3 Discussion

3.1 TF as a therapeutic target

The establishment of TF as a tumour-specific pan-carcinoma marker and the recent assignment of a leukocyte cell differentiation number (CD176 (Karsten, 2002)) underlines the potential of TF as a molecular target in tumour therapy, especially for colon carcinomas. TF fulfils the therapeutic requirements for of a molecular target with respect to distribution, as it is almost absent in normal tissue, but is expressed on a high percentage of carcinomas (Baldus et al., 1998; Baldus et al., 2001; Baldus et al., 2000; Cao et al., 1999; Cao et al., 2000; Cao et al., 1995; Itzkowitz et al., 1989; Stein et al., 1989; Takanami, 1999). Still, TF has not yet been applied as target for radioimmunotherapy or therapy with immunotoxins, mainly because the majority of the established antibodies toward TF are of the IgM class.

An antibody with a possible therapeutic application is usually evaluated based on its pharmacokinetic profile. The evaluation is mainly focused on 5 factors; tumour binding, tumour penetration, tumour retention, serum clearance and tumour to non-tumour ratios. The IgMs are very large molecules (~750 kDa), which hampers their penetration capacity and retains them primarily in the bloodstream. In therapeutic applications, the IgMs will therefore exhibit a very low tumour penetration leading to a low AUC (Area Under Curve, %ID/g as a function of time) for the tumour, and very low tumour to non-tumour ratios – especially in comparison with serum.

Conclusively, smaller antibodies in the form of IgGs or recombinant antibody fragments are needed to enable efficient targeting. Whether recombinant antibodies with high enough affinity could be generated was unpredictable because the apparent requirement for multivalent interaction (established antibodies are of the decavalent IgM type). In general, the intrinsic affinities of single binding sites to short non-charged disaccharide antigens are rather low, which could explain the requirement for multivalent interaction. All the techniques for generation of recombinant antibody fragments are based on monovalency, and thus it was necessary to establish a new technique for selection of antibodies in a multivalent format. A special concern was whether the distance between the binding sites in the recombinant antibody would fit the pattern of TF-expression in order to allow a multivalent interaction.

3.2 Radioimmunotherapy with recombinant antibodies

The engineering of recombinant antibodies by genetic manipulation was initiated about 15 years ago, and has led to a plethora of different antibody constructs with various specificities, affinities and sizes. Many of the techniques for identification of the gene encoding an antigen-specific binding site are based on the scFv format, and the recombinant antibody prototypes are therefore often scFvs (see chapter 1.3.3). A decade ago, the general expectations to the applicability of scFv fragments in therapy were very high. They were expected to have binding characteristics equal to those of other antibodies, but simultaneously it was expected that they would have better pharmacokinetic profiles due to improved tissue penetration and faster serum clearance. However, a survey of the literature shows that these expectations were often not fulfilled (**Figure 42**). The overall tumour-targeting efficacy in the mouse models was moderate with the majority of the studies showing less than 4-5 %ID/g 5 h after injection (**Figure 42a**). The serum clearance was indeed very fast, and the majority of the reports show less than 1%ID/ml 24 h after injection (**Figure 42b**). The exception is the relative good targeting of the EGFRviii receptor in the study of Kuan and co-workers (Kuan et al., 2000), but it should be noted that the high targeting can be accounted for by the injection directly into the tumours, and is therefore different than the other targeting studies.

The disappointing findings with scFvs revealed that the scFv format was not optimal, and that alternative and improved formats were needed. In order to increase the tumour targeting and retention, new constructs were designed with the aim to increase functional affinity. Many of these molecules were simply based on bivalency, and the increased functional affinity was caused by an avidity effect. Many of the bivalent molecules have molecular weights of 50-60 kDa, and have been included in the literature survey in **Figure 43**, which also include data for Fab fragments. Finally the survey was extended to molecules of 75-120 kDa, including minibodies (scFv-C_{H3}), dimeric scDiabodies and $F(ab)_2$ and a scFv-p53 tetrameric construct (**Figure 44**). For comparison a few IgGs have also been included in the survey (**Figure 45**).

In comparison to the scFvs (25-30 kDa), the molecules in the 50-60 kDa range do in general show better overall targeting (**Figure 42a** vs. **Figure 43a**). This effect may be caused by the higher functional affinity, but may also be an effect of the slightly lower serum clearance rate during the first minutes after injection (**Figure 43b** vs. **Figure 42b**). Still, the serum clearance 1 h after injection seems very similar for the two groups. Formats with molecular weights between 75-120 kDa seem superior in the overall targeting (**Figure 44a**). The effect is presumably caused by the slower serum clearance rate (**Figure 44b**), and indicates a significant advantage for molecules in the 75-120 kDa range. The information has been generalised in **Table 18**. It should be noted that the table does not account for the effect of the different antigens with respect to shedding, internalisation and accessibility, nor the stability or electrostatic charge of the antibody fragments. Unfortunately, many

of the studies do not reveal the affinities of the antibodies, which would have been very informative for the comparison of the different studies.

In conclusion, the optimal pharmacokinetic profile should show a high clearance rate to assure high tumour to non-tumour rations, however still allowing efficient binding and penetration of the tumour by the antibody. The scFv format has the lowest potential in RAIT due to the low tumour binding and low tumour retention. The IgG format has the disadvantage of a slow clearance rate resulting in lower tumour to non-tumour ratios. The diabody and scDiabody formats could have applicability for antibodies of exceptional high affinity, but it seems that the antibody formats in the 75-120 kDa range have the highest potential in RAIT.

Pharmacokinetics of recombinant antibodies with MW of 25-30 kDa

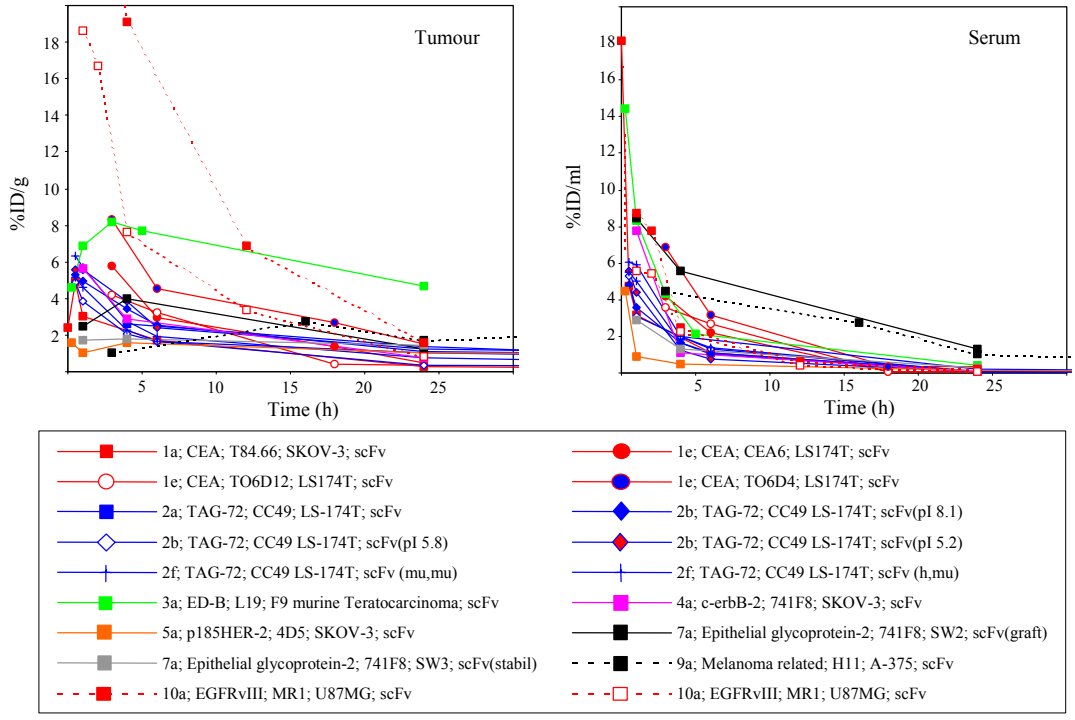

Figure 42

Pharmacokinetics of recombinant antibodies with MW of 50-60 kDa

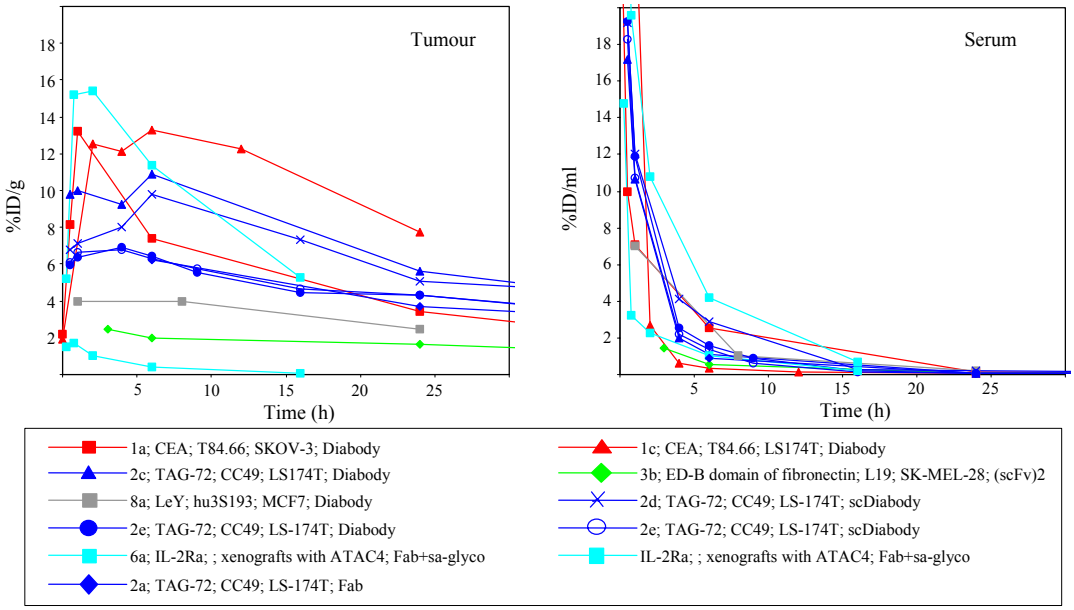

Figure 43

Pharmacokinetics of recombinant antibodies with MW of 75-120 kDa

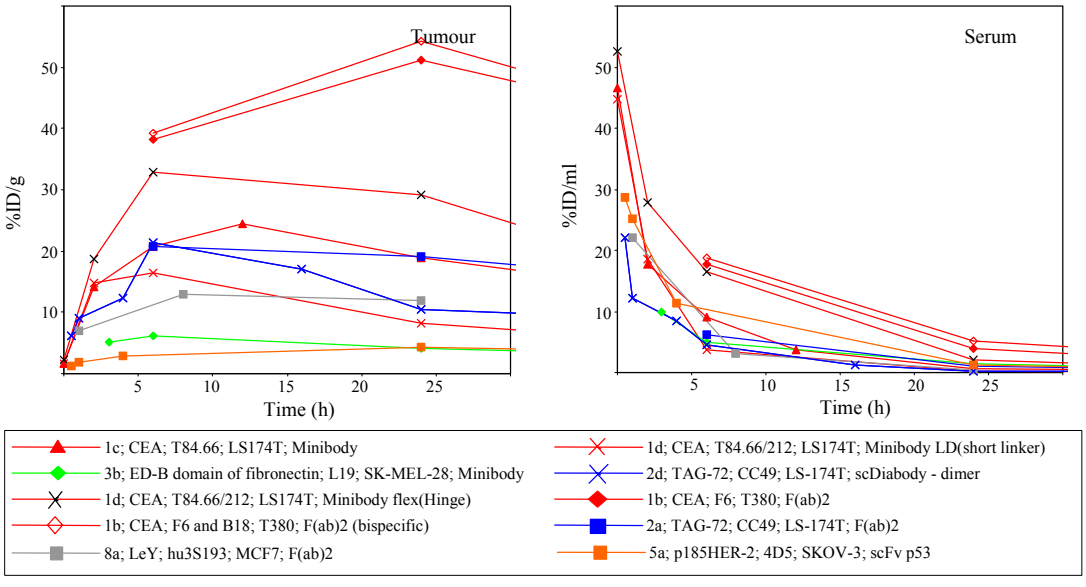

Figure 44

Pharmacokinetics of IgGs (150-160 kDa)

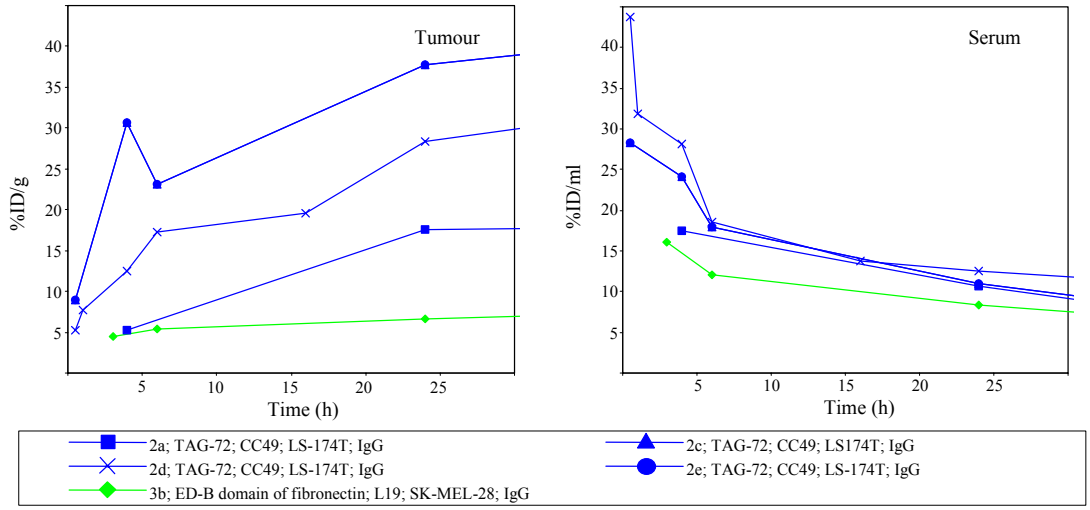

Figure 45

Figure 42-45 Survey of pharmacokinetic data from the literature

The literature was searched for data on pharmacokinetics of recombinant antibody fragments. All studies were performed in tumour models in mice. Studies were selected when more than 2 data sets were provided within 48 h. Studies have not been included when the data only contained 2 time points, or if the data sets only contained information of tumour and serum. The data have been sorted according to the molecular size of the antibody constructs, and the studies have been sorted according to the antigen targeted (numbered 1-10), and listed in the legend followed by information about antibody clone, tumour model and antibody format. The studies included were as follows: CEA: 1a, (Wu et al., 1996); 1b, (Robert et al., 1999); 1c, (Yazaki et al., 2001); 1d, (Hu et al., 1996); 1e, (Jackson et al., 1998). TAG-72: 2a, (Milenic et al., 1991); 2b, (Pavlinkova et al., 1999a); 2c, (Pavlinkova et al., 1999b); 2d, (Goel et al., 2000); 2e, (Beresford et al., 1999); 2f, (Pavlinkova et al., 2000). ED-B domain of fibronectin: 3a, (Tarli et al., 1999); 3b, (Borsi et al., 2002). C-erbB-2: 4a, (Adams et al., 1993). p185HER-2: 5a, (Willuda et al., 2001). IL-2Ra: 6a, (Kobayashi et al., 1999). Epithelial glycoprotein-2: 7a, (Willuda et al., 1999). LeY: 8a, (Tahtis et al., 2001). Melanoma related antigen: 9a, (Reilly et al., 2001). EGFRviii: 10a, (Kuan et al., 2000).

Table 18 Expected pharmacokinetic properties of recombinant antibodies in therapy

	IgG	Tetrabody ScDiabody$_2$ F(ab)$_2$	Triabody Minibody	Diabody ScDiabody Fab	scFv
Size (kDa)	150	100-120	75-90	50-60	25-30
Serum clearance	+	++	++	+++	++++
Tumour binding	++++	++++	++++	++	+
Tumour penetration	+	++	+++	+++	++++
Tumour retention	++++	++++	++++	++	+

Serum clearance should be high to generate high tumour to non-tumour ratios, but also not so high that it compromises the tumour binding or the tumour penetration. The tumour penetration should be good, so that the therapy is not only targeting the cells on the surface of the tumour. The tumour retention is important to ensure that the payload targets the tumour.

The prognostic table was inspired by a poster presented by Peter Hudson at an antibody conference.

3.3 Generation of a TF-specific scFv

The main aim of the work described here was to generate a recombinant antibody fragment with specificity toward TF. The strategy was to select binders from scFv libraries using the phage display technology. Although the observations described above indicates that the scFv format may not be optimal for therapeutic applications, it was assumed that it was optimal for the selection procedure.

Phage display was chosen because the technique has been established as one of the most prominent methods for generating monoclonal antibodies. Over the last 2 decades, it has been used to generate antibodies against various antigens, including some that are difficult to obtain by the hybridoma technique such as self-antigens, toxic antigens and haptens. It was therefore very disappointing that the great expectations to the selections of the Griffin1, and TomlinsonI/J repertoires with respect to TF binders were not fulfilled. Intriguingly, the selections failed although pure antigen was available in the amounts needed. Moreover, the novel helper phage, KM13 (Kristensen & Winter, 1998), was applied in order to decrease the background and make the selections more efficient. Even combinatorial selection strategies with alternating pannings on two different TF-carries failed to ensure successful selection, and therefore could indicate that TF is merely a poor antigen. Again the problem could be the low intrinsic affinity of antibodies toward small non-charged carbohydrate structures.

As an alternative to the naïve repertoires, scFv libraries have been generated from patient donors. In several cases, these libraries have turned out to be a promising source of high affinity antibodies. The rationale is that the patients have been "immunised" or exposed to the antigen, and therefore may have generated an antibody response including antibodies with somatic hypermutations. The strategy was first applied in the search for anti-HIV antibodies (Barbas et al., 1993; Burton et al., 1994), but has also been applied in the search for antibodies against tumour-associated antigens (Cai & Garen, 1995; Graus et al., 1998; Mao et al., 1999).
The potential antibody response in sera from 10 colorectal carcinoma patients was assessed in ELISA against two different TF carriers. However, due to the polyclonal nature of the sera, it was impossible to unambiguously establish the presence of TF-specific antibodies. The presence of TF-specific antibodies can be established following isolation of TF-binding antibodies on a TF-based affinity column (Butschak & Karsten, 2002). Most of the sera exhibited only a limited response, which reflects the very poor prognosis of the patients and the prior treatment with chemotherapy. On the other hand, the PBLs had been stored at -80°C, which may not be the optimal storage temperature for preservation of the mRNA. This can be expected to have a negative effect on the quality and quantity of the produced cDNA. Many of the patients died before the sera screenings, which limited the amount of material available for the screenings and cloning. Nevertheless, a scFv library was cloned and selected

against various tumour-associated antigens including TF. No binders were identified from these selections, although titre increases often indicated enrichment of binding clones.
In retrospect, and especially due to the findings of Butschak (Butschak & Karsten, 2002), it would have been interesting to do an additional screening of sera from healthy individuals including the isolation on the TF-based affinity column. Those with a detectable TF-specific IgG subset could be good choices for donors in the generation of antibody libraries.

However, it also has to be considered that so far the vast majority of the established TF-specific antibodies are of the IgM class, which indicates that multivalency may be the driving force in the binding to TF. This implies that the limited success in the phage library selections was merely due to the monovalent display format. Monovalent display on phage is most frequently preferred because it directs the selection toward high affinity binders and not towards clones that display well and therefore have an avidity advantage (Bass et al., 1990).
Polyvalent display of scFv or Fab fragments on phage particles has been achieved using phage vectors (Cai & Garen, 1995; Clackson et al., 1991; Davies et al., 1995; Huie et al., 2001). The phage vector systems have regained momentum during the last years, mostly because of their potential application in selections for cell internalisation mediated by the cross-linking of certain cell surface proteins via multimeric display on phage (Becerril et al., 1999; Heitner et al., 2001). The drawback of the phage vectors is the relative time-consuming and labour-intensive cloning process, which makes them unsuitable for the generation of immunised libraries with their narrow application range. The alternative is to combine a phagemid system with one of the new pIII-deleted helper phage (Baek et al., 2002; Rondot et al., 2001). The Ex-phage (Baek et al., 2002) with the engineered amber stop codons in the 5' end of gIII seems particular elegant, because no pIII from the helper phage genome is incorporated in the phage particles leading to multivalent display. Unfortunately, many of the well-established libraries (Griffin1, TomlinsonI/J) are cloned in vectors that also incorporate the amber stop codons, and phage particles thus have to be produced in suppressor strains where pIII encoded by the Ex-phage genome will also be produced.
Still, both of these systems would yield scFvs in the classic 18aa-linker format, and a subsequent re-cloning into another format would be required to obtain the desired multivalency and affinity of the soluble antibody derivatives.
The observations on multimerisation of scFvs as a consequence of variations in the linker length (Atwell et al., 1999; Hudson & Kortt, 1999; Kortt et al., 2001; Kortt et al., 1997; Todorovska et al., 2001) were the inspiration for testing the display of multimeric scFvs on phage particles. The gain in functional affinity observed for the scFvs by multimerisation should also be valid for scFvs displayed on phage. In addition, the selected scFvs would simultaneously be selected for functionality with

respect to multimerisation. Based on the observations on scFv multimerisation and the predictions in **Table 18**, it was decided to attempt display of scFvs with 1aa linkers.

As source for library clonings, 3 Balb/c and two C3H/J mice were immunised with asialoglycophorin using an established immunisation strategy (Karsten et al., 1995). Six phage display antibody libraries were generated, 3 in the 1aa-linker format, and 3 in the 18aa-linker format. The libraries were selected for TF-specific antibodies applying a combinatorial selection strategy. Only the libraries in the 1aa-linker format gave rise to clones binding specifically to TF. This result strongly supports the theory on necessity of multivalency of antibodies binding to TF. The format may not only have applicability in the selection towards TF, but should work in selections against other antigens known to associate with low intrinsic affinity, which have so far not been successfully targeted. Sequencing revealed, that all the identified clones encoded the same scFv sequence except for minor variations in the primer encoding regions. This indicates a very narrow sequence variability window for TF-specific antibodies. This hypothesis is further supported by the data from the affinity maturation selections. Especially the change of specificity in the selections from the library, where LCDR3 and HCDR3 were targeted simultaneously demonstrates this point (chapter 2.5) . In addition, the selection of 4F1, which presumably recognises a TF-glycosylated peptide epitope of asialoglycophorin, emphasises both the quality/diversity of the immunised libraries, as well as the hypothesis of the narrow sequence window.

The KM13 helper phage was efficient in minimising the background in the phage library selections, and led to the identification of specific clones after only 2 or 3 rounds of panning. This demonstrates the applicability of the KM13 helper phage in selections of libraries in the 1aa-linker format with efficiencies similar to earlier reports (Goletz et al., 2002; Jensen et al., 2003; Kristensen & Winter, 1998; Ravn et al., 2000; Sanz et al., 2001).

In the investigation of various linker length, a good correlation was found between the binding activities of scFv linker-formats in soluble form and the scFv linker-formats displayed on phage particles. Constructs with shorter linkers, which theoretically direct the formation of higher order multimers, showed an increased binding activity in both systems (**Figure 7, 11 and 15**). This indicates that the rules for multimerisation of scFvs in solution also applies for scFvs displayed on phage, and therefore validates the choice of the 1aa linker format.

Theoretically, multimeric constructs could form from several scFv-pIII-fusion proteins displayed on the same phage, or they could consist of a mixture of scFv-pIII-fusion proteins and non-fused scFv. The presence of non-fused scFv was demonstrated, but there seemed to be no significant difference in amounts observed between the different linker formats. The overall display level was very similar for the different formats, and it was therefore not possible to demonstrate the exact molecular

configuration causing the increased binding activity of the shorter linker formats. Still, the formats with shorter linkers have an increased binding activity, and it can be hypothesised that this enables their detection in screening assays.

3.4 Specificity of selected scFv

The fine-specificity of the scFv(1aa) towards immobilised antigens was established in ELISA, where the antibody was probed for binding to several glycoproteins and a large panel of oligosaccharides coupled to PAA. An essential finding was that the scFv(1aa) binds TF independent of the carrier, whether protein or PAA. Furthermore, the scFv(1aa) exhibited an exquisite specificity toward TF with a minor cross-reactivity to core-2. Models of the TF anomers and of core-2 may give a visual impression of the antigen and indirectly of the steric requirements for the binding pocket (**Figure 46**). The models show the carbohydrate structure (spacefilling) connected to a serine residue (ball and stick). The structures have been aligned so that the plane of the ring-structure of GalNAc is parallel in the upper panel and in the lower panel, respectively. The models may offer a visual explanation to why TFαβ and TFαα are not bound, as well as indicate why both TFβα and core-2 are bound (**Figure 46**). It can be speculated that the antibody-binding pocket interacts with the lower part of the structures, where TFαα, TFαβ and TFββ present a steric differences compared to TFβα and core-2 (indicated by arrows). Extended structures including the core-1 motif but are not bound by the scFv(1aa) also show steric hindrances (models not shown). They further support our assumptions about the extent of the epitope and the binding pocket.

It is difficult to compare the specificity of the scFv(1aa) to any of the established TF-binding antibodies, because most of them have been tested only against TF and a few controls, and have therefore not been proven strictly TF specific. Some of them have even been shown to cross-react with other structures (Karsten et al., 1995; Stein et al., 1989; Steuden et al., 1985). It should be noted that the availability of the carbohydrate-PAA antigens is relatively resent, and it has therefore not always been possible to test the antibodies with regard to carbohydrate fine-specificity to the same extent.

The observed cross-reactivity with core-2 is shared with the mouse antibody, Nemod TF-2, and does not interfere with the tumour specificity of the antibody (unpublished data). To my knowledge no core-2 specific antibodies have been described, and therefore the core-2 biodistribution is not known. Based on immunohistochemical studies done with Nemod TF-2 (data not published) it may be postulated that core-2 is either not expressed as an accessible surface antigen, or that it has a distribution similar to TF.

Structural studies of *O*-glycans from various sources were never able to show a core-2 structure that was not cryptically hidden in longer chains ((Brockhausen, 2000) and references within). This supports the assumption that only core-1is recognised by the scFv(1aa) *in vivo* or in natural conditions.

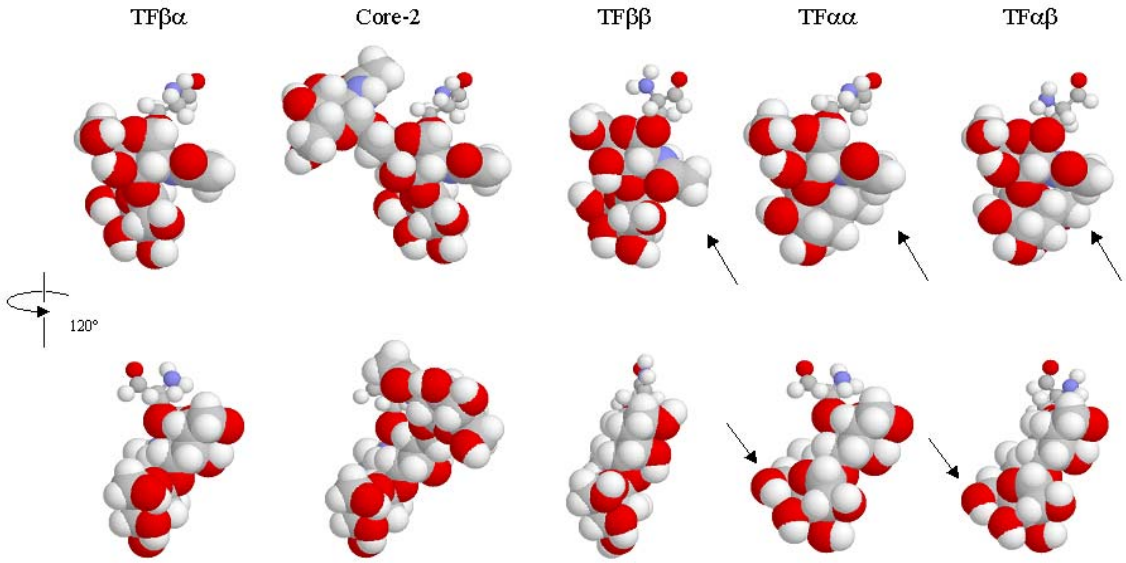

Figure 46 Models of TF anomers and core-2
The carbohydrate structures are shown as (spacefilling) models in extension of a serine residue (as ball and stick), which may indicate the location of the protein moiety. All models were aligned so that the plane of the ring structure of GalNAc is parallel in the upper panel, and have been turned 120° in the lower panel. Arrows indicate steric differences in TFαα, TFβα and TFββ compared to TFαβ and core-2. Models were prepared with the SWEET2 software (http://www.dkfz-heidelberg.de/spec/sweet2/doc/).

The specificity of the scFv(1aa) towards TF exposed on cells was demonstrated by immunocytochemistry and immunohistochemistry, in which the scFv(1aa) specifically stained TF-positive cells and tumour structures, respectively.

3.5 Protein production and purification

To further characterise the scFv(1aa) and for further development, larger amounts of pure protein were needed. Like the majority of scFvs, the scFv(1aa) could only be expressed in minor quantities (250-500 µg/l) using a common *E.coli*-expression system and purification protocol (Ian Tomlinson, personal communication), and thus the first objective was to establish an improved and standardised expression system.

Among the systems tested, the pET11a-derived expression vector in combination with the Rosetta™ seemed the best suited for the purpose. The expression in the system is under tight control because Rosetta™ expresses T7 lysozyme, which inhibits the T7 polymerase synthesised by leaky

transcription. Upon induction, the expression of the recombinant protein is so powerful that it completely takes over the protein production machinery in the bacteria (**Figure 10**). An additional advantage of the T7 lysozyme is that it cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall (Inouye et al., 1973). This renders the bacteria more sensitive to treatments that disrupt the inner membrane. Simple osmotic shock treatment will thus cause complete lysis of the bacteria, and thereby make the retrieval of recombinantly expressed protein comparatively easy. An estimated 2-10 g of recombinant protein is produced per litre culture, but most of this is unfortunately directed into inclusion bodies. Although the protein is readily purified from inclusion bodies, all attempts to refold the protein have failed so far. The strategies have therefore been focused on increasing the amounts of soluble protein. Different strategies have been tested, of which the most prominent was low cultivation temperature (25°C) and supplementation with 50 mM sucrose. In contrast to other reports, where supplement with high concentrations of sucrose had a significant effect on the levels of protein in the culture supernatant (Kipriyanov et al., 1997), only limited effects were observed for the scFv(1aa). At lower concentrations, sucrose had a positive effect on the yield from bacterial extracts (**Figure 12**). The advantage is that more protein can be retrieved from the periplasm upon osmotic shock disruption. Using this expression system, the yield was increased 5 to 10 fold, and an average of 1.3 mg/l highly pure scFv(1aa) was retrieved after IMAC purification as discussed below.

Since expression of eucaryotic proteins in bacteria can be troublesome, expression in COS cells was also attempted, but without any encouraging results with respect to protein yield and binding activity (data not shown).

Expression of antibody fragments in yeast is becoming more frequently reported ((FitzGerald et al., 1997; Goel et al., 2000; Powers et al., 2001) and Ian Tomlinson, personal communication), but has not yet been tested for the TF-antibodies described in this study.

Protein purification was established using 3 different chromatographic methods, of which the first was IMAC. Purification of His_6-tagged proteins from crude bacterial extracts is often associated with co-purification or contamination of IMAC binding proteins from the *E. coli* cytosol. However, optimisation of the washing stringency increased the purity of the eluted protein to more than 95% (judged by SDS-PAGE, **Figure 13**). The total yield of the optimised IMAC-protocol was 15 to 40 % lower that the total yield of scFv without the stringent washing. This loss of protein was accepted due to the increased purity, and the protocol consequently applied as a primary purification step after all expressions. The yield from the IMAC purification among the different linker formats differed slightly, with the scFv(0aa) yielding only 200-300 µg/l, while the scFv(1aa) giving 1.3 mg/l and the scFv(2aa) giving 2.2 mg/l

Ion exchange chromatography and affinity chromatography protocols were established as secondary purification steps to ensure complete homogeneity of the purified protein. The high purity was in particular needed for the conjugation of chelator (DTPA) to the scFvs. Both the chromatographic procedures enabled separation of the proteins into fractions according to their multimerisation grade.

Investigation of the multimerisation grade of the different scFv-linker formats by size exclusion chromatography revealed that the scFv(18aa) formed primarily monomers and dimers as expected. Shorter linkers caused the formation of dimers except for the 1aa-linker that seemed to direct the formation of trimers, while complete deletion of the linker led to formation of tetramers (**Figure 20**). These data are in good agreement with the guidelines in the literature (Hudson & Kortt, 1999; Kortt et al., 2001; Todorovska et al., 2001).

A very interesting finding in this study was the enrichment of trimeric complexes by the AEC-purification of the scFv(1aa) on a HiTrap^{TM} Q HP column (**Figure 24**), and the enrichment of tetrameric complexes by the AC-purification of the scFv(1aa) on a column with immobilised TF (**Figure 25**). There was a good consistency in the observations made with the chromatographic purifications. For example, AC-purified scFv(1aa) could rebind on the affinity column and the AEC-purified scFv(1aa) could rebind on the HiTrap^{TM} Q HP column. However, the AEC-purified scFv(1aa) did not bind on the affinity column as it has been enriched for trimeric complexes, and the affinity chromatography enriches for tetrameric complexes. The reason for the enrichment of tetrameric complexes on the affinity column may be the higher affinity of the tetrameric complexescompared to the dimeric and trimeric complexes. In addition, the distribution of TF on the affinity column may favour the binding of scFv complexes with a binding-site conformation resembling that of the tetrameric complexes and not that of the trimeric complexes.

3.6 Affinities of the scFvs

The affinities of the different scFv constructs towards synthetic TF in the form of asialoglycophorin were determined by surface plasmon resonance (Biacore). The affinity of the scFv(18aa) was very low, whereas the affinities of the dimeric complexes were considerably higher, and even higher for the trimeric-scFv(1aa) complexes and the tetrameric-scFv(0aa) complexes (**Figure 21** and **Table 3**). Again, these results support the assumption of a low intrinsic affinity of a single binding site, and that the binding of TF is mediated through a multivalent interaction. This is further supported by the studies of the effect of competitors on the dissociation rates of the scFv(0aa), scFv(1aa) and scFv(2aa), here it was found that free TF (0.2 M) had no effect, while TF displayed multivalently on

asialoglycophorin or PAA did (50 μM with respect to TF) (**Figure 22**). In contrast to this finding, the effect of soluble LeY in the competition of a LeY IgM was much more dramatic (Rheinnecker et al., 1996).

Interestingly, relatively large differences were observed among the different dimeric constructs, where in particular the scFv(4aa) was not performing well. These findings indicate that the mere formation of dimeric complexes does not guarantee a good affinity/binding activity of the construct, but that an examination of different formats may be a good investment for optimisation of the antibody properties.

For the production, purification, and development of chelated scFv(1aa) is was very important to ensure that the affinity and activity of the product was not hampered in the individual steps. The affinity of the AEC-purified scFv(1aa) measured by Biacore, and the binding activity in ELISA correlated very well with that of the IMAC-purified scFv(1aa), which is in agreement with the expectations according to the similarities in multimerisation grade, and confirms that binding activity and affinity is not lost in the procedure. The AC-purified scFv(1aa), which was enriched for tetrameric complexes, had an increased binding activity in ELISA and a higher affinity measured on the Biacore. This is again in good agreement with the expectations.

The affinity and binding activity of the chelated scFv(1aa) was also determined on the Biacore and by ELISA, confirming the data from the IMAC-purified and AEC-purified scFv(1aa) and opening the way for the radioactivity assays.

The affinities of the scFv(0aa) and scFv(1aa) for synthetic TF (asialoglycophorin) determined by Biacore were in good agreement with the affinities determined by RIA, and was thus the final proof that the protein did not lose activity due to the chelation.

For the generation of a therapeutic candidate the affinity towards natural TF displayed on living cells is more interesting than the affinity towards synthetic TF displayed on proteins coated on a plastic surface. The affinities of the scFv(0aa), scFv(1aa) and the scFv(2aa) towards the cell lines, KG-1, NM-D4, and ZR-75-1 were examined with ^{111}In-labelled scFvs, and revealed that the affinities of the scFvs toward TF displayed on cells were slightly lower (~10 fold, compare **Figure 47 and 48**). It is imperative that the distribution of antigen on the cell surface is not the same as on a sensor chip or in ELISA, and that the binding affinity of a multivalent interaction will depend on the antigen distribution. These results show that the antibodies do indeed bind to natural TF, although the affinity is slightly less than for binding to synthetic TF. For comparison, similar observations were made for the Lewis Y binding antibodies in the study of Clarke *et al* (Clarke et al., 2000).

The results of the affinity investigations in general showed that the scFv(1aa) and the scFv(0aa) had the highest affinity, and in combination with the expected pharmacokinetic profiles of trimeric and tetrameric scFvs, they were therefore the most promising candidates for the further investigation of TF

based tumour targeting. The scFv(1aa) was the primary choice because of the higher yield from expression and purification.

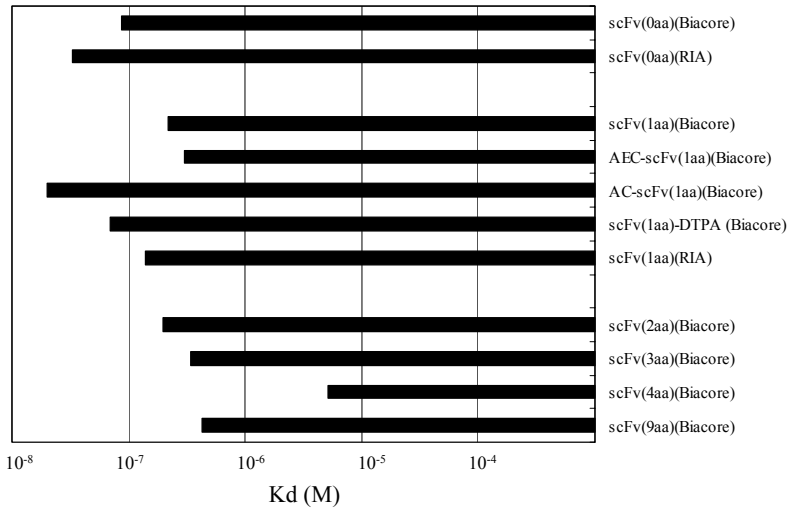

Figure 47 Summary of dissociation constants for the various scFvs

3.7 Radioactive labelling of scFv

The conjugation of a chelator to the antibody and subsequent labelling with radiometals was preferred because of the following advantages: *i*) The labelling reaction is a simple loading of the covalently bound chelator, where the high dissociation constant ensures the stabile binding of the radioisotope. *ii*) In an optimised setting, the radioactivity ($^{111}In^{3+}$ or $^{90}Y^{3+}$) is simply added to the antibody, which has been stored in the labelling buffer at a very high protein concentration. Subsequently, the labelled antibody is diluted in PBS, and can be applied directly for *in vitro* assays or biodistribution trials. In the case of the scFv(0aa), scFv(1aa) and scFv(2aa) it was not possible to reach a sufficiently high protein concentration without severe precipitation of the protein and instead a final washing step by spin filtration was needed to dilute the labelling buffer. Still, the labelling procedure is very straightforward with a good reproducibility.

The labelling strategy was validated through thorough examination of the labelled scFvs. The chemical integrity of the ^{111}In-labelled scFvs was confirmed by TLC, radiography of SDS-PAGE and investigation by size fractionation by spin filtration and size exclusion chromatography.

The *in vitro* binding activities of the ^{111}In-labelled scFvs were evaluated in radioimmunoassays (RIA) and cell binding assays. The cell-binding affinity results are summarised in **Figure 48**, while the RIA results are included in **Figure 47**.

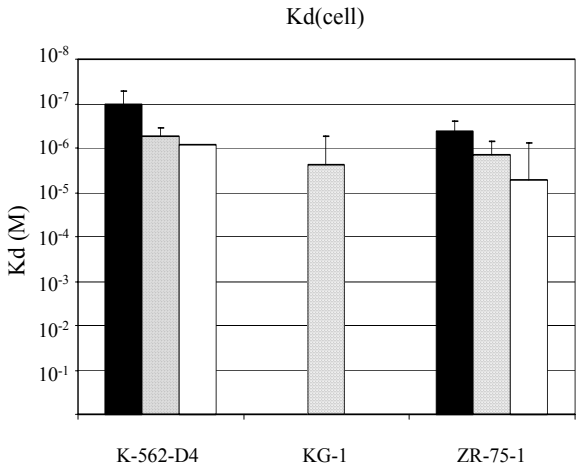

Figure 48 Dissociation constants for cell binding measured with ^{111}In labelled scFvs
Summary of dissociation constants measured by cell binding to NM-D4, KG-1 and ZR-75-1 cells with scFv(0aa) (Black bars), scFv(1aa) (dotted bars), and scFv(2aa) (white bars).

3.8 Biodistributions

The primary objective for the biodistribution investigations is to examine the tumour targeting potential of the antibody. This was investigated using two different mouse models, one with nude mice bearing human colorectal carcinoma xenografts and one with nude mice bearing human breast cancer ZR-75-1 xenografts. It is imperative that the tumours have to display the antigen, which was demonstrated by immunohistochemistry, but it is also important for the validation of the results to know some biological details about the antigen.

With respect to the antigen, it is assumed that the major TF-displaying protein is MUC1, which has been described with respect to internalisation and shedding. Two other proteins have been established as TF- carries: CD43 (Coltart et al., 2002) and a splice variant of CD44 occurring in some colon cancers (Singh et al., 2001).

In the case of ZR-75-1, MUC1 is internalised with a rate of 0.9%/min of the antigen on the cell surface. Furthermore, MUC1 is recycled to the cell surface with a total cycle-time of 143 min. Of these, it is present on the cell surface for around 77 min (Litvinov & Hilkens, 1993). Importantly, no MUC1 was observed in the serum in any of the tumour models (Stahn, personal communication). The scFv(0aa) and the scFv(1aa) have dissociation half-times of 5-8 min (based on the kd determined on the Biacore). If the values from the *in vitro* Biacore binding are assumed to correlate with the *in vivo* binding in the animal models, there is room for some optimisation of the dissociation constant. However, it should be kept in mind that the *in vivo* observations often differ from the theories, exemplified nicely by the biological properties of two anti-MUC1 antibodies on ZR-75-1 (Pietersz et al., 1997). One antibody exhibited a slower internalisation rate than expected, while the other was much faster (~70% internalisation in 15-30 min). Shedding of MUC1 may not be a serious problem as

it seems that the shed MUC1 caries sialylated TF, and therefore is not a target for the TF antibodies ((Litvinov & Hilkens, 1993) and Stahn personal communication).
The turnover of different antigens can vary dramatically. For example, in the case of targeting of erbB, the antigen is internalised with a half-time of 17 min (Worthylake et al., 1999), and there is consequently no reason to engineer antibodies with half-lifes longer than 17 min. The turnover of CEA has been claimed to be largely due to shedding, with a shedding half-life of 3-15 days (Graff & Wittrup, 2003; Shi et al., 1983; Stein et al., 1999). However, significant internalisation within minutes has also been reported (Patrick et al., 1998). With respect to MUC-1 the antigen turnover seems not to be critical based on the unpublished research from Nemod GmbH and Antisoma.

In both tumour models the tumour targeting was addressed 24 h after injecting a dose of 5 μg ^{111}In-labelled scFv(1aa). The targeting efficiency was relatively good with ~5% ID/g in the colon carcinoma model and ~10%ID/g in the ZR-75-1 model. This should be compared to the survey in **Figure 42 – 45**, where the majority of the studies show less than 5% ID/g after 24 h. It has to be stressed that the good tumour uptake in two different models, which has not been reported for any of the published biodistribution trials. It shows that the tumour targeting is relevant for different types of tumours, where differences in TF-expression can be expected, and the result is therefore very important for validation of a carbohydrate target.
The tumour targeting was evaluated with 3 different doses, ranging from 1.7 μg to 15 μg, but without revealing any significant dose dependency (NMD 36 and NMD 41). Moreover, the targeting was evaluated with respect to pharmacokinetic profiles in two different time windows; one with 3 time points from 24 to 72 h (NMD 40) and one with 2 time points from 4 to 24 h (NMD 45). Both revealed an early targeting of the tumour and good tumour retention.
Finally, a comparison of the scFv(0aa) and the scFv(1aa) revealed no considerable difference in the targeting efficiency with respect to antibody size or affinity (NMD 41, 43 and 45). The values for the tumour targeting in NMD 45 were lower than expected (an observation that was shared with a parallel trial targeting of MUC1 using mice from the same cohort), and any conclusions drawn may therefore associated with uncertainties. Still, there seems to be a higher uptake in the liver 4 h after injection for the scFv(0aa), but this is partly cleared after 24 h. There is an indication of an increase in uptake from 4 to 24 h for the scFv(0aa), while no increase is seen for the scFv(1aa). Stretching the interpretation of the data, this is in agreement with the slightly lower serum clearance of the tetrameric scFv(0aa) in comparison to the trimeric scFv(1aa) (**Figure 41)**. This is further supported by the literature survey, revealing that the antibody size may have significant influence on the serum clearance (comparing **Figure 44** with **Figure 42 & 43**). On the other hand, the difference in distribution may be caused by the difference in valency between the scFv(0aa) and scFv(1aa). Increasing the valency of an antibody

has been a popular strategy for increasing the functional affinity and thereby the targeting potential (Adams et al., 1993; Viti et al., 1999; Willuda et al., 2001; Wu et al., 1996).

It should be noted that the affinity of the targeting antibody has been one of the major matter of concern in RAIT of tumours. The heterogeneous distribution of the antibodies in the tumours (Jain, 1987) gave rise to models describing the effect of elevated interstitial pressure on molecular distribution (Jain & Baxter, 1988). Extension of these models lead to the formulation of the "Binding site barrier" hypothesis, the idea that macromolecular ligands could be prevented from penetrating tumours by their successful binding to the target antigens at the periphery of the tumour (Juweid et al., 1992; van Osdol et al., 1991; Weinstein & van Osdol, 1992). However, recent modulations from Graff and Wittrup argue that the "binding site barrier" is merely a transition state in the distribution (Graff & Wittrup, 2003). The antibody will be driven into the tumour as long as there is an antibody concentration gradient between the tumour and the serum. The shape of the antibody profile in the tumour (concentration at time points) will depend on the affinity of the bindings. The modulations indicate an optimal Kd of 1 nM in agreement with the findings of Adams (Adams et al., 2001) (see below). The antibody / antigen distribution in the tumour may be evaluated by immunohistochemistry and microautoradiography of tumour sections thereby revealing the antibody saturation grade of the tumour with respect to the applied dose (Behr et al., 1997).

The relevance of this is that the tumour targeting is always dependent on the antigen concentration and turnover (shedding or internalisation) as well as on the stability, size, valency, and affinity of the antibody.

The affinity requirement for good tumour targeting, with respect to best tumour to non-tumour ratios and not necessarily complete homogenous saturation of the tumour, has been thoroughly debated.

There are many examples in the literature to support the different theories, but most of the theories are not based on well-designed studies. Only a few thorough studies have been made, where antibodies of different affinities were tested in the same model. One problem will always be that the different affinities arise from slightly different sequences, and that these differences may also have effects on other factors such as clearance rate, serum stability and non-specific adsorption to various organs. Still a guideline can be extracted from one thoroughly planned study, performed in SK-OV-3-bearing SCID mice and anti-HER-2/neu scFvs of various affinities (10^{-7} – 10^{-11} M) (Adams et al., 2001). This study revealed an affinity-dependent increase in the tumour targeting at 24 h that reached a plateau at a Kd of approximately 1 nM. The same study also showed that the relative low overall targeting was mainly caused by systemic clearance, as the targeting in anephric mice was significantly higher (15-20 fold at 24 h).

In evaluation of the antibody affinity, it is again important to assess the cell binding characteristics as was found in the investigation of a humanised version of an anti Lewis^{Y} antibody, which revealed an 50 fold reduction of affinity of the humanised version compared to the mouse antibody when measured on the Biacore, but only a 2-3 fold decrease in the affinity when measured in cell binding assays (Scott et al., 2000).

The second important objective with the biodistribution studies was to demonstrate that no other organ was target by the antibodies. The results from the distribution in Nude mice (NMD 29) clearly demonstrated that the kidneys (~50% ID/g) were the only other organs showing significant uptake. In one trial (NMD 36) a considerable uptake in the spleen was observed, but this was not seen in any of the other trials, and seems to be caused by unknown individual conditions.

The kidney burden is a critical problem, as it is often the dose-limiting factor in RAIT. The kidney burden observed for the scFv(1aa) seemed to be independent on the variable parameters such as dose and time (NMD 36, 40 and 45).The high uptake in the kidneys observed at 24 h decreased slowly to time points 48 and 72 h. This result is in agreement with the general findings on renal uptake of small proteins in the kidneys, and in particular uptake and retention of chelator-conjugated proteins (Rogers et al., 1995; Tsai et al., 2001). This is also the conclusion that can be made from the kidney uptake of the antibodies in the literature survey **Figures 42 – 45 (Figure 49)**.

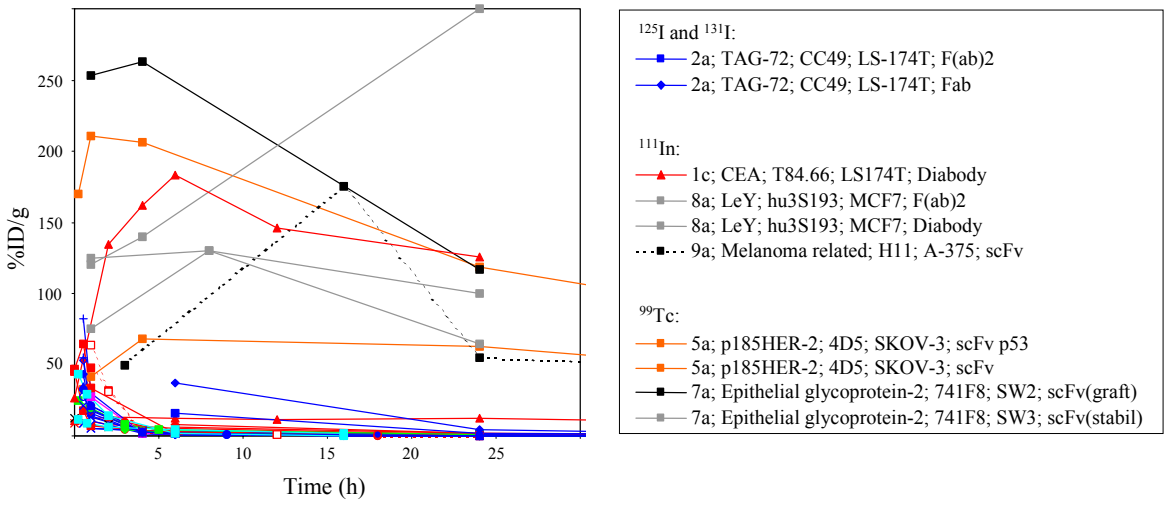

Figure 49 Kidney uptake of recombinant antibodies
The kidney uptake is shown for all the studies applied in **Figure 42-44**. The legend shows only those with significant kidney retention, and the groups them according to the source of radioactivity. All the remaining studies have applied isotopes of Iodine.

All the trials with high uptake have been performed with chelate-conjugated antibodies. It has been demonstrated that the renal uptake is caused by internalisation of the antibodies in proximal tubulus cells after glomerular filtration, followed by degradation of the antibody in the lysosomal

compartments (Rogers et al., 1995; Tsai et al., 2001). Moreover, the lysine-chelator metabolites are retained in the cells, and diffuse only slowly. The size of the scFv(1aa) is on the border of the kidney cut-off, and it was not expected that the scFv(1aa) would be taken up in the kidneys to the extend observed.

Therefore other strategies were needed for lowering the kidney uptake. The co-administration of basic amino acids such as lysine has been applied in many reports in order to overcome nephrotoxicity (renal uptake or kidney uptake) (Behr et al., 1999; Behr et al., 1995; Behr et al., 1997; Kobayashi et al., 1999). It presumably works by lowering the charge interaction between the antibody and the proximal tubulus cells. However, no effect of lysine was observed for the kidney uptake of the scFv(1aa) (NMD 41).

Another strategy for lowering the charge interaction between the antibody and the proximal tubulus cells is to manipulate the isoelectric point of the recombinant antibody. Briefly, this can be achieved by *in vitro* glycosylation or by genetic modification of charged amino acids. Kobayashi and co-workers investigated the glycosylation-mediated change of the isoelectric point of a Fab fragment, and showed that a lower pI decreased the clearance through the kidneys, and led to a 4.6 fold increase of the AUC of the tumour (Kobayashi et al., 1997). Pavlinkova and co-workers engineered scFvs with lower pI by genetic manipulation, but the effect of pI on the investigated scFvs had only minor effect on the distribution (Pavlinkova et al., 1999a).

Even with these studies in mind, the essence of the literature on radiolabelled antibodies clearly shows that the important factors for the kidney uptake are the size of the antibody and presence of a chelator.

The observation on the kidney uptake of the scFv(0aa) was a major breakthrough. A dramatically lower kidney uptake was observed for the scFv(0aa) compared to the scFv(1aa), which is presumably due to the larger size of the scFv(0aa) (NMD 43 and 45). The significance of this finding should not be underscored because the uptake in the kidneys often is dose limiting, and tetrameric scFvs could therefore have great general potential in RAIT. Moreover, the scFv(0aa) shows a fast serum clearance rate, which can be a problem for other large molecules such as the IgGs, because it hampers the tumour to non-tumour ratios.

With respect to targeting TF in RAIT, natural TF and TF-carriers should be considered. Exposed TF has been found on aging erythrocytes on glycophorin A, where its exposure mediates an interaction with the asialoglycoprotein receptor in the liver, and leads to capture of the cells and their subsequent elimination. Furthermore, an increasing degree of O-glycosylation seems to reflect the differentiation states of the erythrocytes (Gahmberg et al., 1984). Theoretically, the ^{111}In-labelled antibodies could

bind to the aging erythrocytes and thereby target the liver, but this does not seem to be a problem for the scFv(1aa) or especially for the scFv(0aa) based on NMD 43 and 45.

Among the investigated scFvs, the scFv(0aa) seems to be the superior for targeting TF in RAIT, and would also be the primary candidate for a more thorough study of the pharmacokinetics aiming at RAIT. In the following evaluation, the biodistribution data would provide information about the targeting efficiency at a particular timepoint, while the pharmacokinetic data would provide information about a therapeutic window. These data are traditionally processed as AUC (Area Under Curve, %ID/g as a function of time). The overall goal is to have a higher AUC for the tumour than for any other organ. The profile of the AUC is also important because it may indicate the position of the therapeutic window, and thus indicate which therapy form will be the most optimal. A higher AUC for the tumour does not necessary indicate a good therapeutic window for RAIT in general. The main area of the AUC should be located at the early time points, simply because there is no need for tumour localisation after the radioactivity has decayed. For the serum clearance similar considerations can be made. In addition, calculations may indicate that a low serum clearance provides a good tumour penetration (Graff & Wittrup, 2003). This is achieved at the expense of having the high radioactivity in the circulation for longer times, potentially causing damage to other organs. It may therefore be beneficial to correlate the AUCs with a decay factor (i.e. factor = 1 at time of injection, and decreasing with decay). This would also provide the possibility of evaluating different radioactivity sources for their effect on the correlated AUCs. Conclusion from such evaluations could be as follows: ^{213}Bi should be suitable in cases where the tumour targeting is extremely fast (scFvs or diabodies) because it has a half-life of only 47 minutes (Adams et al., 2000; Behr et al., 1999). In addition, it should be noticed that ^{213}Bi is an α-emitter with an average tissue penetration track length of 70 μm. It is therefore unlikely that cells in the centre of a solid tumour will receive a sufficient dose. ^{99}Tc could have interest for labelling diabodies and triabodies because it has a half-life of 6 h. However, as a γ-emitter it has no therapeutic effect (Waibel et al., 1999; Willuda et al., 2001). The reason for the interest in ^{99}TC is the labelling protocol that is also applicable for $^{188/186}$Re, which are β-emitters with half-lifes of 17 and 91 h respectively. ^{99}Tc may thus work as a "safer" model for $^{188/186}$Re, similar to the function of ^{111}In for ^{90}Y in chelator labellings. In that respect, ^{90}Y is a β-emitter with a half-life of 66.2 h and therefore applicable when area is added to the AUC of the tumour over several days. It has been used to label many chelator-conjugated antibodies (Behr et al., 1999; Behr et al., 1997; Clarke et al., 2000; King et al., 1994; Klein et al., 1989; Stein et al., 1999; Wong et al., 1995; Wong et al., 2000). Finally, ^{131}I that is a β-emitter with a half-life of 192 h, and the considerations for ^{90}Y are also relevant for ^{131}I (Klein et al., 1989; Pavlinkova et al., 1999b). In comparison to ^{90}Y, some of the

disadvantages of labelling with ^{131}I are the potential variations in labelling efficacy between different preparations, and the dehalogenation *in vivo* leading to accumulation of iodine in the thyroid.
As briefly discussed in the introduction, RAIT may offer the advantage of a bystander effect (radiation of cells that are not bound by radio-labelled antibodies). For the above-mentioned nuclides, such bystander effects are highest for ^{90}Y and ^{188}Re with 12 and 11 mm maximum particle-range in tissue, respectively (Goldenberg, 2003).

3.9 Potential strategies for optimising the antibody

Notwithtanding the limited number of animal studies, the results clearly validate TF as a target for RAIT. The specificity of the selected TF-antibody is outstanding, and it seems valid for tumour targeting. Furthermor, it was an important finding that the larger tetrameric complex (tetrabody) of the scFv(0aa) has an advantage in the biodistribution over the trimeric complex (triabody) of the scFv(1aa). Thus, the antibody format, and a "prototype" sequence have been established. Even though the Biacore measurements of the chelated scFv(1aa) and the AC-purified scFv(1aa) indicate lower dissociation rates with dissociation half-times of 32 min and 28 min, respectively, the affinities measured for the scFv(0aa) and scFv(1aa) for binding to cells are 100 - 1000 fold lower than recommended optimal affinity of 1 nM (Adams et al., 2001; Graff & Wittrup, 2003).
Affinity maturation by phage display is a widely used technology for improving the affinity of antibody fragments. The technique mimics somatic hypermutation as it is based on the construction and subsequent selection of a secondary repertoire, in which diversity is introduced into the V genes of the original antibody. Historically, the CDR regions have been the preferred targets for randomisation (Barbas et al., 1992; Barbas et al., 1994; Crameri et al., 1996; Garrard & Henner, 1993; Jackson et al., 1995; Osbourn et al., 1996; Pini et al., 1997; Yang et al., 1995).
The strategy applied in the generation of secondary repertoires hereinwas based on gene assembly by PCR. Three of the CDRs were chosen for partial randomisation, namely the heavy chain CDR2 and CDR3 as well as the light chain CDR3. The CDR3s were chosen because they are central in the binding pocket and therefore assumed to play a key role in the antigen interactions. The heavy chain CDR2 was chosen because there is a tendency that V_H is more involved in the antigen interaction than V_L. Partial randomisation of the CDRs, also called parsimonious mutagenesis (Balint & Larrick, 1993; Glaser et al., 1992), was chosen based on the assumption that at least some of the original residues are involved in the antigen binding and should be preserved. The parsimonious mutagenesis strategy therefore results in a repertoire that is biased toward binding clones. The primers used for the PCR assembly were designed with a 70% bias for the original base at every position in the targeted CDRs.

The first two secondary repertoires were constructed in the scFv(18aa) format and targeted the heavy chain CDR3 by either parsimonious mutagenesis or complete randomisation including length from 3 to 16 amino acids. The scFv(18aa) format was chosen because monovalent display of scFvs is favourable for selection for high affinity binders (Bass et al., 1990). However, as seen before the monovalent scFv format was not applicable for the selection against TF, and no clones were isolated from these selections.

The last 3 repertoires were generated in the scFv(1aa) and scFv(0aa) formats, which were successfully applied in selections against TF on different carriers. However, so far the selections have only yielded the original sequence. In general the affinity maturation experiments indicated that the sequence of the scFv(1aa) or scFv(0aa) is nearly optimal, and that the sequence variability window is indeed very narrow. It may also indicate that the randomised CDRs are not the right targets for optimisation, and that directed targeting of the CDR regions may not be the optimal strategy.

Alternatively, diversity can be introduced into the antibody genes by random mutations either by error prone PCR (Deng et al., 1994; Hawkins et al., 1992) or by applying bacterial mutator strains (Irving et al., 1996; Low et al., 1996). Selections of the mutated repertoires for high affinity have revealed point mutations in both CDRs and framework regions. Deng *et al.* observed a mutation of a solvent exposed residue in the heavy chain framework 3 that resulted in dramatically increased yields of functional scFv (up to 120 mg/l) (Deng et al., 1994). Low *et al.* observed that the mutations, which gave rise to the greatest increase in affinity, were those in the CDRs, whereas mutations in the framework regions only gave rise to moderate increases in affinity (Low et al., 1996). Conclusively, these reports demonstrate the feasibility of affinity maturation by random point mutagenesis. In addition, and very importantly, stability of the antibody and the expression yields may also be increased because the responsible mutations may be selected from the repertoire due to improved folding or increased display level. It is imperative that increased serum stability of an antibody will have a positive effect on the tumour targeting properties (FitzGerald et al., 1997; Willuda et al., 1999).

Other strategies have been reported for affinity maturation of antibodies, which are not based on the phage display technology. Systems based on bacterial surface display or yeast surface display have been designed (Boder & Wittrup, 1997; Georgiou et al., 1997). Like for the above-mentioned strategies, diversity is introduced into a repertoire, and the displayed repertoire is subsequently selected. The selection is carried out by flow cytometry, which is the applicational advantage of the system. The clones selected may be propagated by simple incubation, and reselected under more stringent conditions (lower target concentration) (Boder et al., 2000; Daugherty et al., 1998). Finally, an *in vitro* system based on ribosome display was used for affinity maturation (Hanes et al., 1998;

Hanes & Pluckthun, 1997). A potential advantage, although not demonstrated, is that diversity can be introduced between rounds of selection by error-prone PCR.

A potential obstacle for the therapeutic application of the selected scFv is the mouse origin because a HAMA response may redirect the pharmacokinetics (DeNardo et al., 2003). It should be noted that the HAMA response primarily targets the Fc region of the mouse antibodies, while the variable regions seem less immunogenic. This point is further supported by the application of many chimeric antibodies in the clinic trials. Still, it is impossible to predict the immunogenicity of a trimeric or tetrameric scFv because they do expose new epitopes. However, having established the 1aa- or 0aa-linker format of phage-displayed scFvs, it seems manageable to generate libraries from human donors and select these for TF-specific binders. It also seems appealing to incorporate the selection strategy of Dahlenborg and co-workers (Dahlenborg et al., 1997), in which PBLs were immortalised with Epstein-Barr virus and selected for binding to antigen coated on magnetic beads. Whether this will enrich for higher affinity antibodies is still an open question. Alternatively, a human variant could be generated from the established libraries using chain shuffling and guided selection. However, this is usually prohibited by the manufactures that do not allow genetic manipulation of the libraries.
Humanisation of the scFv(1aa), using a novel cloning strategy and the herein described multimeric phage display format and selections strategies, has been a project of a diploma student, Delphine Tablet, in our lab. Preliminary results are promising, but the labour-intensive characterisation awaits (Tablet, personal communication).

Multivalent formats based on domain-mediated multimerisation of scFvs seem less optimal for multimerisation of the selected mouse scFv sequence because of the apparent formation of dimers of the scFv(18aa). Therefore, the trimeric and tetrameric formats of scFvs (triabodies and tetrabodies) as described here may be preferable. A similar conclusion has apparently been drawn for a Lewis^Y antibody, where more attention is being focused on triabodies and tetrabodies (Clarke et al., 2000; Power et al., 2001; Power et al., 2003; Scott et al., 2000; Tahtis et al., 2001).

3.10 Potential novel applications of a TF-specific scFv

In extension of the theoretical considerations about choice of radioisotope, scFvs with good pharmacokinetic profiles may also have alternative therapeutic applicabilities.
With respect to the antibody metabolic turnover, different clinical strategies could be developed depending on whether the antibody is internalised or not. If the antibody is internalised, it indicates

that the antibody/antigen pair may be suited for an immunotoxin strategy that relies on internalisation (Pastan, 2003; Trail et al., 2003).
In contrast, if the antibody is not internalised, it could be used in the ADEPT strategy (Antibody-Directed-Enzyme-Prodrug-Therapy). This strategy is based on a labelled enzyme-antibody conjugate that is injected and tracked via the radioactivity. At a maximal tumour to non-tumour ratio, the prodrug is administrated, and consequently converted to a cytotoxic drug selectively in the tumour (Napier et al., 2000). The advantage of the system is the tracing of the enzyme-antibody conjugate, which enables optimisation of the therapeutic window and applicable dose. It is imperative that a very high tumour to non-tumour ratio and a good tumour penetration is needed. The advantage of targeting TF would be that it has been proven exclusively tumour specific.
The immunotoxin strategy or the ADEPT strategy may apply very well to the selected TF-specific scFv trimers or tetramers, because they were isolated by phage display, and therefore proven to be active as fusion proteins.

Even with a moderate pharmacokinetic profile, the scFv(0aa) or scFv(1aa) could have therapeutic application. The reasoning being that the scFvs enable targeting of the periphery of a tumour, while other strategies can target the tumour centre in a combinatorial therapeutic approach.
Among the strategies for targeting the tumour centre are the recently reported inhibitors of angiogenesis. By cutting off the blood supply to the tumour, large and central areas of the tumour may be doomed for necrosis or apoptosis (Folkman, 2003). These areas are characterised by being isolated from the bloodstream, and therefore less targetable with RAIT, which is expected to predominantly hit the “surface” of the tumour. Many strategies have been tested for inhibition of angiogenesis, but only a few will be mentioned as it not within the scope of this thesis,: Inhibition of angiogenesis by targeting vascular endothelial growth factor (VEGF) that mediates angiogenisis has been attempted with antagonistic peptides toward VEGF (Cooke et al., 2001; Fairbrother et al., 1998) or toward the VEGF receptor KDR/FLK1 (Binetruy-Tournaire et al., 2000). A similar strategy was applied in the generation of anti-VEGF scFvs (Afanasieva et al., 2003). Alternatively, markers of angiogenesis can be targeted such as the laminin (Sanz et al., 2003; Sanz et al., 2002; Sanz et al., 2001) or the ED-B domain of fibronectin, (Borsi et al., 2002; Demartis et al., 2001; Halin et al., 2002; Pini et al., 1998; Tarli et al., 1999; Viti et al., 1999).

A novel application of chelator-conjugated recombinant antibodies in diagnostics is positron-emission tomography (PET) with the application of positron-emitting isotopes such as ^{64}Cu (Wu et al., 2000). It will be interesting to follow the development of this new *in vivo* diagnostic method and see if it will

provide an alternative to the classic glucose based PET imaging. Again the high tumour-specificity of TF makes it a promising target for *in vivo* diagnostics in the future.

3.11 Achievements and outlook

In order to provide an outlook for future milestones or strategies, the six major achievements during this PhD project will be briefly summarised.

The first breakthrough was the establishment a new multivalent scFv display format, namely the display of scFvs with dramatically shortened linkers (1aa). The reasoning for investigating the display of multimers was the lack of success in selections against TF using the common scFv libraries and the apparent need for multivalent interaction in the binding of TF. This is presumably also the case for other short non-charged carbohydrates. The short scFv linkers have been shown to direct the formation of multimeric complexes of scFvs in solution and lead to increased functional affinity. A similar increase in functional affinity was demonstrated in this work for scFvs displayed on phage particles, and is therefore assumed to be the driving force in the successful selections of the scFv(1aa) libraries against TF. Although not attempted, it can be assumed that the scFv(1aa) display format is also applicable in selections against other interesting carbohydrate targets, where binding is assumed to depend on a multivalent interaction. Finally, it should not be underscored that the multimeric scFvs generated by selection could be superior to multimeric scFvs generated be genetic manipulation with respect to multimer stability, affinity and production.

The second breakthrough was the generation of TF-specific multimeric scFv with high specificity and affinity. The ELISA analysis revealed an outstanding specificity toward TF, with a minor cross-reactivity to core-2, which is presumably insignificant as the core-2 structure is always masked in longer oligosaccharide chains and has not been found in tissues ((Brockhausen, 2000) and references therein). It should also be noted that no Core-2 antibody has been reported. The *in vitro* affinity examination by surface plasmon resonance revealed dissociation constants in the sub-micromolar range (~88 nM for the scFv(0aa) and 220 nM for the scFv(1aa)), which must be considered good for antibodies towards short non-charged carbohydrates. The affinities were high enough to enable application in immunocytochemistry and immunohistochemistry demonstrating a potential application of the antibodies in diagnostics and prognostics (chapter 1.2). The affinity-purified scFv(1aa) was shown to have an affinity of 20 nM which was the highest affinity measured in this work and close to that of the IgM anti-TF antibodies A78-G/A7, Nemod TF-1 and Nemod TF-2 (Dr. Butschak, personal communication).

The third breakthrough was the successful production and purification of stable multimeric scFvs. It has been generally believed that the multimerisation of scFvs was severely influenced by the concentration of the protein. The examination of affinity of monovalent scFvs has been compromised by the tendency of these to form dimers at high protein concentrations, which leads to a higher functional affinity. In extension of these observations, it was believed that the multimeric scFvs would disintegrate at low protein concentrations, and therefore not have the expected efficacy in therapeutical applications, where the protein is diluted upon administration. Here however, it is demonstrated that the multimeric scFv-complexes are very stable, and do not disintegrate upon dilution. Furthermore, they can be stored at -80°C for at least 6 month without any detectable loss of binding activity or altered elution profile by size exclusion chromatography.

The fourth breakthrough was the establishment of a protocol for the conjugation of DTPA to the scFvs and subsequent labelling with ^{111}In without compromisation of the specificity and binding activity. The scFvs encodes only the binding domains of antibodies, and there is a risk that the conjugation will target the binding site and thereby alter the specificity and affinity, but importantly, this was found not to be the case. Furthermore, the chemical conditions (pH in particular) needed for the conjugation and the labelling are rather harsh ranging from pH 8.7 to pH 4.3, which could also have damaging effects on the activity of the scFv. The successful demonstration of conserved binding activity of the ^{111}In-labelled scFv(1aa) was very important and further indicated the apparent high stability of the multimeric scFvs.

The fifth major breakthrough was the validation of TF as a molecular target for *in vivo* cancer targeting. For the first time, applying two different tumour models, it was established that TF is indeed not only a good tumour marker, but also a valid tumour target for RAIT, which a crucial finding for the further development of RAIT targeting TF on tumours.

The sixth and last major breakthrough was the results of the biodistribution studies. The tumour targeting was very good with 5% ID/g and 10% ID/g in the two different models, which ranks them in the better half of the published tumour targeting studies. In addition, the antibody formats, especially the scFv(0aa), were validated by very low kidney burdens in comparison to other recombinant antibody formats.

Evaluation of these pioneering data enables the design of new milestones and strategies for the project.

Although the tumour targeting with the scFv(0aa) and scFv(1aa) were very satisfying and demonstrate successful targeting of TF *in vivo* for the first time, it would be interesting to investigate additional tumour models, in particular some that enables comparison of targeting with other antibody/antigen pairs (i.e. CEA antigen on LS-174-T xenogafts).

As mentioned before, the next step in the examination of the therapeutic potential of the scFv(0aa) would be a more thorough biodistribution trial, in which the pharmacokinetics should be investigated with more timepoints. The pharmacokinetic profile may reveal a potential therapeutic window (in which the tumour to non-tumour ratios are large), and thereby give indications about optimal radioisotopes. However, certain parameters should be kept in mind. First, the yield of expression and purification of the scFv(0aa) is rather low (~250 µg/l culture), which is sufficient for biodistribution trials, and the initial therapeutical trial, but not in the range for the clinical trials. Second, the affinity of the scFv(0aa) towards the cells tested was 100-1000 fold lower than what is considered optimal for RAIT (Adams et al., 2001; Graff & Wittrup, 2003). Therefore, I would suggest to attempt the selection of a trimeric or tetrameric scFv from a library in the scFv(0aa) or scFv(1aa) format generated from human donors, which is strongly encouraged by the finding of TF-specific IgGs in human sera by Butschak (Butschak & Karsten, 2002). The presence of IgGs can be an indicator that some of the antibodies contain somatic hypermutations, and therefore possibly a higher affinity. The potential of such an investigation is also that new clones may be expressed with higher yields, which seems crucial for the clinical trials. Moreover, a clone of human origin could circumvent any problems associated with a potential HAMA response. Finally, the humanisation of the scFv(1aa) is in process, and the results are of course awaited with impatience and will influence the evaluation