial

<400> 4
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ttctttcatt ccctctctga aaagtattcc aacgtgatat tccttgaagt agatgtggat 180

gactgtcagg atgttgcttc agagtgtgaa gtcaaatgca tgccaacatt ccagtttttt 240

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<210> 5

<211> 711

<212> PRT

<213> Homo sapiens

<400> 5

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Met Lys Leu Val Phe Leu Val Leu Leu Phe Leu Gly Ala Leu Gly Leu
1               5                  10                  15

Cys Leu Ala Gly Arg Arg Arg Arg Ser Val Gln Trp Cys Ala Val Ser
            20                  25                  30

Gln Pro Glu Ala Thr Lys Cys Phe Gln Trp Gln Arg Asn Met Arg Lys
        35                  40                  45

Val Arg Gly Pro Pro Val Ser Cys Ile Lys Arg Asp Ser Pro Ile Gln
    50                  55                  60

Cys Ile Gln Ala Ile Ala Glu Asn Arg Ala Asp Ala Val Thr Leu Asp
65                  70                  75                  80

Gly Gly Phe Ile Tyr Glu Ala Gly Leu Ala Pro Tyr Lys Leu Arg Pro
                85                  90                  95

Val Ala Ala Glu Val Tyr Gly Thr Glu Arg Gln Pro Arg Thr His Tyr
            100                 105                 110

Tyr Ala Val Ala Val Val Lys Lys Gly Gly Ser Phe Gln Leu Asn Glu
        115                 120                 125

Leu Gln Gly Leu Lys Ser Cys His Thr Gly Leu Arg Arg Thr Ala Gly
    130                 135                 140

Trp Asn Val Pro Ile Gly Thr Leu Arg Pro Phe Leu Asn Trp Thr Gly
145                 150                 155                 160

Pro Pro Glu Pro Ile Glu Ala Ala Val Ala Arg Phe Phe Ser Ala Ser
                165                 170                 175

Cys Val Pro Gly Ala Asp Lys Gly Gln Phe Pro Asn Leu Cys Arg Leu
            180                 185                 190

Cys Ala Gly Thr Gly Glu Asn Lys Cys Ala Phe Ser Ser Gln Glu Pro
        195                 200                 205

Tyr Phe Ser Tyr Ser Gly Ala Phe Lys Cys Leu Arg Asp Gly Ala Gly
    210                 215                 220
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Asp Val Ala Phe Ile Arg Glu Ser Thr Val Phe Glu Asp Leu Ser Asp
225                 230                 235                 240

Glu Ala Glu Arg Asp Glu Tyr Glu Leu Leu Cys Pro Asp Asn Thr Arg
                245                 250                 255

Lys Pro Val Asp Lys Phe Lys Asp Cys His Leu Ala Arg Val Pro Ser
            260                 265                 270

His Ala Val Val Ala Arg Ser Val Asn Gly Lys Glu Asp Ala Ile Trp
        275                 280                 285

Asn Leu Leu Arg Gln Ala Gln Glu Lys Phe Gly Lys Asp Lys Ser Pro
    290                 295                 300

Lys Phe Gln Leu Phe Gly Ser Pro Ser Gly Gln Lys Asp Leu Leu Phe
305                 310                 315                 320

Lys Asp Ser Ala Ile Gly Phe Ser Arg Val Pro Pro Arg Ile Asp Ser
                325                 330                 335

Gly Leu Tyr Leu Gly Ser Gly Tyr Phe Thr Ala Ile Gln Asn Leu Arg
            340                 345                 350

Lys Ser Glu Glu Glu Val Ala Ala Arg Arg Ala Arg Val Val Trp Cys
        355                 360                 365

Ala Val Gly Glu Gln Glu Leu Arg Lys Cys Asn Gln Trp Ser Gly Leu
    370                 375                 380

Ser Glu Gly Ser Val Thr Cys Ser Ser Ala Ser Thr Thr Glu Asp Cys
385                 390                 395                 400

Ile Ala Leu Val Leu Lys Gly Glu Ala Asp Ala Met Ser Leu Asp Gly
                405                 410                 415

Gly Tyr Val Tyr Thr Ala Gly Lys Cys Gly Leu Val Pro Val Leu Ala
            420                 425                 430

Glu Asn Tyr Lys Ser Gln Gln Ser Ser Asp Pro Asp Pro Asn Cys Val
        435                 440                 445

Asp Arg Pro Val Glu Gly Tyr Leu Ala Val Ala Val Val Arg Arg Ser
    450                 455                 460
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Asp Thr Ser Leu Thr Trp Asn Ser Val Lys Gly Lys Lys Ser Cys His
465                 470                 475                 480

Thr Ala Val Asp Arg Thr Ala Gly Trp Asn Ile Pro Met Gly Leu Leu
                485                 490                 495

Phe Asn Gln Thr Gly Ser Cys Lys Phe Asp Glu Tyr Phe Ser Gln Ser
            500                 505                 510

Cys Ala Pro Gly Ser Asp Pro Arg Ser Asn Leu Cys Ala Leu Cys Ile
        515                 520                 525

Gly Asp Glu Gln Gly Glu Asn Lys Cys Val Pro Asn Ser Asn Glu Arg
    530                 535                 540

Tyr Tyr Gly Tyr Thr Gly Ala Phe Arg Cys Leu Ala Glu Asn Ala Gly
545                 550                 555                 560

Asp Val Ala Phe Val Lys Asp Val Thr Val Leu Gln Asn Thr Asp Gly
                565                 570                 575

Asn Asn Asn Glu Ala Trp Ala Lys Asp Leu Lys Leu Ala Asp Phe Ala
            580                 585                 590

Leu Leu Cys Leu Asp Gly Lys Arg Lys Pro Val Thr Glu Ala Arg Ser
        595                 600                 605

Cys His Leu Ala Met Ala Pro Asn His Ala Val Val Ser Arg Met Asp
    610                 615                 620

Lys Val Glu Arg Leu Lys Gln Val Leu Leu His Gln Gln Ala Lys Phe
625                 630                 635                 640

Gly Arg Asn Gly Ser Asp Cys Pro Asp Lys Phe Cys Leu Phe Gln Ser
                645                 650                 655

Glu Thr Lys Asn Leu Leu Phe Asn Asp Asn Thr Glu Cys Leu Ala Arg
            660                 665                 670

Leu His Gly Lys Thr Thr Tyr Glu Lys Tyr Leu Gly Pro Gln Tyr Val
        675                 680                 685

Ala Gly Ile Thr Asn Leu Lys Lys Cys Ser Thr Ser Pro Leu Leu Glu
    690                 695                 700
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Ala Cys Glu Phe Leu Arg Lys
705 710

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<211> 315

<212> DNA

<213> Artificial Sequence

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120

tttttccata gtttgtccga aaaatattcg aacgtaatct tccttgaagt cgatgtggat
180

gactgtcagg atgtggcgag cgaatgtgag gtgaaatgca tgccaacttt tcaatttttt
240

aaaaaaggcc agaaagttgg tgaatttagc ggcgccaaca aagaaaaatt agaagcgacg
300

attaatgagc tggtt
315

<210> 7

<211> 109

<212> PRT

<213> Escherichia coli

<400> 7

Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr Asp
1 5 10 15

Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala Glu Trp
20 25 30

Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala Asp
35 40 45

Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp Gln Asn
50 55 60

Pro Gly Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu
65 70 75 80

Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu Ser
85 90 95

Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala
100 105

<210> 8

<211> 114

<212> PRT

<213> Arabidopsis thaliana

<400> 8

Met Ala Ser Glu Glu Gly Gln Val Ile Ala Cys His Thr Val Glu Thr
1 5 10 15

Trp Asn Glu Gln Leu Gln Lys Ala Asn Glu Ser Lys Thr Leu Val Val
20 25 30

Val Asp Phe Thr Ala Ser Trp Cys Gly Pro Cys Arg Phe Ile Ala Pro
35 40 45

Phe Phe Ala Asp Leu Ala Lys Lys Leu Pro Asn Val Leu Phe Leu Lys
50 55 60

Val Asp Thr Asp Glu Leu Lys Ser Val Ala Ser Asp Trp Ala Ile Gln
65 70 75 80

Ala Met Pro Thr Phe Met Phe Leu Lys Glu Gly Lys Ile Leu Asp Lys
85 90 95

Val Val Gly Ala Lys Lys Asp Glu Leu Gln Ser Thr Ile Ala Lys His
100 105 110

Leu Ala

<210> 9

<211> 105

<212> PRT

<213> Artificial Sequence

<220>

<223> Artificial

<400> 9

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Ala
20 25 30

Gly Pro Ala Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Ala Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 10

<211> 105

<212> PRT

<213> Artificial Sequence

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<223> Artificial

<400> 10

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Ala
20 25 30

Gly Pro Ala Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Ala Gln Asp
50 55 60

Val Ala Ser Glu Ala Glu Val Lys Ala Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 11

<211> 105

<212> PRT

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<400> 11

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Cys
20 25 30

Gly Pro Cys Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Ala Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 12

<211> 105

<212> PRT

<213> Artificial Sequence

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<400> 12

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Ser
20 25 30

Gly Pro Cys Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 13

<211> 105

<212> PRT

<213> Artificial Sequence

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<400> 13

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Cys
20 25 30

Gly Pro Ser Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 14

<211> 105

<212> PRT

<213> Artificial Sequence

<220>

<223> Artificial

<400> 14

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Ser
20 25 30

Gly Pro Ser Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 15

<211> 105

<212> PRT

<213> Artificial Sequence

<220>

<223> Artificial

<400> 15

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Ser
20 25 30

Gly Pro Cys Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Ser Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 16

<211> 105

<212> PRT

<213> Artificial Sequence

<220>

<223> Artificial

<400> 16

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Cys
20 25 30

Gly Pro Ser Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Ser Glu Val Lys Cys Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105

<210> 17

<211> 105

<212> PRT

<213> Artificial Sequence

<220>

<223> Artificial

<400> 17

Met Val Lys Gln Ile Glu Ser Lys Thr Ala Phe Gln Glu Ala Leu Asp
1 5 10 15

Ala Ala Gly Asp Lys Leu Val Val Val Asp Phe Ser Ala Thr Trp Cys
20 25 30

Gly Pro Cys Lys Met Ile Lys Pro Phe Phe His Ser Leu Ser Glu Lys
35 40 45

Tyr Ser Asn Val Ile Phe Leu Glu Val Asp Val Asp Asp Cys Gln Asp
50 55 60

Val Ala Ser Glu Cys Glu Val Lys Ser Met Pro Thr Phe Gln Phe Phe
65 70 75 80

Lys Lys Gly Gln Lys Val Gly Glu Phe Ser Gly Ala Asn Lys Glu Lys
85 90 95

Leu Glu Ala Thr Ile Asn Glu Leu Val
100 105