(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2007 (27.09.2007)

PCT

(10) International Publication Number
WO 2007/107774 A2

(51) International Patent Classification:
*G01N 33/50* (2006.01)

(21) International Application Number:
PCT/GB2007/001041

(22) International Filing Date: 22 March 2007 (22.03.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 0605738.4 | 22 March 2006 (22.03.2006) | GB |
| 60/784,445 | 22 March 2006 (22.03.2006) | US |

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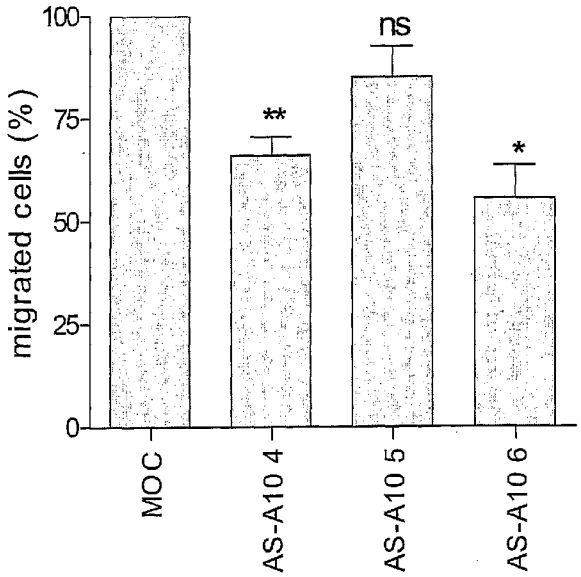
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**
— *of inventorship (Rule 4.17(iv))*

*[Continued on next page]*

(54) Title: NOVEL DIAGNOSTIC AND THERAPEUTIC METHODS




(57) Abstract: The present invention provides the use of a compound comprising a binding moiety capable of binding selectively to integrin alpha- 10 subunit or a heterodimer thereof in the preparation of a (a) a medicament for treating malignant tumours, (b) a diagnostic or prognostic agent for malignant tumours and (c) an agent for detecting and/or imaging malignant tumours cells. Preferably, the tumour is a malignant melanoma. In a preferred embodiment, the compound comprises or consists of an antibody or antigen-binding fragment or derivative thereof. The invention further provides methods for treating, diagnosing and imaging cells of malignant tumours.

WO 2007/107774 A2

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# NOVEL DIAGNOSTIC AND THERAPEUTIC METHODS

## Field of Invention

The present invention relates to methods for the diagnosis and treatment of malignant tumours. In particular, the invention relates to the use of antibodies with specificity for integrin alpha-10 in the diagnosis and treatment of malignant melanomas.

## Introduction

Both benign and malignant tumours are known to express proteins in patterns not found in normal cells. The pattern of proteins exhibited by tumour or malignant cells can reflect the stage of disease (*i.e.*, early stage or metastatic disease). As a malignancy progresses, the cells tend to differ more and more from the tissue from which they originated. As a cancer progresses and becomes increasingly undifferentiated, regardless of the staging schema used to determine the cancer's progression, the cells become more likely to metastasise and/or more refractory to treatment by traditional therapies.

Malignant melanoma is a highly aggressive cancer derived from melanocytes mainly in the epidermis. Some information about processes involved in tumour development is known today but the molecular cause of the disease still remains unsolved. Recent data indicated that loss of cell-cell and cell-matrix contact and changes in the cell cytoskeletal organisation play an important role in early development of the disease (1;2). Additionally, proteins known to be involved in epithelial mesenchymal transition (EMT), such as E-cadherin, MMPs, *etc.*, and several members of the integrin family were found to be deregulated (3-6). Several integrins, such as integrin alphaV/beta3 are known to be strongly

upregulated during melanoma development and progression, which correlates with a more metastatic phenotype (7).

Melanoma cell invasion and metastasis require degradation of interstitial stroma and basement membranes of the ECM. These processes have been shown to depend on proteolytic cascades involving the MMPs and the plasminogen/plasmin system (see Lee *et al.*, 1996, *Circ. Res.* **78**, 44-49; de Vries *et al.*, 1994, *Am. J. Pathol.* **144**, 70-81). Metastasis is a complex multistep process during which tumour cells invade through different ECMs, such as basement membrane and connective tissue, and give rise to new foci at sites distant from the primary tumour. The tumour cell anchors to the ECM via cell surface receptors. The anchored tumour cell next secretes the hydrolytic enzymes which degrade the ECM and causes lysis thereof. The tumour cell then migrates through the ECM. As most cancer patients fail to respond to treatment due to the development of metastasis, it is desirable to inhibit invasion of the normal surrounding tissue by the tumour cells. However, known invasion inhibitors tested to date have been of limited benefit clinically.

Integrins are heterodimeric transmembrane glycoproteins composed of an alpha and a beta subunit which are non-covalently linked. Both subunits consist of a large extracellular domain, a short transmembrane domain and a cytoplasmic domain. The alpha subunit depends of two amino acid chains linked by a disulfide bond (8-10). The N-terminal region binds divalent cations as $Ca^{2+}$ and $Mg^{2+}$ with a seven-fold repeated sequence which contributes to the cation-dependent ligand binding to the integrin (11). The beta subunit has a cysteine rich segment near the transmembrane domain and its C-terminus is necessary for the association with the actin skeleton (9;10). At least 19 different alpha and 8 beta subunits are presently known.

Integrins play an important role as specific cell surface receptors which arrange the contact of tumour cells with extracellular matrix (ECM) proteins (12;13). This contact of the tumour with the environment enables the melanoma cells to convert from a stationary to a migratory and invasive phase. For this transition from radial

growth phase (RGP) to vertical growth phase (VGP) of primary and metastatic melanoma the onset of alphaV/beta3 integrin expression is a specific marker (14-17). Not only the alphaV integrin chain but also the beta1 integrin chain may affect the metastatic potential of melanoma cells (18). For beta1 integrins it has been shown that they play a role in the lymphatic dissemination of cutaneous melanoma (19).

Recently, integrin alpha10 was characterised as a new member of the beta1-integrin family (for example, see WO 99/51639). It is a novel collagen type II-binding integrin expressed by chondrocytes. The predicted amino acid sequence consists of a 1167-amino acid mature protein, including a signal peptide, a long extracellular domain, a transmembrane domain, and a short cytoplasmic domain with homology to other integrins (20;21). A peptide antibody specifically stained chondrocytes in tissue sections of human articular cartilage, showing that alpha10/beta1 is expressed in cartilage tissue. The results of several studies demonstrate that integrin alpha10/beta1 plays a specific role in growth plate morphogenesis and function (22).

Cancers typically begin their growth in only one location in the tissue of origin. As the cancer progresses, the cancer may migrate to a distal location in the patient. For example, a cancer beginning in the skin may migrate to the lung. Other locations common for metastatic disease include metastatic cancer to the brain, lung, liver, and bone.

Once a tumour is diagnosed in a patient, the first question is whether the tumour has progressed and spread to the regional lymph nodes and to distant organs. In the end, most cancer deaths result from metastases that are resistant to conventional cancer therapies. Metastases can be located in different organs and in different regions of the same organ, making complete eradication by surgery, radiation, drugs, and/or biotherapy nearly impossible.

Malignant melanoma is a serious form of skin cancer and the incidence of this disease appears to be increasing, such that currently about 1 in 100 persons in the

United States can expect to develop this cancer in a lifetime. Without treatment it has the tendency to become widely metastatic and result in the demise of the patient.

In people with early-stage melanoma, surgery has a very high chance of completely curing the cancer. The surgery will leave a scar on the skin. However, if the surgery is not done the melanoma is likely to spread into the deeper layers of the skin and then to other areas of the body.

If the melanoma has spread to other parts of the body, treatment cannot cure it, but may be able to control it for a time. This can lead to an improvement in symptoms and a better quality of life. However, for some people in this situation the treatment will have no effect upon the cancer and they will get the side effects without any of the benefit.

Thus, new methods and uses that inhibit tumour metastasis, particularly for malignant melanoma, are needed, which can be used alone or in concert with other agents to treat cancer, especially advanced stage malignant tumours, which typically involve metastases.

**Summary of Invention**

A first aspect of the invention provides the use of a compound comprising a binding moiety capable of binding selectively to an integrin alpha-10 subunit or a heterodimer thereof in the preparation of a medicament for treating a malignant tumour. It will be appreciated by persons skilled in the art that the medicament may be used for prophylactic and/or therapeutic purposes, *i.e.* the medicament may be administered to a subject in need thereof in an amount sufficient to give prophylactic and/or therapeutic effect.

By "tumour" we include an abnormal mass of tissue that results from excessive cell division that is uncontrolled and progressive, also called a neoplasm.

By "malignant" we include the meaning of a tumour having the properties of anaplasia, invasion and/or metastasis.

A second aspect of the invention provides the use of a compound comprising a binding moiety capable of binding selectively to an integrin alpha-10 subunit or a heterodimer thereof in the preparation of a diagnostic or prognostic agent for a malignant tumour.

A third aspect of the invention provides a use of a compound comprising a binding moiety capable of binding selectively to an integrin alpha-10 subunit or a heterodimer thereof in the preparation of an agent for detecting and/or imaging malignant tumour cells in a body of an individual, either *in vivo* or *in vitro*.

Persons skilled in the art will appreciate that the above aspects of the invention may be used for any tumour found to over-express an integrin alpha-10 subunit. Preferably, the tumour is selected from the group consisting of tumours of the breast, bile duct, brain, colon, stomach, reproductive organs, lung and airways, skin, gallbladder, liver, nasopharynx, nerve cells, kidney, prostate, lymph glands and gastrointestinal tract. Most preferably, however, the tumour is a malignant melanoma.

Yet another aspect of the invention provides for a use of a compound comprising a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof in the preparation of an agent for inhibiting metastases or metastatic spread. The agent is administered to a subject in need thereof in an amount sufficient to inhibit metastases, and/or metastatic spread.

Thus, in one aspect of the invention, the present invention can be used to inhibit, prevent or slow down the progression of malignancies. Another aspect of the invention is to inhibit, prevent or slow down metastases or metastatic progression.

The present invention can also be used to inhibit, prevent or slow down the invasion of healthy tissue by malignant tumour cells.

It is yet a further aspect of the invention that the tumour treated is a solid tumour. Solid tissue tumours contemplated for treatment according to the invention include but are not limited to melanomas (*e.g.* cutaneous melanoma, a metastatic melanoma, or an intraocular melanoma), prostate cancers, and metastatic lesions of other primary tumours. Preferably, the tumour is a solid tumour, *e.g.* a melanoma, or a metastatic lesion of other primary tumours.

In a further aspect of the invention, the agent can be administered alone or in combination with other cancer treatment therapies in a multi-treatment format. Examples are where the subject is further treated with a chemotherapy, an immunotherapy, surgery, radiation therapy, hyperthermia, or a drug to ameliorate the adverse effects of a cancer therapy. For example, if the tumour is a melanoma, the subject can be administered the agent after having had the melanoma surgically removed. Further, if the tumour is melanoma, the above agent can be further combined with such cancer therapies as isolated limb perfusion, regional chemotherapy infusion, systemic chemotherapy, or immunotherapy with an antibody (*e.g.*, an anti-GM2 1 ganglioside antibody, anti- GD2 ganglioside antibody, anti-GD3 ganglioside - 4 antibody), or antisera. The chemotherapeutic agent can be any one or more of the following: dacarbazine, carmustine,

lomustine, tauromustine, fotemustine, semustine, cisplatin, carboplatin, vincristine, vinblastine, vindesine, taxol, dibromodulcitol, detorubicin, piritrexim and interferon.

Yet another aspect of the invention provides for a use of a compound comprising a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof in the preparation of an agent for treating metastases to the brain, lung, liver, or bone.

Another aspect of the invention provides for a combination therapy wherein a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof in the preparation of an agent are used in combination with other tumour treatment therapies as known in the art.

A neoplasm, or tumour, is an abnormal, unregulated, and disorganised proliferation of cell growth. A neoplasm is malignant, or cancerous, if it has properties of destructive growth, invasiveness and metastasis. Invasiveness refers to the local spread of a neoplasm by infiltration or destruction of surrounding tissue, typically breaking through the basal laminas that define the boundaries of the tissues, thereby often entering the body's circulatory system. Metastasis typically refers to the dissemination of tumour cells by lymphatics or blood vessels. Metastasis also refers to the migration of tumour cells by direct extension through serous cavities, or subarachnoid or other spaces.

Through the process of metastasis, tumour cell migration to other areas of the body establishes neoplasms in areas away from the site of initial appearance. There are essential steps in the formation of metastasis in all tumours. The steps include the following:

1. After neoplastic transformation, progressive proliferation of neoplastic cells supported by the organ/tissue environment in which the neoplasm is located.
2. Neovascularisation or angiogenesis of the tumour for further growth beyond 1 to 2 mm in diameter.

3. Down-regulation of expression of cohesive molecules wherein the cells have increased motility or ability to detach from the primary lesion.
4. Detachment and embolisation of single tumour cells or cell aggregates, with the vast majority of these cells being rapidly destroyed.
5. Once tumour cells survive the detachment and embolisation step, they must go on to proliferate within the lumen of the blood vessel. The cells will then go on to extravasate into the organ parenchyma by mechanism similar to those operative during invasion.
6. Tumour cells with the appropriate cell surface receptors can respond to paracrine growth factors and hence proliferate in the organ parenchyma.
7. Tumour cell evasion of host defences (both specific and nonspecific immune responses).
8. For a metastasis to proliferate beyond 1 to 2 mm in diameter, the metastases must develop a vascular network.

Thus, if a primary tumour is given enough time to go through these steps, it will form metastatic lesions at a site or sites distant to the primary tumour. The present invention disclosed herein may inhibit, slow down or prevent one or more one or more of these steps in the metastatic process. For additional details on the mechanism and pathology of tumour metastasis, see Isaiah J. Fidler, "Molecular Biology of Cancer: Invasion and Metastasis," in *Cancer: Principles & Practice of Oncology* pp 135-152 (Vincent T. DeVita et al., editors, 5th ea., 1997).

Tumour cell invasion is believed to occur by a three-step process:

1) tumour cell attachment to extracellular matrix;
2) proteolytic dissolution of the matrix; and
3) movement of the cells through the dissolved barrier.

This process can occur repeatedly and can result in metastases, *i.e.* secondary tumours, at sites distant from the original, primary, tumour.

By the term "subject" or "patient" as used herein is meant to include a mammal. The mammal can be a canine, feline, primate, bovine, ovine, porcine, camelid, caprine, rodent, or equine. Preferably the mammal is human.

By the term "primary tumour" is meant the original neoplasm and not a metastatic lesion located in another tissue or organ in the patient's body.

By the terms "metastatic disease", "metastases" and "metastatic lesion" are meant a group of cells which have migrated to a site distant relative to the primary tumour.

As used herein, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an antibody" includes a plurality of such antibodies and reference to "the dosage" includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

By "capable of binding selectively" we include binding moieties which bind at least 10-fold more strongly to integrin alpha-10 subunit or a heterodimer thereof than to another proteins (in particular other integrins, such as alpha11, alpha1 and apha2 having most identity with alpha10); preferably at least 50-fold more strongly and more preferably at least 100-fold more strongly. Advantageously, the binding moiety is capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof under physiological conditions, *e.g. in vivo*. Suitable methods for measuring relative binding strengths include immunoassays, for example where the binding moiety is an antibody (see Harlow & Lane, "Antibodies: A Laboratory", Cold Spring Habor Laboratory Press, New York). Alternatively, binding may be assessed using competitive assays or using Biacore® analysis (Biacore International AB, Sweden).

Most preferably, the binding moiety binds exclusively to an integrin alpha-10 subunit or a heterodimer thereof.

In a preferred embodiment, the binding moiety selectively binds to an integrin alpha-10 subunit. Conveniently, the binding moiety selectively binds to a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1 or natural variants thereof.

MELPFVTHLFLPLVFLTGLCSPFNLDEHHPRLFPGPPEAEFGYSVLQHVGGGQRWMLVGAPWDG
PSGDRRGDVYRCPVGGAHNAPCAKGHLGDYQLGNSSHPAVNMHLGMSLLETDGDGGFMACAP
LWSRACGSSVFSSGICARVDASFQPQGSLAPTAQRCPTYMDVVIVLDGSNSIYPWSEVQTFLRRL
VGKLFIDPEQIQVGLVQYGESPVHEWSLGDFRTKEEVVRAAKNLSRREGRETKTAQAIMVACTEG
FSQSHGGRPEAARLLVVVTDGESHDGEELPAALKACEAGRVTRYGIAVLGHYLRRQRDPSSFLRE
IRTIASDPDERFFFNVTDEAALTDIVDALGDRIFGLEGSHAENESSFGLEMSQIGFSTHRLKDGILFG
MVGAYDWGGSVLWLEGGHRLFPPRMALEDEFPPALQNHAAYLGYSVSSMLLRGGRRLFLSGAP
RFRHRGKVIAFQLKKDGAVRVAQSLQGEQIGSYFGSELCPLDTDRDGTTDVLLVAAPMFLGPQNK
ETGRVYVYLVGQQSLLTLQGTLQPEPPQDARFGFAMGALPDLNQDGFADVAVGAPLEDGHQGAL
YLYHGTQSGVRPHPAQRIAAASMPHALSYFGRSVDGRLDLDGDDLVDVAVGAQGAAILLSSRPIV
HLTPSLEVTPQAISVVQRDCRRRGQEAVCLTAALCFQVTSRTPGRWDHQFYMRFTASLDEWTAG
ARAAFDGSGQRLSPRRLRLSVGNVTCEQLHFHVLDTSDYLRPVALTVTFALDNTTKPGPVLNEGS
PTSIQKLVPFSKDCGPDNECVTDLVLQVNMDIRGSRKAPFVVRGGRRKVLVSTTLENRKENAYNT
SLSLIFSRNLHLASLTPQRESPIKVECAAPSAHARLCSVGHPVFQTGAKVTFLLEFEFSCSSLLSQV
FVKLTASSDSLERNGTLQDNTAQTSAYIQYEPHLLFSSESTLHRYEVHPYGTLPVGPGPEFKTTLR
VQNLGCYVVSGLIISALLPAVAHGGNYFLSLSQVITNNASCIVQNLTEPPGPPVHPEELQHTNRLNG
SNTQCQVVRCHLGQLAKGTEVSVGLLRLVHNEFFRRAKFKSLTVVSTFELGTEEGSVLQLTEASR
WSESLLEVVQTRPILISLWILIGSVLGGLLLLALLVFCLWKLGFFAHKKIPEEEKREEKLEQ

**SEQ ID NO:1**

By "natural variants" we include, for example, allelic variants. Typically, these will vary from the given sequence by only one or two or three, and typically no more than 10 or 20 amino acid residues. Typically, the variants have conservative substitutions.

Also included in "natural variants" is a splice variant of integrin alpha 10, for example as described in WO 99/51639 (see Example 4 therein).

Variants of the above polypeptide sequence include polypeptides comprising a sequence with at least 60% identity to the amino acid sequences of SEQ ID NO: 1 to 32, preferably at least 70% or 80% or 85% or 90% identity to said sequences, and more preferably at least 95%, 96%, 97%, 98% or 99% identity to said amino acid sequences.

Percent identity can be determined by methods well known in the art, for example using the LALIGN program (Huang and Miller, *Adv. Appl. Math.* (1991) **12**:337-357) at the Expasy facility site (http://www.ch.embnet.org/software/LALIGN_form.html) using as parameters the global alignment option, scoring matrix BLOSUM62, opening gap penalty –14, extending gap penalty –4.

Alternatively, the percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally.

In an alternative preferred embodiment, the binding moiety selectively binds to a heterodimer comprising an integrin alpha-10 subunit, such as an alpha-10/beta-1 heterodimer.

The compound and/or binding moiety therein may be a polypeptide.

Conveniently, the binding moiety is not a nucleic acid molecule capable of inhibiting expression of an integrin alpha-10 subunit.

In a particularly preferred embodiment of the first, second and third aspects of the invention, the binding moiety is an antibody or antigen-binding fragment or derivative thereof.

Exemplary antibodies with binding affinity for the integrin alpha-10 subunit are described in International Patent Application No. PCT/SE2004/000580 (Publication No. WO 2004/089990).

By "antibody" we include substantially intact antibody molecules, as well as chimaeric antibodies, humanised antibodies, human antibodies (wherein at least

one amino acid is mutated relative to the naturally occurring human antibodies), single chain antibodies, bispecific antibodies, antibody heavy chains, antibody light chains, homodimers and heterodimers of antibody heavy and/or light chains, and antigen binding fragments and derivatives of the same.

By "antigen-binding fragment" we mean a functional fragment of an antibody that is capable of binding to the integrin alpha-10 subunit or a heterodimer thereof.

Preferably, the antigen-binding fragment is selected from the group consisting of Fv fragments (*e.g.* single chain Fv and disulphide-bonded Fv), Fab-like fragments (*e.g.* Fab fragments, Fab' fragments and $F(ab)_2$ fragments), single variable domains (*e.g.* $V_H$ and $V_L$ domains), domain antibodies (dAbs, including single and dual formats [*i.e.* dAb-linker-dAb]) and nanobodies (for example, see Revets *et al.*, 2005, *Expert Opin Biol Ther.* **5**(1):111-24).

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Moreover, antigen-binding fragments such as Fab, Fv, ScFv and dAb antibody fragments can be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Also included within the scope of the invention are modified versions of antibodies and an antigen-binding fragment thereof, *e.g.* modified by the covalent attachment of polyethylene glycol or other suitable polymer.

Although the antibody may be a polyclonal antibody, it is preferred if it is a monoclonal antibody. In some circumstances, particularly if the antibody is going to be administered repeatedly to a human patient, it is preferred if the monoclonal antibody is a human monoclonal antibody or a humanised monoclonal antibody.

Methods of generating antibodies and antibody fragments are well known in the art. For example, antibodies may be generated via any one of several methods which employ induction of *in vivo* production of antibody molecules, screening of immunoglobulin libraries (Orlandi. *et al*, 1989. *Proc. Natl. Acad. Sci. U.S.A.* **86**:3833-3837; Winter *et al.*, 1991, *Nature* **349**:293-299) or generation of monoclonal antibody molecules by cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the Epstein-Barr virus (EBV)-hybridoma technique (Kohler *et al.*, 1975. *Nature* **256**:4950497; Kozbor *et al.*, 1985. *J. Immunol. Methods* **81**:31-42; Cote *et al.*, 1983. *Proc. Natl. Acad. Sci. USA* **80**:2026-2030; Cole *et al.*, 1984. *Mol. Cell. Biol.* **62**:109-120).

Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982).

Antibody fragments can be obtained using methods well known in the art (see, for example, Harlow & Lane, 1988, *"Antibodies: A Laboratory Manual"*, Cold Spring Harbor Laboratory, New York). For example, antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (*e.g.* Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Alternatively, antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods.

It will be appreciated by persons skilled in the art that for human therapy or diagnostics, humanised antibodies are preferably used. Humanised forms of non-human (*e.g.* murine) antibodies are genetically engineered chimaeric antibodies or antibody fragments having preferably minimal-portions derived from non-human antibodies. Humanised antibodies include antibodies in which complementary determining regions of a human antibody (recipient antibody) are replaced by residues from a complementary determining region of a non human

species (donor antibody) such as mouse, rat of rabbit having the desired functionality. In some instances, Fv framework residues of the human antibody are replaced by corresponding non-human residues. Humanised antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported complementarity determining region or framework sequences. In general, the humanised antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the complementarity determining regions correspond to those of a non human antibody and all, or substantially all, of the framework regions correspond to those of a relevant human consensus sequence. Humanised antibodies optimally also include at least a portion of an antibody constant region, such as an Fc region, typically derived from a human antibody (see, for example, Jones *et al.*, 1986. *Nature* **321**:522-525; Riechmann *et al.*, 1988, *Nature* **332**:323-329; Presta, 1992, *Curr. Op. Struct. Biol.* **2**:593-596).

Methods for humanising non-human antibodies are well known in the art. Generally, the humanised antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues, often referred to as imported residues, are typically taken from an imported variable domain. Humanisation can be essentially performed as described (see, for example, Jones *et al.*, 1986, *Nature* **321**:522-525; Reichmann *et al.*, 1988. *Nature* **332**:323-327; Verhoeyen *et al.*, 1988, *Science* **239**:1534-1536; US 4,816,567) by substituting human complementarity determining regions with corresponding rodent complementarity determining regions. Accordingly, such humanised antibodies are chimaeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanised antibodies may be typically human antibodies in which some complementarity determining region residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be identified using various techniques known in the art, including phage display libraries (see, for example, Hoogenboom & Winter,

1991, *J. Mol. Biol.* **227**:381; Marks *et al.*, 1991, *J. Mol. Biol.* **222**:581; Cole *et al.*, 1985, In: *Monoclonal antibodies and Cancer Therapy*, Alan R. Liss, pp. 77; Boerner *et al.*, 1991. *J. Immunol.* **147**:86-95).

In a further alternative embodiment, the binding moiety is a polypeptide capable of binding selectively to the integrin alpha-10 subunit or a heterodimer thereof. Polypeptide binding moieties can be identified by means of a screen. A suitable method or screen for identifying peptides or other molecules which selectively bind a target protein or polypeptide may comprise contacting the target protein or polypeptide with a test peptide or other molecule under conditions where binding can occur, and then determining if the test molecule or peptide has bound the target protein or peptide. Methods of detecting binding between two moieties are well known in the art of biochemistry. Preferably, the known technique of phage display is used to identify peptides or other ligand molecules suitable for use as binding moieties. An alternative method includes the yeast two hybrid system.

Polypeptide binding moieties and compounds for use in the invention may be made by methods well known to persons skilled in the art (for example, see Sambrook & Russell, 2000, *Molecular Cloning, A Laboratory Manual*, Third Edition, Cold Spring Harbor, New York).

In brief, expression vectors may be constructed comprising a nucleic acid molecule which is capable, in an appropriate host, of expressing the polypeptide binding moiety or compound encoded by the nucleic acid molecule.

A variety of methods have been developed to operably link nucleic acid molecules, especially DNA, to vectors, for example, via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, *e.g.* generated by endonuclease restriction digestion, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerising activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a larger molar excess of linker molecules in the presence of an enzyme that is able to catalyse the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease site are commercially available from a number of sources including International Biotechnologies Inc., New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use PCR. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention or binding moiety thereof. Thus, the DNA encoding the polypeptide may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the compound of the invention or binding moiety thereof. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case or retroviral vectors, RNA) encoding the polypeptide constituting the compound of the invention or binding moiety thereof may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance.

Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the expression vector of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example, *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors typically include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (Ycps).

Other vectors and expression systems are well known in the art for use with a variety of host cells.

The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No. ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CRL 1658 and 293 cells which are human embryonic kidney cells. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and

Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cells, bacterial cells, insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5 PEB using 6250V per cm at 25 μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, *i.e.* cells that contain a DNA construct of the present invention, can be identified by well-known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when

the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity.

Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

The host cell may be a host cell within a non-human animal body. Thus, transgenic non-human animals which express a compound according to the first aspect of the invention (or a binding moiety thereof) by virtue of the presence of the transgene are included. Preferably, the transgenic non-human animal is a rodent such as a mouse. Transgenic non-human animals can be made using methods well known in the art.

Methods of cultivating host cells and isolating recombinant proteins are well known in the art. It will be appreciated that, depending on the host cell, the compounds of the invention (or binding moieties thereof) produced may differ. For example, certain host cells, such as yeast or bacterial cells, either do not have, or have different, post-translational modification systems which may result in the production of forms of compounds of the invention (or binding moieties thereof) which may be post-translationally modified in a different way.

It is preferred that compounds of the invention (or binding moieties thereof) are produced in a eukaryotic system, such as a mammalian cell.

According to a less preferred embodiment, the compounds of the invention (or binding moieties thereof) can be produced *in vitro* using a commercially available *in vitro* translation system, such as rabbit reticulocyte lysate or wheatgerm lysate (available from Promega). Preferably, the translation system is rabbit reticulocyte lysate. Conveniently, the translation system may be coupled to a transcription system, such as the TNT transcription-translation system (Promega). This system has the advantage of producing suitable mRNA transcript from an encoding DNA polynucleotide in the same reaction as the translation.

It will be appreciated by persons skilled in the art that compounds for use in the medicament embodiment of the invention preferably should inhibit one or more biological activities of the integrin alpha-10 subunit or heterodimer thereof. Such inhibition of the biological activity of the integrin alpha-10 subunit or heterodimer thereof by a compound may be in whole or in part. For example, the compound may inhibit the biological activity of the integrin alpha-10 subunit or heterodimer thereof by at least 10%, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, and most preferably by 100% compared to the biological activity of the integrin alpha-10 subunit or heterodimer thereof in melanoma cells which have not been exposed to the compound. In a preferred embodiment, the compound is capable of inhibiting the biological activity of the integrin alpha-10 subunit or heterodimer thereof by 50% or more compared to the biological activity of the integrin alpha-10 subunit or heterodimer thereof in melanoma cells which have not been exposed to the compound. As used herein, "biological activity" refers to the effect of integrin alpha10 or its heterodimer upon a living organism, tissue or cell. Included herein, but not limited to, is binding to its natural ligand(s), as well as down-stream events therefrom, causing direct or indirect effects on a living organism.

Preferably, the compound inhibits one or more biological activities of the integrin alpha-10 subunit selectively. By 'selectively' we mean that the compound inhibits the biological activity of the integrin alpha-10 subunit or heterodimer thereof to a greater extent than it modulates the activity of other proteins in the melanoma cells. Preferably, the compound inhibits only the biological activity of the integrin alpha-10 subunit or heterodimer thereof, although it will be appreciated that the expression and activity of other proteins within the melanoma cells may change as a downstream consequence of a selective inhibition of the integrin alpha-10 subunit or heterodimer thereof. Thus, we exclude agents which have a non-specific effect on gene expression and/or cancer cell growth.

Advantageously, the compound is also selective in the sense that it acts preferentially on the biological activity of the integrin alpha-10 subunit in

malignant tumours, such as malignant melanoma cells (*i.e.* cell-specific inhibition). Preferably, the compound inhibits the biological activity of the integrin alpha-10 subunit in tumour cells only.

Optionally, the compounds used in the present the invention also comprise a further moiety. Such further moiety may be any further moiety which confers on the compound a useful property with respect to the treatment or imaging or diagnosis of malignant melanoma, *e.g.* target cell specific portions, cytotoxic moieties and/or detectable moieties.

Thus, in one embodiment, the compound comprises a target cell specific portion with binding affinity for malignant tumour cells.

By "target cell specific" portion we mean a portion of the compound which comprises one or more binding sites which recognise and bind to entities on the target tumour cell. Upon contact with the target cell, the target cell specific portion may be internalised along with the alpha-10 binding portion.

The entities recognised by the target cell-specific portion are expressed predominantly, and preferably exclusively, on the target tumour cell. The target cell specific portion may contain one or more binding sites for different entities expressed on the same target cell type, or one or more binding sites for different entities expressed on two or more different target cell types.

Preferably, the target cell-specific portion recognises the target cell with high avidity.

By "high avidity" we mean that the target cell-specific portion recognises the target cell with a binding constant of at least $K_d = 10^{-6}$ M, preferably at least $K_d = 10^{-9}$ M, suitably $K_d = 10^{-10}$ M, more suitably $K_d = 10^{-11}$ M, yet more suitably still $K_d = 10^{-12}$ M, and more preferably $K_d = 10^{-15}$M or even $K_d = 10^{-18}$ M.

The entity which is recognised may be any suitable entity which is expressed by tumour cells. Often, the entity which is recognised will be an antigen.

Examples of antigens include those listed in Table 1.

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**Table 1**

Tumour Associated Antigens

| Antigen | Antibody | Existing Uses |
|---|---|---|
| Carcino-embryonic Antigen | C46 (Amersham) 85A12 (Unipath) | Imaging & Therapy of colon/rectum tumours. |
| Placental Alkaline Phosphatase | H17E2 (ICRF, Travers & Bodmer) | Imaging & Therapy of testicular and ovarian cancers. |
| Pan Carcinoma | NR-LU-10 (NeoRx Corporation) | Imaging & Therapy of various carcinomas incl. small cell lung cancer. |
| Polymorphic Epithelial Mucin (Human milk fat globule | HMFG1 (Taylor-Papadimitriou, ICRF) (Antisoma plc) | Imaging & Therapy of ovarian cancer, pleural effusions, breast, lung & other common epithelial cancers. |
| Human milk mucin core protein | SM-3(IgG1)[1] | Diagnosis, Imaging & Therapy of breast cancer |
| β-human Chorionic Gonadotropin | W14 | Targeting of enzyme (CPG2) to human xenograft choriocarcinoma in nude mice. (Searle *et al* (1981) *Br. J. Cancer* **44**, 137-144) |

| | | |
|---|---|---|
| A Carbohydrate on Human Carcinomas | L6 (IgG2a)[2] | Targeting of alkaline phosphatase. (Senter *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4842-4846 |
| CD20 Antigen on B Lymphoma (normal and neoplastic) | 1F5 (IgG2a)[3] | Targeting of alkaline phosphatase. (Senter *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4842-4846 |

[1] Burchell *et al* (1987) *Cancer Res.* **47**, 5476-5482

[2] Hellström *et al* (1986) *Cancer Res.* **46**, 3917-3923

[3] Clarke *et al* (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1766-1770

Other antigens include alphafoetoprotein, Ca-125, prostate specific antigen and members of the epidermal growth factor receptor family, namely EGFR, erb B3 and erb B4.

Where the target cell is a maligant melanoma cell, a target cell-specific portion with binding affinity for the 3G5-reactive ganglioside antigen (3G5 antigen) may be used (see Fiedler et al., 2004, *Am J Dermatopathol.* **26**(3):200-4; Saxton *et al.*, 1988, *Dis Markers* **6**(2):97-108).

Preferably, the target cell specific portion is an antibody or antigen-binding fragment or derivative thereof. Thus, in one embodiment, the compound may comprise a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof and a further binding moiety capable of binding the target tumour cells (*e.g.* malignant melanoma cells), for example a bi-specific antibody or bi-specific fragment or variant thereof.

In an additional embodiment, the further moiety is one which is useful in killing or imaging cells associated with malignant melanoma. Preferably, the further moiety is one which is able to kill the cells to which the compound is able to bind.

Advantageously, the binding moiety and further moiety are covalently attached.

In a preferred embodiment of the invention the further moiety is directly or indirectly cytotoxic. In particular the further moiety is preferably directly or indirectly toxic to malignant melanoma cells.

By "directly cytotoxic" we include the meaning that the moiety is one which on its own is cytotoxic. By "indirectly cytotoxic" we include the meaning that the moiety is one which, although is not itself cytotoxic, can induce cytotoxicity, for example by its action on a further molecule or by further action on it.

For example, the cytotoxic moiety is a cytotoxic chemotherapeutic agent. Suitable cytotoxic chemotherapeutic agents are well known in the art.

Cytotoxic chemotherapeutic agents, such as anticancer agents, include: alkylating agents including nitrogen mustards such as mechlorethamine ($HN_2$), cyclophosphamide, ifosfamide, melphalan (L-sarcolysin) and chlorambucil; ethylenimines and methylmelamines such as hexamethylmelamine, thiotepa; alkyl sulphonates such as busulfan; nitrosoureas such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU) and streptozocin (streptozotocin); and triazenes such as decarbazine (DTIC; dimethyltriazenoimidazole-carboxamide); Antimetabolites including folic acid analogues such as methotrexate (amethopterin); pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2'-deoxycoformycin). Natural Products including vinca alkaloids such as vinblastine (VLB) and vincristine; epipodophyllotoxins such as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorubicin (daunomycin; rubidomycin),

doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin C); enzymes such as L-asparaginase; and biological response modifiers such as interferon alphenomes. Miscellaneous agents including platinum coordination complexes such as cisplatin (*cis*-DDP) and carboplatin; anthracenedione such as mitoxantrone and anthracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N-methylhydrazine, MIH); and adrenocortical suppressant such as mitotane (*o,p'*-DDD) and aminoglutethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen.

Several of these agents have previously been attached to antibodies and other target site-delivery agents, and so compounds of the invention comprising these agents may readily be made by the person skilled in the art. For example, carbodiimide conjugation (Bauminger & Wilchek (1980) *Methods Enzymol.* **70**, 151-159; incorporated herein by reference) may be used to conjugate a variety of agents, including doxorubicin, to antibodies or peptides.

Carbodiimides comprise a group of compounds that have the general formula $R_1$-N=C=N-$R_2$, where $R_1$ and $R_2$ can be aliphatic or aromatic, and are used for synthesis of peptide bonds. The preparative procedure is simple, relatively fast, and is carried out under mild conditions. Carbodiimide compounds attack carboxylic groups to change them into reactive sites for free amino groups.

The water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is particularly useful for conjugating a functional moiety to a binding moiety and may be used to conjugate doxorubicin to tumour homing peptides. The conjugation of doxorubicin and a binding moiety requires the presence of an amino group, which is provided by doxorubicin, and a carboxyl group, which is provided by the binding moiety such as an antibody or peptide.

In addition to using carbodiimides for the direct formation of peptide bonds, EDC also can be used to prepare active esters such as N-hydroxysuccinimide (NHS) ester. The NHS ester, which binds only to amino groups, then can be used to

induce the formation of an amide bond with the single amino group of the doxorubicin. The use of EDC and NHS in combination is commonly used for conjugation in order to increase yield of conjugate formation (Bauminger & Wilchek, *supra*, 1980).

Other methods for conjugating a functional moiety to a binding moiety also can be used. For example, sodium periodate oxidation followed by reductive alkylation of appropriate reactants can be used, as can glutaraldehyde cross-linking. However, it is recognised that, regardless of which method of producing a conjugate of the invention is selected, a determination must be made that the binding moiety maintains its targeting ability and that the functional moiety maintains its relevant function.

In a further embodiment of the invention, the cytotoxic moiety is a cytotoxic peptide or polypeptide moiety by which we include any moiety which leads to cell death. Cytotoxic peptide and polypeptide moieties are well known in the art and include, for example, ricin, abrin, *Pseudomonas* exotoxin, tissue factor and the like. Methods for linking them to targeting moieties such as antibodies are also known in the art. The use of ricin as a cytotoxic agent is described in Burrows & Thorpe (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8996-9000, incorporated herein by reference, and the use of tissue factor, which leads to localised blood clotting and infarction of a tumour, has been described by Ran *et al* (1998) *Cancer Res.* **58**, 4646-4653 and Huang *et al* (1997) *Science* **275**, 547-550. Tsai *et al* (1995) *Dis. Colon Rectum* **38**, 1067-1074 describes the abrin A chain conjugated to a monoclonal antibody and is incorporated herein by reference. Other ribosome inactivating proteins are described as cytotoxic agents in WO 96/06641. Pseudomonas exotoxin may also be used as the cytotoxic polypeptide moiety (see, for example, Aiello *et al* (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10457-10461; incorporated herein by reference).

Certain cytokines, such as TNFα and IL-2, may also be useful as cytotoxic agents.

Likewise, certain radioactive atoms may also be cytotoxic if delivered in sufficient doses. Thus, the cytotoxic moiety may comprise a radioactive atom which, in use, delivers a sufficient quantity of radioactivity to the target site so as to be cytotoxic. Suitable radioactive atoms include phosphorus-32, iodine-125, iodine-131, indium-111, rhenium-186, rhenium-188 or yttrium-90, or any other isotope which emits enough energy to destroy neighbouring cells, organelles or nucleic acid. Preferably, the isotopes and density of radioactive atoms in the compound of the invention are such that a dose of more than 4000 cGy (preferably at least 6000, 8000 or 10000 cGy) is delivered to the target site and, preferably, to the cells at the target site and their organelles, particularly the nucleus.

The radioactive atom may be attached to the binding moiety in known ways. For example EDTA or another chelating agent may be attached to the binding moiety and used to attach $^{111}$In or $^{90}$Y. Tyrosine residues may be directly labelled with $^{125}$I or $^{131}$I.

The cytotoxic moiety may be a suitable indirectly cytotoxic polypeptide. In a particularly preferred embodiment, the indirectly cytotoxic polypeptide is a polypeptide which has enzymatic activity and can convert a relatively non-toxic prodrug into a cytotoxic drug. When the binding moiety is an antibody this type of system is often referred to as ADEPT (Antibody-Directed Enzyme Prodrug Therapy). The system requires that the binding moiety locates the enzymatic portion to the desired site in the body of the patient (*i.e.* the malignant melanoma cells) and after allowing time for the enzyme to localise at the site, administering a prodrug which is a substrate for the enzyme, the end product of the catalysis being a cytotoxic compound. The object of the approach is to maximise the concentration of drug at the desired site and to minimise the concentration of drug in normal tissues (see Senter, P.D. *et al* (1988) "Anti-tumour effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate" *Proc. Natl. Acad. Sci. USA* **85**, 4842-4846; Bagshawe (1987) *Br. J. Cancer* **56**, 531-2; and Bagshawe, K.D. *et al* (1988) "A cytotoxic agent can be generated selectively at cancer sites" *Br. J. Cancer.* **58**, 700-703.)

Clearly, any binding moiety with specificity the integrin alpha-10 subunit or a heterodimer thereof may be used in place of an antibody in this type of directed enzyme prodrug therapy system.

The enzyme and prodrug of the system using a targeted enzyme as described herein may be any of those previously proposed. The cytotoxic substance may be any existing anti-cancer drug such as an alkylating agent; an agent which intercalates in DNA; an agent which inhibits any key enzymes such as dihydrofolate reductase, thymidine synthetase, ribonucleotide reductase, nucleoside kinases or topoisomerase; or an agent which effects cell death by interacting with any other cellular constituent. Etoposide is an example of a topoisomerase inhibitor.

Reported prodrug systems include: a phenol mustard prodrug activated by an *E. coli* β-glucuronidase (Wang *et al*, 1992 and Roffler *et al*, 1991); a doxorubicin prodrug activated by a human β-glucuronidase (Bosslet *et al*, 1994); further doxorubicin prodrugs activated by coffee bean α-galactosidase (Azoulay *et al*, 1995); daunorubicin prodrugs, activated by coffee bean α-D-galactosidase (Gesson *et al*, 1994); a 5-fluorouridine prodrug activated by an *E. coli* β-D-galactosidase (Abraham *et al*, 1994); and methotrexate prodrugs (*e.g.* methotrexate-alanine) activated by carboxypeptidase A (Kuefner *et al*, 1990, Vitols *et al*, 1992 and Vitols *et al*, 1995). These and others are included in the Table 2 below.

**Table 2**

| Enzyme | Prodrug |
|---|---|
| Carboxypeptidase G2 | Derivatives of L-glutamic acid and benzoic acid mustards, aniline mustards, phenol mustards and phenylenediamine mustards; fluorinated derivatives of these |
| Alkaline phosphatase | Etoposide phosphate<br>Mitomycin phosphate |
| Beta-glucuronidase | *p*-Hydroxyaniline mustard-glucuronide<br>Epirubicin-glucuronide |
| Penicillin-V-amidase | Adriamycin-N phenoxyacetyl |
| Penicillin-G-amidase | N-(4'-hydroxyphenyl acetyl) palytoxin<br>Doxorubicin and melphalan |
| Beta-lactamase | Nitrogen mustard-cephalosporin<br>*p*-phenylenediamine; doxorubicin derivatives; vinblastine derivative-cephalosporin, cephalosporin mustard; a taxol derivative |
| Beta-glucosidase | Cyanophenylmethyl-beta-D-gluco-pyranosiduronic acid |
| Nitroreductase | 5-(Azaridin-1-yl-)-2,4-dinitrobenzamide |
| Cytosine deaminase | 5-Fluorocytosine |
| Carboxypeptidase A | Methotrexate-alanine |

(This table is adapted from Bagshawe (1995) *Drug Dev. Res.* **34**, 220-230, from which full references for these various systems may be obtained; the taxol derivative is described in Rodrigues, M.L. *et al* (1995) *Chemistry & Biology* **2**, 223).

Suitable enzymes for forming part of the enzymatic portion a compound of the invention include: exopeptidases, such as carboxypeptidases G, G1 and G2 (for glutamylated mustard prodrugs), carboxypeptidases A and B (for MTX-based prodrugs) and aminopeptidases (for 2-α-aminocyl MTC prodrugs);

endopeptidases, such as *e.g.* thrombolysin (for thrombin prodrugs); hydrolases, such as phosphatases (*e.g.* alkaline phosphatase) or sulphatases (*e.g.* aryl sulphatases) (for phosphylated or sulphated prodrugs); amidases, such as penicillin amidases and arylacyl amidase; lactamases, such as β-lactamases; glycosidases, such as β-glucuronidase (for β-glucuronomide anthracyclines), α-galactosidase (for amygdalin) and β-galactosidase (for β-galactose anthracycline); deaminases, such as cytosine deaminase (for 5FC); kinases, such as urokinase and thymidine kinase (for gancyclovir); reductases, such as nitroreductase (for CB1954 and analogues), azoreductase (for azobenzene mustards) and DT-diaphorase (for CB1954); oxidases, such as glucose oxidase (for glucose), xanthine oxidase (for xanthine) and lactoperoxidase; DL-racemases, catalytic antibodies and cyclodextrins.

Preferably, the prodrug is relatively non-toxic compared to the cytotoxic drug. Typically, it has less than 10% of the toxicity, preferably less than 1% of the toxicity as measured in a suitable *in vitro* cytotoxicity test.

It is likely that the moiety which is able to convert a prodrug to a cytotoxic drug will be active in isolation from the rest of the compound but it is necessary only for it to be active when (a) it is in combination with the rest of the compound and (b) the compound is attached to, adjacent to or internalised in target cells.

When each moiety of the compound is a polypeptide, the two portions may be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al* (1979) *Anal. Biochem.* **100**, 100-108. For example, the binding moiety may be enriched with thiol groups and the further moiety reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

Alternatively, the compound may be produced as a fusion compound by recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two moieties of the compound of the invention either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the compound. Conceivably, the two portions of the compound may overlap wholly or partly.

In a further preferred embodiment, the cytotoxic moiety may be a radiosensitizer. Radiosensitizers include fluoropyrimidines, thymidine analogues, hydroxyurea, gemcitabine, fludarabine, nicotinamide, halogenated pyrimidines, 3-aminobenzamide, 3-aminobenzodiamide, etanixadole, pimonidazole and misonidazole (see, for example, McGinn *et al* (1996) *J. Natl. Cancer Inst.* **88**, 1193-11203; Shewach & Lawrence (1996) *Invest. New Drugs* **14**, 257-263; Horsman (1995) *Acta Oncol.* **34**, 571-587; Shenoy & Singh (1992) *Clin. Invest.* **10**, 533-551; Mitchell *et al* (1989) *Int. J. Radiat. Biol.* **56**, 827-836; Iliakis & Kurtzman (1989) *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 1235-1241; Brown (1989) *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 987-993; Brown (1985) *Cancer* **55**, 2222-2228).

Also, delivery of genes into cells can radiosensitise them, for example delivery of the p53 gene or cyclin D (Lang *et al* (1998) *J. Neurosurg.* **89**, 125-132; Coco Martin *et al* (1999) *Cancer Res.* **59**, 1134-1140).

The further moiety may be one which becomes cytotoxic, or releases a cytotoxic moiety, upon irradiation. For example, the boron-10 isotope, when appropriately irradiated, releases $\alpha$ particles which are cytotoxic (for example, see US 4, 348, 376 to Goldenberg; Primus *et al* (1996) *Bioconjug. Chem.* 7, 532-535).

Similarly, the cytotoxic moiety may be one which is useful in photodynamic therapy such as photofrin (see, for example, Dougherty *et al* (1998) *J. Natl. Cancer Inst.* **90**, 889-905).

The further moiety may comprise a nucleic acid molecule which is directly or indirectly cytotoxic. For example, the nucleic acid molecule may be an antisense oligonucleotide which, upon localisation at the target site is able to enter cells and lead to their death. The oligonucleotide, therefore, may be one which prevents expression of an essential gene, or one which leads to a change in gene expression which causes apoptosis.

Examples of suitable oligonucleotides include those directed at bcl-2 (Ziegler *et al* (1997) *J. Natl. Cancer Inst.* **89**, 1027-1036), and DNA polymerase α and topoisomerase IIα (Lee *et al* (1996) *Anticancer Res.* **16**, 1805-1811.

Peptide nucleic acids may be useful in place of conventional nucleic acids (see Knudsen & Nielsen (1997) *Anticancer Drugs* **8**, 113-118).

In a further embodiment, the binding moiety may be comprised in a delivery vehicle for delivering nucleic acid to the target. The delivery vehicle may be any suitable delivery vehicle. It may, for example, be a liposome containing nucleic acid, or it may be a virus or virus-like particle which is able to deliver nucleic acid. In these cases, the binding moiety is typically present on the surface of the delivery vehicle. For example, the binding moiety, such as a suitable antibody fragment, may be present in the outer surface of a liposome and the nucleic acid to be delivered may be present in the interior of the liposome. As another example, a viral vector, such as a retroviral or adenoviral vector, is engineered so that the binding moiety is attached to or located in the surface of the viral particle thus enabling the viral particle to be targeted to the desired site. Targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Targeted retroviruses are also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into pre-existing viral *env* genes (see Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a review of this and other targeted vectors for gene therapy).

Immunoliposomes (antibody-directed liposomes) may be used in which the binding moiety is an antibody. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)-butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

The nucleic acid delivered to the target site (*i.e.* malignant tumour cell) may be any suitable DNA which leads, directly or indirectly, to cytotoxicity. For example, the nucleic acid may encode a ribozyme which is cytotoxic to the cell, or it may encode an enzyme which is able to convert a substantially non-toxic prodrug into a cytotoxic drug (this latter system is sometime called GDEPT: Gene Directed Enzyme Prodrug Therapy).

Ribozymes which may be encoded in the nucleic acid to be delivered to the target are described in Cech and Herschlag "Site-specific cleavage of single stranded DNA" US 5,180,818; Altman *et al* "Cleavage of targeted RNA by RNAse P" US 5,168,053, Cantin *et al* "Ribozyme cleavage of HIV-1 RNA" US 5,149,796; Cech *et al* "RNA ribozyme restriction endoribonucleases and methods", US 5,116,742; Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction

endonucleases and methods", US 5,093,246; and Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods; cleaves single-stranded RNA at specific site by transesterification", US 4,987,071, all incorporated herein by reference. Suitable targets for ribozymes include transcription factors such as c-fos and c-myc, and bcl-2. Durai *et al* (1997) *Anticancer Res.* **17**, 3307-3312 describes a hammerhead ribozyme against bcl-2.

EP 0 415 731 describes the GDEPT system. Similar considerations concerning the choice of enzyme and prodrug apply to the GDEPT system as to the ADEPT system described above.

The nucleic acid delivered to the target site may encode a directly cytotoxic polypeptide.

Alternatively, the further moiety may comprise a polypeptide or a polynucleotide encoding a polypeptide which is not either directly or indirectly cytotoxic but is of therapeutic benefit. Examples of such polypeptides include anti-proliferative or anti-inflammatory cytokines, and anti-proliferative, immunomodulatory or factors influencing blood clotting which may be of benefit in treating malignant tumours.

The further moiety may usefully be an inhibitor of angiogenesis such as the peptides angiostatin or endostatin. The further moiety may also usefully be an enzyme which converts a precursor polypeptide to angiostatin or endostatin. Human matrix metallo-proteases such as macrophage elastase, gelatinase and stromolysin convert plasminogen to angiostatin (Cornelius *et al* (1998) *J. Immunol.* **161**, 6845-6852). Plasminogen is a precursor of angiostatin.

In a further embodiment of the invention, the further moiety comprised in the compound of the invention is a readily detectable moiety.

By a "readily detectable moiety" we include the meaning that the moiety is one which, when located at the target site following administration of the compound of the invention into a patient, may be detected, typically non-invasively from outside the body and the site of the target located. Thus, the compounds of this embodiment of the invention are useful in imaging and diagnosis.

Typically, the readily detectable moiety is or comprises a radioactive atom which is useful in imaging. Suitable radioactive atoms include $^{99m}$Tc and $^{123}$I for scintigraphic studies. Other readily detectable moieties include, for example, spin labels for magnetic resonance imaging (MRI) such as $^{123}$I again, $^{131}$I, $^{111}$In, $^{19}$F, $^{13}$C, $^{15}$N, $^{17}$O, gadolinium, manganese or iron. Clearly, the compound for use in the invention must have sufficient of the appropriate atomic isotopes in order for the molecule to be readily detectable.

The radio- or other labels may be incorporated in the compound of the invention in known ways. For example, if the binding moiety is a polypeptide it may be biosynthesised or may be synthesised by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as $^{99m}$Tc, $^{123}$I, $^{186}$Rh, $^{188}$Rh and $^{111}$In can, for example, be attached *via* cysteine residues in the binding moiety. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker *et al* (1978) *Biochem. Biophys. Res. Comm.* **80**, 49-57) can be used to incorporate $^{123}$I. Reference ("Monoclonal Antibodies in Immunoscintigraphy", J-F Chatal, CRC Press, 1989) describes other methods in detail.

In a further preferred embodiment of the invention the further moiety is able to bind selectively to a directly or indirectly cytotoxic moiety or to a readily detectable moiety. Thus, in this embodiment, the further moiety may be any moiety which binds to a further compound or component which is cytotoxic or readily detectable.

The further moiety may, therefore be an antibody which selectively binds to the further compound or component, or it may be some other binding moiety such as

streptavidin or biotin or the like. The following examples illustrate the types of molecules that are included in the invention; other such molecules are readily apparent from the teachings herein.

For example, the compound may comprise or consist of a bispecific antibody wherein one binding site comprises the binding moiety (which selectively binds to the integrin alpha-10 subunit or a heterodimer thereof) and the second binding site comprises a moiety which binds to, for example, an enzyme which is able to convert a substantially non-toxic prodrug to a cytotoxic drug.

Alternatively, the compound may comprise an antibody which selectively binds to the integrin alpha-10 subunit or a heterodimer thereof, to which is bound biotin. Avidin or streptavidin which has been labelled with a readily detectable label may be used in conjunction with the biotin labelled antibody in a two-phase imaging system wherein the biotin labelled antibody is first localised to the target site in the patient, and then the labelled avidin or streptavidin is administered to the patient. Bispecific antibodies and biotin/streptavidin (avidin) systems are reviewed by Rosebrough (1996) *Q J Nucl. Med.* **40**, 234-251.

In a preferred embodiment of the invention, the binding moiety and the further moiety are polypeptides which are fused.

The uses of the above aspects of the invention provide agents and medicaments for treating, imaging and/or diagnosing malignant tumours, *e.g.* melanoma, as described in more detail below. In a preferred embodiment, the malignant tumour is metastatic.

Thus, the invention provides medicaments for inhibiting the migration of tumour cells (for example, towards a chemoattractant).

It will be appreciated by persons skilled in the art that compounds used in the above-described aspects of the invention are preferably provided in the form of a pharmaceutical composition comprising the compound and a pharmaceutically

acceptable carrier. By "pharmaceutically acceptable" is included that the formulation is sterile and pyrogen free. Suitable pharmaceutical carriers are well known in the art of pharmacy. The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free; however, other acceptable carriers may be used. Thus, "pharmaceutically acceptable carrier" and "pharmaceutically acceptable excipient" includes any compound(s) used in forming a part of the formulation that is intended to act merely as a carrier, *i.e.*, not intended to have biological activity itself. The pharmaceutically acceptable carrier or excipient is generally safe, non-toxic, and neither biologically nor otherwise undesirable. A pharmaceutically acceptable carrier or excipient as used herein includes both one and more than one such carrier or excipient.

The terms "treating", and "treatment", and the like are used herein to generally mean obtaining a desired pharmacological and physiological effect. Further, it refers to any process, action, application, therapy, or the like, wherein a mammal, including a human being, is subject to medical aid with the object of improving the mammal's condition, directly or indirectly. More specifically, it may be one or more of the following inhibiting, preventing, alleviating malignant melanoma, reduction, slowing, inhibition of melanoma cell migration, loss of metastatic lesions in any solid tumour, inhibited or reduced development of new metastatic lesions in any solid tumour after treatment has started.

The term "inhibition" in the context of neoplasia, tumour growth, metastases, invasiveness, *etc.*, may be assessed by delayed appearance of primary or secondary tumours, slowed development of primary or secondary tumours, decreased occurrence of primary or secondary tumours, slowed or decreased severity of secondary effects of disease, arrested tumour growth and regression of tumours, among others. In the extreme, complete inhibition, is referred to herein as prevention.

The term "prevention" includes either preventing the onset of clinically evident neoplasia , tumour growth, metastases, invasiveness, preventing onset of primary or secondary tumours, etc., altogether or preventing the onset of a preclinically evident stage of neoplasia, tumour growth, metastases, invasiveness, onset of primary or secondary tumours in individuals at risk. Also intended to be encompassed by this definition is the prevention of initiation for malignant cells or to arrest or reverse the progression of premalignant cells to malignant cells. This includes prophylactic treatment of those at risk of developing the neoplasia, tumour growth, metastases, invasive tumours, and onset of primary or secondary tumours.

The compounds for use in the invention can be formulated at various concentrations, depending on the efficacy/toxicity of the compound being used. Preferably, the formulation comprises the agent of the invention at a concentration of between 0.1 μM and 1 mM, more preferably between 1 μM and 100 μM, between 5 μM and 50 μM, between 10 μM and 50 μM, between 20 μM and 40 μM and most preferably about 30 μM. For *in vitro* applications, formulations may comprise a lower concentration of a compound of the invention, for example between 0.0025 μM and 1 μM.

It will be appreciated by persons skilled in the art that the medicaments and agents will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice (for example, see *Remington: The Science and Practice of Pharmacy*, 19th edition, 1995, Ed. Alfonso Gennaro, Mack Publishing Company, Pennsylvania, USA).

For example, the medicaments and agents can be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications. The medicaments and agents may also be administered via intracavernosal injection.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxy-propylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

The medicaments and agents of the invention can also be administered parenterally, for example, intravenously, intra-articularly, intra-arterially, intraperitoneally, intra-thecally, intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be

presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

For oral and parenteral administration to human patients, the daily dosage level of the medicaments and agents will usually be from 1 to 1000 mg per adult (*i.e.* from about 0.015 to 15 mg/kg), administered in single or divided doses.

The medicaments and agents can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, *e.g.* dichlorodifluoromethane, trichlorofluoro-methane, dichlorotetrafluoro-ethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A3 or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, *e.g.* using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, *e.g.* sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose or 'puff' contains at least 1 mg of a compound of the invention for delivery to the patient. It will be appreciated that the overall daily dose with an aerosol will vary from patient to patient, and may be administered in a single dose or, more usually, in divided doses throughout the day.

Alternatively, the medicaments and agents can be administered in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. The compounds of the invention may also be transdermally administered, for example, by the use of a skin patch. They may also be administered by the ocular route.

For application topically to the skin, the medicaments and agents can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Where the medicament or agent is a polypeptide, it may be preferable to use a sustained-release drug delivery system, such as a microsphere. These are designed specifically to reduce the frequency of injections. An example of such a system is Nutropin Depot which encapsulates recombinant human growth hormone (rhGH) in biodegradable microspheres that, once injected, release rhGH slowly over a sustained period.

Sustained-release immunoglobulin compositions also include liposomally entrapped immunoglobulin. Liposomes containing the immunoglobulin are prepared by methods known *per se*. See, for example Epstein *et al.*, *Proc. Natl. Acad. Sci. USA* **82**: 3688-92 (1985); Hwang *et al.*, *Proc. Natl. Acad. Sci. USA* **77**:

4030-4 (1980); U.S. Patent Nos. 4,485,045; 4,544, 545; 6,139,869; and 6,027,726. Ordinarily, the liposomes are of the small (about 200 to about 800 Angstroms), unilamellar type in which the lipid content is greater than about 30 mole percent (mol. %) cholesterol; the selected proportion being adjusted for the optimal immunoglobulin therapy.

Alternatively, polypeptide medicaments and agents can be administered by a surgically implanted device that releases the drug directly to the required site.

Electroporation therapy (EPT) systems can also be employed for the administration of proteins and polypeptides. A device which delivers a pulsed electric field to cells increases the permeability of the cell membranes to the drug, resulting in a significant enhancement of intracellular drug delivery.

Proteins and polypeptides can also be delivered by electroincorporation (EI). EI occurs when small particles of up to 30 microns in diameter on the surface of the skin experience electrical pulses identical or similar to those used in electroporation. In EI, these particles are driven through the stratum corneum and into deeper layers of the skin. The particles can be loaded or coated with drugs or genes or can simply act as "bullets" that generate pores in the skin through which the drugs can enter.

An alternative method of protein and polypeptide delivery is the thermo-sensitive ReGel injectable. Below body temperature, ReGel is an injectable liquid while at body temperature it immediately forms a gel reservoir that slowly erodes and dissolves into known, safe, biodegradable polymers. The active drug is delivered over time as the biopolymers dissolve.

Protein and polypeptide pharmaceuticals can also be delivered orally. One such system employs a natural process for oral uptake of vitamin B12 in the body to co-deliver proteins and polypeptides. By riding the vitamin B12 uptake system, the protein or polypeptide can move through the intestinal wall. Complexes are produced between vitamin B12 analogues and the drug that retain both significant

affinity for intrinsic factor (IF) in the vitamin B12 portion of the complex and significant bioactivity of the drug portion of the complex.

A fourth aspect of the invention provides a method of imaging malignant tumour cells in the body of an individual, the method comprising administering to the individual an effective amount of a compound as defined above.

In a preferred embodiment of this aspect of the invention, the method comprises the further step of detecting the location of the compound in the individual. Detecting the compound or antibody can be achieved using methods well known in the art of clinical imaging and diagnostics. The specific method required will depend on the type of detectable label attached to the compound or antibody. For example, radioactive atoms may be detected using autoradiography or in some cases by magnetic resonance imaging (MRI) as described above.

A fifth aspect of the invention provides a method of diagnosing or prognosing a malignant tumour in an individual, the method comprising administering to the individual an effective amount of a compound as defined above. The method may be one which is an aid to diagnosis, *i.e.* additional tests may be required in order to reach a firm diagnosis.

In a preferred embodiment of this aspect of the invention, the method of diagnosing, or aiding diagnosis of, a malignant tumour in an individual comprises the further step of detecting the location of the compound in the individual.

A sixth aspect of the invention provides a method of treating an individual in need of treatment, the method comprising administering to the individual an effective amount of a compound as defined above. Preferably, the patient in need of treatment has a malignant tumour, *e.g.* a melanoma.

Persons skilled in the art will further appreciate that the medicaments and agents described above have utility in both the medical and veterinary fields. Thus, the medicaments and agents may be used in the treatment of both human and non-

human animals (such as horses, dogs and cats). Preferably, however, the patient is human.

A 'therapeutically effective amount', or 'effective amount', or 'therapeutically effective', as used herein, refers to that amount which provides a therapeutic effect in the treatment of malignant tumours for a given administration regimen. This is a predetermined quantity of active material calculated to produce a desired therapeutic effect, *e.g.* reduced migration of tumour cells, and/or reduction of metastatic lesions as assessed, for example, by radiologic imaging, in association with the required additive and diluent, *i.e.* a carrier or administration vehicle. Further, it is intended to mean an amount sufficient to reduce and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in a host. As is appreciated by those skilled in the art, the amount of a compound may vary depending on its specific activity. Suitable dosage amounts may contain a predetermined quantity of active composition calculated to produce the desired therapeutic effect in association with the required diluent. In the methods and use for manufacture of compositions of the invention, a therapeutically effective amount of the active component is provided. A therapeutically effective amount can be determined by the ordinary skilled medical or veterinary worker based on patient characteristics, such as age, weight, sex, condition, complications, other diseases, *etc.*, as is well known in the art.

Thus, in a preferred embodiment, the method comprises administering to the individual an amount of the compound sufficient to inhibit a biological activity of an integrin alpha-10 subunit or heterodimer thereof in malignant tumour (*e.g.* melanoma) cells.

It will be appreciated by persons skilled in the art that such an effective amount of the compound or formulation thereof may be delivered as a single bolus dose (*i.e.* acute administration) or, more preferably, as a series of doses over time (*i.e.* chronic administration).

Depending on the particular compound used in imaging, diagnosis or treatment, the timing of administration may vary and the number of other components used in therapeutic systems disclosed herein may vary.

For example, in the case where the compound of the invention comprises a readily detectable moiety or a directly cytotoxic moiety, it may be that only the compound, in a suitable formulation, is administered to the patient. Of course, other agents such as immunosuppressive agents and the like may be administered.

In the case of compounds which are detectably labelled, imaging takes place once the compound has localised at the target site.

However, if the compound is one which requires a further component in order to be useful for treatment, imaging or diagnosis, the compound of the invention may be administered and allowed to localise at the target site, and then the further component administered at a suitable time thereafter.

For example, in the case of the ADEPT and ADEPT-like systems above, the binding moiety-enzyme moiety compound is administered and localises to the target site. Once this is done, the prodrug is administered.

Similarly, for example, in respect of the compounds wherein the further moiety comprised in the compound is one which binds a further component, the compound may be administered first and allowed to localise at the target site, and subsequently the further component is administered.

Thus, in one embodiment a biotin-labelled antibody is administered to the patient and, after a suitable period of time, detectably labelled streptavidin is administered. Once the streptavidin has localised to the sites where the antibody has localised (*i.e.* the target sites) imaging takes place.

A seventh aspect of the invention provides a method for monitoring the progression of a malignant tumour in an individual, the method comprising:

(a) providing a sample of malignant tumour cells collected from the individual at a first time point and measuring the amount of integrin alpha-10 subunit protein therein;

(b) providing a sample of malignant tumour cells collected from the individual at a second time point and measuring the amount of integrin alpha-10 subunit protein therein; and

(c) comparing the level of integrin alpha-10 subunit protein measured in steps (a) and (b)

wherein an increased amount of integrin alpha-10 subunit protein measured in step (b) compared to step (a) is indicative of a progression in the malignant tumour.

An eighth aspect of the invention provides a method of identifying cells associated with malignant tumour, the method comprising measuring the amount of integrin alpha-10 subunit protein in a sample of cells to be tested and comparing it to the amount of integrin alpha-10 subunit protein in a sample of known malignant tumour cells.

In a preferred embodiment of the eighth aspect of the invention, the method further comprises comparing the amount of integrin alpha-10 subunit protein in a sample of cells to be tested with the amount of integrin alpha-10 subunit protein in a control sample. Conveniently, the control sample comprises corresponding healthy (*i.e.* non-tumour) cells. For example, when the tumour is a malignant melanoma, the control cells may be normal epidermal melanocytes.

Advantageously, the cells to be tested are identified as malignant tumour cells by the upregulation of integrin alpha-10 subunit protein levels compared to

corresponding normal healthy cells. By "upregulated" we mean that the integrin alpha-10 subunit protein is increased by at least 10% compared to expression of the same gene in normal cells. Preferably, the level of the integrin alpha-10 subunit is protein increased by at least 20%, 30%, 40% or 50%. Most preferably amount of the integrin alpha-10 subunit is increased by at least 100%.

A further aspect of the invention provides a method of distinguishing between different types or stages of malignant tumour, the method comprising measuring the amount of integrin alpha-10 subunit protein in a sample of cells to be tested and comparing it to the amount of integrin alpha-10 subunit protein in a sample of malignant tumour cells of a known type or stage.

Preferably, the known malignant tumour cells are characterised by the upregulation of integrin alpha-10 subunit protein compared to normal non-tumour cells.

The amount of integrin alpha-10 subunit in a sample may be determined using methods well known in the art. Preferred for assaying integrin alpha-10 protein levels in a biological sample are antibody-based techniques. For example, integrin alpha-10 protein expression in tissues can be studied with classical immunohistological methods. In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilize fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, *e.g.* with urea and neutral detergent, for the liberation of integrin alpha-10 protein for westem blot or dot/slot assay (Jalkanen *et al.*, 1985, J. *Cell. Biol.* **101**:976-985; Jalkanen et *al.*, 1987, *J. Cell. Biol.* **105**:3087-3096). In this technique, which is based on the use of cationic solid phases, quantitation of integrin alpha-10 protein can be accomplished using isolated integrin alpha-10 protein as a standard. This technique can also be applied to body fluids.

A ninth aspect of the invention is a method of screening for (*i.e.* identifying) candidate compounds with efficacy in the treatment of malignant tumours, the method comprising the steps of:

(a) contacting a molecule to be tested with an integrin alpha-10 subunit (or a fragment or binding sequence thereof); and

(b) detecting the presence of a complex containing the integrin alpha-10 subunit (or fragment thereof) and the molecule to be tested

the molecule to be tested being identified as a candidate compound if the complex is detected in step(b).

Persons skilled in the art will appreciate that the methods of the above aspects of the invention may be used for any tumour found to over-express an integrin alpha-10 subunit. Preferably, the tumour is selected from the group consisting of tumours of the breast, bile duct, brain, colon, stomach, reproductive organs, lung and airways, skin, gallbladder, liver, nasopharynx, nerve cells, kidney, prostate, lymph glands and gastrointestinal tract. Most preferably, however, the tumour is a malignant melanoma.

In a preferred embodiment, the test molecule is a polypeptide.

Suitable peptide ligands that will bind to an integrin alpha-10 subunit (or fragments or derivatives thereof) may be identified using methods known in the art.

One method, disclosed by Scott and Smith (1990) *Science* **249**, 386-390 and Cwirla *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6378-6382, involves the screening of a vast library of filamentous bacteriophages, such as M13 or fd, each member of the library having a different peptide fused to a protein on the surface of the bacteriophage. Those members of the library that bind to integrin alpha-10 subunit (or a fragment thereof) or a heterodimer thereof are selected using an iterative binding protocol, and once the phages that bind most tightly have been purified, the

sequence of the peptide ligands may be determined simply by sequencing the DNA encoding the surface protein fusion. Another method that can be used is the NovaTope (TM) system commercially available from Novagen, Inc., 597 Science Drive, Madison, WI 53711. The method is based on the creation of a library of bacterial clones, each of which stably expresses a small peptide derived from a candidate protein in which the ligand is believed to reside. The library is screened by standard lift methods using the antibody or other binding agent as a probe. Positive clones can be analysed directly by DNA sequencing to determine the precise amino acid sequence of the ligand.

Further methods using libraries of beads conjugated to individual species of peptides as disclosed by Lam *et al* (1991) *Nature* **354**, 82-84 or synthetic peptide combinatorial libraries as disclosed by Houghten *et al* (1991) *Nature* **354**, 84-86 or matrices of individual synthetic peptide sequences on a solid support as disclosed by Pirrung *et al* in US 5143854 may also be used to identify peptide ligands.

It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. Examples may include cell based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham International) system may be used. For example, an assay for identifying a compound capable of modulating the activity of a protein kinase may be performed as follows. Beads comprising scintillant and a polypeptide that may be phosphorylated may be prepared. The beads may be mixed with a sample comprising the protein kinase and $^{32}$P-ATP or $^{33}$P-ATP and with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for $^{32}$P or $^{33}$P SPA assays. Only $^{32}$P or $^{33}$P that is in proximity to the scintillant, i.e. only that bound to the polypeptide, is detected. Variants of such an assay, for example in which the polypeptide is immobilised on the scintillant beads *via* binding to an antibody, may also be used.

Other methods of detecting polypeptide/polypeptide interactions include ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical

and analytical methods. Fluorescence Energy Resonance Transfer (FRET) methods, for example, well known to those skilled in the art, may be used, in which binding of two fluorescent labelled entities may be measured by measuring the interaction of the fluorescent labels when in close proximity to each other.

Alternative methods of detecting binding of a polypeptide to macromolecules, for example DNA, RNA, proteins and phospholipids, include a surface plasmon resonance assay, for example as described in Plant *et al* (1995) *Analyt Biochem* **226(2)**, 342-348. Methods may make use of a polypeptide that is labelled, for example with a radioactive or fluorescent label.

A further method of identifying a compound that is capable of binding to an integrin alpha-10 subunit is one where the polypeptide is exposed to the compound and any binding of the compound to the said polypeptide is detected and/or measured. The binding constant for the binding of the compound to the polypeptide may be determined. Suitable methods for detecting and/or measuring (quantifying) the binding of a compound to a polypeptide are well known to those skilled in the art and may be performed, for example, using a method capable of high throughput operation, for example a chip-based method. New technology, called VLSIPS™, has enabled the production of extremely small chips that contain hundreds of thousands or more of different molecular probes. These biological chips or arrays have probes arranged in arrays, each probe assigned a specific location. Biological chips have been produced in which each location has a scale of, for example, ten microns. The chips can be used to determine whether target molecules interact with any of the probes on the chip. After exposing the array to target molecules under selected test conditions, scanning devices can examine each location in the array and determine whether a target molecule has interacted with the probe at that location.

Biological chips or arrays are useful in a variety of screening techniques for obtaining information about either the probes or the target molecules. For example, a library of peptides can be used as probes to screen for drugs. The peptides can be exposed to a receptor, and those probes that bind to the receptor

can be identified. See US Patent No. 5,874,219 issued 23 February 1999 to Rava *et al.*

It will be understood that it will be desirable to identify compounds that may modulate the activity of an integrin alpha-10 subunit *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the interactions between the said and the interacting polypeptide are substantially the same as between a said naturally occurring polypeptide and a naturally occurring interacting polypeptide *in vivo*.

It will be appreciated that in the method described herein, the ligand may be a drug-like compound or lead compound for the development of a drug-like compound.

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

Alternatively, the methods may be used as "library screening" methods, a term well known to those skilled in the art. Thus, for example, the method of the invention may be used to detect (and optionally identify) a polynucleotide capable of expressing a polypeptide activator of a protein listed in Table 1. Aliquots of an expression library in a suitable vector may be tested for the ability to give the required result.

Preferably, the compound decreases the activity of the integrin alpha-10 subunit. For example, the compound may bind substantially reversibly or substantially irreversibly to the active site of said protein. In a further example, the compound may bind to a portion of said protein that is not the active site so as to interfere with the binding of the said protein to its ligand. In a still further example, the compound may bind to a portion of said protein so as to decrease said protein's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said protein's activity, for example in the activation of the said protein by an "upstream activator".

Preferred aspects of the invention are described in the following non-limiting examples, with reference to the following figures:

**Figure 1 - Integrin alpha10 expression in melanoma cell lines compared to primary melanocytes**

**A.** By real-time PCR the amount of integrin alpha10 mRNA expression was carefully quantified. All melanoma cell lines showed strong induction of integrin alpha10 expression compared to normal human epidermal melanocytes (NHEM) except of SK Mel 3 and SK Mel 28. For abbreviations, see 'Materials and methods' below.

**B.** The amount of integrin alpha10 mRNA in RNA extracted from tissue samples of malignant melanoma by microdissection was measured by real-time PCR. Expression was compared to normal human epidermal melanocytes (NHEM). Induction of expression of integrin alpha10 was found in all melanoma samples.

Key: Prim tumour = primary tumour, LN met = lymph node metastases, Skin met= skin metastases, lung met= lung metastases, intest.met = intestinal metastases..

**C.** Integrin alpha10 protein expression in chondrocytes, primary human melanocytes and melanoma cell lines detected by western blot analysis. In melanoma cell lines expression was induced. As loading control the blot was counterstained with a ß-actin antibody.

**D.** Immunostaining of integrin alpha10 revealed strong signals in primary melanoma, as observed at a magnification of (i) 200 times and (ii) 400 times.

**E.** Analysis of collagen type II expression in melanoma cell lines by PCR. In all examined melanoma cell lines collagen type II is transcribed.

**Figure 2 - Effect of reduction of integrin alpha10 on migration and invasion**

Analysis of the cell clones expressing less integrin alpha10 revealed a reduction of the migratory potential of the as cell clones 4 and 6 in comparison to Mel Im cells and mock control as shown in Boyden Chamber assays (**A**). The same effect was shown using an inhibitory antibody against integrin alpha10 (mAb365, as described in WO 2004/089990). Mel Im cells which were incubated with the antibody had a less migratory potential compared to untreated Mel Im cells (**B**). There were no significant changes of the invasive potential of the as integrin alpha10 cell clones compared to Mel Im cells and the mock control (**C**).

To analyze the effect of integrin alpha10 on undirectional migration, wound healing assays were performed. No significant differences in undirectional migration could be detected comparing the as cell clones 4, 5 and 6 to the wildtype Mel Im cells and the mock control (**D**).

Assays were performed in triplicate. Asterisks indicate P values ($p<0.05$).

**EXAMPLE A**

***Material and Methods***

Cell culture

The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, Mel Ju, SK Mel 28, SK Mel 3, and HTZ19d were described previously (23). The cell lines Mel Ei, Mel Wei, Mel Ho and Mel Juso were derived from a primary cutaneous melanoma, Mel Im, Mel Ju, SK Mel 28, SK Mel 3, and HTZ19d were derived from metastases of malignant melanomas. Cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycine (50 µg/ml), L-glutamine (300 µg/ml) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and split at a 1:5 ratio every three days. Normal human epidermal melanocytes (NHEM) derived from normal skin were cultivated in melanocyte medium MGM-3 (Promocell, Heidelberg, Germany) under a humified atmosphere of 5% $CO_2$ at 37°C. Cell proliferation was determined using the XTT assay (Roche, Mannheim, Germany).

For the arrays: 5 foreskin melanocytes (FOM), 12 cell lines derived from primary melanomas and 15 cell lined derived from melanoma metastases (previously described in Hsu MY, Elder DE, Herlyn M (1999): Melanoma: The Wistar (WM) melanoma cell lines. In: Human Cell Culture, Vol. 3 (Masters, J.R.W. and (Palsson, B., eds.) Kluwer Acad. Publ., London, pp. 259-274.)

RNA isolation and reverse transcription

Total cellular RNA was isolated from cultured cells or from microdissected tissues using the RNeasy kit (QIAGEN, Hilden, Germany) and cDNAs were generated by reverse transcriptase reaction performed in 20µl reaction volume containing 2 µg of total cellular RNA, 4 µl of 5x first strand buffer (Invitrogen, Groningen, The Netherlands), 2 µl of 0.1 M DTT, 1 µl of $dN_6$-primer (10mM), 1 µl of dNTPs (10mM) and DEPC-water. The reaction mixture was incubated for

10 min at 70°C, 200 units of Superscript II reverse transcriptase (Invitrogen) were added and RNAs were transcribed for 1 hour at 37°C. Reverse transcriptase was inactivated at 70°C for 10 minutes and the RNA was degraded by digestion with 1 µl RNase A (10 mg/ml) at 37°C for 30 minutes.

Microarray-Based Gene Expression Analysis

Total RNA was isolated from cell cultures at 70% confluency by use of TRIzol reagent (Invitrogen, Carlsbad, CA), as suggested by the manufacturer. We used 5 µg of total RNA to synthesize double-stranded cDNA (Superscript Choice System for cDNA Synthesis kit; Invitrogen) and after cleanup (GeneChip Sample Cleanup Module; Qiagen/Affymetrix, Santa Clara, CA), to produce fragmented biotin-labeled cRNA (Enzo RNA Transcript Labeling Kit; Enzo, Farmingdale, NY), using the manufacturers' protocols. Human HG-U133A chips (Affymetrix, Inc.) were hybridized with 15 µg of fragmented labeled cRNA overnight at 45°C, washed (Genechip Fluidics Station 400; Affymetrix), and scanned (GeneArray Scanner; Affymetrix) according to Affymetrix protocols. Scanned images were analyzed with the MAS 5.0 software (Affymetrix), and intensities were scaled to a value of 500. Cluster 3.0 was used for visualization purposes.

Expression analysis

RT-PCR analysis of integrin alpha10 was performed using specific primers (alpha10-forward: 5'- CAT GAG GTT CAC CGC ATC ACT- 3' [**SEQ ID NO:2**] and alpha10-reverse: 5'-AAG GCA AAG GTC ACA GTC AAG G - 3' [**SEQ ID NO:3**] (192 bp fragment)). The PCR reaction was performed in a 50 µl reaction volume containing 5 µl 10x Taq-buffer, 1 µl of cDNA, 0,5 µl of each primer (20 mM), 0.5 µl of dNTPs (10 mM), 1 Units of Taq polymerase and 41,5 µl of water. The amplification reactions were performed by 36 cycles of 1 min at 94°C, 1 min at 64°C and a final extension step at 72°C for 1.5 min. The PCR products were resolved on 1.5% agarose gels.

Analysis of expression by quantitative PCR

Quantitative real time-PCR was performed on a Lightcycler (Roche, Mannheim, Germany). cDNA template (2 µl), 2,4 µl 25mM $MgCl_2$, 0.5 µl (20 mM) of forward and reverse primers and 2µl of SybrGreen LightCycler Faststart Mix in a total of 20 µl were applied to the following PCR program: 10 min 95°C (initial denaturation); 20°C/sec temperature transition rate up to 95°C for 15 sec, 3 sec 64°C, 5 sec 72°C, 86°C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis and checking the PCR products on 1.8% agarose gels.

Western Blot analysis

3 x $10^6$ cells were lysed in 200µl RIPA-buffer (Roche) and incubated for 15 minutes at 4°C. Insoluble fragments were removed by centrifugation at 13000 rpm for 10 minutes and the supernatant lysate was immediately shock frozen and stored at -80°C. RIPA-cell lysate was loaded and separated on SDS-PAGE gradient gels (Invitrogen) and subsequently blotted onto a PVDF membrane. After blocking for 1 hour with 2% BSA / TBST (0,05% Tween) the membrane was incubated for 16 hours with the primary antibody (polyclonal anti-integrin alpha10 antibody (1:2000) and beta-actin (Sigma, 1:2500). Then the membrane was washed three times in TBST, incubated for 1 hour with 1:3000 of an alkaline phosphate-coupled secondary antibody (Chemicon) and then washed again. Finally immunoreactions were visualized by NBT / BCIP (Sigma) staining.

Immunohistochemistry

Cryosections of primary melanoma and metastases of malignant melanomas were screened for integrin alpha10 protein expression by immunohistochemistry. The tissues were fixed and subsequently incubated with primary polyclonal anti-integrin alpha10 antibody (1:120) over night at 4°C (20). The secondary antibody (biotin-labeled anti-rabbit, DAKO, Germany) was incubated for 30 minutes at room temperature, followed by incubation with streptavidin-POD (DAKO) for 30

minutes. Antibody binding was visualized using AEC-solution (DAKO). Finally, the tissues were counterstained by haemalaun solution (DAKO).

Plasmid Constructs

To construct the integrin alpha10 promoter 5'-deletion constructs, the human genomic region was amplified by polymerase chain reaction (PCR) with a 3'-reverse primer (5′-GACAAGCTTGCCTGATCGGTTTCTGTC-3′) [**SEQ ID NO:4**] that bound at -3 relative to the integrin alpha10 translational start site in conjunction with different 5'-forward primers that bound at varying distances within the upstream flanking sequence (-1139: 5′-GACGCTAGCACCTAGCTGAGGAGTTGG-3′ [**SEQ ID NO:5**], -960: 5′-GACGCTAGCGGAGCTGTGTCTTCACAAG-3′ [**SEQ ID NO:6**], -350: 5′-GACGCTAGCGAATCCATCTCCCACTCC-3′ [**SEQ ID NO:7**]). To facilitate subcloning of the amplified fragments, the reverse primer contained a HindIII restriction site adaptor, and the forward primers contained a NheI site. The PCR fragments and the luciferase expression vector pGL3-basic were digested separately with HindIII and NheI before ligation. The nomenclature used for each deletion construct (-1139, -960 and -350) indicates the number of base pairs of the upstream 5'-flanking sequence with respect to the ATG translation start codon.

Transient Transfection and Luciferase Assay

DNA transfection of the Mel Im cells was performed using Lipofectamin plus (Invitrogen, Carlsbad, CA). Briefly, the cells were cultured in 6-well plates. Each cationic lipid/plasmid DNA suspension was prepared by mixing 0.2 μg of the luciferase reporter plasmid and 0.1 μg of the internal control plasmid pRL-TK with transfection solutions according to the manufacturer's instructions. The cells were harvested 24 h later and the lysate was analyzed for luciferase activity with a luminometer using Promega dual-luciferase assay reagent. At least three independent transfection experiments were carried out for each construct.

Stable transfection of melanoma cells with antisense alpha10

A panel of Mel Im cell clones with reduced integrin alpha10 expression was established by stable transfection with an antisense expression plasmid (base -1 - 857 cloned in antisense orientation into pCMX-PL1). Plasmids were cotransfected with pcDNA3 (Invitrogen), containing the selectable marker for neomycin resistance. Controls received pcDNA3 alone. Transfections were performed using lipofectamin plus (Invitrogen). One day after transfection, cells were placed into selection medium containing 50 µg/ml G418 (Sigma). After 25 days of selection, individual G418-resistant colonies were subcloned.

Proliferation assays

Proliferation was measured using the Cell Proliferation Kit II (Roche) according to the manufacturer's protocol.

Migration and invasion assay

Migration and invasion assays were performed using Boyden Chambers containing polycarbonate filters with 8µm pore size (Costar, Bodenheim, Germany), essentially as described previously (23). Filters were coated with gelatine or Matrigel (diluted 1:3 in $H_2O$; Becton Dickinson, Heidelberg, Germany), respectively. The lower compartment was filled with fibroblast-conditioned medium, used as a chemo-attractant. Melanoma cells were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of $2x10^5$ cells/ml and placed in the upper compartment of the chamber. After incubation at 37°C for 4 hours, the filters were collected and the cells adhering to the lower surface fixed, stained and counted.

For the antibody experiments (see Figure 2B), a concentration of 400 ng/ml of the test antibody (mAb365) or the control antibody (IgG2) was used. The cells were pre-incubated for 10 minutes with the antibody before they were used in the Boyden Chamber migration assay.

Anchorage independent growth assay

Cells were seeded into 6-well plates in DMEM, 0.36% agar (Sigma), supplemented with 10% FCS on top of a 0.72% agar bed in similar medium. The cultures were incubated for 14 days and the colonies were measured and photographed. Colony size was measured using a Carl Zeiss microscope (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). For each cell clone the diameter of 20 colonies was determined and statistics was performed.

Migration assays

Migration of cells was assayed by scratch assays. For scratch assays ("wound-healing-assay") cells were seeded in high density into 6-well plates and scratched by a pipette tip in a definite array. Migration into this array was documented and measured after 24 and 48 hours. Each analysis was performed in triplicate.

Statistical analysis

Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using the Student's paired t-test. A p value $<0.05$ was considered statistically significant. All calculations were performed using the GraphPad Prism software (GraphPad software Inc, San Diego, USA).

***Results***

Induction of integrin alpha10 transcription during melanoma development

Using Affimetrix U133A, we investigated the expression of integrin alpha10 in 5 primary cultures of melanocytes isolated from human foreskin (FOM), 12 cell lines derived from primary melanomas and 15 cell lines derived from melanoma metastases. 4 of the 12 primary melanoma cell lines and 3 of the 15 metastatic lines showed high expression of integrin alpha10, whereas the remaining cell lines showed expression similar to that of the melanocytes (data not shown).

To continue on this finding, nine human melanoma cell lines were evaluated for expression of integrin alpha10 mRNA using quantitative PCR and compared to human primary melanocytes (NHEM). Strong induction of expression was found in all melanoma cell lines compared to melanocytes except of SK Mel 3 and SK Mel 28 (Figure 1A).

To locate the induction of integrin alpha10 expression within the development of malignant melanoma, RNA isolated from 4 primary melanomas, 3 lymph node metastases and 5 distant metastasis by micro dissection were screened by quantitative RT-PCR. Induction of transcription of integrin alpha10 was observed in all primary melanomas, lymph node and distant metastases analyzed when compared to expression levels in normal skin and in isolated normal human epidermal melanocytes (NHEM) (Figure 1B). However, the expression was not as strong as in chondrocytes (data not shown).

Seven different melanoma cell lines were further screened for integrin alpha10 protein expression by western blotting using an anti-integrin alpha10 antibody (Figure 1C). Consistent with the induced amount of mRNA expression, expression of integrin alpha10 protein was detected in all melanoma cell lines compared to primary human melanocytes. In summary, both the results from quantitative RT-PCR and western blots indicate an induction of integrin alpha10 expression in melanoma cells. Cell compartments were separated by a fractioning

procedure and cell membrane, cytoplasm and nucleus were analyzed separately for integrin alpha10 expression. Membrane localization of integrin alpha10 was clearly revealed (data not shown).

To examine integrin alpha10 expression in vivo, tissue samples from patients with primary malignant melanomas were immunostained with an anti-integrin alpha10 antibody. Representative sections are presented in Figure 1D. Melanoma cells were positive for integrin alpha10 expression.

In summary, these data show a correlation between protein expression and tumor progression as the amount of integrin alpha10 protein staining increases in metastatic melanoma.

As integrin alpha10 was shown to bind to collagen type II we analyzed collagen type II expression by melanoma cells by RT-PCR. Strong expression was detected in all melanoma cells (Figure 1E).

Functional relevance of integrin alpha10 expression

To analyse the functional role of integrin alpha10 in melanoma cells we downregulated expression of integrin alpha10 in the melanoma cell line Mel Im by stable transfection with an integrin alpha10 antisense expression construct. Successful downregulation of integrin alpha10 in the cell clones was demonstrated by quantitative RT-PCR, whereas no changes of integrin alpha10 expression were seen in control transfected cell clones (data not shown).

Interestingly, migration assays using the Boyden Chamber system showed a significant reduction of the migratory potential in the cell clones 4 and 6 expressing less integrin alpha10 compared to the mock transfected cell clone (Figure 2A).

To verify this finding in Mel Im cells the migration assay was performed using a monoclonal antibody against integrin alpha10 which has an inhibitory capacity. After incubation with the antibody the Mel Im cells had a less migratory potential

compared to untreated Mel Im cells (Figure 2B). To evaluate the effect of integrin alpha10 on invasion, further assays with the Boyden Chamber system to test the invasiveness of cells were performed. The as-integrin alpha10 cell clones (clone 4, 5 and 6) showed no significant changes in invasive potential compared to the mock transfected cell clone and to wild type Mel Im cells (Figure 2C).

To further evaluate the effect of integrin alpha10 on undirectional migration, wound healing assays (scratch assays) were performed. The as-integrin alpha10 clone 4 showed an increased migratory potential compared to the control and to the Mel Im cells, the as clones 5 and 6 had a decreased migratory potential. These effects were not significant and no general trend for the as integrin alpha10 cell clones was detectable (Figure 2D).

Taken together, integrin alpha10 expression does not effect cell proliferation or anchorage-independent growth, but decreases cell migration directed to a chemoattractant.

**EXAMPLE B – METASTASES MODEL**

*Objective*

The objective of this example is to test the role of alpha10 in a lung metastasis model using B16/F1 or B16/F10 (both from ATCC – American Type Culture Collection).

*Cells*

B16/F1 or F10 cells are monoclonal continuous culture, grown as monolayer in DMEM + 10% FBS + Antibiotics + 2mM L-Glutamine; 37°C, 5% CO2. Seed cells at 2-5x$10^5$ cells/$cm^2$ using 0.25% trypsin or trypsin/EDTA.

*Transfection of ITGA10*

The coding region of human ITGA10 cloned into the expression vector pBJ (gift from D. Gullberg) is co-transfected with pcDNA (hygro) into the cell-line. Cells (50-70% confluent) are transfected with the mentioned plasmids using FuGENE® 6 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. 24 hours after transfection the medium is changed to medium containing Hygromycin B (Roche Applied Science). After selection and cloning, cells are checked for ITGA10 expression using FACS and RT-PCR.

*Experimental set-up*

B16/F1 or B16/F10, un-transfected or transfected with ITGA10 (B16-ITGA10, F1 or F10 as of choice), are examples of B16 melanoma cell clones that may be used. The B16 cells are harvested in exponential growth phase by trypsinization or by non-enzymatic cell dissociation solution (to preserve ITGA10 integrity on cell surface). Cells are washed twice with PBS before injection.

Cells (1-10x$10^5$ cells in 200 µl PBS/mouse) are injected (on day 0) into the tail vein of female C57BL/6 (n=8-10 mice/group).

The following groups are tested:

1. Mice are, on day 0, injected i.v. with B16 (or B16-ITGA10) cells pre-incubated with mAb365 (5-10 µg/ml) or A05 (5-10 µg/ml).
2. Mice are, on day 0, injected i.v. with B16 (or B16-ITGA10) cells pre-incubated with isotype control antibody (5-10 µg/ml).
3. Mice are, on day 0, injected i.v. with B16 (or B16-ITGA10) cells.
4. Mice are, on day 0, injected i.v. with B16 (or B16-ITGA10) cells. Mice are treated i.p. with 200-300 µg mAb365 or A05 per mouse on day -1, 2, 5, 7 and 10.

5. Mice are, on day 0, injected i.v. with B16 (or B16-ITGA10) cells. Mice are treated i.p. with 200-300 µg control antibody per mouse on day -1, 2, 5, 7 and 10.

Mice are euthanized on day 12-14 (alternatively not until day 20-21) for inspection of lungs and surface tumour nodules are counted.

*References: B16/F1 or B16/F10 - Lung metastasis model*

Qian F, Zhang ZC, Wu XF, Li YP, Xu Q. Interaction between integrin alpha(5) and fibronectin is required for metastasis of B16F10 melanoma cells. *Biochem Biophys Res Commun.* 2005 Aug 12;**333**(4):1269-75.

Schlereth B, Kleindienst P, Fichtner I, Lorenczewski G, Brischwein K, Lippold S, da Silva A, Locher M, Kischel R, Lutterbuse R, Kufer P, Baeuerle PA. Potent inhibition of local and disseminated tumor growth in immunocompetent mouse models by a bispecific antibody construct specific for Murine CD3. *Cancer Immunol Immunother.* 2006 Jul;**55**(7):785-96.

**EXAMPLE C – SUB-CUTANEOUS, NON-METASTATIC MODEL**

*Objective*

The objective of this example is to test the role of alpha10 in a subcutaneous non-metastatic model using B16/F1 or B16/F10 (both from ATCC – American Type Culture Collection).

*Cells*

As described above.

*Experimental set-up*

B16/F1 or B16/F10, un-transfected or transfected with ITGA10, are examples of B16 melanoma cell clones that could be used. B16 cells are harvested in exponential growth phase by trypsinization or by non-enzymatic cell dissociation solution (to preserve ITGA10 integrity on cell surface). Cells are washed twice with PBS before injection. Cells ($0.1\text{-}2x10^6$ cells in 100 µl PBS/mouse) are injected subcutaneously (on day 0) on the flank of C57BL/6 mice (n=5-10 mice/group age 8-10 weeks).

The following groups are tested:

1. Mice are, on day 0, injected s.c. with B16 (or B16-ITGA10) cells preincubated with mAb365 (5-10 µg/ml) or A05 (5-10 µg/ml).
2. Mice are, on day 0, injected s.c. with B16 (or B16-ITGA10) cells preincubated with isotype control antibody (5-10 µg/ml).
3. Mice are, on day 0, injected s.c. with B16 (or B16-ITGA10) cells.
4. Mice are, on day 0, injected s.c. with B16 (or B16-ITGA10) cells. Mice are treated i.p. with 200-300 µg mAb365 or A05 per mouse on day 1, 5, 9, 13 and 17.
5. Mice are, on day 0, injected s.c. with B16 (or B16-ITGA10) cells. Mice are treated i.p. with 200-300 µg control antibody per mouse on day 1, 5, 9, 13 and 17.

Mice are examined daily for signs of tumour growth and tumour volumes are measured 3 times per week with a calliper in two perpendicular dimensions. Tumour volumes are calculated according to the formula: tumour volume = [(width$^2$ x length)/2]. When the tumour volume reaches 10 mm$^3$ (set by the ethical permission) the animals are euthanized and tumours are collected for histological examination.

*References: B16/F1 or B16/F10 - subcutaneous non-metastatic model*

Singhal SS, Awasthi YC, Awasthi S. Regression of melanoma in a murine model by RLIP76 depletion. *Cancer Res.* 2006 Feb 15;**66**(4):2354-60.

Schlereth B, Kleindienst P, Fichtner I, Lorenczewski G, Brischwein K, Lippold S, da Silva A, Locher M, Kischel R, Lutterbuse R, Kufer P, Baeuerle PA. Potent inhibition of local and disseminated tumor growth in immunocompetent mouse models by a bispecific antibody construct specific for Murine CD3. *Cancer Immunol Immunother.* 2006 Jul;**55**(7):785-96.

**EXAMPLE D – METASTATIC XENOGRAFT MODEL**

*Objective*

The objective of this example is to test the role of alpha10 in a metastatic xenograft model.

*Cells*

MDA-MB-435 from ATCC.

*Experimental set-up*

MDA-MB-435 was originally described as a human mammary carcinoma but has recently been shown to possess characteristics of a malignant melanoma (G Ellison, T Klinowska, R F R Westwood, E Docter, T French, and J C Fox, Mol Pathol. 2002 October; 55(5): 294–299.).

MDA-MB-435 cells, wild-type or ITGA10 transfected, are harvested in exponential growth phase by trypsinization or by non-enzymatic cell dissociation solution (to preserve ITGA10 integrity on cell surface). Cells ($1\text{-}5x10^6$ cells in

100 µl PBS/mouse) are washed twice with PBS before injection into the mammary fat pad of female athymic BALB/c/AnNCr *nu/nu* mice (4-6 weeks old).

The following groups (n=8-10 mice/group) are tested:

1. Mice are, on day 0, injected into mammary fat pad with MDA-MB-435 cells (or MDA-MB-435-ITGA10) cells.
2. Mice are, on day 0, injected into mammary fat pad with MDA-MB-435 cells (or MDA-MB-435-ITGA10) cells. Mice are treated i.p. in four days intervals with 200-300 µg mAb365 or A05 per dose/mouse starting on day 1 until the end of the experiment.
3. Mice are, on day 0, injected into mammary fat pad with MDA-MB-435 cells (or MDA-MB-435-ITGA10) cells. Mice are treated i.p. in four days intervals with 200-300 µg control antibody per dose/mouse starting on day 1 until the end of the experiment.

Mice are examined daily for signs of tumour growth and tumour volumes are measured twice a week with a calliper in two perpendicular dimensions. Tumour volumes are calculated according to the formula: tumour volume = [(width$^2$ x length)/2]. After eight weeks or when the primary tumour volume reaches 1 cm$^3$ (set by the ethical permission) the animals are euthanized. The lungs are fixed in Bouin's liquid and stored in abs. ethanol. After the lungs restored their inherent colour, the tumour nodules can be assessed and counted by macroscopic observation.

*References: MDA-MB-435 - metastatic xenograft model*

Schou M, Brunner N, Spang-Thomsen M, Rygaard J. Mendelian analysis of a metastasis-prone substrain of BALB/c nude mice using a subcutaneously inoculated human tumour. *APMIS*. 2006 Dec;**114**(12):899-907.

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## EXAMPLE E – NON-METASTATIC XENOGRAFT MODEL

*Objective*

The objective if this example is to analyse the role of alpha10 in a non-metastatic xenograft model.

*Experimental set-up*

Mel Im cells (Jacob K, Wach F, Holzapfel U, Hein R, Lengyel E, Buettner R, Bosserhoff AK, Melanoma Res 1998, 8:211–219) are harvested in exponential growth phase by trypsinization or by non-enzymatic cell dissociation solution (to preserve ITGA10 integrity on cell surface). Cells are washed twice with PBS before injection. Cells ($0.1\text{-}2x10^6$ cells in 100 µl PBS/mouse) are injected subcutaneously (on day 0) on the flank of BALB/c *nu/nu* mice (n=5-10 mice/group age 4-6 weeks).

The following groups are tested:

1. Mice are, on day 0, injected s.c. with Mel Im cells pre-incubated with mAb365 (5-10 µg/ml) or A05 (5-10 µg/ml).
2. Mice are, on day 0, injected s.c. with Mel Im cells pre-incubated with isotype control antibody (5-10 µg/ml).
3. Mice are, on day 0, injected s.c. with Mel Im cells.
4. Mice are, on day 0, injected s.c. with Mel Im cells. Mice are treated i.p. in four days intervals with 200-300 µg mAb365 or A05 per dose/mouse starting on day 1 until the end of the experiment.

5. Mice are, on day 0, injected s.c. with Mel Im cells. Mice are treated i.p. in four days intervals with 200-300 μg control antibody per dose/mouse starting on day 1 until the end of the experiment.

Mice are examined daily for signs of tumour growth and tumour volumes are measured 3 times per week with a calliper in two perpendicular dimensions. Tumour volumes are calculated according to the formula: tumour volume = [(width$^2$ x length)/2]. When the tumour volume reaches 1 cm$^3$ (set by the ethical permission) the animals are euthanized and tumours are collected for histological examination.

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## CLAIMS

1. Use of a compound comprising a binding moiety capable of binding selectively to an integrin alpha-10 subunit or a heterodimer thereof in the preparation of a medicament for treating a malignant tumour.

2. Use of a compound comprising a binding moiety capable of binding selectively to an integrin alpha-10 subunit or a heterodimer thereof in the preparation of a diagnostic or prognostic agent for a malignant tumour.

3. Use of a compound comprising a binding moiety capable of binding selectively to an integrin alpha-10 subunit or a heterodimer thereof in the preparation of an agent for detecting and/or imaging malignant tumour cells.

4. The use according to any one of Claims 1 to 3 wherein the tumour is selected from the group consisting of tumours of the breast, bile duct, brain, colon, stomach, reproductive organs, lung and airways, skin, gallbladder, liver, nasopharynx, nerve cells, kidney, prostate, lymph glands and gastrointestinal tract.

5. The use according to any one of Claims 1 to 3 wherein the tumour is a malignant melanoma.

6. The use according to any one of Claims 1 to 5 wherein the binding moiety selectively binds to integrin alpha-10 subunit.

7. The use according to any one of Claims 1 to 6 wherein the binding moiety selectively binds to a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO: 1 or natural variants thereof.

8. The use according to any one of Claims 1 to 7 wherein the binding moiety selectively binds to a heterodimer comprising an integrin alpha-10 subunit.

9. The use according to any one of the preceding claims wherein the binding moiety is an antibody or antigen-binding fragment or derivative thereof.

10. The use according to Claim 9 wherein the antibody or antigen-binding fragment or derivative thereof is selected from the group consisting of Fv fragments, Fab-like fragments, single variable domains, domain antibodies and nanobodies.

11. The use according to Claim 9 or 10 wherein the antibody or an antigen-binding fragment or derivative thereof is humanised.

12. The use according to any one of the preceding claims wherein the compound additionally comprises a further moiety selected from the group consisting of target cell-specific portions, cytotoxic moieties and detectable moieties.

13. The use according to any one of the preceding claims wherein the malignant tumour is metastatic.

14. The use according to any one of the Claims 1 and 4 to 30 wherein the medicament is for inhibiting migration of malignant tumour cells.

15. The use according to Claim 31 wherein the migration of tumour cells is towards a chemoattractant.

16. A method of imaging malignant tumour cells in the body of an individual, the method comprising administering to the individual an effective amount of a compound comprising a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof.

17. A method of diagnosing or prognosing malignant tumours in an individual, the method comprising administering to the individual an effective amount of a compound comprising a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof.

18. A method according to Claim 16 or 17 further comprising the step of detecting the location of the compound in the individual.

19. A method of treating an individual with a malignant tumour, the method comprising administering to the individual an effective amount of a compound comprising a binding moiety capable of binding selectively to integrin alpha-10 subunit or a heterodimer thereof

20. A method according to any one of Claims 16 to 19 wherein the tumour is selected from the group consisting of tumours of the breast, bile duct, brain, colon, stomach, reproductive organs, lung and airways, skin, gallbladder, liver, nasopharynx, nerve cells, kidney, prostate, lymph glands and gastrointestinal tract.

21. A method according to any one of Claims 16 to 20 wherein the tumour is a malignant melanoma.

22. A method according to any one of Claims 16 to 21 wherein the compound inhibits migration of tumour cells.

23. A method for monitoring the progression of a malignant tumour in an individual, the method comprising:

(a) providing a sample of malignant tumour cells collected from the individual at a first time point and measuring the amount of integrin alpha-10 subunit protein therein;

(b) providing a sample of malignant tumour cells collected from the individual at a second time point and measuring the amount of integrin alpha-10 subunit protein therein; and

(c) comparing the amount of integrin alpha-10 subunit protein measured in steps (a) and (b)

wherein an increased amount of integrin alpha-10 subunit protein measured in step (b) compared to step (a) is indicative of a progression in the malignant tumour.

24. A method of identifying cells associated with a malignant tumour, the method comprising measuring the amount of integrin alpha-10 subunit protein in a sample of cells to be tested and comparing it to the amount of integrin alpha-10 subunit protein in a sample of known malignant tumour cells.

25. A method of distinguishing between different types or stages of a malignant tumour, the method comprising measuring the amount of integrin alpha-10 subunit protein in a sample of cells to be tested and comparing it to the amount of integrin alpha-10 subunit protein in a sample of malignant tumour cells of a known type or stage.

26. A method according to any one of Claims 23 to 25 wherein the tumour is selected from the group consisting of tumours of the breast, bile duct, brain, colon, stomach, reproductive organs, lung and airways, skin, gallbladder, liver, nasopharynx, nerve cells, kidney, prostate, lymph glands and gastrointestinal tract.

27. A method according to any one of Claims 23 to 26 wherein the tumour is a malignant melanoma.

28. A method according to any one of Claims 24 to 27 wherein the known malignant tumour cells are characterised by the upregulation of the amount of integrin alpha-10 subunit protein compared to corresponding normal (non-tumour) cells.

29. A method according to Claim 28 wherein the known malignant tumour is a malignant melanoma characterised by the upregulation of the amount of integrin alpha-10 subunit protein compared to normal epidermal melanocytes.

30. A method according to any one of Claims 23 to 29 further comprising comparing the amount of integrin alpha-10 subunit protein in a sample of cells to be tested with the amount of integrin alpha-10 subunit protein in a control sample.

31. A method according to Claim 30 wherein malignant tumour is a melanoma and the control sample comprises normal epidermal melanocytes.

32. A method according to any one of Claims 16 to 31 wherein the cells to be tested are identified or distinguished as malignant tumour cells if the amount of integrin alpha-10 subunit protein is upregulated compared to corresponding normal (non-tumour) cells.

33. A method according to any one of Claims 16 to 32 wherein the binding moiety selectively binds to integrin alpha-10 subunit.

34. A method according to any one of Claims 16 to 33 wherein the binding moiety selectively binds to a polypeptide comprising or consisting of the amino acid sequence of SEQ ID NO 1 or natural variants thereof.

35. A method according to any one of Claims 16 to 33 wherein the binding moiety selectively binds to a heterodimer comprising an integrin alpha-10 subunit.

36. A method according to any one of Claims 16 to 35 wherein the binding moiety is an antibody or antigen-binding fragment or derivative thereof.

37. A method according to Claim 36 wherein the antibody or antigen-binding fragment thereof is selected from the group consisting of Fv fragments, Fab-like fragments, single variable domains, domain antibodies and nanobodies.

38. A method according to Claim 36 or 37 wherein the antibody or an antigen-binding fragment thereof is humanised.

39. A method according to any one of Claims 16 to 38wherein the compound additionally comprises a further moiety selected from the group consisting of target cell-specific portions, cytotoxic moieties and detectable moieties.

40. A method of screening for candidate compounds with efficacy in the treatment of a malignant tumour, the method comprising the steps of:

(a) contacting a molecule to be tested with an integrin alpha-10 subunit (or a fragment or binding sequence thereof); and

(b) detecting the presence of a complex containing the integrin alpha-10 subunit (or fragment thereof) and the molecule to be tested

the molecule to be tested being identified as a candidate compound if the complex is detected in step(b).

41. A method according to Claim 40 wherein the tumour is selected from the group consisting of tumours of the breast, bile duct, brain, colon, stomach,

reproductive organs, lung and airways, skin, gallbladder, liver, nasopharynx, nerve cells, kidney, prostate, lymph glands and gastrointestinal tract.

42. A method according to Claim 40 or 41 wherein the tumour is a malignant melanoma.

43. A method according to any one of Claims 40 to 42 further comprising step (c) of testing the selected candidate compound for efficacy in one or more additional models of malignant melanoma.

44. A method of treating an individual with a malignant tumour substantially as described herein.

45. A method of diagnosing or prognosing a malignant tumour in an individual substantially as described herein.

46. A method of imaging malignant tumour cells substantially as described herein.

FIGURE 1A

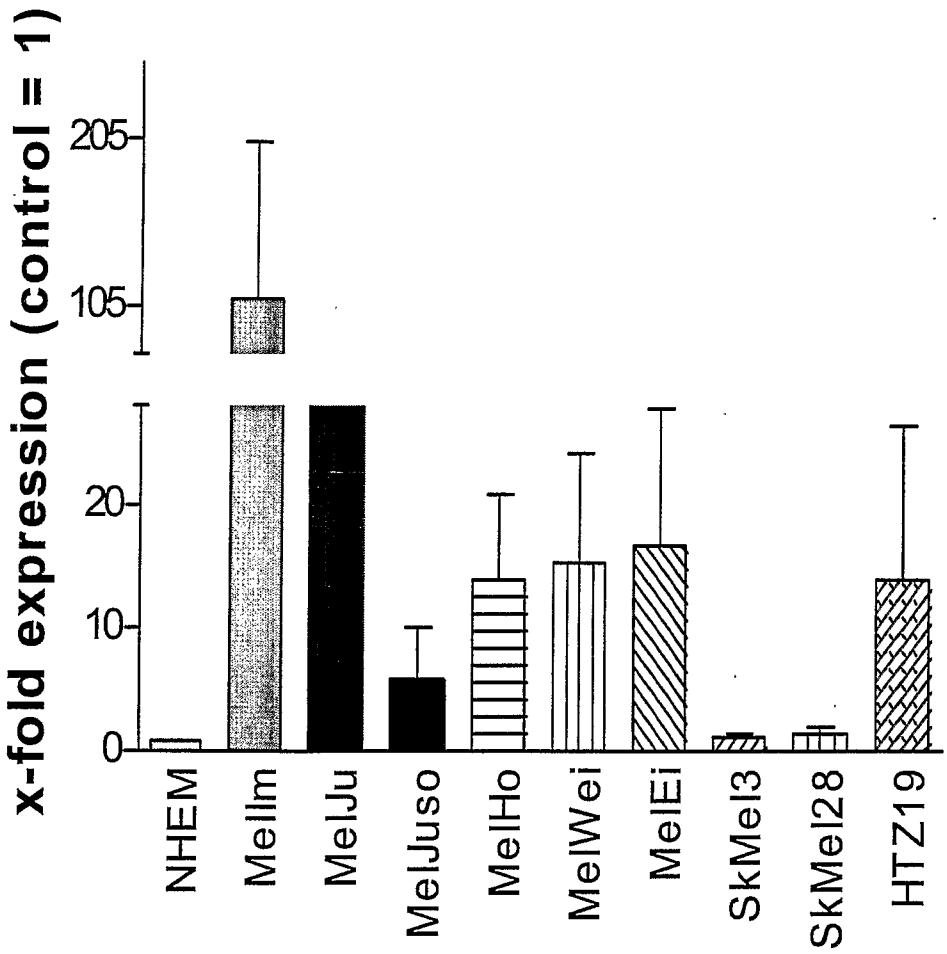

x-fold expression (control = 1)
205
105
20
10
0
NHEM
MelIm
MelJu
MelJuso
MelHo
MelWei
MelEi
SkMel3
SkMel28
HTZ19

FIGURE 1B

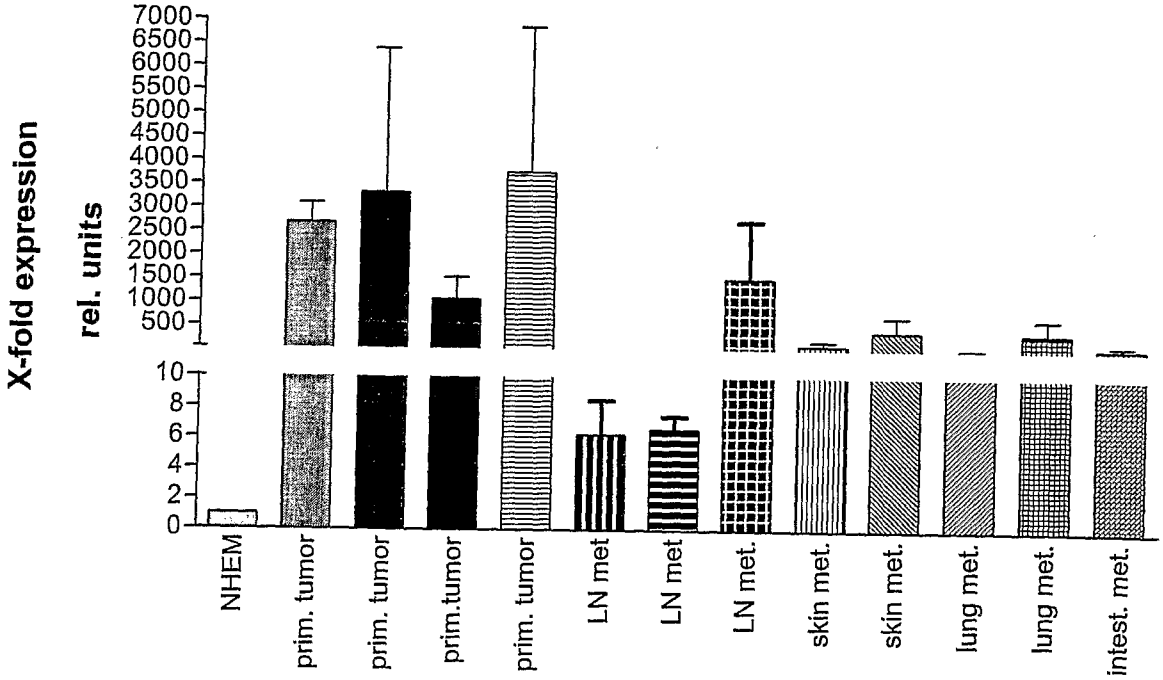

X-fold expression
rel. units
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
10
8
6
4
2
0
NHEM
prim. tumor
prim. tumor
prim.tumor
prim. tumor
LN met
LN met
LN met.
skin met.
skin met.
lung met.
lung met.
intest. met.

FIGURE 1C

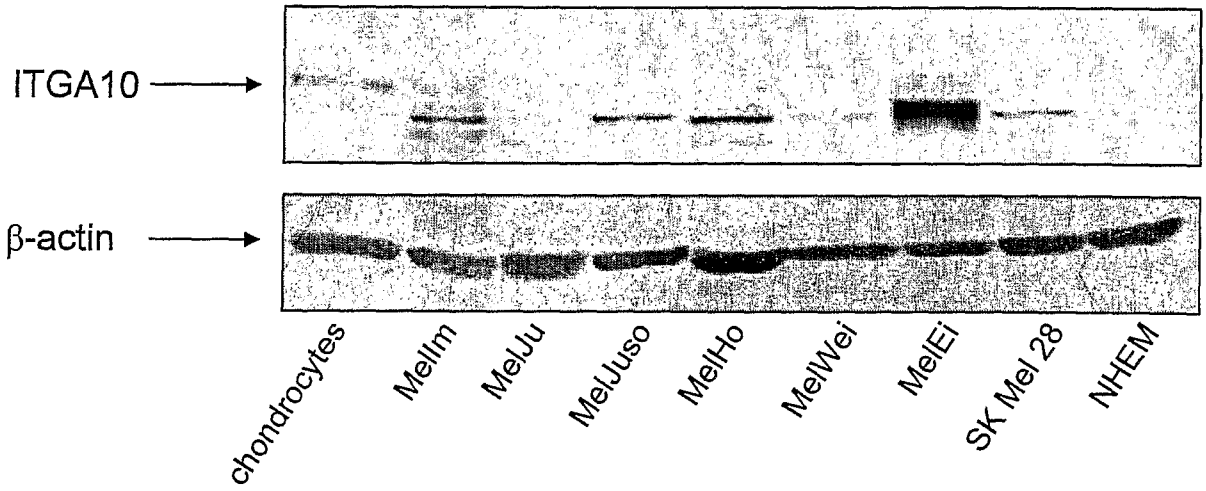
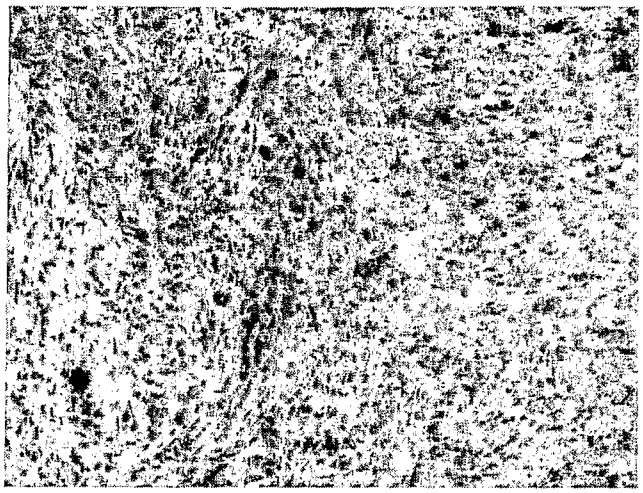

ITGA10
β-actin
chondrocytes
MelIm
MelJu
MelJuso
MelHo
MelWei
MelEi
SK Mel 28
NHEM

**FIGURE 1D**

(i) 200 X



(ii) 400 X

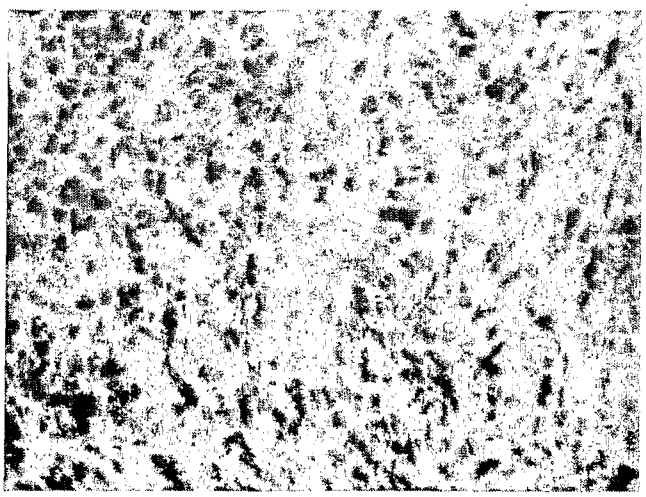


FIGURE 1E

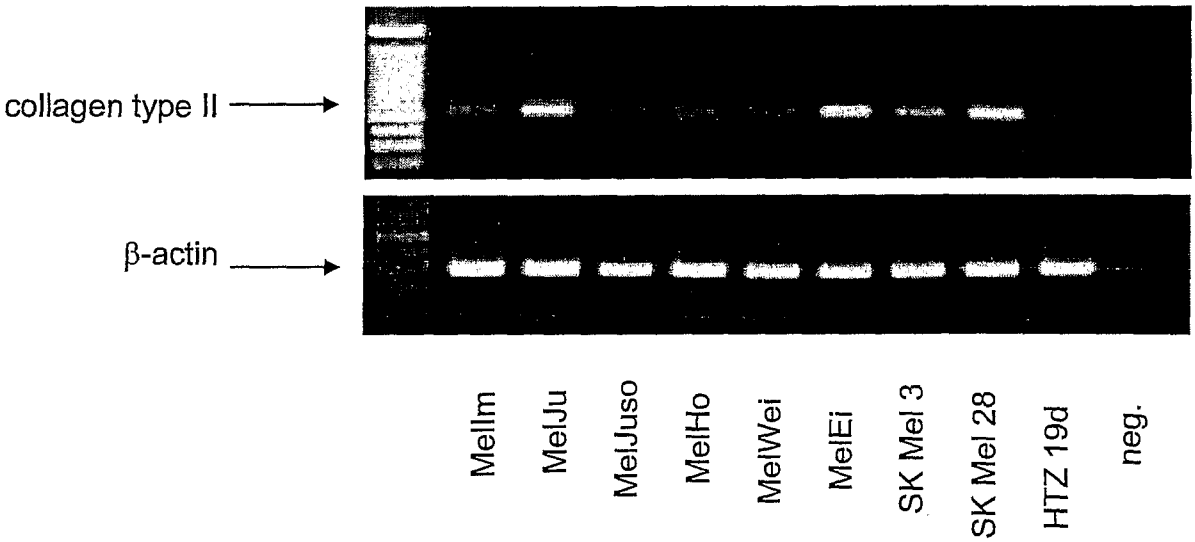

collagen type II
β-actin
MelIm
MelJu
MelJuso
MelHo
MelWei
MelEi
SK Mel 3
SK Mel 28
HTZ 19d
neg.

FIGURE 2A

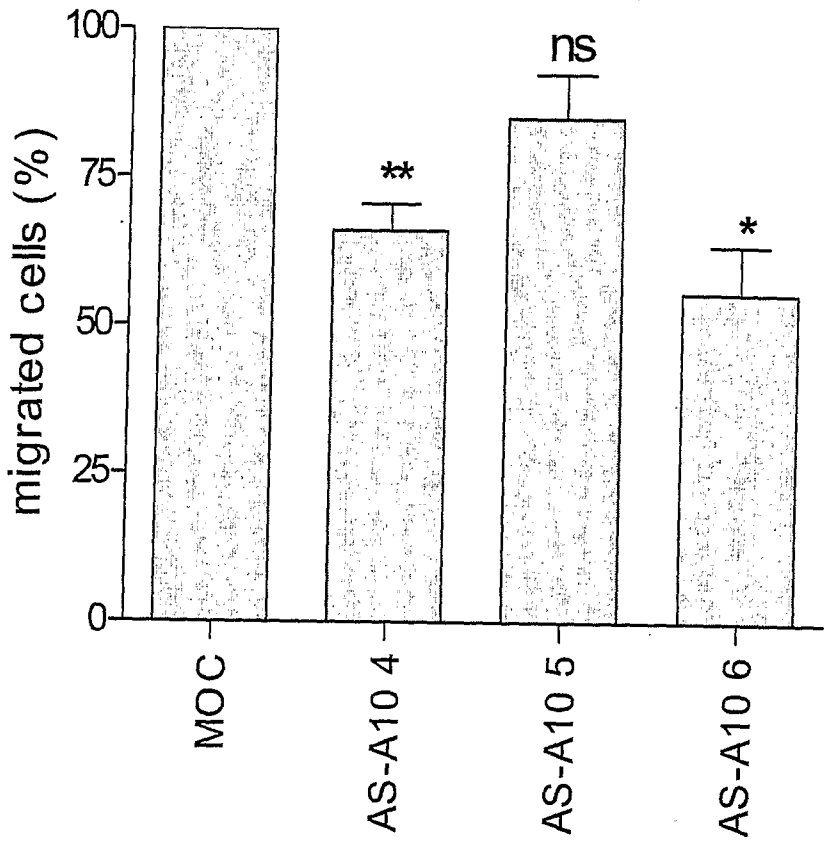


FIGURE 2B

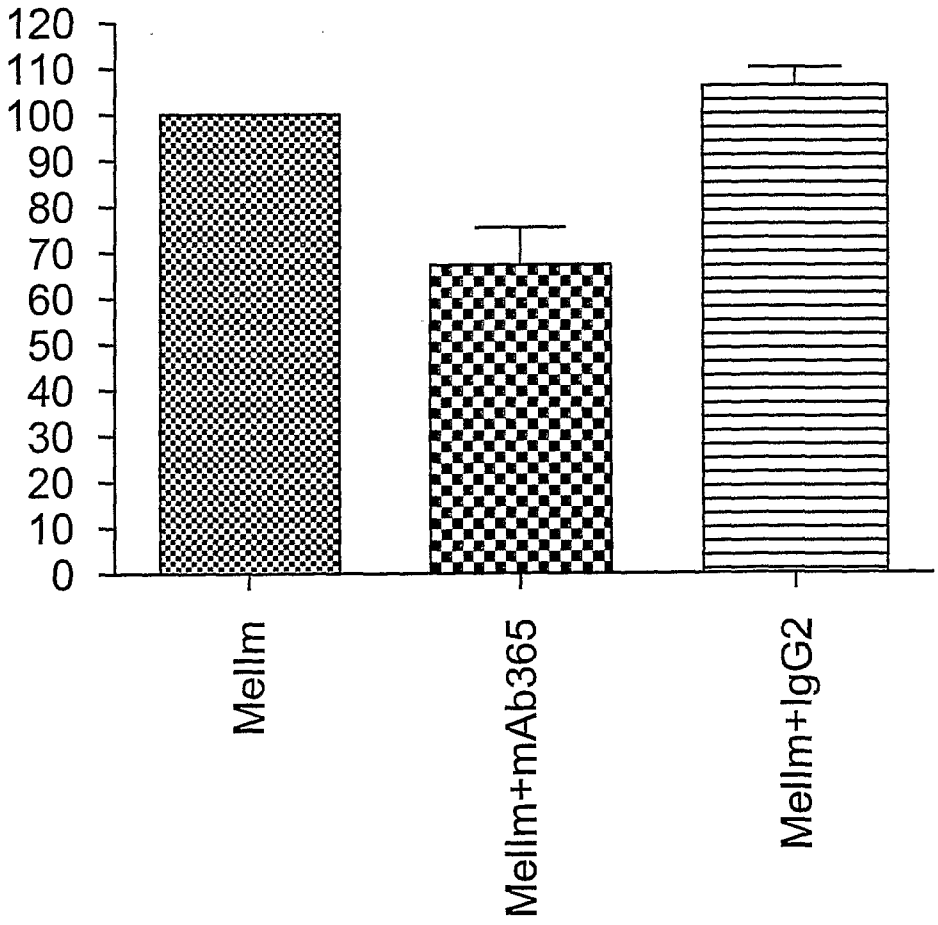

120
110
100
90
80
70
60
50
40
30
20
10
0
MelIm
MelIm+mAb365
MelIm+IgG2

FIGURE 2C

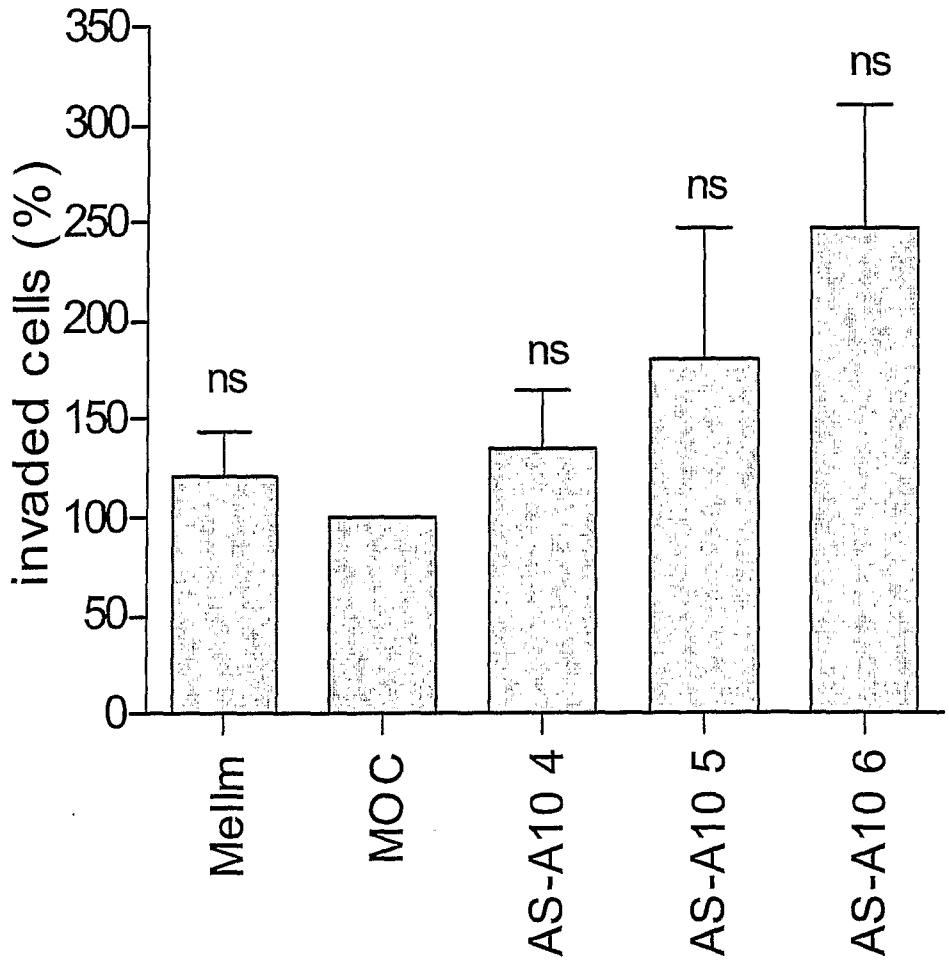

invaded cells (%)
350
300
250
200
150
100
50
0
ns
ns
ns
ns
MelIm
MOC
AS-A10 4
AS-A10 5
AS-A10 6

FIGURE 2D