Technical Advance

A Human Immunoglobulin G1 Antibody Originating from an *in Vitro*-Selected Fab Phage Antibody Binds Avidly to Tumor-Associated MUC1 and Is Efficiently Internalized

Paula Henderikx,* Nicole Coolen-van Neer,*
Anita Jacobs,* Edith van der Linden,[†]
Jan-Willem Arends,[†] Jürgen Müllberg,* and
Hennie R. Hoogenboom*[†]

From Dyax s.a.,* Liège, Belgium; and the Department of
Pathology,[†] Maastricht University, Maastricht, The Netherlands

We describe the engineering and characterization of a whole human antibody directed toward the tumor-associated protein core of human MUC1. The antibody PH1 originated from the *in vitro* selection on MUC1 of a nonimmune human Fab phage library. The PH1 variable genes were reformatted for expression as a fully human IgG1. The resulting PH1-IgG1 human antibody displays a 160-fold improved apparent kd (8.7 nmol/L) compared to the kd of the parental Fab (1.4 μ mol/L). In cell-binding studies with flow cytometry and immunohistochemistry, PH1-IgG1 exhibits staining patterns typical for antibodies recognizing the tumor-associated tandem repeat region on MUC1, eg, it binds the tumor-associated glycoforms of MUC1 in breast and ovarian cancer cell lines, but not the heavily glycosylated form of MUC1 on colon carcinoma cell lines. In many tumors PH1-IgG1 binds to membranous and cytoplasmic MUC1, with often intense staining of the whole-cell membrane (eg, in adenocarcinoma). In normal tissues staining is either absent or less intense, in which case it is found mostly at the apical side of the cells. Finally, fluorescein isothiocyanate-labeled PH1-IgG1 internalizes quickly after binding to human OVCAR-3 cells, and to a lesser extent to MUC1 gene-transfected 3T3 mouse fibroblasts. The tumor-associated binding characteristics of this antibody, its efficient internalization, and its human nature, make PH1-IgG1 a valuable candidate for further studies as a cancer-targeting immunotherapeutic. (*Am J Pathol* 2002, 160:1597–1608)

Whole antibodies to tumor-associated targets or targets overexpressed on tumor cells, such as CD20, EpCAM, and Her2/neu have been shown to mediate a strong clinical benefit to the patient.^{1–3} The mucin MUC1 is a tumor-associated antigen in adenocarcinoma, studied particularly in ovarian, breast, and bladder cancer. It is a highly glycosylated transmembrane protein containing a variable number of tandem repeats of 20 amino acids.⁴ Because of its overexpression, lower glycosylation, and loss of polar expression in tumor tissues, it is accepted as a candidate for active as well as for passive immunotherapy.⁵ In adenocarcinoma, new epitopes of the MUC1 core protein become accessible on the membrane of the tumor cells. Peptide-specific antibodies can target these epitopes, differentiating normal from tumor tissue.⁶

This differential targeting can be useful in immunotherapy or diagnosis of adenocarcinoma, but when injecting murine monoclonals human anti-mouse antibody responses occur. The human anti-mouse antibody response can diminish the efficiency of the antibody in later administrations. Completely human antibodies against tumor-associated antigens can solve this problem.⁷ By means of phage display technology, human antibody fragments can be presented on the tip of a phage and selected for their specificity.⁷ These antibody fragments are then reformatted to a desired shape, isotype, fusion protein, and so forth.⁸ When human V-gene sources are used, the resulting antibodies are completely human in sequence. When used for therapy in humans, such an antibody would cause no human anti-mouse antibody responses and therefore could be used repeatedly without substantially affecting therapeutic efficacy. Nevertheless, such human antibodies may evoke anti-idiotypic

Accepted for publication February 14, 2002.

Present address of J.-W. A: Dept. of Pathology, Free University, Slotervaartziekenhuis, Amsterdam, The Netherlands.

Address reprint requests to Prof. Dr. Hennie R. Hoogenboom, Dyax s.a., Building 22, Boulevard du Rectorat 27B, Sart Tilman, 4000 Liège, Belgium. E-mail: hhoogenboom@dyax.com.

antibody responses as proposed by Jerne,⁹ which in their turn can mimic the antigen and therefore can lead to active immunotherapy.¹⁰ This side-effect could have a positive effect in immunotherapy.¹¹

We used the phage display method to select a MUC1-specific antibody (PH1-Fab) from a very large phage library displaying 3.7×10^{10} Fab fragments (P Henderikx, unpublished data).¹² The Fab antibody had a very low affinity of 1.4 μ M on biotinylated MUC1 peptide in BIAcore analysis (BIAcore AB, Uppsala, Sweden). By changing the format from the single binding site of a Fab to two binding sites of an IgG1, we aimed to increase the apparent affinity of the antibody for the peptide and cellular MUC1. We therefore reformatted the PH1-Fab to a completely human PH1-IgG1 antibody by recloning the V_H and V_L genes into two vectors of a mammalian expression vector system, containing the human kappa constant domain or the human γ -1 heavy chain constant region.¹³ The vectors were co-transfected into mammalian CHO-K1 cells for expression and production of the fully IgG identified. The apparent affinity increase was measured in BIAcore. To fully characterize the antibody for possible use in immunotherapy, we used the human PH1-IgG1 in extensive fluorescence-activated cell sorting and immunohistochemical analysis. To understand the differences between the binding pattern for this antibody versus other MUC1 antibodies, we compared our PH1-IgG1 with HMFG1, which is used in a phase III clinical trial.¹⁴ Finally, to determine which tumor-targeting format would be optimal for this antibody, we studied the *in vitro* internalization of PH1-IgG1 with fluorescein isothiocyanate (FITC)-labeled antibody.

Materials and Methods

Cloning of PH1-IgG1 into a Mammalian Expression Vector and Transfection into CHO-K1 Cells

The heavy and the light chain of the human PH1-Fab were recloned into the mammalian VHexpress and VKexpress expression vectors, respectively, to be reformatted for expression as a whole human γ -1/kappa antibody.¹³ The VH-fragment of PH1 was amplified by polymerase chain reaction using specific the oligos *VH1C back eukaryotic* (5'-GGA CTA GTC CTG GAG TGC GCG CAC TCC CAG GTC CAG CTG GTG CAG TCT GGG GGA GGC TTG GTA CAG-3') and *M13* primer (Amersham Pharmacia, Uppsala, Sweden), and introduced into the VHexpress vector as *Bss*HII/*Bst*EII fragment.

An *Apa*LI/*Pa*cl fragment of PH1-VL was generated by polymerase chain reaction using the specific oligos VKexpress *MUC-for* (5'-GCG CTC GCA TTT GCC TGT TAA TTA AGT TAG ATC TAT TCT ACT CAC GTT TGA TAT CCA CTT TGG TCC CAG GGC C-3') and *MUC1-VL-Back-APA* (5'-CCA GTG CAC TCC GAA ATT GTG CTG ACT CAG TCT CC-3'), and inserted into VKexpress.

Transfections of CHO-K1 (ATCC, Manassas, VA) cells were performed using the nonliposomal transfection re-

agent FuGene 6 (Roche, Brussels, Belgium) according to the manufacturer's instructions. Forty-eight hours after transfection limiting dilutions were performed into medium containing 700 μ g/ml of G418. Cells were plated in 96-well plates at 10, 100, and 1000 cells per well.

Screening of Cell Culture Supernatants in an Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants of clones growing on medium containing selection markers were tested in ELISA for antibody binding to MUC1 and to determine VH/VL production levels.

MUC1 Peptide Binding

For MUC1-binding tests, we used an adapted method described earlier.¹⁵ Incubation volumes are 100 μ l. MUC1 was immobilized indirectly with 0.5 μ g/ml of biotinylated MUC1 60-mer via coated biotinylated bovine serum albumin and streptavidin in a flexible microtiter plate. Coating of MUC1 60-mer was done overnight at 4°C. After three washes with phosphate-buffered saline (PBS), plates were blocked for 30 minutes at room temperature with 2% (w/v) skimmed milk powder (Marvel (Premier Brands, UK) in PBS. Plates were washed two times with PBS-0.1% Tween-20 and 1 $\times$ PBS, and supernatants were then incubated for 1.5 hours at room temperature while shaking [diluted 1:2 in 4% (w/v) Marvel/PBS]. Subsequently, plates were washed five times with PBS-0.1% Tween-20 and one time with PBS. Bound IgG was detected with rabbit anti-human IgG horseradish peroxidase (1:6000 diluted in 2% Marvel/PBS). After the last incubation, staining was performed with tetramethylbenzidine and H_2O_2 as substrate and stopped by adding 0.5 volume of 2 N H_2SO_4 ; the optical density was measured at 450 nm.

Production of Human IgG

To determine the amount of human PH1-IgG1 produced, a plate was coated for 1 hour at 37°C with 0.25 μ g/ml of rabbit anti-human $V\kappa$ Ig in PBS. After three washes with PBS, plates were blocked for 30 minutes at room temperature with 2% (w/v) semi-skim milk powder (Marvel) in PBS. Plates were washed two times with PBS-0.1% Tween-20 and 1 $\times$ PBS, and supernatants were then incubated for 1.5 hours at room temperature while shaking [diluted 1:2 in 4% (w/v) Marvel/PBS]. A twofold dilution series of hulgG was used as a standard, starting with a concentration of 500 ng/ml. Subsequently, plates were washed five times with PBS-0.1% tween-20 and one time with PBS and bound IgG was detected with rabbit anti-human IgG horseradish peroxidase (1 μ g/ml in 2% Marvel/PBS). After the last incubation, staining was performed with tetramethylbenzidine and H_2O_2 as substrate and stopped by H_2SO_4 to a final concentration of 1 mol/L; the optical density was measured at 450 nm.

Figure 1. **A:** Silver staining of purified PH1-IgG1. **Lane 1**, molecular weight marker; **lanes 2 and 3**, 5 μ l and 2 μ l of the purified antibody. **B:** Western blot of purified PH1-IgG1, 1 μ g was loaded.

Production and Purification of the PH1-IgG1 from Culture Media of CHO-K1 Clone 7F Cells

Approximately 3×10^8 -transfected CHO-K1 cells (clone 7F) were cultured in T175 triple-layer flasks in a humidified incubator at 37°C for 3 weeks. The culture medium contained 0.5% fetal calf sera and was exchanged once a week. From each harvest ~ 1 L of culture supernatant was obtained. Anti-MUC1 antibody was purified with Protein A. Briefly, 1 L of culture supernatant was loaded onto a 5-ml HiTrap Protein A column (Amersham/Pharmacia) at a flow rate of 5 ml/minute. The column was extensively washed with PBS. Bound MUC1 antibody was eluted with 12.5 mmol/L of citric acid and neutralized with 0.5 mol/L HEPES (pH 9). Protein-containing fractions were combined, dialyzed against PBS (16 hours, 4°C), and sterile filtered. Purified anti-MUC1 antibody was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis plus silver staining (Figure 1). Therefore, PH1-IgG1 (100 to 200 ng), purified with Protein A, was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel under reducing conditions and protein bands were visualized by silver staining.¹⁶ For Western blot purified PH1-IgG1 was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel under reducing conditions and transferred onto nitrocellulose. PH1-IgG1 heavy chain and light chain were simultaneously detected with a horseradish peroxidase-conjugated polyclonal antibody against human IgG and a horseradish peroxidase-conjugated monoclonal antibody against human kappa chain, respectively. Production amount was measured in a human IgG ELISA described above. Total protein concentrations were measured using a bicinchoninic acid microprotein assay (Pierce, New York, NY).

Surface Plasmon Resonance

The selected PH1-IgG1 and the PH1-Fab were evaluated for their binding characteristics by surface plasmon res-

onance on a BIAcore 2000 apparatus (BIAcore AB, Uppsala, Sweden). A Sensor Chip SA was coated with a biotinylated MUC1 15-mer, containing the epitope PAP [Ac-PDTRPAPGSTAPPAK-NH₂ (with a K as last amino acid for amino group-based biotinylation), 50 RU and 320 RU] and 60-mer [NH₂-(VTSAPDTRPAPGSTAPPAHG)₃-COOH,¹⁷ 50 RU] in Hepes Buffered Saline-EDTA P20 HBS-EP buffer (BIAcore). A surface, blocked with biotin (15 RU), was used as a negative control. The PH1-Fab and PH1-IgG1 were injected in HBS-EP buffer. To minimize rebinding of the antigen-binding fragments a flow rate of 30 μ l/sec was used. Affinity calculation was performed with the BIA Evaluation program 3.0.1. provided by BIAcore. Fitting was accepted when chi-square was lowest, on the two channels with a nonsaturated amount (50 RU) of MUC1 peptide bound. The affinity for the PH1-Fab was calculated at steady state. Because of the two binding sites on PH1-IgG1, the avidity was calculated as an apparent affinity constant using 1:1 determination with mass transfer limitation.

Flow Cytometric Analysis

Cell lines used were the mouse fibroblast cell line 3T3, the MUC1-transfected cell line 3T3-MUC1 (ETA),¹⁸ the breast carcinoma lines T47D and MCF-7, the ovarian carcinoma line OVCAR-3, the colon cancer cell lines LS174T and CaCo2, and the T-cell line Jurkat (nontransfected cell lines were provided by ATCC).

Cellular MUC1 binding was tested in flow cytometry, with purified PH1-IgG1 and with HMFG1 (Autogen Bioclear, Wiltshire, UK). Approximately 500,000 cells were used in each experiment. After trypsinization, cells were washed one time in RPMI 10% fetal calf serum, 0.01% NaN₃ (incubation buffer). To confirm the specificity, antibodies were incubated without or with 100 μ g/ml of MUC1 60-mer for 1 hour at room temperature. The same amounts (10 μ g/ml) of specific (PH1-IgG1, HMFG1) or nonbinding human antibody (hIgG1) were used. Then the samples were added to the cells and left for 1 hour at room temperature. Cells were spun down by centrifugation for 3 minutes at $611 \times g$. Between incubations, cells were washed twice with incubation buffer. Anti-human IgG1 antibody was added to the cells and incubated for 1 hour at room temperature. Then rabbit anti-mouse-FITC (1/20 dilution; DAKO, Glostrup, Denmark) was added to all tubes for 30 minutes. Detection of bound antibodies was performed by means of flow cytometry on a FACSCalibur (Becton Dickinson, Oxnard, CA) and results were analyzed with the CELLQuest program (Becton Dickinson).

Biotinylation and FITC Labeling of PH1-IgG1

PH1-IgG1 in 50 mmol/L of NaHCO₃, pH 8.5, at a concentration of 250 μ g/ml was treated with Sulfo-NHS-LC-Biotin (Pierce) for 1 hour at room temperature under gentle agitation. Four μ g of biotin ester was used for 100 μ g of the antibody. The reaction was stopped by treatment with Tris/HCl, pH 7.5, at a final concentration of 50 mmol/L, for 30 minutes. To separate the biotinylated antibody from

free biotin the reaction mixture was dialyzed against PBS. Biotinylation of PH1-IgG1 was verified by binding of the antibody to MUC1-positive OVCAR-3 cells and ETA cells and MUC1-negative 3T3 cells with flow cytometry analysis.

FITC labeling was performed according to the manufacturer with 200 μ g of PH1-IgG1 in 200 μ l of reaction mixture using a FITC protein-labeling kit (Molecular Probes, Leiden, The Netherlands). Labeling was checked on MUC1-positive and MUC1-negative cell lines using flow cytometry analysis (3T3, ETA, OVCAR-3).

Immunohistochemistry

A variety of formalin-fixed normal and tumor tissues were tested for the binding pattern of the PH1-IgG1. Tissues were chosen with a preference for diagnosed adenocarcinoma. HMFG-1 was used as a control for a limited number of tumor tissues. The biotinylated PH1-IgG1 antibody was used. Five- μ m sections of paraffin-embedded tissues were deparaffinized, rehydrated, hydrogen peroxide-treated, (0.3% H_2O_2 in PBS), and preincubated with PBS, 15% fetal calf serum, and 5% human serum for 20 minutes. Antibodies were diluted to a concentration of 17 μ g/ml in PBS and 10% human serum and incubated for 1 hour at room temperature. For PH1-IgG1, slides were then incubated with an avidin-biotin-complex (ABC, DAKO) for 30 minutes. For HMFG1, slides were first incubated with biotinylated sheep anti-mouse (RAMPO, DAKO) in PBS, 0.1% Tween 20, and 1% bovine serum albumin for 30 minutes and then with the avidin-biotin-complex. For each tissue biotinylated (irrelevant) human IgG was used as a control. Between antibody incubation slides were washed three times for 5 minutes in PBS. Staining was detected by diaminobenzidine and H_2O_2 . The peroxidase reaction was stopped with water and slides were counterstained with hematoxylin. The epithelial tissues were evaluated for their binding reactivity (sporadic, <10%; focal, 10% < f < 80%; diffuse, > 80%) and their localization in the cell (a, apical, polar; c, cytoplasmic, depolarized; m, abundant expression on the whole-cell membrane).

Evaluation of Internalization Using a Confocal Microscope

Antibody was FITC-labeled according to the manufacturer's instructions (see above). For internalization studies the human tumor cell line OVCAR-3 and the MUC1-transfected mouse fibroblast 3T3 cell line, ETA, were used. As negative control the colon cell line CaCo2 was used. FITC-labeled antibody was added to the cells (10 μ g/ 10^6 cells at a concentration of 100 μ g/ml) for an incubation period of 1 hour on ice. The cells were washed and put on ice or at 37°C to check whether the antibody stayed bound to the membrane and to study internalization in both temperature conditions. Every time point (1, 3, and 6 hours and overnight) cells were checked on confocal microscope for membrane binding and internalization. Fc binding was checked by competition with human IgG1. Staining patterns (membranous or intracellular) were

evaluated with a confocal microscope (Asciophat, Zeiss; Atto Instrument, Rockville, MD).

Results

Cloning of PH1-IgG1 into a Mammalian Expression Vector and Selection of Transfectants

The variable region antibody genes encoding the human Fab directed to MUC1 (PH1-Fab) was recloned for expression as fully human γ -1/kappa antibody into the mammalian VHexpress and VKexpress expression vectors¹³ as described in Materials and Methods. These vectors allow rapid recloning of phage antibodies and expression of full IgG.¹³

After co-transfection of CHO cells with the two heavy and light chain gene-containing plasmids, cells were grown on selective media as indicated in Materials and Methods. On the plate with 100 cells/well seeded, 37.5% of the wells showed cell growth after 5 days in culture; at 10 cells/well, no growth was detected. Supernatants of the cultures were assayed for the presence of human IgG and binding to MUC1 peptide in ELISA. A third of the wells with selected cells were positive for binding to MUC1, with levels of human IgG between 5 and 75 ng/ml. We chose line 7F (which secreted at 75 ng/ml) for further subcloning and production of PH1-IgG1.

Production and Purification of the PH1-IgG1

The MUC1-specific PH1-IgG1 antibody was purified from cell line 7F grown in 0.5% fetal calf serum containing culture media as described in Materials and Methods. Under these conditions more than 95% pure PH1-IgG1 protein was obtained (based on silver staining; Figure 1), at a yield of 0.5 mg of PH1-IgG1 per L of culture after purification. The results of a human IgG1-specific ELISA and a bicinchoninic acid total protein detection assay were in agreement (data not shown), indicating minimal contamination with bovine IgG.

Affinity Analysis

The affinity of antibody PH1-IgG1 was determined using BIAcore. Affinities of the PH1-Fab calculated were 1.47 (15-mer) and 1.40 (60-mer) μ mol/L with a mean of 1.43 μ mol/L (Figure 2, A and B). Mean apparent affinity of PH1-IgG1 (8.7 nmol/L) was calculated with the BIAcore software from binding curves on low-density surfaces being 8.3 nmol/L (15-mer) and 9.06 nmol/L (60-mer) (Figure 2C). Thus under the conditions used, binding of PH1-IgG1 was 160 times stronger than of the PH1-Fab.

Comparative Flow Cytometric Analysis

Because differences in the fine-specificity of MUC1 antibodies can lead to differences in the panel of tissues and tumors recognized, we compared antibody PH1-IgG1

Figure 2. BIAcore analysis of PH1-Fab (**A** and **B**) and PH1-IgG1 (**C**). Binding pattern of PH1 (**A**) and its analysis at steady state [response units equilibrium (RUeq)] (**B**). **C**: Binding pattern of IgG1-PH1, fitted with the BIAevaluation program (version 3.01). Fitting curves at Langmuir with mass transfer limitation) are superimposed (**dashed line**) to the concentration curves (**straight line**).

with a frequently used for murine antibody, HMFG1, for their binding pattern in flow cytometry. PH1-IgG1 recognizes the minimal PAP epitope as determined by epitope fingerprinting of the PH1-Fab, whereas HMFG1 recognizes the PDTR epitope (P Henderikx, unpublished data).¹⁵ The two antibodies were tested on different tumor cell lines (Figure 3). Both antibodies bind with the same binding pattern to most of the cell lines, except for the ovarian carcinoma cell line OVCAR-3 that seems to expose more of the PH1-IgG1 epitope than of the HMFG1 epitope. Both antibodies bind a very small subpopulation

of the LS174T colon tumor cell line and of the T-cell line Jurkat, which can be inhibited by MUC1 60-mer. No binding to the CaCo2 cell line was observed. Binding of the MUC1-specific antibodies to cells can be competed-off with MUC1 peptide. This study indicates that there is a difference in the spread and/or density of the various MUC1 epitopes or a differential accessibility of these epitopes because of residual glycosylation. To understand the abundance of the PH1-IgG1 MUC1-epitope, we considered it necessary to carry out immunohistochemical analysis on a large set of tissues and tumors, as discussed in the next paragraph.

Immunohistochemical Analysis of PH1-IgG1

We performed immunohistochemical analysis on a large set of tissues and tumors (Table 1, Figure 4). Tumors predominantly showed cytoplasmic staining (depolarized staining), often together with abundant membranous staining of the whole-cell membrane (membranous) and almost no apical (polarized) staining. In contrast, normal tissues predominantly demonstrate apical staining and rarely cytoplasmic staining or whole-cell membrane staining (Figure 3). Staining intensity was higher in tumor tissues than in normal tissues (see Figure 4).

Normal bladder was negative in all cases tested. Tumor tissues of the bladder displayed different staining patterns in which both adenocarcinoma tissues had a depolarized staining pattern. In colon cancer, normal tissues were negative; the mucinous tumor tested showed a cytoplasmic staining pattern. In the endometrium some normal glands also demonstrate a depolarized (membranous) localization. In the normal kidney the staining pattern was always the same with no staining in glomeruli and proximal tubes, focal apical staining in distal tubes, and diffuse apical staining in collecting ducts. In contrast with normal lung (negative), adenocarcinomas of the lung were intensively MUC1-positive in a depolarized (membranous) manner. In most tumors an extensive staining of the whole-cell membrane was found. Not all tumor cells were reacting with the antibody (focal staining). In breast and ovarian adenocarcinoma tissues there was a differential staining between normal and adenocarcinoma, being polarized in normal and cytoplasmic with membranous staining in adenocarcinoma (six of six for breast, four of seven for ovarian adenocarcinoma). Intensity of staining was less in normal tissue than in tumor tissue. The reactivity was diffuse to focal in tumor tissues and focal in normal tissues. Pancreas adenocarcinoma showed a cytoplasmic staining pattern, normal acini, however, expressed MUC1 in an apical or cytoplasmic pattern. In normal tissues of the endometrium and sebaceous gland of the skin, depolarized membranous expression was noticed. The only normal tissue with membranous staining is the sebaceous gland; the expression of membranous MUC1 is described to be restricted to mature sebocytes.¹⁹

Taken together, a differential expression between normal tissue and tumor was found in bladder, lung, breast, ovary, pancreas, parathyroid, and prostate. Apical stain-

Figure 3. Flow cytometry. Comparison between HMFG1 and PH1-IgG1 for their binding characteristics to tumor cell lines. **Thick line**, binding with the antibody; **thin line**, nonspecific binding control; **dotted line**, inhibition with MUC1 60-mer.

ing was detected in normal tissues; depolarized cellular staining is most frequently observed in tumors and aberrant staining of the whole-cell membrane is only demonstrated in tumors with the exception of the sebaceous glands of the skin.

Comparison with HMFG1 for a limited number of tissues is shown in Table 2. Normal tissues were stained mainly focally apical, except for endometrium tissue that was stained cytoplasmic with PH1-IgG1. The binding pattern was similar but not identical; for example, in tumor tissues, diffuse staining was observed in five ovarian and breast cancer tumors with HMFG1 and three cases with PH-IgG1; cytoplasmic staining was detected in three tumors stained with HMFG1 and six cases with PH-IgG1 staining; membranous staining of tumors was noticed in four HMFG cases and in five PH-IgG1 cases.

Evaluation of Internalization of PH1-IgG1

To analyze the extent with which PH1-IgG1 after binding would be internalized; we performed an internalization study using FITC-labeled antibody in confocal microscopy. The FITC-labeled antibody bound in fluorescence-activated cell sorting analysis to the OVCAR-3 and ETA cell lines, and not to the negative 3T3 cell line (data not shown). After incubation on ice with the human antibody PH1-IgG1, membranous binding was observed on the MUC1-expressing OVCAR-3 and ETA cell lines (Figure 5A). As in flow cytometry, the intensity of staining was more pronounced for the ETA cell line as compared with the OVCAR-3 cell line. No autofluorescence was observed, and no fluorescence was visible on the negative control, the CaCo2 cell line. At 37°C, the internalization of

Table 1. Immunohistological Staining of Normal and Tumor Epithelial Tissues with PH1-IgG1

Tissue	Normal tissues			Tumor tissues		
	Reactivity	Localization	Frequency	Reactivity	Localization	Frequency
Bladder	—	—	2/2	s	a	1/4
				f	a	1/4
				f	a, c	1/4
				d	c, m	1/4
Colon	—	—	3/3	f	a, c	1/1
Endometrium	f	a	2/6	f	a, c, m	1/1
	f	c	1/6			
Epididymis	d, f	a, c	2/6	f	a, c, m	1/1
	f	a	3/3			
Kidney						
glomeruli	—	—	5/5			
prox. tub.	—	—	5/5			
dist. tub.	f	a	5/5			
coll. ducts	d	a	5/5			
Liver	—	—	3/3	—	—	1/1
bile duct	s	a	1/1			
Lung	—	—	6/6	f	c, m	2/5
				f	a, m	1/5
				f	a, c, m	1/5
				f	a	1/5
Breast	f	a	4/5	f	a, c, m	3/7
				d	a, c, m	2/7
				d	a, m	1/7
				f	a	1/7
Ovarian	f	a	2/2	d	c, m	2/8
				f	a, c, m	1/8
				d	a, c, m	1/8
				f	c	2/8
				d	c	1/8
				f	a	1/8
Pancreas	d	a (c)	5/5	f	a, c	1/2
				d	a, m, c	1/2
acini	—	—	5/5			
isl.	—	—	5/5			
Langerhans						
Parathyroid	—	—	3/3	f	a, c	1/2
Prostate	—	—	5/6	f	c	1/2
	s	a	1/6	—	—	1/3
				d	a, c	1/3
Salivary gland				d	c, m	1/3
ducti	d-f	a-c	2/2			
acini	f	a	2/2			
Skin						
sebaceous gland	d	m	2/2			
	f	a				
sweat gland	—	—	2/2			
hair follicle	—	—	2/2			
Testes	—	—	3/3	—	—	1/1
Tuba	f	a	2/2			
Thyroid gland	—	—	2/2	—	—	1/1
Spermatic duct	f	a	1/1			

s, sporadic staining (<10%); f, focal staining (10 < s < 80%); d, diffuse staining (>80%); a, polarized apical; c, depolarized cytoplasmic; m, abundantly present on whole-cell membrane.

the PH1-IgG1-FITC became visible for both the ETA cells and the OVCAR-3 cells. After 1 hour, more than 50% of OVCAR-3 cells revealed internalized antibody in vesicles (Figure 5B), whereas the ETA cells kept mainly membrane-bound antibody. After 3 hours of incubation, the OVCAR-3 cells internalized more than 80% of the FITC-labeled antibody: vesicles were visible but also cells with a low level of intracellular fluorescence, were noticeable

(Figure 5D). After 6 hours, all OVCAR-3 cells had internalized the antibody, and most cells lost the vesicle internalization pattern but exhibited a low cytoplasmic fluorescence only. At either 3 or 6 hours, OVCAR-3 cells kept on ice had the antibody still bound to the membrane only (Figure 5C). The ETA cells had internalized less than 3% of the antibody after 3 hours, but after overnight incubation, we observed that the surviving cells had in-

Figure 4. Immunohistochemical staining with PH1-IgG1 of cancer and normal tissues. P, Proximal tubuli; D, distal tubuli; LH, loop of Henle.

Table 2. Comparison in Immunohistochemistry between Human PH1-IgG1 and the Mouse HMFG1

	HMFG1		PH1		Frequency
	Distribution	Localization	Distribution	Localization	
Bladder (N)	—	—	—	—	1
Breast (N)	f	a	f	a	3
Breast (T)	d	a	f	a, c	1
Breast papiloma	—	—	f	a	1
Breast (T)	f	m	d	a, c, m	1
Breast (T)	d	m, c	f	a	1
Liver	—	—	—	—	1
Parathyroid (T)	f	a	d	a, m	1
Tuba (N)	f	a	f	a	1
Endometrium (N)	f	a	f	c	1
Ovary (T)	f	c, m	f	c, m	1
Ovarian (T)	d	a	f	a, c, m	1
Ovarian (N)	—	—	—	—	1
Ovarian (T)	d	a	f	a, c	1
Ovarian (T)	f	a	f	a	1
Ovarian (T)	d	c, m	d	c, m	2

T, Tumor tissue; N, normal tissue; a, polarized apical; c, depolarized cytoplasmic; m, abundantly present on whole-cell membrane; s, sporadic staining (<10%); f, focal staining (10 < s < 80%); d, diffuse staining (>80%).

ternalized the antibody and no membrane-bound antibody was left; in contrast cells kept overnight on ice showed membranous staining.

Discussion

We succeeded in producing a fully human anti-MUC1 IgG1 antibody, by recloning the variable regions of the MUC1-specific phage Fab antibody PH1 for expression in mammalian cell expression vector. This is one of the first detailed studies of a fully human antibody directed

against a tumor-associated antigen, and which originates from an *in vitro*-selected phage antibody. For immunotherapy, it is important to know the characteristics of an antibody to choose a therapy that fits the antibody or visa versa. Indeed, differences in affinity or epitope recognition are responsible for differences between MUC1 antibodies in immunohistochemical staining and in internalization behavior.^{20–22}

First, the affinity of the antibody is a major determining factor in establishing how fast it will bind to a tumor cell and how quickly it will release from the antigen-bearing tumor cell. We compared the apparent affinity of the newly generated bivalent antibody with the affinity of the original monovalent Fab in BIAcore. The apparent affinity of the PH1-IgG1 increased 160 times when compared with the PH1-Fab (Figure 2). This is solely because of the change from one to two binding sites (avidity), because binding was comparable on the 60-mer and 15-mer peptide for both Fab and IgG. Comparison between diabodies obtained from scFvs to ErB2 with different affinities showed that the magnitude of the increase in the apparent affinity constant for the bivalent molecule was inversely proportional to the affinities of the scFvs.²³ In keeping with this observation, our Fab antibody has a low affinity and the improvement of apparent affinity for the IgG molecule is subsequently very high, confirming the above observation from Nielsen and colleagues.²³

In flow cytometric analysis we compared PH1-IgG1 with HMFG1 that is reported to recognize a different glycosylation-sensitive epitope.^{20,24} The binding pattern on tumor cell lines did not differ significantly between both antibodies except for the OVCAR-3 cell line, which was stained less by HMFG1 probably because of the different epitope recognition. On colon cancer cell lines, both antibodies hardly showed any binding. The MUC1 on colon cancer cells is highly glycosylated and therefore glycosylation-sensitive antibodies rarely stain colon cells or colon tumors.^{25,26} This suggests that our antibody PH1-IgG1 recognizes MUC1 in a differential glycosyla-

Figure 5. Confocal microscope pictures for internalization studies of PH1-IgG1-FITC in OVCAR-3 cells. **A:** Zero hours after incubation with antibody. **B:** After 1 hour, at 37°C. **C:** After 3 hours, at 4°C. **D:** After 3 hours, at 37°C, showing the feinting of the FITC signal.

tion form, which is expected to be tumor associated. The antibodies did bind well to the T47D breast cancer cell line known to express different glycoforms of MUC1.²⁷

Immunohistochemical staining revealed a differential staining between tumor tissues and normal tissues, the staining being apical or absent in normal tissues and depolarized in tumor tissues as described for glycosylation-sensitive antibodies.^{6,20} Cao and colleagues²⁰ showed that the absence of reactivity to the colon/small intestine and apical reactivity with breast, as observed with our antibody, is mostly because of glycans that mask the conformational epitope of MUC1. The only normal tissue with membranous staining was the sebaceous gland; the expression of membranous MUC1 is described to be restricted to mature sebocytes.¹⁹ The antibody binds specifically and preferentially to differentially glycosylated MUC1 in immunohistochemistry. Thus, although the epitope of the PH1 antibody may fall outside the amino acids that are putatively glycosylated, its binding may still be affected because of steric hindrance or carbohydrate-induced conformational changes of the epitope within the tandem repeat region, as described by Spencer and colleagues²⁸ for the antibody C595 that binds to the RPAP epitope.

The differential staining between normal tissue and adenocarcinoma was found in bladder, lung, breast, ovary, pancreas, parathyroid, and prostate. Consequently, the corresponding cancers may be putative candidates for immunotherapy with a suitable format of the PH1 antibody. Despite the apical expression of MUC1 in normal tissues, glycosylation-sensitive murine antibodies localize MUC1-expressing tumors in radioimmunoscintigraphy in humans also when injected intravenously.^{29–32} Limiting factors in such targeting studies are serum MUC1 binding and human anti-mouse antibody responses.^{33,34} Because of the absence of staining with our antibody in for example, bladder tissues, local injection of antibody could display a higher tumor/normal tissue ratio than obtained with the known mouse monoclonals.³³ Summarized, the staining pattern of the PH1 epitope is only moderately different from staining patterns of other glycosylation-sensitive antibodies, it thus can be expected that the PH1-IgG1 antibody will specifically bind to tumor *in vivo*.⁶ As for other MUC1 antibodies, a heterogeneous (focal) expression of the MUC1 epitope in tumor tissue was observed, as well as variability in the level of membranous MUC1 as revealed by the PH1 immunohistochemistry. Therefore, it could be beneficial to use a form of immunotherapy that has a bystander effect on surrounding tumor cells, eg, radioimmunotherapy, the combination of radioimmunotherapy and immunotoxins, or the usage of fusion proteins stimulating tumor-infiltrating lymphocytes.^{35,36}

Internalization studies demonstrated that both OVCAR-3 and ETA cells internalize the FITC-labeled antibody, although with a different rate. First, the internalization pattern was almost exclusively in vesicles; later the vesicle structure was less abundant and faint staining was found in the cytoplasm. The same observation was made by Calafat and colleagues³⁷ using a mucin-specific antibody resin. In this study the antibody resin was

found back in the cytosol after leaving the vesicles. It has been described that the MUC1 antigen recycles 0.9% of surface fraction/minute; this study confirms our observation that after 1 hour more than 50% of the cells have internalized the antibody.³⁸ Internalization of MUC1 antibodies is not always the same and may depend on the epitope. Pietersz and colleagues²² compared two antibodies for their internalization rate: for the antibody CTMO1 (epitope RPAP), after 1 hour 70% of the bound antibody was internalized; this in contrast with the antibody BC2 (epitope APDTR), where only 6% internalization was observed. We have demonstrated that 50% of the PH1-IgG1 antibody (epitope PAP) internalizes into vesicles after 1 hour. Our observation confirms Pietersz and colleagues²² suggestion that internalization of MUC1 antibodies is influenced by the epitope specificity; from our data it is clear that in particular antibodies recognizing the (R)PAP epitope are efficiently internalized. The MUC1-transfected 3T3 cell line ETA, internalized the FITC-labeled PH1-IgG1 much more slowly. At first sight this could be because of the fact that mouse cells normally do not express human MUC1 and that the internalization machinery is not effective for a xenogenic protein. Some transfected cell lines may internalize better, others used the MUC1-transfected 3T3 cell line MOR5-CF2 for their internalization studies.²² To aid in increasing the efficacy of antibodies to kill solid tumors, many different effectors have been tried, sometimes with major success.³⁹ For example, internalizing antibodies are suitable for immunotherapy when conjugated to prodrugs or drugs and possibly for gene therapy and so forth.⁴⁰ Although for radioimmunotherapy it is not strictly necessary that a radiolabel internalizes, this process may also positively influence tumor cell killing. After internalization of the radioactively labeled antibody there is a retention of the radiolabel inside the cells, even after degradation of the antibody, which makes the tumor targeting more efficient in radioimmunoscintigraphy as well as in radioimmunotherapy.^{22,41,42} Alternatively, provided the antibody PH1 does not bind to serum MUC1 (studies ongoing), the MUC1-binding site may be combined with immunomodulatory molecules (immunocytokines) or made into a bispecific antibody for retargeting T cells. As before, internalization of the antibody-antigen complex may aid in the efficacy of the recombinant drug. For example, immunocytokines and diabodies against the internalizing transferrin receptor have an anti-tumor effect.⁴³ Indeed, we have recently made a bispecific diabody consisting of PH1 combined with an anti-CD3 molecule; this bispecific antibody kills MUC1-expressing tumor cells efficiently *in vitro* (C. Vos, Department of Gynecology, Maastricht University, Maastricht, The Netherlands, P. Henderikx and H.R. Hooganboom, Dyax s.a., Liège, Belgium, F. Snijdwint, University Hospital, Vrije Universiteit, Amsterdam, The Netherlands, R. Hoet, Dyax s.a., Liège, Belgium, unpublished results).

In conclusion, the human antibody PH1-IgG1 recognizes tumor-associated MUC1 in adenocarcinoma. It avidly binds to tumor cells and it is internalized, and therefore could be useful for tumor-targeting applications including radioimmunotherapy, radioimmunoscintigraphy

phy, conjugation to cytotoxic reagents, immune cell stimulation, and so forth, and this particularly in lung, bladder, ovarian, prostate, and breast adenocarcinoma.

Acknowledgments

We thank Dr. Silvia von Mensdorff-Pouilly for providing us with the MUC1 60-mer peptide, Drs. Dennis Rupa and Adriaan de Bruïne for their advice on the immunohistochemistry, Dr. Jos Broers for help with confocal microscopy, and Dr. Mark Vanhove for his assistance.

References

- Grillo-Lopez AJ, White CA, Varns C, Shen D, Wei A, McClure A, Dallaire BK: Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma. *Semin Oncol* 1999, 26:66–73
- Riethmuller G, Holz E, Schlimok G, Schmiegel W, Raab R, Hoffken K, Gruber R, Funke I, Pichlmaier H, Hirche H, Buggisch P, Witte J, Pichlmayr R: Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol* 1998, 16:1788–1794
- Baselga J: Clinical trials of Herceptin (trastuzumab). *Eur J Cancer* 2001, 37(Suppl 1):S18–S24
- Gendler S, Taylor-Papadimitriou J, Duhig T, Rothbard J, Burchell J: A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. *J Biol Chem* 1988, 263:12820–12823
- Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M: MUC1 and cancer. *Biochim Biophys Acta* 1999, 1455:301–313
- Zotter S, Hageman P, Lossnitzer A, Mooi WJ, Hilgers J: Tissue and tumor distribution of human polymorphic epithelial mucin. *Cancer Res* 1988, 48:11–12:56–101
- Hoogenboom HR, Marks JD, Griffiths AD, Winter G: Building antibodies from their genes. *Immunol Rev* 1992, 130:41–68
- de Haard H, Henderikx P, Hoogenboom HR: Creating and engineering human antibodies for immunotherapy. *Adv Drug Deliv Rev* 1998, 31:5–31
- Jerne NK: Towards a network theory of the immune system. *Ann Immunol (Paris)* 1974, 125:373–389
- Mittelman A, Chen GZ, Wong GY, Liu C, Hirai S, Ferrone S: Human high molecular weight-melanoma associated antigen mimicry by mouse anti-idiotypic monoclonal antibody MK2–23: modulation of the immunogenicity in patients with malignant melanoma. *Clin Cancer Res* 1995, 1:705–713
- Kosmas C, Epenetos AA, Courtenay-Luck NS: Activation of cellular immunity after intracavitary monoclonal antibody therapy of ovarian cancer. *Cancer* 1994, 73:3000–3010
- de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderikx P, de Bruïne AP, Arends JW, Hoogenboom HR: A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J Biol Chem* 1999, 274:18218–18230
- Persic L, Roberts A, Wilton J, Cattaneo A, Bradbury A, Hoogenboom HR: An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. *Gene* 1997, 187:9–18
- Nicholson S, Gooden CS, Hird V, Maraveyas A, Mason P, Lambert HE, Meares CF, Epenetos AA: Radioimmunotherapy after chemotherapy compared to chemotherapy alone in the treatment of advanced ovarian cancer: a matched analysis. *Oncol Rep* 1998, 5:223–226
- Henderikx P, Kandilogiannaki M, Petrarca C, von Mensdorff-Pouilly S, Hilgers JH, Krambovitis E, Arends JW, Hoogenboom HR: Human single-chain Fv antibodies to MUC1 core peptide selected from phage display libraries recognize unique epitopes and predominantly bind adenocarcinoma. *Cancer Res* 1998, 58:4324–4332
- Laemmli UK, Beguin F, Gujer-Kellenberger G: A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J Mol Biol* 1970, 47:69–85
- von Mensdorff-Pouilly S, Gourevitch M, Kenemans P, Verstraeten A, van Kamp G, Kok A, van Uffelen K, Snijdwint F, Paul M, Meijer S, Hilgers J: An enzyme-linked immunosorbent assay for the measurement of circulating antibodies to polymorphic epithelial mucin (MUC1). *Tumor Biol* 1998, 19:186–195
- Acres RB, Hareuveni M, Balloul JM, Kieny MP: Vaccinia virus MUC1 immunization of mice: immune response and protection against the growth of murine tumors bearing the MUC1 antigen. *J Immunother* 1993, 14:136–143
- Metze D, Soyer HP, Zelger B, Neumaier M, Grunert F, Hartig C, Amann U, Bhardwaj R, Wagener C, Luger T: Expression of a glycoprotein of the carcinoembryonic antigen family in normal and neoplastic sebaceous glands. Limited role of carcinoembryonic antigen as a sweat gland marker. *J Am Acad Dermatol* 1996, 34:735–744
- Cao Y, Karsten U, Hilgers J: Immunohistochemical characterization of a panel of 56 antibodies with normal human small intestine, colon, and breast tissues. *Tumour Biol* 1998, 19(Suppl 1):S88–S99
- Baldus SE, Goergen D, Hanisch F, Dienes HP: Epitope-dependent differential immunoreactivities of anti-MUC1 monoclonal antibodies in human carcinomas. *Int J Oncol* 2001, 18:507–512
- Pietersz GA, Wenjun L, Krauer K, Baker T, Wreschner D, McKenzie IF: Comparison of the biological properties of two anti-mucin-1 antibodies prepared for imaging and therapy. *Cancer Immunol Immunother* 1997, 44:323–328
- Nielsen UB, Adams GP, Weiner LM, Marks JD: Targeting of bivalent anti-ErbB2 diabody antibody fragments to tumor cells is independent of the intrinsic antibody affinity. *Cancer Res* 2000, 60:6434–6440
- Burchell J, Taylor-Papadimitriou J: Effect of modification of carbohydrate side chains on the reactivity of antibodies with core-protein epitopes of the MUC1 gene product. *Epithelial Cell Biol* 1993, 2:155–162
- Sikut R, Sikut A, Zhang K, Baekstrom D, Hansson GC: Reactivity of antibodies with highly glycosylated MUC1 mucins from colon carcinoma cells and bile. *Tumour Biol* 1998, 19(Suppl 1):S122–S126
- Blockzijl A, Nilsson K, Nilsson O: Epitope characterization of MUC1 antibodies. *Tumour Biol* 1998, 19(Suppl 1):S46–S56
- Hanisch FG, Stadie TR, Deutzmann F, Peter-Katalinic J: MUC1 glycoforms in breast cancer—cell line T47D as a model for carcinoma-associated alterations of O-glycosylation. *Eur J Biochem* 1996, 236:318–327
- Spencer DI, Missailidis S, Denton G, Murray A, Brady K, Matteis CI, Searle MS, Tendler SJ, Price MR: Structure/activity studies of the anti-MUC1 monoclonal antibody C95 and synthetic MUC1 mucin-core-related peptides and glycopeptides. *Biospectroscopy* 1999, 5:79–91
- van Hof AC, Molthoff CF, Davies Q, Perkins AC, Verheijen RH, Kenemans P, den Hollander W, Wilhelm AJ, Baker TS, Sopwith M, Frier M, Symonds EM, Roos JC: Biodistribution of (111)indium-labeled engineered human antibody CTMO1 in ovarian cancer patients: influence of protein dose. *Cancer Res* 1996, 56:5179–5185
- Rosner D, Nabi H, Wild L, Ortman-Nabi J, Hreshchysyn MM: Diagnosis of breast carcinoma with radiolabeled monoclonal antibodies (MoAbs) to carcinoembryonic antigen (CEA) and human milk fat globulin (HMFG). *Cancer Invest* 1995, 13:573–582
- Biassoni L, Granowska M, Carroll MJ, Mather SJ, Howell R, Ellison D, MacNeill FA, Wells CA, Carpenter R, Britton KE: 99mTc-labelled SM3 in the preoperative evaluation of axillary lymph nodes and primary breast cancer with change detection statistical processing as an aid to tumour detection. *Br J Cancer* 1998, 77:131–138
- Kramer EL, Liebes L, Wasserheit C, Noz M, Blank EW, Zabalegui A, Melamed J, Furmanski P, Peterson JA, Ceriani RL: Initial clinical evaluation of radiolabeled MX-DTPA humanized BrE-3 antibody in patients with advanced breast cancer. *Clin Cancer Res* 1998, 4:1679–1688
- van Hof AC, Molthoff CF, Davies Q, Perkins AC, Verheijen RH, Kenemans P, den Hollander W, Wilhelm AJ, Baker TS, Sopwith M, Frier M, Symonds EM: Biodistribution of (111)indium-labeled engineered human antibody CTMO1 in ovarian cancer patients: influence of protein dose. *Cancer Res* 1996, 56:5179–5185
- DeNardo SJ, Kramer EL, O'Donnell RT, Richman CM, Salako QA, Shen S, Noz M, Glenn SD, Ceriani RL, DeNardo GL: Radioimmuno-

- therapy for breast cancer using indium-111/yttrium-90 BrE-3: results of a phase I clinical trial. *J Nucl Med* 1997, 38:1180–1185
35. Wei BR, Ghetie MA, Vitetta ES: The combined use of an immunotoxin and a radioimmunoconjugate to treat disseminated human B-cell lymphoma in immunodeficient mice. *Clin Cancer Res* 2000, 6:631–642
36. Lode HN, Xiang R, Becker JC, Gillies SD, Reisfeld RA: Immunocytokines: a promising approach to cancer immunotherapy. *Pharmacol Ther* 1998, 80:277–292
37. Calafat J, Molthoff C, Janssen H, Hilken J: Endocytosis and intracellular routing of an antibody-ricin A chain conjugate. *Cancer Res* 1988, 48:3822–3827
38. Litvinov SV, Hilken J: The epithelial sialomucin, episialin, is sialylated during recycling. *J Biol Chem* 1993, 268:21364–21371
39. Reff ME, Heard C: A review of modifications to recombinant antibodies: attempt to increase efficacy in oncology applications. *Crit Rev Oncol Hematol* 2001, 40:25–35
40. Syrigos KN, Deonarian DP, Epenetos AA: Use of monoclonal antibodies for the diagnosis and treatment of bladder cancer. *Hybridoma* 1999, 18:219–224
41. Kwa HB, Wesseling J, Verhoeven AH, van Zandwijk N, Hilken J: Immunoscintigraphy of small-cell lung cancer xenografts with anti neural cell adhesion molecule monoclonal antibody, 123C3: improvement of tumour uptake by internalisation. *Br J Cancer* 1996, 73:439–446
42. Press OW, Shan D, Howell-Clark J, Eary J, Appelbaum FR, Matthews D, King DJ, Haines AM, Hamann P, Hinman L, Shochat D, Bernstein ID: Comparative metabolism and retention of iodine-125, yttrium-90, and indium-111 radioimmunoconjugates by cancer cells. *Cancer Res* 1996, 56:2123–2129
43. Dreier T, Lode HN, Xiang R, Dolman CS, Reisfeld RA, Kang AS: Recombinant immunocytokines targeting the mouse transferrin receptor: construction and biological activities. *Bioconjug Chem* 1998, 9:482–489