Title: MIA (MELANOMA INHIBITORY ACTIVITY) INHIBITORS FOR DETECTING, PREVENTING AND CURING VITILIGO

Abstract: Peptides, modified peptides and antibody or antibody fragment, which inhibit the activity of MIA(Melanoma Inhibitory Activity), for detecting, preventing and curing vitiligo are disclosed.
MIA (MELANOMA INHIBITORY ACTIVITY) INHIBITORS FOR DETECTING, PREVENTING AND CURING VITILIGO

DESCRIPTION

The present invention refers to the field of pharmaceuticals, in particular preparations for detecting, preventing and curing vitiligo.

Background of the invention

Vitiligo, also named as common generalized vitiligo, is an acquired pigmentary disorder of the skin and mucous membranes, and it is characterized by circumscribed depigmented macules and patches. Vitiligo is a progressive disorder in which some or all of the melanocytes in the affected skin seem to be selectively destroyed. Vitiligo affects 0.5-2% of the world population, and the average age of onset is 20 years.

Non-segmental vitiligo is the most common sub-type of vitiligo. Non-segmental vitiligo is an acquired chronic pigmentation disorder characterized by white patches, often symmetrical, which usually increase in size with time, corresponding to a substantial loss of functioning epidermal and sometimes hair follicle melanocytes (Taieb A, Picardo M; VETF Members. The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. Pigment Cell Res 2007; 20: 27-35).

Despite many studies performed on vitiligo skin, the exact pathogenesis of this dermatosis is still to be clarified and several pathogenetical mechanisms have been proposed during years, involving autoimmunity, cytotoxic metabolites, neural and genetic components; no convincing model describing the interplay of all these contributing factors has been formulated (Schallreuter KU, Bahadoran P, Picardo M, et al. Vitiligo pathogenesis: autoimmune disease, genetic defect, excessive reactive oxygen species, calcium imbalance, or what else? Exp Dermatol 2008; 17: 139-40). So far, two main features can be unequivocally ascribed to vitiligo: histologically there is lack of inflammation and clinically there is association with other autoimmune disorders. Recently, it has been suggested that the major and possible primary predisposing factor in vitiligo development could be a defective adhesion of melanocytes and that, based on in vivo and in vitro observations (Gauthier Y, Cario-Andre M, Lepreux S, Pain C, Taieb A. Melanocyte detachment after skin friction in non lesionai skin of patients with generalized vitiligo. Br J Dermatol 2003; 148: 95-101; Cario-Andre’ M, Pain C, Gauthier Y, Taieb A. The melanocytorrhagic hypothesis of vitiligo tested on pigmented, stressed, reconstructed epidermis. Pigment Cell Res 2007; 20: 385-393), mechanical trauma and various chemical stressors could represent the main precipitating events. This theory considers vitiligo as a disease caused by the chronic detachment and named melanocytorrhagy the trans-epidermal loss of melanocytes (Gauthier Y, Cario-Andre M, Lepreux S, Pain C,

Several therapies can be considered in treatment of vitiligo (Sehgal VN, Srivastava G. Vitiligo treatment options: an evolving scenario. J Dermatolog Treat 2006; 17: 262-75).

None of the single vitiligo therapies produces predictably good results in all patients; the response to single therapy is highly variable. Generally, the treatment must be individualized, and patients should be made aware of the risks associated with therapy. The most common treatments for non-segmental vitiligo are:

- Narrow-Band Ultra Violet B (UVB-NB) phototherapy: widely used with good clinical results, based on narrow-band fluorescent tubes with an emission spectrum of 310-315 nm and a maximum wavelength of 311 nm. Treatment frequency is 2-3 times weekly, but never on consecutive days. This treatment can be safely used in children, pregnant women, and lactating women. Short-term adverse effects include pruritus and xerosis. Long-term adverse effects are not well defined since the carcinogenic potential of UVB is still to be clarified. The therapy achieved the best results on vitiligoid patches of face and trunk; very poor results usually are achieved on vitiligo of hands and feet.

- Corticosteroid therapy: corticosteroids are used topically, systemically or intralesionally; topical steroids are often chosen first to treat localized vitiligo but the results of therapy have been reported as moderately successful, particularly in patients with localized vitiligo and/or an inflammatory component to their vitiligo, even if the inflammation is subclinical. The use of topic, systemic or intra-lesional steroids may have side effects like toxicity (systemic) or cutaneous atrophy (topical or intra-lesional); in addiction very poor results usually are achieved on vitiligo of hands and feet.

- Topical calcineurin inhibitors (tacrolimus and pimecrolimus): tacrolimus and pimecrolimus could be successful used to cure vitiligoid patches of face and neck; recently, Food and Drug Administration (FDA) imposed a "black box warning" on the topical calcineurin inhibitors, because of a theoretic risk of oncogenesis because of the trivial systemic absorption of these agents; in addiction very poor results usually are achieved on vitiligo of hands and feet.
Surgical alternatives exist for the treatment of vitiligo; however, because of the time-consuming nature of surgical therapies, these treatment regimens are limited to segmental or localized vitiligo.

So far, anyway, none of these therapies could reach a complete re-pigmentation of the vitiliginous patches in all the patients.

The precise mechanism of action of these therapies is still unknown and it is generically attributed to their immunosuppressive activity. It is remarkable, however, that UVB-NB radiation increase alpha5beta1 integrin expression on melanocytes after exposure (Neitmann M, Alexander M, Brinckmann J, Schlenke P, Tronnier M. Attachment and chemotaxis of melanocytes after ultraviolet irradiation in vitro. Br J Dermatol 1999; 141: 794-801).


Active detachment and alpha5beta1 integrins are very interesting features also in another and more severe melanocytic disorder such as malignant melanoma. Malignant melanoma is the most severe skin cancer and its metastatic form is associated with the poorest prognosis (Thompson JF, Scolyer RA, Kefford RF. Cutaneous melanoma. Lancet 2005; 365 :687-701).

Recently, it seems that the metastatic dissemination of malignant melanoma (a melanocyte-derived tumor) could be mediated by an active detachment of tumor cells and that this detachment can be caused by a small protein secreted from malignant melanoma cells called melanoma inhibitory activity - MIA (Bosserhoff AK, Stoll R, Sleeman JP, Bataille F, Buettner R, Holak TA. Active detachment involves inhibition of cell-matrix contacts of malignant melanoma cells by secretion of melanoma inhibitory activity. Lab Invest 2003; 83: 1583-94). It has been demonstrated that MIA interacts with integrin alpha5beta1 (Bauer R, Humphries M, Fassler R, Winklmeier A, Craig SE, Bosserhoff AK. Regulation of integrin activity by MIA. J Biol Chem 2006; 281: 11669-77). So it seems that
the interactions between a malignant melanocytes self-produced protein (MIA) and a self-
melanocyte adhesion molecule (alpha5beta1 integrin) would be responsible of an active
detachment of neoplastic melanocyte cells in malignant melanoma.

WO 03/64457 discloses peptides, modified peptides and antibody or antibody fragment
inhibiting the activity of MIA and their use for treating solid tumors, leukemia and
degenerative disorders.

The technical problem of the present invention is to provide compounds which avoid the
side effects of the known therapies, such as pruritus and xerosis or cutaneous atrophy,
toxicity and potential carcinogenesis or oncogenesis.

At the same time is highly desirable to provide compounds leading to complete re-
pigmentation irrespective of the area to be treated, thus providing good results even on
hands and feet, generally poorly recovered.

A further technical problem is to provide a marker usable for the early detecting and
further follow-up of the disease.

There is also a strong felt need for active agents capable to inhibiting the onset of the
disease in patients being prone to it.

The same inventor surprisingly and unexpectedly found that MIA protein is present in
patients affected by vitiligo and not in normal people and it is involved in the real
pathogenetic mechanism which lead to formation of vitiliginous patches.

The presence of MIA explains why a clear clinical inflammation is lacking in vitiligo
patches. The detachment of melanocyte does not activate the inflammation pathway, as
there is no cellular necrosis or apoptosis near the basal membrane.

Nowadays, targeted therapies are not available for vitiligo as a target molecule has never
been found.

MIA inhibitors represent a targeted therapy for non-segmental vitiligo.

Due to their specific binding to MIA, these molecules do not raise the side-effects
commonly reported for actual therapies as cutaneous atrophy, pruritus, toxicity or
oncogenetic potential.

Moreover, the inhibition of MIA, by avoiding damages towards melanocytes leads to re-
pigmentation defiant of treated the anatomic area, thus implementing the treatment of
difficult areas such as hands and feet.

The therapy-resistant site for vitiligo, such as hands and feet, are said to be due to the
lack of melanocytes reservoir in these sites; our data shows a high presence of MIA
against the melanocytes of these sites, probably due the continuous friction of these
zones. Inhibitors of MIA, by removing all molecules of MIA, successfully treat these areas.
Therefore peptides, modified peptides and antibody or antibody fragment inhibiting the activity of MIA can be used for treating vitiligo by inducing re-pigmentation. Said compounds can also be used useful as early markers of vitiligo development and as tools for follow-up of vitiligo patients and also for preventing the appearance of vitiliginous patches.

Said compounds can also be used for making the already known therapies more effective. Is therefore object of the present invention the use of at least one peptide with sequence ID No. 1 to 49 for detecting, preventing and curing vitiligo.

In a further object of the present invention, in said peptides one or more amino acid is substituted by a natural amino acid or a non-natural amino acid.

In a further object of the present invention, the non-natural amino acid is a modified natural amino acid wherein the modification is a substitution of one or more atoms with a functional group comprising 1 to 12 atoms selected from C, H, N, S, O, P, F, Cl, Br, I, Se.

In a further object of the present invention, the above peptides comprise an additional amino acid or one amino acid is deleted.

In a further object of the present invention, the modification of amino acid of the peptide with sequence ID No. 1 to 49 means glycosylation, acetylation, hydroxylation (hydroxyproline), carboxylation (gamma-carboxyglutamate), phosphorylation, alkylation, myristoylation (N-terminal), palmitoylation and prenylation) as well as non-naturally occurring amino acids including, trans-3- methylproline, 2, 4-methanoprolime, cis-4-hydroxyproline, trans-4- hydroxyproline, N-methylglycine, allo-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamin, pipercolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3- azaphenylalanine, 4 azaphenylalanine, and 4-fluorophenylalanine.

It is also object of the present invention the use of at least one anti-MIA antibody or fragment thereof for detecting, preventing and curing vitiligo.

Preferred anti-MIA antibody is selected from the group consisting of: anti-alpha 4-integrin (A4-PUJ1, UBI), anti-alpha 4-integrin (P1 H4, Chemicon), anti-alpha 5-integrin (A5-PUJ5, UBI), anti-alpha 5-integrin (P1 D6, Chemicon).

Further characteristics of the present invention would be clear from the following detailed description with reference to the experimental examples and the attached sheets of drawings.

**Brief Description of the figures**

Figure 1 shows the mechanism of action of alpha5beta1 integrin and MIA in melanocytes leading to vitiligo. In panel A the normal adhesion of melanocyte with the basal membrane, mediated by alpha5beta1 integrins is shown. In panel B a vitiligious
melanocyte attacked by MIA, which binds to alpha5beta1 integrins, is shown. In panel C: it is shown that, after the binding of MIA with alpha5beta1 integrins, the presence of other precipitating factors as oxidative stress, physical trauma or autoantibodies lead to a partial detachment of the melanocyte. In panel D the complete detachment of melanocyte and formation of vitiligo patches on the skin (melanocytorrhagy) is shown. In panel E an anti-MIA drug binding to MIA and inactivating it is shown. In panel F the vitiliginous melanocyte keeping the adhesion with the basal membrane even in the presence of precipitating factors is shown.

**Detailed description of the Invention**

**Definitions**

Within the meaning of the present invention, vitiligo means an acquired progressive pigmentary disorder of the skin and mucous membranes characterized by circumscribed depigmented macules and patches.

Within the meaning of the present invention, a peptide inhibits the activity of MIA by binding to MIA.

Within the meaning of the present invention, natural amino acids are the 20 amino acids occurring in natural proteins and peptides.

Within the meaning of the present invention, non-natural amino acids are non-genetically-coded amino acids that either occur naturally or are chemically synthesized.

Within the meaning of the present invention, modified amino acids means amino acids modified by glycosylation, acetylation, hydroxylation (hydroxyproline), carboxylation (gamma-carboxyglutamate), phosphorylation, alkylation, myristoylation (N-terminal), palmitoylation and prenylation.

Object of the present invention is the use of peptides, modified peptides and antibody or antibody fragment, which inhibit the activity of MIA, for detecting, preventing and curing vitiligo.

Said peptides inhibiting the activity of MIA are:

SEQ ID NO: 01 VPHIPPN
SEQ ID NO: 02 MPPTQVS
SEQ ID NO: 03 QMHPWPP
SEQ ID NO: 04 QPPFWQF
SEQ ID NO: 05 TPPQQLA
SEQ ID NO: 06 IPPYNTL
SEQ ID NO: 07 AVRPAAPL
SEQ ID NO: 08 GAKPHPQ
SEQ ID NO: 09 QQLSPLP
SEQ ID NO: 10 GPPPSPV
SEQ ID NO: 11 LPLTPLP
SEQ ID NO: 12 QLNVNHQARADQ
SEQ ID NO: 13 TSASTRPELHYP
SEQ ID NO: 14 TFLPHQMHPWPP
SEQ ID NO: 15 VPHIPPNSMALT
SEQ ID NO: 16 RLTLVLLIMPA

SEQ ID NO: 17 YNLPKVSSNLSP
SEQ ID NO: 18 MPPTQVSKFRLI
SEQ ID NO: 19 ANIDATPLFLRA
SEQ ID NO: 20 LLRTTETLPM FL
SEQ ID NO: 21 SALEPLV
SEQ ID NO: 22 GSPTPNA
SEQ ID NO: 23 APSHATH
SEQ ID NO: 24 TTVGHD
SEQ ID NO: 25 THFSTFT
SEQ ID NO: 26 SLLLDTS
SEQ ID NO: 27 SVAMKAHKPLL
SEQ ID NO: 28 NTIPGFASKSLD
SEQ ID NO: 29 VSNYKFYSTTSS
SEQ ID NO: 30 VSRHQSWHPHDQ
SEQ ID NO: 31 HLNILSTLWKYR
SEQ ID NO: 32 HNASPSWGSPVM
SEQ ID NO: 33 SHPWNAQRELSD
SEQ ID NO: 34 HHWPFWRTLPLS
SEQ ID NO: 35 WHTKFLPRLPS
SEQ ID NO: 36 NNTSFTWPSVP
SEQ ID NO: 37 SHLSTWKWWO
SEQ ID NO: 38 FHWHPRLWPLPS
SEQ ID NO: 39 WHWYGWRPPAM
SEQ ID NO: 40 FHRYLPLQPPG
SEQ ID NO: 41 WHWPLFIIPNTA
SEQ ID NO: 42 WHNGIWWHYGR
SEQ ID NO: 43 HHLNYLWPWTRV
SEQ ID NO: 44 FWHRWSTFPEQP
In the peptides from ID 1 to 49 one or more amino acid can be substituted by a natural or 
a non-natural amino acid. The peptides from ID 1 to 49 can comprise an additional amino acid or one amino acid is 
deleted.

the peptides from ID 1 to 49 may comprise at least one amino acid modified by 
glycosylation, acetylation, hydroxylation (hydroxyproline), carboxylation (gamma- 
and prenylation) as well as non-naturally occurring amino acids including, trans-3-
methylproline, 2, 4-methanoproline, cis-4-hydroxyproline, trans-4- 
methylglycine, allo-threonine, methylthreonine, hydroxyethylcysteine, 
hydroxyethylhomocysteine, nitroglutamine, homo- glutamin, piperolic acid, tert-leucine, 
norvaline, 2-azaphenylalanine, 3- azaphenylalanine, 4 azaphenylalanine, and 4-
fluorophenylalanine.

It is also object of present invention, the use of at least one anti-MIA antibody or fragment 
thereof for detecting, preventing and curing vitiligo.

Preferred anti-MIA antibody is selected from the group consisting of anti-alpha 4-integrin 
(A4-PUJ1, UBI), anti-alpha 4-integrin (P1 H4, Chemicon), anti-alpha 5-integrin (A5-PUJ5, 
UBI), anti-alpha 5-integrin (P1 D6, Chemicon).

Are further objects of the present invention the pharmaceutical composition comprising at 
least one of the above peptides and/or at least one of the above anti-MIA antibodies 
solubilized and/or vehicle by pharmaceutically acceptable excipients and/or diluents.

**Example**

Bioptic samples

10 bioptic samples were collected from edges injured areas of patients with conclamed 
non-segmental vitiligo, also samples of control skin were collected.

Macroscopically normal pigmented skins on the volar surface of the forearm of five 
healthy subjects were chosen for the experiments as sample controls.

All the patients have a negative history of melanoma and were screened with skin exams 
for absence of this cutaneous neoplasia.
Macroscopic and microscopic analyses were performed on the pathologic and normal specimens. All samples were fixed in 10% formalin in 0.1-mol/L (pH = 7.4) phosphate buffer, dehydrated in a series of rising alcohol concentrations, and then embedded in paraffin for light microscopy. Four micrometer-thick sections were stained with hematoxylin and eosin, Heidenheim modified Azan-Mallory and examined by two independent pathologists.

Immunohistochemical staining with antibodies against leucocytes (CD45, clone 2B1 1+PD7/6., dil 1/200, DAKO), lymphocytes (CD43 clone DF-T1 dil 1/200: DAKO, CD45RO clone CLB-UCHL1 dil: Monosan, CD20 clone L26 dil:1/200 DAKO), and macrophages (CD68 clone KP1 dil 1/200 DAKO), were performed. Sections obtained from each skin-biopsy were prepared, treated, and stained for immunohistochemistry according to standard procedures. For the detection of premelanosomes and mature melanosomes we used S100 polyclonal rabbit antibody and HMB45 (clone HMB45 dil 1/100 DAKO).

Briefly, slides were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity, then washed with phosphate-buffered saline and incubated in buffered normal horse serum to prevent nonspecific Ab binding. Sections were incubated with the primary Abs for 1 hour at room temperature. After washing, a biotin-labeled secondary Ab was applied, followed by an avidin-peroxidase conjugate. Diaminobenzidine was used as a chromogen.

Two antibodies in two different sequential sections of patients and controls with anti-human integrin alpha5beta1 (Chemicon, by Millipore, MA, USA Clone JB 1/50), and melanoma inhibitory activity (MIA, Santa Cruz Biotechnology, CA, USA, polyclonal antibody IgG dil:1/20) were tested and visualized with peroxidase diaminobenzidine (DAB). Double immunostaining to illustrate co-localization of MIA protein with integrin alpha5beta1 slides was done simultaneously on the same section with a sequential immuno-staining technique with anti-human integrin alpha5beta1 and melanoma inhibitory activity and visualizing the outcome with anti-mouse or anti-rabbit fluorescein isothiocyanate- or rhodamine-conjugated secondary antibodies (Chemicon, by Millipore, MA, USA). Nuclei were stained with TO-PRO 3 (Invitrogen, Molecular Probes; distributed by Invitrogen, Milan, Italy).

Microphotographs were taken using a TCS-SL laser scanner confocal microscope (Leica, Wetzar, Germany).

Results

Nine on ten of the biotpic samples were positive for MIA (table 1). The only MIA-negative sample derived from a patient suffering from segmental vitiligo, whereas all the other MIA-
positive samples were derived from patients suffering from non-segmental vitiligo. All skin samples used as control were negative for the presence of MIA. The anti-alpha5beta1 antibody perfectly co-localized with anti-MIA antibody and that this feature where present also in melanocyte already detached from basal membrane and found in the upper epidermis.

Table I

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age</th>
<th>Type of vitiligo</th>
<th>Biopsy from</th>
<th>Presence of MIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>45</td>
<td>NON-SEGMENTAL</td>
<td>HAND</td>
<td>YES</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>56</td>
<td>NON-SEGMENTAL</td>
<td>ABDOMEN</td>
<td>YES</td>
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<tr>
<td>3</td>
<td>M</td>
<td>62</td>
<td>SEGMENTAL</td>
<td>BACK</td>
<td>NO</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>35</td>
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</tr>
<tr>
<td>5</td>
<td>F</td>
<td>59</td>
<td>NON SEGMENTAL</td>
<td>ABDOMEN</td>
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<tr>
<td>6</td>
<td>F</td>
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<tr>
<td>7</td>
<td>M</td>
<td>48</td>
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<td>8</td>
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</tr>
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<td>F</td>
<td>61</td>
<td>NON SEGMENTAL</td>
<td>BACK</td>
<td>YES</td>
</tr>
</tbody>
</table>

The presence of MIA in non-malignant melanocytes was completely unexpected, as normal melanocytes have already been investigated for MIA with negative results.

The data demonstrate that MIA is involved in the pathogenetic mechanism of non-segmental vitiligo. The formation of the vitiliginous patches is consequent to a melanocytorrhagy attributed to the interaction of MIA with alpha5beta1 integrins, causing the elimination of pigments in vitiliginous skin.

The use of MIA inhibitors as targeted therapy for blocking the action of MIA lead to a complete re-pigmentation in every anatomic area.

MIA inhibitors prevent the development of vitiligo in patients already suffering of this dermatosis and in clinical remission.

The presence of MIA in vitiligoid melanocytes is an effective marker for histological definition of the disease in skin specimen.
CLAIMS

1. Use of peptides selected from the group consisting of from ID No. 1 to ID No. 49 for detecting, preventing and curing vitiligo.

2. Use according to claim 1 wherein vitiligo is non-segmental vitiligo.

3. Use according to claim 1 wherein in the peptides from ID No. 1 to ID No. 49 one or more amino acid can be substituted by a natural or a non-natural amino acid.

4. Use according to claim 1 wherein the peptides from ID 1 to 49 can comprise an additional amino acid.

5. Use according to claim 1 wherein in the peptides from ID No. 1 to ID No. 49 one amino acid is deleted.

6. Use according to claim 1 wherein the peptides from ID 1 to 49 comprise at least one amino acid modified by glycosylation, acetylation, hydroxylation (hydroxyproline), carboxylation (gamma-carboxyglutamate), phosphorylation, alkylation, myristoylation (N-terminal), palmitoylation and prenylation.

7. Use according to claim 1 wherein the peptides from ID 1 to 49 comprise at least one amino acid modified by non-naturally occurring amino acids selected from the group consisting of trans-3-methylproline, 2, 4-methanoproline, cis-4-hydroxyproline, trans-4-hydroxyproline, N-methylglycine, allo-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homo-glutamin, pextendil acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4 azaphenylalanine, and 4-fluorophenylalanine.

8. Use of anti-MIA antibody or fragment thereof for detecting, preventing and curing vitiligo.

9. Use according to claim 8 wherein vitiligo is non-segmental vitiligo.

10. Use according to claim 8 wherein the anti-MIA antibody is selected from the group consisting of anti-alpha 4-integrin, anti-alpha 4-integrin, anti-alpha 5-integrin, anti-alpha 5-integrin.

11. Composition comprising at least one peptide according to any one of claims 1-6 and/or at least one anti-MIA antibody of claims 8-10 and pharmaceutically acceptable excipients and/or diluents.