4 Materials and Methods

4.1 General materials

4.1.1 Antigens

Glycophorin and Asialoglycophorin from human blood type MN (Sigma-Aldrich, Steinheim, Germany). The glycosylation of glycophorin A has been thoroughly investigated. Glycophorin A contains 15 *O*-glycosylations and a single *N*-glycosylation (Tomita & Marchesi, 1975). The majority of the *O*-glycosylations are TF structures masked with sialic acid. Most frequently, TF is disialylated (78% - see **Figure 1**), but also monosialylated TF (17%) and trisialylated TF (5%) are found (Fukuda et al., 1987). Upon treatment with neuraminidase, all the sialic acids may be cleaved off resulting in the exposure of up to 15 TF disaccharides on asialoglycophorin.

PAA conjugates: Oligosaccharides conjugated to poly[N-(2-hydroxyethyl)acrylamide] (Lectinity Holdings, Inc, www.lectinity.com)

BSA: Albumin Fraktion V, Bovine (Carl Roth GmbH & Co., Karlsruhe, Germany)

4.1.2 Phage

Griffin.1, TomlinsonI and TomlinsonJ scFv libraries from the MRC, Cambridge Centre for Protein Engineering, Cambridge England were kindly provided by Prof. Greg Winter and Dr. Ian Tomlinson, respectively.

KM13 Helper phage (Kristensen & Winter, 1998) – supplied by Peter Kristensen and propagated as described in the MRC phage display protocols

4.1.3 Bacteria

E. coli TG1: Suppressor strain was used for cloning and for amplification of phage between rounds of panning (Supplied by MRC, Cambridge, UK)

E. coli BL21(DE3)pLysS and Rosetta™(DE3)pLysS (Novagen): Used for expression of protein from the pET11a expression vector (Novagen).

4.1.4 Cell lines

KG-1: Homo sapiens, bone marrow, acute myelogenous leukaemia

K-562:	Homo sapiens, bone marrow, chronic myelogenous leukaemia
NM-D4, NM-F9:	Derived from K-562 by selection for TF
ZR-75-1:	Homo sapiens, asctes ductal carcinoma (adherent)
ZR-75-1(Nemod):	Homo sapiens, asctes ductal carcinoma (non-adherent)

4.1.5 Vectors

Schematic representations of pRI(pHEN2) and the modified pET11a are shown in **Figure 50**. pRI arose from pHEN2. The difference is the addition of the two blunt end restriction enzyme sites, SrfI and PmeI. The sites enable blunt-end insertion of cDNA libraries, but as this application of the vector has not been described in this thesis, the vector has been cited as pHEN2 to honour Hennie Hoogenboom. The pET11a was modified to include the PelB leader, the restriction sites for cloning variable genes, and the His-tag and Myc-tag for protein purification and detection. Modifications were introduced applying NdeI and EcoRI. Several factors ensure a very tight control of expression with the pET11a vector. Supplementing the media with 1% glucose inhibits the production of cAMP that mediate derepression of lacUV5 at the stationary growth phase. The lac repressor (lacI) further blocks transcription. Finally, the bacteria may express T7 lysozyme that is an inhibitor of the T7 polymerase.

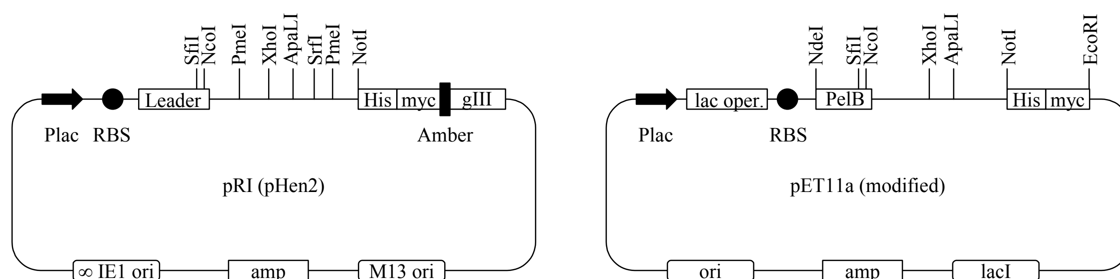

Figure 50 Vector maps
Schematic representation of vectors for phage display and protein expression. Variable genes were inserted using NcoI and XhoI (V_H) and ApaLI and NotI (V_L). See main text for further information.

4.1.6 Primers

All primers were synthesised according to the degenerate primer sequences of Zhou and co-workers (Zhou et al., 1994), but adapting the restriction site sequences in pHEN2. Primer mixtures were always 10 µM.

Primers used for construction of scFv repertoires with 1aa linkers are listed in **Table 11**.

Table 11 Primers used for the generation of scFv repertoires with 1aa linkers

MKVB1(1aa)	GGGCCCGAAGACAAAGCG**gatgttttgatgacccaaact**
MKVB2(1aa)	GGGCCCGAAGACAAAGCG**gatattgtgatgacgcaggct**
MKVB3(1aa)	GGGCCCGAAGACAAAGCG**gatattgtgataacccag**
MKVB4(1aa)	GGGCCCGAAGACAAAGCG**gacattgtgctgacccaatct**
MKVB5(1aa)	GGGCCCGAAGACAAAGCG**gacattgtgatgacccagtct**
MKVB6(1aa)	GGGCCCGAAGACAAAGCG**gatattgtgctaactcagtct**
MKVB7(1aa)	GGGCCCGAAGACAAAGCG**gatatccagatgacacagact**
MKVB8(1aa)	GGGCCCGAAGACAAAGCG**gacatccagctgactcagtct**
MKVB9(1aa)	GGGCCCGAAGACAAAGCG**caaattgttctcacccagtct**
MLVB1(1aa)	GGGCCCGAAGACAAAGCG**caggctgttgtgactcaggaa**
MHVF1(1aa)	GGGCCCGAAGACAACGC***T*****gcagagacagtgaccagagt**
MHVF2(1aa)	GGGCCCGAAGACAACGC***Tgaggagactgtgagagtggt***
MHVF3(1aa)	GGGCCCGAAGACAACGC***Tgaggagacggtgactgaggt***
MHVF4(1aa)	GGGCCCGAAGACAACGC***Tgaggagacggtgaccgtggt***

Primers used for the affinity maturation strategies have not been revealed to protect patent and IP's.

4.1.7 Antibodies

Anti-Human/HRP Goat anti-Human Ig HRP (Jackson Immunoresearch Lab. Inc, PA, USA)

Anti-Mouse/HRP: P 0260 Rabit Anti-mouse HRP, Ig (DAKO, Glostrup, Denmark)

Anti-M13/HRP: (Amersham Biosciences, Freiburg, Germany)

Anti-pIII/HRP: PSKAN3 (MoBiTec, Göttingen, Germany)

Anti-His/HRP: Tetra His^{TM} HRP conjugate (Qiagen GmbH, Hilden, Germany)

Anti-Myc: 9E10, Mouse Ascites Fluid (Sigma, Saint Louis, Missouri, USA)

9B11, (Cell Signaling Technology, Inc, Frankfurt am Main, Germany)

Anti-mouse IgG/HRP: Goat anti-Mouse IgG (Fcγ) HRP (Jackson Immunoresearch Lab. Inc, PA, USA)

Anti-Human IgG/HRP: Goat anti-Human IgG, $F(ab')_2$ HRP (Jackson Imm. Lab. Inc, PA, USA)

Anti-mouse IgG/POD: Goat anti-Mouse IgG (Fcγ) POD (Jackson Immunoresearch Lab. Inc, PA, USA)

Anti-Human IgG/POD: Goat anti-Human IgG, $F(ab')_2$ POD (Jackson Imm. Lab. Inc, PA, USA)

Anti-IgG Cy3: Goat anti-Mouse IgG (Fcγ) Cy3 (Jackson Immunoresearch Lab. Inc, PA, USA)

Anti-IgM Cy3: Goat anti-Mouse IgM (μ) Cy3 (Jackson Immunoresearch Lab. Inc, PA, USA)

4.2 Phage display protocols

For extended protocols for selection of phage display libraries see
http://ww.mrc-cpe.cam.ac.uk/g1p.php?menu=1808.
http://www.ghmp.mrc.ac.uk/geneservice/reagents/products/datasheets/scFv/tomlinsonIJ.pdf.

4.2.1 Phage amplification and precipitation

Libraries or monoclonals were prepared fresh from –80°C master stocks. Briefly for polyclonal stocks, 200 ml cultures of 2 × TY supplemented with amp (100 µg/ml) and 1% glucose were inoculated with master stock to obtain $OD_{600\ nm}$ ~ 0.05 and then incubated at 37 °C until $OD_{600\ nm}$ ~ 0.6 was reached. Cultures were superinfected with helper phage KM13 (MOI 20) at 37 °C for 30 min. Subsequently, the bacteria were harvested by centrifugation and resuspended in 200 ml 2 × TY supplemented with amp (100 µg/L) and kana (50 µg/L) and incubated overnight at 23 °C. The phage supernatants were cleared by centrifugation, and the phage precipitated with PEG/NaCl (3.5%vol and 425 mM, respectively) for 2 h at 4 °C, followed by centrifugation at 4600 rpm for 60 min. Precipitated phage particles were dissolved in PBS, and applied directly in selections or ELISA.
Phage was produced at 23°C due to the observation that display level and binding activity in general were better at lower temperatures. The highest display level was observed at 18°C, but this limits the overall production of phage particles due to lower growth rates.

4.2.2 Selection

Selections were carried out using approximately 5×10^{12} cfu, which were pre-incubated in 1% BSA-PBS for 30 min at RT.
Pannings were performed in immunotubes coated with antigen (10 µg/ml in PBS, overnight at 4 °C). Tubes were blocked with 2% BSA-PBS at RT for 1 h and rinsed trice with PBS before the incubation with the phage library for 1 h. Phage library were pre-incubated in 1% BSA-PBS (final concentration) before applying in pannings. After extensive washing (20 times PBS and 3 times with 50 mM Tris pH 8.0 1 mM $CaCl_2$) phage was eluted with trypsin (1 mg/ml, 50 mM TrisHCl, 1 mM $CaCl_2$, pH 8 for 30 min).
Biopannings were performed by incubating the biotinylated antigen with the pre-incubated phage in solution for one h, followed by a 10 min capture on BSA-PBS blocked streptavidin-conjugated magnetic beads. After washing 8 times with PBS, captured phage was eluted with trypsin in 200 µl (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$.

Finally, the eluted phage was incubated with TG1 in log phase to allow infection, and bacteria plated on 2 × TY supplemented with amp and glucose.

4.2.3 Phage screening

Monoclonal phage particles were screened for binding in ELISA as described by Marks (Marks et al., 1991). ELISA plates were coated overnight at 4°C with 100 µl antigen (10 µg/ml in PBS), and blocked with 400 µl 2% BSA-PBS for at least 60 min at RT. Fifty microliter 2% BSA-PBS and 50 µl phage supernatant were mixed in the well and incubated for 60 min. Plates were washed 6 times with PBS and bound phage detected with anti-M13/HRP diluted 1:2500 in 1% MPBS. Following another 6 washes with PBS, the ELISA was developed according to the standard ELISA protocol.

4.3 Cloning

4.3.1 Sequencing

Sequences were obtained as “Extended Hot Shot” from Sequence Laboratories (Göttingen, Germany).

4.3.2 Library construction form human donors

PBLs from the 10 patients were pooled ($\Sigma=2\times10^7$). Total RNA was extracted using the Qiagen RNAeasy Midi kit, eluted in 300 µl DEPC-treated water. Secondary structures of the RNA were destroyed by heating to 65 °C for 2 min, and the mRNA captured on oligo (dT)25 Dyna beads (Dynal) in a final volume of 600 µl mRNA binding buffer (10^8 beads; 10 mM Tris HCl; pH 7.5; 500 mM LiCl; 2 mM EDTA). After rotating 10 min, the beads were collected and washed twice with 1 ml mRNA washing buffer (10 mM Tris HCl; pH 7.5; 150 mM LiCl; 1 mM EDTA) at room temperature and twice with 1 ml ice-cold DEPC-treated water. The captured mRNA was reverse transcribed to ss-cDNA using the oligo (dT)25 beads as primer and the Omniscript Reverse Transcriptase (16 units, 0.5 mM dNTPs and 40 units RNase inhibitor in a total volume of 80 µl) by incubation at 37 °C for 2 h.
Variable heavy-chain genes were amplified by PCR using V_H-For primer mix and V_H-Back primer mix and all the ss-cDNA coupled beads as template in a total volume of 400 µl. Subsequently, the cDNA coupled beads were retrieved, washed in TE, and applied as template in the PCR amplification of the variable light-chain genes using the V_L-For primer mix and V_L-Back primer mix.
The amplified genes were purified using the Qiagen PCR purification kit, and digested overnight with the various restriction enzymes needed for cloning into pHEN2 (refer **Figure 50**). Purified V_L inserts

were ligated into pHEN2 to yield a V_L-library, from which new plasmid was retrieved and used as vector for cloning the V_H inserts, resulting in a scFv library.

4.3.3 Construction of scFv libraries from immunised mice

Mice were immunised with asialoglycophorin, and spleen cells harvested 4 days after boost. Cultivation of spleen cells was done in RPMI 1640 supplemented with 10% FCS and 5% HCF (Origen Hybridoma Cloning Factor, TEBU Frankfurt/M). Total RNA was extracted from ~10^7 cells using the Qiagen RNAeasy Midi kit, and a cDNA library constructed as described above.

Variable heavy chain genes were amplified by PCR using V_H-For(1aa) primer mix and V_H-Back primer mix and all the ss-cDNA coupled beads as template in a total volume of 400 µl. Subsequently, the cDNA coupled beads were retrieved, washed in TE, and applied as template in the PCR amplification of the variable light chain genes using the V_L-For primer mix and V_L-Back(1aa) primer mix.

The amplified genes were purified using the Qiagen PCR purification kit, and digested overnight with BbsI. The digests were separated on a 2% agarose gel, and the desired DNA fragments excised and extracted. V_H and V_L genes were joined by ligation (T4 DNA ligase (NEB)) and amplified by PCR using the V_H Back and V_L For primers. Finally, the PCR product was purified and cloned into pHEN2 using NcoI and NotI, resulting in repertoires of average size 5×10^6.

Libraries in the scFv(18aa) format were cloned into pHEN2 using standard procedures as described above.

4.3.4 Construction of secondary libraries

Insert for the affinity maturation libraries were generated by SOE-PCR as outlined in **Figure 29**. The inserts were digested with NcoI and NotI and ligated into pHEN2 using T4 DNA ligase.

4.3.5 Construction of multimers

The scFv(0aa), scFv(2aa) and scFv(3aa) constructs were generated by overlapping PCR extension using connecting primers, 0-aa: accacggtcaccgtctcctcagatatccagatgacacagact, 2aa: accacggtcaccgtctcctca**gccgcc**gatatccagatgacacagact, and 3aa: accacggtcaccgtctcctca**gcctcggcc**gatatccagatgacacagact, respectively. The PCR products were inserted in pET11a(PelB) with restrictions enzymes NcoI and NotI and electroporated into TG1. The scFv(18aa) construct was cloned using the standard restriction enzymes for cloning in pHEN2. The

constructs scFv(4aa) – scFv(9aa) were cloned by replacing the V_L and the linker of the scFv(18aa) construct. V_L was PCR amplified using a V_L-For primer in combination with primers 4aa: gcctcgagtgcagatatccagatgacacagact, 5aa: gcctcgagttctgcagatatccagatgacacagact, 6aa: gcctcgagttcatc tgcagatatccagatgacacagact, 7aa: gcctcgagtggctcatctgcagatatccagatgacacagact, 8aa: gcctcgagtggcggct catctgcagatatccagatgacacagact, 9aa: gcctcgagtggctcgggctcatctgcagatatccagatgacacagact, respectively. The PCR products were inserted into pET11a(scFv(18aa)) by cut and paste with XhoI and NotI and electroporated into TG1 for plasmid preparation.

4.4 Protein expression and purification

4.4.1 Expression and harvesting

Briefly, 50 µl electrocompetent *E. coli* Rosetta(DE3)pLysS were transformed with pET11a plasmid encoding a scFv, and an overnight culture in 2 × TY supplemented with amp, chloramphenicol and 1% glucose. The overnight culture was diluted 1:100 and incubated at 25°C in 2 ×TY further supplemented with 50 mM Sucrose. At $OD_{600\ nm}$ ~ 0.6 the culture was induced with 500 µM IPTG and incubated overnight at 25°C. Bacteria were harvested by centrifugation, and TES fractions prepared by resuspending the bacteria in icecold TES buffer (50 mM TrisHCl, 1 mM EDTA, 20% sucrose, pH 8, 1/20 culture volumen) and stirring at 4°C for 30 min. Subsequently, the same volume of ice-cold 5 mM $MgSO_4$ was added and incubated for 30 min. Finally, the TES fraction was cleared by centrifugation (4600 rpm for 2 h) and dialysed overnight against phosphate buffer “10 mM” (50 mM phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0).

4.4.2 Immobilised Metal Affinity Chromatography (IMAC)

The scFvs were purified from the TES fractions by IMAC applying an open column with 5 ml Chelating Sepharose™ Fast Flow (Amersham Biosciences) loaded with $NiSO_4$. After loading of the TES fractions by gravity flow, the column was washed with 50 ml phosphate buffer “**50 mM**” (50 mM phosphate, 300 mM NaCl, **50 mM imidazole**, pH 8.0) and 10 ml phosphate buffer “**70 mM**” (50 mM phosphate, 300 mM NaCl, **70 mM imidazole**, pH 8.0). The protein was eluted with phosphate buffer “**300 mM**” (50 mM phosphate, 300 mM NaCl, **300 mM imidazole**, pH 8.0), and dialysed against PBS overnight at 4°C.

4.4.3 Anion Exchange Chromatography (AEC)

The IMAC purified scFv(1aa) was diluted 10 fold in carbonate buffer to yield final concentrations of 50 mM Na_2CO_3, pH 8.7 and 50 mM NaCl. The protein was loaded on a 5 ml HiTrap Q HP column (Pharmacia) at a high flow-rate (5 ml/min). Column was washed with 15 ml (50 mM Na_2CO_3, pH 8.7, 50 mM NaCl) before eluting by increasing the NaCl concentration to 300 mM. Eluted protein was re-buffered into PBS using centricon spin-tubes (Amicon), and equilibrated to 500 µg/ml before it was stored at -80°C, or applied directly for conjugation of chelator (DTPA).

4.4.4 Affinity chromatography (AC)

A column was packed with 5 ml TF-sepharose (Lectinity, TF is coupled to sepharose via a flexible linker). The IMAC purified scFv(1aa) in PBS was loaded on the column at a moderate flow-rate (1 ml/min). After washing the column with 15 ml PBS, bound protein was recovered by basic elution (PBS, pH 11). The eluted protein was neutralised by addition of citric acid or by re-buffering into PBS using centricon spin-tubes.

4.4.5 Size exclusion chromatography

ScFv constructs were analysed on a custom made G-200 column. The column was calibrated with molecular-weight marker proteins and equilibrated in PBS (pH 7.0). Samples were run at a flow-rate of 0.1 ml/min.

4.5 Protein characterisation assays

4.5.1 ELISA

ELISA plates were coated overnight at 4°C with 100 µl antigen (10 µg/ml in PBS), and blocked with 400 µl 2% BSA-PBS for at least 60 min at RT.

The primary reagents (antibodies) were added in concentrations stated in the individual experiments, or as recommended by the manufactures. Secondary and tertiary reagents were applied as recommended by the manufactures.

Washing was performed between each incubation step by filling wells with ~400 µl PBS (submerging) and “slamming” it out.

ELISA's were developed by adding 100 µl TMB substrate (3´.3´.5´.5´.-tetramethylbenzidine in dimethyl sulfoxide) and incubating for 20 min. Reactions were quenched with 50 µl 2.5N H_2SO_4, and readings taken at 450 nm with 630 nm as control.

4.5.2 Western blots

Phage samples were run on 15% SDS-PAGE, and electroblotted on nitrocellulose membranes using a semi-dry blotting device (Biorad). Membranes were blocked overnight in 2% BSA-PBS at 4°C, and blots performed in 50 ml disposable tubes. Incubations were performed in 10 ml 1% BSA-PBS with antibodies 9E10 (Sigma), Tetra-His (Qiagen) or anti pIII (Mo Bi Tec) as recommended by the manufacturers. Secondary reagent was the anti-mouse/HRP conjugate, and the blots were developed using the chemiluminescence kit (SuperSignal, Pierce, USA).

4.5.3 Surface plasmon resonance studies

Measurements were performed on a BIAsensor machine (BIAcore2000, Pharmacia) in PBS (pH 7.0) with a flow rate of 5 µl/min at 25°C. Asialoglycophorin was immobilised covalently on the dextran matrix of a CM5 sensor chip using the standard amine immobilization procedure according to BIAcore protocols. A high surface density of asialoglycophorin (16800 RU) was immobilised. For analysis of the scFv constructs, sensorgrams at 5 different concentrations (28 µg/ml, 17 µg/ml, 11 µg/ml, 7 µg/ml and 4 µg/ml) were obtained. Dissociation was analysed by injection of PBS. After each measurement, the chip surface was regenerated with 2 volumns of 10 µl PBS (pH 11) followed by PBS (pH 7.0).

4.5.4 Immunocytology

Multitest slides (10 wells, Roth) were prepared from several cell lines. Cells were cultured on the slides in RPMI 1640 medium, supplemented with 10 % fetal calf serum, in an incubator with 8 % CO_2 for 24 h. Then the medium was carefully sucked away, and the slide was air-dried. At this stage, the slides were wrapped and stored at - 80 °C. After thawing, cells were briefly fixed with formalin (acid-free for histology, Merck, 5 % in PBS, 5 min). Incubation with the scFv(1aa) preparation (HiTrap-purified) at a concentration of 10 µg/ml in PBS was done for 90 min at 4 °C, followed by incubation with mouse Tetra-His antibody or mouse 9B11 antibody for 30 min, then with goat anti-mouse immunoglobulin (H+L) antibody, Cy3-labeled (Jackson/Dianova, 1:100) for 30 min, and finally briefly with DAPI (0.5 µg/ml, 1 min). Between incubations, wells were washed with PBS (3 times). Slides were mounted with a buffer containing 50 % glycerol and a trace of p-phenylenediamine as

anti-fading agent. They were examined with an Axioplan 2 microscope equipped with the digital camera AxioCam (Zeiss).

4.5.5 Immunohistology

The slides with cryo sections were provided by Dr. Kemner (MDC, Berlin, Germany) or by EPO GmbH, (Berlin, Germany) and stored at -80°C. After thawing, sections were briefly fixed with formalin (acid-free for histology, Merck, 5 % in PBS, 5 min). Endogenous POD was blocked by 3% H_2O_2 in PBS for 30 min, and Fc-receptors and other unspecific binding sites blocked with TF-absorbed rabbit serum for 30 min. The primary antibodies (L36, scFv(1aa), Chimeric Ab or A78-G/A7) were incubated for 60 min. Secondary antibodies (Goat anti-mouse IgG/POD, Goat anti-Human IgG/POD, Rabbit anti-mouse Ig/POD) were incubated for 30 min. After a final wash, colour development was achieved with the DAKO Liquid DAB+ SubstrateChromogen System (Dako, Denmark). Sections were counter stained with MAYERS Hämalaunlösung (Merck, Darmstadt, Germany) and finally embedded in Entallan (Merck, Darmstadt, Germany). Pictures were prepared with an Axioplan 2 microscope equipped with the digital camera AxioCam (Zeiss).

4.6 Radioactivity assays

4.6.1 Labelling

Protein that had been stored in a 300 mM acetate buffer, pH 4.3 at -80°C was thawed on ice. ^{111}In ($InCl_3$) was diluted to 40 µCi/µl with 50 mM HCl, and added directly to the protein. The labelling reactions was incubated at room temperature for ~2 h. Subsequently, the protein was diluted in PBS, and further washed with PBS using spin filtration.
Finally, the yield, concentration and actual binding activity were determined.

4.6.2 RIA

RIA resembles simple ELISA. Coating of antigens and subsequent blocking was performed as described for ELISA. The ^{111}In-labelled scFvs were added in fixed concentrations, 100 µl in each well, and binding was allowed by incubation at room temperature for ~ 60 min. Subsequently, the supernatants were aspirated, and the wells washed twice with PBS. Bound protein was released by adding 100 µl 500 mM NaOH, 1% SDS and incubating for 10 min at 50°C. Solutions were retrieved and radioactivity measured.

Data was processed by Scatchard blot analysis.

4.6.3 Cell binding

Unless otherwise stated, cell bindings were performed with 2×10^6 cells in final volumes of 200 µl. The ^{111}In-labelled scFvs were added in fixed concentrations, and binding was allowed by incubation at 4 °C for ~ 60 min. Subsequently, the cells were spun 4000 rpm for 4 min and the supernatants aspirated. The cells were re-suspended in 400 µl PBS by *VERY* low speed vortex, spun again, and supernatant removed (repeated twice). Finally, the radioactivity in cell-pellet was measured and the data processed by Scatchard plot analysis.

4.6.4 Biodistribution

^{111}In-labelled protein was sterile filtered and diluted. Injection aliquots were 200 µl in PBS supplemented with 0.1% FCS. Actual radioactivity was determined, and probes handed to EPO GmbH (Berlin, Germany). Injection, sacrifice and dissection of mice were performed by EPO GmbH. The radioactivity in the dissected organs was measured. Data was processed as %ID/g (% Injected Dose per gram organ)

Mouse models:
Both models were in nude scid mice and performed at EPO GmbH.
The Human colorectal carcinoma model was prepared by xenotransplants, while the ZR-75-1 model was prepared by injection of 10^7 cells. Tumours would reach 50 to 500 mm^2 within 3-5 weeks.