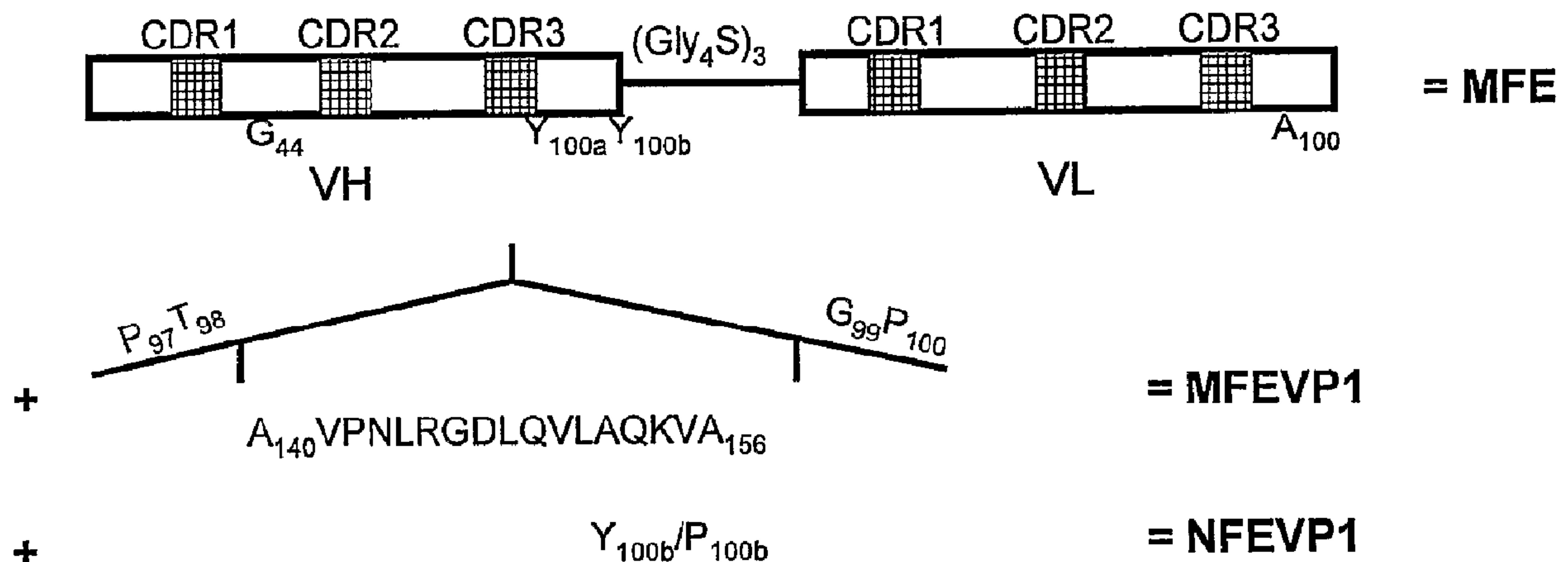




(86) **Date de dépôt PCT/PCT Filing Date:** 2008/09/26
(87) **Date publication PCT/PCT Publication Date:** 2009/04/02
(45) **Date de délivrance/Issue Date:** 2018/12/04
(85) **Entrée phase nationale/National Entry:** 2010/03/26
(86) **N° demande PCT/PCT Application No.:** GB 2008/003283
(87) **N° publication PCT/PCT Publication No.:** 2009/040550
(30) **Priorité/Priority:** 2007/09/26 (GB0718843.6)

(51) **Cl.Int./Int.Cl. C07K 16/00** (2006.01),
C07K 16/28 (2006.01)
(72) **Inventeurs/Inventors:**
CHESTER, KERRY, GB;
KOGELBERG, HEIDE, GB;
MARSHALL, JOHN F., GB
(73) **Propriétaire/Owner:**
CANCER RESEARCH TECHNOLOGY LIMITED, GB
(74) **Agent:** BERESKIN & PARR LLP/S.E.N.C.R.L., S.R.L.

(54) **Titre : MATERIAUX ET PROCEDES SE RAPPORTANT A LA MODIFICATION DE LA LIAISON D'ANTICORPS**
(54) **Title: MATERIALS AND METHODS RELATING TO MODIFYING THE BINDING OF ANTIBODIES**



(57) **Abrégé/Abstract:**

The invention relates to materials and methods for modifying the binding of antibodies, and more particularly to antibodies that are obtainable by inserting an amino acid sequence capable of binding to a target into a complementarity determining region of a parent antibody so that the antibody thus obtained is capable of binding to the target. The invention further relates to the uses of the antibodies for therapy, diagnosis or imaging, and to methods of producing the antibodies.

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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
2 April 2009 (02.04.2009)

PCT

(10) International Publication Number
WO 2009/040550 A1

(51) International Patent Classification:
C07K 16/00 (2006.01) *C07K 16/28* (2006.01)

(74) Agents: KIDDLE, Simon et al.; Mewburn Ellis LLP, 33 Gutter Lane, London EC2V 8AS (GB).

(21) International Application Number:
PCT/GB2008/003283

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, 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, ME, 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, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date:
26 September 2008 (26.09.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0718843.6 26 September 2007 (26.09.2007) GB

(71) Applicant (*for all designated States except US*): **CANCER RESEARCH TECHNOLOGY LIMITED** [GB/GB]; Sardinia House, Sardinia Street, London Greater London WC2A 3NL (GB).

(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, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

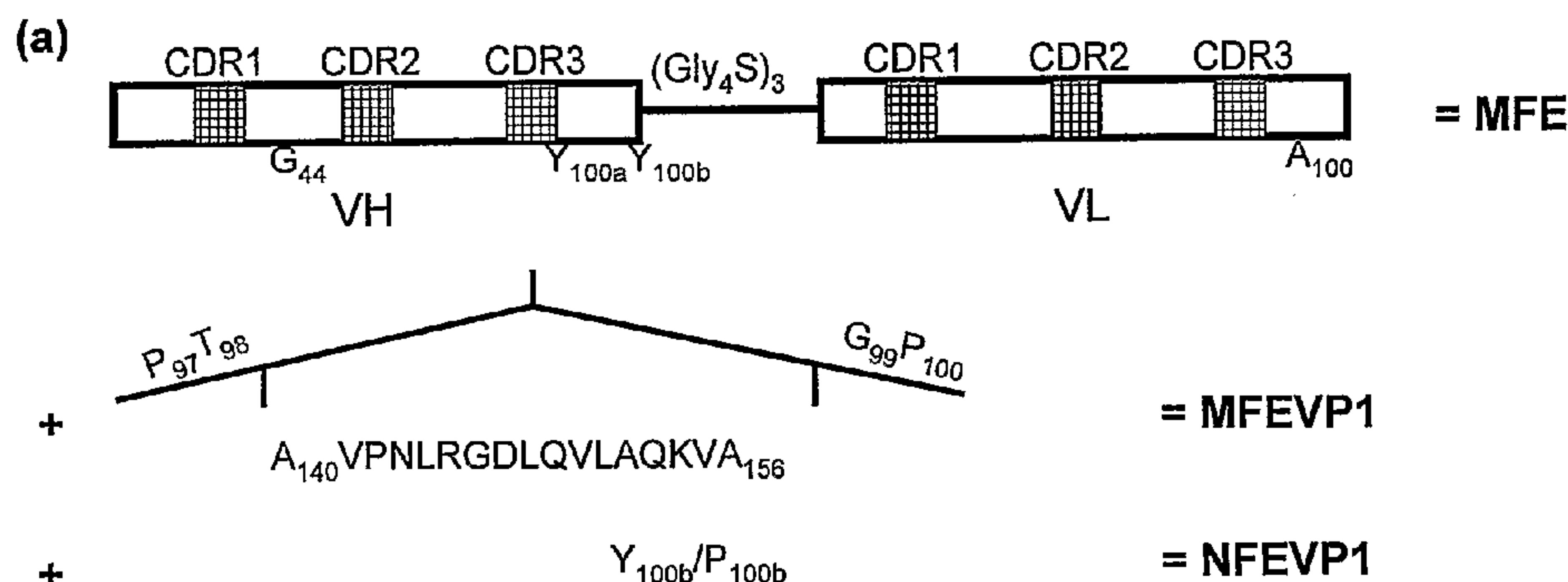
(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **CHESTER, Kerry** [GB/GB]; Department of Oncology, Hampstead Campus, Royal Free and University College Medical School, UCL, Rowland Hill Street, London Greater London NW3 2PF (GB). **KOGELBERG, Heide** [—/GB]; Department of Oncology, Hampstead Campus, Royal Free and University College Medical School, UCL, Rowland Hill Street, London Greater London NW3 2PF (GB). **MARSHALL, John F.** [GB/GB]; Barts and The London Queen Mary's School of Medicine and Dentistry, Ground Floor, John Vane Science Centre, Charterhouse Square (GB).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

(54) Title: MATERIALS AND METHODS RELATING TO MODIFYING THE BINDING OF ANTIBODIES



(57) Abstract: The invention relates to materials and methods for modifying the binding of antibodies, and more particularly to antibodies that are obtainable by inserting an amino acid sequence capable of binding to a target into a complementarity determining region of a parent antibody so that the antibody thus obtained is capable of binding to the target. The invention further relates to the uses of the antibodies for therapy, diagnosis or imaging, and to methods of producing the antibodies.

WO 2009/040550 A1

**Materials and Methods Relating to Modifying the Binding of
Antibodies**

Field of the Invention

5 The present invention relates to materials and methods for
modifying the binding of antibodies, and more particularly
to antibodies that are obtainable by inserting an amino acid
sequence capable of binding to a target into a
complementarity determining region of a parent antibody so
10 that the antibody thus obtained is capable of binding to the
target. The present invention further relates to the uses
of the antibodies for therapy, diagnosis or imaging and to
methods of producing the antibodies.

15 **Background of the Invention**

Integrins are a family of heterodimeric class I
transmembrane receptors. Individual integrins comprise an α
and β subunit in non covalent association and there are
known to exist at least 18 α subunits and 8 β subunits that
20 can form 24 different heterodimers. They are involved in
numerous cell-matrix and cell-cell interactions and
facilitate cell adhesion, proliferation, migration and
invasion. These processes occur in several normal and
pathological processes, including wound healing,
25 inflammation and tumour growth and metastasis.

$\alpha v \beta 6$ is an epithelial cell-restricted integrin and has been
shown to be expressed in malignant but not in normal
epithelium. *De novo* expression of this integrin has been
30 reported in oral squamous cell carcinomas (SCC) and ovarian
cancer tissues and cancer cell lines and over-expression in
adenocarcinomas of the breast, and ovarian cancer, colon
carcinoma, oral squamous cell carcinoma and in
gastroenteropancreatic adenocarcinomas, in particular in

pancreatic ductal adenocarcinomas. It has also been shown that expression of $\beta 6$ into a poorly invasive SCC cell line increased migration on fibronectin and invasion through the reconstituted basement membrane, suggesting a primary role for this integrin in oral SCC invasion and metastasis. The transcriptional activation of $\beta 6$ and subsequent expression of $\alpha v\beta 6$ has been observed during the epithelial-mesenchymal transition (EMT), which allows colon carcinoma cells to acquire a more aggressive phenotype. Moreover, analysis of colorectal carcinoma samples revealed that the elevated expression is associated with a significant reduced survival time of patients.

WO2007/039728 (Cancer Research Technology Limited) discloses experiments in which $\alpha v\beta 6$ peptide ligands comprising the sequence motif RGD~~L~~XXL/I, wherein LXXL/I is contained within an alpha helical structure are investigated. The use of this peptide motif arose from studies in which $\alpha v\beta 6$ expression was involved in activation of autocrine TGF- β in post-EMT cells. The Latency Associated Protein (LAP) of the latent LAP-TGF β 1 complex is a known ligand for $\alpha v\beta 6$ and binding has a role in the activation of TGF- β 1. The LAP protein contains the arginine-glycine-aspartic acid (RGD) sequence, a known binding motif for most integrins. In addition, a further ligand for $\alpha v\beta 6$ is the viral protein 1 (VP1) of the foot-and-mouth disease virus (FMDV), which also contains the RGD motif. FMDV uses $\alpha v\beta 6$ to attach to host cells and the integrin most likely also plays a role in virus uptake into endosomes. The binding of VP1 specifically to $\alpha v\beta 6$ is mediated via residues immediately following and including the aspartic acid of the RGD motif; the DLXXL sequence has been identified as an additional $\alpha v\beta 6$ binding motif from its ability to inhibit $\alpha v\beta 6$ -fibronectin

interactions. Peptides in which either of the two leucine residues were mutated were less good as inhibitors of FMDV C-S8c1 to recognize and infect susceptible cells. The highly related RGDLXXI motif is present in the LAP protein and would be predicted to be also involved in binding with high affinity to $\alpha v\beta 6$.

A previous study engineered a RGD motif and three RGD repeats into the CRD3 loop of an immunoglobulin human/mouse chimeric heavy chain antibody and showed that the antibody recognized specifically the integrin $\alpha v\beta 3$ (20). Similarly, a gp120 binding antibody was created by inserting a peptide from the CD4 receptor into the CRD3 loop (21) and a DNA-binding antibody by replacing the CDR3 loop with a sequence from a class B basic helix-loop-helix protein (22). More recently peptide sequences of the prion protein that are known epitopes for monoclonal antibodies that inhibit prion disease formation were grafted into the CD3 loop of the heavy chain of an IgG antibody specific for the envelope glycoprotein of HIV-1 (23). The resulting PrP-IgGs bound specifically to disease-associated conformations of PrP but not to the HIV envelope.

Summary of the Invention

Broadly, the present invention concerns antibodies that are obtainable by inserting an amino acid sequence that is capable of binding to a target into the complementarity determining region (CDR) of a parent antibody so that the antibody thus obtained is capable of binding to the target. Thus, this approach may be used to introduce a new binding specificity to the antibody. As the insertion is made in one of the CDRs of the parent antibody, and is preferably made in CDR H3, the insertion often has the effect of reducing or abolishing the binding of the parent antibody

for the antigen to which it initially bound. The antibodies of the present invention are useful as they are able to bind to the target, such as $\alpha v \beta 6$ integrin, while retaining one or more of the useful properties of the parent antibody, such as interacting with the immune system (e.g. recruitment of complement), a pharmacological property such as stability and/or half-life, e.g. when the antibody is administered in vivo and especially when compared to the corresponding peptide, and/or ease of production in recombinant host cells. In the present invention, the parent antibodies are based on MFE-23 antibodies and variants and derivatives thereof as discussed in more detail below.

Accordingly, in a first aspect, the present invention provides an antibody which is capable of binding to a target as obtainable by inserting an amino acid sequence capable of binding to the target into a complementarity determining region of a parent antibody, wherein the parent antibody comprises the following complementary determining regions (CDRs):

- (a) Heavy Chain CDR 1: Gly Phe Asn Ile Lys Asp Ser; and/or
- (b) Heavy Chain CDR 2: Asp Pro Glu Asn Gly Asp; and/or
- (c) Heavy Chain CDR 3: Thr Pro Thr Gly Pro Tyr Tyr Phe Asp; and/or
- (d) Light Chain CDR 1: (i) Ser Ser Ser Val Pro, or (ii) Ser Ser Ser Val Ser; and /or
- (e) Light Chain CDR 2: (i) Ser Thr Ser, or (ii) Leu Thr Ser; and/or
- (f) Light Chain CDR 3: Arg Ser Ser Tyr Pro Leu.

In some embodiments, the antibody may have an amino acid sequence capable of binding to the target inserted into more than one of the complementarity determining regions of the

parent antibody. Additionally or alternatively, the antibody may have an amino acid sequence capable of binding to the target grafted onto a complementarity determining region of the parent antibody.

5

As discussed further herein, the above CDRs are derived from MFE-23 antibodies and amino acid sequence for binding to the target is preferably inserted into the heavy chain CDR3 of the parent antibody, and more preferably between amino acids
10 Thr and Gly residues of CDR H3 of the parent antibody, i.e. between residues 98 and 99 as shown in the sequence of MFE-23 provided herein.

In another preferred embodiment, the target is inserted into
15 the heavy chain CDR2 of the parent antibody, preferably between amino acids Glu and Asn of CDR H2 of the parent antibody, i.e. between residues 53 and 54 of the sequence of MFE-23.

20 In a further aspect, the present invention provides an antibody as described herein for use in therapy, diagnosis or imaging.

In a further aspect, the present invention provides the use
25 of an antibody as described herein for the preparation of a reagent for imaging or diagnosis using a detectable group conjugated or linked to the antibody.

In a further aspect, the present invention provides the use
30 of an antibody as described herein for the preparation of a medicament for the treatment of a condition characterised by diseased cells which express the target, for example a condition in which the cells overexpress the target and/or

display the target on the cell surface and/or which is a disease mediated by the target.

5 Examples of such conditions are provided below and include cancer, for example by making use of the antigens expressed on the surface of cancer cells. In some embodiments, the conditions include $\alpha v \beta 6$ -mediated diseases or diseases in which cells overexpress $\alpha v \beta 6$, such as cancer, chronic fibrosis, chronic obstructive pulmonary disease (COPD), lung
10 emphysema or chronic wounding skin disease.

In a further aspect, the present invention provides a method for diagnosing or imaging a condition characterised by diseased cells which express the target and/or which is a
15 disease mediated by the target, the method comprising (a) administering to a patient suspected of having the disease an antibody as described herein which is linked to a detectable moiety and (b) detecting the detectable moiety to diagnose or image the condition.

20 In a further aspect, the present invention provides a method of treating a condition characterised by diseased cells which express the target and/or which is a disease mediated by the target, the method comprising administering to
25 patient a therapeutically effective amount of an antibody as described herein.

In further aspect, the present invention provides nucleic acid sequences, expression vectors and host cells for
30 producing antibodies according to the present invention.

Embodiments of the present invention will now be further described by way of example and not limitation with reference to the accompanying figures and tables.

Brief Description of the Figures

Figure 1. Schematic presentation of the construction of MFEVP1 and NFEVP1. (A) Insertion of the RGD containing peptide sequences of VP1 (A₁₄₀ to A₁₅₆) into the CDR3 loop (between T98 and G99) of the VH chain of MFE-23 gives MFEVP1. Y_{100b} to P_{100b} mutation of MFEVP1 gives NFEVP1. (B) Ribbon diagram of the X-ray structure of MFE-23. CDR3 loop residues P₉₇ to P₁₀₀ of the VH chain of MFE are shown in stick presentation and the site of peptide insertion in MFE-23 is indicated by an arrow. Y_{100b} that was mutated to P_{100b} to give NFEVP1 is shown in ball-and-stick presentation.

Figure 2. *E. coli* expressed NFEVP1 separated into monomeric and dimeric forms on size-exclusion chromatography (Superdex75). The *E. coli* protein was applied after Ni²⁺-affinity and the *P. Pastoris* after EMD-IMAC and Ni²⁺-affinity chromatographies. 12% Tris-glycine reducing SDS-PAGE shows dimeric (D) and monomeric (M) fraction of the *E. coli* expressed protein.

Figure 3. MFEVP1 showed concentration dependent binding to immobilized $\alpha v \beta 6$ in ELISA and on cells and inhibited cell migration of VB6 cells through LAP coated wells. (a) MFEVP1 (various concentrations) and MFE (10 μ g/ml) were applied to immobilized $\alpha v \beta 6$ and control Tris-buffered wells. Binding was detected with rabbit anti-MFE followed by goat horseradish peroxidase (HRP)-linked secondary anti-rabbit IgG antibody. (b) MFE or MFEVP1 were allowed to bind to A375P $\beta 6$ and A375Ppuro cells. Bound scFvs were detected by Flow Cytometry with mouse anti-Tetra-His IgG followed by Alexa Fluor®-conjugated anti-mouse Fc. Grey solids, non-immune IgG; black lines, 10D5 (anti- $\alpha v \beta 6$). MFEVP1 is denoted by red lines (50 μ g/ml), orange lines (5 μ g/ml), green lines

(0.5 $\mu\text{g/ml}$) and blue lines (0.05 $\mu\text{g/ml}$). Concentrations lower than 50 $\mu\text{g/ml}$ are omitted for MFE, and all A375Ppuro experiments. 50 $\mu\text{g/ml}$ MFEVP1 binding to A375P β 6 overlapped with the histograms for 5 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$ and is not shown. Data is representative of three independent experiments with similar results. (c,d) VB6 cells were allowed to invade through LAP-coated polycarbonate filters in the presence of the proteins. Inhibition of cell migration was observed for MFEVP1, NFEVP1 (both at 50 $\mu\text{g/ml}$) and 10D5 (c) and in a concentration-dependent manner for MFEVP1 (d). W632, designated control, and 10D5 antibodies were used at 1:100 and 10 $\mu\text{g/ml}$, respectively. The data represent the mean of triplicate measurements and error bars represent the standard deviation at each data point (a,c and d).

Figure 4. MFEVP1 and NFEVP1 bound to immobilized $\alpha\text{v}\beta$ 6 and not to $\alpha\text{v}\beta$ 3 by ELISA. A375Ppuro and A375P β 6 expressed $\alpha\text{v}\beta$ 8, $\alpha\text{v}\beta$ 5, $\alpha\text{v}\beta$ 3 and α 5 β 1 at similar levels. $\alpha\text{v}\beta$ 6 expression is only detected by the A375P β 6 cells. (a) Binding of MFEVP1, NFEVP1 and MFE [all at 20 $\mu\text{g/ml}$] was detected with rabbit anti-MFE followed by goat horseradish peroxidase (HRP)-labelled anti-rabbit IgG antibodies and, to detect integrin immobilization, wells were also incubated with mouse anti- αv followed by sheep HRP-labelled anti-mouse IgG. The data represent the mean of triplicate measurements and error bars represent the standard deviation at each data point. (b) A375Ppuro and A375P β 6 cells were incubated with antibodies. Negative controls (white histograms) had secondary antibody only.

Figure 5. MFEVP1 showed residual binding to immobilized CEA and bound to CEA-expressing LS-174T cells. CEA binding was eliminated by the Y_{100b} to P_{100b} mutation. (a) MFEVP1 and NFEVP1 (at three different concentrations) were added to

immobilized CEA and PBS wells. Binding was detected with rabbit anti-MFE followed by goat horseradish peroxidase(HRP)-labelled anti-rabbit IgG. The data represent the mean of triplicate measurements and error bars represent the standard deviation at each data point. (b) Cells were incubated with MFEVP1, NFEVP1 and MFE (all at 50 µg/ml). Binding was detected by Flow Cytometry with rabbit anti-MFE IgG followed by R-phycoerythrin(R-PE)-labelled goat anti-rabbit IgG. The Omission Control is the MFE experiment without rabbit anti-MFE IgG. Results are representative of three independent experiments. % Gated Fluorescence Intensities (7×10^1 - 10^4 , as indicated) are mean values from three separate experiments of which the mean control values have been subtracted.

15

Figure 6. NFEVP1(P) is internalized by β6-transfected A375P cells as revealed by indirect fluorescence confocal microscopy. β6-transfected and non-transfected(puro) A375P cells were incubated with NFEVP1 for 1hr at 4°C, the scFv was removed and cells were shifted to 37°C for the times indicated. Cell surface bound and internalized NFEVP1 was detected with rabbit anti-mouse IgG followed by Alexa Fluor®546 labelled goat anti-rabbit IgG(red). In the control experiment NFEVP1 was omitted. Blue reveals nuclear staining with Hoechst.

25

Figure 7. NFEVP1 and MFE had very similar secondary structure elements and denaturation curves as determined by FT-IR spectroscopy. (a) Second derivative FT-IR spectra of NFEVP1 and MFE were obtained from the absorbance spectra recorded at 30°C after buffer control subtraction. (b) For the denaturation curve both proteins were heated from 25°C to 85°C and the FT-IR spectra were measured. The midpoints of denaturation were obtained from fitting of the peak

30

intensity at 1635 cm^{-1} of the 2nd derivative spectra to a sigmoidal curve as 47°C for MFE and 45°C for NFEVP1.

Figure 8. HFEVP1 eluted almost exclusively as dimer on size-exclusion chromatography (Superdex 75), bound to immobilized $\alpha\text{v}\beta 6$ in ELISA and inhibited the adhesion of $\alpha\text{v}\beta 6$ -expressing 3T3 $\beta 6.19$ cells to LAP. (a) *P. Pastoris* expressed HFEVP1 was applied after EBD-IMAC and Ni^{2+} -affinity chromatographies. Twelve percent Tri-glycine SDS-PAGE under reducing and non-reducing conditions is shown of the dimeric fraction. (b) NFEVP1, HFEVP1 and MFE were applied at $20\mu\text{g/ml}$ to immobilized $\alpha\text{v}\beta 6$ and control Tris buffered wells. Binding was detected with mouse anti-Tetra-His IgG followed by sheep anti-mouse horseradish peroxidase(HRP)-linked secondary antibody. The data represent the mean of triplicate measurements and error bars represent the standard deviation at each data point. (c) Radiolabelled [^{51}Cr]3T3 $\beta 6.19$ cells in various concentrations of MFE, NFEVP1, HFEVP1 or 10D5 were added to 96-well plates coated with $50\mu\text{l}$ ($0.25\mu\text{g/ml}$) LAP. Data show the mean and standard deviations of quadruplet wells.

Figure 9. IMAC purification of hMFE, hMFE23-RGD and hMFE23-RGE. Western blotting of hMFE (A), hMFE23-RGD (B) and hMFE23-RGE (C) using anti-His₄. Samples were applied as follows: supernatant (lane 1), PBS wash (lane 2), 40 mM imidazole wash (lane 3), 200 mM imidazole wash (lane 4), 200 mM imidazole wash after dialysis against PBS (lane 5) and EDTA wash (lane 6).

30

Figure 10. Binding of hMFE, hMFE23-RGD and hMFE23-RGE to CEA. hMFE, hMFE23-RGD and hMFE23-RGE as neat supernatant, 1:10 dilution and 1:100 dilution for hMFE and 2YT were added

to immobilized CEA. Binding was detected with mouse anti-His₄ and HRP-conjugated sheep anti-mouse IgG.

Figure 11. Detection of His-tagged hMFE, hMFE-RGD and hMFE-RGE proteins binding to immobilised CEA, rs α v β 6 or PBS control. 96-well plates were coated with 1 μ g/ml CEA, 1.5 μ g/ml rs α v β 6 or PBS, and residual non-specific binding sites blocked with 1% TweenTM 20, 5% BSA. Purified proteins were diluted 1/50, added to the wells, and allowed to bind for one hour before washing and detection with Qiagen TetraHis (mouse anti-4xHis) followed by peroxidase-conjugated anti-mouse. Bound peroxidase was visualised by the colour change reaction on addition of the TMB+ reagent (DAKO), and quantitated by the absorbance at 450nm after the reaction had been stopped with 1N H₂SO₄. TetraHis and anti-mouse were omitted from some control wells, as shown. Data shown are the results of duplicate wells.

Detailed Description

20 *Antibodies*

In general, the present invention provides antibodies in which a peptide sequence capable of binding to a target, such as α v β 6 integrin, is inserted into at least one complementarity determining region of a parent antibody in order to modify the parent antibody so that it is capable of binding to the target, while retaining other useful properties of the parent antibody, such as the antibody's properties in interacting with the immune system (e.g. recruitment of complement), stability and half-life when administered in vivo, especially when compared to the corresponding peptide, and ease of production. Multiple target-binding sequences may be inserted into different complementarity determining regions of the parent antibody. Additionally or alternatively, in addition to the inserted

sequence(s), a target-binding sequence may be grafted onto one or more of the complementarity determining regions of the parent antibody. The presence of multiple target binding sequences may permit the formation of dimeric or
5 multimeric forms of the antibody. Examples of targets and the peptide sequences that are capable of binding to them are discussed in more detail below.

In particular, the present invention employs MFE-23 antibody
10 scaffolds and variants thereof as the parent antibodies into which the targeting peptides are inserted. As used herein, MFE-23 antibodies include the following examples from this family as parent antibody scaffolds that may be used in the present invention. MFE-23 was originally a scFv isolated
15 from a murine phage display library and selected for specific binding to carcinoembryonic antigen (CEA) (Chester et al., Lancet 343: 455-456, 1994). MFE-23 has been humanised in order to reduce the likelihood of immunogenicity (hMFE) and the humanised antibody has been
20 affinity matured to produce the mutant scFv sm3E. A stabilised humanised form of MFE-23 is referred to as shMFE. MFE-23 antibodies, and derivatives thereof, are disclosed in WO95/15341 or EP 0733072A and US7232888, which are expressly referenced in this context for their disclosure of these
25 antibodies, including their complete sequences including CDRs and properties. The antibodies have also undergone successful pre-clinical, and in some cases clinical, studies support its potential for use in targeted cancer therapies (e.g., radioimmunotherapy, cellular immunotherapy, gene
30 therapy, targeted cytokine therapy) and as an imaging agent. These include Phase I studies of radiolabelled MFE-23 for use as an imaging agent, for radioimmunoguided surgery and as the tumour-targeting moiety of an antibody directed enzyme prodrug therapy.

MFE-23 antibodies comprise the following six CDRs, as shown in SEQ ID NO: 6 of US7232888 (with the exception of CDR (d) (ii) disclosed elsewhere in the patent [and CDR (e) (i) disclosed elsewhere in the patent]):

- (a) Heavy Chain CDR 1: Gly Phe Asn Ile Lys Asp Ser;
- (b) Heavy Chain CDR 2: Asp Pro Glu Asn Gly Asp;
- (c) Heavy Chain CDR 3: Thr Pro Thr Gly Pro Tyr Tyr Phe Asp;
- (d) Light Chain CDR 1: (i) Ser Ser Ser Val Pro, or (ii) Ser Ser Ser Val Ser;
- (e) Light Chain CDR 2: (i) Ser Thr Ser, or (ii) Leu Thr Ser;
- (f) Light Chain CDR 3: Arg Ser Ser Tyr Pro Leu.

In Light Chain CDR2, (i) is derived from the sequence of the CDR of the murine MFE-23 antibodies and (ii) is derived from the sequence of the CDR of humanised MFE-23.

Since CDR loops are the accessible regions for interaction with the epitopes of the antigen to which the parent antibody was specific, it is generally desirable in the present invention to insert the targeting peptide sequences into these regions. While any CDR of the parent MFE-23 antibodies may be suitable, it is preferred that the peptide sequence capable of binding to the target is inserted into the CDR H3 region of the parent antibody, and preferably between residues Thr98 and Gly99. This is because the present inventors realised that in the parent MFE-23 antibodies, CDR H3 is the longest and most variable CDR and is essential for antigen binding. The site between Thr98 and Gly99 was selected as the sequence is at the end of a protruding loop structure and therefore means that the inserted targeting peptide should be available for binding to the target.

In one embodiment, the antibody of the present invention is a scFv or a diabody which comprises a linker having the sequence $(\text{Gly}_4\text{Ser})_n$, wherein n is between 1 and 4. A preferred example of a scFv or diabody having this general structure is represented in Figure 1.

The insertion may also be combined with mutation of one or more amino acid residues of the CDRs or framework regions of the parent antibody to modify or otherwise improve the properties of parent antibody, for example to reduce or eliminate the binding of the parent antibody to the antigen to which it was raised or was initially capable of specifically binding or to humanise the antibody or to increase affinity for the target. However, in other embodiments, it may be useful to retain the specificity of the parent antibody so that the antibody of the present invention is bispecific. Examples of other changes made to the amino acid sequence of the parent antibody include mutation of G44 to C44 of the VH domain and A100 to C100 of the VL domain to introduce two cysteine residues to form an inter-molecular stabilising disulphide bridge in a diabody and Y_{100b} to P_{100b} of the VH domain to help to reduce or eliminate any remaining residual binding to CEA. Further examples of other changes to the amino acid sequence of the parent antibody include mutations to improve affinity, specificity or stability and methods for achieving this are well known in the art. For example, if affinity maturation or increased specificity is required, residues in the CDR H3 could be varied using PCR mutagenesis or site directed mutagenesis. In addition, the remaining CDRs could be varied using PCR mutagenesis, site directed mutagenesis or rational addition/replacement of peptides (Hoogenboom, (2005) and references therein). The resulting mutated genes would be cloned and expressed as scFvs in filamentous

bacteriophage libraries. The libraries would be screened for phage displaying scFvs with improved binding and biological efficacy. If increased stability or yield is required the whole DNA could be varied using PCR

5 mutagenesis, site directed mutagenesis or growth in mutator strains of bacteria (Hoogenboom, (2005) and references therein). The resulting mutated genes would be cloned and expressed as scFvs in filamentous bacteriophage libraries. The libraries would be screened for phage displaying scFvs

10 with high expression levels and stability. In some embodiments it may also be useful to reduce the immunogenicity. Potential immunogenicity of the scFv, for example HFEVP1 or affinity matured variant, could be addressed by identification and modification of T-cell

15 epitopes and methods for this are well known in the art. This is based on the rationale that T cell help is required in order to mount a long-lived, isotype switched and high affinity antibody response [Chester KA, et al (2005)]. The approach requires the identification of short peptides

20 contained within the protein sequence that have a capacity to bind to the MHC class II binding groove and stimulate a subsequent T-cell response. Key residues in these peptides that contribute to T-cell epitope formation can be identified by determining whether the side chains interact

25 with key binding pockets in the MHC class II binding groove or with the T-cell receptor (TCR). Subsequent amino acid substitutions at these key residues can inhibit T-cell epitope formation by reducing the peptide affinity for MHC class II or preventing TCR recognition of the peptide MHC

30 complex. An experimental approach using a T-cell proliferation assays could be used as this takes into account factors that contribute to epitope formation, such as antigen processing and TCR binding. In vitro studies could be performed with peptides spanning the whole of the

scFv sequence, for example, 15mer peptides overlapping by 12 amino acids. The peptides could be tested for ability to stimulate T-cells in T-cell proliferation assays.

Typically, T-cells from healthy donors expressing a wide
5 range of HLA alleles would be used in addition to T-cells obtained from any patients demonstrating an immune response to the scFv. The precise location of key residues responsible for T-cell activation could be determined by residue replacement (usually with alanine) and testing the
10 resultant peptides in T-cell proliferation assays. *In silico* analysis could also be used to predict amino acid substitutions that should disrupt peptides binding to MHC class II. In the final stage, *in silico* modelling could be applied to predict those amino acid substitutions likely to
15 eliminate immune reactivity to the scFv without disruption of scFv structure or loss of scFv function. The gene for the preferred variant could be manufactured by recombinant DNA technology.

20 In the present invention, "MFE-23 antibody" includes antibodies which comprise the CDRs of the scFv MFE-23, fragments or derivatives thereof. It also includes the CDRs when incorporated into other antibody framework as described above, e.g. to produce a complete antibody structure, a
25 diabody or another antibody. These include antibody fragments which comprise an antigen binding domain are such as Fab, scFv, Fv, dAb, Fd and diabodies. It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or
30 chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework

regions, of a different immunoglobulin. See, for instance, EP 0 184 187 A, GB 2,188,638 A or EP 0 239 400 A.

Antibodies can be modified in a number of ways and the term
5 "antibody" should be construed as covering any specific
binding member or substance having an antibody antigen-
binding domain with the required specificity. Thus, this
term covers antibody fragments and derivatives, including
any polypeptide comprising an immunoglobulin binding domain,
10 whether natural or wholly or partially synthetic. Chimeric
molecules comprising an immunoglobulin binding domain, or
equivalent, fused to another polypeptide are therefore
included. Cloning and expression of chimeric antibodies are
described in EP 0 120 694 A and EP 0 125 023 A.

15

It has been shown that fragments of a whole antibody can
perform the function of binding antigens. Examples of
binding fragments are (i) the Fab fragment consisting of VL,
VH, CL and CH1 domains; (ii) the Fd fragment consisting of
20 the VH and CH1 domains; (iii) the Fv fragment consisting of
the VL and VH domains of a single antibody; (iv) the dAb
fragment (Ward, E.S. et al., Nature 341, 544-546 (1989))
which consists of a VH domain; (v) isolated CDR regions;
(vi) F(ab')₂ fragments, a bivalent fragment comprising two
25 linked Fab fragments (vii) single chain Fv molecules (scFv),
wherein a VH domain and a VL domain are linked by a peptide
linker which allows the two domains to associate to form an
antigen binding site (Bird et al, Science, 242; 423-426,
1988; Huston et al, PNAS USA, 85: 5879-5883, 1988); (viii)
30 bispecific single chain Fv dimers (WO93/11161) and (ix)
"diabodies", multivalent or multispecific fragments
constructed by gene fusion (WO 94/13804; Holliger et al,
P.N.A.S. USA, 90: 6444-6448, 1993). Fv, scFv or diabody
molecules may be stabilised by the incorporation of

disulphide bridges linking the VH and VL domains (Reiter et al, Nature Biotech, 14: 1239-1245, 1996). Minibodies comprising a scFv joined to a CH3 domain may also be made (Hu et al, Cancer Res., 56: 3055-3061, 1996).

5

In terms of utility of these antibodies, this is primarily therapeutic with imaging/diagnosis being a secondary utility. In terms of therapeutic use, antibodies have advantages over peptides as it is generally appreciated in the art that peptides tend to have short serum half-life in vivo. In contrast, antibody therapeutics tend to possess greater stability and longer half-life. Additional advantages of preferred antibodies of the present invention include combining high affinity binding properties of the $\alpha v \beta 6$ binding peptides together with advantages of the MFE antibody scaffold, namely stability, good production yields, e.g. in *Pichia pastoris*, and for humanised versions of the MFE antibody scaffold, low predicted immunogenic potential.

20 *Targeting Peptides*

The present invention involves the insertion of amino acid sequences generally referred to herein as "targeting peptides" that are capable of binding to a target. Generally, the inserted amino acid sequences capable of binding to the target are between 8, 10 or 12 amino acids and 24 or 30 amino acids in length, and more preferably are between 12 and 20 amino acids in length. In one embodiment, the target is an antigen present on diseased cells, to which the antibodies of the present invention are capable of binding using the targeting peptide. By way of illustration, the diseased cells may be surface antigen on, for example, cancer cells. In a preferred embodiment, the targeting peptide is capable of binding to an integrin, such as $\alpha v \beta 6$ integrin which is known to be involved in normal and

pathological processes such as wound healing, inflammation and tumour growth and metastasis.

More generally, the targets for the antibodies of the present invention may be targets that (i) are overexpressed or de novo expressed in diseased cells (and more specifically cancer cells) and/or (ii) are expressed in diseased cells and mediate the disease (and more specifically cancer). Thus, the present invention includes antibodies which can be used as tumour targeting antibodies, where target is overexpressed or de novo expressed in diseased cell, and/or function-modulating antibodies, e.g. whereby the target is expressed on the diseased cell and the antibody modulates the activity of the target and/or the targets ability to interact with its binding partners. In addition to $\alpha v\beta 6$ integrin, some specific examples include tumour associated antigens and/or antigens involved in mediated disease such as MUC1, 5T4, VEGFR (Hofmeister V et al., 2007), Tie2, endoglin (CD105) (Hofmeister V et al., 2007; Munoz R et al., 2007), uPA receptor (uPAR) (Li Y et al., 2007), PSMA (Baccala A et al., 2007, Buhler P et al., 2007)^{4,5}, and members of the ErbB (Her) family (Johnston JB et al., 2006).

Further targets may be identified using techniques well known in the art. These might include the following approaches.

In one approach, preferred peptides would include epitopes of the antigen-binding protein that are located on surface-exposed loops, making use of the three-dimensional structure of the antigen-binding protein. For example, in design of the MFE/shMFE/VP1 constructs we used the knowledge that the inserted 17-mer VP1 peptide is part of a long highly mobile

loop that forms a self contained unit in the VP1 protein of FMDV (Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman, J., Parry, N., (1993) Structure of a major immunogenic site on foot-and-mouth disease virus *Nature* 362:566-568). It is a general feature that residues of the ligand that bind to $\alpha v\beta 6$ are part of a surface exposed loop. In fibronectin also, which is a further ligand for $\alpha v\beta 6$, the RGD binding motif is part of a highly mobile loop, which protrudes from the rest of the tenth type III module (Main, A. L., Harvey, T. S., Baron, M., Boyd, J., and Campbell, I. D. (1992) The three-dimensional structure of the tenth type III module of fibronectin: An insight into RGD-mediated interactions *Cell* 71:671-678).

15

In a second approach, preferred peptides could be rationally designed using three-dimensional structures of the ligand-antigen complex if these are available. In this case the ligand residues that are involved in binding to the cell surface antigen are known from the detail of the ligand-antigen interaction.

20

In a third approach, preferred peptides could be those which are potent in inhibiting antigen/antigen-binding protein interactions as shown *in vitro* in ELISA or cell-based assays. For example, a 17-mer peptide that contained the 17-mer VP1 peptide sequence minus the N-terminal alanine and a 20-mer peptide that contained the 17-mer peptide sequence used to create MFEVP1 were potent inhibitors of FMDV binding to purified $\alpha v\beta 6$ and $\alpha v\beta 6$ -expressing cells (Burman, A., Clark, S., Abrescia, N. G., Fry, E. E., Stuart, D. I., and Jackson, T. (2006) Specificity of the VP1 GH Loop of Foot-and-Mouth Disease Virus for αv Integrins *J. Virol.* 80:9798-9810) and of $\beta 6$ -transfected fibroblast cells to LAP

30

(Dicara, D., Rapisarda, C., Sutcliffe, J. L., Violette, S. M., Weinreb, P. H., Hart, I. R., Howard, M. J., and Marshall, J. F. (2007) Structure-function analysis of Arg-Gly-Asp helix motifs in alpha v beta 6 integrin ligands *J. Biol. Chem* 282:9657-9665), respectively.

In a fourth approach, preferred peptides could be those that are selected *in vitro* or *in vivo* from peptide libraries displayed on filamentous bacteriophage. This method has been used to obtain peptides with high affinity and specificity for tumour targets (Landon, L.A., Deutscher, S.L. (2003) Combinatorial Discovery of Tumor Targeting Peptides Using Phage Display. *J Cell Biochem.* 90:509-517). Peptide libraries consist of random cyclic and linear peptide sequences. *In vitro* selection is performed using purified recombinant proteins or cells that express the target protein.

For example, the 17-mer peptide VP1 peptide sequence inserted into MFE-23 to create MFEVP1 contains the DLXXL sequence, which has specificity for $\alpha v \beta 6$, and has also been identified from screening 12-mer peptide libraries displayed on phage using recombinant transmembrane truncated soluble receptor. (Kraft, S., Diefenbach, B., Mehta, R., Jonczyk, A., Luckenbach, G. A., and Goodman, S. L. (1999) Definition of an Unexpected Ligand Recognition Motif for $\alpha v \beta 6$ Integrin *J. Biol. Chem.* **274**:1979-1985).

In another example, a random phage library consisting of linear 12 amino acid peptides identified peptides that bound specifically to recombinant prostate-specific membrane antigen (PMSA), an attractive candidate for targeted therapy for prostate and other solid tumours (Aggarwal, S., Singh, P., Topaloglu, O., Isaacs, J.T., Denmeade, S.R. (2006) A

Dimeric Peptide That Binds Selectively to Prostate-Specific Membrane Antigen and Inhibits its Enzymatic Activity *Cancer Res.* 66:9171-9177). A pIII-displayed disulphide-constrained heptamer peptide library was screened on purified
 5 extracellular PSMA to identify PSMA binding peptides.

In another example, the epidermal growth factor receptor, type 2 (ErbB-2) tyrosine kinase receptor that shows increasing promise as a target for cancer diagnosis and
 10 therapy has been used as bait. In this case, a random 6 amino acid peptide bacteriophage display library was selected against purified ErbB-2 extracellular domain to identify peptide binding sequences (Karasseva, N.G., Glinsky, V.V., Chen, N.X., Komatireddy, R., Quinn, T.P.
 15 (2002) Identification and Characterization of Peptides that Bind Human ErbB-2 Selected from a Bacteriophage Display Library *J. Protein Chem.* 21:287-296).

Peptide sequences can also be selected by display of peptide
 20 libraries on phage that bind to carbohydrate antigens, which show different composition in malignant transformations. For example, an in vitro phage display was employed to find peptides that bind to the Thomsen-Friedenreich (TF) tumour-associated antigen (Peletskaya, E.N., Glinsky, V.V.,
 25 Glinsky, G.V., et al. (1997) Characterisation of peptides that bind the tumour-associated Thomson-Friendenreich antigen selected from bacteriophage display libraries *J. Mol. Biol.* 270:374-384).

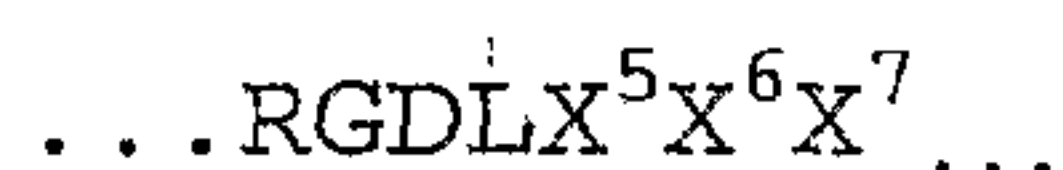
30 Peptide library selection on cells was used to identify a further $\alpha v \beta 6$ binding peptide sequence. A 20-mer peptide library fused to bacteriophage coat protein pIII selected a peptide that bound specifically to a lung adenocarcinoma cell line and was shown subsequently to bind to $\alpha v \beta 6$

(Elayadi, A.N., Samli, K.N., Prudkin, L., Liu, Y.-H., Bian, A., Xie, X.-J., Wistuba, I.I., Roth, J.A., McGuire, M.J., Brown, K.C. (2007) A Peptide Selected by Biopanning Identifies the Integrin $\alpha v \beta 6$ as a Prognostic Biomarker for
5 Nonsmall Cell Lung Cancer. *Cancer Res.* 67:5889-5895).

Peptides that bind to tumour and tumour-associated vasculature have also been successfully selected from phage peptide libraries *in vivo* (Landon, L.A., Deutscher, S.L.
10 (2003) Combinatorial Discovery of Tumor Targeting Peptides Using Phage Display. *J Cell Biochem.* 90:509-517). This approach allows for targeting of novel antigens in addition to known targets. Using as a target the RIP1-Tag2 mouse model of multistage tumorigenesis involving the pancreatic
15 islets of Langerhans, allowed the identification of several peptides that discriminate between the vasculature of the premalignant angiogenic islets and the fully developed tumours (Joyce, J.A., Laakkonen, P., Bernasconi, M., Bergers, G., Ruoslahti, E., Hanahan, D. (2003) Stage-
20 specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis *Cancer Cell* 4:309-403). A similar study isolated peptides selective for angiogenic progenitors and solid tumours from the skin of a transgenic mouse model involving the human papillomavirus
25 type 16 oncogene (Hoffman, J.A., Giraudo, E., Singh, M., Zhang, L., Inoue, M., Porkka, K., Hanahan, D., Ruoslahti, E. (2003) Progressive vascular changes in a transgenic mouse model of squamous cell carcinoma *Cancer Cell* 4:383-391). Peptides specific for human vasculature and human tissue
30 have been derived from peptide phage library selection on human tissue transplanted into SCID mice (George, A.J.T., Lee, L., Pitzalis, C. (2003) Isolating ligands specific for human vasculature using *in vivo* phage selection *Trends Biotech.* 21:199-203).

By way of example, the targeting peptides that may be used in accordance with the present invention include those disclosed in our earlier application, WO2007/039728. These
5 peptides include peptides which comprise the amino acid sequence motif $\text{RGDLX}^5\text{X}^6\text{X}^7$, wherein X^5 , X^6 and X^7 independently represent any amino acid residue, and preferably independently selected from Glu, Ala, Leu, Met, Gln, Lys, Arg, Val, Ile, Trp, Phe, Asp, His and Thr. While the
10 peptides disclosed in WO2007/039728 generally comprise an alpha helical structure induced by the amino acids following the RGD sequence, this is not a requirement of the peptides of the present invention, in particular as they are inserted into the CDR of a parent antibody and may not therefore be
15 able to adopt this secondary structural feature.

Preferred examples of peptides capable of binding to $\alpha\text{v}\beta 6$ integrin include peptides which comprise amino acid sequences represented by the following general formulae,
20 based on the peptides disclosed in WO2007/039728:



wherein

- X^5 is selected from Glu, Ala or Gln;
25 X^6 is selected from His, Val, Thr or Glu;
 X^7 is selected from Leu or Ile.

These peptide sequence above may be part of a longer sequences, for example having one, two, three, four, five,
30 ten or more additional amino acids linked to the N- and/or C-terminus of the peptide, while optionally conforming to the preferred ranges of lengths of peptide sequence set out above. A particularly preferred targeting peptide sequence has the amino acid sequence AVPNLRGDLQVLAQKVA.

In some embodiments, the peptides may comprise an "alpha helical structure" as disclosed in WO2007/039728, that is a sequential group of amino acids in a peptide that interact
5 with a particular hydrogen bonding pattern and thus define a helical structure. For example, the hydrogen bonding pattern in a standard alpha helix is between the carbonyl oxygen of residue n and the amide hydrogen of residue $n+4$. For the 3_{10} -helix, this hydrogen bonding pattern is between
10 residues n and $n+3$ and for a pi-helix it is between residues n and $n+5$. The number of residues per turn in each alpha-helix is 3.6, 3.0 and 4.4 for the standard alpha-helix, 3_{10} -helix and pi-helix respectively, see for example WO95/00534.

15 *Variants and uses of the antibodies*

In one aspect, the antibodies of the present invention may be linked to a detectable moiety. The term "detectable moiety" relates to a group that, when located at the target site following administration of the antibodies of the
20 present to a patient, may be detected, typically non-invasively from outside the body and the site of the target located. Thus, the antibodies of the present invention are useful in imaging and diagnosis. Detectable moiety are entities that are detectable by imaging techniques such as
25 Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS), Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) and optical imaging. Preferably, imaging moieties are stable, non-toxic entities that retain their properties
30 under *in vitro* and *in vivo* conditions. Examples of such moieties include but are not limited to radioactive moieties, for example radioactive isotopes. Suitable radioactive atoms include technetium-99m or iodine-123 for scintigraphic studies. Other readily detectable moieties

include, for example, spin labels for MRI such as iodine-123 again, iodine-131, indium-111, fluorine-18, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron and optical moieties which include Cy5.5 and quantum dots.

5

Alternatively or additionally, the antibodies of the present invention may be conjugated or linked to a therapeutically active moiety, for example a moiety that is cytotoxic.

10 A further class of groups that can be incorporated into the antibodies of the present invention are affinity tags that can be introduced into the antibodies to enable them to be manipulated or detected in one or more subsequent steps. A wide range of affinity tags are known in the art suitable
15 affinity tags include members of specific binding pairs, antibodies and antigens, biotin which binds to streptavidin and avidin, polyhistidine (e.g. hexa-His or tri-His tags) or amino di- or tri-carboxylates which bind to metal ions such as Ni^{2+} or Co^{2+} , Flag or Glu epitopes which bind to anti-Flag
20 antibodies, S-tags which bind to streptavidin, calmodulin binding peptide which binds to calmodulin in the presence of Ca^{2+} ; ribonuclease S which binds to aporibonuclease S; and c-Myc which recognises anti-c-Myc antibody. Examples of other affinity tags that can be used in accordance with the
25 present invention will be apparent to those skilled in the art. Antibodies including these affinity tags can be easily purified and manipulated.

The term "therapeutically active moiety" encompasses a
30 moiety having beneficial, prophylactic and/or therapeutic properties..

In one embodiment the therapeutically active moiety is a cytotoxic chemotherapeutic agent. Cytotoxic

chemotherapeutic agents are well known in the art and include anti-cancer agents such as:

Alkylating agents including nitrogen mustards such as mechlorethamine (HN2), cyclophosphamide, ifosfamide,

5 melphalan (L-sarcolysin) and chlorambucil; 10 ethylenimines and methylnelamines such as hexamethylnelamine, thiotepa; alkyl sulphonates such as busulfan; nitrosoureas such as carmustine (BCNU), lomustine (CCNLJ), semustine (methyl-CCN-U) and streptozoein (streptozotocin); and triazenes such as
10 decarbazine (DTIC; dimethyltriazenoimidazolecarboxamide); Antimetabolites including folic acid analogues such as methotrexate (amethopterin); pyrimidine analogues such as fluorouracil (5- fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine
15 arabinoside); and purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2'-deoxycofonnycin). Natural Products including vinca alkaloids such as vinblastine (VLB) and vincristine; epipodophyllotoxins such
20 as etoposide and teniposide; antibiotics such as dactinomycin (actinomycin D), daunorabycin (daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin Q; enzymes such as L-asparaginase; and biological response modifiers such as
25 interferon alphenomes. Miscellaneous agents including platinum coordination complexes such as cisplatin (cis-DDP) and carboplatin; anthracenedione such as mitoxantrone and antbracycline; substituted urea such as hydroxyurea; methyl hydrazine derivative such as procarbazine (N-
30 methylhydrazine, MIH); and adrenocortical suppressant such as mitotane (o, p'-DDD) and aminogluthethimide; taxol and analogues/derivatives; and hormone agonists/antagonists such as flutamide and tamoxifen.

Methods of conjugating antibodies to therapeutic agents are well known in the art.

In further embodiments, the antibodies of the present
5 invention may be formulated using particle based delivery systems such as nanoparticles and lipid-based vesicles such as liposomes or other similar structures composed of lipids. Liposomes are a spherical vesicles comprising a phospholipid bilayer that may be used as agents to deliver materials such
10 as drugs or genetic material. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (egg phosphatidylethanolamine) or of pure components like DOPE (dioleoylphosphatidylethanolamine). The synthesis and use of liposomes is now well established in the art. Liposomes
15 are generally created by sonication of phospholipids in a suitable medium such as water. Low shear rates create multilamellar liposomes having multi-layered structures. Continued high-shear sonication tends to form smaller unilamellar liposomes. Research has also been able to
20 enable liposomes to avoid detection by the immune system, for examples by coating the liposomes with polyethylene glycol (PEG). It is also possible to incorporate species in liposomes, such as the peptides of the invention to help to target them to a delivery site, e.g. in cells or *in vivo*.

25

The use of nanoparticles as delivery agents for materials associated with or bound to the nanoparticles is known in the art. Some types of nanoparticle comprises a core, often of metal and/or semiconductor atoms, to which ligands of one
30 or more different types may be linked, including, for example, one or more of the peptides of the present invention, see for example WO02/32404, WO2005/10816 and WO2005/116226. Other types of nanoparticle may be formed from materials such as liposomes. In some instances, the

nanoparticles may be derivatised or conjugated to other ligands may be present to provide the nanoparticles with different properties or functions. In some embodiments, the nanoparticles may be quantum dots, that is nanocrystals of
5 semiconducting materials which have the striking chemical and physical properties that differ markedly from those of the bulk solid (see Gleiter, Adv. Mater. 1992, 4, 474-481). Now that their quantum size effects are understood, fundamental and applied research on these systems has become
10 increasingly popular. An interesting application is the use of nanocrystals as luminescent labels for biological systems, see for example Brucher et al, Science 1998, 281, 2013-2016, Chan & Nie, Science, 1998, 281, 2016-2018, Mattousi et al, J. Am. Chem. Soc., 2000, 122, 12142-12150,
15 and Alivisatos, Pure Appl. Chem. 2000, 72, 3-9. The quantum dots have several advantages over conventional fluorescent dyes: quantum dots emit light at a variety of precise wavelengths depending on their size and have long luminescent lifetimes.

20

In a further embodiment, the cytotoxic moiety is a cytotoxic peptide or polypeptide moiety by which we include any moiety which leads to cell death.

25 Cytotoxic peptide and polypeptide moieties are well known in the art and include, for example, ricin, abrin, *Pseudomonas* exotoxin, RNase, tissue factor and the like.

The use of ricin as a cytotoxic agent is described in Burrows & Thorpe, P.N.A.S. USA 90: 8996-9000, 1993,

30

and the use of tissue factor, which leads to localised blood clotting and infarction of a tumour, has been described by Ran et al., Cancer Res. 58: 4646-4653, 1998 and Huang et al., Science 275: 25 547-550, 1997. Tsai et al., Dis. Colon Rectum 38:

1067- 1074, 1995 describes the abrin A chain conjugated to a monoclonal antibody.

Other ribosome inactivating proteins are described as cytotoxic agents in WO 96/06641. *Pseudomonas* exotoxin may
5 also be used as the cytotoxic polypeptide moiety (see, for example, Aiello et al, P.N.A.S. USA 92: 10457-10461, 1995.

Certain cytokines, such as TNF α and IL-2, may also be useful as cytotoxic and/or therapeutic agents.

10

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
15 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
20 density of radioactive atoms in the antibody of the invention are such that a dose of more than 4000 cGy, and more 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
25 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
30 ¹¹¹In or ⁹⁰Y. Tyrosine residues may be labelled with ¹²⁵I or ¹³¹I.

In a further embodiment, the present invention provides a polypeptide is linked to viral coat protein other than FMDV

to change the tropism of the virus for delivery of DNA encoding therapeutic genes.

Alternatively, any of these systems can be incorporated into
5 a prodrug system. Such prodrug systems are well known in the art and include ADEPT systems in which an antibody according to the present invention is conjugated or conjugatable or fused to an agent capable of converting a prodrug to a cytotoxic moiety is an enzyme for use in
10 antibody directed enzyme prodrug therapy.

In a further aspect, the present invention provides a pharmaceutical composition comprising peptide and/or nucleic acid and/or expression vector as defined above and a
15 pharmaceutical acceptable carrier.

The term "pharmaceutically acceptable carrier" generally includes components that are compatible with the peptide, nucleic acid or vector and are not deleterious to the
20 recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free. However, other acceptable carriers may be used. Typically, the pharmaceutical compositions or formulations of the invention are for parenteral administration, more particularly for
25 intravenous administration.

In a further aspect, the present invention provides the use of an antibody as described herein, or nucleic acid encoding the antibody or a vector comprising the nucleic acid, for
30 the preparation of a medicament for the treatment of a condition characterised by diseased cells which express the target, for example a condition in which the cells overexpress the target and/or display the target on the cell

surface and/or which is disease that is mediated by the target.

Examples of such conditions are provided below and include
5 cancer, for example by making use of the antigens expressed
on the surface of cancer cells. In some embodiments, the
conditions include $\alpha v \beta 6$ -mediated diseases or diseases in
which cells overexpress $\alpha v \beta 6$, such as cancer, chronic
fibrosis, chronic obstructive pulmonary disease (COPD), lung
10 emphysema or chronic wounding skin disease, such as
epidermolysis bullosa. As mentioned herein, these
conditions also include the treatment of wound healing and
inflammation.

15 The medicament or pharmaceutical composition of the present
invention as defined above may usefully be administered to a
patient who is also administered other medicaments, as it
will be known to those skilled in the art. For example, in
the case of cancer, the medicament or pharmaceutical
20 composition of the present invention may be administered to
a patient before, after or during administration of the
other anti-tumour agent(s), for example before, after or
during chemotherapy. Treatment with the antibody after
chemotherapy may be particularly useful in reducing or
25 preventing recurrence of the tumour or metastasis. For
example, the anti-tumour agent can be covalently linked
directly or indirectly (via liposomes/nanoparticles) to an
antibody of the present invention.

30 In a further aspect, the present invention provides a method
of imaging epithelial cells overexpressing $\alpha v \beta 6$ in the body
of an individual, the method comprising administering to the
individual an effective amount of an antibody as defined
herein. The method is particularly useful for the imaging

of chronic fibrosis, chronic obstructive pulmonary disease (COPD), lung emphysema, chronic wounding skin disease (e.g. epidermolysis bullosa) or epithelial tumour cells. For example, the method of imaging may include linking the
5 targeting antibody to a fluorescent probe and incorporate into a mouth-wash, chewing gum, spray or other emolument such that the antibody-probe conjugate may be visualised by its fluorescent tag.

10 Examples

The experimental examples set out below demonstrate that the specificity of a parent antibody can be modified by inserting a $\alpha v \beta 6$ binding peptide sequence into the CDR of the parent antibody. In the examples, the parent antibody
15 is a single-chain Fv antibody fragment (scFv), which consists of the variable heavy and variable light chain regions tethered by a flexible linker that retains the complete antibody's binding properties. By way of example, the scFv may be based on a MFE-23 scFv, a scFv developed by
20 phage technology that binds with high affinity to the carcinoembryonic antigen, CEA (14). CEA is a tumour selective marker that is highly expressed on most gastrointestinal carcinomas and on a number of breast, lung and ovarian carcinomas. Iodinated MFE has been used in
25 patients for imaging (15) and radioimmunoguided surgery in colorectal cancer (16). MFE has also shown promise for cancer therapy when used as a fusion protein with carboxypeptidase G2 in antibody-directed enzyme prodrug therapy (ADEPT) (17); (18).

30

In this embodiment of the present invention, a scFv against $\alpha v \beta 6$ was produced rationally by antibody engineering by inserting the peptide binding motifs of the known $\alpha v \beta 6$ peptide ligands, such as VP1 proteins, into the loop region

of MFE. The third complementarity-determining region (CRD3) of the variable heavy chain (VH) of MFE provides the major site of interaction with CEA, as assessed by mutagenesis, and accordingly was chosen as a preferred site for such an
5 insertion.

In the experiments set out below, the insertion of an $\alpha v\beta 6$ -binding peptide of VP1 from the FMDV strain O₁ BFS into the CRD3 loop of the VH domain of MFE is described. A 17-mer
10 peptide and a 20-mer peptide corresponding to this region of VP1 have previously been shown to be potent inhibitors of FMDV binding to purified $\alpha v\beta 6$ and $\alpha v\beta 6$ -expressing cells (24) and of $\beta 6$ -transfected fibroblast cells to LAP (25), respectively. The addition of a 17-mer peptide of VP1,
15 equivalent in sequence to the inhibitory peptides, to MFE changed binding specificity of the scFv from CEA to $\alpha v\beta 6$, as shown by ELISA, cell-binding and inhibition in a migration assay. Additional mutation of Y_{100b} to P_{100b} of the VH domain as in NFEVP1 eliminated all remaining residual binding to
20 CEA.

Materials and Methods

3D Protein visualisation

25 The X-ray structure of MFE (pdb code 1QOK) (26) was visualised in Insight II (Accelrys) on a Silicon Graphics workstation.

Antibodies

30 Murine monoclonal antibodies to $\alpha v\beta 3$ (LM609), $\alpha v\beta 6$ (10D5) and $\alpha 5\beta 1$ (P1D6) were purchased from Chemicon International, Harrow, UK whereas those to $\alpha v\beta 5$ (P1F6) and $\alpha v\beta 8$ (14E5) were generous gifts from Drs. Dean Sheppard and Steve Nishimura

(UCSF), respectively. The secondary antibody was Alexafluor-488 conjugated rabbit anti-mouse antisera (Molecular Probes) unless otherwise stated.

5 *Construction of plasmids for expression in E. coli*

Construction of MFE-RGD and MFE-RGE plasmid, MFE-RGD/pUC119 and MFE-RGE/pUC119

MFE-RGD and MFE23-RGE were constructed by site-directed mutagenesis using the MFE-RGD forward

10 (5'_CTACTGCAACGAAGGGACAGCTAGAGGTGATTGGCTACTTTGTTTCGACTACTGGG GACAAG_ 3') and MFE-RGD reverse

(5'_CTTGTCCCCAGTAGTCGAACAAAGTAGCCAAATCACCTCTAGCTGTCCCTTCGTTG CAGTAG _3') primers or MFE-RGE forward (

5'_GAAGGGACAGCTAGAGGTGAATTGGCTACTTTGTTTCGACTACTG_3') and MFE-

15 RGE reverse (

5' _CAGTAGTCGAACAAAGTAGCCAAATTCACCTCTAGCTGTCCCTTC _3')

respectively. The PCR reactions used the phMFEhis₁₁₉

plasmid as template, which incorporated the humanised form of MFE-23 (hMFE) and a C-terminal 6xHis tag enabling

20 purification by Immobilised Metal Ion Affinity Chromatography (IMAC).

Construction of MFE and HFE MFE VH CDR3 loop variant plasmid, MFEVP1/pUC119 and HFEVP1/pUC119

25 MFEVP1 and HFEVP1, which contain the MFE of HFE sequences respectively, and a 17-mer $\alpha\beta$ 6-binding peptide of VP1 in the CRD3 loop of the heavy chain (Figure 1), were

constructed from three PCR reactions. First, the 5' end were constructed with the VH MFEVP1 (5' CATGCCATGGCCCAGGTGAAACTG)

30 or VH HFEVP1 (5' CATGCCATGGCCCAAGTTAAACTGGAACAG TCC) sense primers and the MFEVP1

(5' GCGCCAGCACCTGCAGATCACCTCGCAGATTCGGAACTGCAGTCGGAGTCCCCTCAT TAC) or HFEVP1

(5' GAGCCAGCACCTGCAGATCACCTCGCAGATTCGGAACTGCAGTTGGTGTCCCTTCGT

TGC) anti-sense primers, respectively, that contained parts of the additional peptides of the $\alpha\beta 6$ -binding motif of VP1 (shown underlined in the primes). Second, the 3'ends were constructed with the VL MFEVP1 (5'

5 ATAGTTTAGGCGGCCGCCCGTTTCAGCTC) or VL HFEVP1 (5' ATAGTTTAGGCGGCCGCCAGCCTTGATTTC) anti-sense primers and the MFEVP1

(5' CTGCGAGGTGATCTGCAGGTGCTGGCGCAGAAAGTTGCAGGGCCGTACTACTTTGAC TACTG) or HFEVP1

10 (5' CTGCGAGGTGATCTGCAGGTGCTGGCTCAGAAAGTTGCAGGTCCTTAC**CCT**TTTCGAC TACTGGGGACAAGG) sense primers, respectively which contain part of the additional $\alpha\beta 6$ -binding motif and in the case of HFEVP1 also introduced the Y100b to P100b mutation (shown bold and underlined in the primer; amino acid numbering is

15 given with the Kabat nomenclature). The PCR reactions for MFEVP1 used the MFE/puc119 as template and those for HFEVP1 the HFE/pCTCON plasmid (27) as templates. Third, the PCR products from the first two reactions, were used as templates and amplified with the VH MFEVP1 sense and VL

20 MFEVP1 anti-sense or VH NFEVP1 sense and VL HFECP1 anti-sense primers to give the PCR product for MFEVP1 or HFEVP1 respectively. The VH sense and VL anti-sense primers introduced NcoI and NotI sites (shown in bold and underlined in the primers) into the PCR products, respectively. Thus,

25 the third PCR products and the puc119 plasmid were treated with these restriction enzymes and ligated to yield either MFEVP1/pUC119 or HFEVP1 plasmids. Correctness of the DNA sequences was verified by DNA sequencing.

30 *Introduction of the VH-Y_{100b} to VH-P_{100b} mutation in MFEVP1 plasmid*

The Y_{100b} to P_{100b} mutations of the VH domain of the MFEVP1 was introduced by site-directed mutagenesis. The Pro mutation was introduced in MFEVP1/pUC119 with

5 'GTTGCAGGGCCGTACCCGTTTGACTACTGGGGC 3' as the sense and
 5 'GCCCCAGTAGTCAAACGGGTACGGCCCTGCAAC 3' as the anti-sense
 primers to give NFEVP1/pUC119 (the Pro nucleotide sequence
 is shown in bold). DNA sequencing verified these Pro
 5 mutations.

*Construction of NFEVP1/pICZαBHis and HFEVP1/pCIZαBCysHis
 plasmid for expression in yeast*

The NFEVP1/pUC119 and HFEVP1/pUC119 plasmid were digested
 10 with SfiI and NotI and cloned into an equally digested
 pICZαBHis or pPICZαBCysHis vectors respectively, for
 expression in yeast. The modified pICZαBHis and
 pICZαBCysHis vectors, when compared to the original pPICZαB
 vector (Invitrogen, Karlsruhe, Germany), do not contain the
 15 myc-tag but the His tag is present. The pPICZαBCysHis vector
 in addition contains a Cys immediately before the six His
 residues.

*Expression and purification of MFE-RGD and MFE-RGE in E.
 20 coli*

The MFE-RGD/pUC119 and MFE-RGE/pUC119 plasmids were
 electroporated into competent *E. coli* TG1 cells and grown on
 2xYT, containing ampicillin (100µg/ml) and 1% glucose plates
 at 37°C. Single colonies were used to inoculate 10ml of
 25 2xYT, containing ampicillin (as above) and 1%glucose media
 and after 1:500 dilution were grown in 50ml of 2xYT,
 ampicillin (as above) and 0.1% glucose at 37°C until the
 OD_{600nm} was 0.9. Protein expression and secretion into the
 media was induced by addition of 1mM isopropyl-β-D-
 30 thiogalactoside (IPTG, Sigma) and grown at 30°C O/N. The
 supernatant was separated from the cells by centrifugation
 at 4,000rpm for 20 min.

Purification by Qiagen Ni-NTA.

MFE-RGD and MFE-RGE proteins were purified under native conditions from bacterial supernatant using the Qiagen Ni-NTA Spin Column kit broadly according to the manufacturer's instructions. Briefly, a spin column holding a nickel-containing resin was equilibrated with "Lysis Buffer" (see below) before supernatant containing the 6xHis-tagged protein was spun through and the column washed to remove non-specifically bound material. During this procedure exposed his-tags bind to the nickel resin, and are therefore retained specifically in the column. His-tagged proteins were then eluted by two spins with an imidazole buffer. The manufacturer's instructions were followed with amendments as follows: bacterial supernatant was loaded directly onto the equilibrated column (thereby avoiding the lysis steps) and this step repeated until approximately 4.8ml bacterial supernatant had passed through each column. Buffer recipes were altered as follows: "Lysis Buffer": PBS, 300mM sodium chloride; "Wash Buffer": PBS, 300mM sodium chloride, 20mM imidazole; "Elution Buffer": PBS, 300mM sodium chloride, 250mM imidazole. Bound protein was eluted with 2 x 200µl Elution Buffer and the two fractions dialysed separately against PBS using Slide-a-lyser dialysis cassettes (Perbio Science UK Ltd, Cramlington, UK) according to the manufacturer's instructions. Dialysed protein was removed from the Slide-a-lyser and stored at -80°C.

Purification by Streamline

Hundred fifty ml of clarified supernatant was dialysed against three changes of PBS and NaCl was added to a final concentration of 1M. The proteins were purified by Streamline™ Chelating (Amersham Biosciences). The matrix was charged with 5 volumes of 0.1M CuSO₄ for 5min, excess CuSO₄ was washed off with 5 volumes of dH₂O and equilibrated with 10 volumes of binding buffer (PBS/1M NaCl). The

dialysed supernatant was mixed with the 1.5 ml of charged matrix and poured into a small column. Non-specific bound proteins were washed off the column with PBS/1M NaCl followed by 40mM Imidazole/PBS/1M NaCl. The proteins were
5 eluted with 200mM Imidazole/PBS/1M NaCl. The matrix was then washed with 5 volumes of 0.1M EDTA. Each washing and elution step was done with 4.5 ml volumes. Fractions of interest were pooled and dialyzed against PBS. Protein yields in the 200 mM imidazole fraction measured after dialysis were as
10 follows: hMFE, 0.181 mg/ml, hMFE23-RGD, 0.101 mg/ml and hMFE23-RGE, 0.108 mg/ml. Washing, eluted and dialysed samples revealed by Western Blotting are shown in Fig. 9 A-C.

15 *Expression and purification of MFEVP1 and NFEVP1 in E. coli*
The MFEVP1/pUC119 and NFEVP1/pUC119 plasmids were electroporated into competent *E. coli* TG1 cells and grown on 2xYT, containing ampicillin (50µg/ml) and 1% glucose plates at 37°C. Single colonies were used to inoculate 5ml of
20 2xYT, containing ampicillin (as above) and 1%glucose media and after 1:500 dilution were grown in 2x500ml of 2xYT, ampicillin (as above) and 0.05% glucose at 37°C until the OD_{600nm} was 0.9. Protein expression and secretion into the media was induced by addition of 1mM isopropyl-β-D-
25 thiogalactoside (IPTG, Sigma) and grown at 30°C O/N. The supernatant was separated from the cells by centrifugation at 16,000 g for 25 min and was further clarified by filtration through 0.2 µm membranes (Nalgene) and subsequently dialysed three times against PBS..
30 Purification of MFEVP1 and NFEVP1 was by immobilized metal-affinity chromatography (IMAC). Ten ml of Cu-charged StreamlineTM chelating resin (GE Healthcare) were incubated with the supernatant, after addition of 1M NaCl, at RT for 1h. The resin was collected, washed with 1M NaCl/PBS, 40mM

imidazole and bound proteins were eluted with 200mM imidazole. The 200mM imidazole protein containing fractions were dialysed against TBS, concentrated by an Amicon stirred cell with a YM3 membrane (Millipore) and further purified by
 5 size-exclusion chromatography. Size-exclusion chromatography was performed on Superdex75 column in Tris buffered saline, pH 7.5 (TBS) at 1.5 ml/min. MFEVP1 and NFEVP1 eluted as two peaks, representing the monomeric (67 ml) and dimeric (56 ml) forms. Their molecular weights were
 10 estimated from molecular weight standards, Ovalbumin (44 kDa), Carbonic Anhydrase (29kDa), and Myoglobin (17kDa) and the monomeric and dimeric forms of MFE.

15 *Expression and purification of NFEVP1 and HFEVP1 in P. pastoris*

For expression of NFEVP1 and HFEVP1 in *P. pastoris* the NFEVP1/pPICZ α BHis and HFEVP1/pPICZ α BCysHis plasmids, respectively, were linearized with PmeI and transformed into electrocompetent X33 cells (Invitrogen) by electroporation.
 20 Transformants were grown on YPDS/100 μ g/ml Zeocin (Invitrogen, Karlsruhe, Germany) plates. Single colonies were screened for protein expression and for inserts by PCR with the 5'AOX and 3'AOX primers. The colony with highest protein expression was stored in 20% glycerol at -80°C.
 25 NFEVP1 and HFEVP1 were produced by fermentation and initial purification involved expanded bed adsorption immobilized metal affinity chromatography (EBA-IMAC), which also concentrates the proteins, following previously described procedures.(22;23) The 200 mM imidazole EBA-IMAC eluate
 30 fraction, containing either NFEVP1 or HFEVP1, were dialysed into PBS. To the concentrate 1M NaCl was added and this was applied to a Ni²⁺ charged HiTrapTM Chelating HP 1ml affinity column (GE Healthcare) for further concentration. Elution in 1ml fractions was by 500mM imidazole/1M NaCl/PBS. The

diabody containing eluate (2ml) was further purified by size-exclusion chromatography on a Superdex 75 column in PBS, with a flow rate of 1.5ml/min.

5 *SDS-Polyacrylamide gel electrophoresis (PAGE) and Western Blot Analysis*

Proteins were analysed by SDS/PAGE using Tris/Glycine Gels (Invitrogen) and stained with Coomassie brilliant blue.

10 *Western blot analysis*

Proteins separated by SDS-PAGE were transferred to a PVDF membrane (Bio-Rad) at 125mA for 90min. For detection with specific antibodies, the membrane was blocked with 5% milk proteins (Marvel)/PBS for 2-16h at RT. Detection was
15 performed by incubation with mouse anti-His4 (1:1000 dilution) followed by incubation with HRP-conjugated sheep anti-mouse IgG (1:1000 dilution, GEHealthcare). Both antibodies were diluted in 1% milk proteins/PBS (w/v) and incubation was for 1h at RT. Final staining was achieved by
20 incubation with 0.25mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with H₂O₂ (1/2000). Washing steps consisted of five washes with 0.1% Tween 20/PBS (v/v) followed by three PBS washes.

25 *Binding of MFE CDR3 VH loop variants and anti- αv antibody to immobilized $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 3$ by ELISA*

Ninety-six-well plates (Nunc-ImmunoTM Plates, Maxi Sorp, Nalge Nunc International) were coated with 100 μl /well of 1 $\mu\text{g}/\text{ml}$ of $\alpha\text{v}\beta 6$ or 3 $\mu\text{g}/\text{ml}$ of $\alpha\text{v}\beta 3$ (Chemicon International, Harrow, UK) in Tris buffered saline (TBS), pH 7.5 at RT for
30 1h or TBS as a control. The plate was washed 2 times with 0.1 % Tween 20 in TBS followed by 8 washes with TBS, and 150 μl /well of 5% Marvel in TBS was added for 1h at RT to block non-specific binding. The plate was washed as above but the TBS solutions contained 1mM MgCl₂, 1mM MnCl₂ and 1mM CaCl₂

(TBSM), and dilutions of MFE, MFEVP1, NFEVP1 and HFEVP1 and mouse anti- α v (1:1000, Chemicon International, Harrow, UK) in 1% Marvel in TBSM were added (100 μ l/well). The plate was incubated for 1h at RT, washed, and incubated for 1h with 100 μ l/well of rabbit anti-MFE and with anti- α v for mouse anti- α v wells (1:1000), washed and incubated for 1 h with 100 μ l/well of HRP-conjugated goat anti-rabbit IgG (Sigma, 1:1000 dilution) and with sheep HRP-labelled anti-mouse IgG (GE Healthcare, 1:1000 dilution) for anti- α v wells. Bound samples were detected by applying 100 μ l of the substrate o-phenylenediamine dihydrochloride (OPD, Sigma) in citrate buffer pH 5.0; the reaction was stopped with 100 μ l of 4M HCl and the absorbance read at 490 nm on a Dynex Technologies Plate Reader. In experiments testing the metal dependence of MFE CDR3 loop variants binding, the diluent was TBS containing 10 mM EDTA (pH 7.5) and all washing steps included 10 mM EDTA.

Binding of MFE CDR3 VH loop variants and hMFE23-RGD, hMFE23-RGE and hMFE to immobilized CEA by ELISA

Ninety-six-well plates (as under Binding of MFE CDR3 VH loop variants to immobilized α v β 6 by ELISA) were coated with 100 μ l/well of CEA at 1 μ g/ml in PBS or PBS as a control, washed with twice with PBS on an automatic plate washer (Thermo Labsystems) and blocked with 5% Marvel in PBS. MFEVP1, NFEVP1, MFE, hMFE23-RGD, hMFE23-RGE and hMFE were diluted in 1% Marvel in PBS and 100 μ l added to the wells in triplicate, washed as above, incubated with rabbit anti-MFE (1:1000 dilution) for MFEVP1, NFEVP1, MFE and with mouse anti-His4 (1:1000 dilution, Qiagen Ltd.) for hMFE23-RGD, hMFE23-RGE and hMFE washed once with 0.1% Tween 20/PBS and four times with H₂O, and incubated with HRP-conjugated goat anti-rabbit IgG (1:1000 dilution) for MFEVP1, NFEVP1, MFE and with HRP-conjugated sheep anti-mouse IgG (1:1000 dilution, GE

Healthcare) for hMFE23-RGD, hMFE23-RGE and hMFE. After washing as above with 0.1% Tween-20/PBS and H₂O binding was detected with OPD and absorbance read at 490 nm (as under Binding of MFE CDR3 VH loop variants to immobilized $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 3$ by ELISA).

Flow cytometric analysis of MFE and MFE loop variants' binding to LS-174T cells

LS-174T cells were washed twice with PBS and detached with trypsin/EDTA (Cambrex). On average 5×10^5 cells were incubated with 50 $\mu\text{g}/\text{ml}$ of MFEVP1, NFEVP1 and MFE and washed with PBS. Detection of binding was first by incubation with rabbit anti-MFE IgG (1:100 dilution), washing with PBS and second by incubation with 1 μg of R-Phycoerythrin (R-PE)-conjugated goat anti-rabbit IgG (Invitrogen, Karlsruhe, Germany) followed by washing with PBS. All incubation steps were carried out for 60 min at 4°C in 100 μl PBS containing 0.1% (w/v) Bovine Serum Albumin (BSA) and 0.1% (w/v) sodium azide. In control experiments the rabbit anti-MFE IgG was omitted. Cells were fixed (IntraStain kit, DakoCytomation) and analysed by flow cytometry on a FACSCalibur™ cytometer (Becton Dickinson, Oxford, UK).

Flow cytometric analysis of MFEVP1 and MFE binding to the $\alpha\text{v}\beta 6$ expressing cell line, A375P $\beta 6$ and the parent cell line, A375Ppuro

A375P $\beta 6$ and A375Ppuro cells (generated as described previously(25)) were washed once in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% (w/v) Bovine Serum Albumin (BSA) and 0.1% (w/v) sodium azide (DMEM 0.1/0.1) and re-suspended in an appropriate volume. 50 μl of this suspension containing approximately 2×10^5 cells was transferred to individual wells of V-bottomed 96-well plates and mixed with 50 μl of MFEVP1, MFE and 10D5 (Chemicon International, Harrow, UK) at various concentrations. After

incubation at 4°C for 60 minutes, cells were washed two times with 150µl DMEM 0.1/0.1. Cells were re-suspended in 50µl mouse Tetra-His antibody (Qiagen, Crawley, UK) diluted 1:100 in DMEM 0.1/0.1 and incubated for a further 35 minutes
5 at 4°C. Cells were then washed twice with 150µl DMEM 0.1/0.1 as above. Alexa-488-conjugated goat anti-mouse (1:200 dilution in DMEM 0.1/0.1; Molecular Probes) was then added and cells incubated for a further thirty minutes at 4°C. Cells were washed three times as above and transferred
10 to 5ml centrifuge tubes (BD Falcon 352054, supplied by VWR, UK). Cells were analysed on an LSR-1 FACS flow cytometer (Becton Dickinson, Oxford, UK) using CellQuest software.

Flow cytometric analysis of anti- $\alpha v\beta 6$, anti- $\alpha v\beta 8$, anti- $\alpha v\beta 5$, anti- $\alpha v\beta 3$ and $\alpha v\beta 1$ binding to the $\alpha v\beta 6$ expressing cell line, A375P $\beta 6$ and the parent cell line, A375Ppuro.
Flow cytometric analysis of RGD-directed integrin expression
- A375Ppuro and A375P $\beta 6$ puro cells were detached with trypsin/EDTA, resuspended in DMEM 0.1/0.1 to $2 \cdot 10^5$
20 cells/50µl and mixed with 50µl of anti-integrin antibodies (at 10 µg/ml). After 45 minutes at 4°C the cells were washed twice with DMEM 0.1/0.1 and bound antibodies detected with 50µl of 1:200 dilutions of Alexafluor-488 conjugated anti-mouse antibodies for 30 minutes at 4°C. After two washed
25 samples were analysed by flow cytometry as above. Negative controls received similar concentrations of mouse IgG (Dako).

Immunofluorescence confocal microscopy of internalisation of
30 *NFEVP1.*

A375P $\beta 6$ and A375Ppuro cells were trypsinized, re-suspended in DMEM, containing L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum. Cells ($\sim 2 \times 10^5$) were seeded

in 2ml of the above media in 24mm² dishes containing glass coverslips and allowed to attach for 48 hrs at 37°C. The media was removed and exchanged with DMEM containing 1% heat-inactivated fetal bovine serum and 50µg/mL of NFEVP1 and either directly incubated at 10min, 30min, 1 hr and 3 hr at 37°C or first pre-incubated at 4°C for 1hr, upon which the scFv was removed, and the cells were shifted to 37°C. After incubation cells on the coverslips were washed with PBS, containing 2mM Ca²⁺ and 1mM Mg²⁺, fixed in 4% paraformaldehyde/PBS for 20min on ice, washed with PBS and incubated with 10mM ammonium chloride for 10 min at RT, washed, permeabilized with 0.1% Triton X-100TM for 5min on ice, washed and blocked with 3% (w/v) BSA/PBS for 20min at RT. Cells were washed, and stained with 10µg/mL Affini Pure Rabbit anti-mouse IgG (H+L, Jackson Immune Research) in 1% (w/v) BSA/PBS, washed and stained with 10µg/mL Alexa Fluor 546® labelled Goat anti-rabbit IgG (H+L, Molecular Probes, Invitrogen), containing Hoechst trihydrochloride (1:5000) in 1% (w/v) BSA/PBS, washed three times with PBS and one time with H₂O. All washes were three times with PBS if not indicated otherwise. Coverslips were mounted on slides using ProLong Gold antifade (Molecular Probes, Invitrogen). Cells were visualized with a Olympus® confocal scanning microscope (Olympus, London, UK).

25

Migration assays

Haptotactic cell migration assays were performed using matrix coated polycarbonate filters (8 µm pore size, Transwell®, Becton Dickinson, Oxford, UK). The membrane undersurface was coated with LAP (0.5 µg/ml) in α-MEM for 1 hour at 37°C and blocked with migration buffer (0.1% BSA in α-MEM) for 30 minutes at 37°C. For blocking experiments, cells were incubated with MFEVP1, NFEVP1 and 10D5 antibody (at 10µg/ml, Chemicon International, Harrow, UK) for 60

minutes at 4°C prior to seeding. The lower chamber was filled with 500µl of migration buffer, following which cells were plated in the upper chamber of quadruplicate wells, at a density of 5×10^4 in 50 µl of migration buffer and
5 incubated at 37°C for 20 hours. Following incubation, the cells in the lower chamber (including those attached to the undersurface of the membrane) were trypsinised and counted on a Casy 1 counter (Sharfe System GmbH, Germany).

10 *Fourier-transform infrared (FT-IR) spectroscopy of NFEVP1 and MFE*

FT-IR spectra were recorded using a Perkin-Elmer 1750 FT-IR spectrometer equipped with a fast recovery TGS type detector. NFEVP1 at 0.47mg/ml, MFE at 0.59mg/ml and PBS
15 control were dialysed with three buffer changes at 4°C into 20mM Phosphate buffer, pH7.5 and subsequently lyophilized. NFEVP1 and MFE were dissolved in $^2\text{H}_2\text{O}$ to a final concentration of 10mg/ml and control at an equivalent volume. Eight µl of each protein and control were placed
20 into the 6µm recess on one of the two specialists-made CaF_2 windows (Feinoptische Werkstatt, Berlin, Germany) that was mounted inside a Beckman FH-01 CFT micro-cell. For denaturation experiments the cell was exposed to temperatures from 25°C to 85°C in steps of 2-5°C using an
25 attached waterbath. Before each spectrum acquisition samples were maintained at the desired temperature in order to stabilize the temperature inside the cell (10 min). A total of 200 scans were acquired at each temperature for the denaturation measurements, whereas for comparison of NFEVP1
30 and MFE secondary structural elements 1000 scans were acquired at 30°C each. The absorbance spectra were first subtracted by their respective buffer control followed by calculation of the second derivative spectra using a 13 data point Savitsky-Golay smoothing function.

Inhibition of cell adhesion

The ability of modified and unmodified scFv antibodies to inhibit the $\alpha v \beta 6$ -specific adhesion of [^{51}Cr]-labelled

5 3T3 $\beta 6.19$ fibroblast cells to LAP was performed as described previously(31).

Results

Construction, expression in *E. coli* and purification of scFv
10 MFE VH loop variants, hMFE23-RGD (also known as hMFE-RGD) and hMFE23-RGE (also known as hMFE-RGE) and binding of hMFE23-RGD and hMFE23-RGE to CEA and $\alpha v \beta 6$.

The MFE antibody has no $\alpha v \beta 6$ -binding capability. Initial attempts to confer affinity for $\alpha v \beta 6$ using site-directed
15 mutagenesis to delete the CDR 3 loop residues 123-128 of the VH chain of MFE, which are known to be critical for CEA binding (Boehm et al., 2000b), and replace them with the peptide sequence RGD Δ LATL, an RXDLXXL motif based on the sequence of TGF β 1 Latency Associated Peptide (LAP), failed.
20 In addition, it was decided to insert one alanine residue prior to the RGD, as bioinformatic modeling suggested that this might improve the presentation of the motif by increasing its solvent accessibility. As the aspartate residue is critical to integrin binding (Humphries, 1990),
25 an ARGELATL recombinant was also constructed as a control. The proteins were expressed in *E. coli* and secreted into the supernatant. Proteins were purified from the bacterial supernatants using Nickel spin columns and visualised by SDS-PAGE followed by Western blotting and detection with an
30 anti-4xHis monoclonal antibody (Figure 9).

The parent scFv, MFE, binds to CEA so it was important to establish whether hMFE23-RGD and hMFE23-RGE would remain some of this binding activity. Results showed that hMFE23-

RGD and hMFE23-RGE showed some residual binding to immobilised CEA (Figure 10). The ability of hMFE23-RGD and hMFE23-RGE to bind $\alpha\beta 6$ was then investigated. Results showed that both hMFE23-RGD or hMFE23-RGE failed to show any measurable binding to immobilised $\alpha\beta 6$. Although there was a high level of background, the hMFE23-RGD gave no signal over and above that of the negative control hMFE23 (Figure 11).

Construction, expression in E. coli and the yeast, P. pastoris, and purification of scFv MFE VH loop variants, MFEVP1, NFEVP1 and shMFE(P)CDR2VP1

For construction of a $\alpha\beta 6$ -binding MFE the peptide sequence from A₁₄₀ to A₁₅₆ of the viral coat protein VP1 of the Foot-and-Mouth disease virus (FMDV) was inserted at the tip of the CDR3 loop of the VH chain of MFE, between T98 and G99 (using Kabat nomenclature as shown in Figure 1). This VH loop variant of MFE was named MFEVP1. The DNA sequence of MFEVP1 was generated by overlapping PCR as described in Material and Methods. The protein was expressed in *E. coli* and secreted into the supernatant at comparable levels and similar size to the parent molecule, MFE. Initial purification and concentration of the protein was obtained by IMAC chromatography. The protein was further purified by size-exclusion chromatography (Fig. 2a). MFEVP1 eluted as two distinct peaks, 56 ml and 67 ml, representing the dimeric and monomeric forms, respectively. A further scFv MFE loop variant, NFEVP1, containing the $\alpha\beta 6$ -binding sequence of VP1 and a Y_{100b} to P_{100b} mutation in the VH domain was constructed (see Fig. 1a) from MFEVP1 by site-directed mutagenesis. Expression and purification in *E. coli* of this protein was similar to MFEVP1. The DNA sequence of NFEVP1 was also cloned into a yeast vector for expression in *P. pastoris*. NFEVP1 was obtained at 56mg/L after initial EDB-IMAC chromatography, using previously described

procedures.(22, 23). NFEVP1 was concentrated and final purification was by size-exclusion chromatography (Fig. 2b). The chromatographic profile of the yeast expressed protein was virtually super imposable to that obtained from *E. coli*.

5

A further MFE VH loop variant, shMFE(P)CDR2VP1, was constructed by inserting the A₁₄₀ - A₁₅₆ VP1 peptide between the DNA sequences of VH CDR2 residues E53 and N54 of the shMFE² sequence (which contains the Y_{100b}P mutation). This DNA sequence was cloned into a yeast vector and expressed in *P. pastoris*. The expressed protein was analysed by SDS-PAGE and Western blotting, which showed that the protein was stably expressed in yeast.

10

15 *Binding of MFEVP1 and NFEVP1 to $\alpha v\beta 6$ and inhibition of migration*

The ability of pure monomeric forms of MFEVP1 and NFEVP1 to bind to $\alpha v\beta 6$ was investigated. MFEVP1 showed concentration dependent binding to $\alpha v\beta 6$ immobilized plates in ELISA when probed with anti-MFE followed by anti-rabbit labelled HRP IgG (Fig. 3a). In contrast, the absorbance reading for parent MFE, tested under identical conditions, was similar to background levels. In agreement with metal dependent integrin-ligand binding MFEVP1 did not bind in the presence of EDTA (data not shown).

20

MFEVP's ability to bind to $\alpha v\beta 6$ when expressed on cells was also investigated. MFEVP1 showed concentration dependent binding to $\beta 6$ -transfected A375P $\beta 6$ cells (a retrovirally with $\beta 6$ cDNA transfected melanoma cell line(25)) by Flow Cytometry when monitored with mouse anti-His followed by Alexa488-conjugated anti-mouse Fc (Fig. 3b). Observed fluorescence shifts were similar for the 5 μ g/ml and 0.5 μ l/ml

25

30

concentrations, indicating that MFEVP1 reached almost saturation levels of binding at 0.5µg/ml. The αvβ6-specific antibody, 10D5, was included as a positive control whereas MFE, the negative control, was not shifted beyond background levels. Neither 10D5, MFEVP1 nor MFE showed binding to the non-transfected parent cell, A375Ppuro (transfected with empty vector), confirming that the observed binding of MFEVP1 to the β6-transfected cell line was specifically to αvβ6.

10

The VP1-inserted scFvs' ability to functionally inhibit migration of αvβ6-expressing cells was investigated next. VB6 cells, a well characterised retrovirally with β6 cDNA transfected oral SCC cell line(27), were allowed to migrate towards the αvβ6-specifically binding ligand, LAP, which is coated underneath a Transwell filter. Addition of 50µg/ml of MFEVP1 and the Y_{100b} to P_{100b} mutant, NFEVP1, to VB6 cells considerably inhibited their migration when compared to MFE (Fig. 3c). The function blocking αvβ6-specific 10D5 antibody added at 10µg/ml showed similar levels of inhibition of cell migration of VB6 cells as the scFvs. The inhibition of migration was concentration dependent as shown for MFEVP1 (Fig. 3d).

25 *Investigation of binding of scFv MFE VH loop variants to integrins other than αvβ6*

Cross reactivity of MFEVP1 and NFEVP1 with integrins other than αvβ6 was first investigated by ELISA, using immobilized αvβ3 and αvβ6, and by cell binding studies using cells expressing a variety of integrins. Results showed that MFEVP1 and NFEVP1 did not bind to immobilized αvβ3 in ELISA when probed with rabbit anti-MFE followed by HRP-labelled anti-rabbit IgG (Fig. 4a) whereas binding to αvβ6 was

observed. Immobilization of both integrins to the wells was confirmed with an anti- α v antibody. Selectivity of MFEVP1 and NFEVP1 for α v β 6 was also in agreement with the cell binding studies described under "Binding of scFv MFE VH loop variants to α v β 6 and inhibition of migration" (shown in Fig. 3b). Because both scFvs did not bind to the non-transfected A375Ppuro cell line, which express α v β 3 at similar levels to the β 6-transfected A375P β 6 cell line (Fig. 4b), this agrees with the ELISA results that MFEVP1 and NFEVP1 did not bind at detectable levels to α v β 3. In addition, the A375Ppuro cells also expressed α v β 8, α v β 5 and α 5 β 1 at similar levels to those found on A375P β 6 cells (Fig. 4b) which showed that MFEVP1 and NFEVP1 did also not bind at detectable levels to these integrins.

15

Binding of MFEVP1 and NFEVP1 to CEA

The parent scFv, MFE, binds to CEA so it was important to establish whether MFEVP1 would remain some of this binding activity. Results showed that MFEVP1 showed residual binding to immobilized CEA in ELISA in a concentration-dependent manner, although considerably below that seen for MFE (Fig. 5a) when revealed with rabbit anti-MFE and HRP-labelled goat anti-rabbit IgG. The Tyr_{100b} to Pro_{100b} mutant, NFEVP1, showed no residual binding on ELISA to immobilized CEA.

25 This same mutation when introduced into MFE had been shown previously to completely abolish binding to CEA(19). The ability of MFEVP1 to bind to CEA when expressed on cells was also investigated. These experiments showed that MFEVP1 bound to the human colon adenocarcinoma cell line LS174T, which is known to express high levels of CEA, when revealed by Flow Cytometry with rabbit anti-MFE followed by goat phycoerythrin-labelled anti-rabbit IgG (Fig. 5b). The shift in fluorescence intensity was only slightly below that of

30

the parent molecule, MFE, as measured by gated fluorescence intensity, 53.8% for MFE and 42.5% for MFEVP1. Consistent with the ELISA results the Tyr_{100b} to Pro_{100b} mutant, NFEVP1, showed no binding to these cells, the observed fluorescence intensity was equal to that of the omission control. The Tyr_{100b} to Pro_{100b} mutation was thus able to eliminate all residual binding of NFEVP1 to CEA as has been seen previously for the parent molecule, MFE.

10 *Internalization of NFEVP1 into β 6-transfected A375P cells*
Having shown binding of NFEVP1 to α v β 6 on cells, we then investigated whether binding of NFEVP1 to α v β 6 on the cell surface resulted in internalization of the scFv in these cells. β 6-transfected A375P cells were therefore incubated
15 at 4°C with NFEVP1 for 1 hr, the scFv was removed and cells were incubated at various temperatures (described under Material and Methods and shown in Fig. 6) at 37°C. After 10 minutes incubation at 37°C the scFv was seen as a well defined thin line surrounding, at a distance, the nucleus
20 (Hoechst staining and shown in blue) when revealed with rabbit anti-mouse IgG followed by Alexa Fluor® 546 labeled goat anti-rabbit IgG and shown in red; typifying its location in the plasma membrane. After 3 hrs incubation the scFv was diffused and speckled around the nucleus,
25 identifying its location inside the cells. The scFv was also found inside the cells 30 minutes and 1 hrs after incubation of the cells at 37°C (data not shown). When compared with the equivalent 10 min and 3 hr incubation experiments of NFEVP1 with the A375Ppuro cells and the
30 control experiment where the scFv was omitted it can be concluded that the fluorescence levels were significantly above background level; experiments were recorded and depicted under identical conditions. It was assured that internalization is mediated entirely by α v β 6 and not some

other mechanism by pre-incubating the scFv at 4°C and removing it before incubation at 37°C as Flow Cytometry results (see above) showed that NFEVP1 bound only to the β6-transfected cells and not the puro cells. However, similar
5 results were obtained when cells were incubated without prior incubation at 4°C and removal of the scFv (data not shown).

Fourier-transform infrared (FT-IR) spectroscopy of NFEVP1.

10 Having obtained pure, monomeric NFEVP1, identified αvβ6 binding and inhibition of migration of αvβ6 expressing cells, it was investigated whether insertion of the 17-mer VP1 peptide had affected the structure and stability of the protein when compared with the parent molecule, MFE. The
15 2nd derivative FT-IR spectra of NFEVP1 and MFE were virtually super imposable. NFEVP1 showed a strong band at 1635 cm⁻¹ in agreement with a protein whose structure consists mainly of β-sheet and shown previously in the x-ray structure of MFE (25). The secondary structural elements
20 associated with the FT-IR bands have been assigned previously for MFE.(26) The intensity of the β-sheet band at 1635 cm⁻¹ was used to monitor stability of the protein; with increasing temperature this band will reduce in intensity while the protein denatures. Recording of the denaturing
25 curve and fitting to a sigmoidal curve gave a midpoint of denaturation of 45°C for NFEVP1 (Fig. 7b). For MFE this temperature was 47°C, identical to the previously reported value(28). Therefore, insertion of the VP1 peptide did not affect the structure of the protein and NFEVP1 had very
30 similar stability when compared to MFE.

Expression, purification, αvβ6-binding and inhibition of cell binding of stabilized humanized NFEVP1 (HFEVP1)

Having established that the murine scFv, MFE, can be used as a scaffold for introduction of the $\alpha v\beta 6$ -binding peptide, VP1, the same strategy was applied but using the previously described stabilized humanized MFE (HFE) (27) as a scaffold.

5 The protein expressing vector was obtained by overlapping PCR identical to the murine analogue with the Y100b to P100b mutation in the heavy chain that eliminated CEA binding. Stabilized humanized NFEVP1 (HFEVP1) was expressed at 115mg/L by *P. Pastoris*, determined after initial EDB-IMAC

10 chromatography. The protein formed almost exclusively a dimer as revealed by size-exclusion chromatography (Figure 8(a)). Fractions, containing dimer were separated and used for subsequent experiments. HFEVP1 bound to immobilized $\alpha v\beta 6$ in ELISA when probed with anti-His followed by anti-

15 mouse labelled HRP IgG (Figure 8(b)). HFEVP1 also inhibited the adhesion of $\alpha v\beta 6$ -expressing 3T3 $\beta 6.19$ cells towards LAP (Figure 8(c)). HFEVP1 was the most potent inhibitor tested in the assay. IC₅₀ values determined from the assay were 21.97 $\mu\text{g/ml}$ (768 μM) for NFEVP1, 8.42 $\mu\text{g/ml}$ (56.1 μM for 10D5

20 and 2.55 $\mu\text{g/ml}$ (45.14 μM) for HFEVP1.

Discussion

Whole antibodies with specificity for their target have been

25 classically obtained by hybridoma screening technology after immunization with the antigen(30). A further well established approach that selects for scFvs is phage display technology where repertoires of scFvs are displayed on the surface of filamentous bacteriophage and screened for

30 binding to antigen(31). In addition, antibodies with binding specificities for their targets can be generated by a rational structure based approach, grafting of a target-binding sequence from a ligand into the CDR region of the antibody.

Here we describe the generation of the scFv, MFEVP1, with $\alpha v\beta 6$ binding specificity by a rational structure based approach by insertion of the binding region of the viral coat protein, VP1, of Foot-and-mouth disease virus (FMDV), serotype O, into the CDR3 loop region of the VH domain of MFE. Previous structural data of VP1 have identified the integrin-binding RGD motif on the tip of the GH loop(32;33). A further motif, DLXXL, was identified by displaying peptide libraries on phage as crucial for $\alpha v\beta 6$ binding (12). In VP1 this motif includes the D residue of the RGD sequence and the two L residues are arranged in a DLXXL motif.

The resulting scFv loop variant, named MFEVP1, was expressed in *E. coli* and secreted into the media at comparable levels to the parent MFE. Purification was via the His-tag followed by size-exclusion chromatography to obtain the monomeric form.

We showed by ELISA that the monomeric scFv bound to immobilized $\alpha v\beta 6$ and by Flow cytometry when expressed on cells. In agreement with integrin-ligand interactions the binding was metal dependent and was abolished in the presence of EDTA. Furthermore, MFEVP1 inhibited the migration of $\alpha v\beta 6$ -expressing cells towards its ligand, LAP-1.

MFEVP1 was specific for the integrin $\alpha v\beta 6$ and did not show binding at detectable levels to immobilized $\alpha v\beta 3$ in ELISA and to $\alpha v\beta 3$, $\alpha v\beta 8$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ expressed on cells.

Insertion of the binding region of VP1 of FMDV of O type strain into MFE was predicted to be a promising strategy in

order to develop a scFv with specificity for $\alpha v\beta 6$ because previous studies have shown that in $\beta 6$ -transfected cells $\alpha v\beta 6$ functions as the major receptor for virus attachment, whereas other epithelial expressed integrins, namely $\alpha 5\beta 1$ or
5 $\alpha v\beta 5$, appear not to have a role(34).

MFEVP1 had the desired specificity for $\alpha v\beta 6$, however, also residual binding to the parent target, CEA. This binding could be eliminated when the VH residue Y_{100b} was mutated to
10 P_{100b}, thus generating NFEVP1. Previous work has shown that binding of MFE to CEA was abolished when this mutation was introduced in MFE(19). MFE-CEA interactions were predicted from the way a MFE molecule interacts with another MFE molecule in the X-ray structure and this highlighted the
15 importance of Y_{100b}(26). This was further elaborated by a subsequent modelling study of the interaction of MFE with CEA(35).

The $\alpha v\beta 6$ -binding peptide did not affect the structure of the
20 scFv and maintained a similar midpoint of denaturation when compared to MFE, NFEVP1, 45°C, MFE, 47°C, as determined by FT-IR spectroscopy.

A link between $\alpha v\beta 6$ expression and carcinoma progression has
25 been suggested due to its ability to modulate invasion, inhibit apoptosis, regulate protease expression and activate TGF- $\beta 1$ (36). $\alpha v\beta 6$ has also been highlighted as a promising cancer target due to its *de novo* expression on various cancerous tissues(36). A 12^{mer} $\alpha v\beta 6$ -binding peptide has
30 shown promise in mediating T cell killing of $\alpha v\beta 6$ expressing ovarian tumour targets when fused to human IgG4 hinge-Fc extracellular domain and to the cytoplasmic tail of CD3- ξ (37).

Although scFvs might not be in itself good targeting agents due to their rapid blood clearance they will allow genetic fusion to toxins, increasing the size and slower blood clearance, in particular when the toxin forms dimeric or multimeric arrangements, which will introduce avidity. Delivery of scFv-toxin fusions would benefit from their ability to internalize. NFEVP1 was able to internalize in $\alpha\beta 6$ -expressing A375P $\beta 6$ cells. scFvs can also be multimerically attached to drug carrying vehicles, such as liposomes and polymers. scFvs can be converted into whole antibodies for dimeric presentation, exploiting the intrinsic toxic functions, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). A construct which provides good tumour penetration, high target retention, due to its dimeric binding, and rapid blood clearance is the diabody(38). The diabody is double the size of the scFv and considerable smaller than a whole antibody. It is generated by shortening the scFv (Gly₄Ser)₃ VH-VL linker to a Gly₄Ser linker, which forces the VH and VL domains from different chains to pair(39).

A major problem in cancer therapy is immunogenicity of the antibody therapeutic in particular in repeated treatment. NFEVP1 is of murine origin and is likely to result in immunogenetic reactions in humans. Hence previous studies have addressed this problem by converting MFE into a humanized version.(27) Comparison of the x-ray structure of MFE with a human analogue allowed identification of 28 surface residues for humanization of MFE.(25) These residues when introduced into MFE combined with three additional mutations for stabilization identified by yeast display expression maturation produced stabilized humanized MFE(27). In this study the stabilized humanized MFE was

used as a scaffold to insert the VP1 peptide and combined with the Y100b to P100b mutation gave HFEVP1. The protein was expressed almost exclusively as a dimer in *P. Pastoris* as shown by size-exclusion chromatography. HFEVP1 bound to $\alpha v\beta 6$ in ELISA and inhibited the adhesion of $\alpha v\beta 6$ expressing cells to LAP thus mimicking the behaviour of the murine analogue. HFEVP1 was a slightly better inhibitor than the commercially available whole antibody, 10D5; IC50 value for HFEVP1 was 2.55 $\mu\text{g/ml}$ (45.14 μM) and for 10D5 was 8.42 $\mu\text{g/ml}$ (56.1 μM).

In conclusion, a $\alpha v\beta 6$ -binding scFv was generated by insertion of the RGD-containing VP1 peptide of FMDV into MFE that had no binding for MFE's target, CEA, when combined with the VHY_{100b} to VHP_{100b} mutation. This study has shown that MFE-23 (including the humanised variants) are good scaffolds for peptide insertion to alter binding specificity of the scFv. The MFE-23 antibody (including the humanised variants) could thus be envisaged to be used to obtain binding to other tumour targeting antigens using a similar approach.

References

All publications, patent and patent applications cited herein or filed with this application, including references filed as part of an Information Disclosure Statement.

5

10

15

20

25

30

1. Hynes, R. O. (2002) *Cell* **110**, 673-687
2. Ruoslahti, E. (1991) *J. Clin. Invest* **87**, 1-5
3. Giancotti, F. G. and Mainiero, F. (1994) *Biochim. Biophys. Acta* **1198**, 47-64
4. Breuss, J. M., Gallo, J., DeLisser, H. M., Klimanskaya, I. V., Folkesson, H. G., Pittet, J. F., Nishimura, S. L., Aldape, K., Landers, D. V., Carpenter, W., and . (1995) *J. Cell Sci.* **108** (Pt 6), 2241-2251
5. Ahmed, N., Pansino, F., Clyde, R., Murthi, P., Quinn, M. A., Rice, G. E., Agrez, M. V., Mok, S., and Baker, M. S. (2002) *Carcinogenesis* **23**, 237-244
6. Arihiro, K., Kaneko, M., Fujii, S., Inai, K., and Yokosaki, Y. (2000) *Breast Cancer* **7**, 19-26
7. Sipos, B., Hahn, D., Carceller, A., Piulats, J., Hedderich, J., Kalthoff, H., Goodman, S. L., Kosmahl, M., and Kloppel, G. (2004) *Histopathology* **45**, 226-236
8. Ramos, D. M., But, M., Regezi, J., Schmidt, B. L., Atakilit, A., Dang, D., Ellis, D., Jordan, R., and Li, X. (2002) *Matrix Biology* **21**, 297-307
9. Bates, R. C., Bellovin, D. I., Brown, C., Maynard, E., Wu, B., Kawakatsu, H., Sheppard, D., Oettgen, P., and Mercurio, A. M. (2005) *J. Clin. Invest* **115**, 339-347
10. Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pittet, J. F., Kaminski, N., Garat,

- C., Matthay, M. A., Rifkin, D. B., and Sheppard, D. (1999) *Cell* **96**, 319-328
11. Jackson, T., King, A. M., Stuart, D. I., and Fry, E. (2003) *Virus Res.* **91**, 33-46
- 5 12. Kraft, S., Diefenbach, B., Mehta, R., Jonczyk, A., Luckenbach, G. A., and Goodman, S. L. (1999) *J. Biol. Chem.* **274**, 1979-1985
13. Mateu, M. G., Valero, M. L., Andreu, D., and Domingo, E. (1996) *J. Biol. Chem.* **271**, 12814-12819
- 10 14. Chester, K. A., Begent, R. H., Robson, L., Keep, P., Pedley, R. B., Boden, J. A., Boxer, G., Green, A., Winter, G., Cochet, O., and . (1994) *Lancet* **343**, 455-456
- 15 15. Begent, R. H., Verhaar, M. J., Chester, K. A., Casey, J. L., Green, A. J., Napier, M. P., Hope-Stone, L. D., Cushen, N., Keep, P. A., Johnson, C. J., Hawkins, R. E., Hilson, A. J., and Robson, L. (1996) *Nat. Med.* **2**, 979-984
- 20 16. Mayer, A., Tsiompanou, E., O'Malley, D., Boxer, G. M., Bhatia, J., Flynn, A. A., Chester, K. A., Davidson, B. R., Lewis, A. A., Winslet, M. C., Dhillon, A. P., Hilson, A. J., and Begent, R. H. (2000) *Clin. Cancer Res.* **6**, 1711-1719
17. Chester, K. A., Bhatia, J., Boxer, G., Cooke, S. P., Flynn, A. A., Huhalov, A., Mayer, A., Pedley, R. B., Robson, L., Sharma, S. K., Spencer, D. I., and Begent, R. H. (2000) *Dis. Markers* **16**, 53-62
- 25 18. Chester, K. A., Mayer, A., Bhatia, J., Robson, L., Spencer, D. I., Cooke, S. P., Flynn, A. A., Sharma, S. K., Boxer, G., Pedley, R. B., and Begent, R. H. (2000) *Cancer Chemother. Pharmacol.* **46 Suppl**, S8-12
- 30 19. Read, D. A., Chester, K. A., Keep, P. A., Begent, R. H. J., Pedersen, J. T., and Rees, A. R. (1995) *Br. J. Cancer* **71**,

20. Lanza, P., Felding-Habermann, B., Ruggeri, Z. M., Zanetti, M., and Billetta, R. (1997) *Blood Cells Mol. Dis.* **23**, 230-241
- 5 21. Lanza, P., Billetta, R., Antonenko, S., and Zanetti, M. (1993) *Proc. Natl. Acad. Sci. U. S. A* **90**, 11683-11687
22. McLane, K. E., Burton, D. R., and Ghazal, P. (1995) *Proc. Natl. Acad. Sci. U. S. A* **92**, 5214-5218
- 10 23. Moroncini, G., Kanu, N., Solforsini, L., Abalos, G., Telling, G. C., Head, M., Ironside, J., Brockes, J. P., Burton, D. R., and Williamson, R. A. (2004) *Proc. Natl. Acad. Sci. U. S. A* **101**, 10404-10409
24. Burman, A., Clark, S., Abrescia, N. G., Fry, E. E., Stuart, D. I., and Jackson, T. (2006) *J. Virol.* **80**, 9798-9810
- 15 25. Dicara, D., Rapisarda, C., Sutcliffe, J. L., Violette, S. M., Weinreb, P. H., Hart, I. R., Howard, M. J., and Marshall, J. F. (2007) *J. Biol. Chem.*
- 20 26. Boehm, M. K., Corper, A. L., Wan, T., Sohi, M. K., Sutton, B. J., Thornton, J. D., Keep, P. A., Chester, K. A., Begent, R. H., and Perkins, S. J. (2000) *Biochem. J.* **346 Pt 2**, 519-528
27. Thomas, G. J., Lewis, M. P., Whawell, S. A., Russell, A., Sheppard, D., Hart, I. R., Speight, P. M., and Marshall, J. F. (2001) *J. Invest Dermatol.* **117**, 67-73
- 25 28. Lee, Y. C., Boehm, M. K., Chester, K. A., Begent, R. H., and Perkins, S. J. (2002) *J. Mol. Biol.* **320**, 107-127
29. Tolner, B., Smith, L., Begent, R. H. J., and Chester, K. A. (2006) *Nat. Protocols* **in press**,
- 30 30. Kohler, G. and Milstein, C. (1975) *Nature* **256**, 495-497
31. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) *Annu. Rev. Immunol.* **12**, 433-455

32. Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D., and Brown, F. (1989) *Nature* **337**, 709-716
 33. Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., King, A., Lea, S., Lewis, R., Newman, J., Parry, N., and . (1993) *Nature* **362**, 566-568
 34. Jackson, T., Sheppard, D., Denyer, M., Blakemore, W., and King, A. M. (2000) *J Virol.* **74**, 4949-4956
 35. Boehm, M. K. and Perkins, S. J. (2000) *FEBS Lett.* **475**, 11-16
 36. Thomas, G. J., Nystrom, M. L., and Marshall, J. F. (2006) *J. Oral Pathol. Med.* **35**, 1-10
 37. Pameijer, C. R., Navanjo, A., Meechoovet, B., Wagner, J. R., Aguilar, B., Wright, C. L., Chang, W. C., Brown, C. E., and Jensen, M. C. (2007) *Cancer Gene Ther.* **14**, 91-97
 38. Holliger, P. and Hudson, P. J. (2005) *Nat. Biotechnol.* **23**, 1126-1136
 39. Holliger, P., Prospero, T., and Winter, G. (1993) *Proc. Natl. Acad. Sci. U. S. A* **90**, 6444-6448
 40. FitzGerald, K., Holliger, P., and Winter, G. (1997) *Protein Eng* **10**, 1221-1225
 41. Graff, C. P., Chester, K., Begent, R., and Wittrup, K. D. (2004) *Protein Eng Des Sel* **17**, 293-304
- Chester KA, Baker M and Mayer A (2005) Overcoming the immunological response to foreign enzymes in cancer therapy *Expert Rev. Clin. Immunol.* 1: 549-559
- Hoogenboom, (2005). Selecting and screening recombinant antibody libraries. *Nat Biotechnology*, 23, 1105-1116
- Hofmeister V, Schrama D, Becker JC, , Anti-cancer therapies targeting the tumor stroma, *Cancer Immunol Immunother.* 2007 Jul 27; in press

- Li Y, Cozzi PJ, Targeting uPA/uPAR in prostate cancer.
Cancer Treat Rev. 2007, 33(6):521-7.
- Muñoz R, Arias Y, Ferreras JM, Rojo MA, Gayoso MJ, Nocito M,
Benítez J, Jiménez P, Bernabéu C, Girbés T., Targeting a
5 marker of the tumour neovasculature using a novel anti-human
CD105-immunotoxin containing the non-toxic type 2 ribosome-
inactivating protein nigrin b. Cancer Lett. 2007 Oct
18;256(1):73-80.
- 10 Baccala A, Sercia L, Li J, Heston W, Zhou M. Expression of
prostate-specific membrane antigen in tumor-associated
neovasculature of renal neoplasms. Urology. 2007
Aug;70(2):385-90.
- Bühler P, Wolf P, Gierschner D, Schaber I, Katzenwadel A,
15 Schultze-Seemann W, Wetterauer U, Tacke M, Swamy M, Schamel
WW, Elsässer-Beile U. A bispecific diabody directed against
prostate-specific membrane antigen and CD3 induces T-cell
mediated lysis of prostate cancer cells. Cancer Immunol
Immunother. 2007 Jun 20; in press
- 20 Johnston JB, Navaratnam S, Pitz MW, Maniate JM, Wiechec E,
Baust H, Gingerich J, Skliris GP, Murphy LC, Los M.
Targeting the EGFR Pathway for cancer therapy. Curr Med
Chem. 2006, 13:3483-3492.

Claims:

1. An antibody which binds to $\alpha v\beta 6$ integrin as obtainable by inserting the amino acid sequence
5 AVPNLRGDLQVLAQKVA or the amino acid sequence NAVPNLRGDLQVLAQKVART between adjacent amino acid residues Thr98 and Gly99 (Kabat numbering) of heavy chain complementary determining region (CDR) 3 of a parent antibody, wherein the parent antibody comprises the
10 following complementary determining regions (CDRs):
 (a) Heavy Chain CDR 1: Gly Phe Asn Ile Lys Asp Ser (SEQ ID NO: 20); and
 (b) Heavy Chain CDR 2: Asp Pro Glu Asn Gly Asp (SEQ ID NO: 21); and
15 (c) Heavy Chain CDR 3: Thr Pro Thr Gly Pro Tyr Tyr Phe Asp (SEQ ID NO:22); and
 (d) Light Chain CDR 1: (i) Ser Ser Ser Val Pro (SEQ ID NO: 23), or (ii) Ser Ser Ser Val Ser (SEQ ID NO: 24); and
 (e) Light Chain CDR 2: (i) Ser Thr Ser, or (ii) Leu Thr Ser; and
20 (f) Light Chain CDR 3: Arg Ser Ser Tyr Pro Leu (SEQ ID NO: 25).
- 25 2. The antibody of claim 1, wherein the antibody is humanised.
3. The antibody of claim 1 or claim 2, wherein the antibody is a scFv or a diabody.
- 30 4. The antibody of claim 3, wherein the scFv or diabody comprises a linker having the sequence (Gly₄Ser)_n, wherein n is between 1 and 4.

5. The antibody of any one of claims 1 to 4, wherein insertion of the amino acid sequence into the CDR of the parent antibody reduces the binding of the parent antibody to the antigen to which the parent antibody initially bound.
6. The antibody of any one of claims 1 to 5, wherein the parent antibody comprises one or more mutations selected from the group consisting of: G44C, A100C and Y100bP (Kabat numbering).
7. The antibody of any one of claims 1 to 6, wherein the inserted amino acid sequence for binding to $\alpha v\beta 6$ integrin is the amino acid sequence NAVPNLRGDLQVLAQKVART.
8. The antibody of any one of claims 1 to 6, wherein the inserted amino acid sequence for binding to $\alpha v\beta 6$ integrin comprises the amino acid sequence AVPNLRGDLQVLAQKVA.
9. The antibody of any one of claims 1 to 8, wherein the antibody is conjugated to a detectable moiety.
10. The antibody of claim 9, wherein the detectable moiety is detectable by Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS), Single Photon Emission Computed Tomography (SPECT), Positron Emission Tomography (PET) or optical imaging.
11. The antibody of claim 9 or claim 10, wherein the detectable moiety is a radioactive moiety.

12. The antibody of any one of claims 1 to 8, wherein the antibody is conjugated to a therapeutically active moiety.
- 5 13. The antibody of claim 12, wherein the therapeutically active moiety is an anti-cancer agent.
14. The antibody of any one of claims 1 to 8, wherein the antibody is conjugated to a tagging group for use in
10 purification or detection.
15. The antibody of claim 14, wherein the tagging group is c-Myc or a poly-histidine group.
- 15 16. The antibody of any one of claims 1 to 8, wherein the antibody is directly or indirectly conjugated or linked to one or more of a cytotoxic moiety, an agent capable of converting a prodrug to a cytotoxic moiety, a radiosensitiser and/or a radioactive atom.
20
17. The antibody of claim 16, wherein the cytotoxic moiety is a chemotherapeutic agent or a cytotoxic polypeptide.
- 25 18. The antibody of claim 16, wherein the agent capable of converting a prodrug to a cytotoxic moiety is an enzyme.
19. Use of the antibody of claim 18 in antibody directed
30 enzyme prodrug therapy (ADEPT).

20. The antibody of any one of claims 1 to 8, 12, 13 and 16-18 for use in therapy of a cancer in which cells overexpress $\alpha v\beta 6$.
- 5 21. The antibody of any one of claims 9 to 11 for use in diagnosis or imaging.
22. Use of the antibody of any one of claims 1 to 8, 12, 13 and 16 to 18, or a nucleic acid encoding an antibody
10 of any one of claims 1 to 8 or a vector comprising said nucleic acid, for the preparation of a medicament for the treatment of a cancer in which cells overexpress $\alpha v\beta 6$.
23. Use of the antibody of any one of claims 1 to 8 for
15 the preparation of a reagent for imaging or diagnosis using a detectable group conjugated or linked to the antibody, wherein the imaging or diagnosis is for a cancer in which cells overexpress $\alpha v\beta 6$.
- 20 24. A nucleic acid molecule that encodes the antibody of any one of claims 1 to 8.
- 25 25. An expression vector comprising the nucleic acid molecule of claim 24, operably linked to control sequences to direct its expression.
26. A host cell transformed with the expression vector of claim 25.
- 30 27. The host cell of claim 26 which is *Pichia pastoris* or *Escherichia coli*.
28. A method for producing the antibody of any one of

claims 1 to 8 which comprises culturing the host cell of claim 26 or claim 27 and isolating the antibody thus produced.

5 29. A pharmaceutical composition comprising the antibody of any one of claims 1 to 18 and a pharmaceutically acceptable carrier.

30. A chimeric antibody molecule comprising the antibody
10 of any one of claims 1 to 18 fused to another polypeptide.

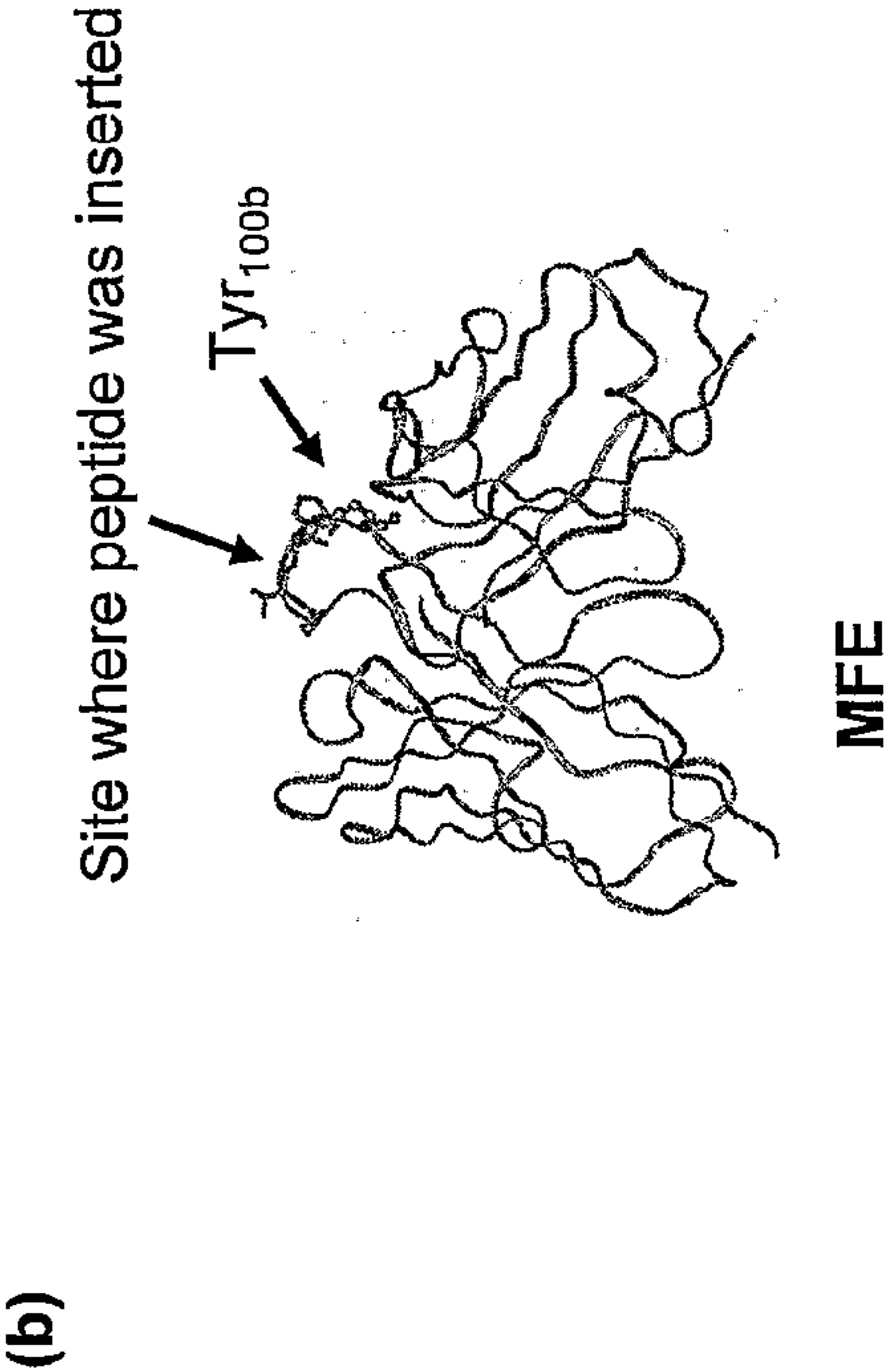
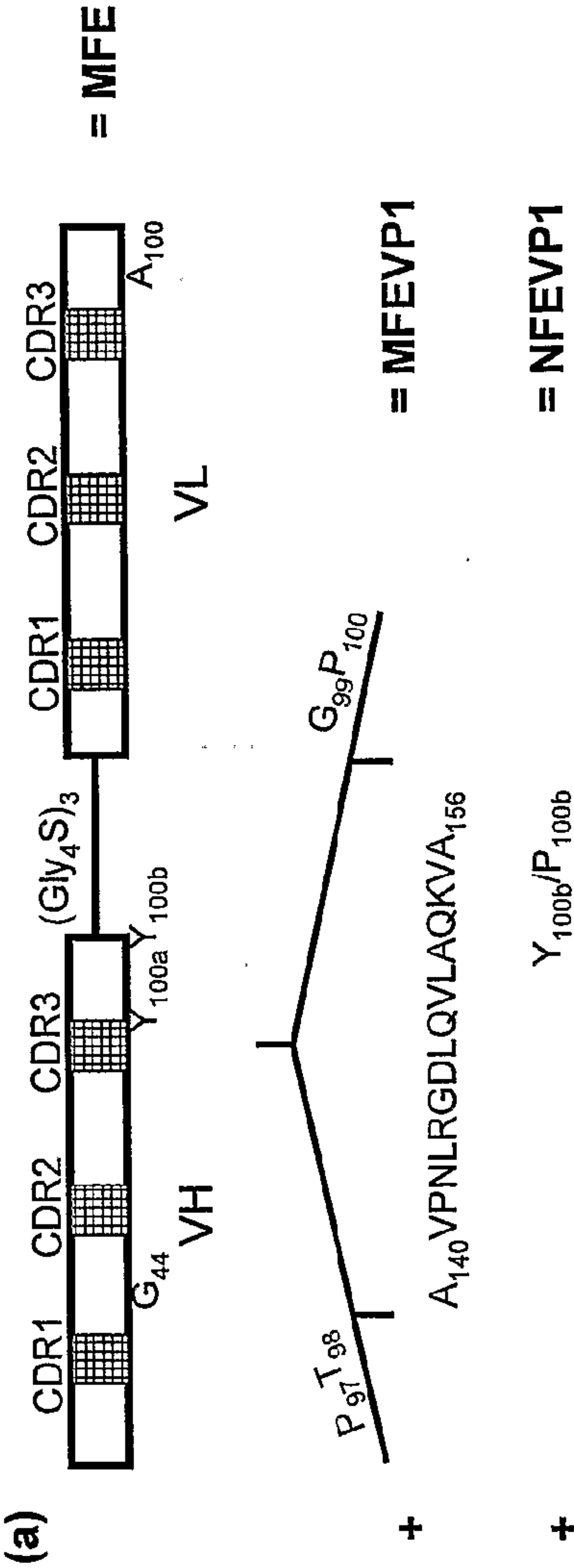


Figure 1

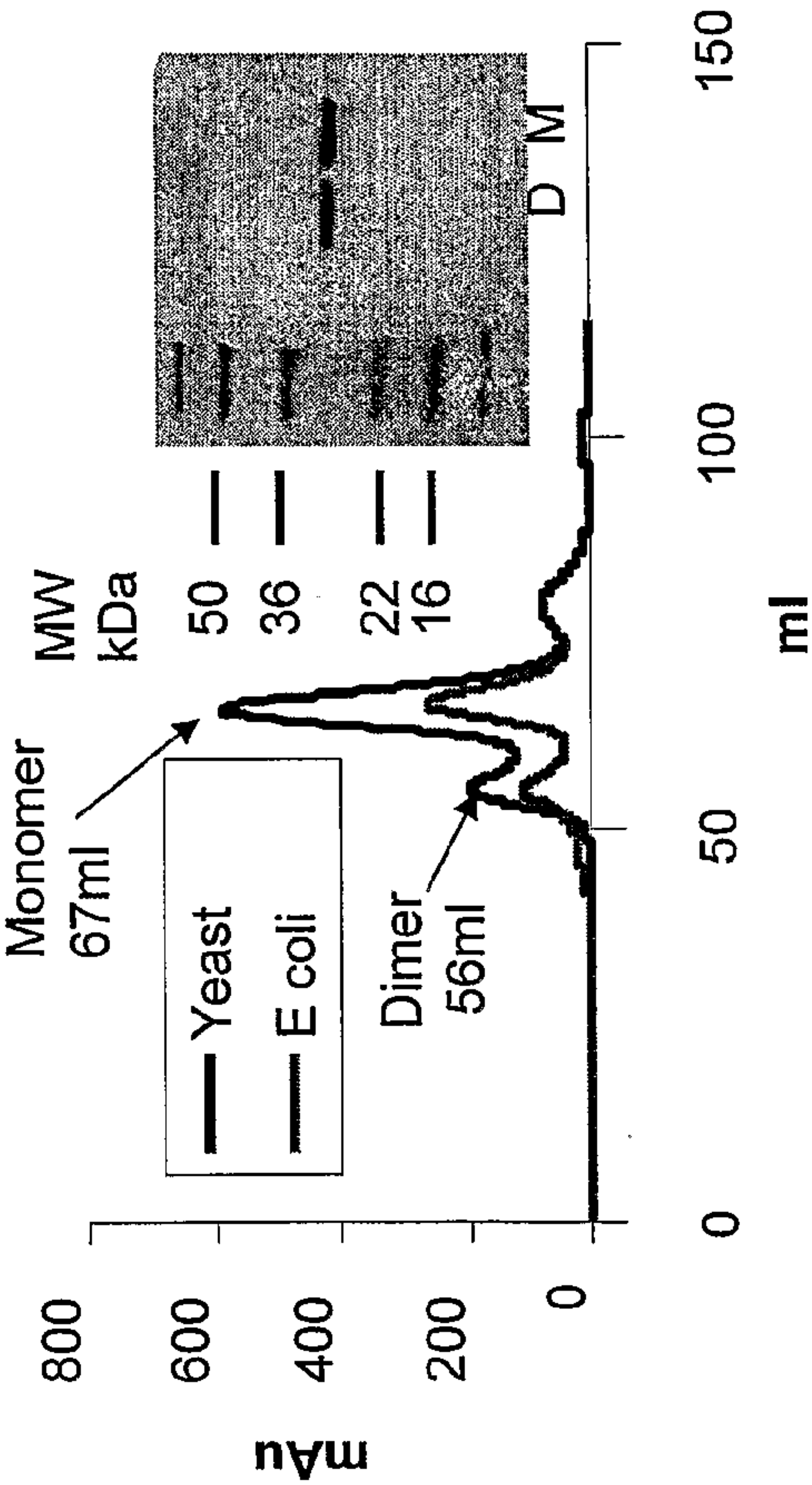


Figure 2

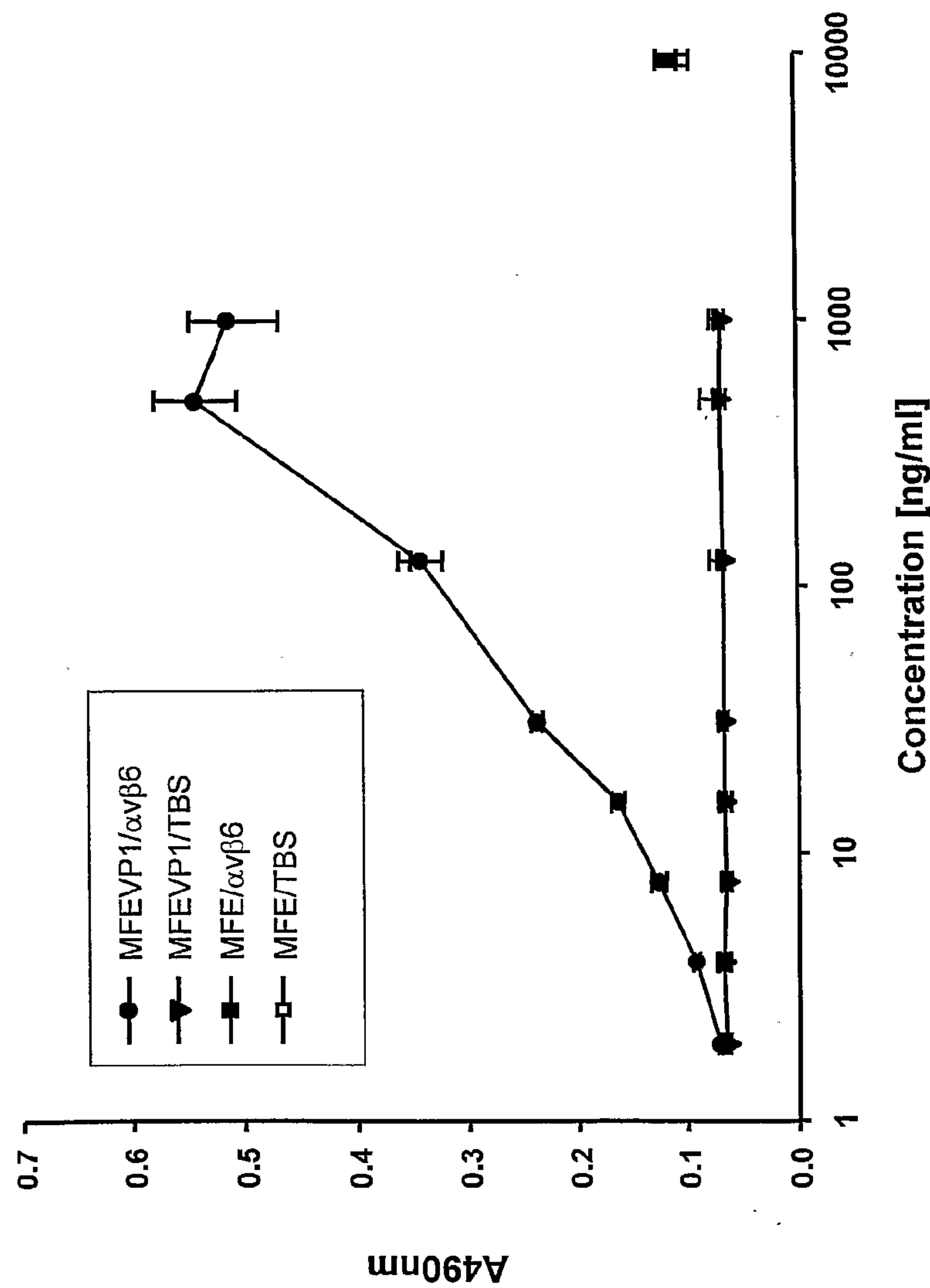


Figure 3a

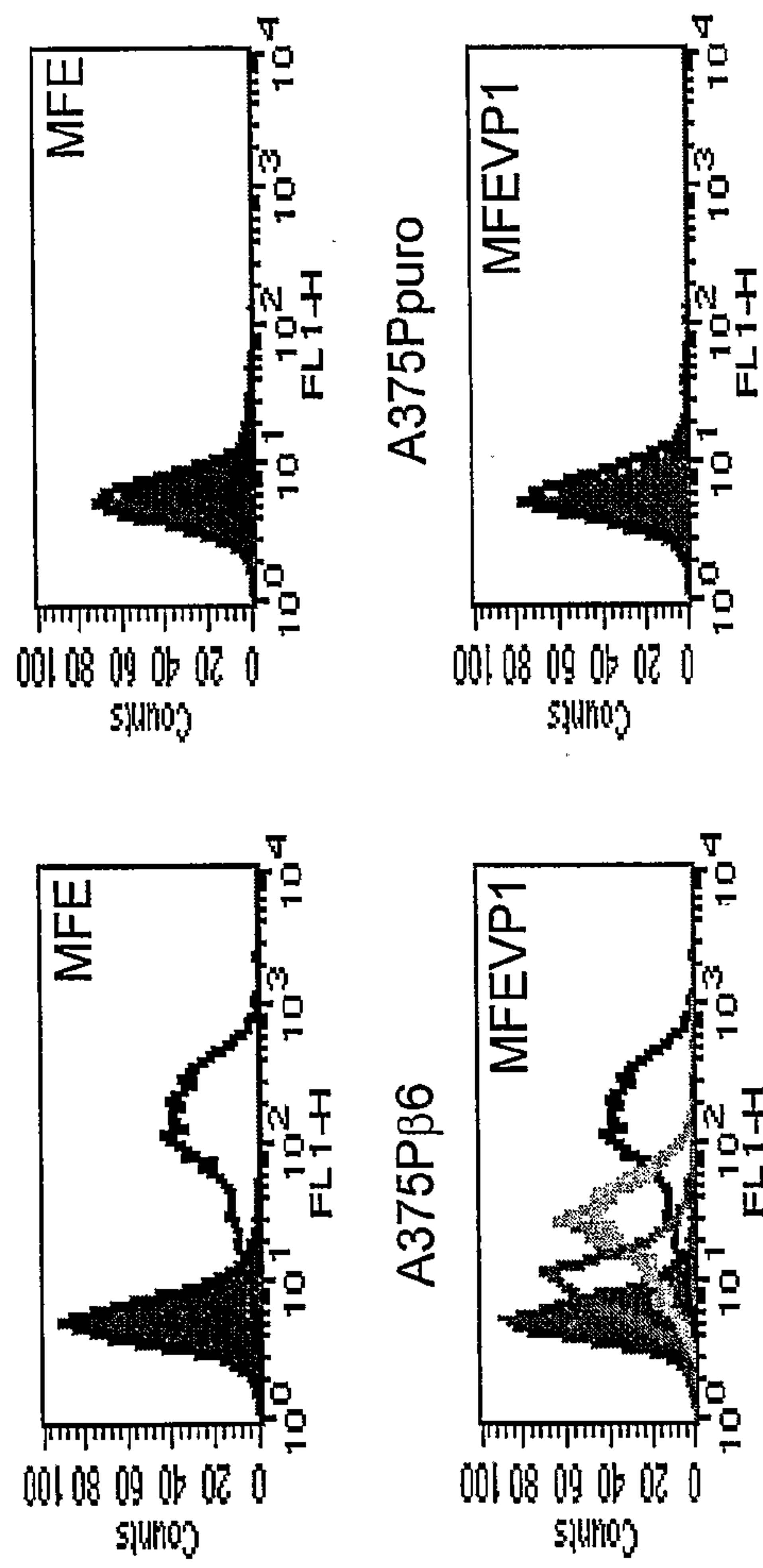


Figure 3b

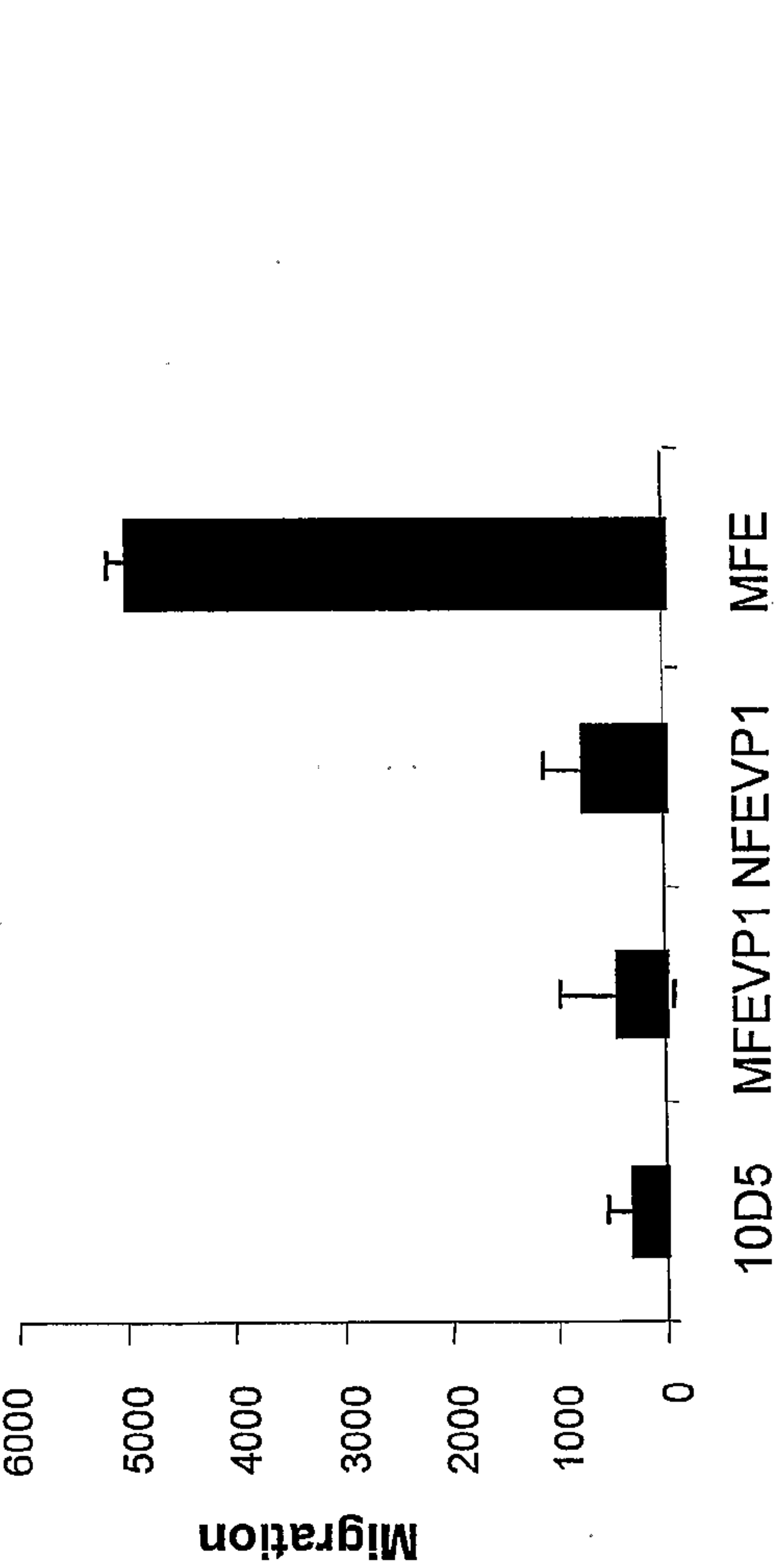


Figure 3c

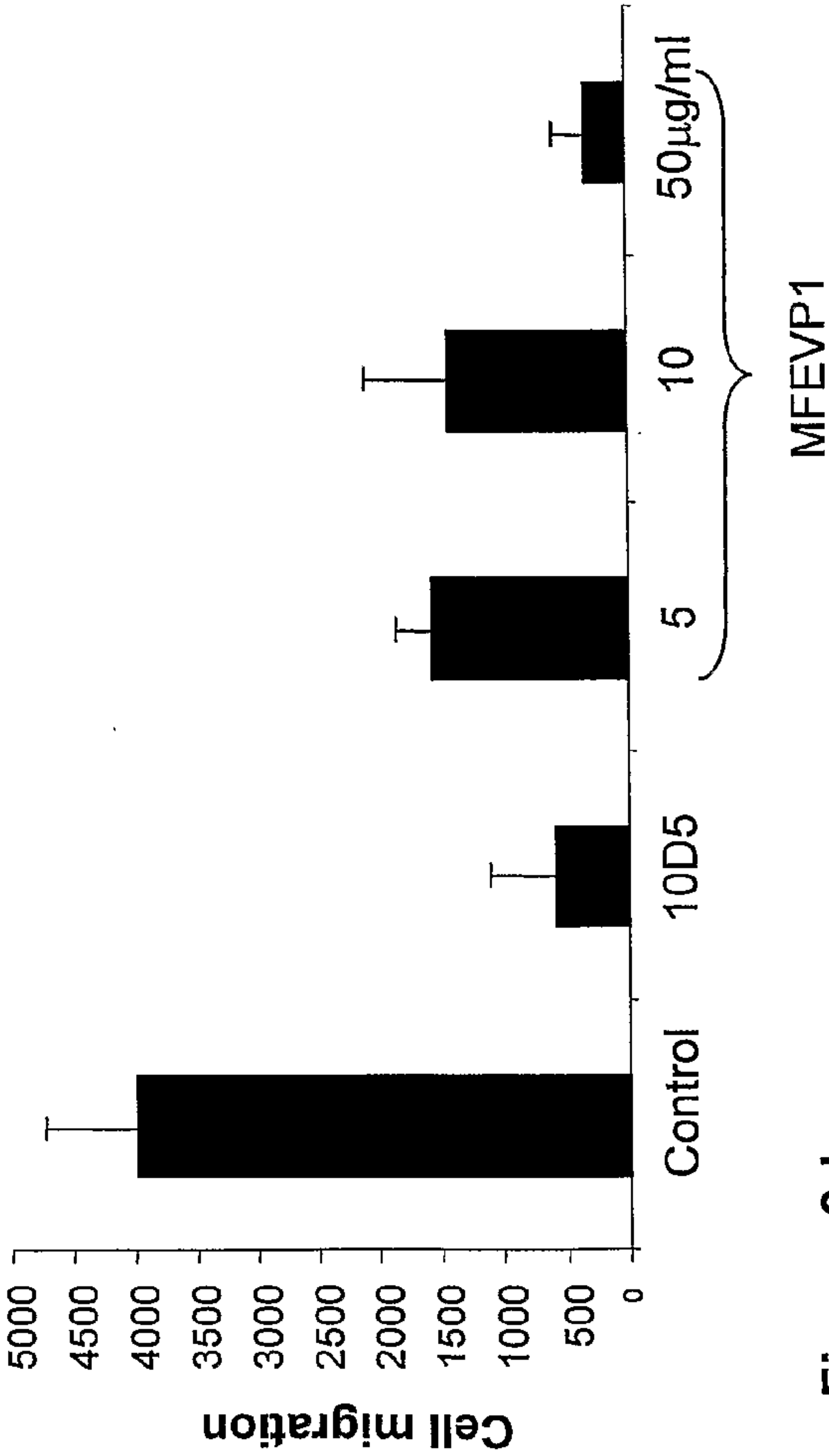


Figure 3d

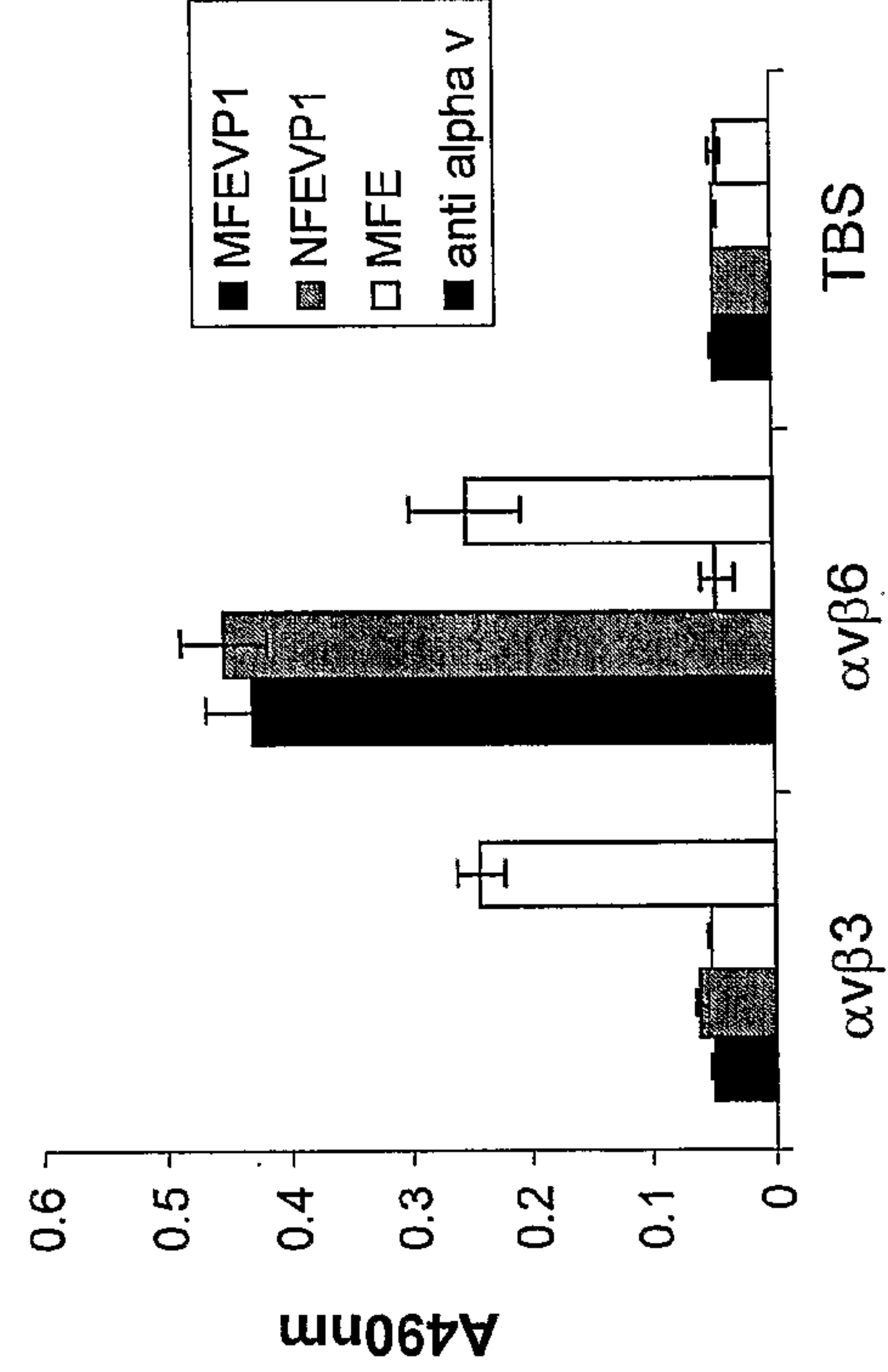


Figure 4a

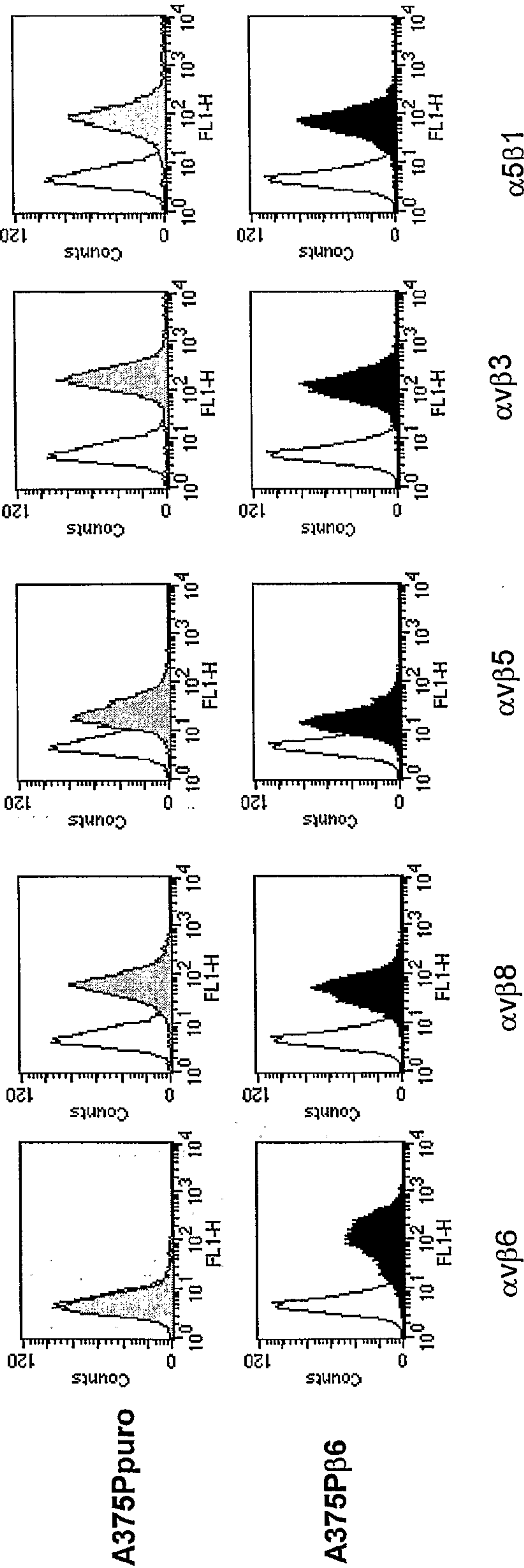


Figure 4b

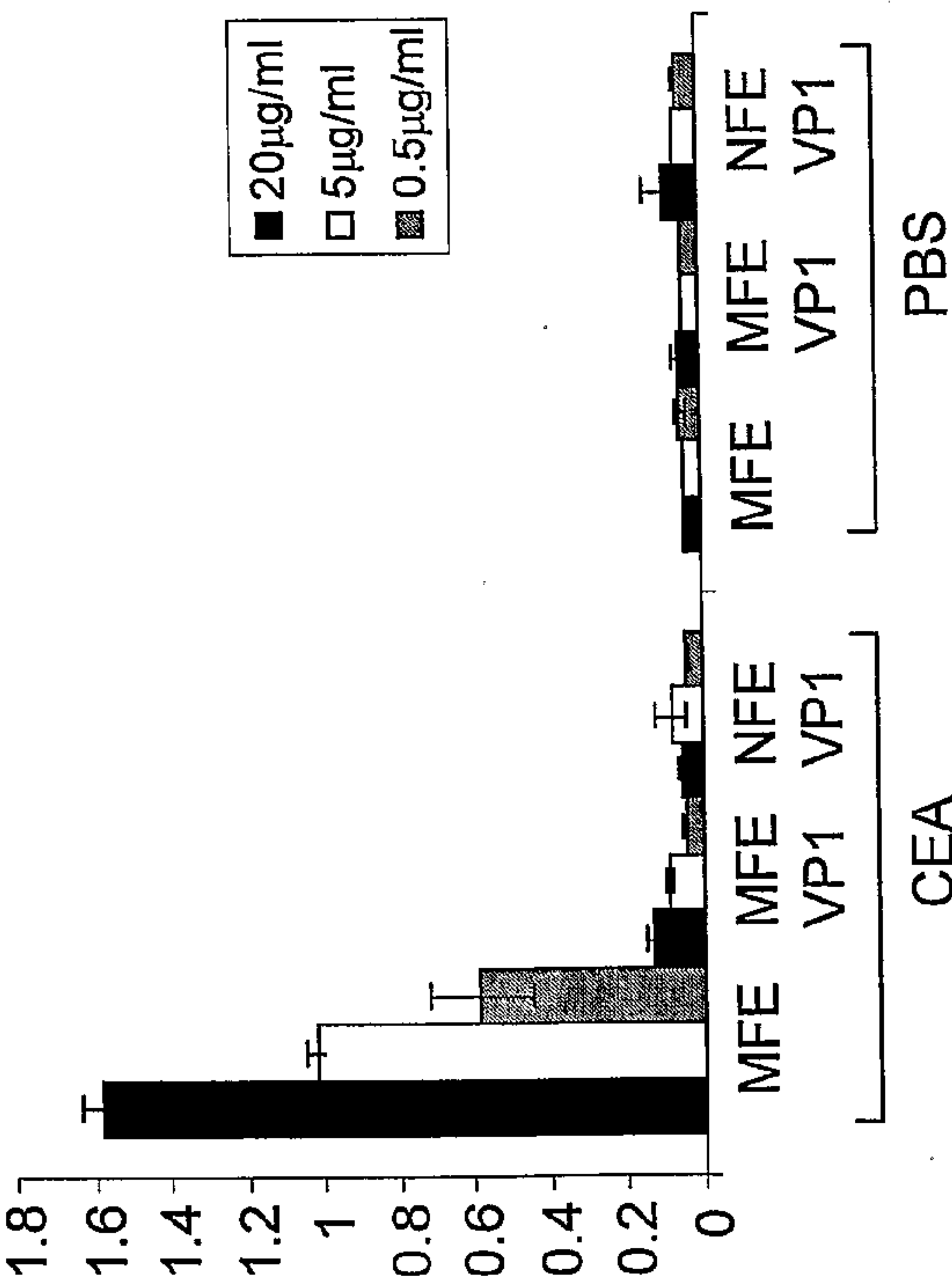


Figure 5a

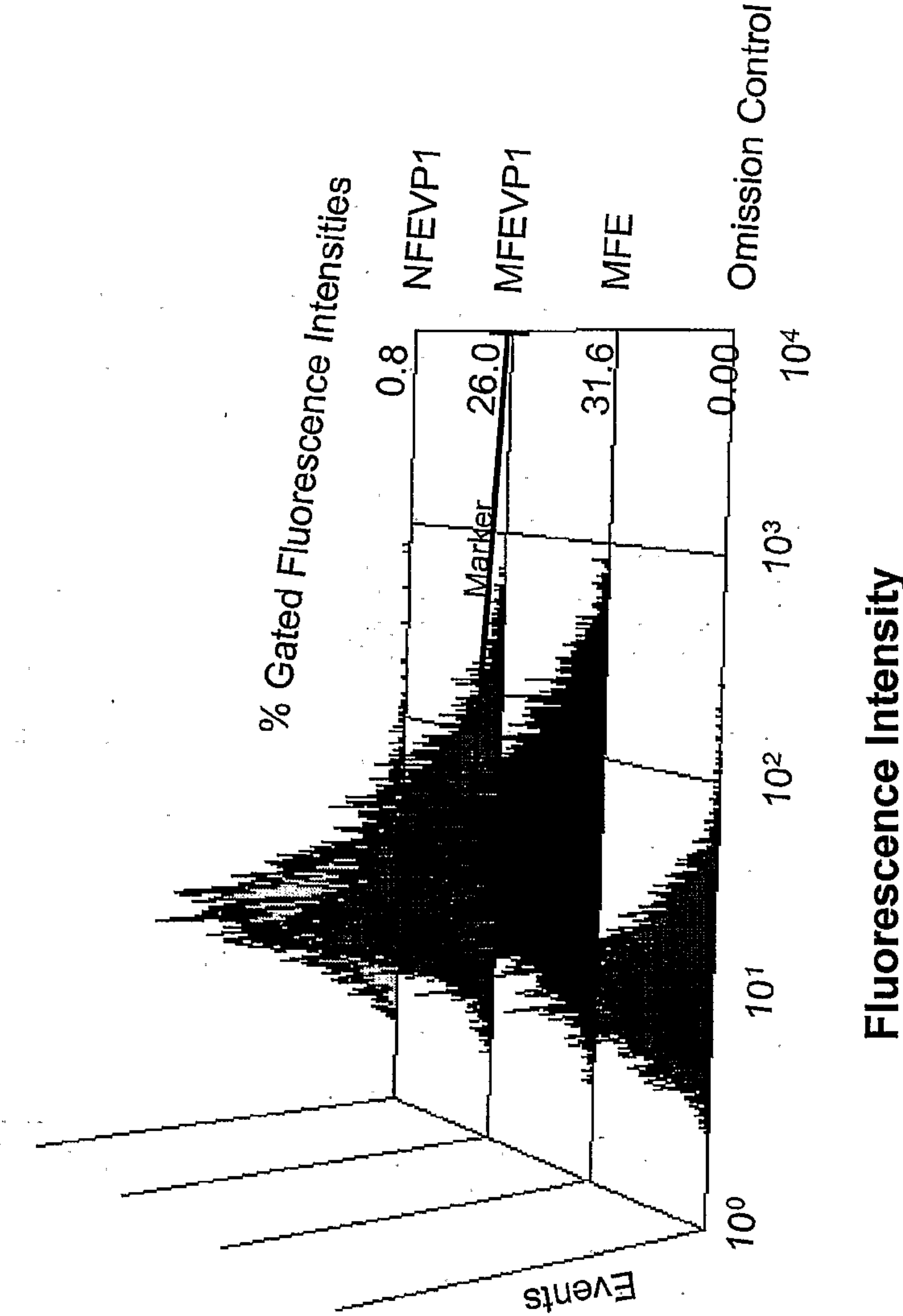


Figure 5b

Figure 6

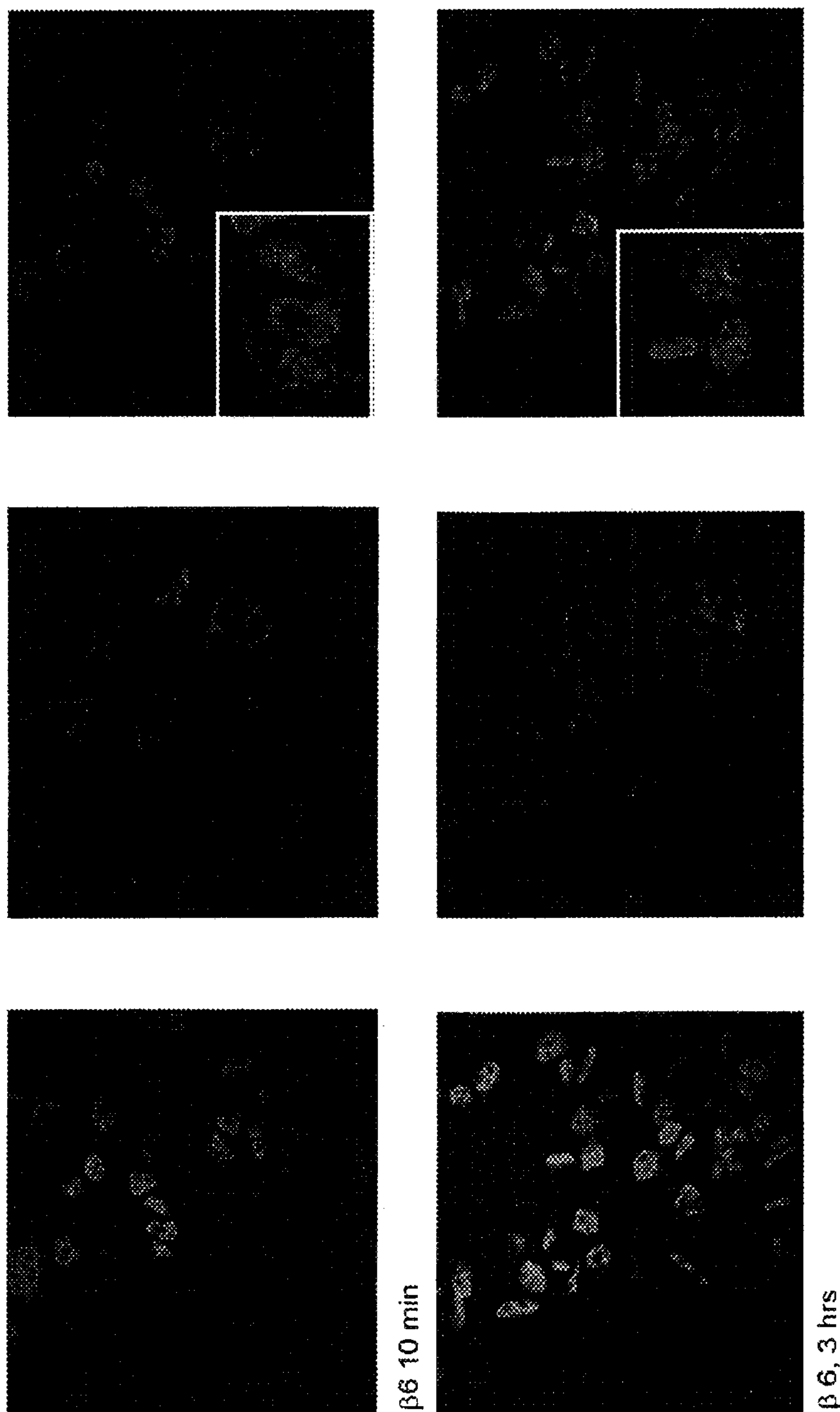


Figure 6

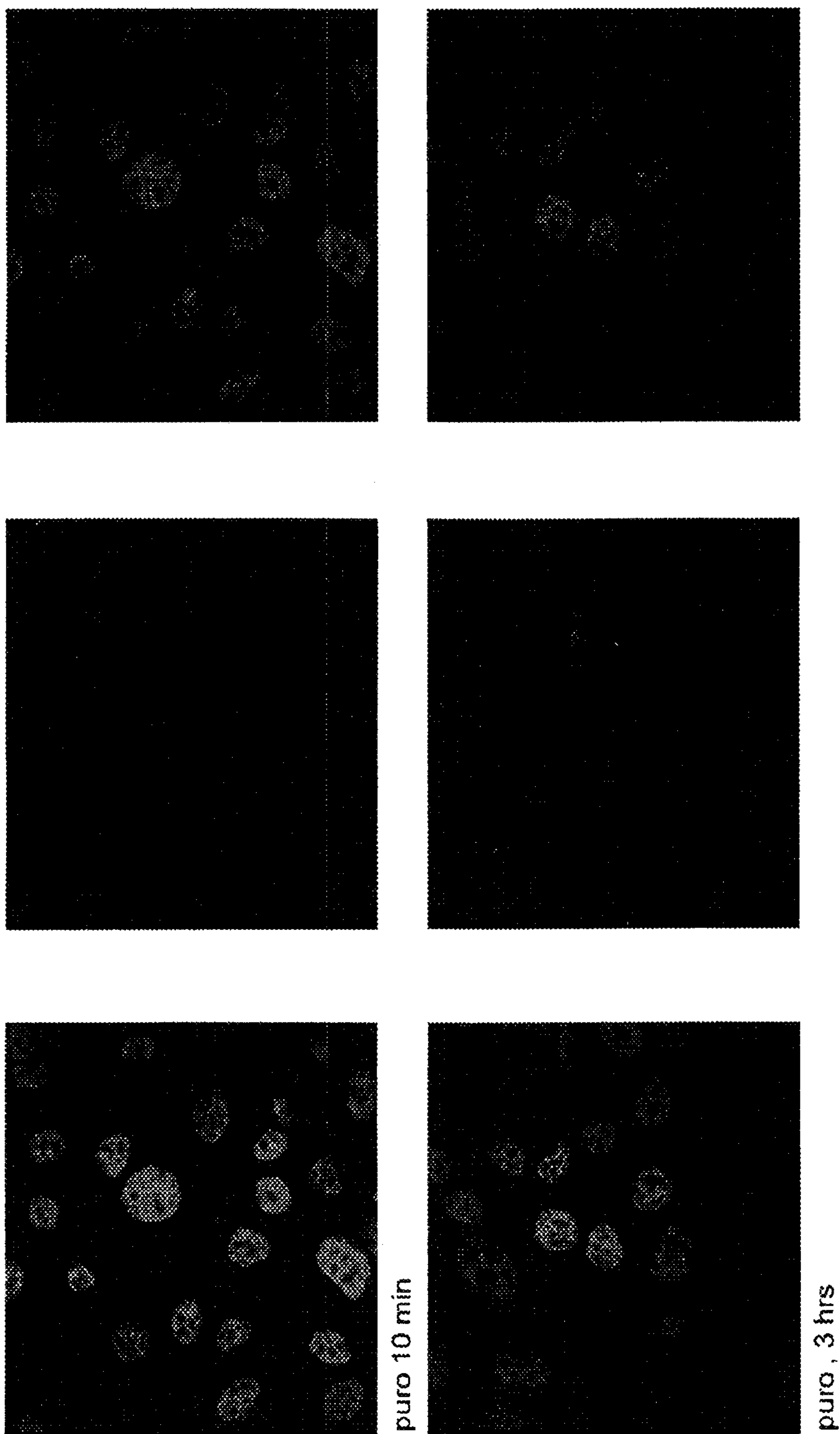
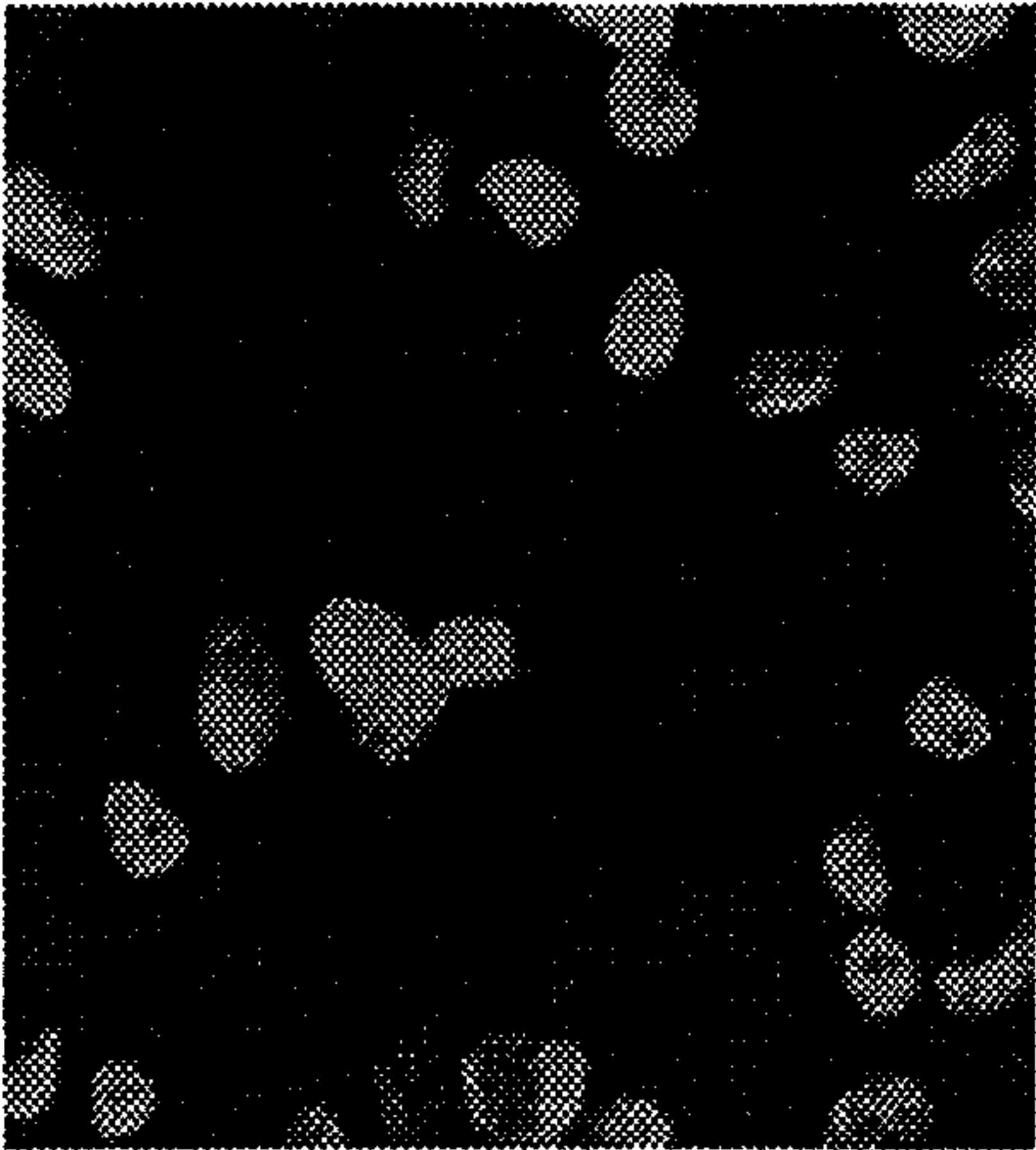
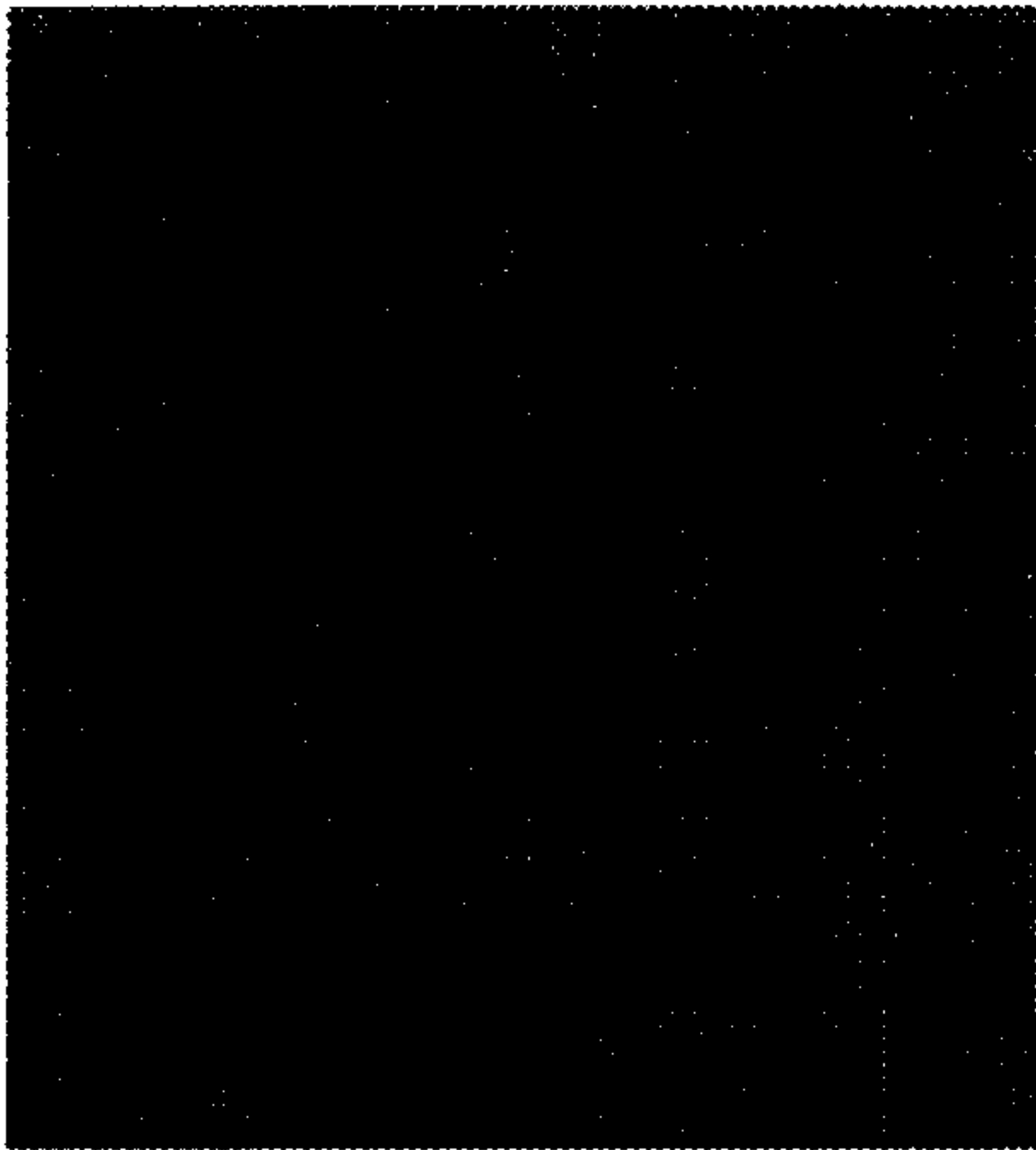
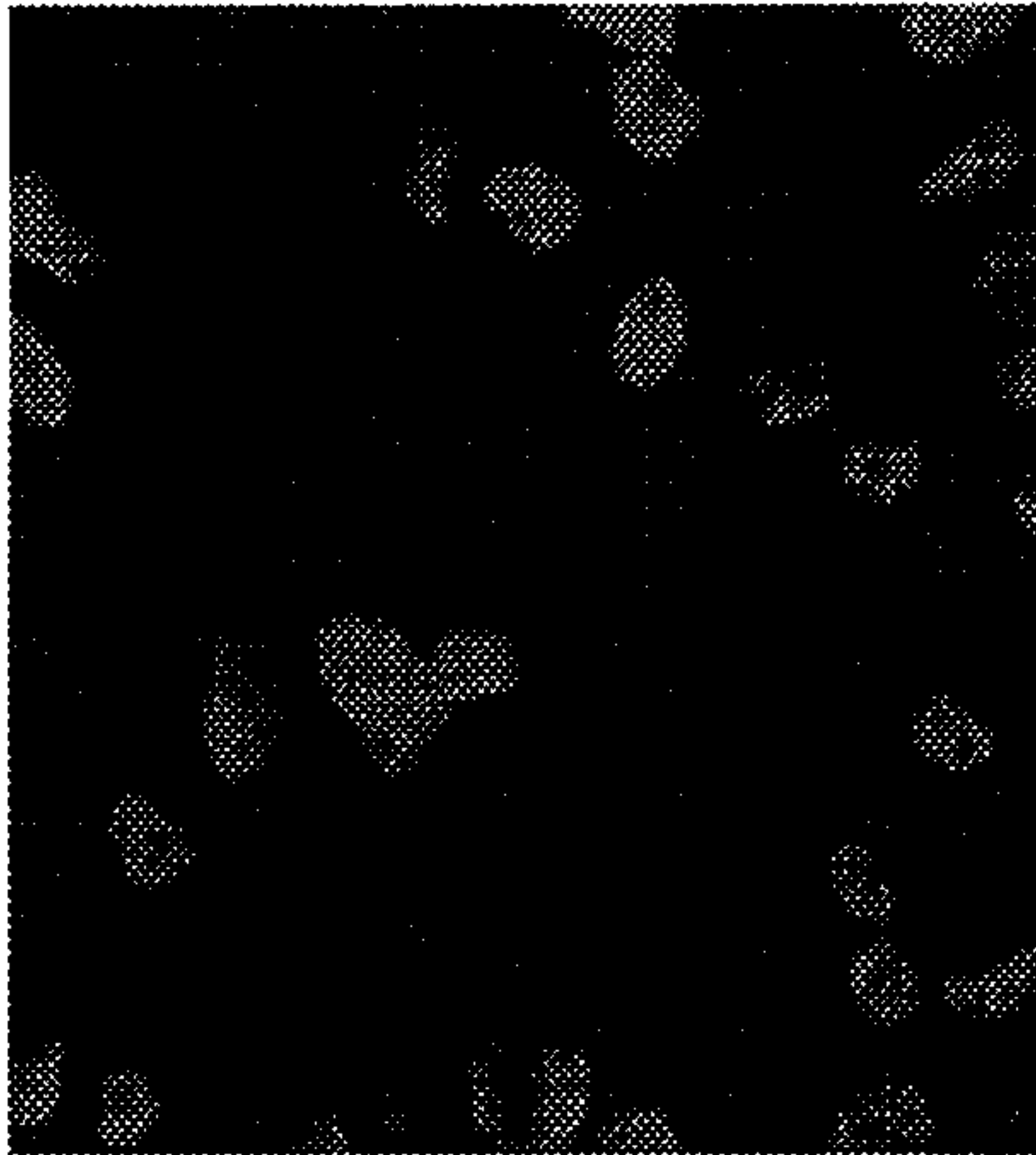


Figure 6



β6, Control

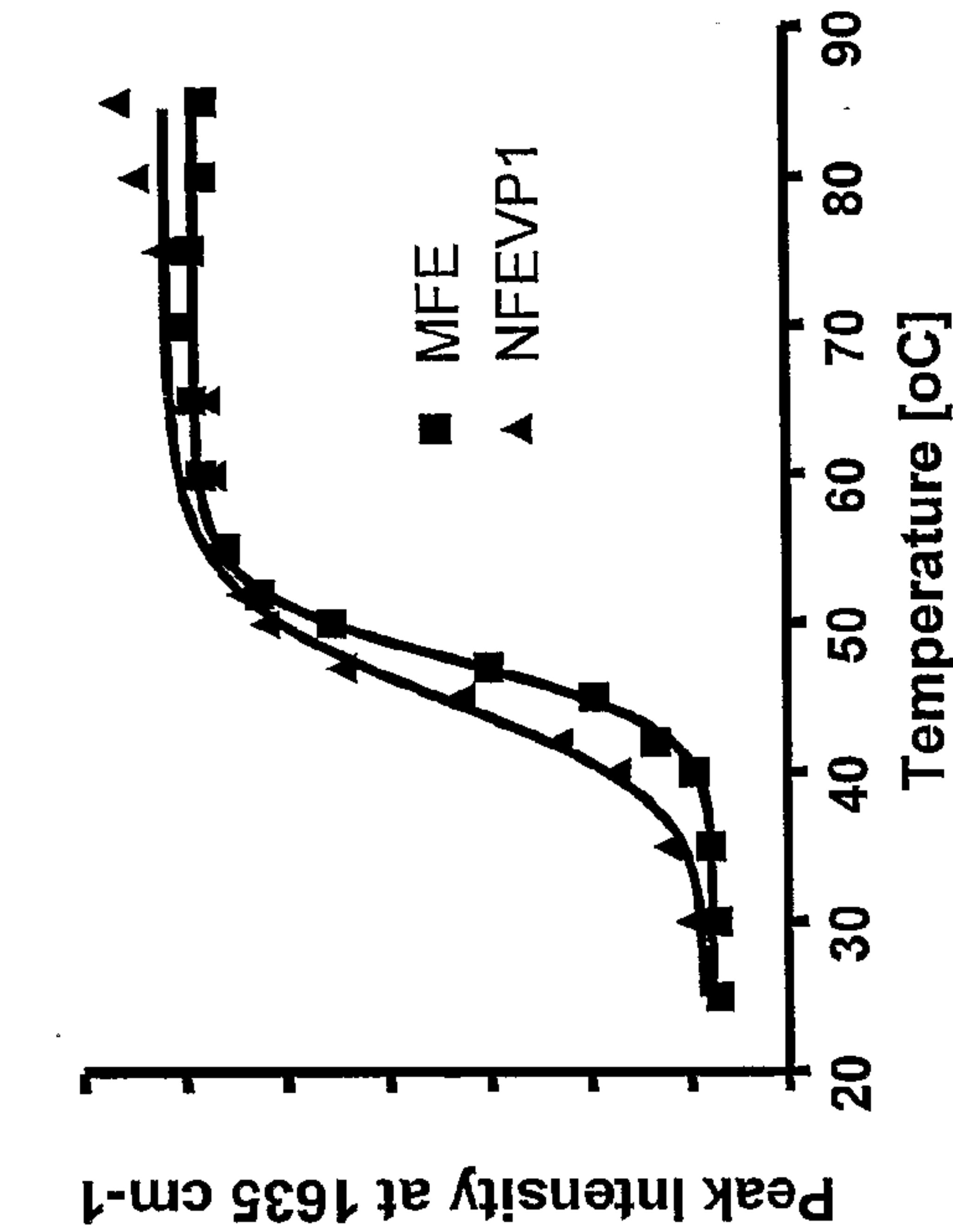


Figure 7(b)

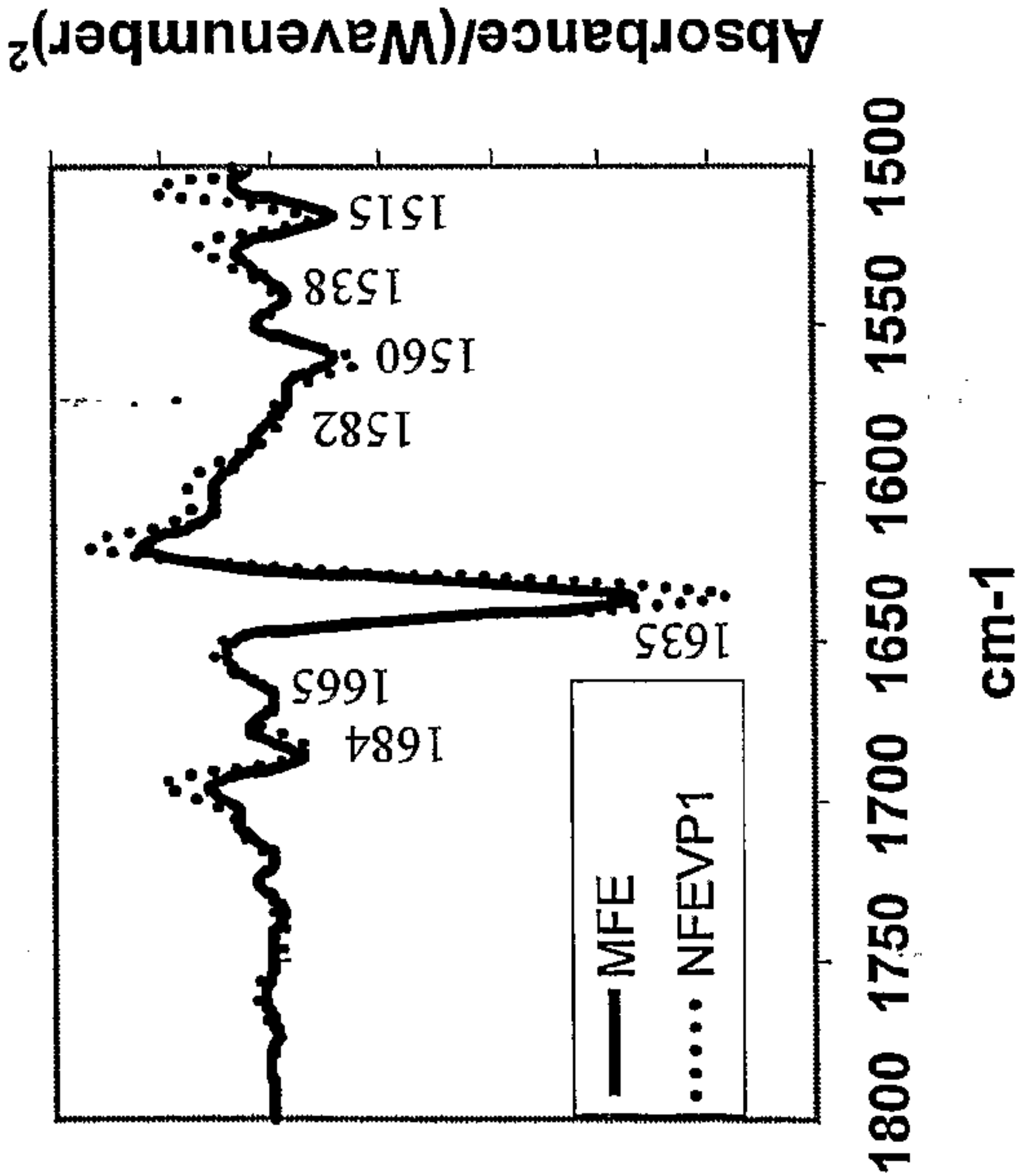


Figure 7(a)

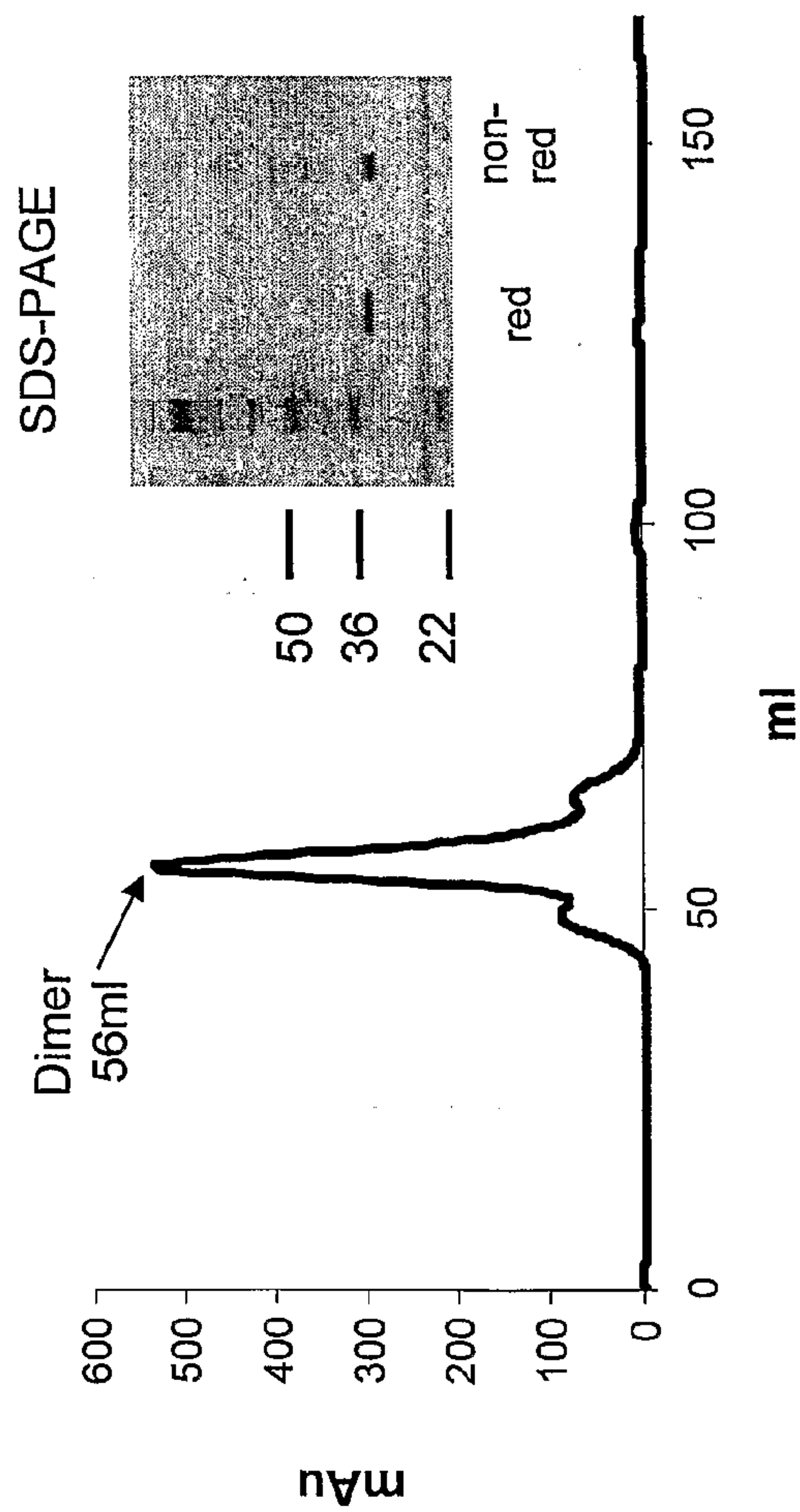


Figure 8(a)

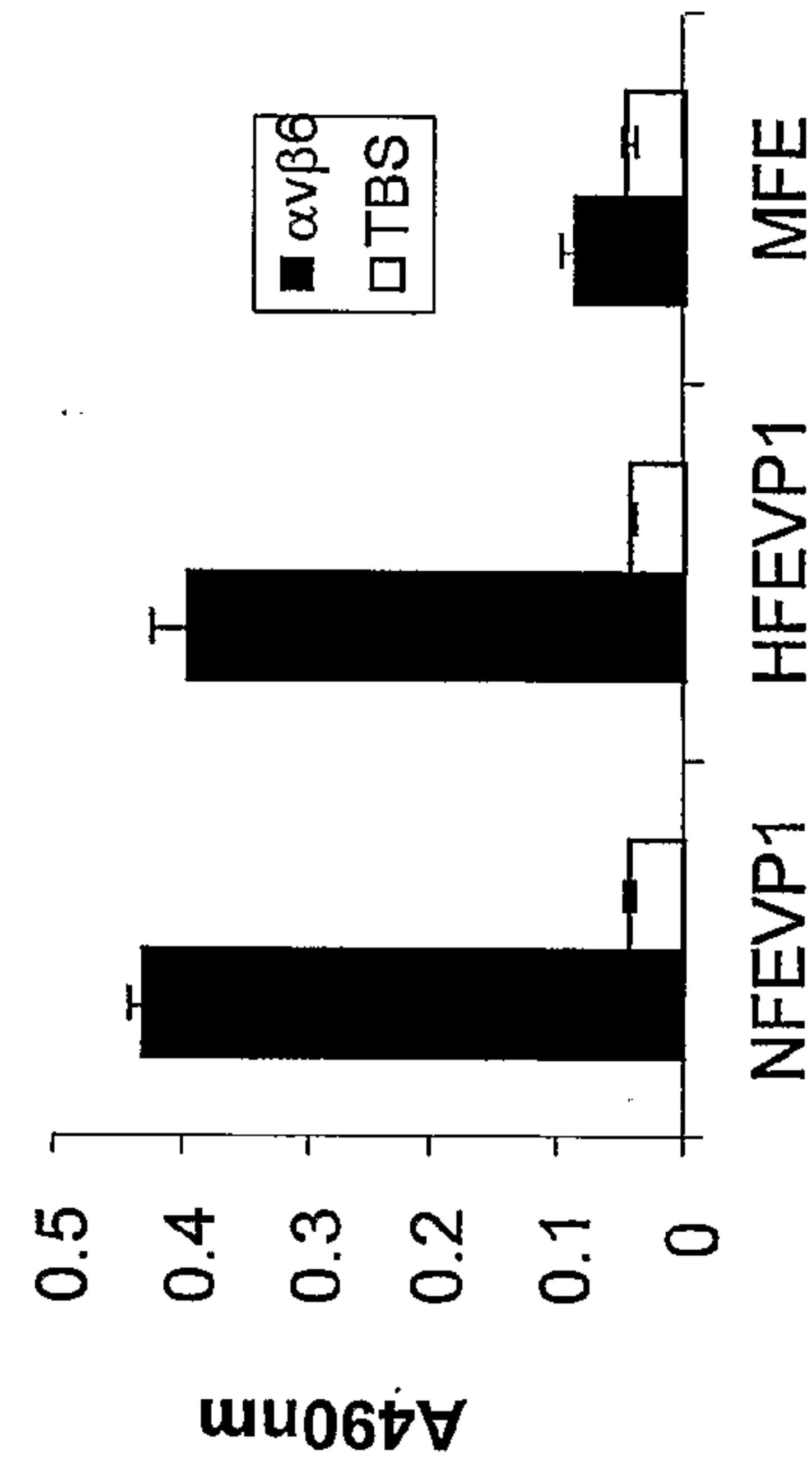


Figure 8(b)

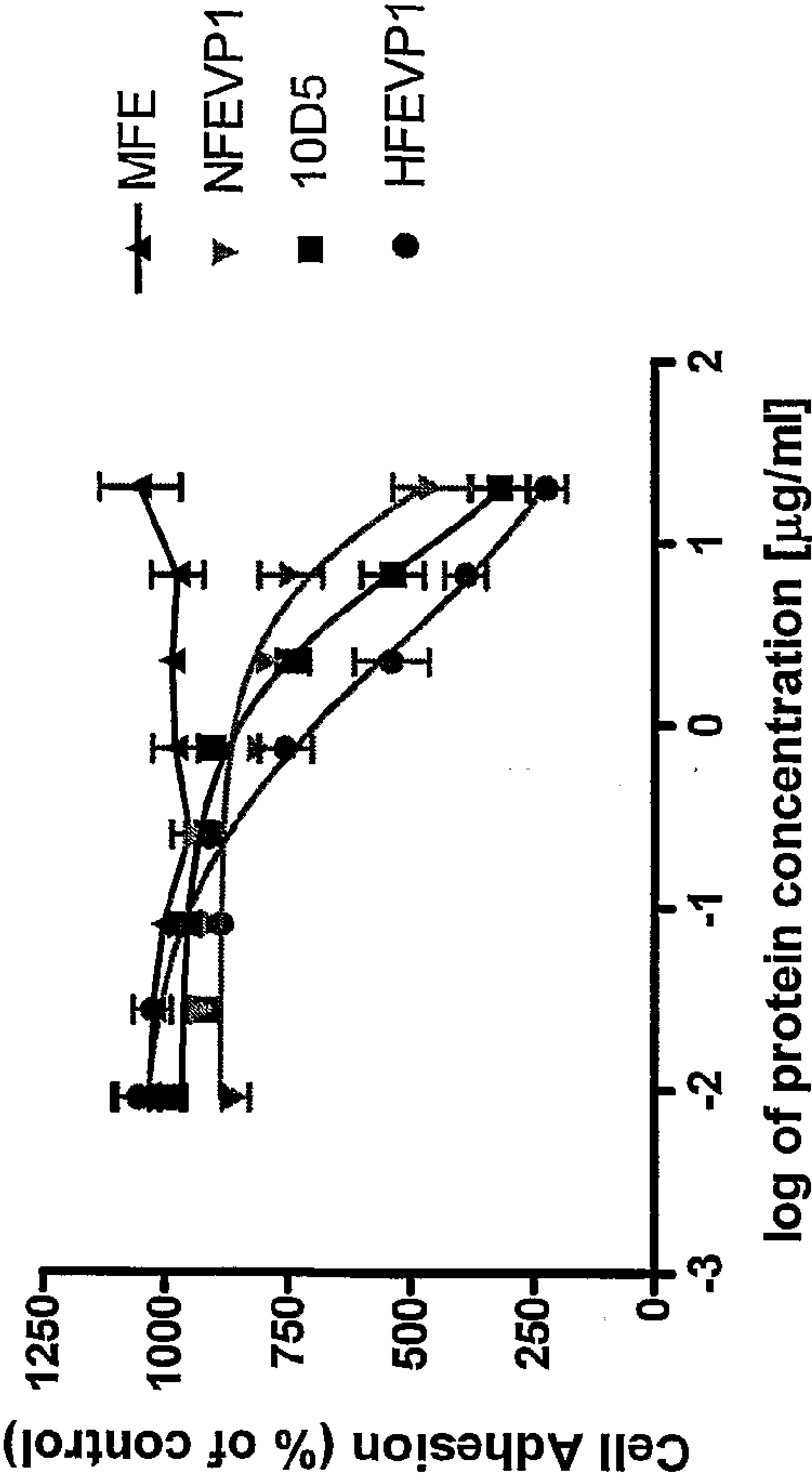


Figure 8(c)

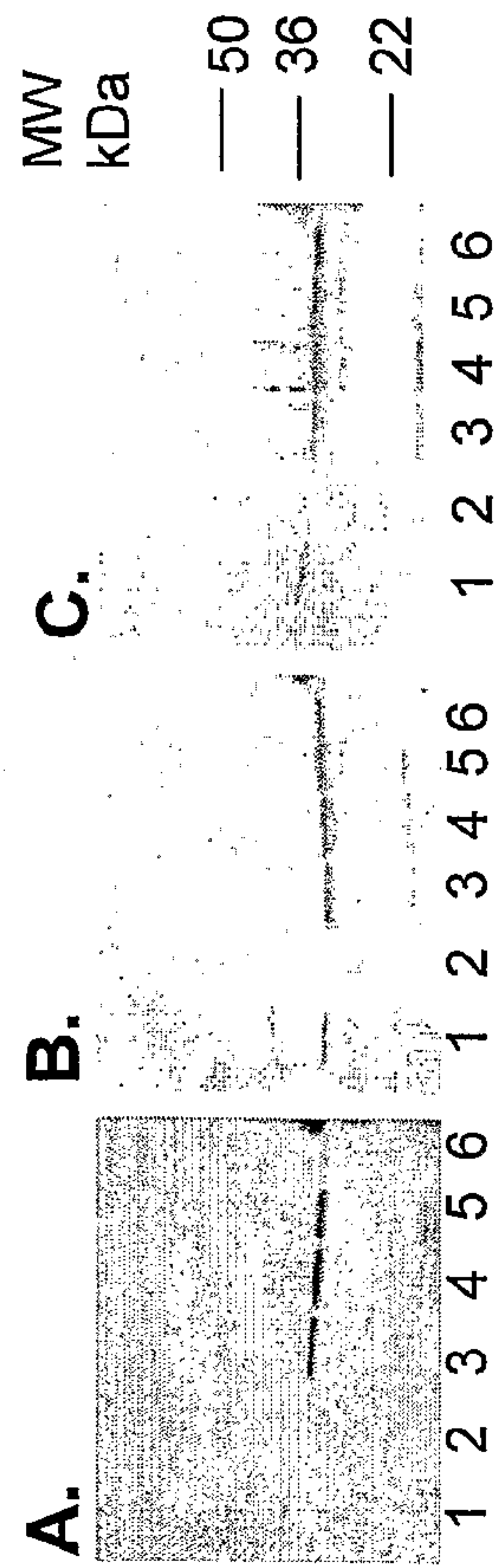


Fig. 9

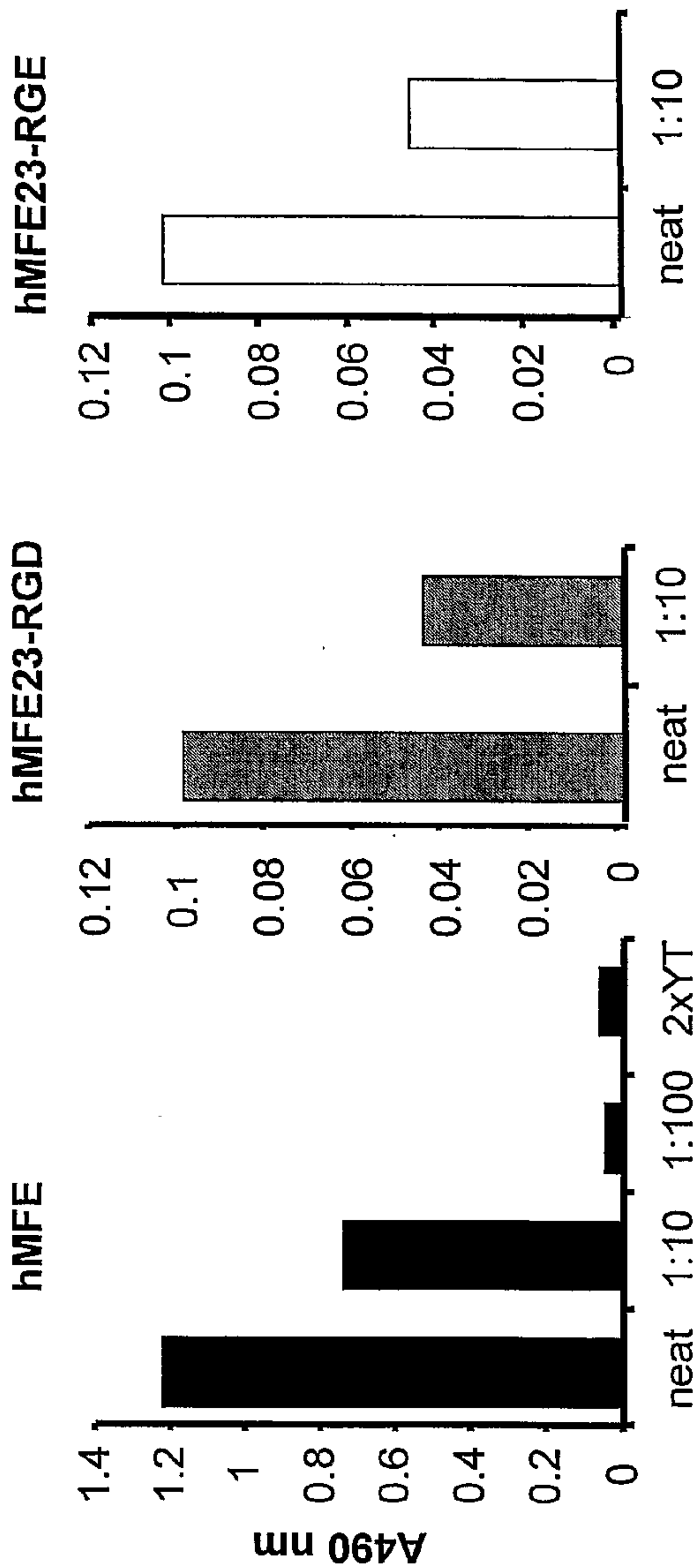


Fig. 10

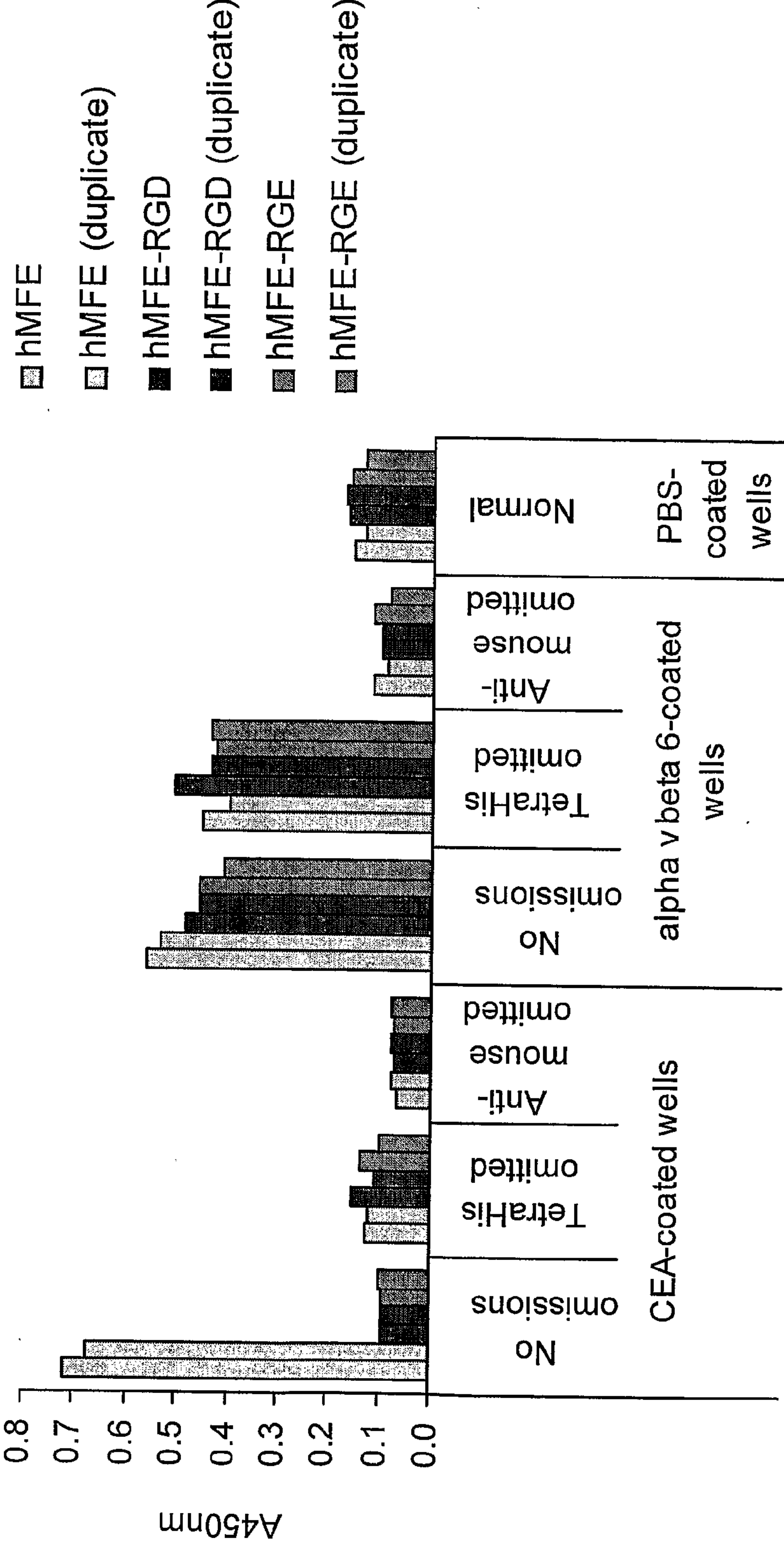
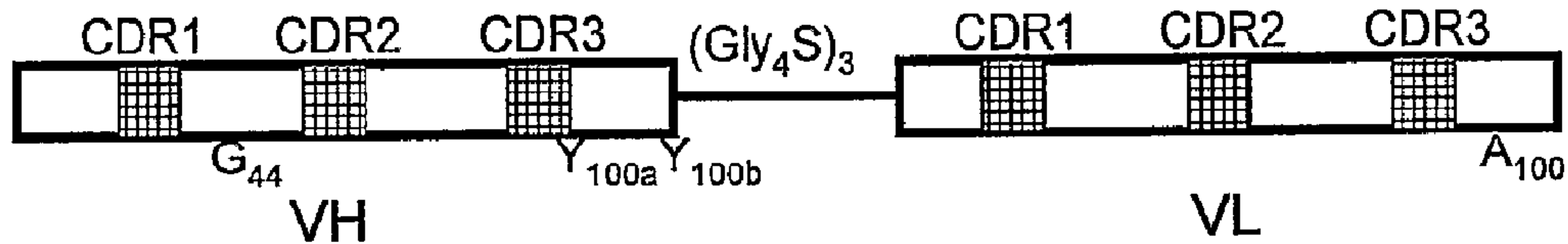
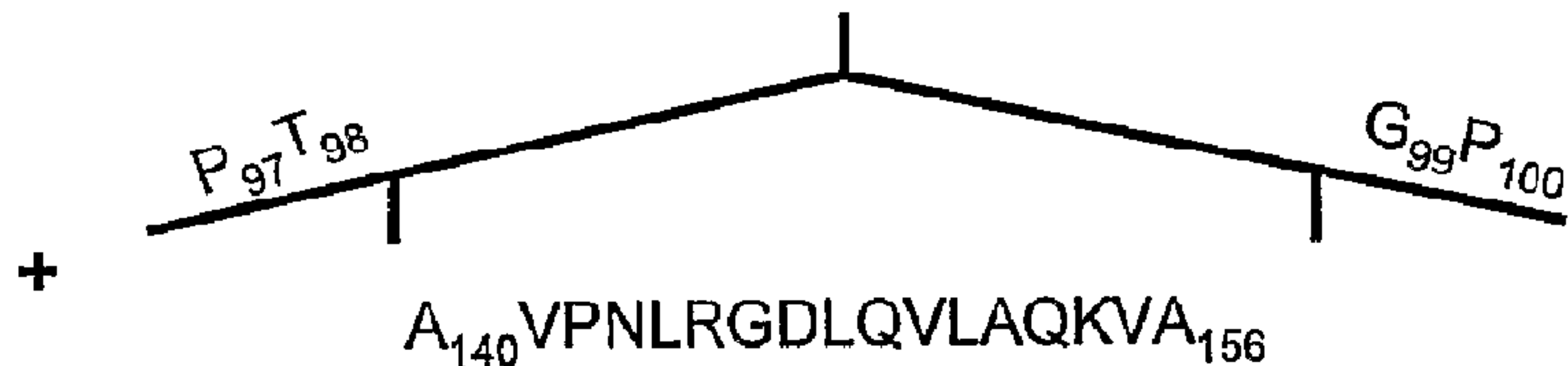


Figure 11.



= MFE



= MFEVP1



= NFEVP1