TARGETING MELANOMA USING SPECIFIC ANTIBODY FRAGMENTS

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Abstract

Targeting tumours for diagnostic or therapeutic purposes using monoclonal antibodies or their fragments has shown great promise. However, the optimal characteristics of molecules for such interventions have yet to be defined. Melanoma presents an increasing clinical burden and a clear need for improved diagnostic and therapeutic strategies is evident. Numerous anti-melanoma monoclonal antibodies have been developed. Unfortunately, these molecules have failed to reach routine clinical use.

In this thesis, the in vitro and in vivo characteristics of a series of anti-melanoma single chain antibody fragments (scFvs) targeting high molecular weight melanoma-associated antigen (HMW-MAA) are investigated. Attempts are made to optimise the production and purification processes of the recombinant antibody fragments. V-domain-swapped and chain-shuffled affinity variants are produced by PCR and antibody phage display techniques to allow study of the effect of these modifications in vitro and in vivo in a murine model. The coadministration of cationic amino acids such as lysine has been shown to reduce renal accumulation of larger tumour-targeting antibody fragments and the efficacy of this strategy with scFv is investigated.

These experiments show the V-domain-swapping of the scFv studied increases protein yield by 50% without hindering tumour targeting in vitro or in vivo. Immunoaffinity purification similarly increases protein yield by 50%. Problematic scFv aggregation is
noted at higher concentrations and appears independent of buffer conditions. However, the inclusion of imidazole in the storage buffer solution increases scFv solubility considerably. Increasing affinity by means of chain shuffling results in a clear increase in tumour targeting efficacy \textit{in vivo} and it is likely that further increase in affinity will, in this model, result in further benefit. Coadministration of L-lysine reduces renal scFv accumulation 18 hours post injection by over 80\% and could be employed to clarify imaging studies or reduce renal toxicity associated with scFv-derived therapies.
For my parents, my brother, Brian

and my best friend and darling, Julie
Statement

I attest that the work contained in this thesis is entirely my own with the exception of those contributions acknowledged below. The scFvs LHM2 and aCD18 and the chain-shuffled chimaeric antibody-phage scFv library used in these experiments were developed by Dr. Jorg Kupsch. The sub-cloning of scFv RAFT2 and initial characterisation of RAFT3 scFv were performed by Dr. Norbert Kang. All DNA sequencing was carried out by Mrs Nimesha Patel.
Acknowledgement

I would like to thank Dr Jörg Kupsch at the RAFT Institute, Northwood for his help in the preparation of this thesis. These studies would not have been possible without his advice and unceasing encouragement. I would also like to thank Professor Roy Sanders (RAFT Institute, Northwood) and Professor Colin Green (Northwick Park Institute for Medical Research, Harrow) for their help and support both specifically and in general. I remain grateful to Mr Norbert Kang (Plastic Surgery Dept., Mount Vernon Hospital) for passing on the lessons he learned the hard way!

I am deeply indebted to Dr George Wilson, Mr Peter Russell and the animal house staff at the Gray Laboratory for their help with the animal studies which form the core of this thesis. I wish to acknowledge the assistance of Dr Sue Douglas, Dr Marjorie Girling and Mr Scott Denver (Department of Nuclear Medicine, Mount Vernon Hospital, Northwood) for their help in producing and in the safe handling of the radioactive material used in my experiments.

Finally, I would like to offer my deepest thanks to the other research fellows, PhD/sandwich students, technical/administrative staff and volunteers at the RAFT Institute and the Gray Lab. Their help and good-natured sarcasm helped me through the darker moments in this research.
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Table 5.3.2d  T:NT for $^{125}$I Labelled RAFT3 scFv
Table 5.3.2e  T:NT for $^{125}$I Labelled Anti-CD18 scFv
Table 5.3.2f  T:NT for $^{125}$I Labelled RAFT3 scFv & Lysine

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Table 7.3.6  Summary of Radio-iodination Results
Table 7.3.7  Scatchard Summary
Table 7.3.8.1  Plasma Clearance Summary
Table 7.3.8.2a  %ID/g for $^{125}$I Labelled RAFT3 scFv
Table 7.3.8.2b  %ID/g for $^{125}$I Labelled RAFT3DB scFv
Table 7.3.8.2c  %ID/g for $^{125}$I Labelled Anti-CD18 scFv
Table 7.3.8.2d  T:NT for $^{125}$I Labelled RAFT3 scFv
Table 7.3.8.2e  T:NT for $^{125}$I Labelled RAFT3DB scFv
Table 7.3.8.2f  T:NT for $^{125}$I Labelled Anti-CD18 scFv
Table 7.3.8.2g  Radio-localisation Index $^{125}$I Labelled RAFT3 scFv
Table 7.3.8.2h  Radio-localisation Index $^{125}$I Labelled RAFT3DB scFv
Chapter 1

1. Introduction
1. Introduction

Descriptions of melanoma first appeared in the literature around 200 years ago and the credit for the earliest description remains controversial. In 1799 John Hunter, at the Royal College of Surgeons in London, described a “cancerous fungous excrescence” behind the jaw of a 35 year old man thought to represent melanoma. Laennec, a pupil of Dupuytren, lectured on “melanose” in 1804 with a subsequent publication in 1806 (Laennec, 1806). The first definite description in English was provided by Norris in 1820 (Norris, 1820) and Carswell coined the word “melanoma” in 1838 (Carswell, 1838). Numerous publications followed over the 19th century until Handley delivered his landmark paper on the pathology of melanoma which was to shape the surgical management of the disease for over 50 years (Handley, 1907).

1.1 The Clinical Problem

1.1.1 Epidemiology of Melanoma

Melanoma continues to be an increasing clinical problem across the Western world. A rapidly increasing trend was first noted in the 1950s and 60s when the US incidence rose by 72% in 18 years (Lee and Carter, 1970). Between 1979 and 1994 the age-adjusted incidence of melanoma in Scotland rose by 120% for males and 83% for females (MacKie et al., 1997) whilst in the USA between 1973 and 1994 the overall incidence of melanoma rose by 120.5% (Hall et al., 1999). Once considered a rare tumour, melanoma is now the eighteenth commonest cancer in the UK, the tenth commonest in the USA and the fourth commonest in Australia (Serraino et al., 1998). An estimated 40,300 new cases presented in the US in 1997 with 7,300 deaths (Parkin, 1998). The lifetime risk of melanoma of a child born in the USA in 2000 has been estimated at 1 in 75, rising from 1 in 1500 for those born in 1935 (Landis et al., 1998). These increases are largely restricted to white populations with little change in the incidence in black men and women (Fig. 1.1.1a). Whilst melanoma is relatively rare in
Fig. 1.1.1a  Age-adjusted Incidence of Melanoma by Race and Sex (US)


Fig. 1.1.1b  Age-adjusted Mortality of Melanoma by Race and Sex (US)

black populations, the overall survival figures are considerably poorer than those for white populations (SEER Cancer Statistics Review 1974-1997).

The male preponderance seen in the US is the reverse of that seen in the UK, though the rate of increase in incidence in males in the UK exceeds that in females (MacKie et al. 1997). Of more concern still is the likelihood that incomplete reporting of melanoma makes these figures an underestimate of the real problem (Karagas et al. 1991; Melia et al. 1995).

This dramatic increase in incidence has been accompanied by a continuing if less striking increase in the mortality rate (Fig. 1.1.1b). This smaller increase in mortality is due, at least in part, to an increasing proportion of thin melanomas being diagnosed which may relate to increased public awareness (MacKie and Hole, 1992; MacKie, 1993; Rhodes, 1995). Indeed, some studies have suggested that, were this trend to continue, the overall mortality from melanoma in the US may peak in the early decades of the 21st century (Scotto et al. 1991; Roush et al. 1992). However, until and unless these predictions are proved correct, malignant melanoma will present an increasing challenge and burden to our stretched healthcare resources.

1.1.2 Aetiology of Melanoma

The major risk factor for melanoma is sun exposure to which more than half of all melanomas world-wide and in excess of 80% in white populations have been attributed (Armstrong and Kricker, 1993). This association was first clearly demonstrated in the 1950s when Lancaster described the link between incidence of melanoma and latitude for white European populations across the globe (Lancaster, 1956). The same relationship has also been noted in terms of population UV exposure (Brozena et al., 1993) and radiation in the ultraviolet B range (280 to 320nm) is thought to be the most important (Koh et al., 1990). Recently, the association with latitude in the US has become less pronounced, perhaps in response to greater mobility in the population (Lee, 1997). Intense, intermittent sun exposure has been shown to play a much greater role than cumulative sun exposure (Elwood and Jopson, 1997). Indeed, childhood sunburn
has frequently been implicated in the aetiology of melanoma and seems particularly important (Zanetti et al., 1992; Autier and Doré, 1998). In contrast, basal cell and squamous cell carcinoma are thought to be associated with chronic, cumulative UV exposure. The potential reasons for these differences have recently been studied (Gilchrest et al., 1999; Halachmi and Gilchrest, 2001). Keratinocytes, which proliferate easily, readily undergo apoptosis (or programmed-cell death) following UV-induced DNA damage, thus protecting the individual from potential malignant change. Melanocytes, however, have a limited proliferative ability and, therefore, need to be more resistant to apoptosis to retain their numbers. This is achieved by their expression of higher levels of the anti-apoptotic proto-oncogene Bcl-2 (Plettenberg et al., 1995; Ramsay et al., 1995). They are therefore more reliant on their DNA-repair mechanisms to reverse any episodes of UV-induced DNA damage in order to prevent malignant change. These DNA-repair mechanisms have been shown to be inducible by frequent low UV doses. Further evidence exists that certain DNA-repair enzymes can also stimulate melanogenesis (Gilchrest et al., 1993). Sudden, excessive exposure to UV radiation may, therefore, lead to malignant change before induction of sufficient DNA-repair ability has occurred. Melanocytes are far more vulnerable to this due to their resistance to undergoing apoptosis as described above. Intermittent exposure to high UV doses is, therefore, more likely to result in melanomas in relatively young patients. Non-melanoma skin cancer is more likely to occur following multiple low dose UV exposures, since high doses of UV radiation are more likely to lead to the death of the cell involved by apoptosis. It therefore tends to occur in older patients who have acquired a sufficient number of lower-dose UV exposures.

The problem of Ozone depletion leading to increasing UVB levels may lead to an increase in melanoma and other skin cancers in the future though no direct evidence exists as yet. Similarly, some preliminary evidence has linked melanoma to sun beds and tanning parlours (Swerdlow and Weinstock, 1998).

A number of individual factors are also associated with melanoma (Table 1.1.2). These primarily relate to increased sun sensitivity, a family predisposition to the disease and to the presence of multiple simple or atypical naevi on the skin. The presence of atypical
<table>
<thead>
<tr>
<th>Factor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fair skin</td>
<td>Beral 1983</td>
</tr>
<tr>
<td>Blonde hair</td>
<td>Beral 1983</td>
</tr>
<tr>
<td>Family history of melanoma</td>
<td>Greene 1985b</td>
</tr>
<tr>
<td>Familial atypical mole melanoma syndrome</td>
<td>Lynch 1978, Greene 1985a</td>
</tr>
<tr>
<td>Xeroderma pigmentosum</td>
<td>Tullis 1984</td>
</tr>
<tr>
<td>Previous melanoma</td>
<td>Rhodes 1987</td>
</tr>
<tr>
<td>Previous non-melanoma skin cancer</td>
<td>Evans 1988, Lindelof 1991</td>
</tr>
<tr>
<td>Large number of benign naevi</td>
<td>Greene 1985b, Green 1985, Augustsson 1991, Kruger 1992</td>
</tr>
<tr>
<td>Giant congenital pigmented naevi</td>
<td>Reed 1965, Trozak 1975, Rhodes 1981</td>
</tr>
</tbody>
</table>
naevi on the skin correlates with melanoma risk (Schneider et al., 1994). Kruger described an association between simple benign naevi on the trunks of males or lower limbs in females and the development of melanoma in those areas (Kruger et al., 1992). However, these may simply be a direct result of cumulative sun exposure. Such associations between different risk factors have often made definitive identification of the most important ones difficult.

1.1.3 Metastatic Melanoma and Its Management

Melanoma staging is generally described using the AJCC (American Joint Committee on Cancer) system shown in Table 1.1.3. A correlation between tumour thickness and survival was first established in 1970 (Breslow, 1970). Since then, numerous reports have demonstrated that tumour thickness is the single most important prognostic factor in Stage I and II melanoma (Balch et al., 1978; Drzewiecki and Andersen, 1982; Karakousis et al., 1989; Schultz et al., 1990; Balch et al., 1992). Thin melanomas (<1.5mm) have over 97% 5 year survival, dropping to 72.5% for intermediate thickness lesions (1.51-4.0mm) and 56.3% for thick lesions (>4mm) (Buzaid et al., 1997). Metastasis of melanoma either to regional lymph nodes or more distantly (Stage III – IV) has a grave effect on prognosis. Involvement of loco-regional lymph nodes reduces 5 year survival to less than 40% (the prognosis worsening with increasing number of nodes involved) (Balch, 1992) whilst distant metastases reduce it to around 6% (Barth et al., 1995).

Of an estimated $563 million spent treating melanoma in the US in 1997, 90% was attributable to the 20% of patients falling into stages III and IV (Tsao et al., 1998). Despite this massive expenditure, little improvement in survival figures for these patient groups has been achieved over much of the past century.

Melanoma metastatic to regional lymph nodes is managed by surgical excision. Histopathological analysis of the involved nodes gives further prognostic data with the absolute number of involved nodes being the most important factor (Balch, 1992) (Fig. 1.1.3). Elective lymph node dissection (ELND) of a clinically disease-free nodal basin
### Melanoma Staging

#### TNM DEFINITIONS

**Primary tumour (pT)**

<table>
<thead>
<tr>
<th>pTX:</th>
<th>Primary tumour cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTO:</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>pTis:</td>
<td>Melanoma in situ (atypical melanocytic hyperplasia, severe melanocytic dysplasia), not an invasive lesion (Clark's Level I)</td>
</tr>
<tr>
<td>pT1:</td>
<td>Tumour 0.75 mm or less in thickness and invades the papillary dermis (Clark's Level II)</td>
</tr>
<tr>
<td>pT2:</td>
<td>Tumour more than 0.75 mm but not more than 1.5 mm in thickness and/or invades to papillary-reticular dermal interface (Clark's Level III)</td>
</tr>
<tr>
<td>pT3:</td>
<td>Tumour more than 1.5 mm but not more than 4 mm in thickness and/or invades the reticular dermis (Clark's Level IV)</td>
</tr>
<tr>
<td>pT3a:</td>
<td>Tumour more than 1.5 mm but not more than 3 mm in thickness</td>
</tr>
<tr>
<td>pT3b:</td>
<td>Tumour more than 3 mm but not more than 4 mm in thickness</td>
</tr>
<tr>
<td>pT4:</td>
<td>Tumour more than 4 mm in thickness and/or invades the subcutaneous issue (Clark's Level V) and/or satellite(s) within 2 cm of the primary tumour</td>
</tr>
<tr>
<td>pT4a:</td>
<td>Tumour more than 4 mm in thickness and/or invades the subcutaneous tissue</td>
</tr>
<tr>
<td>pT4b:</td>
<td>Satellite(s) within 2 cm of the primary tumour</td>
</tr>
</tbody>
</table>

**Regional lymph nodes (N)**

<table>
<thead>
<tr>
<th>NX:</th>
<th>Regional lymph nodes cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0:</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1:</td>
<td>Metastasis 3 cm or less in greatest dimension in any regional lymph node(s)</td>
</tr>
<tr>
<td>N2:</td>
<td>Metastasis more than 3 cm in greatest dimension in any regional lymph node(s) and/or in-transit metastasis</td>
</tr>
<tr>
<td>N2a:</td>
<td>Metastasis more than 3 cm in greatest dimension in any regional lymph node(s)</td>
</tr>
<tr>
<td>N2b:</td>
<td>In-transit metastasis</td>
</tr>
<tr>
<td>N2c:</td>
<td>Both (N2a and N2b)</td>
</tr>
</tbody>
</table>

Note: In-transit metastasis involves skin or subcutaneous tissue more than 2 cm from the primary tumour but not beyond the regional lymph nodes.

**Distant metastasis (M)**

<table>
<thead>
<tr>
<th>MX:</th>
<th>Distant metastasis cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0:</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1:</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a:</td>
<td>Metastasis in skin or subcutaneous tissue or lymph node(s) beyond the regional lymph nodes</td>
</tr>
<tr>
<td>M1b:</td>
<td>Visceral metastasis</td>
</tr>
</tbody>
</table>

#### Group Staging

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I a</td>
<td>pT1 N0 M0</td>
</tr>
<tr>
<td>I b</td>
<td>pT2 N0 M0</td>
</tr>
<tr>
<td>II a</td>
<td>pT3 N0 M0</td>
</tr>
<tr>
<td>II b</td>
<td>pT4 N0 M0</td>
</tr>
<tr>
<td>III a</td>
<td>Any pT N1 M0</td>
</tr>
<tr>
<td>III b</td>
<td>Any pT N2 M0</td>
</tr>
<tr>
<td>IV</td>
<td>Any pT Any N M1</td>
</tr>
</tbody>
</table>
### Prognostic Markers in Regional Metastatic Melanoma
(Balch et al. 1992)

<table>
<thead>
<tr>
<th>Prognostic Parameter</th>
<th>Five Year Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal positivity</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>2-4</td>
<td>38</td>
</tr>
<tr>
<td>&gt;4</td>
<td>18</td>
</tr>
<tr>
<td>Extracapsular spread</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>26</td>
</tr>
<tr>
<td>Absent</td>
<td>37</td>
</tr>
<tr>
<td>Ulceration of primary</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>20</td>
</tr>
<tr>
<td>Absent</td>
<td>39</td>
</tr>
<tr>
<td>Thickness of primary</td>
<td></td>
</tr>
<tr>
<td>&lt;4mm</td>
<td>38</td>
</tr>
<tr>
<td>&gt;4mm</td>
<td>24</td>
</tr>
<tr>
<td>Age of patient</td>
<td></td>
</tr>
<tr>
<td>&lt;50 years</td>
<td>36</td>
</tr>
<tr>
<td>&gt;50 years</td>
<td>24</td>
</tr>
</tbody>
</table>
has been practised widely, particularly in the US. This strategy is aimed at arresting micrometastatic disease before further progression. Early non-randomised retrospective trials suggested a possible survival advantage using ELND (Wanebo et al., 1975; Milton et al., 1982; Reintgen et al., 1983; McCarthy et al., 1985; Drepper et al., 1993; Rompel et al., 1995). However, two large prospective randomised studies demonstrated no survival benefit in patients treated in this way ELND (Veronesi et al., 1977; Veronesi et al., 1982; Sim et al., 1986; Sim et al., 1997). It seems likely that this failure to improve patient outcome resulted from a combination of unnecessary surgery in patients without nodal involvement and ineffective surgery in patients already harbouring distant metastases. One more recent trial has suggested a survival advantage in a subsets of patients with intermediate thickness melanomas, ulcerated primaries and / or limb melanomas (Balch et al., 2000). This study is discussed in more detail in Section 1.1.4.6.

The efficacy of therapy in distant metastases has been disappointing (Balch et al., 1983; Barth et al., 1995). Melanoma is relatively resistant to both radiotherapy and chemotherapy. Most treatment regimes have focussed on dacarbazine alone (Lee, 1995) or combination chemotherapy involving agents such as dacarbazine, cisplatin and vinblastine (Atkins, 1997). Response rates are typically in the 24-48% range and generally have not improved on that seen with dacarbazine alone (Lee, 1995; Rusthoven et al., 1996; Atkins, 1997; Chapman et al., 1999; Serrone et al., 2000). In these cases, responses are typically of short duration (median 3-6 months) and long-term complete responses are seen in only 1-2% of patients (Hill et al., 1984).

Recently, however, the advent of Interferon-α (IFN-α) as an immuno-modulatory agent has given some cause for optimism. Used as a single agent, response rates of around 16% have been demonstrated in patients with stage IV melanoma receiving high dose interferon-α2b over a one year period (Kirkwood, 1994; Argawala, 1996). Up to one half of the patients showed a complete response though this was largely confined to patients with low-volume, soft tissue disease. Morbidity, however, is significant with high dose interferon and a clearly significant survival advantage superior to conventional therapy remains to be demonstrated. Early reports of combination therapy
using conventional chemotherapeutic regimes together with IFN-α ("Biochemotherapy") have been encouraging (Legha et al., 1997). Interestingly, improved survival has also been described in “high risk” melanoma patients (those with thick primary tumours or resected nodal disease) receiving similar interferon doses (Kirkwood et al., 1996; Kirkwood et al., 2000). In the second study, high and low-dose interferon regimens were included. High dose interferon produced an increase in disease free survival (44%, 40%, and 35% at 5 years for high dose, low dose and observation arms, respectively) but no statistically significant increase in overall survival during the course of the study. These findings, together with the not inconsiderable systemic toxicity associated with interferon therapy increases the importance of careful patient selection and accurate staging in maximising any overall benefit of this therapy.

These clinical data have intensified efforts to more accurately stage the disease and, in particular, disease in the lymph node basin. Detection of micrometastatic disease would allow selection of patients for LND or any potential adjuvant therapy whilst better detection of distant metastatic disease would spare some patients surgery to the lymph node basin and the resulting morbidity when no benefit would follow.

1.1.4 Diagnosis of Metastatic Melanoma

Ideally any technique used in the detection of metastasis should show high sensitivity and specificity whilst allowing easy whole body tumour detection. In clinical practice, metastases are currently more likely to be indicated by patient history or physical examination than be detected by investigation (Weiss et al., 1995). Currently, chest radiography, computed tomography (CT) and ultrasound are the mainstays of diagnosis with Magnetic Resonance Imaging (MRI) being used less frequently. Positron Emission Tomography (PET) and sentinel lymph node biopsy (SNB) are becoming more widely used, though at present SNB is principally limited to clinical trials. The usefulness and limitations of these various techniques is discussed briefly below.
1.1.4.1 Chest Radiography

Chest X-rays (CXR) are the most frequently used imaging modality in melanoma. Whilst used commonly in assessing lungfields in newly diagnosed melanoma, the detection rate for secondary tumour deposits is low. In one study of 876 patients with clinically localised melanoma, 130 (15%) had a suspicious CXR meriting further investigation. Of these only 1 patient (0.1%) had pulmonary metastases (Terhune et al., 1998). In an earlier post-mortem study, 9 of 17 patients with metastatic melanoma (53%) had pulmonary melanoma deposits not evident on recent CXRs (Simeone et al., 1977). These findings suggest that CXR is of little value in asymptomatic patients with clinically localised melanoma.

1.1.4.2 Computed Tomography

Computed tomography (CT) is effective in detecting pathologically enlarged structures >2cm in diameter but does not confirm the presence of metastasis (Buzaid et al., 1993). Micrometastatic deposits ([2mm (Siegel, 1996)) would fall below the size necessary for CT diagnosis and makes its usefulness in screening clinically localised primary melanoma limited. The absorbed radiation dose for CT is not inconsiderable at around 8.0 mSv for an abdominal CT (POPUMET Ionising Radiation Regulations 1988). This is not critical in patients likely to have metastatic disease but would be a high dose for a “screening” procedure in lower-risk patients.

1.1.4.3 Ultrasound

Ultrasound (U/S) is a non-invasive and relatively inexpensive modality and is the mainstay for detecting intra-abdominal pathology. This technique is heavily dependent on the experience and expertise of the operator (Wojak et al., 1995) and individual patient factors such as obesity can make assessment of the intra-abdominal organs difficult (Oria, 1998). Whole body ultrasound scanning presents difficulties since the technique is time-consuming and allows for visualisation of only small volumes of tissue at any one time.
Ultrasound has been shown to be useful in assessing regional lymph node basins in primary melanoma and to give more accurate results than clinical palpation (Rossi et al., 1997; Blum et al., 2000). However, in this setting, ultrasound is non-specific and any identified node would require biopsy confirmation of the diagnosis.

1.1.4.4 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI), like ultrasound, has the advantages of being non-invasive and free from ionising radiation. MRI is a non-specific modality but has the ability to identify specific tissues and substances by characteristic patterns. Areas of melanin content, haemorrhage and intra-tumour necrosis may be highly suggestive of melanoma (Premkumar et al., 1996). Indeed MRI has been suggested as a tool capable of distinguishing cutaneous melanoma from benign cutaneous melanocytic lesions (Takahashi and Kohda, 1992). However, the degree of discrimination has proved to be relatively poor (Maurer et al., 1995).

MRI scanners tend to be claustrophobic and high quality images can take many minutes to acquire. Indeed, patient anxiety can be a significant problem in MRI scanning (Goyen and Klewer, 1997; McIsaac et al., 1998), affecting up to 40% of patients, and resultant motion artefact can affect the quality of the images obtained (Dantendorfer et al., 1997).

These problems, together with the high cost of the procedure (over £700 for a whole body scan, Mount Vernon Hospital Radiology Dept. figure) have prevented the widespread use of MRI for whole body screening in melanoma. In screening for loco-regional metastasis, MRI has been shown to offer similar results to CT scanning (Buzaid et al., 1995).

1.1.4.5 PET Scanning

Positron Emission Tomography (PET) is a relatively new and exciting tool in the detection of metastatic melanoma (Strauss and Conti, 1991; Spratt, 1998; Schwimmer et
The positron-emitting radioisotope Fluorine-18 is incorporated in 18F-fluoro-2-deoxy-D-glucose (18FDG). The 18FDG is taken up by melanoma to a much greater extent than by normal tissues due to its higher metabolic rate (Kern, 1991) and allows the detection of metastases. 18F has a short half-life (108 minutes) which reduces problems associated with disposal of radioactive waste and patient radiation dosage but additionally necessitates a close proximity of scanning equipment to the production facility.

Whilst a positive PET scan in the setting of suspected metastatic melanoma is highly suggestive of secondary spread (Wagner et al., 1997), the investigation is not without its potential pitfalls. Since PET-detection of melanoma relies on its high metabolic rate, other highly metabolic tissues such as cardiac muscle, tense musculature, some other tumours and inflammatory lesions can provide false positive results (Hoh et al., 1993; Engel et al., 1996). Recently, Tyler et al. reported on 106 clinically Stage III melanoma patients undergoing PET scanning (Tyler et al., 2000). Of 144 positive areas on PET, 37 (39%) proved to be false positives, though a significant proportion of these could be attributed to clinically-evident non-malignant conditions. Reviewing data from 13 studies, however, Schwimmer et al. conclude that PET has a sensitivity of 92% and a specificity of 90% for metastatic melanoma (Schwimmer et al., 2000). It is impressive results such as these which have rapidly established PET as a valuable investigative tool in melanoma.

1.1.4.6 Sentinel Lymph Node Biopsy

The concept of a sentinel lymph node representing the first draining node in a regional lymph node basin was suggested in 1977 by Cabanas in the context of penile carcinoma (Cabanas, 1977). Cochrane and Morton have proposed the method of intraoperative lymphatic mapping and sentinel node biopsy in melanoma (Cochran et al., 1992; Morton et al., 1992). The method involves identifying the sentinel lymph node, which is the first in the regional chain to drain the area of skin affected by melanoma and a reliable indicator of nodal status in the whole basin (Morton et al., 1992; Ross et al., 1993; Reintgen et al., 1994; Thompson et al., 1995). This is performed pre-operatively.
by lymphoscintigraphy using $^{99m}$Tc-colloid (Alex and Krag, 1993; Krag et al., 1995) and per-operatively following intra-dermal injection of patent-blue dye (Wong et al., 1991) which is taken up by the sentinel node. This node is then excised. Morton originally recommended frozen-section examination with the patient still under anaesthetic. However, subsequent experience has suggested that full histological analysis is necessary. If occult micrometastases are identified, formal block dissection is performed (Selective Lymph Node Dissection, SLND). If histology reveals no evidence of tumour, then no dissection is performed and the patient followed up routinely.

SLNB has potential both from a staging and a treatment perspective. The technique initially evolved from controversy over the place of elective lymph node dissection (ELND) in malignant melanoma. It has been suggested that intermediate thickness melanoma patients (1-4mm), having a significant risk of developing regional lymph node metastases without distant disease might benefit from ELND to remove micrometastases (Balch et al., 1979; Balch, 1988). A number of retrospective studies suggested a possible benefit for these intermediate thickness melanoma patients undergoing ELND (Wanebo et al., 1975; Milton et al., 1982; Reintgen et al., 1983; McCarthy et al., 1985; Drepper et al., 1993; Rompel et al., 1995) whilst others did not (Elder et al., 1985; Slingluff et al., 1994; Coates et al., 1995). Prospective randomised trials were established to try to clarify the issue. The WHO and Mayo Clinic trials showed no survival benefit from ELND (Veronesi et al., 1977; Veronesi et al., 1982; Sim et al., 1986; Sim et al., 1997) but suffered from criticism of the patient stratification and the lack of subset analysis. Two subsequent prospective studies have been undertaken to answer these criticisms. The WHO Trunk Trial showed no overall survival benefit in patients with trunk melanoma thicker than 1.5mm undergoing ELND but that patients with nodal disease probably benefited (Cascinelli et al., 1998). Interim results from the Intergroup Melanoma Committee Trial suggested a small subset of patients might benefit from ELND (Balch et al., 1996). The final 10 year follow up demonstrated that ELND benefited patients with non-ulcerated tumours, patients with tumours 1-2mm in thickness and patients with limb primaries (Balch et al., 2000).
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The survival advantages in the latter two studies were not dramatic and involved subsets of patients only. The authors in both studies suggested that sentinel lymph node biopsy might allow the selection of high risk patients (i.e. those with nodal micrometastases) for ELND and prevent unnecessary morbidity in lower risk patients and reveal a higher overall benefit in patients selected for ELND. Several prospective multicentre trials have been established to investigate the effect of SNB and SLND on survival. One such trial, comparing SNB/SLND to ELND has now been reported and the authors conclude that SNB/SLND is equivalent to ELND but without the resultant morbidity for a large number of patients (Essner et al., 1999). However, of more interest will be those studies comparing SNB with or without LND to simple observation alone.

SNB may prove as important in terms of staging and selection of patients for adjuvant therapy. Two components are required for accurate staging using SNB – accurate localisation of the sentinel node and reliable histological evaluation of the tissue obtained. The former has been clearly demonstrated. Utilising vital blue dye and immunoscintigraphy, sentinel lymph nodes can be successfully identified in over 96% of patients (Albertini et al., 1996; Gershenwald et al., 1998). Metastasis in the lymph node basin beyond a negative sentinel node occurs in less than 1% of cases (Morton et al., 1992), indicating that the sentinel node does indeed provide an accurate picture of the nodal status in the whole basin. Routine histological examination of the node may underestimate the presence of micrometastases and serial sectioning and immunostaining may be required (Robert et al., 1993). Polymerase chain reaction (PCR) techniques identifying tyrosinase mRNA in lymph nodes of melanoma patients may prove more accurate still but the clinical relevance of PCR-positive, histology-negative lymph nodes has yet to be clearly established (Wang et al., 1994). With these points in mind, SNB clearly has a role to play in staging melanoma and may allow more accurate stratification and selection of patients for trials of adjuvant therapy.

Whatever its merits as a therapeutic modality and staging investigation, however, SNB gives no information about status of distant organs and therefore can only form one small part of the staging process in malignant melanoma.
1.1.5 Conclusion

It is clear from this brief overview of melanoma, its treatment and investigation that a gap exists in our ability to stage patients rapidly and accurately whilst minimising expense, patient discomfort and risk. The need for a single, simple investigation to determine the extent of metastatic disease is increasingly important when new therapeutic options are emerging. Optimal staging is crucial to patient selection and to accurate analysis of data from therapeutic trials, when survival advantages may be small.

Current treatment modalities for advanced or high-risk melanoma patients remain disappointing in their results. The need for more effective targeting of metastatic and even micrometastatic disease is clear.

Antibody tumour localisation is one strategy which may help fill both these voids. Radiolabelled antibodies can be used for tumour imaging whilst a similar therapeutic approach can be used to target malignant deposits with antibodies linked to radio-chemicals, toxins or immuno-therapeutic moieties. Previous attempts to target and image melanoma using antibodies have met with mixed success. However, modern molecular biology techniques may allow improvement on these early results and the development of clinically useful tools in combating melanoma. The results of such studies to date and the potential of these molecular biology techniques are discussed in the next section.


1.2 Radio-immunoscintigraphy and Tumour Targeting

1.2.1 Overview

Radio-immunoscintigraphy (RIS) is a technique wherein radiolabelled antibodies or their fragments are used to localise pathological processes in an antigen-specific manner. Simple antigen-antibody interactions cause preferential localisation of the radiotracer-antibody conjugate in the abnormal region and allow detection by means of gamma camera imaging. Tumour-associated antigens have formed the main target for this technique which became feasible with the advent of hybridoma technology in the 1970s (Köhler and Milstein, 1975). Murine monoclonal antibodies were raised against tumour antigens and hybridomas formed which provided a reliable source of anti-tumour immunoglobulins. Over the past twenty years, much work has been carried out in a variety of tumours without RIS moving forward to routine clinical practice. Numerous strategies for improving antibody tumour-localisation have been devised and are discussed below (Section 1.3).

1.2.2 Melanoma as a Target for RIS

Melanoma seems to be a highly immunogenic tumour and a strong anti-tumour response in the form of intense lymphocytic infiltration is often seen at histological examination (Crowley and Seigler, 1990). Indeed, this host response has been shown to correlate with a better prognosis (Tefany et al., 1991) and spontaneous remission has been described (McGovern, 1975; Bodurtha et al., 1976; Menzies and McCarthy, 1997). This anti-tumour response is at least in part due to the wide range of tumour-associated antigens found on melanoma cells. Over 40 melanoma-associated antigens (MAAs) have been described (Kang et al., 1997) and show varying degrees of tumour specificity. Several of these exhibit consistent expression across all stages of the disease (Herlyn, 1988) and thus make ideal candidates for antibody targeting studies. High molecular weight melanoma-associated antigen (chondroitin sulphate proteoglycan, HMW-MAA) is a major MAA which has proved to be highly immunogenic in mice and thus has frequently given rise to monoclonal antibodies produced by hybridoma
techniques (Reisfeld and Cheresh, 1987; Kupsch et al., 1995). In over 90% of primary melanoma cell cultures 80 to 100% of cells express this antigen (Herlyn et al., 1980; Herlyn et al., 1985) making it a promising target for radio-immunodetection and targeted therapy.

1.2.3 HMW-MAA Expression in Normal Tissues

The ideal radioimmunopharmaceutical is not a single entity but a combination of individual factors relating to the tumour itself, the antigenic target and indeed the targeting molecule. Some of these factors are summarised in Table 1.2.3. HMW-MAA has been extensively studied as a potential antigen for tumour targeting. As noted above (Sections 1.2.2 & 1.2.3), it is reliably expressed across the whole spectrum of melanoma (Pluschke et al., 1996; Kang et al., 1997; Kupsch et al., 1999). Immunohistochemical studies have shown its expression to be largely restricted to melanoma cells. In fact, HMW-MAA has been found on cultured melanocytes but is not seen in normal epidermal melanocytes (Hellström et al., 1983). It has also been detected at low levels on some small blood vessels (Hellström et al., 1983) and guinea pig studies have shown low levels of expression in hair follicles and some basal keratinocytes (Liao, 1987). Interestingly, a minor degree of expression on human basal keratinocytes has also been reported (Kupsch et al., 1995). While these data show this antigen to be only relatively melanoma-specific, cross reactivity with normal tissues in these studies has been very low. Indeed, Pluschke’s study revealed very little HMW-MAA expression in normal tissues by RNA blot (Pluschke et al., 1996). A low level of binding to blood vessels is unlikely to interfere greatly with melanoma targeting. These data therefore suggest that a radioimmunopharmaceutical directed at HMW-MAA will bind to melanoma in a reliable fashion with very little cross-reactivity with normal tissues.
### Table 1.2.3 Characteristics of an Ideal Radiopharmaceutical

1) Antigen against which it is directed must:

a) be specific for melanoma (i.e. no distribution on normal tissues or other tumours)
b) be expressed in melanoma in all patients
c) be expressed on all forms of melanoma
d) be expressed on primary and metastatic lesions
e) be expressed uniformly throughout the tumour 
f) be expressed at a high level in each cell and
g) not exhibit altered expression after chemo- or radiotherapy

2) The antibody or antibody fragment must:

a) have adequate affinity for the antigen 
b) retain full antigen binding activity after radiolabelling 
c) be inexpensive and easy to produce 
d) be easy to purify and refine to clinical grade 
e) be easy to radiolabel 
f) be non-toxic to the patient 
g) be non-immunogenic

3) For immunoscintigraphy, the radiopharmaceutical should:

a) exhibit sufficient levels of uptake in the tumour to be detected 
b) show little or no accumulation in normal tissues 
c) localise to the tumour rapidly and be retained for sufficient time for image capture
1.2.4 Level of Expression of HMW-MAA on Melanoma Cells

The level of antigen expression (i.e. the absolute number of HMW-MAA molecules per cell) is an important determinant of the ease with which a particular radioimmunopharmaceutical "lights up" a given tumour deposit and therefore of the efficacy of imaging or therapeutic studies based on these molecules. This simply relates to the fact that the number of available binding sites is a major factor in determining the amount of targeting molecule that accumulates in and around the lesion. For immunoscintigraphy, uniformity of antigen expression within a given tumour deposit would not be absolutely necessary since any "hotspot" would be diagnostic.

Heterogeneity of distribution is likely to be more important in therapeutic studies where it is important to target as many tumour cells as possible. The degree of antigenic heterogeneity for HMW-MAA in metastatic melanoma deposits is relatively low in comparison to other tumour-associated antigens (Natali et al., 1985; Kageshita et al., 1991). Furthermore, there is relatively low variation in HMW-MAA expression in separate deposits of melanoma in individual patients (Natali et al., 1985) and HMW-MAA is expressed to a similar extent in both primary and metastatic melanomas (Kageshita et al., 1991). These characteristics make HMW-MAA a promising candidate for both imaging and therapeutic targeting studies.

In vivo data in humans using the anti-HMA-MAA monoclonal ZME-018 for ISG have given some indication of the necessary number of antibody molecules which must bind to each melanoma cell for successful imaging to occur. In one study (Macey, 1988) the maximum accumulation of the IgG in tumour deposits occurred 3 days after injection. Studies of tumour deposits led to an estimated 35000 antibody molecules binding per tumour cell at this peak timepoint. The authors conclude that for successful ISG, the antigen should be present in at least these numbers per cell. In fact, in vitro studies on the number of antigenic sites per cell have shown that HMW-MAA is present at levels of between $2 \times 10^4$ and $1.8 \times 10^6$ molecules per melanoma cell (Giacomini et al., 1985; Shockley et al., 1990). The density of HMW-MAA on a wide variety of both cultured melanoma cells and melanoma specimens is comparable to that of other potential target
antigens such as p97 \((5 \times 10^4 \text{ to } 5 \times 10^6 \text{ molecules per melanoma cell})\) (Houghton, 1992) and gangliosides GM2, GD2, GT3 and 9-O-Ac-GD3 \((8 \times 10^6 \text{ to } 3 \times 10^8 \text{ molecules per melanoma cell})\) (Hamilton et al., 1993). These data suggest that a radioimmunopharmaceutical directed at HMW-MAA should be able to bind melanoma cells in sufficient amounts to be detected by ISG. This has been demonstrated in extensive immunoscintigraphic studies targeting HMA-MAA in melanoma patients (Buraggi et al., 1985; Lotze et al., 1986; Cerny et al., 1987; Rentsch et al., 1989; Schaling et al., 1990; Siccardi et al., 1990).

1.2.5 Effect of Chemotherapy on HMW-MAA Expression

Several studies have confirmed that cellular expression of HMW-MAA appears to be largely unaffected by treatment with immunotherapeutic and chemotherapeutic agents both in vitro (Taramelli, 1986; Murray et al., 1988) and in vivo (von Stamm et al., 1993). These findings would suggest that targeting agents directed against HMW-MAA would retain their efficacy for imaging or therapy even when these agents have been used.

1.2.6 Clinical Experience

Over 55 clinical radio-immunoscintigraphic studies in melanoma have been published. Despite this wide degree of investigation, RIS has not progressed to being a routine clinical tool in the management of malignant melanoma. These studies show a wide variation in the overall sensitivity for metastases, ranging from 48% (Jaffe et al., 1988) to 98% (Santos and Godinho, 1995). Investigations have mainly focussed on two antigens, HMW-MAA (48 studies) and p97 (10 studies). Sensitivity appears to be largely independent of the antigen studied. Authors reported significant accumulation of radiotracer in normal tissues hampering identification of metastatic deposits (Cerny et al., 1987; Boni et al., 1995). Studies using enzymatically-produced antibody F(ab')\(_2\) and Fab fragments failed to show consistently greater sensitivity (26 to 83% for F(ab')\(_2\) (Feggi et al., 1993; Boni et al., 1995), 58 to 93% for Fab (Lotze et al., 1986; Blend et al., 1992). Direct comparisons between studies are hampered by the wide variety of
protocols involved and the varying data collected and reported. Even when the same antibody carrying the same radiolabel has been used, considerable variation appears between studies in overall sensitivity (e.g. (Loeffler et al., 1994) (80%) and (Santos and Godinho, 1995) (98%)). The sensitivities described in these in vivo studies above are at least comparable to those found in more conventional imaging studies such as CT (Elliott et al., 1989), MRI (Buzaid et al., 1995) and PET (Schwimmer et al., 2000). Indeed, in the more successful studies, the sensitivity approaches or exceeds that of PET (92%) (Schwimmer et al., 2000).

Whilst not achieving ideal detection of metastases in these patients and hindering firm conclusions by the variety of protocols chosen, these studies demonstrate the potential efficacy of immunoscintigraphy in melanoma and the problems associated with non-specific accumulation in the tissues. From this starting point, we must consider the possibilities for improving the tumour to normal tissue contrast in melanoma antibody localisation studies.
1.3 Strategies to Improve Tumour Localisation

Numerous strategies have been proposed and attempted in order to improve the tumour specificity and tumour retention of antibodies and their derivatives. Fundamental to these approaches is an understanding of the factors which hinder the efficient, specific accumulation of targeting molecules in tumours:

1.3.1 Factors Impeding Tumour Targeting

1.3.1.1 Abnormal Tumour Vascularity

Tumour vasculature is typically of a heterogeneous nature with areas of relative ischaemia and even tumour necrosis evident (Solesvik et al., 1982; Jain, 1988; Konerding et al., 1989). Such varying tumour blood flow results in varying delivery of an antibody molecule to the antigenic target in the tumour (Epenetos et al., 1986; Dykes et al., 1987).

Whilst a tendency to increased capillary permeability is seen in tumours (Jain, 1987a) and results in non-specific accumulation of immunoglobulin (Sands et al., 1988; Kalofonos et al., 1990), this is counteracted by the absence of functioning lymphatic vessels (Gullino, 1975; Jain, 1987b). The absence of lymphatics results in reduced drainage of interstitial fluid from the tumour and a relative increase in tumour interstitial pressure (Jain, 1987a; Jain, 1987b; Jain, 1990). This increased interstitial pressure has been demonstrated clinically in melanoma patients (Boucher et al., 1991). The net hydrostatic result is to retard the ingress of molecules by simple diffusion. This is particularly important in the case of larger molecules such as monoclonal antibodies (150 kDa). A relative flow of interstitial fluid from the centre to the periphery of a tumour compounds this situation and results in "pooling" of molecules peripherally (Jain and Baxter, 1988).

These factors result in very slow tumour penetration by intact antibody molecules. Mathematical modelling has predicted intra-tumoural diffusion of an IgG molecule to
be limited to about one millimetre in two days (Jain and Baxter, 1988) and this is supported by experiments in vivo using quantitative autoradiography (Yokota et al., 1992). Such slow penetration may certainly hamper the efficacy of therapeutic constructs when an even dose to the whole tumour deposit would be desirable.

1.3.1.2 Accumulation in Normal Tissues

1) Binding of IgG to Fc receptors on normal tissues

Binding of IgG to receptors present on normal tissues has been proposed as a major factor in increasing non-specific accumulation of tumour-targeted immunoglobulin (Herlyn et al., 1983; Buchegger et al., 1986; Herlyn et al., 1986). This in turn results in a reduction in the tumour to normal tissue contrast achieved and the efficiency of imaging or therapeutic studies. These receptors are naturally present in many tissues including hepatic tissue, the spleen, renal glomeruli, the alveolar wall in the lung, villi of the intestine and lymph nodes (Mizoguchi et al., 1979; Sancho et al., 1984). However, this interaction represents only part of the story. Numerous studies investigating the normal organ accumulation of intact tumour-targeting antibodies and their fragments (lacking the Fc) have demonstrated only a partial improvement (see (Adams, 1998) for review).

2) Persistence of antibody in circulation

Long circulating half-lives seen with murine monoclonal IgG molecules (t1/2α typically ~ 0.7 to 2.6 h and t1/2β ~ 50-113 h) (see (Adams, 1998) for review) result in prolonged antibody retention in serum and a corresponding delay in achieving high tumour to blood ratios of antibody concentration. Antibody concentrations will also remain high in blood-rich organs such as liver, spleen and kidney whilst serum levels remain high. The reverse situation may be found in tumours where blood flow is highly variable and areas of relative ischaemia are common (Solesvik et al., 1982; Jain, 1988; Konerding et al., 1989). This may be particularly important when trying to minimise radiation dose to normal tissues in imaging studies or toxicity in therapeutic interventions.
3) Human Anti-mouse Antibody Reactions (HAMA)

Most monoclonal antibodies are murine in origin and elicit immune responses in human subjects (Rosen et al., 1987; Schulz et al., 1988; Van Kroonenburgh and Pauwels, 1988; Klee, 2000). Two to three murine Mab administrations will lead to a HAMA response in more than 90% of patients (Reynolds et al., 1989; Seccamani et al., 1989). These responses result in the formation of immune complexes which are deposited in normal tissues (lung, liver, spleen, kidney) and hamper efficient tumour localisation (Van Kroonenburgh and Pauwels, 1988; Torres et al., 1993). This becomes an increasing problem with repeated administration of monoclonal antibodies (Torres et al., 1993), limiting the number of imaging or therapeutic doses. Such HAMA responses seem to be dependent on protein dose (Colcher et al., 1990b) and are less problematic when antibody fragments are employed (Reynolds et al., 1989). Humanised monoclonal antibodies (Kashmiri et al., 1995) and human antibody fragments produced by newer recombinant techniques (Clackson et al., 1991; Winter et al., 1994) should eliminate the problems of the HAMA response inherent in murine antibodies.

1.3.2 Improving Tumour Targeting

A wide variety of approaches have been investigated in a bid to improve tumour targeting. The most important of these are discussed below.

1.3.2.1 Modification of Tumour Milieu

Increasing the vascularity of the tumour may increase blood volume and thereby enhance the non-specific accumulation of a radiopharmaceutical in the tumour. Tumour-specific increases in blood volume have been achieved in animal studies using vasoactive moieties (including IL-1, IL-2, Leukotriene-B4 and TNF) conjugated to monoclonal antibody (Khawli et al., 1994). In that study, the authors demonstrated enhanced antibody targeting of cervical carcinoma xenografts in a murine model as a result both of increased tumour blood volume and increased vascular permeability.
However the results have yet to be confirmed in the clinical setting. Similarly, decreasing the interstitial pressure of the tumour should increase the amount of radiopharmaceutical gaining access. This has been achieved with steroids (Kristjansen et al., 1993), external beam irradiation (Znati et al., 1996), systemic administration of tumour necrosis factor (Kristensen et al., 1996) and photodynamic therapy (Fingar et al., 1991). Many vasoactive agents have been used in an attempt to reduce the interstitial pressure (including adrenaline, noradrenaline, hydralazine, glyceryl trinitrate and nicotinamide) but the success of such manipulations has been highly variable and difficult to predict from first principles (Zlotecki et al., 1995). For example, both noradrenaline and angiotensin II are potent vasoconstrictors. However, in one study the former was shown to reduce tumour blood flow while the latter increased it in a murine model (Zlotecki et al., 1993).

The accumulation of radioimmunopharmaceuticals in tumours has also been enhanced by increasing tumour vascular permeability through systemic administration of vasoactive drugs such as histamine and leukotriene (Hennigan et al., 1991), tumour necrosis factor (Rowlinson-Busza et al., 1995) and interleukin-2 (Hennigan et al., 1991; Jekunen et al., 1996). However, the effect of such vasoactive drugs has yet to be clearly demonstrated in humans. In contrast, the systemic toxicity of such drugs is well described (Sherman et al., 1988; Ardizzoni et al., 1994). The use of tumour-specific immunoconjugates (Le Berthon et al., 1991; Khawli et al., 1994) may limit the systemic toxicity of these drugs while simultaneously increasing the accumulation of the antibody in the tumour.

At present these strategies have thus far failed to improve significantly tumour localisation of radio-immunoconjugates and it seems likely that other methods must be employed to enhance targeting.

1.3.2.2 Antibody Modification

Modern molecular biological techniques have provided solutions to many of the limitations of hybridoma-derived monoclonal antibodies. Large combinatorial antibody
Fragments of Whole IgG

**F(ab')\textsubscript{2} Fragments**

F(ab')\textsubscript{2} fragments consist of the two antigen binding fragments (Fabs) of the whole IgG molecule linked by a thiol group through the C\textsubscript{H1} domain of the heavy chain constant
Fig. 1.3.2.2  Antibody-based Targeting Molecules

IgG and fragments

- Whole IgG
- F(ab')2
- Fab
- scFv
- Isolated VH
- Isolated CDR3

ScFv-derived

- scFv
- Thiol-linked (scFv)2
- Non-covalent dimer (Diabody)
- Non-covalent trimer (Triabody)
- Minibody

Schematic diagram of antibody-based targeting molecules. Antigen is represented by green triangles. Redrawn and modified from Adams 1998 with permission.
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Size (kDa)</th>
<th>Valency</th>
<th>Antigen</th>
<th>T1/2α (min)</th>
<th>T1/2β (h)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDR</td>
<td>1.5</td>
<td>1</td>
<td>*MUC-1</td>
<td>3.6</td>
<td>0.9</td>
<td>Sivolapenko 1995</td>
</tr>
<tr>
<td>VH</td>
<td>12</td>
<td>1</td>
<td>*Lysozyme</td>
<td>No data</td>
<td>No data</td>
<td>Ward 1989</td>
</tr>
<tr>
<td>scFv</td>
<td>25</td>
<td>1</td>
<td>HER2, CEA</td>
<td>2.4-8.4</td>
<td>1.5-3.9</td>
<td>Adams 1993, Wu 1996</td>
</tr>
<tr>
<td>Fab</td>
<td>50</td>
<td>1</td>
<td>HER2, CEA</td>
<td>9-14.8</td>
<td>1.5</td>
<td>Colcher 1990, McCartney 1995</td>
</tr>
<tr>
<td>(scFv')_2</td>
<td>55</td>
<td>2</td>
<td>HER2</td>
<td>13</td>
<td>2.4</td>
<td>Weiner 1995, Adams 1993</td>
</tr>
<tr>
<td>Minibody</td>
<td>80</td>
<td>2</td>
<td>CEA</td>
<td>35-72</td>
<td>4.8-5.3</td>
<td>Hu 1996</td>
</tr>
<tr>
<td>F(ab')_2</td>
<td>100</td>
<td>2</td>
<td>HER2, CEA</td>
<td>24-30</td>
<td>6-12</td>
<td>Colcher 1990a</td>
</tr>
<tr>
<td>IgG</td>
<td>150</td>
<td>2</td>
<td>HER2, CEA</td>
<td>42-156</td>
<td>61-113</td>
<td>Colcher 1990a</td>
</tr>
</tbody>
</table>

Pharmacokinetics of various antibody fragments and whole IgG in comparison to molecular size. All data from murine studies except CDR (human). *No HER2 / CEA studies available.
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Antigen</th>
<th>Valency</th>
<th>Affinity/avidity (Kₐ, 1/mol)</th>
<th>Max %ID/g</th>
<th>T:B1 (24h)</th>
<th>Ref</th>
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<td>741F8 scFv</td>
<td>HER2</td>
<td>1</td>
<td>0.5 x 10⁸</td>
<td>5.64</td>
<td>11.7</td>
<td>Adams</td>
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<td>HER2</td>
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<td>0.5 x 10⁸</td>
<td>4.10</td>
<td>10.0</td>
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</tr>
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<td>2.90</td>
<td>8.75</td>
<td></td>
</tr>
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<td>741F8 (scFv')₂</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1995</td>
</tr>
<tr>
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<td>1</td>
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<td>13.3</td>
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<td>21.48</td>
<td>1996</td>
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<td>212 dimer &amp;</td>
<td>CEA</td>
<td>1 &amp; 2</td>
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<td>23.23</td>
<td></td>
</tr>
<tr>
<td>monomer mixture</td>
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<td></td>
<td></td>
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<td></td>
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<td>212 scFv dimer</td>
<td>CEA</td>
<td>2</td>
<td>819 x 10⁸</td>
<td>13.68</td>
<td>48.69</td>
<td>Wu</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1999</td>
</tr>
<tr>
<td>C6.5 scFv</td>
<td>HER2</td>
<td>1</td>
<td>0.4 x 10⁸</td>
<td>4</td>
<td>1.32*</td>
<td>Nielsen</td>
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<td>HER2</td>
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<td>6.25 x 10⁸</td>
<td>N/A</td>
<td>6.48*</td>
<td>2000</td>
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<td>1</td>
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<td>1.25 x 10⁸</td>
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<td>10 x 10⁸</td>
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<td>2</td>
<td>27.8 x 10⁸</td>
<td>8</td>
<td>3.18*</td>
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</tbody>
</table>

Studies investigating affinity / valency effect on targeting in comparable models. Maximum percentage of injected dose per gram of tumour (%ID/g) and tumour to blood ratio at 24 hours (T:B1 (24h) are shown). *24 hour T:B1 not given - figures are %ID/g in tumour at 24 hours.
region (Fe) (Milenic et al., 1991; Molthoff et al., 1992). These antibody fragments are easily produced by pepsin digestion of whole monoclonal antibody. Absence of C{sub H}2 and C{sub H}3 domains reduces their immunogenicity and gives F(ab'){sub 2} fragments a molecular weight of around 100 kDa whilst retaining the molecule's bivalent nature.

**Fab Fragments**

Further enzymatic digestion or reduction of IgG yields 50 kDa moieties consisting of only the Fab region of the whole molecule. This molecule retains the full antigen specificity of the parent though, naturally, possesses only a single antigen binding site. Fab fragment antibody phage display libraries have now been produced which allow selection of recombinant Fab fragments for a specific antigen in the same way as that described for scFv (Gram et al., 1992; Zebedee et al., 1992) (see below). Since scFv can show a tendency to spontaneous multimerisation, Fab phage display libraries have the potential advantage over scFv phage display libraries of purely monomeric antigen binding. This allows for the selection of high affinity clones without the likelihood of isolating multimeric targeting molecules inadvertently.

**Single-chain Fvs**

Single chain Fvs (scFvs) consist of the V{sub H} and V{sub L} domains of an Fv joined by a short peptide linker which permits folding of the single continuous polypeptide strand and reconstitution of the antigen-binding site. The single stranded nature of the peptide facilitates production as recombinant protein and increases stability of the molecule in comparison to isolated Fvs lacking a linker peptide (Glockshuber et al., 1990). ScFvs are highly versatile and modifications can be readily made in a site-directed fashion even at a single amino-acid level (Hoogenboom and Winter, 1992; Neri et al., 1996; Adams et al., 1998a). Tumour-specific scFvs can be isolated either by selecting a combinatorial phage display library on a target antigen (Marks et al., 1991) or be produced by cloning the appropriate gene segments from a specific hybridoma (Huston et al., 1988). Phage selection on antigen generally requires pure tumour-associated antigen of which relatively few examples are available. CEA and c-erbB-2 have been
the principal tumour antigens used for this purpose (Schier et al., 1995; Verhaar et al., 1995; Osbourn et al., 1996; Schier et al., 1996b; Jackson et al., 1998). ScFv construction from monoclonal antibodies has tended to result in loss of affinity in comparison to the parent molecule (Huston et al., 1988; Wu et al., 1996). More recently, Kupsch and co-workers have shown that highly tumour-specific scFvs can be isolated by phage selection using whole tumour cells (Kupsch et al., 1999). The presence of a complete antigen-binding site maintains the specificity and affinity of an intact monoclonal antibody though their monovalent nature limits the avidity of the molecule for the antigen. Various dimeric derivatives of scFvs have been described which attempt to address the avidity problem for circumstances where this may be important (see below).

Variable Domains

Isolated heavy (V\textsubscript{H}) or light (V\textsubscript{L}) variable domains retain one half of the antigen binding site of the parent molecule. These can be produced as recombinant protein (Ward et al., 1989) and, in contrast to isolated complementarity determining regions (CDRs), retain 3 CDRs on a supportive framework which might reduce the likelihood of cross-reactivity and improve binding to epitopes requiring specific spatial orientation.

Complimentarity Determining Regions

The six complementarity determining regions of a complete antigen binding site are directly responsible for the antibody's interaction with its target molecule. Single CDRs can be isolated by genetic engineering techniques and produced as recombinant protein (Sivolapenko, 1995). These small peptides (around 15 amino acids, ~1.5kDa) can maintain reactivity with the antigen. The heavy chain CDR3 is of particular interest since this region is often responsible for the dominant antibody : antigen interaction in terms of binding energy (Novotny et al., 1989; Hawkins et al., 1993; Kelley and O'Connell, 1993). Reduction in the number of CDRs interacting with the target, however, might reduce antigen specificity and increase the possibility of undesirable cross-reactivity with normal tissues. In addition, isolated CDRs lack the supportive
framework of the parent molecule and this increased flexibility may hinder binding to epitopes requiring specific spatial conformations.

**ScFv Derivatives**

**Disulphide-linked Dimers**

Disulphide-linked scFv dimers (covalent dimers, (scFv')₂) are manufactured by introducing cysteine residues at appropriate points in the V₇ / V₈ domain interface of the construct (Glockshuber *et al.*, 1990; Shalaby *et al.*, 1992) or at the C-terminus (Cumber *et al.*, 1992; Kostelny *et al.*, 1992; Pack and Plückthun, 1992; Kipriyanov *et al.*, 1994). The scFvs are then expressed as dimers in eukaryotic cells (FitzGerald *et al.*, 1997; Plückthun and Pack, 1997) or produced as monomer in *E.coli* prior to dimerisation *in vitro* (Glockshuber *et al.*, 1990; Cumber *et al.*, 1992; Kipriyanov *et al.*, 1994). The dimeric molecule is relatively flexible, mimics the avidity of a whole monoclonal yet maintains a small size (~55kDa) (Adams *et al.*, 1993; McCartney *et al.*, 1995).

**Diabodies & Larger Multimeric ScFvs**

ScFv diabodies (non-covalent dimers) can be produced by altering the length of the peptide linker lying between the V domains of the scFv fragment. This prevents refolding of the chains into a normal, paired orientation. Consequently, pairing occurs between chains of different scFv molecules in a non-covalent manner producing stable dimeric molecules with two antigen-binding sites (Holliger *et al.*, 1993; Holliger and Winter, 1993). These dimeric scFvs can be produced as homo- or heterodimeric molecules depending on the V-domains included in each scFv chain. Heterodimeric scFvs are bispecific, targeting two different antigens or antigenic epitopes (Atwell *et al.*, 1996; Holliger *et al.*, 1996).
The typical linker length of 15 amino acids has shown a tendency to form mixtures of monomeric, dimeric and trimeric scFv in several studies (Essig et al., 1993; Whitlow et al., 1993; Wu et al., 1996). Modification of the linker length has a significant effect on the multimerisation seen in scFvs. Shorter linkers (<14 amino acids) tend to promote multimer formation (Kortt et al., 1997) and longer linkers (>17 or 25 amino acids) tending to produce monomeric scFv (Whitlow et al., 1993; Desplancq et al., 1994; Wu et al., 1996).

As noted above, shortening the linker length used in scFv production can induce not only dimer production but also larger trimeric or tetrameric molecules resulting in greatly increased functional avidity for the antigen (Plückthun and Pack, 1997). This increased avidity is combined with increased size (~90 and 120kDa for trimers and tetramers, respectively) bringing the molecules above the “renal threshold” (Rennke et al., 1978). Greater size, therefore, results in reduced renal clearance and accumulation compared to monomeric scFv but at the cost of slower tumour penetration. The linker lengths provoking levels of multimerisation seem to vary between different scFvs (Kortt et al., 1997; Pei et al., 1997; Atwell et al., 1999; Le Gall et al., 1999) and may need to be determined empirically in each case. Subsequent studies have suggested that V-domain orientation is also important in multimer formation (Plückthun and Pack, 1997; Dolezal et al., 2000). The production of scFv multimers has recently been reviewed in detail (Hudson and Kortt, 1999).

Minibodies and Miniantibodies

By fusing the DNA encoding a human IgG1 C\textsubscript{H}3 domain to that encoding the scFv, a single stranded protein terminating in the C\textsubscript{H}3 domain is produced. The very high affinity between C\textsubscript{H}3 domains (Schiffer et al., 1988) results in spontaneous formation of 80kDa dimeric molecules known as minibodies (Hu et al., 1996). These minibodies may have sufficient mass to surpass the renal threshold and avoid first pass elimination. Similarly, some researchers have utilised a truncated murine IgG3 hinge region together with Fos or Jun leucine zippers to produce spontaneous dimerisation of scFv forming...
Variation in the size of antibody-derived molecules has implications both for clearance of the molecule from circulation and for tumour penetration. The renal threshold lies around the 65kDa mark and uncharged peptides smaller than this are rapidly excreted by the renal route and show a tendency to accumulate in the kidneys (Maack et al., 1979; Silbernagl, 1988). Larger molecules exhibit reduced clearance with prolonged circulatory half-lives. Clearly, the rapidity of circulatory clearance has an effect on tumour penetration and localisation. Buchegger et al. demonstrated the enhanced tumour penetration seen in smaller molecules using a model wherein the mice had been rendered surgically anephric (Buchegger et al., 1986). In this study, overall tumour retention of F(ab')2 and Fab fragments clearly exceeded that seen in a whole monoclonal antibody. This is in contrast to the results seen with functioning renal excretion where the fragments' more rapid removal from circulation results in reduced tumour retention overall (see (Adams, 1998) for review).

1.3.2.2 Enhanced Antigen-antibody Interactions - Affinity

The necessity for affinity between an antibody and its target is obvious. However the optimal affinity in a targeting molecule is as yet unclear. The natural assumption that greater affinity will result in better targeting has been challenged by Weinstein and co-workers. Weinstein suggested that very high affinity antibodies would bind irreversibly to the first target antigen encountered, producing a rim of antibody molecules around the tumour blood vessels and hindering further tumour penetration (Weinstein et al., 1987; Fujimori et al., 1989). This has been described as the “binding site barrier effect”. Conflicting studies have failed to clarify the issue, some suggesting the benefit of higher affinity (Colcher et al., 1988), whereas others did not (Juweid et al., 1992). More recently, two studies have apparently supported the “binding site barrier effect” hypothesis in scFvs (Adams et al., 1998a; Jackson et al., 1998), suggesting that affinity enhancement beyond a Kd of 10^-9 M would be unlikely to improve tumour targeting in vivo. The main difficulty in this controversy lies in separating the effect of affinity from
the many other factors which may influence tumour localisation. Ideally, one would compare two antibodies targeting the same antigenic epitope in the same model. Antibodies with similar chemical characteristics (e.g. molecular charge) would help allow definitive conclusions to be drawn. This controversy is discussed in more detail in Chapter 4.

*In vivo*, improved antibody affinity for the antigen occurs by means of affinity maturation where serial exposure to the antigen results in the selection of antibody clones with greater binding properties (Milstein *et al.*, 1986; Kocks and Rajewsky, 1988; Berek *et al.*, 1991; Jacob *et al.*, 1991). This concept has been mimicked *in vitro* using scFvs by site-directed mutagenesis, random mutagenesis and by chain shuffling. Site-directed mutagenesis relies on random mutation of critical amino acids in the CDRs of the scFv by PCR or other techniques to obtain a higher affinity (Yang *et al.*, 1995; Schier *et al.*, 1996b; Thompson *et al.*, 1996; Adams *et al.*, 1998a). Random mutagenesis allows indiscriminate mutation within the scFv DNA for the same purpose (Marks *et al.*, 1992; Irving *et al.*, 1996; Low *et al.*, 1996). In chain shuffling, the V<sub>H</sub> or V<sub>L</sub> of the molecule is combined with a library of many different V<sub>L</sub> or V<sub>H</sub> domains to produce a large pool of varying molecules (Clackson *et al.*, 1991; Ohlin *et al.*, 1996; Schier *et al.*, 1996a; Thompson *et al.*, 1999; Park *et al.*, 2000). The library can then be selected by antibody phage display techniques, allowing the identification of high affinity variants. Subsequently, the remaining original V region can be replaced by the same process to increase the affinity still further.

1.3.2.3 *In vivo* experience with antibody modification

When comparing differing targeting molecules *in vivo* it is important to compare antibodies and fragments in the same model, ideally targeting the same antigenic epitope. Unfortunately, no standardised model for comparison has been devised or agreed and a great many disparate studies targeting different molecules in varying models have been reported (Adams, 1998; Colcher *et al.*, 1998). This makes interpretation of the data difficult. It seems reasonable to concentrate on those studies where comparable targets have been selected. Currently, antibodies and fragments
targeting the tumour-associated antigens carcino-embryonic antigen (CEA) and HER-2 (c-erbB-2) have been the most extensively studied in a consistent murine model (Colcher et al., 1990a; Adams et al., 1993; McCartney et al., 1995; Weiner et al., 1995; Hu et al., 1996; Wu et al., 1996; Wu et al., 1999). The principal comprehensive studies targeting these antigens are summarised in Table 1.3.2.3a.

It is evident from the studies shown in this table that smaller molecules clear more rapidly from circulation as demonstrated by their shorter equilibration half-lives ($t_{\alpha}$) and elimination half-lives ($t_{\beta}$). Isolated CDR3 fragments clear very rapidly from circulation, which would probably severely limit the absolute tumour retention achieved and in turn their potential for eventual tumour targeting. Larger molecules prolong the circulatory time and enhance the degree of tumour localisation at the cost of greater non-specific localisation and blood pooling (reduced tumour : normal tissue ratios) (Colcher et al., 1990a). The renal elimination threshold (around 65kDa) is only exceeded by Minibodies (80kDa), F(ab')$_2$ and whole IgG and results in prolonged time in circulation (Colcher et al., 1990a; Hu et al., 1996). Renal accumulation of smaller molecules is typical and the tumour to kidney ratios achieved are generally inferior to those for whole IgG or F(ab')$_2$ molecules. This predicted renal accumulation has not, however, been reported by all authors (Adams et al., 1998a). These pharmacokinetic attributes are primarily mechanical and independent of tumour biology save in the case of shed antigens. Shed antigens may form circulating complexes with the targeting molecules, prolonging residence time in the blood pool and increasing hepatic accumulation (Douillard et al., 1985; Beatty et al., 1990).

Increasing the affinity or avidity of the molecule for antigen has the potential to enhance tumour localisation and several studies have addressed this issue (Adams et al., 1993; Adams et al., 1995; Wu et al., 1996; Adams et al., 1998a; Wu et al., 1999; Nielsen et al., 2000). The major studies targeting the same antigens in a murine model are summarised in Table 1.3.2.3b. Adams et al. failed to demonstrate any clear improvement in targeting CEA using disulphide-stabilised dimers and similar scFv monomers (Adams et al., 1993). However, Wu et al. in 1996 demonstrated the increased tumour accumulation of a scFv monomer / dimer mixture and then
subsequently demonstrated clearly superior tumour targeting with pure dimeric scFv (Wu et al., 1996; Wu et al., 1999). t½α and t½β were roughly similar for monomers and dimers (7.2 min vs. 15 min and 288 min vs. 174 min.) This suggests that the bivalent nature of the dimer (and hence its increased avidity for the antigen) rather than its increased mass is responsible for the improved tumour localisation.

Altering the antigen affinity of monomeric scFvs was investigated by Adams when variants of the same scFv targeting the same antigen were compared after site-directed mutagenesis of a critical CDR. Enhanced tumour localisation and tumour to normal tissue contrast were clearly related to antigen affinity (Adams et al., 1998a). Nielsen et al. have recently reported on 3 monomeric scFvs targeting HER2 and their corresponding dimers in a murine model (Nielsen et al., 2000). The authors show that higher affinity is associated with superior tumour localisation in the monomeric scFvs and the dimeric scFvs do indeed exhibit superior tumour localisation in comparison to their monomeric parents. Interestingly, this superiority in the dimers appears to be independent of the avidity of the individual dimers for the antigen with the lowest avidity dimer producing the best targeting profile. The authors postulate that perhaps lower affinity dimers dissociating more rapidly from the antigen when bound monovalently frees binding sites for neighbours which are in a position to bind bivalently (and more permanently). However, this is merely hypothesis and the interactions are complex and poorly understood.

1.3.2.4 Alteration of molecular charge

The net charge on a protein molecule can significantly influence its plasma clearance (Maack et al., 1979; Silbernagl, 1988). Protein molecules are neutral in charge at the pH corresponding to their isoelectric point (pI) (Purtell et al., 1979). Above this pH the protein would act as a base and below it would act as an acid. Reducing the pI of a molecule into the acidic range, therefore, results in a molecule with a net negative charge at physiological pH. This anionisation can be achieved by chemical modification (Tarburton et al., 1990) or, in the case of recombinant molecules, by manipulation of the amino acid content of the protein chain (Tan et al., 1998). Cell surfaces are generally
positively charged whilst the renal tubular basement membrane is negatively charged (Maack et al., 1979; Silbernagl, 1988). Cells would, therefore, be typically expected to attract negatively charged molecules whilst renal tubular cells would repel them (and reduce renal reabsorption). In one study, manipulation of the pI of a Fab fragment into the acidic range reduced renal accumulation and circulatory clearance in comparison with the control without affecting tumour localisation (Tarburton et al., 1990). On the other hand, chemical cationisation of a whole monoclonal markedly enhanced both renal clearance and uptake of the molecule by tumour cells (Pardridge et al., 1995). These techniques might be applied in a tailor-made fashion to individual molecules since the manipulations required to optimise targeting in different antibody-derived species clearly vary. Modification of recombinant fragments is particularly promising since site-specific alterations can be made to alter the pI without significantly interfering with antigen binding (Tan et al., 1998; Pavlinkova et al., 1999a). This topic is discussed extensively in Chapter 5.

1.3.2.5 Pretargeting

Pretargeting is a concept aimed at permitting the combination of the high quantitative tumour localisation achievable with whole antibodies and the high tumour-specificity seen with some antibody fragments (Hnatowich et al., 1987; Goodwin et al., 1988; Paganelli et al., 1988). A three-stage procedure is usually involved. Initially, for example, the tumour-targeting molecule may be administered after coupling to a streptavidin tag. After sufficient time has passed for adequate tumour localisation to occur, circulating antibody-streptavidin is removed using a clearing agent (e.g. biotinylated albumin) which binds to circulating antibody and promotes its removal. The clearing agent is incapable of leaving the vascular compartment to interfere with tumour-localisation. Finally, a therapeutic agent which binds specifically to the antibody is administered resulting in specific activity in the tumour (Hnatowich et al., 1987; Paganelli et al., 1988).

A similar approach involves coupling the targeting antibody to an enzyme capable of activating a cytotoxic prodrug (e.g. etoposide phosphate) (Deonarain et al., 1995). The
Chapter 1

antibody-enzyme conjugate is administered and allowed to localise to the tumour. Sufficient time is allowed for circulating antibody to be largely eliminated or a specific antiserum can be used to promote clearance. The prodrug is then injected and activated preferentially in the tumour by the high local concentration of the activating enzyme. In this strategy, the clearance of circulating antibody allows very high tumour to blood ratios (and, therefore, activated cytotoxic drug) to be obtained (see (Deonarain et al., 1995) for review).

Early use of scFvs in pre-targeting have been encouraging. Denardo et al. have described the selection of metal chelate (Cu-TETA and Y-DOTA) binding scFvs intended for use in bispecific tumour-targeting molecules. The bispecific scFv would be administered and localise to tumour then the $^{67}$Cu-TETA or $^{90}$Y-DOTA radiochemical would be administered resulting in enhanced accumulation around the tumour (DeNardo et al., 1999). In vivo results have yet to be published. Goshorn et al. have demonstrated the efficacy of streptavidin-tagged scFv tetramers in pretargeting carcinoma xenografts in a nude mouse model. The pretargeting molecule was administered followed by a single dose of 800 $\mu$Ci of 90Y-DOTA-biotin. Cures in both small cell lung and colonic carcinoma xenografts were achieved (Goshorn et al., 2001).

1.3.3 Radio-label Selection and Technique

The choice of radiolabel for imaging or localisation studies should be closely related to the pharmacokinetic properties of the targeting molecule (Williams et al., 1995). Molecules with short circulatory half-lives are suited to short-lived isotopes such as $^{99m}$Tc, where most of the radio-active decay would take place while the molecule remains in the body. Longer-circulating molecules such as whole IgG, where effective tumour to normal tissue contrast may take days to develop, require longer-acting isotopes such as $^{111}$In or $^{131}$I.

The radiolabelling technique employed may have a significant effect on the behaviour of the targeting molecule (Hnatowich, 1990). Several techniques (e.g. Iodogen™ (Fraker and Speck, 1978) or Chloramine-T radio-iodination (Hunter and Greenwood, 1962))
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involve a non-specific approach where residues suitable for labelling are utilised randomly. Should these residues be associated with the CDRs of the antibody or fragment, antigen affinity might be reduced, resulting in a decrease in targeting efficacy. Additionally, the procedure involved may require somewhat harsh conditions which might result in a degree of denaturation of the protein. These considerations become increasingly important in the case of antibody fragments which are often inherently more fragile and unstable than the parent immunoglobulin (Wörn and Pluckthun, 1999). The smaller size of the fragment results in a reduction in potential labelling sites (e.g. IgG-associated carbohydrate) and a greater possibility of interfering with the CDRs. Several authors have attempted to overcome these problems by incorporating specific labelling sites into the recombinant protein, permitting site-directed labelling (George et al., 1995; Kipriyanov et al., 1995; Verhaar et al., 1996).

Stability of the radio-label-antibody conjugate can be important for in vivo studies since the imaging or distribution data rely on localisation of the radioisotope rather than the antibody or fragment per se. Radioiodine is the most commonly utilised labelling isotope for localisation experiments. However, data may be skewed by a tendency to dehalogenation of the antibody after uptake into tumour (and other) cells with the radioiodine subsequently diffusing out of the cell (Zalutsky et al., 1985; Carrasquillo, 1989). Some degree of iodine dissociation is also evident in circulation. Adams et al. demonstrated this very effectively when comparing two separate iodination techniques using the same murine model and antibody fragment (Adams et al., 1995). Increased stability of the radiolabel resulted in significantly enhanced targeting and imaging properties. Catabolism of radio-metal labelled antibodies or their derivatives can lead to significant accumulation of the radio-isotope in the reticulo-endothelial cells of the liver and spleen and hence reduced tumour to normal tissue contrast (Carrasquillo et al., 1987; Adams et al., 1995).
1.4 Conclusion

It is clear from this exploration of the increasing problem of melanoma and the difficulties faced in clinical practice, especially in advanced disease, that new methods are required for the efficient diagnosis and treatment of this disease.

Malignant melanoma presents an attractive prospect for antibody-mediated targeting and previous studies have demonstrated the potential of this approach for imaging and possibly therapy. Antibody fragments have been shown to overcome many of the problems which have hindered the use of whole immunoglobulins in melanoma and these molecules present an exciting opportunity to study the factors influencing efficient targeting.

Two clinical scFv studies targeting colonic carcinoma have been published to date with encouraging results (Begent et al., 1996; Larson et al., 1997). More recently the potential usefulness of radiolabelled anti-tumour scFvs in guiding surgical excision of tumours ("Radioimmunoguided Surgery"), again in colonic carcinoma (Mayer et al., 2000). As yet, the potential of such molecules in malignant melanoma remains largely unexplored.

We believe that improving the targeting efficiency of anti-melanoma scFvs by means of molecular biology techniques will allow the development of more effective imaging and therapy in melanoma.

This thesis presents the results of the in vitro production and characterisation of affinity and chain-shuffled modified scFvs together with their in vivo assessment as targeting molecules in an animal melanoma model. It explores strategies aimed at bringing scFv production levels and solubility to clinically useful levels. In addition, it addresses strategies to reduce the degree of renal accumulation using these targeting molecules. These results will be of value in the construction and use of antibody fragments in the diagnosis and therapy of melanoma.
Chapter 1

1.5 Aims

1.5.1 Hypothesis

Our hypothesis states that the modification of anti-melanoma antibody fragments (scFvs) in terms of domain orientation, valency and affinity for the antigen allows the selection of molecules of improved targeting efficiency in comparison to the parent anti-melanoma scFv RAFT2.

1.5.2 Experimental Plan

To confirm this hypothesis, a series of new scFvs based on the parent RAFT2 scFv were developed by means of molecular biology techniques. ScFvs of reversed V-domain orientation were produced and investigated. Attempts were made to produce multimeric scFv by manipulation of the linker region of the RAFT2 scFv. ScFvs of varying affinity for the parent antigen were produced by means of antibody phage display and chain shuffling techniques. Each of the novel scFv series was characterised in vitro and then studied in vivo after radiolabelling using iodine-125. Pharmacokinetics and biodistribution were studied in a nude mouse model using human melanoma xenografts and the targeting efficiency of the various molecules examined in comparison to the parent molecule. Finally, the efficacy of amino acid coadministration in reducing problematic renal scFv accumulation was investigated.
Chapter 2

2. Materials and Methods
2. Materials and Methods

Unless otherwise specified, all "standard methods", "established techniques" or "standard formulations" referred to in sections 2.1 and 2.2 were obtained from "Molecular Cloning: A Laboratory Manual" (Sambrook et al., 1989). Unless otherwise specified, all "standard methods", "established techniques" or "standard formulations" referred to in subsequent sections were obtained from "Antibodies: A Laboratory Manual" (Harlow and Lane, 1988). Similarly, chemicals were obtained from BDH Laboratory Supplies except where noted. Restriction enzymes were produced by Boeringer Mannheim and custom primers by Life Technologies.

2.1 General Molecular Biological Techniques

2.1.1 Plasmid Extraction

Plasmid extraction was carried out using the Wizard-Miniprep™ System (Promega).

1.5 to 2 ml of an overnight culture of the relevant bacterial clone was centifuged in a microcentrifuge to obtain a cell pellet (13,000 rpm, 1 min, room temperature (RT)). The pellet was resuspended in 200 μl of buffered glucose solution. 200 μl of NaOH and SDS solution was added to lyse the cell. The cell lysis solution was neutralised using 200 μl of buffer containing potassium acetate and acetic acid. The suspension was then centrifuged to remove cellular debris (13000 rpm, 10 min, RT). The cleared supernatant was removed to a further tube and mixed with 1 ml of DNA-binding resin. The sample was then loaded onto a 2 ml mini-column and suction applied using a vacuum manifold. The resin/DNA mix was washed with 2 ml of column wash solution (Tris-buffer and 58% ethanol). 50 μl of distilled H₂O was added and the column allowed to stand for 5 to 10 mins. The released DNA was then recovered from the column by centrifugation at 13000 rpm for 30 s.
2.1.2 Plasmid and DNA Purification

Plasmid and DNA samples from restriction digest or PCR reactions were purified using agarose gel separation and Wizard PCR DNA Purification Resin (Promega). Protein in the sample was denatured by heating to 68°C for 10 minutes in a heating block. The DNA sample was loaded on a 1.5% TAE gel in 1X TAE buffer and separated at 100V for ~40 min. The band of interest was identified using UV light and cut out of the gel. The gel fragment was then heated in 1ml DNA Purification Resin at 68°C until melted and the DNA recovered as per the manufacturer's protocol. The purity and yield of DNA was then confirmed by loading a small sample on a further 1.5% TAE gel and running as above.

2.1.3 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were carried out according to established techniques (Molecular Cloning: A Laboratory Manual 1989). Appropriate 3' and 5' primers were designed and obtained from GibcoBRL at 100µM concentration and diluted to 10µM before use. 100µM dNTP stock was prepared from stocks of the constituent nucleotides (Boeringer Mannheim). A master "PCR mix" was produced containing

- 22.5µl 10X Taq polymerase buffer (Boeringer Mannheim)
- 11.25µl Taq polymerase (Boeringer Mannheim)
- 36µl dNTP stock
- 11.25µl DMSO (Sigma Aldrich)
- 105µl dH₂O

PCR was carried out using 10µl 5' and 3' primer (10µM), 39µl "PCR mix" and 1µl of template DNA. Two drops of mineral oil (Sigma Aldrich) were placed on the top of the solution to prevent evaporation during thermocycling.
Chapter 2

The reaction was achieved using a static thermoblock (Biometra) set to:

<table>
<thead>
<tr>
<th>Step</th>
<th>Duration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 min</td>
<td>94°C</td>
</tr>
<tr>
<td>2</td>
<td>1 min</td>
<td>94°C</td>
</tr>
<tr>
<td>3</td>
<td>2 min</td>
<td>50°C</td>
</tr>
<tr>
<td>4</td>
<td>3 min</td>
<td>72°C</td>
</tr>
<tr>
<td>5</td>
<td>5 min</td>
<td>72°C</td>
</tr>
<tr>
<td>End</td>
<td></td>
<td>(Hold 4°C)</td>
</tr>
</tbody>
</table>

Cycled 30X

2.1.4 Restriction Digest

Restriction digests were carried out using appropriate restriction enzymes and buffer (Boeringer Mannheim). DNA samples were prepared in dH2O and enzyme and buffer added. Reactions were carried out at temperatures appropriate for the particular enzyme until complete digestion was demonstrated on a TAE gel. The reaction was terminated by heating to 68°C for 10 min.

2.1.5 DNA Ligation

Ligation of DNA fragments was carried out according to standard techniques. Fragments for ligation were made up to a maximum volume of 10 μl of which 1 μl (5 units) was ligase (Boehringer Mannheim) and 1 μl was 10X ligase buffer (Boehringer Mannheim). The ligation mixture was incubated at 16°C overnight in a water bath.

2.1.6 Bacterial Culture Media and Agar Plates

Luria Broth base (GibcoBRL) was used for all bacterial culture. 25g of broth base was added per litre and autoclaved at 15psi, 120°C for >20min before use.

Agar plates were prepared using Luria Agar (GibcoBRL) at 17g of agar per litre of distilled H2O. The agar was sterilised in the same manner as the broth above. The agar was melted in a conventional microwave oven and cooled to ~60°C in a water bath.
before adding glucose, IPTG or antibiotic where appropriate and poured immediately.
The plates were allowed to set and stored at 4°C for up to weeks. Before use, the plates
were dried at 37°C for one hour.

2.1.7 Bacterial Transformation

Electrocompetent *E. coli* cells (gift of Mrs N. Patel, RAFT Institute, Northwood) stored
at -80°C were thawed slowly on ice. 1-2 μg of scFv plasmid in a volume of 2μl dH2O
was added in a sterile Eppendorf tube and allowed to sit on ice for 5 minutes. The 52μl
of solution was then transferred to a 2mm electroporation cuvette (Invitrogen) and
pulsed using an electroporation device (Bio-Rad) at 2500V, 201Ω, 25μF for 5ms. 1ml
of SOC medium (GibcoBRL) was added immediately and the cell suspension incubated
at 37°C for 1 hour with shaking at 250rpm. The cell suspension was then spread at
dilutions on Luria agar plates containing 2% dextrose and the appropriate selection
marker (e.g. ampicillin) and incubated overnight at 30°C.

2.1.8 Agarose Gels and Electrophoresis Buffers

All DNA electrophoresis was carried out in TAE buffer following standard methods.
All agarose gels were run in 1X concentration TAE buffer made from a 50X
concentration stock solution made as tris-base (242 g/L) (BDH), 100 ml of 0.5 M EDTA
(BDH) pH 8.0 + 600 ml distilled H2O. The pH was adjusted to pH 7.7 with glacial
acetic acid (BDH) and the volume adjusted to 1l with more distilled H2O.

A standard 1% agarose electrophoresis gel was made as 1.0g agarose powder (Gibco) +
100 ml 1X TAE buffer + 5.0 μl ethidium bromide (Gibco). The components were
heated in a microwave oven with the top off the bottle to melt the agarose and poured
into a mould.

The resuspension buffer for plasmid extraction (see Section 2.1.1) was composed of 5
ml of 1 M D-glucose + 2.5 ml of 1 M tris-HCl + 2.0 ml of 0.5 M EDTA made up to 100
ml with 90.5 ml of distilled H2O. Cell lysis buffer for extraction of plasmid DNA was
Table 2.2.1: Formulation of SDS-PAGE Gels

<table>
<thead>
<tr>
<th>Component</th>
<th>12%</th>
<th>4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis (30%)</td>
<td>4.0ml</td>
<td>1.3ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>3.3ml</td>
<td>6.0ml</td>
</tr>
<tr>
<td>3.0 M Tris-HCl, pH 8.8</td>
<td>2.5ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100μl</td>
<td>100μl</td>
</tr>
<tr>
<td>Ammonium persulfate (100mg/ml)</td>
<td>50μl</td>
<td>50μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5μl</td>
<td>10μl</td>
</tr>
<tr>
<td>TOTAL VOLUME</td>
<td>10ml</td>
<td>10ml</td>
</tr>
</tbody>
</table>

Fig. 2.2.4: Semi-Dry Blotter Used for Western-blot Transfer
composed of 2 ml of 5 M NaOH + 5 ml of 10% SDS + 43 ml of distilled H₂O. The lysate was neutralised with a buffered solution composed of 29.5 g potassium acetate in 50 ml of distilled H₂O adjusted to pH 4.8 with glacial acetic acid and then adjusted to 100 ml final volume with distilled H₂O.

2.2 SDS-PAGE and Western Blot Analysis

2.2.1 Gels

Mini-Protean™ gel electrophoresis equipment (Bio-Rad) was used for all protein electrophoresis experiments. Samples were loaded on a 4% SDS-polyacrylamide gel stacking layer and separated on a 12% gel. Gel constituents are shown in Table 2.2.1.

2.2.2 Buffers

Samples were prepared in sample buffer consisting of 1% glycerol, 2% SDS, 1.2% Dithiothreitol (DTT), 1.0 ml Tris-HCl pH 6.8 and 0.05% bromophenol blue. Electrophoresis was carried out in running buffer (25 mM Tris-base, 192 mM glycine, 0.1% SDS). Separated protein samples were transferred to nitrocellulose paper using transfer buffer (25mM Tris-base, 192mM glycine, 20% methanol in distilled water). Blocking of non-specific binding was achieved using 3% skimmed milk solution (Marvel™, Tesco Supermarkets) in phosphate-buffered saline (PBS) containing 0.05% sodium azide (Sigma). PBS contained 26.5 mM KCl, 147 mM KH₂PO₄, 80.6 mM NaCl and 8.06 mM NaHPO₄·2H₂O in distilled water. All antibodies used in staining the filters were diluted in incubation buffer containing 0.02% Bovine Serum Albumin (BSA) and 0.1% Tween-20 in PBS. Wash buffer for Western blots consisted of 0.05% Tween-20 in PBS.

2.2.3 SDS-PAGE

The method employed for SDS-PAGE analysis of protein was as previously published (Molecular Cloning, A Laboratory Manual, 1989). Typically, 20µl of the protein
solution was mixed with 5μl of sample buffer and heated to 95°C for 3 minutes. After loading, the electrophoresis bath was run at 200V, 100mA for approximately 1 hour.

Protein staining was achieved using Coomassie Blue Dye or Gelcode Blue (Pierce). Coomassie Blue was prepared by dissolving 1g of Coomassie brilliant dye in 200 ml 10% acetic acid / 45% dH2O / 45% methanol. Gels were stained for approximately 20 minutes before destaining using the same acetic acid / water / methanol solution in the absence of Coomassie brilliant blue dye.

Protein fragment size was estimated in comparison to Kaleidoscope™ Prestained Protein Markers (Bio-Rad) or Low Range Molecular Weight Standards (Biorad).

A quantitative assessment of the protein loaded was obtained using standard ovalbumin (Sigma Aldrich) samples.

2.2.4 Western Blotting

Western blotting of protein samples was carried out using previously published methods (Molecular Cloning, A Laboratory Manual, 1989). Gels for blotting were obtained using the methods described above (Sections 2.2.1 to 2.2.3). Proteins were transferred onto nitrocellulose paper (Hybond™, Amersham International) using a semi-dry blotter (EF200B Cambridge Electrophoresis). 2 pieces of Whatman™ 3MM filter paper pre-soaked in transfer buffer were placed on the semi-dry blotter, followed by the gel, the nitrocellulose and finally 2 more pre-soaked pieces of filter paper (Fig. 2.2.4). The semi-dry blotter lid is then placed in position and a 100V 200mA transfer current applied for 1 hour.

The nitrocellulose filter was then removed and shaken overnight in blocking buffer at room temperature. Detection of scFvs on the filter was achieved using 9E10 monoclonal antibody which is directed against the c-myc tail present in the antibody fragments. The filter was successively stained with 9E10 hybridoma supernatant, goat anti-mouse IgG-biotin conjugate (Sigma Aldrich) and, extravidin-alkaline phosphatase conjugate (Sigma...
Aldrich). Each reagent was diluted 1/1000 in incubation buffer with the exception of 9E10 which was applied neat. 1 hour incubations were employed with three 10 minute washes in wash buffer between each. The colour change on the filter was achieved using alkaline phosphatase conjugate substrate (BioRad) which was prepared from its two components as per the manufacturer’s instructions. Alternatively, Vector Red alkaline phosphatase was used (Vector Labs) again as per the manufacturer’s instructions. The colour change was observed and terminated by washing the filter in tapwater.

2.3 Antibody Phage Display Techniques

2.3.1 Rationale

Antibody phage display techniques allow for the selection of highly specific antibody fragments. An affinity matured scFv phage library can then be re-selected to identify the most effective clones.

2.3.2 Transformation of E. coli strain TG1

Minipreps of scFv in vector pCantab6 were used to transform 50µl of electrocompetent E. coli TG1. 1-2µg of plasmid in 2µl of dH2O was used to transform the bacterial cells as previously described (Section 2.1.7). The cell suspension was then spread onto Luria Agar (GibcoBRL) plates containing 100mM ampicillin and 2% D-glucose. The plate was dried at 37°C for 1 hour before the addition of the sample and further air-dried before incubation. Overnight incubation was carried out at 30°C on plates spread with 10, 50, 100 or 300µl of cell suspension to allow calculation of a phage titre.

2.3.3 Recovery of Phage

After approximately 18 hours’ incubation transformed colonies were evident. Colonies were counted to provide an estimate of the phage titre. Sample colonies were picked for overnight incubation and subsequent induction of scFv production (Section 2.4.2). The
remaining colonies were resuspended in 20ml Luria Broth medium and a phage lysate obtained.

A phage lysate was prepared by inoculating 50ml Luria Broth containing 2% D-glucose and 100µg ampicillin (Boeringer Mannheim) per ml (LBGA) using the resuspended transformed colony solution to an optical density of 0.3. The solution was shaken at 37°C for 1½ hours. 500µl M13K07 helper phage solution (gift of Mrs. N. Patel) was added and the solution shaken at 37°C for a further hour. The solution was spun for 10min at 3000rpm in a benchtop centrifuge and the supernatant discarded. The pellet was resuspended in 500ml LBGA containing 10µg kanamycin (Boeringer Mannheim) per ml and shaken overnight at 37°C. The following morning the 500ml sample was spun at 9000rpm at 4°C for 15min and the supernatant retained and filtered through a 0.2µM filter (Dow Corning). 0.15 volumes of 16.7% PEG6000 (Sigma) in 3.3M NaCl was added and the solution stored overnight at 4°C. The following morning the solution was spun at 8500rpm at 4°C for 40min with the centrifuge brake set to slow. The supernatant was discarded and the visible PEG pellet (containing the rescued phage) dried off on a benchtop. The pellet was resuspended in 2ml PBS and stored at 4°C until required.

2.3.4 Phage Selection on Peripheral Blood Monocytes and Melanoma Cells

Human peripheral blood monocytes (PBMCs) were prepared as follows:

50ml of fresh human blood was obtained from a healthy volunteer and 1ml of anticoagulant 0.5M EDTA together with 50ml PBS added immediately. 15ml of sterile Lymphoprep™ (GibcoBRL) were then placed in each of 4 sterile 50ml centrifuge tubes and 25mls of the blood/PBS mix added gently to each. The tubes were spun at 2500rpm at room temperature in a benchtop centrifuge with the brake in the “off” position. The PBMCs were then recovered from the interphase in the tubes using a Pasteur pipette and topped up to 50ml using PBS. The PBMCs then spun down in a benchtop centrifuge (15min, 2500rpm, room temperature) and the supernatant discarded. The PBMCs were washed twice in 5mls blocking solution (Section 2.2.2), spinning the cells down at
2500rpm for 10min after each wash. Finally the cells were resuspended in 900μl blocking solution and briefly stored at 4°C until used.

Prior to selection, 100μl phage solution were added to the PBMCs in 900μl blocking solution and rotated gently at room temperature for one hour. The sample was spun at 7000rpm for 10min in a benchtop centrifuge and the supernatant containing the "pre-cleared" phage (i.e. that not binding to general cellular antigens) decanted to a new Eppendorf tube and stored at 4°C until used.

Recovered, "pre-cleared" phage was selected on one T175 culture flask of A375-M melanoma cells (approx. 3 x 10^7 cells) after the cells had been detached from the flask using Versene (GibcoBRL) (Section 2.5.1). Non-specific binding sites on the tumour cells were blocked by rotation in blocking solution for 30 min at room temperature. The melanoma cells were spun down (1500rpm, 5 min) and resuspended in blocking solution. After the third spin, cells were resuspended in 900μl blocking solution and transferred to 1.5ml Eppendorf tube. 100μl of phage lysate was then added and the sample rotated for 1 hour at RT. The sample was then spun down (1500 rpm, 5 min, RT) and the cells resuspended in 10ml PBS. The suspension was allowed to sit at room temperature for 20mins to allow detachment of any phage not firmly bound and the spin-resuspension cycle repeated 6 to 8 times. Finally, the cells were spun down and resuspended in 100μl 100mM citric acid pH 7.6 and left to stand on the bench-top for 5 minutes. The suspension was spun at 13000 rpm for 5 min on a bench-top centrifuge and the supernatant removed to a separate tube. This supernatant containing the eluted phage was neutralised using 100μl HCl and the resulting phage suspension stored at 4°C.

2.3.5 Colony Lifting

Suitable plates for colony lifting were identified as those with the highest colony titre where discrete colonies could still be easily identified. From a pre-prepared template, a circular piece of nitrocellulose filter paper (Hybond™, Amersham International) was cut to size. The filter was then carefully placed on the colonies on the plate. Its position
was noted by piercing the filter and agar at the 12 and 3 o'clock positions using a sterile needle. The filter was then removed, inverted and placed colony side up on a further agar plate containing 1mM isopropyl thio-galactoside (IPTG, BDH) and 2% D-glucose to induce scFv production. The plates were then incubated overnight at 30°C. The original plates were incubated at 30°C for 8 hours to allow regeneration of the colonies before storage at 4°C. The following morning colonies were visible on the transferred filter which was then removed for colony lysis and staining. Solutions used in this procedure were SDS solution (10% sodium dodecyl sulphate), denaturing solution (0.5M NaOH; 1.5M NaCl), neutralisation solution (1.5M NaCl; 0.5M Tris-HCl pH 7.4) and 2X SSC solution (made as 20X: 87.65g NaCl; 50.25g trisodium citrate.2H2O in 500ml dH2O). The colony lift filter was placed, colonies up, on Whatman™ 3MM filter paper soaked in these solutions as follows (at room temperature):

1) SDS Solution 10 min
2) Denaturing Solution 5 min
3) Neutralisation Solution 5 min
4) Neutralisation Solution 5 min
5) 2X SSC Solution 15 min

The nitrocellulose filter was then placed colony side up in blocking buffer and shaken gently at room temperature for 4 hours. The colonies were then gently rubbed off and the filter returned to blocking buffer and left shaking overnight. Thereafter the colony lift filter was processed in the same fashion as filters produced in Western blotting (Section 2.2.4).

**2.3.6 Bacterial Transfection**

An overnight culture of TG1 *E.coli* (Section 2.4.2) was diluted 1/100 and the resulting solution incubated a further 6 hours at 30°C on a rotatory shaker incubator. 100μl of the selected phage (Section 2.3.4) were added to 2ml of the cultured bacteria and incubated at 37°C for 1 hour with shaking. The transfected bacteria were then plated out on LBGA agar plates in varying dilutions and incubated overnight at 30°C. The following morning
an estimate of the phage titre (in terms of colony forming units) could be made and phage rescue commenced (Section 2.3.3).

2.4 Protein Production and Purification Techniques

2.4.1 Storage of Bacterial Clones

All bacterial clones were stored at -80°C as glycerol stocks. 500μl of an overnight bacterial culture was mixed with 500μl of sterile glycerol, vortexed briefly to mix, then stored in a Cryovial™ (Nalgene) at -80°C.

2.4.2 Overnight Bacterial Cultures

Overnight cultures of bacterial clones were prepared by picking bacterial colonies from an agar plate or by stab-sampling a glycerol stock of the relevant clone. The bacterial sample was resuspended in 1.5 ml of LB (Luria Broth, Life Technologies) containing 2% D-glucose and, where appropriate, 100μg ampicillin (Boeringer Mannheim) per ml (LBGA). This suspension was then shaken at 250rpm, 30°C overnight.

2.4.3 Preparation of Bacterial Supernatants

All scFvs were isolated from secreted proteins in bacterial supernatants. Overnight cultures were diluted 1/100 in the morning in LBGA and shaken at 250rpm for 6 hours at 30°C to an OD_{600} of approximately 1.5. For small-scale inductions a volume of 1.5ml was used and for large-scale inductions a 500ml culture was produced from a 10ml overnight culture. Induction of protein production was carried out using isopropyl-thiogalactoside (IPTG) in the absence of glucose. The cells were spun down (3000rpm, 10min, RT) and the supernatant poured off. For small-scale inductions, the bacteria were resuspended in LB containing 100μg ampicillin per ml and 500μM IPTG (LB_{AI500}). For large-scale inductions, the bacteria were resuspended in 1 litre of LB containing 100μg ampicillin per ml and 100μM IPTG (LB_{AI100}). A 2.5 litre baffled flask
containing 1 litre of broth was used. Cultures were then shaken overnight (for 16 hours) at 30°C and 250rpm.

Small-scale induction supernatants were analysed without purification after pelleting the bacteria at 13000rpm for 5min on a benchtop centrifuge.

For large inductions, 0.05% sodium azide was added as a bacteriostatic agent and 200μM phenyl-methyl-sulphonyl-fluoride to reduce proteolysis. The culture solution was then spun at 18500 g at 4°C for 1 hour in a cold centrifuge (Beckman, JA-10500 rotor). The supernatant was passed through a 0.2μm filter (Dow Corning) and subsequently concentrated to around 60mls using a tangential flow filtration device (Fig. 2.4.3) with a 10 kDa filtration threshold (Ultrasette™, Filtron). The ultrafiltrate was tested by Western Blot analysis to ensure no scFv was lost during the process. The sample was then dialysed overnight at 4°C against 2 litres of PBS pH 7.5.

2.4.4 Low Pressure Liquid Chromatography

All liquid chromatography was carried out using a Biologic LP Chromatography Platform™ (Bio-Rad). This equipment included a programmable peristaltic pump and fraction collector, conductivity and UV absorbance meters and an electronic mixer valve for accurate proportioning of reagents (Fig. 2.4.4). All purifications were carried out at room temperature.

2.4.5 Purification of scFvs by Immobilised Metal-ion Affinity Chromatography

Two column systems were employed for protein purification by Immobilised Metal-ion Affinity Chromatography (IMAC):

1) An IMAC column was prepared using Chelating Sepharose™ agarose gel (Phamacia Biotech) which has the ability to chelate metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}). ScFvs subcloned into the vector pUC119 His 6 Xba possess a hexahistidine tail at their C-terminus which has a particular affinity for these ions and this affinity forms the basis of this technique.
Bacterial supernatant is pumped through the Ultrasette™ filter. Back pressure produced by the clamp forces H₂O and proteins (< 10 kDa) to flow across the membrane producing an ultra-filtrate. The scFv is too large to pass through the pores in the membrane and is retained (retentate). Adapted image - original courtesy of NV Kang.
Schematic diagram of the different components making up the Biologic LP™ Platform. The red arrows indicate the direction of flow of buffers or protein solutions through the assembly.
A 50ml Econocolumn™ (Bio-Rad) was loaded with 40 ml of Chelating Sepharose™ gel. The gel was packed and washed with distilled H₂O as per the manufacturer's instructions. 60ml of 0.1 M CuSO₄ was then loaded onto the column at 2ml per minute, turning the column blue, then washed through with 60ml of dH₂O to remove free metal ions. The column was then equilibrated with 100 ml of PBS pH 7.5.

Concentrated bacterial supernatants (Section 2.4.3) were taken from PBS dialysis and NaCl added to a 1M concentration. This served to reduce non-specific binding of protein to the column. The bacterial supernatant was then loaded at 1.0ml per minute and the column thereafter washed with 2 column volumes (100ml) of PBS containing 1M NaCl to remove unbound material. The “run-through” was retained for subsequent analysis.

Elution of the scFv was carried out using a linear gradient of imidazole (Sigma Aldrich) and 1M NaCl in PBS pH 7.5 at 1 ml/min. The gradient rose from 20 to 70mM imidazole over 50 ml, from 70 to 90mM over 150 ml, then from 90 to 200mM over 50 ml.

The eluted fractions were collected in 10ml aliquots and analysed by SDS-PAGE and Western blotting to identify the protein fraction. ScFvs typically eluted at a volume of 140-190mls. This fraction containing the scFv was concentrated for use as detailed below (Section 2.4.9).

2) 1ml Hi-trap™ columns (Pharmacia Biotech) were used for purification all scFvs. Columns were prepared according to the manufacturer's instructions and charged with 0.5ml 0.1M nickel, copper or zinc sulphate. Unbound metal ions were washed away using 5 column volumes of distilled water (5ml). The column was equilibrated with 5ml start buffer (10mM imidazole, 0.5M NaCl in PBS pH 7.4) and the bacterial supernatant loaded at 4ml/min. The column was again washed with 5ml start buffer then sequentially with 5 ml of 0.5 M NaCl/ PBS pH 7.4 containing 30mM, 40mM, 50mM, 100mM and 200mM imidazole. The metal ions and any remaining protein on the column was then freed using 5ml 0.1M EDTA. All samples were collected and
concentrated as below (Section 2.4.9). This technique is discussed in more detail in Section 3.2.5.2.

2.4.6 Purification of scFvs by Recombinant Protein-L Affinity Chromatography

Recombinant Protein L (rPL, ACTIgen) is derived from *Peptostreptococcus magnus* and possesses a very high affinity for human kappa light chains. An immunoaffinity column was prepared by coupling protein L to CNBr-activated Sepharose™ gel. 3g of the sepharose powder was swollen in 30ml 1mM HCl then washed on a sintered glass filter using 600ml 1mM HCl. 1mg of rPL was dissolved in 1ml of coupling buffer (sodium carbonate buffer pH 8.3 containing 0.5M NaCl). The Protein L and Sepharose were mixed at room temperature for one hour then excess ligand was washed away using 5 gel volumes of coupling buffer (approx. 40mls), spinning down the gel at 1000rpm for 10mins and discarding the supernatant. Blocking of remaining active groups was achieved by adding 30ml 0.1M Tris-HCl pH 8.0 and rotating at room temperature for 2 hours. The gel was then washed in three cycles of exchange buffer 1 (0.1M Sodium acetate buffer / 0.5M NaCl pH 4.0) then exchange buffer 2 (0.1M Tris-HCl / 0.5M NaCl pH 8.0) using 5 gel volumes in each wash. The gel was spun down the gel before each wash to allow the supernatant to be discarded. Finally, the gel wash washed in PBS containing 0.05% sodium azide before storage.

For use the gel was packed in a 10ml Econocolumn™ (Biorad) and washed with 40ml PBS without sodium azide. Bacterial supernatant containing scFv for purification was then loaded at 1ml per minute and the column washed with 50ml PBS. Bound scFv was then eluted using 15ml 0.1M glycine pH 2.0 an 2ml per min and the column again washed with 50ml PBS. The first 15ml of the elution volume were collected directly into a beaker containing 1M Tris base pH 7.5 to neutralise the acid. The elute was then concentrated to a volume of ~1ml and dialysed overnight at 4°C against PBS pH 7.5. Elution of bound protein was monitored using the UV and conductivity meters and recorded on the chart recorder (Section 2.4.4). The column was washed again with 50ml of PBS and the run-through retained for subsequent analysis. For storage, the column was washed with 50ml of PBS containing 0.05% sodium azide. All eluted fractions
were then analysed by SDS-PAGE and Western blot (Section 2.2) to assess the protein yield and purity.

2.4.7 Purification of RAFT3 scFv by Ion-Exchange Chromatography

Ion exchange chromatography was carried out using a Q-Sepharose™ Hi-Trap™ 1ml ion-exchange columns (Pharmacia). Samples were loaded at 1ml per minute and elution carried out with a stepwise gradient of PBS containing increasing concentrations of NaCl. 5ml volumes of PBS with 0.05, 0.1, 0.2, 0.5 and 1M NaCl were used. Fractions were collected in 5ml aliquots and dialysed overnight against PBS before analysis by SDS-PAGE.

2.4.8 Analysis of ScFv by Gel Filtration

The monomeric or multimeric nature of the scFv constructs was investigated using gel filtration. A pre-packed HiPrep™ S-300 column (Pharmacia Biotech) was equilibrated using 2 column volumes of PBS as per the manufacturer’s instructions. The column was then calibrated using 250μg protein standards of varying molecular weight – chymotrypsinogen, ovalbumin, ribonuclease and albumin (Pharmacia Biotech). 250μg of each protein was pooled in a 1ml PBS sample and loaded onto the column at 0.3ml/min using PBS as running buffer. Two identical calibrations were carried out and the filtration profile recorded on a chart recorder linked to a UV absorbance sensor. The four peaks corresponding to the proteins were identified and the elution volumes ($V_e$) calculated. A standard curve was generated using the means of the calibration runs and this curve used to estimate the molecular weight of the scFv.

100μg of scFv was loaded onto the column in 1ml of PBS at 0.3ml/min and fractions collected at volumes corresponding to the molecular weight of monomeric, dimeric, and trimeric scFv. These samples were then subjected to Western blot analysis.
2.4.9 Concentration of Purified ScFv

Purified scFv was concentrated in 12kDa cut-off dialysis tubing (Mericel) against polyethylene-glycol molecular weight 6000 or 20000 (PEG-6000/20000) to a volume of ~ 1 ml then dialysed overnight at 4°C in PBS pH 7.5. 0.05% sodium azide was added for bacteriostasis and the samples stored at -20°C.

2.5 Tissue Culture

All cultured hybridoma and melanoma cell lines were grown in RPMI medium (Life Technologies) supplemented with 2mM L-glutamine (Gibco) and 10% foetal calf serum (FCS, Gibco). To retard bacterial growth, Penicillin and streptomycin were added to the media for cells intended for in vitro work. No antibiotics were added to the media when cells were intended for in vivo use. Tissue culture procedures were carried out under a Class II Hood (Greiner). This mixture is hereafter referred to as “culture media”. All culture flasks were incubated at 37°C in a tissue culture incubator (Jencons) in an air atmosphere supplemented to 5% CO₂.

2.5.1 Maintenance of A375-M Cell Line

The human melanoma cell line A375-M was used for all in vitro and in vivo experiments involving cultured melanoma cells. The cell line was obtained from the European Catalogue of Human and Animal Cell Culture (ECACC) and grown using standard tissue culture techniques. Briefly, frozen stocks (10⁶ cells) in 1ml dimethyl sulfoxide (Sigma Aldrich) / FCS (1:9) were thawed rapidly in a 37°C waterbath, resuspended in 10ml of culture media and pelleted at 1000rpm for 5min in a benchtop centrifuge. The pellet was resuspended in 1ml of culture media and added to a T25 culture flask (Greiner). At confluence, the culture media was aspirated off and the cells released by incubation in 1:5000 Versene (Life Technologies) at 37°C for ~ 10min. An equal amount of PBS was added and the cells pelleted at 1000rpm for 5 min. When passaging cells, the pellet was resuspended in a small volume of culture media prior to seeding T-75 flasks (Greiner) containing 15ml of culture media. Typically, cells were
diluted 1:5 before seeding the flasks. When larger cell numbers were required, the cells were passaged into T175 flasks (Greiner) containing 40ml culture media.

2.5.2 Preparation of Cell ELISA Plates

In vitro analysis of scFv-antigen interaction was carried out using cell ELISA plates seeded with A375-M melanoma cells, since purified HMW-MAA is not readily available.

Cells from a single T-75 flask at confluence (approx. 8 x 10^6 cells) were harvested and resuspended in 50ml culture media. This suspension was used to seed 10 96-well flat-bottomed tissue culture plates (Falcon) aliquoting 50μl per well (~8 x 10^3 cells). The plates were incubated for 3-4 days (37°C, 5% CO₂) until slightly over-confluent. The culture medium was then aspirated off and the plates dried overnight in a 37°C incubator before storage at 4°C.

2.5.3 Cell ELISA Technique

Cell ELISA plates were typically set up using two-fold dilutions of antibody or scFv in RPMI medium (GibcoBRL) containing 10% FCS and 0.05% sodium azide (Fig. 2.5.3). Total volumes of 50μl per well were employed. Outer lanes on the plate were not used due to inconsistency in the results obtained in these wells.

Antibody-loaded plates were incubated for 1 hour at room temperature before washing three times 200μl of the same media per well. Between washes the wells were emptied by inverting the plate and gently tapping it on some tissue paper. For scFvs, 50μl 9E10 supernatant was added to each well and incubated for one hour. The 9E10 supernatant wash washed off in the same manner and 50μl of a 1:1000 dilution of Rabbit Anti-mouse Horseradish Peroxidase Conjugate (Dako) added. After a further 1 hour incubation, the HRP conjugate was washed off using 3 two-minute washes with 200μl plain RPMI medium per well. Following this wash, all remaining media was aspirated from the wells and 100μl of OPD solution (Sigma Aldrich) added as substrate for the reaction. The plates were observed and the reaction terminated using 50μl 2M sulphuric acid.
Fig. 2.5.3  
Technique of Cell ELISA

All 96 wells are loaded with 50 µl media (RPMI (Gibco) + 10% FCS + 0.05% NaN₃).

Wells in first column are loaded with scFv

Volume in each well of first column is now 100 µl.

Mix gently with multichannel pipette 3-4 times.

Take out 50 µl aliquot from each well of first column and add this to the next well in the second column. Repeat the process until column 12 is reached.

Load each well with 50 µl of 9E10 hybridoma supernatant.

Wash three times with 100 µl/well media (blot off excess media with tissue paper at the end of each wash).

Load 50 µl/well anti-mouse IgG-HRP conjugate (Dako) at 1:1000 dilution in media without NaN₃.

Load 100 µl/well Horseradish peroxidase substrate (OPD) (Sigma Aldrich)

Let colour change over 5-10 min.

Stop reaction with 2.0 M H₂SO₄.

Read plate at 490 nm.
acid per well. The extent of the brown colour change was assessed on a plate reader (BioRad) at 490nm with a mix time of 5s and a reference filter of 430nm. When whole murine monoclonal antibodies were used as the primary antibody, the procedure was identical save that the 9E10 supernatant step was omitted.

2.5.4 Production of 9E10 Hybridoma Supernatant

9E10 monoclonal antibody directed against the c-myc epitope was prepared as a hybridoma supernatant. The cell line was obtained from the American Tissue Culture Collection (ATCC) and maintained according to established culture techniques. Cells were cultured from frozen stocks by the same technique employed for melanoma cells (Section 2.5.1). Cells were passaged from T-25 to T-75 then T-175 flasks containing 100ml of culture media. The hybridoma cells were only mildly adherent and could be detached from the flask using gentle tapping without the need for Versene. Once in T-175 flasks, the media was harvested when depleted and replaced with a further 100ml of culture media and the process repeated. By this technique, many litres of hybridoma supernatant could be prepared. The harvested supernatant was filter sterilised (0.2μ filter, Dow-Corning) and 0.05% sodium azide added as a bacteriostatic agent before storage at 4°C.

2.6 Antibody Radiolabelling

2.6.1 Columns and buffers

Phosphate buffer (pH 7.5) was used for all iodination reactions. To make 100 ml of 1 M phosphate buffer, 81 ml of 1 M Na₂PO₄ was mixed with 19 ml of 1 M NaHPO₄ and adjusted with NaOH to pH 7.5 (Iodination buffer). Iodogen stop buffer was composed of 2.0 mg/ml D-tyrosine (Sigma Aldrich) in 0.05 M phosphate buffer pH 7.5 + 10% glycerol (BDH) + 0.1% xylene cyanol (BDH). Unincorporated radio-iodine was removed using PD-10 gel filtration columns (Pharmacia). These were prepared by running through the column with 60ml 1% BSA in PBS then 60ml PBS alone in a bid to reduce non-specific protein binding.
2.6.2 ScFv Radio-iodination

All reactions were carried out in a dedicated fume hood lined with 3 mm of lead-sheet and a gamma shield (Scot Lab). A lead apron and double layers of latex gloves were worn for all iodination reactions. Standard safety precautions for the safe disposal of all radioactive waste were observed in accordance with regulations for Mount Vernon Hospital, Northwood, Middlesex.

All reactions were carried out at room temperature. Approximately 50µg of scFv (25 - 75µg) in 50-100µl of PBS was placed in a 1.5 ml polypropylene Eppendorf tube. Iodine-125 (Amersham International) as NaI was added to a tube pre-coated with Iodogen (Iodogen Tubes, Pierce) which has been rinsed with Iodination Buffer. In general 10-14MBq of $^{125}$I was used for each labelling. The reaction was allowed to proceed for 6 minutes and then the activated radiolabel transferred to the Eppendorf tube containing the scFv. The mixture was then gently mixed at room temperature for 9 minutes. 50µl of stop buffer was than added and the sample mixed for 5 minutes. The mixture was then transferred to a previously prepared PD10 column (G-25 Sephadex™, Pharmacia Biotech) to separate iodinated protein from unincorporated iodine (see Section 2.6.3).

2.6.3 Separation of Unincorporated Iodine-125

To separate unincorporated iodine from the iodinated protein, the mixtures from iodination reactions were loaded on to the PD10 columns (Section 2.6.1). A tension net at the top of each PD10 column prevented the gel from drying out and ensured that the same volume of fluid loaded onto the top of the column dripped out at the bottom of the column. Aliquots of PBS (300 µl) were used to run the mixture through the PD10 column. A total of 20 fractions were collected from each column and the radioactivity in each fraction was measured.
2.6.4 Estimation of Incorporation of Iodine-125

The fractions collected from the PD10 column with the highest counts (usually fractions 10-15) were assumed to contain the labelled protein and were pooled. Counts in these fractions were expressed as a percentage of the total counts in all fractions + the PD10 column (Fig. 2.6.4). The amount of radioactivity incorporated into the protein sample was assumed to be the same percentage of the total iodine used in the reaction.

2.7 Determination of Antibody Affinity

2.7.1 Antibody Preparation

Radiolabelled antibodies were prepared as previously described (Section 2.6). The radiolabelled scFv were diluted to 20 µg/ml (1µg in 50 µl) in media (RPMI pH 7.4 (Gibco) + 10% FCS (Gibco) + 0.05% NaN₃ - see Section 2.5). Unlabelled LHM2-IgG was diluted to 10 µg/ml (500 ng in 50 µl) in media while scFv were diluted to 40 µg/ml (2 µg in 50 µl).

2.7.2 Preparation of Cultured Melanoma Cells

Cultured melanoma cells (cell line A375-M) prepared as previously described (see Section 2.5.1) were used for all experiments. All cells were allowed to reach confluence before use in antibody binding experiments.

2.7.3 Scatchard Analysis

In order to determine antibody affinity and antigen density, a cell suspension technique (Mariano et al., 1995) was used to allow the antibody to interact with melanoma surface antigens in a controlled and reproducible system. The objective was to measure the amount of radiolabelled antibody or antibody fragments binding to the melanoma cells at equilibrium. Excess amounts of unlabelled intact monoclonal antibody LHM2 were
### Results of Sample Iodination of RAFT3 scFv

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Volume (μl)</th>
<th>cps at 10 cm</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>0</td>
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<tr>
<td>2</td>
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<td>20</td>
<td>300</td>
<td>35</td>
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</tbody>
</table>

Iodine-125 used in this reaction = 10 MBq

\[
\text{Total counts} = \frac{3540 \text{ cps}}{4045} \times 100\% = 800 \text{ cps}
\]

\[
\text{Activity attained} = \frac{1.1 \text{ MBq}}{50 \mu g} = 0.022 \text{ MBq/μg}
\]

Retained on column (@ 30cm) = 700 cps
All fractions (@ 30cm) = 100 cps
Total counts = 800 cps

\[
\text{% Incorporation} = \frac{3540}{4045} \times 100\% = 8.7\% (1.1 \text{ MBq})
\]
used to competitively inhibit binding of the radiolabelled scFv in order to determine the specific and non-specific proportions.

A number of assumptions were made in these experiments. It was assumed that the steady state was reached after two hour incubation at room temperature. At the steady state, it was assumed that the amount of scFv remaining in the supernatant represented the unbound or "free" scFv while any activity associated with the cells recovered from the solution represented scFv "bound" to the cells. For all calculations, the molecular weights of dimeric and monomeric scFv were assumed to be 52 kDa and 27 kDa respectively.

10^6 A375-M cells from confluent culture flasks (Section 2.5.1) were placed in 20 Eppendorf tubes in RPMI medium/10% FCS to a volume of 0.4ml and 100μl PBS was added. The tubes were divided into two sets of 10 and double volume was placed in the 1st tube of each together with 2μg of ^125I-labelled scFv. Serial two-fold dilutions of iodinated scFv were made across two sets of 10 Eppendorfs. In the control set, a >10-fold excess of inhibiting LHM2 IgG was added to prevent any specific binding and give an indication of non-specific binding. The entire experiment was set up in triplicate to allow average values to be obtained.

The Eppendorfs were then rotated at room temperature for 2 hours to allow equilibrium to develop.

400μl of each sample was then added to 200μl of dibutyl phthalate mixture (4:1 diputyl phthalate : phthalic acid dinonyl ester (Sigma)) in Eppendorf tubes and the sample centrifuged on a benchtop centrifuge at 7000rpm for 1 minute. The phthalate oil mixture allowed the cells (and bound scFv) to sink to through the oil to the bottom of the tube while retaining the supernatant (and unbound scFv) in the upper (aqueous) phase. 100μl of supernatant was then removed and placed in a scintillation tube. The Eppendorfs were then placed carefully in liquid nitrogen and the contents frozen. This cells and bound scFv were then separated from the supernatant by cutting off the bottom of the tube (and the cell pellet) into a scintillation tube.
Chapter 2

Scintillation tubes were placed directly into a gamma counter with no further processing. Accumulated radioactivity in each sample was measured in a CompuGamma CS gamma counter (LKB Wallace) programmed to measure a gamma radiation peak of between 35-102 keV for iodine-125. Counts were accumulated over 2 minutes. An average value for cpm (counts per minute) was calculated by the machine.

Saturation binding curves were then produced by charting bound vs. unbound fractions of scFv for the various dilutions. Specific binding was estimated by subtracting the non-specific binding seen in the inhibited vials from the binding measured in the uninhibited samples. Scatchard plots were then produced and affinities of the various antibodies estimated.

The Scatchard plot was made by plotting the ratio of scFv specifically bound (SB) : unbound (U) in the supernatant against scFv specifically bound (SB). A straight-line was fitted to the data points using Origin™ software (version 4.0, Microcal™). The slope of the curve gave the association constant \( K_a \) in 1/mol. Errors for \( K_a \) were automatically calculated by the software package.

2.8 Animal Model

2.8.1 Tumour Cell Preparation

A375-M tumour cells were used for all animal experiments. Cells were cultured as described in Section 2.5 in the absence of antibiotic. Cells were harvested from confluent flasks under sterile conditions less than 1 hour prior to inoculation.

2.8.2 Tumour Production in Mouse Model

Immuno-deficient Balb/c nu female mice were used for all experiments. Mice were obtained from existing breeding stock at the animal house (Gray Laboratory, Mount Vernon Hospital). Standard aseptic techniques were used in all procedures involving
immuno-compromised mice according to the animal house protocols. The mice were housed in MB1 plastic cages (North Kent Plastics) holding a maximum of 20 mice and fed on a diet of standard expanded pellets (B & K Universal). Mice were implanted with tumour cells when they reached between 7 to 10 weeks of age.

Hands and forearms were scrubbed with antiseptic agents before touching the animals. All clothing except underwear was removed before entering the animal house and surgical hats, surgical masks, dedicated (clean) overalls, latex gloves and operating boots were worn at all times.

A clean operative field was established by laying tissue paper onto the bench-top and spraying this with 70% ethanol. The cell suspension was drawn up into a 1 ml syringe (NHS supplies) and a 23 G x 1” (Terumo™ “blue-hub”) needle was fixed on the end of the syringe.

Ten million cells were used for each animal to be inoculated. Cells were resuspended in sterile PBS to a volume of 50μl per 10 million cells and injected subcutaneously in to the flank of a Balb/c nu mouse under Metofane™ anaesthesia (methoxyfluorane, Janssen Pharmaceutical). A small subcutaneous bleb was raised by the tumour bolus at the site of inoculation on the right flank. The animals were then returned to their cages after recovery from anaesthesia and observed daily until the tumours had reached a size of approx. 7mm mean geometric diameter (after ~ 3 weeks) before localisation experiments were undertaken. (Fig. 2.8.2)

2.9 Antibody Biodistribution and Pharmacokinetics

2.9.1 Experimental Design

Freshly radiolabelled antibody or antibody fragment was injected by tail vein injection after filtering the solution through a 2μ filter (Millipore). Each mouse was injected with approx. 0.5 μg of antibody fragment at an activity of around 3MBq per μg. Efforts were made to ensure that the absolute amount of radiolabelled antibody used in each
Balb/c nu mouse 3 weeks after subcutaneous injection with $1 \times 10^5$ A375-M (human) melanoma cells into right flank. The tumour measures 7 mm (+/- 1 mm).
Photo courtesy of N.V. Kang.
experiment was similar. However, there was still some variation due to differences in
the specific activity achieved with each iodination reaction.

No specific steps were carried out to remove endogenous pyrogen, trace amounts of
Cu\(^{2+}\) (or other ligand) or NaN\(_3\) in the radiolabelled preparations before injection.
However, no adverse effects of any kind (directly attributable to the injected solutions)
were observed in the mice for the duration of any of the experiments.

For localisation experiments, time points were selected which reflected the expected
distribution of antibody and antibody fragments based on previously published data
(Colcher et al., 1990a). For our scFvs, 4 time points were selected: 1 hour, 3 hours, 6
hours and 18 hours after injection.

For pharmacokinetic experiments, time points were selected which reflected the
expected blood clearance of antibody fragments based on previously published data
(Colcher et al., 1990a). At these time points, groups of between 3 and 5 mice were
sacrificed by cervical dislocation. Time points of 1 minute, 15 minutes, 30 minutes, 60
minutes, 90 minutes, 3 hours, 6 hours and 18 hours after injection were selected.

2.9.2 Injection of Radio-labelled Antibody

Mice were warmed for 2 to 3 min under a 250W infrared lamp (Salamander) at 30cm to
increase tail vein dilation. Metofane™ anaesthesia used and tail vein injections carried
out using a 26 G needle (Microlance).

In order to ensure reproducibility of the administered dose, all radiolabelled antibody
fragments were diluted to a volume of 100 μl/mouse. After injection, haemostasis of
the venepuncture site was achieved by digital pressure applied for 1 - 2 minutes. The
mice were returned to their cages and left on tissue paper in the prone position to
recover from the anaesthetic.
2.9.3 Sample Collection

At the pre-determined timepoints, mice were sacrificed by cervical dislocation and tissue specimens obtained immediately. The whole tumour, both kidneys, both lungs, the right lobe of the liver, the left quadriceps muscles, the left femur and the spleen were collected. A blood sample was obtained by cardiac puncture. Samples were placed into pre-weighed scintillation tubes (BDH) for analysis.

2.9.4 Sample Analysis

Sample weights were determined and the scintillation tubes were loaded onto a gamma counter (CompuGamma CS, LKB Wallace). The activity in each tube was measured in counts per minute (cpm) over 3 minutes with a window set at 111 to 140keV. A calibration sample of the radio-labelled antibody or antibody fragment equal to that injected per animal was also loaded to allow calculation of the percentage of the injected dose in each sample (%ID).

After counting the sample weight was determined and the %ID/g of tissue calculated:

\[
\%\text{ID/g} = \frac{\text{cpm in sample} \times 100}{\text{cpm in calibration sample (100\%ID)} \times \text{sample weight (g)}}
\]
3. ScFv Production and Purification
3. ScFv Production and Purification

3.1 Introduction

This chapter details the backgrounds of the original scFvs used in our studies and our attempts to optimise the production and purification of these scFvs. ScFvs consist of only the isolated Fv segment of a whole antibody molecule. The linking of the C-terminus of one V domain of the Fv to the N-terminus of the other results in a single continuous polypeptide which can be produced as a recombinant protein (Bird et al., 1988; Bird and Walker, 1991). These molecules are scFvs and are demonstrated diagrammatically in Figure 3.1. The scFv has a mass of approximately 27kDa in comparison to 150kDa for the whole IgG molecule. The short peptide linker incorporated in the protein allows refolding of the protein strand with reconstitution of the three dimensional structure of the Fv and therefore the antigen binding site.

When produced as recombinant protein, scFv yields have been noted to vary greatly depending on the individual molecule (Deng et al., 1994; Kipriyanov et al., 1997a; Kipriyanov et al., 1997b; Merk et al., 1999). ScFvs can be recovered from bacterial cytoplasm, periplasm or supernatant (Plückthun, 1991). Some scFvs are produced in relatively small quantities (Anand et al., 1991; Harper et al., 1999) and this was indeed the case with the anti-melanoma scFv LHM2, the parent molecule in our studies. In addition, much of the protein may be produced in a non-native, unfolded form which is insoluble or does not retain the antigen-binding capability of the folded peptide. The use of plasmids incorporating a secretion leader allows the bacterial cells to export the protein into the supernatant in the properly folded state, though often in much reduced amounts (Harper et al., 1999). Alternatively, periplasmic or intracellular non-native (unfolded) protein can be isolated and attempts made at refolding in vitro (Whitlow, 1991; Skerra, 1993; Kipriyanov et al., 1995). Whilst this technique can significantly increase yields, it can be problematic. Refolding can be a time-consuming and difficult process. Functional, native scFv is present in the periplasmic space but the yield is limited by the rate at which periplasmic folding takes place (Knappik et al., 1993). In
Fig. 3.1  ScFv Structure in Relation to Whole IgG Molecule

Fv

scFv (27kDa)

Fab Region

Vh and Vk domains of Fv joined by peptide linker

Fc

Whole Immunoglobulin (~150kDa)
addition, without a supply of antigen or anti-idiotype monoclonal antibody to test binding (e.g. in immunoaffinity purification), it is difficult to assess the efficiency of the refolding process. In our case, High Molecular Weight Melanoma-Associated Antigen was not available and makes refolding a less appealing prospect.

To use a recombinant molecule such as a scFv in clinical practice, one must not only demonstrate the efficacy of the molecule in targeting but also develop a method of production and purification capable of producing large amounts of clinical grade material. Since the original LHM2 scFv showed very low expression and purification yield (less than 50μg per litre of bacterial supernatant), we concentrated on methods aimed at increasing the yield and optimising the purification process in order to address these problems. In addition, relatively low solubility was problematic and required further investigation. This chapter details the background to the development of the scFvs under study and our attempts to combat the problems of yield, purification and solubility which were originally apparent.

3.1.1 Rationale

1) Bacterial scFv yield

As noted above, single-chain Fvs can be recovered from the bacterial supernatant, periplasm or cytoplasm of bacteria (Plückthun, 1991). However, the yield of scFvs recovered in the supernatant is relatively low varying between 0.2 and 16.5 mg per litre of bacterial culture (Skerra et al., 1991; Kipriyanov et al., 1997a). Yields of scFv from the periplasm or cytoplasm are typically higher, varying between 5-120 mg/l from the periplasm (Deng et al., 1994; Kipriyanov et al., 1994) and 10-50 mg/l from whole cell extracts (Anand et al., 1991; Yang et al., 1995). Our previous data comparing the yields of LHM2 scFv from the supernatant, periplasm and whole cell extracts are in agreement with this pattern (Kang, 1998). This same work demonstrated that secreted soluble scFv was the easiest to purify in a functional state and therefore our subsequent studies have concentrated on this method of scFv production.
Manipulation of the conditions of protein induction can significantly affect protein yield. By altering the temperature, bacterial density, inducing agent concentration and length of induction, we sought to optimise the yield of scFv. It has been shown that the incorporation of osmotic agents such as sucrose into the induction medium can increase yield (Kipriyanov et al., 1997a) and this strategy was also investigated.

Finally, it has been suggested that the orientation of the V domains in the plasmid ($V_hV_l$ or $V_lV_h$) and, therefore the eventual scFv, can influence the eventual recombinant protein yield (Anand et al., 1991; Tsumoto et al., 1994; Merk et al., 1999). We therefore investigated the effect of reversing the orientation of our scFv in the plasmid on eventual protein yield and on the scFv’s in vitro and in vivo properties.

2) Purification

A wide range of purification methods are available for isolating specific proteins from bacterial supernatants (Doonan, 1996). Previous work had demonstrated the efficacy of Immobilised Metal-Ion Affinity Chromatography (IMAC) in purifying our scFvs when histidine-tagged (Kang, 1998). We investigated a variety of techniques including IMAC using a variety of immobilised ligands, Immunoaffinity Purification using recombinant Protein L and Ion Exchange Chromatography to improve upon previous yields and to identify the most efficient methods for our purposes.

3) Improving scFv solubility

The production of recombinant scFvs whether by the isolation of variable regions of a hybridoma-derived monoclonal antibody or by phage selection yields protein molecules which are not found in vivo. This “detachment” of the Fv from the remainder of the immunoglobulin reveals amino acids within the chain which would normally be “concealed” in the folds of the protein. Whilst naturally occurring IgG is highly water-soluble at physiological pH, the exposure of hydrophobic amino acid moieties by this mechanism may result in relatively insoluble protein (Nieba et al., 1997). A review of the literature reveals very little detail on the typical solubilities of scFvs. Some authors,
however, have addressed the issue for individual scFvs and have attempted to increase
the solubility of the molecules either by the addition of hydrophilic amino acids onto the
end of the protein chain (Tan et al., 1998) or by the replacement of uncovered
hydrophobic patches with hydrophilic residues (Nieba et al., 1997). Similarly,
Riechman described the enhancement of isolated $V_H$ domain solubility by the
replacement of hydrophobic residues normally associated with the (now absent) $V_L$ by
mimicking the structure of camelid heavy chains (Riechmann, 1996). Camel heavy
chains are naturally devoid of light chains and therefore do not carry hydrophobic
residues at these positions.

Our scFvs demonstrated limited solubility at higher concentrations which limited our
ability to purify highly concentrated antibody and the efficacy of experimental assays
requiring higher concentrations of the recombinant protein. The solubility of the scFv
was studied and attempts made to increase the solubility by modifying the buffer
solution used and its pH.

The general methods involved are detailed in Chapter 2 and more specific methods
follow.
3.2 Methods

3.2.1 Design of LHM2 scFv and Anti-CD18 scFv

Four scFvs are used in these experiments – LHM2 scFv, RAFT3 scFv, RAFT3DS scFv and anti-CD18 scFv. Details of RAFT2 scFv (Chapter 4) are included in this chapter for completeness and clarity. Two of these scFvs (LHM2 scFv and anti-CD18 scFv) were designed and constructed by Dr. Jörg Kupsch at the London Hospital Medical College and the RAFT Institute for Plastic Surgery. RAFT2 scFv was developed by Dr. N. Kang at the RAFT Institute. Each of the other scFvs is based on the original LHM2 molecule. Appropriate details on the subcloning or construction of all five scFv are included in this thesis.

The LHM2 scFv (London Hospital Medical College clone 2 scFv) was designed and constructed by Dr Jörg Kupsch (Kupsch et al., 1995; Kupsch et al., 1999). It is directed against the high molecular weight melanoma associated antigen (HMW-MAA) (Section 1.2.2). The antibody fragment was produced by isolating the \( V_L \) and \( V_H \) gene segments of the anti-HMW-MAA mouse monoclonal antibody LHM2 IgG. The \( V \) gene segments were cloned using mouse \( V \) region-specific PCR primers. The \( \approx 350 \) bp PCR fragments were isolated, purified and then mixed together with appropriate primers in a further PCR reaction to introduce a 15 amino acid linker and to assemble the two \( V \)-segments using splicing by overlap extension (Horton et al., 1989). This LHM2 cassette was then subcloned into plasmid pCantab5 (Fig. 3.2.1a). The subcloned plasmid was designated LHM2 scFv (Fig. 3.2.1b). The LHM2 scFv construct includes an N-terminal secretion signal which directs transport of the scFv into the bacterial periplasm. From here the scFv is released into the supernatant facilitating recovery of the scFv (Plückthun, 1990; Skerra et al., 1991). It also includes a C-terminal \( c\text{-}myc \) sequence for detection by Western blot. The \( c\text{-}myc \) tag can be used for immunoaffinity purification of the LHM2 scFv using the anti-\( c\text{-}myc \) antibody 9E10 (Section 2.5.4). However, purification by immunoaffinity chromatography using this tag proved to be difficult (Kang, 1998). For this reason, the LHM2 scFv was subcloned into a different vector containing a hexahistidine tail (pUC119 His 6 \( myc \) Xba) to simplify purification (Section 3.2.2)
**Fig. 3.2.1a** Polycloning Site of pCantab5

- **Sfi I**: V_{H}
- **V_{L}**: Not I
- **g3 signal**: Hind III (2235)
- **c-myc**: P_{usc}
- **V_{H}** Linker **V_{L}**: for Western blot analysis

**Fig. 3.2.1b** ScFv in pCantab5

- P
- **Gene III**
- **V_{H}**
- **Linker**
- **V_{L}**
- **c-myc**

- **Secretion Signal**
- **LHM2 scFv cassette**
- **c-myc tag** for Western blot analysis
Chapter 3

The anti-CD18 scFv was made in a similar manner by Dr Jörg Kupsch and is derived from a whole, humanised, anti-CD18 monoclonal antibody (Sims et al., 1993). In order to simplify purification, the anti-CD18 scFv was also subcloned into plasmid pUC119 His 6 myc Xba for purification by IMAC (Section 2.4.5).

3.2.2 Sub-cloning of RAFT2 scFv

Purification of the scFv by immobilised metal-ion affinity chromatography (IMAC) was made possible using the plasmid pUC119 His 6 myc Xba (Hawkins et al., 1994), a gift from Dr G. Winter (Cambridge) (Fig. 3.2.2a). Details of the technique of IMAC are given in Section 2.4.5. In brief, it relies on the affinity of certain metals for a poly-histidine tail. The construct in plasmid pUC119 His 6 myc Xba was cloned with the N-terminal secretion signal which allows the scFv to be recovered from the supernatant. It also introduces a C-terminal c-myc tag for detection by Western blot followed by six histidine residues for IMAC purification.

The LHM2 cassette was produced by PCR using the original LHM2 scFv as a template. The primers used generated a 980bp PCR fragment which included the bacterial secretion signal (geneIII) derived from pCantab5. The PCR fragment was gel purified and double digested with Not I and Hind III producing an 860bp fragment for ligation with Not I/Hind III linearised pUC119 His 6 myc Xba (Fig. 3.2.1a). The ligation was used to transform E.coli TG1. Successful insertion of the LHM2 cassette into pUC119 His 6 myc Xba was demonstrated by double digestion of the plasmid DNA with Hind III and Not I.

The subcloned plasmid was designated RAFT2 (Fig. 3.2.2b). Expression of the RAFT2 scFv is under the control of the lac promoter and was tested by Western blot analysis of a small-scale (2ml) culture (Section 2.4.3). The RAFT2 scFv is secreted from E.coli as soluble protein which can be recovered from the supernatant. Intracellular expression of scFv was determined by preparing whole cell extracts for analysis in Western blot.

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Fig. 3.2.2a
Polycloning Region in pUC119 His 6 myc Xba

Fig. 3.2.2b
ScFv in pUC119 His 6 myc Xba
Western blot analysis of the supernatant indicated that a 27 kDa protein, similar in size to the LHM2 scFv was produced. The Western blot showed that the RAFT2 scFv was present in both the supernatant and the whole cell extract. The results also demonstrated that the relative yields of RAFT2 scFv and LHM2 scFv in the supernatant appeared to be similar. Further analysis by cell ELISA showed that the RAFT2 scFv derived from the supernatant bound to human melanoma cells. RAFT2 scFv from periplasm and whole-cell extracts was not used in further experiments because the results from previous experiments with LHM2 scFv revealed that scFv from the periplasm had reduced binding while that derived from the whole-cell extracts failed to bind melanoma at all (Kang, 1998).

These data, therefore, suggested that pUC119 His 6 myc Xba was the most suitable vector. Consequently, all subsequent experiments were carried out using scFv which had been subcloned into this vector.

3.2.3 Design of RAFT3 scFvs

A human-mouse chimeric scFv library was designed and constructed by Dr. Jörg Kupsch using the technique of chain-shuffling. One antibody fragment selected from that library with the same melanoma specificity as the original LHM2 scFv (designated RAFT3 scFv) was used in further studies in this chapter. This process is described in detail in Chapter 4 Section 4.2.2 and is briefly summarised below.

The LHM2 and RAFT2 scFvs are derived from mouse V_H and V_L-regions. human anti-mouse antibody (HAMA) reactions are likely to be either low or non-existent when scFv are administered to humans, an attempt was made to fully humanise the LHM2 scFv through the technique of chain-shuffling (Marks et al., 1992). In this technique, first one, and then the other mouse V region is substituted with the sequences for human V regions. This process is demonstrated stylistically in Fig. 3.2.3.

Antibody phage display was employed to select phage expressing anti-melanoma scFv by panning the selected library against A375M melanoma cells. By retaining the
Fig. 3.2.3
Chain-Shuffling to Replace Mouse $V_{\kappa}$ Light Chains With Human $V_{\kappa}$

LHM2 scFv in pCantab (Mouse)  

- $V_H$  
- $V_L$  
- PCR Fragment 300-400 bp  
- $V_H$ specific for melanoma

$V_{L(\kappa)}$-Library in pUC19 (Human)  

- $V_L$  
- PCR Fragments 300-400 bp  
- $V_{L(\kappa)}$ with random antigen specificity

PCR Reaction

- PCR Reaction to introduce linker

$V_H$ Mouse Derived  
$V_L$ Human Derived

Ligated

Subcloned into pCantab for Selection by Phage-Display
original mouse $V_H$ region specific for HMW-MAA, the specificity of the chimeric construct for the same antigen was conserved. The use of phage display for selection of scFv had the further advantage of selecting clones with increased affinity for the antigen (discussed in detail in Chapter 4). It is generally accepted that the expression of scFv is toxic to bacteria (Hayhurst, 2000). Therefore, those bacteria infected by phage encoding toxic scFv sequences would be competitively disadvantaged and those containing less toxic scFv would predominate. This would favour the selection of higher-yield clones.

The new scFv cassettes were subcloned into pUC119 His 6 myc Xba in a manner analogous to the RAFT2 scFv (Section 3.2.2) in order to simplify purification by IMAC (Section 2.4.5). Twenty clones were studied and the two performing best in cell ELISA binding melanoma cells and in Western blot analysis of scFv yield were chosen. The two mouse / human chimeric scFvs in this vector selected for subsequent study were named RAFT3 scFv and RAFT2-11 scFv (see Chapter 4).

3.2.4 Optimising Expression of scFv

Expression of our RAFT series scFvs in *E.coli* is low. Several variables were investigated in order to optimise the yield. Experiments were carried out using the RAFT3 scFv.

3.2.4.1 Optimising Induction Conditions

Small overnight cultures of scFv clones were prepared as described in Section 2.4.2. The following morning these were diluted in a ratio of 1:100 in 1.5ml of LB$_{GA}$ media (Section 2.4.3). After 6hrs incubation, the cells were pelleted and resuspended LB$_{AI}$ (i.e. containing ampicillin and IPTG). The most effective bacterial concentration at induction was assessed by varying the starting optical density (OD$_{600}$) of the solution. OD$_{600}$s of 0.25, 0.5, 1.0, 1.5, 2.0 and 3.0 were set up in 1.5ml LB$_{AI}$ cultures containing 100mM IPTG. The optimum concentration of induction agent was investigated by resuspending bacteria in medium containing 10, 50, 100, 300 or 500µM IPTG to induce scFv production. They were then incubated in a rotary incubator at 308C. Similarly, induction times of 1, 3, 6, 12, 18, 24 and 48hrs were studied to assess the optimum
incubation time. The influence of induction temperature was established using 100µM IPTG either at room temperature, 30°C or 37°C. Finally, overnight inductions at 30°C in 100µM IPTG were carried out with and without the addition of 1 molar D-sucrose. Samples collected were assayed by Western blot using the c-myc detection tag to assess the scFv yield (Section 2.2).

3.2.4.2 Domain Swapping

As described above, it has been suggested that the orientation of the scFv V domains in the plasmid might affect the ability of the bacterial cells to manufacture the recombinant protein (Anand et al., 1991; Merk et al., 1999). This was investigated using the RAFT3 scFv which was originally cloned in the V\textsubscript{H} domain-V\textsubscript{L} domain orientation. The strategy is outlined diagrammatically in Figure 3.2.4.2a. Specific primers were designed to allow the V domains to be isolated separately from the plasmid using PCR techniques (Section 2.1). The cloning strategy and primers used are indicated in Figure 3.2.4.2b. The V domains were joined in the V\textsubscript{L}-V\textsubscript{H} orientation by digesting the DNA at a BamH\textsubscript{I} site in the linker region and ligating the fragments. The ligation was then used as a template for the PCR using the outer primers. Correct fragment size was confirmed and the DNA purified by TAE gel electrophoresis (Sections 2.1.5 and 2.1.8). The DNA fragment was then re-ligated with the parent plasmid pUC119 His 6 myc Xba (Section 2.1.5) and transformed into E. coli TG1 by electroporation (Section 2.1.7). The successfully transformed bacteria were identified by selection on LB agar plates containing ampicillin, resistance to which is encoded by the plasmid. Colonies were then picked and small-scale inductions made (Section 2.4.3). The supernatants of these inductions were assayed in cell ELISA against melanoma cells and Western blot to confirm anti-melanoma scFv production. The best binding clone was selected for subsequent analysis and named RAFT3DS (domain swap).

Large scale inductions (1 litre) of the RAFT3 and RAFT3DS scFvs were then made (Section 2.4.3) to allow comparison of yield and of their in vitro and in vivo properties.
RAFT3 in pUC119 His 6 myc Xba

- Isolated by PCR
- Linker digested
- Orientation reversed in ligation
- Religated into plasmid
The RAFT3 V<sub>K</sub> was amplified with R230 and R228 and the fragment reamplified with R229 and R228. This introduces a BamHI site in the linker and a c-terminal NotI site. The RAFT3 V<sub>H</sub> was amplified with R231 (SfiI site) and R233 (BamHI site). The two fragments were digested with BamHI, ligated and the ligation used as template for a PCR with primers R231 and R228. The resulting ~750 bp fragment was cloned SfiI/NotI into pUC119 his 6 myc Xba.

**Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>R228</td>
<td>CAAGAGCCGCCHCCTCGTGAGAGGAGACGGTG</td>
<td>NotI</td>
</tr>
<tr>
<td>R229</td>
<td>TGACTGATACGGGAAGTGCACGGTTGGGATCTCTGCAGAT</td>
<td>BamHI</td>
</tr>
<tr>
<td>R230</td>
<td>AGGTGGCGGATCTCAGATCCAATTGG</td>
<td></td>
</tr>
<tr>
<td>R231</td>
<td>TATGCGGCCCAGCAGCCGAATTGTGCTGAC</td>
<td>SfiI</td>
</tr>
<tr>
<td>R233</td>
<td>TCATGCGGATCTCCGCCACCACGGTTGATTTCC</td>
<td>BamHI</td>
</tr>
</tbody>
</table>
3.2.4.2.1 Production and Purification of RAFT 3 and RAFT3DS scFvs

One litre cultures of the *E. coli* TGI incorporating the modified scFv expression plasmid were made and induced to produce recombinant protein as described in Section 2.4.2. After dialysis of the concentrated, filtered bacterial supernatant against PBS at 4°C, the scFv was purified using Immobilised Metal Ion Affinity Chromatography (IMAC) on Hi-trap Sepharose Columns™ and a stepwise gradient of imidazole (Section 2.4.5). Collected fractions were then assayed by SDS-PAGE and Western blot under reducing conditions to establish the fractions containing scFv and confirm its purity (Section 2.2).

3.2.4.2.2 Confirmation of Epitopic Specificity

In order to demonstrate that the various clones still bound the same epitope of the High Molecular Weight Melanoma Associated Antigen, a competition cell ELISA was set up. The wells were initially loaded with 500ng of scFv per well and serial dilutions of LHM2 IgG (the parent mouse monoclonal antibody) were carried out across the plate from 500 to 1ng per well. LHM2 IgG binding was detected using rabbit anti-mouse IgG-HRP conjugate (Dako) which does not cross-react with scFv. The ELISA was completed as previously described (Section 2.5.3) and the plate read at 490nm.

3.2.4.2.3 Radiolabelling

Radiolabelling was carried out using the Iodogen™ method (Pierce) and $^{125}$I as the radiolabel (Section 2.6).

Immuno-reactivity after labelling was assessed by comparing equivalent amounts of scFv to cold antibody in cell ELISA (Section 2.5.3).
3.2.4.2.4 Affinity Studies

The affinity constants of the scFvs were assessed in order to identify and quantify any variance in affinity for the antigen in vitro. Saturation binding and Scatchard plots were obtained after incubating the radiolabelled antibody with melanoma cells as described in Section 2.7.3. Affinity and dissociation constants were calculated using Graphpad Prism™ analysis software.

3.2.4.2.5 In Vivo Studies

Pharmacokinetic and biodistribution data were obtained in Balb/c nu mice bearing human melanoma xenografts (Section 2.8) for RAFT3 scFv, RAFT3DS scFv and control anti-CD18 scFv. 0.5µg of iodine-125 labelled scFv in 100µl PBS was injected via the tail vein and the mice euthanased at appropriate timepoints. Three to five mice were used for each timepoint. Anti-CD18 scFv was used as a negative control for all experiments.

3.2.4.2.5.1 Biodistribution

Mice were sacrificed at 1, 3, 6 and 18 hours after injection. A blood sample was obtained by cardiac puncture and organs removed for weighing and gamma-counting. The tumour, left quadriceps, left femur, spleen, liver, kidneys and lungs were counted.

3.2.4.2.5.2 Analysis of Biodistribution Results

Results were expressed in terms of percentage of the injected dose per gram of tissue (%ID/g) and from this tumour to normal tissue ratios (T:NT) were calculated. Comparison to the negative control anti-CD18 scFv allowed calculation of the radio-localisation index (RI) which represents the tumour-specific localisation of the antibody fragment in question.
3.2.5 Optimising Protein Purification

All scFvs were purified from bacterial supernatants (Section 2.4).

3.2.5.1 Protein Concentration

Bacterial supernatant was concentrated by ultrafiltration (Section 2.4.3). In order to confirm that scFv was not lost through the ultrafiltration membrane, the retentate and ultrafiltrate were subjected to Western blot analysis (Section 2.2). The concentrated supernatant was then dialysed overnight against PBS at 4°C.

3.2.5.2 Purification of RAFT3 scFv using Immobilised Metal Ion Affinity Chromatography (IMAC)

The RAFT3 scFv in vector pCantab5 (used for antibody phage display) was subcloned into pUC119 His 6 myc Xba which incorporates 6 histidine residues at the C-terminus of the scFv (Fig. 3.2.2b). All the scFvs described in this section were produced after a standard induction and purified from one litre of supernatant concentrated to ~100 ml as previously described (Section 2.4.3). All elution fractions obtained from IMAC columns were concentrated to ~1ml by dialysis against PEG. This was followed by dialysis against PBS overnight at 4°C and analysis by SDS-PAGE and Western blot.

Previous work had focussed on using large (50ml) IMAC columns charged with copper ions (Kang, 1998). This technique was compared to purification using proprietary mini-columns containing highly cross-linked sepharose charged with a variety of metal ions (copper, nickel and zinc).

Large scale IMAC columns were prepared and used as described in Section 2.4.5. SDS-PAGE and Western blot analysis was carried out on all fractions obtained after loading bacterial supernatant containing RAFT3 scFv.
Small highly cross-linked IMAC columns were obtained pre-prepared (Hi-Trap Chelating Sepharose™ HP columns, Pharmacia). 1ml columns were used. The concentrated bacterial supernatant was adjusted to 0.5M NaCl and 0.01M imidazole at pH 7.5. ScFv purification was then carried out as follows:

1) The columns were washed using 5 column volumes of dH₂O (5ml) to remove storage buffer.
2) The columns were charged using 0.5ml of 0.1M metal-ion solution (CuSO₄, ZnSO₄ or NiSO₄).
3) Excess ions were removed by washing with 5 column volumes of dH₂O.
4) The columns were equilibrated using 5 column volumes of Start Buffer (0.01M imidazole, 0.5M NaCl in PBS, pH 7.5).
5) Concentrated induction supernatant containing scFv was loaded at 2ml per minute using the Biologic LP platform.
6) The columns were washed using 5 column volumes of Start Buffer.
7) Step-wise protein elution was carried out using 5ml volumes of increasing concentrations of imidazole (30, 40, 50, 100 and 200mM) in PBS at pH 7.5 containing 0.5M NaCl.
8) Metal ions and any protein remaining on the column were stripped using 5ml 0.1M EDTA in PBS.
9) The columns were washed using 5 column volumes of dH₂O (5ml) before re-use.

All eluted samples were collected for subsequent analysis and the process repeated using all 3 metal ions in the study. It was noted that considerable stripping of metal ions occurred in the case of nickel and copper ions during loading of the bacterial supernatant (zinc ions are colourless). Extensive dialysis of the concentrated supernatant against PBS before loading failed to resolve the problem. This effect seemed likely to markedly reduce the efficiency of purification and was overcome by placing 3 separate Hi-Trap™ columns in series and carrying out the purification steps using threefold increases in the buffer volumes (Fig. 3.2.5.2).
Fig. 3.2.5.2 Series HiTrap™ Columns

Single column

Bacterial supernatant loaded

Bacterial supernatant causes leeching of metal ions

All of metal ions and scFv lost

3 columns in series

Bacterial supernatant loaded

Metal ions on 1st column only

Bacterial supernatant causes leeching of metal ions

Metal ions & scFv trapped on subsequent columns
3.2.5.3 Purification of RAFT3 scFv by Immunoaffinity Chromatography

Protein L derived from *Peptostreptococcus magnus* has been demonstrated to bind human kappa light chains (Akerstrom and Bjorck, 1989; Nilson *et al.*, 1992). This protein has recently been produced in recombinant form (rProtein L™, Actigen) and has been used to purify a wide range of immunoglobulins by immunoaffinity chromatography (De Chateau *et al.*, 1993; Nilson *et al.*, 1993). Its potential in isolating recombinant scFvs efficiently and to a high level of purity has also been noted (Akerstrom *et al.*, 1994). Whilst the original LHM2 scFv contained only mouse-derived light chains, the chimaeric nature of the RAFT3 scFv offered the possibility of utilising recombinant protein L to improve protein retention and to simplify purification.

3.2.5.3.1 Preparation of Recombinant Protein L™ Sepharose Column

An immunoaffinity column was prepared by coupling protein L to CNBr-activated Sepharose™ gel (Pharmacia) as described (Section 2.4.6).

3.2.5.3.2 ScFv Purification

All immunoaffinity purification was carried out on the Biorad Biologic LP Chromatography platform (Section 2.4.4).

The Protein L column was washed with 5 column volumes (50ml) of PBS at 2ml/minute. The concentrated & dialysed bacterial supernatant containing the scFv (~100ml) was then loaded onto the column at 1ml/min. The column was washed with a further 50ml of PBS at 2ml/min and the run-through retained for subsequent analysis. The bound protein was then eluted using 15ml of 0.1M glycine pH 2.0 at 2ml/min. The eluate was continuously mixed with 1ml of 1M Tris/HCl pH 7.5 to minimise the effect of the acidic pH on the eluted protein. Elution of bound protein was monitored using the UV and conductivity meters and recorded on the chart recorder (Section 2.4.4). The column was washed again with 50ml of PBS and the run-through retained for subsequent analysis. For storage, the column was washed with 50ml of PBS containing...
0.02% sodium azide. Eluted fractions were then analysed by SDS-PAGE and Western blot (Section 2.2) to assess the protein yield and purity.

### 3.2.5.4 Purification by Ion-Exchange Chromatography

A prominent ≈ 25 kDa impurity is present following IMAC purification of RAFT3 scFv in some of the eluted fractions (see Section 3.3.2.1). Ion-exchange chromatography (Section 2.4.7) was therefore investigated as a means to remove this impurity.

Approximately 250 µg of RAFT3 scFv containing the 25kDa impurity in 5 ml of PBS was loaded onto a Q-Sepharose ™ Hi-Trap™ 1ml ion-exchange column (Pharmacia). The column was washed with 5ml PBS and the run-through was retained for analysis. The column was eluted with a stepwise gradient of PBS containing increasing concentrations of NaCl. 5ml volumes of PBS with 0.05, 0.1, 0.2, 0.5 and 1M NaCl were used. Fractions were collected in 5ml aliquots and dialysed overnight against PBS before analysis by SDS-PAGE.

### 3.2.6 ScFv Solubility Studies

A clear tendency to form insoluble aggregates on storage at higher concentration was noted with the RAFT series scFvs. As pointed out in Section 3.1, this is a potential problem of recombinant proteins of this nature. A number of strategies were employed to attempt to increase the stability and solubility of the scFv.

#### 3.2.6.1 Assessment of RAFT3 ScFv Solubility

Higher concentrations of RAFT3 scFv in PBS (>500µg/ml) at pH 7.5 were stored at 4°C for several weeks. The samples were then centrifuged at 13000rpm for 10 mins in a benchtop centrifuge to remove any precipitate. The supernatant was then studied in SDS-PAGE to assess the concentration of soluble scFv remaining and allow comparison with the original concentration pre-storage.
3.2.6.2 Effect of Buffer Modification

A number of buffer additives have long been known to be useful in increasing the stability of proteins in solution. Aliquots of 100μl of RAFT3 scFv at 400μg/ml in PBS were studied after the addition of Tween-20, 1 M sucrose, 1 M glycine, 1 M alanine, 1 M glutamic acid, 1 M arginine-HCl, 1 M glycerol and 0.4 M PEG or using 1M Tris pH7.4 as the buffer. Samples were stored at 4°C for 7 days and then the samples were centrifuged at 13000rpm for 10min in a benchtop centrifuge. The supernatant was decanted to another tube and aliquots studied by SDS-PAGE and Western blot analysis to allow assessment of concentration of soluble protein remaining and comparison of the relative efficacies of the additives.

3.2.6.3 Effect of pH Modification

The iso-electric point (pI) of dissolved protein molecules reflects the pH at which the molecule would possess a net charge of zero. The farther the pH of the buffer solution from the pI of the protein, the greater the net charge exhibited. It has been suggested that one factor in the relatively poor solubility of some scFvs may be the proximity of the pI of the molecules to the physiological pH of the buffers in which they are stored (Tan et al., 1998). The effect of buffer pH and its relationship to the pI of the RAFT3 scFv were therefore investigated.

The estimated pI of the scFv was calculated using the ProtParam tool from ExPASy™ (Expert Protein Analysis System, Swiss Institute of Bioinformatics. http://expasy.cbr.nrc.ca/tools/protparam.html) which is freely accessible.

The effect of varying buffer pH was assessed using aliquots of 100ul of RAFT3 scFv at 400μg/ml in PBS at pH 2, 4, 6, 7.5, 9, 11 and 13. The samples were stored at 4°C for 7 days and then centrifuged at 13000rpm for 10min in a benchtop centrifuge. The supernatants were retained and aliquots of each studied in SDS-PAGE and Western blot analysis to assess the effect of pH on solubility.
3.2.6.4 Effect of Imidazole and Sodium Chloride on ScFv Solubility

During the process of scFv purification using IMAC, the eluted samples containing scFv were generally collected in buffer containing 50-200mM imidazole. After concentration of the eluted fractions against PEG (Section 2.4.9), the samples were typically dialysed overnight at 4°C against PBS to remove excess NaCl and imidazole from the solution. A small precipitate noted at this stage (not present pre-dialysis) suggested that removing either the sodium chloride or imidazole from the solution might be involved in aggregate formation.

The effect of imidazole and NaCl were studied using aliquots of 100μl of RAFT3 scFv at 400μg/ml in PBS. In two parallel experiments, varying concentrations of imidazole (50, 100 and 200mM) or NaCl (100, 500 or 1000mM) were added whilst maintaining the pH at 7.5. The samples were stored for 3 days at 4°C, centrifuged at 13000rpm for 10min and then the supernatants containing soluble scFv were analysed as described in Section 3.2.5.4.

3.2.6.5 Re-solubilising ScFv using Imidazole

The effect of imidazole in re-solubilising precipitated scFv was also assessed by adding PBS containing 200mM imidazole to isolated scFv precipitate and assessing the degree of re-solubilisation by SDS-PAGE and Western blot in comparison to scFv precipitate resuspended in PBS.

In practice, a 400μl sample of RAFT3 scFv originally at 400μg/ml in PBS in which considerable precipitation had occurred was divided in two after resuspension of the precipitate by agitation. The two aliquots were then centrifuged in a benchtop centrifuge at 13000 rpm for 10min to pellet the precipitate. The supernatant was decanted and the pellet resuspended in either 200μl of PBS or 200μl of PBS containing 200mM imidazole. The samples were then stored overnight at 4°C. The following day, the samples were re-centrifuged to remove any precipitate and the supernatants removed for
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analysis by SDS-PAGE and Western. By this method, the re-solubilising effect of the imidazole could be assessed.

Once the concentration of any re-solubilised scFv was ascertained, its immuno-reactivity was assessed in comparison to a fresh sample of unprecipitated scFv in PBS by cell ELISA (Section 2.5.3).

3.2.6.6 Effect of Imidazole on Radiolabelling and Biodistribution

Since storage in buffer containing 200mM imidazole might adversely alter the radiolabelling and tumour targeting properties of the scFv (and therefore its clinical usefulness), it was necessary to establish any detrimental effect.

RAFT3 scFv stored in 200mM imidazole / PBS was studied in comparison to RAFT3 scFv in PBS alone in terms of the efficacy of radio-iodination and bio-distribution in a mouse tumour xenograft model. Radiolabelling and in vivo analysis was carried out as described for RAFT3DS scFv (Sections 3.2.4.2.3 and 3.2.4.2.5) using an 18 hour timepoint only.
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3.3 Results

3.3.1 Optimising scFv Expression

A variety of strategies were investigated in an attempt to improve on the rather poor RAFT3 scFv yield (150μg per litre of bacterial supernatant) obtained in previous work in this laboratory (Kang, 1998).

3.3.1.1 Optimising Induction Conditions

Induction conditions have been shown to have a significant impact on eventual recombinant protein yield. It is therefore essential to optimise the factors which might influence bacterial protein production, particularly in relatively low yield proteins such as the RAFT scFv series.

The effect of varying induction conditions on the concentration of secreted soluble scFv in bacterial supernatant was studied by varying the starting bacterial concentration (optical density, OD), varying the concentration of IPTG as inducing agent, altering the incubation temperature and by adding 1M sucrose to the culture medium (Section 3.2.4.1).

Bacterial OD at induction was studied at starting ODs at 600nm of 0.25, 0.5, 1.0, 1.5, 2.0 and 3.0. Increasing the starting OD to 1.5 resulted in increasing scFv concentration after overnight induction. However, further increase in the starting bacterial concentration did not enhance scFv yield and a starting OD of 1.5 was chosen for further experiments.

Altering the concentration of inducing agent and the length of induction were studied similarly and the results are shown in Fig. 3.3.1.1a. Increasing the concentration of IPTG from 10μM to 100μM results in increased scFv secretion into the supernatant but further increase to 500μM IPTG does not increase the yield of scFv, presumably since maximal production has been achieved. 100μM IPTG was therefore used for all
**Fig. 3.3.1.1a**  Differing IPTG Concentration at Induction

Western blot of small scale RAFT3 scFv inductions at differing IPTG concentrations

**Fig. 3.3.1.1b**  ScFv Induction over Time

Western blot of a single small scale RAFT3 scFv inductions at differing timepoints
subsequent scFv inductions. The duration of induction seems important for yield. Induction up to 12 hours results in increasing scFv yield. Thereafter, scFv yield appears to plateau and indeed decreases from the 24 hour time point (Fig. 3.3.1.1b). On the basis of this experiment, an overnight induction (approximately 16 hours) was selected for scFv induction both for likely maximum yield and for convenience.

Inductions in parallel at room temperature (22°C, 30°C and 37°C studied the influence of temperature on scFv yield (Section 3.2.4.1). The result of the subsequent Western blot is shown in Fig. 3.3.1.1c. It is apparent that the greatest scFv yield is obtained at 30°C and this temperature was used for all further experiments.

The effect of the inclusion of the osmotic agent sucrose at 1M concentration in the induction media is also shown in Figure 3.3.1.1c. No increase in scFv secretion was demonstrated and therefore 1M sucrose was not used in subsequent studies.

These results suggested an optimal induction using a starting bacterial OD of 1.5, 100μM IPTG, incubated overnight at 30°C.

3.3.1.2 Domain Swapping

Reversal of the orientation of the V-domains of the RAFT3 scFv in the plasmid was carried out in order to investigate its effect on scFv yield. The production of the new scFv and investigation of any effect on its in vivo properties are detailed below.

3.3.1.2.1 Design and Bacterial Transformation

Purification of the PCR-amplified V-domain DNA of the RAFT3 scFv was confirmed by TAE gel electrophoresis and the isolated DNA fragments ligated overnight with pUC119 His 6 myc Xba and transformed into E.coli TG1 by electroporation. This yielded 93 colony forming units of ampicillin-resistant bacteria, confirming incorporation of the plasmid.
Western blot of small scale RAFT3 scFv inductions at differing temperature and with 1M D-sucrose
3.3.1.2.2 Production and Purification

Ten colonies were picked and small-scale (1.5ml) inductions studied in cell ELISA. The results are shown in Figure 3.3.1.2.2a. The cell ELISA demonstrated that one clone exhibited clear melanoma cell binding (Clones #8). The same ten supernatants containing scFv were analysed by Western blot which suggested that clone #8 had the highest scFv yield in this experiment. This clone was therefore designated RAFT3DS (domain swap) scFv and was used for all further experiments. The correct orientation was confirmed by DNA sequencing carried out by Dr. J. Kupsch and Mrs N. Patel.

A large scale induction of the RAFT3DS scFv was made. The resultant Coomassie-stained SDS-PAGE gel and Western blot of the purified fractions (Figs. 3.3.1.2.2b) demonstrated a 50% increased yield of scFv in comparison to the parent RAFT3 scFv (900µg per litre vs. 600µg).

3.3.1.2.3 Confirmation of Epitope Specificity

Competition cell ELISA was employed to demonstrate that the new RAFT3DS scFv targeted the same epitope of the antigen as the parent molecule. Inhibition of binding of LHM2 IgG, the mouse monoclonal antibody parent of RAFT3, was assayed and the results are shown in Figure 3.3.1.2.3. Clear inhibition of binding is demonstrated by both scFvs and roughly equivalent degrees of inhibition at each concentration suggest similar affinities for the target antigen. Inhibition of binding of LHM2 IgG indicates retention of LHM2 epitope specificity in the modified scFv.

3.3.1.2.4 Radiolabelling

It was necessary to demonstrate that alteration of the domain orientation of the scFv did not adversely affect its radiolabelling properties by masking sites suitable for radiolabel incorporation.
Cell ELISA on A375-M melanoma cell line. 25μl of bacterial supernatant from a 2 ml induction was loaded. Results are representative of 3 cell ELISAs.

* indicates clearly positive clones.
Coomassie-stained SDS-PAGE gel showing purified RAFT3DS fractions and Western blot of pooled 50, 100 and 200mM imidazole fractions scFv from same experiment.
LHM2 at increasing dilution was incubated in the presence or absence of 500ng scFv/well. LHM2 was detected using anti-mouse IgG-HRP conjugate. Results are representative of 3 cell ELISAs.
The results of radio-iodination of the domain swap scFv in comparison to the RAFT3 scFv are shown in Table 3.3.1.2.4. Immunoreactivity of the radio-labelled scFv was studied in comparison to unlabelled scFv in cell ELISA (Fig. 3.3.1.2.4). Whilst incorporation of radio-iodine varied dramatically between the two scFvs (RAFT3 scFv 26.9% vs. 9.2% for RAFT3DS), the specific activities achieved were similar and low (0.038 and 0.074 MBq/μg for RAFT3 scFv and RAFT3DS respectively). The immunoreactivity was also comparable at 90.6 % for RAFT3 scFv and 87.5% for RAFT3DS after labelling.

3.3.1.2.5 Affinity Studies

Affinity studies were carried out to allow assessment of the effect of domain orientation on antigen binding. Any reduction in affinity for the antigen would negate the benefit of increased yield in the domain-swapped scFv.

The affinity of the RAFT3DS scFv for the target antigen on melanoma cells was studied in a saturation binding experiment using A375M melanoma cells (Section 3.2.4.2.4). Saturation binding and Scatchard plots were made using the data (Figs. 3.3.1.2.5a&b) and the association and dissociation constants calculated (Section 2.7). Affinity constants ($K_a$) of $3.19 \times 10^7$ and $4.13 \times 10^7$ l/mol were obtained for RAFT3 scFv and RAFT3DS scFv respectively (Table 3.3.1.2.5). The data demonstrate equivalent affinities for the antigen, indicating that the reversal of the V-domains in the construct has not adversely affected antigen binding in vitro.

3.3.1.2.6 In Vivo Studies

Domain-swapped RAFT3 scFv was produced in an attempt to increase the scFv yield and, therefore, make the scFv more suitable for a patient trial. It was necessary, however, to confirm the tumour targeting properties of the scFv were not adversely affected.
Fig. 3.3.1.2.4  Immuno-reactivity of Radio-iodinated ScFv

Immunoreactivity of unlabelled □ and labelled ■ scFvs assessed in cell ELISA against A375-M melanoma cells. 0.5μg of each scFv was loaded. Anti-CD18 scFv is shown as a negative control ■.

Table 3.3.1.2.4  Summary of Radio-iodination Results

<table>
<thead>
<tr>
<th>scFv</th>
<th>Incorporation (%)</th>
<th>Average Specific Activity (MBq/microg)</th>
<th>Immunoreactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT3</td>
<td>26.9</td>
<td>0.038</td>
<td>90.6</td>
</tr>
<tr>
<td>RAFT3DS</td>
<td>9.2</td>
<td>0.074</td>
<td>87.5</td>
</tr>
<tr>
<td>anti-CD18</td>
<td>4.1</td>
<td>0.008</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Fig. 3.3.1.2.5a  RAFT3DS Saturation Binding Plot

![Saturation Binding Plot](image)

- ScFv bound to cells (M)
- ScFv free in supernatant (M)
- Red dots: Uninhibited
- Blue dots: Inhibited

Fig. 3.3.1.2.5b  RAFT3DS Scatchard Plot

![Scatchard Plot](image)

Specifically Bound (M) vs. Specifically Bound/Unbound (SB/UB)

Table 3.3.1.2.5  Scatchard Summary

<table>
<thead>
<tr>
<th>scFv</th>
<th>$K_A$ (l/mol)</th>
<th>$K_D$ (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT3</td>
<td>$3.19 \times 10^7 (+/-0.74)$</td>
<td>$3.13 \times 10^{-8}$</td>
</tr>
<tr>
<td>RAFT3DS</td>
<td>$4.13 \times 10^7 (+/-0.16)$ (Error = SEM)</td>
<td>$2.42 \times 10^{-8}$</td>
</tr>
</tbody>
</table>
In order to demonstrate that the modification of the RAFT3 scFv by domain swapping did not adversely affect its ability to target melanoma \textit{in vivo}, the radiolocalisation properties of the parent and domain-swapped scFv were studied in a murine model using radio-iodinated scFv and human melanoma xenografts.

\textit{In vivo} studies of tumour and normal tissue biodistribution at 1, 3, 6 and 18 hour time points are shown in Tables 3.3.1.2.6a-c. RAFT3 scFv and RAFT3DS scFv are compared and anti-CD18 scFv is used as a negative control. The same data are shown graphically in Figures 3.3.1.2.6a-c. Tumour localisation was comparable with a peak localisation of 2.5\%ID/g for RAFT3 scFv at 1 hour compared to 2.2\%ID/g for the RAFT3DS scFv. Renal accumulation was significant for both scFvs and is consistent with the tendency to kidney accumulation seen in molecules of this size. Accumulation in other normal organs was again comparable though the 1 hour lung accumulation seen with RAFT3DS was markedly greater than that of the RAFT3 scFv. This accumulation was not evident at later timepoints and is probably artefactual. No tumour-specific localisation was seen in the case of the anti-CD18 control.

The contrast achieved between tumour and normal tissues is summarised in Tables and Figures 3.3.1.2.6.d-f. The ratio between the tumour and normal tissue accumulation is shown for each normal tissue. Tumour to normal tissue contrast increases with time for both scFvs. Only in the case of the kidney does the tumour accumulation fail to exceed that in the normal tissue (maximum T:NT 0.201 and 0.204 for RAFT3 scFv and RAFT3DS scFv, respectively). The tumour to normal tissue ratios in the other tissues are comparable for both the RAFT scFvs studied. The only clear differences are muscle where the T:NT ratios at 6 hours are 5.075 and 10.798 for RAFT3 scFv and RAFT3DS scFv and bone where the T:NT ratios at 18 hours are 21.649 and 12.266. These discrepancies are discussed below. Anti-CD18 scFv showed no tumour-specific localisation and the tumour to normal tissue ratios decreased over the time course of the experiment in contrast to the increases seen with the anti-melanoma scFvs. This demonstrates the tumour accumulation seen with the RAFT scFvs is antigen-specific. The similar blood accumulation for both RAFT3 scFv and RAFT3DS scFv implies equivalent circulatory
### Table 3.3.1.2.6a
**%ID/g for $^{125}$I Labelled RAFT3 scFv**

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bu</th>
<th>Sp</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.510</td>
<td>16.210</td>
<td>1.702</td>
<td>1.980</td>
<td>0.802</td>
<td>0.591</td>
<td>1.772</td>
<td>3.428</td>
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<tr>
<td>3</td>
<td>1.220</td>
<td>8.820</td>
<td>0.853</td>
<td>0.933</td>
<td>0.602</td>
<td>0.407</td>
<td>0.669</td>
<td>1.267</td>
</tr>
<tr>
<td>6</td>
<td>0.750</td>
<td>6.712</td>
<td>0.307</td>
<td>0.209</td>
<td>0.134</td>
<td>0.178</td>
<td>0.297</td>
<td>0.217</td>
</tr>
<tr>
<td>18</td>
<td>0.370</td>
<td>2.089</td>
<td>0.144</td>
<td>0.070</td>
<td>0.041</td>
<td>0.019</td>
<td>0.087</td>
<td>0.073</td>
</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

---

### Table 3.3.1.2.6b
**%ID/g for $^{125}$I Labelled RAFT3DS scFv**

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bu</th>
<th>Sp</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
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<td>1.511</td>
<td>8.944</td>
<td>0.867</td>
<td>0.385</td>
<td>0.281</td>
<td>0.487</td>
<td>0.818</td>
<td>0.884</td>
</tr>
<tr>
<td>6</td>
<td>0.799</td>
<td>5.600</td>
<td>0.285</td>
<td>0.220</td>
<td>0.074</td>
<td>0.175</td>
<td>0.201</td>
<td>0.225</td>
</tr>
<tr>
<td>18</td>
<td>0.292</td>
<td>1.680</td>
<td>0.102</td>
<td>0.060</td>
<td>0.035</td>
<td>0.024</td>
<td>0.091</td>
<td>0.047</td>
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</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

---

### Table 3.3.1.2.6c
**%ID/g for $^{125}$I Labelled anti-CD18 scFv**

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bu</th>
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<td>0.235</td>
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<td>0.182</td>
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<td>18</td>
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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 3.3.1.2.6a  %ID/g for $^{125}$I Labelled RAFT3 scFv

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</tr>
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<td>Lu</td>
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<td>Mu</td>
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<td></td>
<td></td>
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</tr>
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<td>Bn</td>
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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 3.3.1.2.6b  %ID/g for $^{125}$I Labelled RAFT3DS scFv

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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 3.3.1.2.6c  %ID/g for $^{125}$I Labelled anti-CD18 scFv

<table>
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</tr>
<tr>
<td>Li</td>
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<tr>
<td>Lu</td>
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<td></td>
</tr>
<tr>
<td>Mu</td>
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</tr>
<tr>
<td>Bn</td>
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</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
### Table 3.3.1.2.6d

**T:NT for $^{125}$I Labelled RAFT3 scFv**

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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<td>1.501</td>
<td>1.372</td>
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<td>3.145</td>
<td>1.913</td>
<td>1.010</td>
</tr>
<tr>
<td>6</td>
<td>0.101</td>
<td>2.215</td>
<td>3.254</td>
<td>5.075</td>
<td>3.820</td>
<td>2.290</td>
<td>3.134</td>
</tr>
<tr>
<td>18</td>
<td>0.201</td>
<td>2.917</td>
<td>5.983</td>
<td>10.244</td>
<td>21.649</td>
<td>4.828</td>
<td>5.753</td>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 3.3.1.2.6e

**T:NT for $^{125}$I Labelled RAFT3DS scFv**

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<th>Hours after injection</th>
<th>Ki</th>
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<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.181</td>
<td>1.542</td>
<td>0.543</td>
<td>5.136</td>
<td>2.233</td>
<td>1.430</td>
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<tr>
<td>3</td>
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<td>1.744</td>
<td>3.929</td>
<td>5.378</td>
<td>3.104</td>
<td>1.847</td>
<td>1.709</td>
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<tr>
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<td>0.166</td>
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<td>3.643</td>
<td>10.798</td>
<td>4.601</td>
<td>4.067</td>
<td>3.596</td>
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<tr>
<td>18</td>
<td>0.204</td>
<td>2.844</td>
<td>4.832</td>
<td>8.437</td>
<td>12.266</td>
<td>3.221</td>
<td>6.259</td>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 3.3.1.2.6f

**T:NT for $^{125}$I Labelled Anti-CD18 scFv**

<table>
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<tr>
<th>Hours after injection</th>
<th>Ki</th>
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<th>Mu</th>
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<th>Sp</th>
<th>Bl</th>
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</thead>
<tbody>
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<td>0.150</td>
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<td>4.537</td>
<td>9.218</td>
<td>1.113</td>
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<td>2.113</td>
<td>0.532</td>
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<tr>
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<td>0.050</td>
<td>0.180</td>
<td>0.803</td>
<td>2.873</td>
<td>1.817</td>
<td>1.066</td>
<td>0.615</td>
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</table>

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 3.3.1.2.6d  T:NT for $^{125}$I Labelled RAFT3 scFv

![Graph showing T:NT for $^{125}$I Labelled RAFT3 scFv.](image)

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 3.3.1.2.6e  T:NT for $^{125}$I Labelled RAFT3 DS scFv

![Graph showing T:NT for $^{125}$I Labelled RAFT3 DS scFv.](image)

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 3.3.1.2.6f  T:NT for $^{125}$I Labelled anti-CD18 scFv

![Graph showing T:NT for $^{125}$I Labelled anti-CD18 scFv.](image)

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
half-lives. The actual values were, therefore, not studied in detail in these experiments but are addressed for RAFT3 scFv specifically in Chapter 4.

In summary, our data show that domain swapping can improve RAFT3 scFv yield without compromising its in vitro and in vivo melanoma targeting properties.

3.3.2 Optimising Protein Purification

A Western blot of filtered bacterial supernatant before and after ultrafiltration showed that no scFv passed through the membrane (Fig. 3.3.2). This indicated that no scFv was being lost at this stage in purification. Therefore, attempts to improve scFv retention and purity were concentrated on the downstream process.

3.3.2.1 IMAC Purification

A variety of columns and chelating metal ions are available for IMAC purification. These experiments studied the efficacy of each in order that the most efficient might be identified for subsequent protein purification.

Previous work had suggested a RAFT3 scFv yield of 150μg/ml could be achieved (Kang, 1998). This yield was obtained using a 1/100 dilution of an overnight bacterial culture incubated for 6 hours followed by a 1/100 dilution of the bacterial culture into the induction medium. This procedure would result in a starting bacterial optical density at 600nm (OD₆₀₀) of around 0.015. Since our small scale induction with varying bacterial density suggested scFv yield would increase up to a starting OD₆₀₀ of 1.5, a comparison was made using the former technique and a 1 litre induction with a starting OD₂₀₀ of 1.5. This increase in starting bacterial concentration resulted in an increase in RAFT3 scFv yield from 150 to 500μg/l. The higher starting OD₆₀₀ was, therefore, employed for all further experiments.

A comparison of purifications using self-assembled 50ml IMAC column or highly cross-linked Hi-Trap Chelating Sepharose™ HP (Hi-Trap) columns charged with nickel,
Western blot of large scale RAFT3 scFv inductions supernatant showing control (left lane), concentrated scFv (centre) and absence of scFv in ultrafiltrate (right lane).
copper or zinc ions. The results are shown as Coomassie gels and Western blots in Figures 3.3.2.1a-d. It is clear from these assays that a higher level of purity is attained using the small Hi-Trap columns. Purification results using nickel and zinc ions with the Hi-Trap columns revealed differing purification properties from the copper-loaded column (Fig. 3.3.2.1b-d). Zinc ions, while producing a relatively high yield of scFv failed to differentiate between scFv (27kDa) and the lower molecular weight contaminant of around 25kDa. Nickel ions yielded relatively pure scFv without the 25kDa contaminant. However, the toxicity associated with this ion in vivo and difficulty in detecting ion leeching from the column (nickel ions are colourless) rendered nickel unsuitable for further use. Copper ions were therefore selected for use in standard purification runs. The yields obtained by each method are summarised in Table 3.3.2 and are comparable at 500-600µg/l. Large IMAC column and Hi-Trap Zinc purification required an additional Ion-Exchange Chromatography step which reduced the eventual yield. The yields remain inferior to the 900µg/l obtained with RAFT3DS scFv.

3.3.2.2 Immunoaffinity Chromatography

Recombinant protein L binds human kappa light chains including those forming part of scFv molecules and offers the potential of a one-step purification process with low protein loss and high purity. It’s usefulness in purifying RAFT3 scFv was assessed in these experiments.

Figure 3.3.2.2a shows the elution peaks obtained purifying RAFT3 scFv on the large IMAC and r-Protein L immunoaffinity columns. A clear protein elution peak is evident with the rPL column but absent or obscured by the general protein load in the case of the large IMAC column.

The Coomassie stained SDS-PAGE gel and Western blot of the eluted fractions of the immunoaffinity column demonstrate a very pure sample of scFv with a yield of approximately 900µg RAFT3 scFv per litre of bacterial supernatant (Fig. 3.3.2.2.b).
**Fig. 3.3.2.1a**

**RAFT3 scFv IMAC Purification**

Coomassie-stained SDS-PAGE analysis of eluted IMAC fractions and corresponding Western blot

**Fig. 3.3.2.1b**

**RAFT3 scFv Hi-trap™ IMAC Purification: Cu²⁺ Loaded Column**

Coomassie-stained SDS-PAGE analysis of imidazole-eluted Hi-Traptm IMAC fractions and corresponding Western blot
Coomassie-stained SDS-PAGE analysis of eluted IMAC fractions and corresponding Western blot of pooled 50-200mM imidazole fractions
## Table 3.3.2: ScFv Yield

<table>
<thead>
<tr>
<th>scFv</th>
<th>Purification Method</th>
<th>Yield (µg per litre)</th>
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<td>RAFT3</td>
<td>Econocolumn IMAC</td>
<td>500 (400)</td>
</tr>
<tr>
<td>RAFT3</td>
<td>HT Zinc</td>
<td>500 (400)</td>
</tr>
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<td>RAFT3</td>
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<tr>
<td>RAFT3</td>
<td>HT Copper</td>
<td>600</td>
</tr>
<tr>
<td>RAFT3DS</td>
<td>HT Copper</td>
<td>900</td>
</tr>
</tbody>
</table>

* Yield following necessary ion-exchange step shown in brackets
Fig. 3.3.2.2a

**IMAC & Recombinant Protein L Elution Peak Profiles**

**rPL. RAFT3 scFv elution profile.**

Red line indicates UV absorbance & blue line conductivity. Single clear UV peak corresponds to scFv elution from immunoaffinity column.

Full scale deflection 200mV
Paper speed 2cm/min

**IMAC RAFT3 scFv elution profile.**

Red line indicates UV absorbance & blue line conductivity. Large peak corresponds to general protein load being eluted from column and no discrete scFv peak is discernable.

Full scale deflection 2V
Paper speed 2cm/h
**Fig. 3.3.2.2b RAFT3 scFv : Immunoaffinity Purification**

Coomassie-stained SDS-PAGE and Western Blot analysis of r-Protein L purified RAFT3 scFv
3.3.2.3 Ion-Exchange Chromatography

The persisting 25kDa contaminant seen in some fractions of IMAC-purified scFv (Section 3.3.2.1) was removed successfully using Ion-Exchange Chromatography. Coomassie-stained SDS-PAGE gels of samples before and after ion-exchange chromatography demonstrate that the scFv does not bind to the column whilst the contaminant does (Fig. 3.3.2.3). A Western blot of the samples confirms the position of the scFv in the run-through. Whilst these results demonstrate that Ion-Exchange Chromatography can be used successfully to remove the contaminant 25kDa band, in practice it proved more convenient simply to use those IMAC fractions free of contaminant and re-load the remainder with the next IMAC purification run.

3.3.3 ScFv Solubility Studies

Adequate scFv solubility is important both for use in laboratory based experiments and eventually because of the deleterious effect of low solubility on stability, yield, radiolabelling efficiency and the potential utility of the molecule in a clinical setting.

3.3.3.1 Assessment of ScFv Solubility

The solubility limit of the RAFT3 scFv in PBS pH 7.4 at 4°C proved to be around 150μg/ml. Storage of several samples at 4°C in varying concentrations of scFv showed a tendency to form precipitates until a level of dissolved scFv at this level was reached.

3.3.3.2 Effect of Buffer Modification

A number of different buffer solutions and co-solvents have previously used to enhance the solubility (stability) of peptides and reduce aggregation (Timasheff and Arakawa, 1997).

The results of storage of RAFT3 scFv in various different buffers are shown in the form of a Coomassie-stained SDS-PAGE of the samples (Fig. 3.3.3.2). It is clear from the gel
**Fig. 3.3.2.3 RAFT3 scFv : Ion-exchange Purification**

Original nickel-charged Hi-Trap™ column IMAC purification of RAFT3 scFv. Fractions eluted at varying imidazole concentration are shown. Fractions eluted at imidazole concentrations of 40-200mM were pooled and further purified by ion-exchange chromatography (below).

Ion-exchange purification fractions of RAFT3 scFv pooled from gel above. ScFv is noted in the run-through (load) fraction whilst contaminant bands bind to the column and are eluted at higher NaCl concentrations.

Western blot analysis confirms the presence of scFv in the load sample and the absence of scFv in the pooled remaining samples.

Coomassie-stained SDS-PAGE and Western Blot analysis of ion-exchange purified RAFT3 scFv.
Coomassie-stained SDS-PAGE analysis of RAFT3 scFv stored in various modified buffers. Samples were stored at 4°C for 2 weeks prior to analysis.
that the addition of 1 M D-sucrose, 1 M glycine, 1 M L-alanine, 1 M glutamic acid, 1 M arginine-HCl, 1 M glycerol or 0.4 M PEG_6000 and the use of 1M Tris/HCl pH7.4 as the storage buffer all failed to maintain the scFv in solution to any greater extent than the PBS buffer alone. Similarly, the addition of the detergent 0.05% Tween-20 to the buffer did not prevent precipitation (gel not shown).

3.3.3.3 Effect of pH Modification

A three dimensional model of the RAFT3 scFv was built using the Swiss-Prot™ protein modelling software and confirmed the expected V-domain orientation with a protruding c-myc detection tag (Fig. 3.3.3.3a). The iso-electric point (pI) of the molecule was predicted using ExSpasy™ protein analysis software and estimated the pI to be 8.6. A Western blot of RAFT3 scFv samples stored in PBS at different pHs demonstrated that altering the pH away from the iso-electric point resulted only in a marginal increase in solubility (Fig. 3.3.3.3b), suggesting that the pH of the storage buffer is only one factor in the tendency of this scFv to aggregate.

3.3.3.4 Effect of Imidazole and Sodium Chloride on ScFv Solubility

The observation that scFv eluted in buffer containing imidazole and 1M NaCl remained in solution but precipitated after the imidazole and NaCl were dialysed out led to the suspicion that imidazole or high NaCl concentration might be helping maintain the scFv in solution. The effect of differing concentrations of imidazole or NaCl on scFv solubility was investigated.

A Coomassie-stained SDS-PAGE gel of RAFT3 scFv stored at differing concentrations of imidazole and NaCl demonstrated that the inclusion of 50mM imidazole could maintain scFv in solution at 400µg/ml whilst differing concentrations of NaCl in the buffer showed no effect on scFv solubility (Fig. 3.3.3.4). In the NaCl samples, precipitation reduced the scFv concentration to <200µg/ml after 2 weeks. This finding suggested a clear effect of imidazole on the solubility of the scFv and its potential as a stabilising agent for storing scFv.
Fig. 3.3.3.3a RAFT3 scFv Modelling

Predicted protein structure using Swissprot™ Protein Modelling Software
Coomassie-stained SDS-PAGE analysis of RAFT3 scFv stored in various modified buffers. Samples were stored at 4°C for 2 weeks prior to analysis.
Coomassie-stained SDS-PAGE analysis of RAFT3 scFv stored in various modified buffers. Samples were stored at 4°C for 2 weeks prior to analysis. 20μl of each sample was loaded.
An attempt was therefore made to resolubilise precipitated scFv in buffer containing imidazole.

### 3.3.3.5 Re-solubilising ScFv using Imidazole

SDS-PAGE analysis of scFv precipitate resolubilised in 200mM imidazole by Coomassie staining and Western blotting demonstrated that a considerable degree of resolubilisation could be achieved in comparison to the PBS control (Figs 3.3.3.5a). A cell ELISA using imidazole-resolubilised RAFT3 scFv demonstrated melanoma cell binding approaching (but inferior to) that seen with fresh RAFT3 scFv (Fig. 3.3.3.5b). Since the immuno-reactivity of the re-solubilised scFv could not be easily assessed, it was not used in further experiments.

### 3.3.3.6 Effect of Imidazole on Radiolabelling and Biodistribution

Having demonstrated that imidazole increases the stability of RAFT3 scFv in PBS at pH 7.4, it was then necessary to explore the effect of such stabilisation on the radiolabelling and tumour targeting properties of the scFv.

The immuno-reactivity of radio-iodinated RAFT3 scFv (fresh, not resuspended) stored in PBS containing 200mM imidazole was comparable to that of RAFT3 scFv stored in plain PBS (88.1% vs. 90.6% respectively) as demonstrated in cell ELISA (Fig. 3.3.3.6a). The specific activities of the radiolabelled scFv obtained were also comparable at 0.038MBq/μg for RAFT3 scFv in PBS and 0.021MBq/μg for RAFT3 scFv in PBS with imidazole (Table 3.3.3.6a).

The radio-labelled RAFT3 scFv which had been stored in PBS with 200mM imidazole was then assayed in vivo to allow assessment of the effect of imidazole storage on biodistribution. Figures 3.3.3.6b&c show a comparison of biodistribution and tumour to normal tissue ratios at 18 hours for RAFT3 scFv stored in PBS or PBS plus imidazole. The same results are shown in tabular form in Table 3.3.3.6b. Tumour localisation was
Coomassie-stained SDS-PAGE and Western blot analysis of precipitated RAFT3 scFv sample after “resolubilisation” in PBS with or without 200mM imidazole. Samples were stored at 4°C for 2 weeks prior to analysis. 20μl of each sample was loaded.
Immunoreactivity of fresh and imidazole-resolubilised scFvs assessed in cell ELISA against A375-M melanoma cells. 0.25ug of each scFv was loaded. Anti-CD18 scFv is shown as a negative control. Results represent the average of 3 wells per scFv.
Immunoreactivity of unlabelled and labelled scFvs assessed in cell ELISA against A375-M melanoma cells. 0.5ug of each scFv was loaded. Anti-CD18 scFv is shown as a negative control.

<table>
<thead>
<tr>
<th>scFv</th>
<th>Incorporation (%)</th>
<th>Average Specific Activity (MBq/microg)</th>
<th>Immunoreactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT3</td>
<td>26.9</td>
<td>0.038</td>
<td>90.6</td>
</tr>
<tr>
<td>RAFT3 IMID</td>
<td>13.1</td>
<td>0.021</td>
<td>88.1</td>
</tr>
<tr>
<td>anti-CD18</td>
<td>4.1</td>
<td>0.008</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Fig. 3.3.3.6b  Tissue Localisation of RAFT3 scFv in PBS and Imidazole : %ID/g (18 hours)

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 3.3.3.6c  Tissue Localisation of RAFT3 scFv in PBS and Imidazole : T:NT (18 hours)

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
### Table 3.3.3.6b %ID/g and T:NT for RAFT3 scFv IMID / PBS

<table>
<thead>
<tr>
<th>Data</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>%ID/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>0.370</td>
<td>2.089</td>
<td>0.144</td>
<td>0.070</td>
<td>0.041</td>
<td>0.019</td>
<td>0.087</td>
<td>0.073</td>
</tr>
<tr>
<td>IMID</td>
<td>0.294</td>
<td>1.340</td>
<td>0.109</td>
<td>0.063</td>
<td>0.043</td>
<td>0.016</td>
<td>0.070</td>
<td>0.043</td>
</tr>
<tr>
<td>T:NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>1.000</td>
<td>0.201</td>
<td>2.917</td>
<td>5.983</td>
<td>10.244</td>
<td>21.649</td>
<td>4.828</td>
<td>5.753</td>
</tr>
<tr>
<td>IMID</td>
<td>1.000</td>
<td>0.219</td>
<td>2.697</td>
<td>4.637</td>
<td>6.837</td>
<td>18.125</td>
<td>4.203</td>
<td>6.887</td>
</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood, (Imid) Imidazole
0.37 %ID/g for scFv in PBS and 0.29 %ID/g for scFv in imidazole with tumour to kidney ratios of 0.20 and 0.22, respectively. Tumour to blood ratios reached 5.75 and 6.89 for the PBS and imidazole samples. It is evident that the results for both samples are very similar with little difference evident either in the accumulation in organs or tumour and in the tumour to normal tissue contrast achieved. The only clear difference is in the tumour to normal tissue ratio achieved in muscle where the PBS sample achieved a T:NT of 10.2 in comparison to 6.8 for the imidazole sample.

These results demonstrate that the scFv could not be solubilised by the traditional methods tested but that following imidazole solubilisation, the scFv continued to target melanoma \textit{in vitro} and \textit{in vivo}. This finding is discussed in detail below.
Chapter 3

3.4 Discussion

3.4.1 Optimising scFv Expression

The yield of RAFT series scFvs has previously been disappointing with around 150μg of pure scFv being obtained per litre of bacterial supernatant (Kang, 1998). Such low yields meant that the production process was very labour intensive even when aimed only at producing quantities suitable for preliminary, small-scale laboratory studies. Patient imaging or therapeutic trials would require much larger quantities. Indeed, the two previous scFv studies in patients have used 500μg per patient (Begent et al., 1996) or 5 mg per patient (Larson et al., 1997). The use of large-scale fermentation systems has been capable of increasing scFv yield over 10-fold (King et al., 1993). However, this apparatus was not available in this laboratory. An attempt to optimise the yield of the scFv was therefore essential to facilitate subsequent studies. Furthermore, any improvement in scFv yield would be amplified when large-scale fermentation might eventually be employed.

3.4.1.1 Optimising Induction Conditions

The induction conditions used in the production of recombinant protein are clearly important. Our data demonstrate that increasing the optical density of bacteria in culture prior to induction of recombinant protein production has a significant effect on protein production. A high concentration of healthy, dividing bacteria would be expected to be beneficial to recombinant protein production. The bacteria grow very rapidly in media containing 2% glucose prior to induction. However, whilst bacterial density does increase after resuspension in media containing the induction agent IPTG without glucose, this is much less rapid and probably reflects the toxicity of the recombinant protein to the dividing bacteria. (Hayhurst, 2000). Further increase in the concentration of bacteria in our experiments above an optical density of 1.5 does not lead to an increased yield of soluble scFv in the supernatant. This probably represents a decreased efficiency of bacterial protein production as the bacteria compete for an inadequate
supply of nutrients in increasingly high concentrations of toxic waste products. Therefore, a starting optical density approaching 1.5 before induction seemed optimal.

The concentration of the inducing agent in the bacterial supernatant similarly has a clear influence on the yield of soluble scFv. As noted above, an IPTG concentration of less than 0.1 mM resulted in lower scFv yield. Higher concentrations of IPTG do not appear to increase recombinant protein production further. These results indicate that maximal stimulation of scFv production is obtained with 100 μM IPTG and further increases cannot stimulate induction of any further gene expression. Sawyer et al. investigated this same variable in the CC49 scFv targeting a pancarcinoma antigen TAG-72 (Sawyer et al., 1994). In their study, an IPTG concentration of 0.02 to 0.03 mM was optimal, a level of a similar order of magnitude to our own findings. The difference observed may represent variation in the properties of the individual scFv or in the plasmid used (pFLAG-1 vs. pUC119 His 6 myc Xba). Bowden and Georgiou, producing β-lactamase, described a tendency to aggregated protein in the insoluble fraction of cells grown in the presence of >0.05 mM IPTG (Bowden and Georgiou, 1990).

Similarly, lengthening the duration of induction results in increased scFv production up to 12 hours, reflecting the prolonged time available for recombinant protein synthesis. Thereafter, the failure to increase scFv content of the bacterial supernatant can be explained by the build up of toxic metabolites (including scFv) and a reduction in the number of viable bacteria (including those entering stationary phase). At time points 24 hours and later, the scFv content of the supernatant was noted to decrease. This finding is consistent with decreasing levels of scFv production together with scFv degradation by proteolytic enzymes released into the supernatant.

The temperature at which induction is carried out can have a profound influence on the production and secretion of recombinant protein (Kipriyanov et al., 1997a). The temperature used for incubation of E. coli during induction of scFv production varies in the literature between 22 and 37°C (Chester et al., 1994; Sawyer et al., 1994; Osbourn et al., 1996; Wu et al., 1999). Generally, no reasoning for the choice of a particular
temperature is indicated in scFv papers, though it is probably safe to assume the authors have optimised the induction conditions for the protein concerned.

Lower temperatures reduce the efficiency of metabolic processes but may protect bacteria from potentially toxic metabolites. Studies have shown that reduced temperatures can reduce periplasmic aggregation and increase soluble antibody protein yield (Skerra and Plückthun, 1991). It has been suggested that at higher temperatures (e.g. 37°C) plasmids place a greater metabolic strain on the bacterium and may become unstable, thus reducing the number of bacteria carrying the gene for the desired recombinant protein (Kupsch, unpublished). Two forms of plasmid instability are recognised. Segregational instability involves the loss of the whole plasmid. This is unlikely in the case of a high copy number vector such as pUC119His especially with the necessity of antibiotic resistance but could potentially occur when all the ampicillin has been degraded during overnight incubation. By then, most of the bacteria would have ceased to produce scFv. Deletional instability is of more concern and involves loss of the scFv insert gene in the plasmid. This process has been observed in an OKT3 scFv and LHM2 scFv in the pCantab vector (Kupsch, personal communication).

However, it has also been suggested that bacterial cell walls show greater permeability at higher temperatures and secretion of recombinant protein is facilitated (Kipriyanov et al., 1997a). It seems that the ideal induction temperature for any given recombinant protein is best determined empirically. Our data suggest that the optimal temperature for RAFT scFv production lies at 30°C which could represent a compromise between the protective effect of lower temperatures and the higher efficiency of protein synthesis at higher temperatures. Sawyer et al. also reported increased soluble scFv yield at 30°C in comparison to 37°C (Sawyer et al., 1994). Interestingly, the same study reported superior protein yields at the higher temperature when protein production was induced in the presence of 0.4M sucrose. Certainly, secretion is the rate limiting step in soluble scFv production (Kang, 1998) and enhanced protein production at higher temperatures would be likely to result in the build up of toxic scFv intracellularly.
The addition of non-metabolisable sugars such as sucrose to the induction medium can increase the yield of soluble scFv by an osmotic effect in encouraging transit of recombinant protein into the supernatant (Bowden and Georgiou, 1990; Sawyer et al., 1994; Kipriyanov et al., 1997a) and reducing protein aggregation in the periplasmic space (Kiefhaber et al., 1991). The addition of sucrose did not have any beneficial effect on the yield of RAFT3 scFv. We conclude that perhaps the osmotic gradient does not play an important part in the secretion of this particular scFv.

In summary, optimising the induction of the RAFT3 scFv suggested conditions of 100µM IPTG, at 30°C over 12-18 hours with a starting OD₆₀₀ approaching 1.5 would be likely to improve on the eventual yield of scFv after purification. The eventual yields obtained are discussed below.

### 3.4.1.2 Domain Swapping

It has been suggested that the orientation of the V domains of a scFv in the plasmid can have a profound effect on the yield of recombinant protein (Anand et al., 1991; Tsumoto et al., 1994; Merk et al., 1999). Altering the sequence of the variable domains ("domain swapping") might therefore improve the eventual yield of RAFT3 scFv.

#### 3.4.1.2.1 Design and Bacterial Transformation

Reversal of the V domain orientation or the RAFT3 scFv from VᵥHᵥL to VᵥLᵥH was achieved by PCR techniques. Cell ELISA confirmed the maintenance of melanoma cell binding. This is to be expected since the eventual conformation of the protein should theoretically be unchanged other than the repositioning of the c-myc and hexahistidine tails (Fig. 3.1.1). Similarly, Anand et al. demonstrated maintenance of antigen binding in domain-swapped scFvs targeting Salmonella O-polysaccharide (Anand et al., 1991).
Whilst Western blot analysis of the RAFT3DS scFv small scale induction supernatants suggested a relatively high yield, only after a large scale induction and purification could this be directly compared to the parent RAFT3 scFv. In fact, the increase in yield amounted to approximately 50% (900ug/l vs. 600ug/l). This is a significant improvement. Anand et al. demonstrated that a similar reversal of domain order in another scFv (targeting Salmonella O-polysaccharide antigen) resulted in a 20-fold enhancement in scFv secretion (Anand et al., 1991). However, no overall increase in scFv production was noted with the total cellular scFv content (as inclusion bodies) remaining constant. This suggests that the reversal of the V-domain sequence enhances secretion rather than production. The mechanism involved is poorly understood but the authors postulated that it may mirror the situation in mammalian cells where immunoglobulin secretion is mediated by light chains and reduced when light chain availability is limiting (Morrison and Scharff, 1979). Similarly, free light chains are readily secreted (Mosmann and Williamson, 1980) whilst free heavy chain is generally retained in mammalian cells (Capon et al., 1989). However, a study by Merk et al. investigating differences in the yield of two scFvs with reverse V-domain orientation suggested the lower yield of the V_{H}V_{L} version resulted from mistranslation or ribonucleolytic cleavage of the transcript (Merk et al., 1999). As with many aspects of recombinant protein production, the factors involved may be specific to the individual molecule and may need to be determined empirically. Indeed, domain swapping of two other anti-melanoma scFvs (B3 and B4) did not result in increased yield (J. Kupsch, unpublished).

Having demonstrated an enhanced yield of scFv following reversal of the V-domain orientation in the plasmid, it becomes necessary to establish that the modified construct has maintained the desirable properties of the parent molecule both in vitro and in vivo.
Chapter 3

3.4.1.2.3 In Vitro Properties

Epitopic Specificity

Competition cell ELISA has demonstrated that the RAFT3 scFv and its domain-swapped counterpart are equally capable of inhibiting binding of LHM2 IgG (the parent monoclonal antibody of the RAFT scFv series) to melanoma cells. This confirms that re-orientating the V-domains of the scFv does not interfere with the epitopic specificity of the antibody fragment towards HMW-MAA. Maintenance of antigen binding after domain swapping has been described (Anand et al., 1991) but is not universal (Tsumoto et al., 1994). In Tsumoto’s study, however, the \( V_LV_H \) orientation construct bound poorly to the antigen and this was subsequently demonstrated to relate to mistranslation of the transcript (Merk et al., 1999) and does not truly reflect a comparison of two domain swapped scFvs.

Radiolabelling

The radiolabelling properties of the RAFT3DS scFv showed no loss of its ability to incorporate radio-iodine. This suggests that tyrosine residues available for labelling remain exposed in the new construct. The maintenance of labelling efficiency is essential if the new construct’s superior yield is to be of use in subsequent studies and eventual patient trials. Some variation in the % incorporation of the radiolabel was present (RAFT3 scFv 26.9% vs. 9.2% for RAFT3DS). However, approximately twice the amount of RAFT3 scFv was used (60\( \mu \)g vs. 30\( \mu \)g) and this variations are probably consistent with the different concentrations of protein and amounts of radioactivity (8MBq vs. 11MBq) being used. It is possible that the altered conformation of the protein could have some influence but, if so, this is probably relatively minor. The specific activities attained are similar and comparable to that reported previously for RAFT3 scFv using both Chloramine-T and Iodogen labelling methods (Kang, 1998).
Affinity Studies

Scatchard analysis of the RAFT3 scFv and the RAFT3DS scFv confirmed comparable affinities for the parent antigen ($K_D \ 1.51 \times 10^{-8}$ vs. $2.42 \times 10^{-8}$M) suggesting that not only did the alteration in V domain orientation preserve antigenic specificity but also maintained the strength of binding to the antigen. Anand et al reported somewhat greater variation (up to one log) in the affinities of their domain-swapped scFvs targeting Salmonella O-polysaccharide (Anand et al., 1991). Interestingly, Plückthun and Pack have suggested that the V-domain orientation is can have a profound effect on multimerisation in scFvs (Plückthun and Pack, 1997). The authors suggest that the $V_h$-linker-$V_L$ orientation tended to favour monomers to a greater extent than the reverse orientation. This was explained in terms of the difficulty in folding $V_L$-linker-$V_H$ constructs into a monomeric conformation. Conversely, Dolezal et al. show that both $V_H V_L$ and $V_L V_H$ orientated anti-neuraminidase scFvs containing 15 amino acid linkers maintained monomeric form (Dolezal et al., 2000). In Dolezal’s study, differences in multimerisation behaviour between the two orientations only became apparent at shorter linker lengths. The multimer status of the RAFT3 DS scFv construct was not specifically studied in these experiments. Whilst the RAFT3 scFv has been shown by gel filtration to be a pure monomer (Dr. J. Odidi, personal communication) the possibility that the domain swapped variant dimerises remains. This would seem unlikely, however, in that the affinities of the two constructs are almost identical (there is no apparent avidity effect which one would expect from a scFv multimer) and their in vivo characteristics are highly similar (see below). Nevertheless, excluding a degree of multimerisation could require further studies which could not be performed during the timecourse of these experiments. However, it will be interesting to see if Plückthun and Pack’s assertions will be refuted in the case of this scFv.

In summary, these in vitro studies would suggest that the in vivo properties of the higher yield modified construct would be likely to parallel those of the parent molecule. However, minor modification of a protein’s sequence can markedly affect its final structure and properties. Therefore, before proposing a modified scFv for human trials, it was necessary to confirm its tumour-targeting properties in vivo.
3.4.1.2.4 In Vivo Studies

The values obtained for RAFT3 scFv are comparable with our previous experience (Kang, 1998).

The data from biodistribution studies of both RAFT3 scFv and RAFT3DS scFv (Tables and Figures 3.3.1.2.6a-f) demonstrate that the reversal of V-domains does not affect the eventual organ localisation of the radio-iodinated scFvs. This is consistent with their similar properties in vitro. Specifically, the levels of tumour localisation in terms of %ID/g are equivalent across the four timepoints. Renal accumulation remains significant for both constructs and blood concentrations decrease rapidly to similar levels. In terms of tumour to normal tissue contrast achieved (T:NT), the key factor in imaging studies, both constructs achieve equivalent contrast to liver, lung, spleen and blood. The largest differences exhibited lie in the T:NT ratios produced with respect to muscle and bone. These differences can be explained in terms of experimental error. The very small samples of muscle and bone retrieved in this mouse model (~50mg) and the lack of appreciable accumulation in these tissues resulted in a very low total number of counts per second from each sample. A small error in weighing the tissue would, therefore, be greatly amplified when eventual cps/g were calculated. We would, therefore, contend that these results are unreliable in this model. In subsequent studies, it would be possible to retrieve larger masses of muscle in order to minimise any possible error. However, it will remain difficult to obtain significantly larger samples of bone in this animal model.

In comparison to the control antibody fragment, anti-CD18 scFv, the RAFT scFvs exhibited clear tumour accumulation in excess of that seen in normal tissues. This demonstrates that tumour targeting by these antibody fragments is specific and not merely a function of non-specific mechanisms as has been described (Stya et al., 1987).

In summary, the manipulation of the RAFT3 scFv by domain swapping demonstrated that the orientation of the V-domains can indeed influence the eventual yield of
recombinant protein in *E.coli*. Furthermore, this manipulation increased the yield without adversely affecting the tumour-binding properties of the molecule *in vitro* or its tumour-localising properties *in vivo*.

3.4.2 Optimising Protein Purification

A major component of efficient recombinant protein production is the isolation of the material of interest to a suitably high degree of purity. However, several steps are usually required in this purification process, each of which will inevitably involve some loss of the protein. Attaining a sample of very high purity (such as would be required for clinical work) may involve considerable losses during purification. These losses will reduce the eventual yield and, whilst they may not be critical where large amounts of protein are easily produced, such losses may reduce the usefulness of low-yield proteins. In the case of the RAFT scFv series, the low yields attained in the laboratory necessitated maximising the efficiency of the purification process.

3.4.2.1 IMAC Purification & Ion Exchange Chromatography

Immobilised Metal-ion Affinity Chromatography (IMAC) is a technique which separates proteins on the basis of their affinity for certain metal ions (Porath *et al.*, 1975; Porath, 1992). In the case of our scFvs, the hexahistidine tail incorporated into the protein by the plasmid pUC119 His 6 *myc* Xba has a high affinity for copper, nickel and zinc ions and this property is exploited for purification.

The usefulness of IMAC for purification of RAFT series scFvs in the vector pUC119 His 6 *myc* Xba using copper ions as the ligand has already been demonstrated (Kang, 1998). Our efforts concentrated on improving on the results of this previous work.

Increasing the starting bacterial OD₆₀₀ from 0.015 to 1.5 increased the purified protein yield from 150 to 500µg/l (400µg/l if an ion-exchange step was employed). The use of smaller, highly cross-linked Hi-Trap™ sepharose columns proved clearly more efficient than the larger 50ml sepharose columns we had used previously (Kang, 1998). The 20%
improvement in yield (500 to 600μg/l) was particularly beneficial in that no ion-
exchange step was required (and therefore no concomitant protein loss). This increased
yield and the increase in purity were sufficient to make the Hi-trap™ columns our
method of choice for purification. The small size of these columns and the relatively
small volumes and simple procedures involved in their use made the system ideal for
our small scale protein production. The time required for a purification run on the
columns was reduced from around 24 to 2 hours, enormously reducing the overall
production time for pure scFv. Indeed, this reduction in the time required for
purification may be a factor in increasing yield; scFv rapidly purified will spend less
time in the bacterial supernatant and, therefore, be less susceptible to proteolytic
degradation. Our observation that columns in series could prevent protein loss as a
result of bacterial supernatant stripping the chelated metal ions was especially useful
since such stripping would have negated the usefulness of these columns.

The use of different ions as ligands, namely copper, zinc and nickel proved interesting.
Our finding that copper ions were the most useful coincides with the results of a study
by Casey et al. in which the differences in yield and purity of His₆ tagged MFE-23 scFv
using IMAC columns charged with these ions was assessed (Casey et al., 1995).

The failure of zinc ions to differentiate between the scFv and a 25kDa contaminant band
is surprising. A similar contaminant band has been described in E.coli which could not
be removed by IMAC (Wulfing et al., 1994). In Wulfing’s study, a 196 amino acid
E.coli protein was identified which showed a very high affinity for zinc and nickel ions
but lesser affinity for copper ions. These findings do not entirely mirror our results in
that nickel ions were the most effective in separating scFv from the contaminant. The
exact nature of the contaminant in our system has not yet been determined. That the
25kDa protein is a breakdown product of the scFv remains a possibility. This might
explain its similar purification characteristics in IMAC. However, since it is not stained
using the anti-c-myc monoclonal antibody 9E10 in Western blot, it is clear that no c-myc
(and hence hexahistidine) tail is present. If this is the case, then one might expect the
IMAC purification characteristics of a breakdown product to differ markedly from the
scFv. In any event, this contaminant protein was successfully removed during
purification using copper ions with or without subsequent ion exchange chromatography. Definitive identification of the protein might be achieved by purification then N-terminal sequencing with subsequent comparison to known amino acid sequences. However, this undertaking was not essential for the purposes of this project and therefore no further investigation of the contaminant protein was undertaken. Finally, as described above, nickel ions were rejected due to the difficulty in assessing their retention on the column due to their colourless nature and to the toxicity which has been attributed to nickel contamination (Savolainen, 1996; Barceloux, 1999).

Ion Exchange Chromatography was able to separate the scFv from the 25kDa contaminant band in those samples containing both. The failure of the RAFT3 and RAFT3DS scFvs to bind to the ion exchange column in PBS at pH 7.5 is consistent with the proximity of this pH to the isoelectric points of the molecules (pI 8.56). The scFvs would be nearly neutral in charge at this pH which would minimise their interaction with the Ion Exchange column. The fact that the contaminant band binds to the column at this pH suggests a differing pI from the scFv (whose pI is slightly alkaline) but does not shed any light on its nature. Bacterial proteins tend to have pIs in the acid range (Link et al., 1997) whilst a breakdown product of a scFv might exhibit a charge differing from its parent molecule. In practical terms, it was simpler to reload these impure IMAC fractions on the IMAC column during the next purification run rather than introduce another step into the production process. Ion Exchange Chromatography was, therefore, not routinely used during scFv purification.

### 3.4.2.2 Immunoaffinity Chromatography

Recombinant protein-L (Bjorck, 1988) has been used for the purification of IgG and Fab fragments (De Chateau et al., 1993; Kouki et al., 1997). Very few descriptions of its use for scFv purification have yet reached the literature (Akerstrom et al., 1994). Its high affinity for human kappa light chains and some evidence of scFv binding made it a promising candidate for the purification of the chimaeric RAFT3 scFv. The yield of scFv using rPL was impressive and highlights some of the inefficiencies still present in
our IMAC purification protocol. The fact that a very clear elution peak could be obtained on UV monitoring allowed highly efficient collection of the scFv fraction with a minimal volume. Such a peak could not be identified on IMAC purification since the scFv eluted over a fairly large volume which reduced the amplitude of any peak. A very pure sample was obtained and reflects the very high specificity of this technique for the target light chain.

One drawback of immunoaffinity purification techniques such as this lies in the need to elute the protein of interest using very acidic conditions (pH 2.0 in this case). These low pHs can potentially damage protein material and may denature pH sensitive molecules, rendering the protein non-functional. Indeed, a dense precipitate rapidly formed in the purified scFv samples. This precipitation was originally attributed to acid-induced denaturation of the protein. However, subsequent assessment of the protein’s solubility at higher concentrations (discussed below) raised the possibility that this effect was related to the very high protein concentration achieved using rPL (up to 2.5mg per ml) rather than any acid-induced damage. In fact, reducing the pH of more dilute scFv samples to pH 2.0 for a brief period and then neutralising the solution did not appear to adversely affect protein binding in cell ELISA (data not shown). This would appear to support the suggestion that precipitation of the scFv after rPL purification was indeed simply a function of protein concentration. The expense of rPL (over £80 per mg) would be likely to preclude its use in large scale purification for a patient trial. The reduced yield using IMAC purification would suggest that additional losses are present in that technique. These would be expected where a technique requires further purification steps. In the case of the RAFT series scFvs, solubility limitations and a tendency to aggregation might play an important part since IMAC purification is a significantly longer process. The solubility of the scFv is discussed in detail below.
3.4.3 ScFv Solubility Studies

3.4.3.1 Enhancing Solubility

The limited solubility of RAFT3 scFv at around 150μg/ml in PBS pH 7.5 posed several problems. Firstly, a low solubility reduced the usefulness of highly efficient purification methods such as immunoaffinity using recombinant Protein L™. Secondly, many useful techniques (e.g. technetium labelling) require a protein sample at a relatively high concentration (e.g. 1-2mg/ml) which could not be attained by RAFT3 scFv. Few authors give any details on the solubility of scFvs they have studied. However, as noted in the introduction to this chapter, it has been suggested that hydrophobic residues normally “buried” in IgG molecules may be surface exposed in scFvs and lead to reduced solubility in aqueous buffers (Nieba et al., 1997). The simplest methods of increasing the amount of dissolved protein in a sample rely on manipulating the buffer itself. The method of choice may need to be determined empirically for a given protein and in the case of RAFT3 scFv we employed a range of detergents and co-solvents in a bid to improve solubility. Sugars (Frigon and Lee, 1972), uncharged and charged amino acids (Arakawa and Timasheff, 1984), glycerol (Na and Timasheff, 1981) and PEG (Arakawa and Timasheff, 1985) have been used successfully as co-solvents to improve protein stability in solution. Additionally, the incorporation of detergents such as Tween-20 (Kreilgaard et al., 1998) has been able to limit molecular interaction and aggregation with a similar stabilising effect. However, the failure of these manoeuvres to enhance the solubility of RAFT3 scFv does not clarify the underlying problem with this molecule.

Buffer pH can have a significant effect on solubility (Harris, 1989) and a pH close to the pI of a protein results in a near neutral charge on the molecule. This in turn reduces the repulsive force between similarly charged molecules and increases the likelihood of aggregation and precipitation. This mechanism forms the basis of some techniques for purification by precipitation (isoelectric precipitation). In the case of the RAFT3 scFv, the proximity of its pI (8.56) to the pH of the buffer (7.5) raised the possibility that a similar effect may have at least some part to play and suggested that moving the pH of
the buffer away from 7.5 might increase solubility. However, the slight increase in solubility observed at extremes of pH would not markedly affect the overall solubility. In addition, prolonged storage of scFv at such extremes of pH would be likely to result in denaturation and reduced immunoreactivity. The small rise in solubility seen at extremes of pH would imply that the pH of the buffer plays only a minor part in the solubility of RAFT3 scFv.

Various strategies of scFv modification to increase solubility have been attempted. Riechman described the enhancement of isolated Vh domain solubility by the replacement of hydrophobic residues normally associated with the (now absent) VL by mimicking the structure of camelid heavy chains (Riechmann, 1996). Camel heavy chains are naturally devoid of light chains and therefore do not carry hydrophobic residues at these positions. Camelid heavy chains compensate for the absence of a light chain by possessing a very large CDR3 and attempts to mimic this structure may be difficult without adversely affecting affinity for the antigen. One attempt to camelise an anti-melanoma scFv produced clones which no longer bound melanoma (J. Kupsch, personal communication). In any event, camelisation requires mouse/human subgroup III VH and the RAFT series scFvs are subgroup VII. It would therefore be unlikely be useful for these scFvs. Nieba et al. investigated the replacement of uncovered hydrophobic patches with hydrophilic residues (Nieba et al., 1997). Whilst this strategy increased the yield of soluble scFv over 25-fold, no increase in the eventual scFv solubility was obtained.

Interestingly, Tan et al. attempted to increase the solubility of an anti-renal cell tumour scFv by adding 5 glutamic acid residues to the end of the scFv chain (Tan et al., 1998). In fact this succeeded in increasing solubility in physiological buffer from 100µg/ml to 15mg/ml. However, whilst the authors contend that this increase is due to moving the pI of the protein away from the pH of the buffer (from 7.5 to 6.1), it may, strictly speaking, relate to the incorporation of more hydrophilic residues rather than a direct pH effect. Indeed, work in this lab subsequent to the time course of this project has demonstrated that incorporation of 5 glutamic acid residues into the RAFT3 scFv increases its solubility from 150 to 2000µg/ml (J. Odili, personal communication). Such molecular
manipulation may offer a more permanent solution to the solubility problems affecting our series of scFvs. We would contend, however, that this increase is not solely a matter of charge since we would have then expected a similar effect when modifying the buffer pH to that seen when altering the pI of the scFv.

3.4.3.2 Imidazole Solubilisation

The discovery that imidazole enhanced solubility of the scFv in PBS at physiological pH was surprising. This finding was prompted by the observation that eluted RAFT3 scFv in 50-200mM imidazole / PBS buffer stayed soluble at concentrations greater then 150µg/ml but subsequent dialysis against PBS (removing the imidazole) provoked aggregation. No other reference to a similar effect has been reported in the literature. Imidazole corresponds to the ring structure found in histidine and this similarity forms the basis of its function in competitively eluting histidine-tagged proteins from IMAC columns (Porath, 1992). We postulate that interactions between histidine tails or between histidine tails and other residues may be responsible for the aggregation of scFv seen at concentrations greater than 150µg/ml. The imidazole in solution might, therefore, interfere with these interactions and prevent aggregation and precipitation. Ideally, one might demonstrate this effect by purifying concentrated scFv lacking a hexahistidine tail. If our hypothesis were correct, less aggregation and greater solubility would be exhibited. Initial observations suggesting increased solubility in the absence of the hexahistidine tail during the timecourse of this thesis have prompted further investigation. Detailed experiments were, however, outwith the time constraints of this thesis but subsequent work has demonstrated that manipulated scFvs based on RAFT3 scFv but lacking the histidine tail show greater solubility (J. Odili, personal communication). This hypothesis would also, perhaps, explain the inefficacy of other buffer manipulations in reducing aggregation. Moreover, scFvs can be modified to incorporate an enzymatically cleavable hexahistidine tail using TEV protease (Parks et al., 1992; Parks et al., 1994) and this strategy is being currently investigated in our laboratory.
That resolubilisation of the scFv could be achieved using histidine is also interesting. This suggests that at least some of the precipitation is reversible, since functional scFv is obtained (as shown in cell ELISA). However, as noted in the results section, the proportion of functional scFv is not easily ascertained and any subsequent experiments using this re-solubilised scFv would be difficult to interpret. Were the resolubilised scFv to be shown to maintain its antigen-binding properties, then precipitated scFv might provide an ideal method of storage.

It is encouraging that scFv maintained soluble in imidazole performs comparably to scFv stored in PBS alone in terms of radiolabelling and \textit{in vivo} biodistribution. This would suggest that imidazole-stored scFv would be similarly useful for \textit{in vivo} imaging or tumour targeting. No toxicity was noted in the animal model and this is to be expected since, firstly, most or all the imidazole would be removed during gel filtration of the labelled scFv and, secondly, imidazole-like compounds have indeed been used as systemic anti-fungal agents without harm (Wade \textit{et al.}, 1979; Sohn, 1982). Whilst efforts are made to enhance the solubility of these scFvs by molecular manipulation, the maintenance of solubility in histidine has proved useful in allowing examination of the \textit{in vitro} and \textit{in vivo} properties of these molecules and may perhaps be applicable to clinical use. However, one must bear in mind that imidazole could interfere with chemical modifications targeting aromatic amino acids such as the carbonyl-based radio-technetium labelling recently described (Waibel \textit{et al.}, 1999). The mechanism by which histidine maintains RAFT3 scFv in solution is yet to be determined and is the subject of ongoing studies in this laboratory. If the key is the hexahistidine tail, then this would be important for a wide range of recombinant proteins containing such tags in addition to scFvs.

\subsection*{3.4.4 Conclusion}

The experimental work in this chapter has demonstrated that modification of the induction conditions and purification technique of the RAFT series scFvs results in significant improvement in the scFv yield. In addition, the important influence of the V-domain sequence on recombinant protein production has been identified. Domain-
swapped RAFT3 scFv shows clearly increased yield whilst maintaining its melanoma-targeting properties. This increase in the production of purified scFv has facilitated the preliminary *in vitro* and *in vivo* characterisation of the scFvs and made the production of clinically useful quantities of scFv by batch fermentation a possibility.

Our studies of purification methods have demonstrated that both IMAC and immunoaffinity purification are effective. IMAC purification using copper ion loaded columns in series provides a useful compromise between efficiency and cost.

Limited solubility of these scFvs remains problematic and simple manipulation of the buffer conditions has proved ineffective. However, the incorporation of imidazole in the buffer maintains solubility without adversely influencing tumour targeting. This finding merits detailed further study since the mechanism of action may be of relevance to other scFvs with limited solubility in physiological buffers. Eventual modification of the scFv itself may represent the way forward in overcoming these solubility issues.

Overall, the data contained in this chapter have demonstrated that useful amounts of soluble anti-melanoma scFv can be produced and that clear tumour targeting can be achieved. The subsequent chapters in this thesis will examine strategies aimed at improving the efficacy of tumour targeting and reducing non-specific organ accumulation.
Chapter 4

4. Single Chain Fv Affinity Maturation
4. ScFv Affinity Maturation

4.1 Introduction

Adequate tumour to normal tissue contrast is essential both for imaging and therapeutic applications of targeting molecules. The experiments in this chapter explore one strategy to improve the contrast achieved using single chain Fvs and to assess the impact of scFv affinity on tumour localisation.

Tumour targeting \textit{in vivo} relies on a variety of factors including:

1) Adequate delivery of the targeting molecule to the tumour cells
2) Availability of specific antigen for binding
3) Antigen specificity
4) Clearance of targeting molecule from the normal tissues
5) Effective antigen binding

These points in relation to our anti-melanoma scFvs are considered in turn:

1) Single chain Fvs have been shown in a wide variety of studies to exhibit more efficient tumour localisation than whole monoclonal antibodies (Adams \textit{et al.}, 1993; Colcher \textit{et al.}, 1998) together with more rapid tumour penetration (Yokota \textit{et al.}, 1992).

2) High-molecular-weight melanoma-associated antigen (HMW-MAA) is well characterised and is expressed on the surface of most melanoma cells (Hellström \textit{et al.}, 1983). Indeed a number of clinical targeting studies have shown the potential of imaging melanoma using anti-HWM-MAA monoclonal antibodies (e.g. (Carrasquillo \textit{et al.}, 1988; Loffler \textit{et al.}, 1994; Boni \textit{et al.}, 1995)).

3) The antigen specificity of the RAFT scFv series has previously been demonstrated on a panel of normal tissues (Fig. 4.1) in frozen sections using
immunohistochemical techniques (Kupsch et al., 1999). Indeed, improved specificity was clearly demonstrated in comparison to the parental LHM2 whole monoclonal antibody (Kupsch et al., 1995).

4) Rapid clearance of scFv from normal tissues, with the exception of the kidney, is the norm (Colcher et al., 1998). This is largely a function of the small size of these molecules (~27kDa) which causes them to slip through the "glomerular sieve" and enhances blood clearance (Chang et al., 1975; Maack et al., 1979). This rapid renal excretion together with a tendency to renal reabsorption is responsible for the high kidney localisation typical of scFvs. Whilst scFvs tend to achieve lower tumour accumulation than whole antibody, this rapid normal tissue clearance can increase the tumour to normal tissue contrast achieved to a level greater than that obtained with intact immunoglobulin (Adams, 1998; Colcher et al., 1998).

5) Effective antigen binding in terms of affinity for the antigen is obviously essential for tumour-specific localisation. However, the relationship is not clear and some authors have suggested that higher affinities may not improve targeting in a directly proportional manner (Juweid et al., 1992).

A number of strategies aimed at improving tumour localisation have been proposed and investigated (see (Adams, 1998) for review). These relate primarily to improving upon the affinity or avidity of the antibody fragment for the antigen. This chapter focuses on our investigation of the effect of monomeric antigen affinity on tumour localisation and our attempts to increase the affinity of our anti-melanoma scFvs. Attempts were also made to manufacture a scFv dimer based on the RAFT series scFvs. This strategy, due to an external sequencing error proved problematic. However, useful data was obtained and the rationale and preliminary results are discussed in appendix Chapter 7 for completeness.

**Affinity and Targeting**

The relationship between affinity and targeting efficiency would, intuitively, appear likely to be directly proportional and one might expect that very high affinity targeting
molecules would exhibit improved tumour to normal tissue contrast. However, experimental evidence has not yet conclusively confirmed this inference.

The natural assumption that greater affinity will result in better targeting has been challenged by Weinstein and co-workers. Their group postulated that, whilst a certain level of antigen affinity was essential, very high affinity clones might suffer from a "binding site barrier effect" (Weinstein et al., 1987; Fujimori et al., 1989). This hypothesis suggests that very high affinity molecules (e.g. $K_D \sim 10^{-9} \text{M}$) would bind preferentially (and effectively irreversibly) to the first target antigen lying close to the vasculature, inhibiting further ingress of antibody into the tumour. Conflicting studies have failed to clarify the issue, some suggesting benefit of higher affinity (Colcher et al., 1988), while others have not confirmed this finding (Juweid et al., 1992). Juweid et al., comparing a high affinity targeting antibody ($K_D \sim 6 \times 10^{-11} \text{M}$) to an irrelevant antibody control showed more uniform tumour penetration by the control and a tendency for the study molecule to localise close to the vasculature. One major difficulty lies in separating the effect of affinity from the many other factors which may influence tumour localisation. Ideally, one would compare two antibodies targeting the same antigenic epitope in the same model. Antibodies with similar chemical characteristics would help allow definitive conclusions to be drawn. Many studies involving antibodies of differing affinity have studied antibodies targeting unrelated epitopes of the antigen, making interpretation of the influence of affinity difficult (see (Adams, 1998) for review).

**ScFv Affinity**

The earliest scFvs (and the RAFT series described in this thesis) were produced by isolating the variable region genes from established hybridoma cell lines (Huston et al., 1988). A decrease in affinity for the antigen in comparison to the parental intact immunoglobulin was typical (Huston et al., 1988). This can be explained in terms of loss of avidity due to the monomeric nature of the scFv. Conversely, the greater affinity in the parent molecule relies on the bivalent immunoglobulin being able to
bind two target antigenic epitopes simultaneously. This point is clearly demonstrated in two separate monoclonal antibody-derived scFvs. CC49 monoclonal antibody and its Fab' and scFv progeny are specific for a repeated clustered carbohydrate epitope on the TAG-72 pancarcinoma antigen (Milenic et al., 1991). The parent molecule binds to the target antigen with an apparent affinity of \(2 \times 10^{-9}\) M, eight-fold higher than both the Fab' and scFv. The equivalent affinities of the scFv and Fab' suggest that valency is the most important factor in the altered affinity. On the other hand, the affinities of anti-CEA monoclonal antibody B6.2 and its Fab' and scFv progeny are almost identical \((K_D \sim 3 \times 10^{-9}\) M\) (Colcher et al., 1990a) suggesting, perhaps that only monovalent binding is possible.

More recently, antigen-specific scFvs have been isolated from antibody phage display libraries (Griffiths et al., 1993). These libraries allow filamentous phage displaying the encoded scFv to be selected on purified antigen or intact cells. Large phage libraries (over \(10^9\) transformants) have allowed the selection of antibody fragments with affinities as high as \(10^{-10}\) M (Griffiths et al., 1993; Winter et al., 1994).

Our anti-melanoma scFvs have exhibited only moderate affinities for the antigen \((K_D \sim 10^{-8}\) M\) (Kang, 1998; Kang et al., 2000)), though no greater scFv affinities for HMW-MAA have been reported. The nature of the plasmid encoding the scFv makes its manipulation \textit{in vitro} relatively simple and, therefore, we felt these antibody fragments would be ideal for the further investigation of the effect of affinity on targeting. Indeed, the results obtained would be especially interesting in that the modified scFvs could be selected to target the same antigenic epitope.

**Modifying ScFv Affinity**

Affinity maturation of (monomeric) scFvs isolated from hybridoma or phage display technology has been attempted using three main strategies – chain shuffling, site-directed mutagenesis and random mutagenesis. These techniques involve modifying
the basic structure of the scFv in a bid to improve the strength of its antigenic interaction.

Chain shuffling is based on the observation that light and heavy antibody chains can pair with more than one corresponding heavy or light chain to produce different antigen binding sites. The technique involves the retention of the gene for either the V<sub>H</sub> or V<sub>L</sub> of the antigen-specific scFv and its combination with a library of different complementary V domains (Marks <i>et al.,</i> 1992). The library thus produced is then selected on antigen using antibody phage display to allow the retention of tumour-binding variants of higher affinity. The process may then be repeated to replace the remaining original V-domain to further improve the affinity (or to fully humanise a murine scFv (Marks <i>et al.,</i> 1992; Rader <i>et al.,</i> 1998)). Chain shuffling has been successfully used to manipulate several phage-selected scFvs, resulting in up to 300-fold increase in affinity (Clackson <i>et al.,</i> 1991; Ohlin <i>et al.,</i> 1996; Schier <i>et al.,</i> 1996a; Thompson <i>et al.,</i> 1999; Park <i>et al.,</i> 2000).

Site-directed mutagenesis aims to modify specific amino acid residues in one or more of the CDRs of the scFv (usually CDR3, which has the greatest degree of contact with the antigen) to allow selection of clones with higher affinity (Schier <i>et al.,</i> 1996b; Adams <i>et al.,</i> 1998a). Randomisation of the CDRs can be combined with antibody phage display techniques to allow easier selection of higher affinity clones (Yang <i>et al.,</i> 1995; Thompson <i>et al.,</i> 1996). By combining various advantageous mutations on one scFv, affinity increases of up to 1200-fold have been produced (Schier <i>et al.,</i> 1996b).

Affinity maturation by random mutagenesis has also been employed. This technique uses <i>E.coli</i> strains which are error prone in DNA replication (mutator strains) to introduce random mutations into the scFv (Irving <i>et al.,</i> 1996; Low <i>et al.,</i> 1996). Several rounds of mutation and antibody phage display selection are carried out to achieve increasing affinity for the antigen. This technique mimics the somatic hypermutation seen <i>in vivo</i> in B lymphocytes on repeated exposure to antigen and has
produced affinity increases of up to 1000-fold (Marks et al., 1992; Low et al., 1996). This technique and chain shuffling share the advantage over site-directed mutagenesis of allowing variations in non-CDR regions of the scFv which may enhance affinity.

Our desire was to improve the affinity of our scFvs and, in so doing, study the effect of affinity on melanoma targeting. Neither chain-shuffling nor random mutagenesis had previously been described in hybridoma-derived scFvs. Since V-domain libraries were readily available in our laboratory, we decided to employ chain shuffling and antibody phage display techniques for affinity maturation. All three strategies are discussed in the context of our scFvs at the end of this chapter. Additionally, it was our hope that clones displaying enhanced yield might also be obtained.

**Experimental Rationale**

As noted above, the replacement of heavy or light chains of a scFv molecule is termed chain shuffling and has previously been used as a means to improve affinity for the target antigen (Clackson et al., 1991; Ohlin et al., 1996; Schier et al., 1996a; Thompson et al., 1999; Park et al., 2000). In our study, a combinatorial library of $V_K$ segments was used to produce a scFv consisting of the parent $V_H$ and a large variety of $V_K$s. This library was then screened using antibody phage display techniques wherein filamentous bacteriophage "displaying" the different scFvs on their surface were selected on melanoma cells to identify scFvs of increasing affinity for the antigen. These scFvs were produced as recombinant protein in *E.coli* and then assessed both *in vitro* and *in vivo* to investigate the effect of altered affinity on tumour localisation.
4.2 Methods

4.2.1 ScFv Development

The parent LHM2 mouse monoclonal antibody was a gift of Professor I. Leigh (Queen Mary and Westfield Medical College, London). Dr. Jörg Kupsch isolated LHM2 scFv from this monoclonal antibody by PCR techniques (Kupsch et al., 1995). RAFT2 scFv was produced by sub-cloning LHM2 scFv into pUC119 His 6 c-myc Xba, a gift of Dr. G. Winter (Cambridge). This plasmid incorporates a c-myc tag for in vitro detection using mouse monoclonal antibody 9E10 and a hexahistidine tail for protein purification by Immobilised Metal-ion Affinity Chromatography (IMAC) (see Fig. 4.2.1).

4.2.2 Chain Swapping of LHM2 ScFv

Chain swapping of LHM2 scFv was carried out by Dr. Jörg Kupsch. The technique is outlined stylistically in Figure 4.2.2a. DNA from a LHM2 scFv clone (#7-8) was linearised at the NarI site (Section 2.1.4) previously introduced in the linker region (Kupsch et al., 1999). A human V\textsubscript{K} library in the plasmid pUClSAod (Kupsch et al., 1999) was similarly linearised at the NarI site located at the 5’ end of the V regions.

The linearised scFv and the human V\textsubscript{K} library were ligated (Section 2.1.4) and the reaction used as a PCR template with custom made primers 5’CAACGTGAAAAAATTATTATTTCGC3’ and 5’TGCTCAGTACCAGGCGGAT3’ binding in the gene 1 leader and 330 bp 3’ from the NotI site in pCantab-5, respectively (Section 2.1.3). The asymmetric positioning of the PCR primers allows the discrimination of V\textsubscript{K}V\textsubscript{K} and V\textsubscript{H}V\textsubscript{H} homodimers and V\textsubscript{H}V\textsubscript{K} heterodimers by size (1350, 850 and 1150 bp). The 1150 bp heterodimer fragment was gel purified (Section 2.1.2), SfiI/NotI digested (Section 2.1.4) and cloned back into pCantab-5 (Section 2.1.5). ScFv insertion into pCantab5 is illustrated in Figure 4.2.2b. The plasmid was then transformed into \textit{E.coli} TG1 (Section 2.1.7). The bacteria were plated on Luria Agar plates containing 2% glucose and 100\mu g/ml ampicillin (Section 2.1.6) to allow
Fig. 4.2.2a

Chain-Shuffling to Replace Mouse $V_K$ Light Chains With Human $V_K$

LHM2 scFv in pCantab (Mouse)

$V_H$

PCR Fragment 300-400 bp $V_H$ specific for melanoma

$V_L$

$V_L(\kappa)$-Library in pUC19 (Human)

$V_L$

PCR Fragments 300-400 bp $V_L(\kappa)$ with random antigen specificity

PCR Reaction

PCR Reaction to introduce linker

Ligated

Subcloned into pCantab for Selection by Phage-Display

$V_H$ Mouse Derived

$V_L$ Human Derived
Fig. 4.2.2b

Plasmid pCantab5

scFv in pCantab5. Anti-HMW-MAA scFv.

- **P** geneIII
- **VH**
- **Linker**
- **VL**
- **c-myc**

- Secretion Signal
- LHM2 scFv cassette
- c-myc tag for Western blot analysis
selection of bacteria resistant to ampicillin and, therefore, incorporating the plasmid. A small library of $5.2 \times 10^5$ clones was obtained. The transformed bacteria were resuspended in Luria Broth and a phage lysate prepared (Section 2.3.3), the rescued filamentous phage incorporating and displaying the encoded scFvs (scFv-fusion phage).

### 4.2.3 Screening of Clones

The rescued phage were then positively selected on cultured melanoma cells (Section 2.3.4) and "negatively" on human peripheral blood monocytes (PBMCs) (Kupsch et al., 1999) to enrich for those binding strongly to melanoma and remove those binding normal cells (Fig. 4.2.3). The selected phage were then used to transfect *E.coli* TG1 cells (Section 2.3.6) and the plating and selection process repeated a further three times (four rounds in total).

Pooled bacteria from rounds 3 and 4 were used to isolate plasmid DNA (Section 2.1.1). The scFv-encoding DNA was isolated in bulk by *HindIII/NotI* digest (Section 2.1.4) and gel purified (Section 2.1.2). The scFv DNA fragments were then subcloned into pUC119 His6 myc Xba (Fig. 4.2.1). *E.coli* TG1 were again transformed and plated out overnight at varying dilutions on Luria Agar plates as before. The following morning plates containing discrete colonies were selected and colony lifts performed (Section 2.3.5). Development of the nitrocellulose filters produced by colony lifting using the anti-c-myc monoclonal 9E10 as the primary antibody allowed the identification of c-myc-positive colonies. These were the colonies potentially producing viable scFv. 96 positive colonies from each of rounds 3 and 4 were picked and overnight cultures made in 96 well microtitre plates. Inductions of these overnight cultures were then made and the raw supernatants assayed in duplicate in cell ELISA for melanoma cell binding (Section 2.5.3). The 19 best candidate clones in terms of ELISA signal (8 from round 3 and 11 from round 4) were retested in cell ELISA and Western blot to confirm melanoma binding and assess the scFv yield.
RNA hybrid analysis was then carried out by Dr J. Kupsch to identify identical clones. In short, DNA was PCR amplified from miniprep DNA using the sequencing primers modified to contain T3 and T7 RNA polymerase promoter sequences. RNA hybrid analysis was used to identify identical clones (Myers, 1985; Winter, 1985) using a commercially available kit (Mutation Finder Kit, FMC). Purified PCR fragments were used as templates for the in vitro transcription of RNA by T3 or T7 RNA polymerase. Transcripts of opposite strands were hybridised, digested with RNAses and the digests analysed on 2% agarose gels. Two non-identical clones that had been identified by preliminary sequencing were used as positive controls. RNA hybrid analysis identified 8 non-identical clones. 4 from selection round 3 and 4 from selection round 4). The two most promising clones in terms of melanoma binding and yield were selected and termed RAFT3 scFv and RAFT2-11 scFv. In subsequent experiments these were compared to the original unshuffled RAFT2 scFv.

4.2.4 Production and Purification of ScFvs

One litre cultures of the E.coli TG1 incorporating the unmodified (RAFT2) and modified (RAFT3 scFv and RAFT2-11 scFv) scFv plasmid were induced to produce recombinant protein as described in Section 2.4.2. After dialysis of the concentrated, filtered bacterial supernatant against PBS at 4°C, the scFv was purified using Immobilised Metal Ion Affinity Chromatography (IMAC) on Hi-trap Chelating Sepharose Columns™ and eluted using a stepwise gradient of imidazole (Section 2.4.5). Fractions collected were then assayed by SDS-PAGE and Western blotting under reducing conditions to establish the fractions containing scFv and confirm its purity (Section 2.2). Analysis of the gels demonstrated that pure scFv was obtained in the 50 to 200 mM imidazole fractions. Impure fractions were retained for reloading with the next purification run.
4.2.5 Confirmation of Epitopic Specificity

In order to demonstrate that the various clones isolated continued to bind the same epitope of the High Molecular Weight Melanoma Associated Antigen, a competition cell ELISA was set up. The wells were initially loaded with 500ng of scFv per well and serial dilutions of LHM2 IgG (the parent mouse monoclonal antibody) were carried out across the plate from 500 to 1ng per well. The ELISA was completed as previously described (Section 2.5.3) and the plate read at 490nm.

4.2.6 Radiolabelling

Radiolabelling was carried out using the Iodogen™ method (Pierce) and $^{125}$I as the radiolabel (Section 2.6).

Immuno-reactivity after labelling was assessed by comparing equivalent amounts of radio-iodinated scFv and cold antibody in cell ELISA on A375-M melanoma cells (Section 2.5.3).

4.2.7 Affinity Studies

The affinity constants of the scFvs were assessed in order to identify and quantify varying affinity for the antigen \textit{in vitro} due to the differing V\textsubscript{K}s of the molecules. Saturation binding and Scatchard plots were obtained after incubating the radiolabelled antibody with melanoma cells as described in Section 2.7.3. Affinity and dissociation constants were calculated using Graphpad Prism™ analysis software.

4.2.8 In Vivo Studies

Pharmacokinetic and biodistribution data were obtained in Balb/c nu mice bearing human melanoma xenografts (Section 2.8) for the three scFvs of varying affinity. One half $\mu$g of iodine-125 labelled scFv in 100$\mu$l PBS was injected via the tail vein and the
mice euthanased at appropriate timepoints. Three to five mice were used for each timepoint. Radio-iodinated anti-CD18 scFv was used as a negative control for all experiments.

4.2.8.1 Pharmacokinetics

One half μg of iodine-125 labelled scFv in 100μl PBS was injected via the tail vein and the mice were sacrificed at 1 min, 5 mins, 15 mins, 30 mins, 60 mins, 180 mins, 6 hours and 18 hours after injection. Blood samples were obtained by cardiac puncture, weighed and the radioactivity measured in a gamma counter. Results were expressed as percentage of the injected dose per gram and the 1 min timepoint used as the 100% value. Equilibrium and clearance half-lives were calculated from the curve using Microcal Origin® software.

4.2.8.2 Biodistribution

Mice were sacrificed at 1, 3, 6 and 18 hours after injection of one half μg of iodine-125 labelled scFv in 100μl PBS. A blood sample was obtained by cardiac puncture and organs removed for weighing and measurement of radioactivity. The tumour, left quadriceps, left femur, spleen, liver, kidneys and lungs were counted.

4.2.8.3 Analysis of Biodistribution Results

Results were expressed in terms of percentage of the injected dose per gram of tissue (%ID/g) and from this tumour to normal tissue ratios (T:NT) were calculated. Comparison to the negative control anti-CD18 scFv allowed calculation of the radio-localisation index (RI) which represents the tumour-specific localisation of the antibody or fragment in question.
4.3 Results

4.3.1 PCR Production of Modified Clones

A minilibrary of chimaeric scFvs consisting of the mouse $V_h$ and a human $V_k$ repertoire was made as described in materials and methods. This contained of $5.2 \times 10^5$ colony-forming units before selection on melanoma cells.

4.3.2 Screening of Bacterial Clones

As noted in the methods section, after selection of the library of chimaeric scFvs on melanoma cells, the 19 best binding clones of the 192 picked by colony lifting were assessed by RNA hybrid analysis. This revealed 8 different scFvs in the pool of 19. The cell ELISA and Western blot of these 8 clones are shown in Figures 4.3.2a&b. Two clones (3A5 and 3A11) appeared superior to the others and were selected for subsequent analysis. As noted above, these clones were termed RAFT3 and RAFT2-11.

4.3.3 Production and Purification of ScFvs

Purification of bacterial supernatants by IMAC revealed pure scFv in the fractions eluted using between 50 and 200mM imidazole in the elution buffer as shown by Coomassie Brilliant Blue staining of SDS-PAGE gels made from the eluted fractions. The nature of the protein band was confirmed by Western blotting of an identical gel using anti-c-myc as the primary antibody (9E10) in order to identify scFv.

Comparison with ovalbumin standards demonstrated that around $100\mu g$ of pure RAFT2 scFv was produced per litre of bacterial supernatant. This was considerably less than the $600\mu g$ per litre of culture supernatant for the RAFT3 scFv and $450\mu g$ per litre of RAFT2-11 scFv.
Chapter 4

4.3.4 Confirmation of Epitopic Specificity

Confirmation of epitopic specificity was achieved by competitive inhibition of binding of LHM2 IgG, the parental mouse monoclonal antibody of the RAFT scFv series. All three scFvs in this study competitively inhibited binding of LHM2 indicating that all three shared a common epitope. The results of the competition cell ELISA are shown in Figure 4.3.4.

4.3.5 Radiolabelling

The results of iodinations are summarised in Table 4.3.5. These results demonstrate equivalent labelling efficiency in megabequerels/microgram of protein for all three scFvs. The differing percentage incorporations reflects differing initial concentrations of protein during labelling.

The effect of labelling on immuno-reactivity was assessed in cell ELISA by comparing the melanoma binding exhibited by equivalent amounts of labelled and unlabelled scFv. The results are shown in Figure 4.3.5 and indicate that approximately 90% of the immuno-reactivity was retained (Table 4.3.5).

4.3.6 Affinity Analysis

Saturation binding plots were made for RAFT2, RAFT3 and RAFT2-11 scFvs and the results used to produce Scatchard plots for analysis of binding affinity. The plots for RAFT2-11 scFv are shown as an example (Figs. 4.3.6a & 4.3.6b) and the results for all three scFvs summarised in Table 4.3.6. These figures reveal affinities (association constants, $K_A$) of $2.89 \times 10^5$, $3.19 \times 10^7$ and $1.01 \times 10^8$/mol for the RAFT2, RAFT3 and RAFT2-11 scFvs respectively. This provides a useful range of affinities for in vivo analysis and demonstrates that chain shuffling can improve the affinity of the scFv above that of the parental monoclonal antibody LHM2 ($K_A = 4.0 \times 10^7$ l/mol, (Kang, 1998)).
### Table 4.3.5

**Summary of Radio-iodination Results**

<table>
<thead>
<tr>
<th>scFv</th>
<th>Average Specific Incorporation Activity (MBq/microg)</th>
<th>Immunoreactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT3</td>
<td>0.038</td>
<td>92.1</td>
</tr>
<tr>
<td>RAFT2</td>
<td>0.033</td>
<td>91.2</td>
</tr>
<tr>
<td>RAFT2-11</td>
<td>0.043</td>
<td>86.4</td>
</tr>
<tr>
<td>anti-CD18</td>
<td>0.008</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Fig. 4.3.5**

**Immuno-reactivity of Radio-iodinated scFv**

- **Absorbance @ 490 nm**
  - RAFT3
  - RAFT2-11
  - RAFT2
  - aCD18
  - Blank

Immuno-reactivity of unlabelled and labelled scFvs assessed in cell ELISA against A375-M melanoma cells. 0.5ug of each scFv was loaded. Anti-CD18 scFv is shown as a negative control.
Fig. 4.3.6a  RAFT2-11 Saturation Plot

![RAFT2-11 Saturation Plot](image)

Fig. 4.3.6b  RAFT2-11 Scatchard Plot

![RAFT2-11 Scatchard Plot](image)

Table 4.3.6  Scatchard Summary

<table>
<thead>
<tr>
<th>scFv</th>
<th>$K_A$ (l/mol)</th>
<th>$K_D$ (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT2</td>
<td>2.89x10^5 (+/-0.83)</td>
<td>3.46x10^-6</td>
</tr>
<tr>
<td>RAFT3</td>
<td>3.19x10^7 (+/-0.74)</td>
<td>3.13x10^-8</td>
</tr>
<tr>
<td>RAFT2-11</td>
<td>1.01x10^8 (+/-0.63)</td>
<td>9.91x10^-9</td>
</tr>
</tbody>
</table>

(Error shown as SEM)
4.3.7 In Vivo Studies

4.3.7.1 Pharmacokinetics

Blood clearance curves for RAFT2 scFv, RAFT3 scFv and RAFT2-11 scFvs together with the control anti-CD18 scFv are shown in Figure 4.3.7.1. Values for $t_{1/2\alpha}$ and $t_{1/2\beta}$, representing equilibrium and clearance half-lives respectively, were calculated from the curve using Microcal Origin software and are shown in Table 4.3.7.1. All three scFvs and the negative control exhibit comparable and rapid clearance with $t_{1/2\alpha}$s of 7-9 min and $t_{1/2\beta}$s of 175-206 minutes. The negative control anti-CD18 scFv exhibited the most rapid clearance with $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 7 and 175 min, respectively.

4.3.7.2 Biodistribution

Biodistribution results in normal organs and tumour are shown as %ID/g in Tables 4.3.7.2a-d and expressed graphically in Figures 4.3.7.2a-d. Maximal tumour accumulation at the timepoints measured was found at 1 hour for all four scFvs studied. RAFT2-11 scFv achieved 4.071%ID/g localising to tumour at 1 hour in comparison to 2.510 and 1.533 for RAFT3 scFv and RAFT2 scFv, respectively. This higher tumour accumulation persisted through all timepoints and at 18 hours the RAFT2-11 scFv exceeded RAFT2 and RAFT 3 scFvs tumour localisation 7 and 3-fold respectively (1.186%ID/g vs. 0.160 and 0.370). Normal organ accumulation was maximal in the kidney and dropped rapidly over the 18 hours of the experiment. The results for normal organ accumulation were comparable for all 3 scFvs studied but lower in the case of the control scFv. At no time did tumour accumulation exceed renal accumulation for any scFv.

The tumour to normal tissue ratios achieved are shown in Tables 4.3.7.2e-h and Figures 4.3.7.2e-h. The control anti-CD18 scFv shows no tendency to relative tumour accumulation over the 18 hours of the experiment with all values resting below one or,
### Table 4.3.7.2a

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.510</td>
<td>16.210</td>
<td>1.702</td>
<td>1.980</td>
<td>0.802</td>
<td>0.591</td>
<td>1.772</td>
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<td>1.220</td>
<td>8.820</td>
<td>0.853</td>
<td>0.933</td>
<td>0.602</td>
<td>0.407</td>
<td>0.669</td>
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</tr>
<tr>
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<td>0.750</td>
<td>6.712</td>
<td>0.307</td>
<td>0.209</td>
<td>0.134</td>
<td>0.178</td>
<td>0.297</td>
<td>0.217</td>
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<tr>
<td>18</td>
<td>0.370</td>
<td>2.089</td>
<td>0.144</td>
<td>0.070</td>
<td>0.041</td>
<td>0.019</td>
<td>0.087</td>
<td>0.073</td>
</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2b

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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</thead>
<tbody>
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<td>2.974</td>
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<td>1.411</td>
<td>2.490</td>
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<tr>
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<td>9.147</td>
<td>1.186</td>
<td>1.203</td>
<td>0.547</td>
<td>0.461</td>
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<tr>
<td>6</td>
<td>2.959</td>
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<td>1.040</td>
<td>0.870</td>
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<td>0.687</td>
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<td>0.026</td>
<td>0.168</td>
<td>0.112</td>
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</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2c

<table>
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<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
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<td>1.463</td>
<td>2.683</td>
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<td>6.991</td>
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<td>0.453</td>
<td>0.491</td>
<td>0.237</td>
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<td>0.058</td>
<td>0.052</td>
<td>0.127</td>
<td>0.084</td>
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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2d

<table>
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<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
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<td>0.300</td>
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<td>0.235</td>
<td>2.070</td>
<td>0.727</td>
<td>0.087</td>
<td>0.037</td>
<td>0.045</td>
<td>0.150</td>
<td>0.477</td>
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<td>1.192</td>
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<td>0.024</td>
<td>0.044</td>
<td>0.086</td>
<td>0.343</td>
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<td>0.038</td>
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<td>0.211</td>
<td>0.047</td>
<td>0.013</td>
<td>0.021</td>
<td>0.036</td>
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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 4.3.7.2a %ID/g for $^{125}$I Labelled RAFT2 scFv

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 4.3.7.2b %ID/g for $^{125}$I Labelled RAFT3 scFv

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 4.3.7.2c  %ID/g for $^{125}$I Labelled RAFT2-11 scFv

<table>
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<th>%ID/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td></td>
</tr>
<tr>
<td>3h</td>
<td></td>
</tr>
<tr>
<td>6h</td>
<td></td>
</tr>
<tr>
<td>18h</td>
<td></td>
</tr>
</tbody>
</table>

Organ

Tu, Ki, Li, Lu, Mu, Bn, Sp, Bl

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 4.3.7.2d  %ID/g for $^{125}$I Labelled Anti-CD18 scFv

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>%ID/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td></td>
</tr>
<tr>
<td>3h</td>
<td></td>
</tr>
<tr>
<td>6h</td>
<td></td>
</tr>
<tr>
<td>18h</td>
<td></td>
</tr>
</tbody>
</table>

Organ

Tu, Ki, Li, Lu, Mu, Bn, Sp, Bl

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
### Table 4.3.7.2e T:NT for $^{125}$I Labelled RAFT3 scFv

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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</thead>
<tbody>
<tr>
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<td>0.660</td>
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<td>1.372</td>
<td>2.126</td>
<td>3.145</td>
<td>1.913</td>
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</tr>
<tr>
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<td>3.820</td>
<td>2.290</td>
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<td>21.649</td>
<td>4.828</td>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2f T:NT for $^{125}$I Labelled RAFT2-11 scFv

<table>
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<th>Hours after injection</th>
<th>Ki</th>
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<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
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<td>46.694</td>
<td>7.152</td>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2g T:NT for $^{125}$I Labelled RAFT2 scFv

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<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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</thead>
<tbody>
<tr>
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<td>0.081</td>
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<tr>
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<td>1.905</td>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2h T:NT for $^{125}$I Labelled Anti-CD18 scFv

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<th>Bn</th>
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<td>0.050</td>
<td>0.180</td>
<td>0.803</td>
<td>2.873</td>
<td>1.817</td>
<td>1.066</td>
<td>0.615</td>
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</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 4.3.7.2e T:NT for $^{125}$I Labelled Anti-CD18 scFv

Time after injection
- 1h
- 3h
- 6h
- 18h

Organ

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 4.3.8.2f T:NT for $^{125}$I Labelled RAFT2-11 scFv

Time after injection
- 1h
- 3h
- 6h
- 18h

Organ

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 4.3.8.2g T:NT for $^{125}$I Labelled RAFT2 scFv

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 4.3.8.2h T:NT for $^{125}$I Labelled RAFT3 scFv

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
in the case of bone and muscle, rapidly dropping towards one from early highs of over 5. RAFT2 scFv shows little tumour localisation in comparison to normal tissues until the 18 hour timepoint when all values except liver and kidney exceed 1. This is evidence of poor tumour localisation. RAFT3 and RAFT2-11 scFvs show much more convincing tumour to normal tissue contrast with ratios rapidly increasing with time to values much in excess of 1. By 18 hours, RAFT3 scFv shows T:NT ratios over 2.9 for all organs except kidney and highest in bone at 21.649. RAFT2-11 scFv achieves values generally two-fold greater than those of RAFT3 scFv with T:NT ratios from 4.120 for liver to 55.236 for muscle. It should be pointed out that the values for bone may be subject to considerable experimental error since very small samples of bone were obtained and very low counts were measured, compounding any sampling error. In addition, the very high muscle value achieved is somewhat skewed by one mouse having a very low %ID/g of activity in muscle (almost zero) which almost certainly represents a sampling error. If that mouse is excluded, the tumour to bone ratio is slightly greater than 17 at this timepoint. Nevertheless, a tendency to clearly higher T:NT with increasing affinity for the antigen was evident.

The radiolocalisation indices represent the ratio of melanoma-specific scFv to control scFv localising to the organ in question. The RI values indicate tumour-specific localisation for the RAFT3 and RAFT2-11 scFvs in comparison to the anti-CD18 control (Tables 4.3.7.2i-k & Figs. 4.3.7.2i-k). RAFT3 scFv and RAFT2-11 scFvs show a trend to increasing RIs for tumour and decreasing RIs for normal tissues consistent with a tumour-specific localisation mechanism (retention in the tumour) and non-specific retention in the other tissues (clearing rapidly). RAFT2 scFv shows much less convincing tumour-specific localisation with the RI value for tumour only exceeding those for normal organs at 18 hours. At 18 hours the tumour RI for RAFT2 scFv was 4.211 in comparison to values of 9.737 and 31.216 for RAFT3 scFv and RAFT2-11 scFv, respectively. Relatively high normal tissue RIs are seen for all 3 melanoma-specific scFvs which can be accounted for by the rapid pharmacokinetics of the control scFv and therefore its rapid clearance from normal tissues. Furthermore, this normal
### Table 4.3.7.2i

<table>
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<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
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<td>1</td>
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<td>0.763</td>
<td>5.177</td>
<td>10.894</td>
<td>7.880</td>
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<td>1.464</td>
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<tr>
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<td>3.598</td>
<td>5.504</td>
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<td>3.443</td>
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</tr>
<tr>
<td>18</td>
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<td>3.099</td>
<td>0.927</td>
<td>2.440</td>
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</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2j

<table>
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<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
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<tr>
<td>18</td>
<td>31.216</td>
<td>3.796</td>
<td>1.366</td>
<td>1.273</td>
<td>4.958</td>
<td>1.246</td>
<td>4.716</td>
<td>1.813</td>
</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 4.3.7.2k

<table>
<thead>
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<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
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<tbody>
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<td>6</td>
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<td>5.863</td>
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<td>5.687</td>
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<td>3.004</td>
<td>1.226</td>
<td>3.931</td>
<td>2.486</td>
<td>3.562</td>
<td>1.358</td>
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</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Radiolocalisation index (RI) = \( \frac{\%\text{ID/g of specific scFv}}{\%\text{ID/g of control scFv (anti-CD18)}} \)

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood.
tissue accumulation in the case of the anti-melanoma scFvs tends to fall rapidly, suggesting it is indeed non-specific.

Some fluctuation in RI values are evident over time and probably represents the amplification of any inherent errors in the %ID/g values (since any errors in the specific and control scFvs’ %ID/g values are combined). However, the most important factor in radiolocalisation indices is the trend with increasing tumour RI and diminishing normal tissue RI over time, confirming tumour-specific localisation.
4.4 Discussion

The purpose of these experiments was to produce and assess, *in vitro* and *in vivo*, a series of anti-melanoma scFvs of varying affinity for the target High Molecular Weight Antigen (HMW-MAA). In particular, we wished to study a series of antibody fragments targeting the same epitope of the antigen in order to remove at least some of the confounding variables which have hampered other studies. As reviewed in the introduction to this chapter, several previous studies have considered the effect of affinity on targeting but there is still considerable debate as to the impact of affinity maturation (Fujimori *et al.*, 1989; Adams, 1998). These studies, to our knowledge, represent the first assessment of the impact of affinity in antibody fragment targeting of malignant melanoma.

4.4.1 Selection of ScFvs of Varying Affinity

Selection of scFvs of varying affinity using chain shuffling and affinity maturation proved quite effective. The poor affinity of the parent RAFT2 scFv left ample room for improvement. Whilst the affinity achieved by the RAFT2-11 scFv \((K_D = 9.91 \times 10^{-9} \text{M})\) was not as high as achieved in some maturation studies where picomolar dissociation constants have been reached (Schier *et al.*, 1996b), it remains higher than those previously reported in melanoma (Cai and Garen, 1995; Desai *et al.*, 1998) and is higher than that of the parental murine monoclonal antibody LHM2 \((K_D = 1.6 \times 10^{-8} \text{M})\) (Kang, 1998). The range of affinities obtained represented almost 3 log and presented a useful range for study *in vivo*. This improvement in affinity achieved by chain shuffling is slightly greater than that typically reported (Marks *et al.*, 1992; Schier *et al.*, 1996a; Park *et al.*, 2000). In the case of the RAFT2 scFv, the original chain-shuffled chimaeric library showed clear melanoma binding in about 5% \((\sim 10^4)\) of the clones (J. Kupsch, personal communication). This high proportion would imply that the RAFT2's \(V_H\) domain can pair with many human \(V_K\)s while maintaining melanoma binding. The \(V_H\), therefore, is probably the major functional unit in binding to the antigen. A subsequent attempt to replace the murine \(V_H\) in the chimaeric clones (and
thus fully humanise the scFv) failed to yield any positive clones (J. Kupsch, submitted) and strongly supports the suggestion of the dominance of the $V_H$. However, an almost 3-log improvement in affinity by chain shuffling is evidence of a rather large contribution from the $V_K$ to antigen binding.

4.4.2 ScFv Production

The higher yield of scFv achieved in the affinity-matured RAFT2-11 and RAFT3 scFvs in comparison to the RAFT2 scFv (450μg/l vs. 600μg/l vs. 100μg/l respectively) is related to improved production of the scFv by the bacteria (since it is evident in the supernatant prior to purification). This possibly indicates increased synthesis of the protein due to a more favourable amino acid sequence. Without studying the scFv content of inclusion bodies and periplasm for the various scFvs it would not be possible to exclude altered secretion as the source of the increased yield. Nevertheless, the yields obtained are comparable to those reported for secreted scFvs of 150μg to 16mg per litre of bacterial supernatant (Skerra et al., 1991; Kipriyanov et al., 1997a; Kipriyanov et al., 1997b; Klimka et al., 2000). Whilst our yields might be considered moderate, the amounts produced would be suitable for a clinical trial after scaling up production. Previous clinical studies of scFv have utilised patient doses of 500μg to 5mg scFv (Begent et al., 1996; Larson et al., 1997) which should be achievable using our scFvs in a fermenter-based system.

4.4.3 In Vitro Characteristics

4.4.3.1 Antigen Epitope Specificity

Confirmation of a shared epitopic target as indicated by the competition cell ELISA (Fig. 4.3.4) demonstrated that the process of affinity maturation has not significantly altered the epitope targeted by the RAFT3 and RAFT2-11 scFvs. This finding is reinforced by tissue normal tissue staining characteristics (in frozen section) which demonstrated identical staining of RAFT2 and RAFT3 scFvs, that is pre and post chain
shuffling (J. Kupsch, personal communication). This is important since previous work has suggested that epitopic specificity can be lost in chain-shuffled targeting molecules (Ohlin et al., 1996; Watzka et al., 1998).

4.4.3.2 Affinity

As noted above (Section 4.4.1), the 3 log variation in affinity achieved provides a good basis for assessing its effect on targeting. The relatively low affinity of the RAFT2-11 scFv (in comparison to picomolar dissociation constant scFvs) may remove the likelihood of seeing a "binding site barrier effect", since that hypothesis suggests that only very high affinity targeting molecules (e.g. $K_D < 10^{-9}$M) would be impeded (Weinstein et al., 1987; Fujimori et al., 1989) (See Sections 1.3.2.2.2 and 4.1). Other studies using chain shuffling have achieved smaller increases in affinity. Shuffling immune rearranged $V_H$ and $V_L$ genes of gp120 binding Fabs resulted in novel molecules of "similar apparent binding constants" (Collet et al., 1992; Barbas et al., 1993) whilst Klimke et al. produced a humanised scFv of inferior affinity to its murine parent (Klimka et al., 2000). The use of chain shuffling produced only a six-fold increase in affinity of an anti-c-erbB-2 scFv (Schier et al., 1996a). However, it should be noted that the parent scFv had a (relatively) high affinity with a $K_D$ of $1.6 \times 10^{-8}$M. This higher starting point would be expected to make significant increases more difficult to achieve. The authors of that study shuffled both heavy and light chains but preserved the CDR3 of the original $V_H$ since it is responsible for most antigenic contact. An attempt to similarly chain shuffle the heavy chain of the RAFT3 scFv was unsuccessful and no melanoma-binding clones were identified (J. Kupsch, personal communication), perhaps because no attempt was made to preserve the CDR3. Repeating the experiment with this modification might allow higher affinity clones to be selected.

The use of antibody phage display is a very convenient and rapid tool for identifying clones which continue to bind melanoma. Some modification of our methodology in panning on melanoma cells might have allowed us to select higher affinity clones.
During panning, washing and subsequent phage rescue, it is possible that clones poorly represented in the chain-swapped library might be lost. It has been suggested that higher amounts of antigen should be employed in the initial round of selection to preserve rarer clones. Reducing the amount of antigen used in subsequent selection rounds would then favour the selection of higher affinity over lower affinity clones (Schier et al., 1996a; Schier et al., 1996b; Sheets et al., 1998). Furthermore, modification by increasing the stringency of washing conditions and incubation times during panning might be employed to favour higher affinity clones (Adams and Schier, 1999).

Other techniques have allowed greater improvements in affinity. Numerous studies have employed site-directed mutagenesis of CDR3s in combination with antibody phage display (Yang et al., 1995; Schier et al., 1996b; Thompson et al., 1996; Adams et al., 1998a). Using the same anti-c-erbB-2 scFv as in the study detailed above, Schier et al. explored the use of site-directed mutagenesis aimed at altering specific residues in the V\textsubscript{H} and V\textsubscript{L} CDR3 of the antigen binding site (Schier et al., 1996b). The authors reported a nine-fold increase in affinity by a single mutation and a 1230-fold increase when mutations were combined. The maximum affinity achieved reached a K\textsubscript{D} of 1.1x10\textsuperscript{-10}M. Adams et al. reported a 320-fold increase in the antigen affinity of anti-HER2/neu scFv using this technique (K\textsubscript{D} 3.2x10\textsuperscript{-7} to 1.0\textsuperscript{-9}M) (Adams et al., 1998a). One potential disadvantage of this strategy is that modifications highly beneficial to affinity but lying outside the CDR would not be identified (such as those described in some chain-shuffling studies (Schier et al., 1996a)).

Irving et al. utilised a different technique, random mutagenesis, to modify an scFv targeting glycophorin A (Irving et al., 1996). Mutations were achieved by transfecting the phagemid containing the scFv DNA into a mutator E. coli strain mutD5-FIT, which is deficient in DNA repair. Strongly binding clones were isolated by antibody phage display techniques. After four rounds of mutation/selection a 1000-fold increase in affinity (to K\textsubscript{D} 6x10\textsuperscript{-8}mol/l) was achieved. Low et al. used a similar technique to obtain
Chapter 4

a 100-fold increase in affinity of an anti-2-phenyl-5-oxazolone scFv (Low et al., 1996). These increases are comparable to those found in our study.

Chain shuffling in our experiments produced a critical increase in scFv yield in the best-yielding clones. Other authors have reported reduced yields after shuffling (Schier et al., 1996a; Beiboer et al., 2000). It would seem that such manipulations are likely to affect yield on the basis of the shuffled chain’s amino acid sequence and therefore would be likely to be specific for individual molecules. Our clone screening, assessing the cell ELISA signal using unpurified supernatant, would select clones on the basis of yield and binding affinity and it is not, therefore, surprising that we selected higher-yield clones. Schier et al. sought higher affinity alone and merely comment that the higher affinity mutant had a lower yield (Schier et al., 1996a).

These studies demonstrate that a variety of techniques are available to increase scFv affinity for the antigen. The mutation / selection techniques most closely mimic the mechanisms present in B lymphocytes in vivo and may be the most efficient way to achieve increases in affinity whilst maintaining target specificity. Site-directed mutagenesis is very labour-intensive but is, as yet, the only affinity maturation technique to produce mutants with picomolar dissociation constants. However, it is worth noting that in Low’s study, the most important random mutation identified fell outside the CDR and was located in the framework itself (Low et al., 1996). This mutation would be likely to be missed in any site-directed mutation strategy. Chain shuffling generally seems to produce relatively low improvements in affinity which is in contrast to our experience. However, despite the large improvement obtained in our experiments, the low affinity of the parent molecule resulted in only moderate affinity for the antigen in the best clone produced. Chain shuffling may not be useful in isolation for producing very high affinity scFvs. Site-directed mutagenesis and chain shuffling have been used in combination to achieve an additive 12-fold increase in the affinity of an anti-CEA scFv (Osborn et al., 1996). Perhaps a combination of random mutation / selection and site-directed mutagenesis might be employed to produce the highest affinity constructs. This combination strategy would allow an increase in the
diversity of the starting material (including regions outside the CDRs) before site-directed mutagenesis and, therefore, the possible eventual range of mutations.

Balint and Larick have speculated on the maximal affinity that might be attained (Balint and Larrick, 1993). Reasoning that most of the binding energy of an antigen binding site is contributed by about 30% of the contact residues and that the remainder are inert or indeed “repulsive”, the authors suggest that up to three logs of potential affinity is lost. Since the tertiary immune response \textit{in vivo} produces affinities of up to \(10^{-16}\)M, affinities of up to \(10^{-13}\)M may be possible using site-directed mutagenesis. However, these improvements remain, as yet, theoretical. Moreover, the binding site barrier effect (if it exists) might render such very high affinity targeting molecules redundant.

\textbf{4.4.4 In Vivo Studies}

\textbf{4.4.4.1 Overview}

The purpose of these experiments was to determine the effect of increasing affinity on the biodistribution and pharmacokinetics of our anti-melanoma scFvs. Distribution in tumour and a variety of normal organs was assessed together with blood clearance. Crucially, unlike many studies, all the scFvs studied have been shown to target the same epitope of the antigen.

Any alteration in the behaviour of the molecule \textit{in vivo} should, therefore, be primarily a function of its affinity for the antigen.

\textbf{4.4.4.2 Pharmacokinetics}

Radio-immunopharmaceuticals typically display a biphasic pattern of clearance from the circulation. (Milenic \textit{et al.}, 1991). The primary phase represents the development of equilibrium in all bodily tissues. This first phase has a half-life designated \(t_{\beta\alpha}\) and
is usually rapid (Adams, 1998). The secondary phase is less rapid and reflects the gradual release of intact radiopharmaceuticals dissociating from the antigen and non-specifically from normal tissues. The half-life of the secondary phase is termed $t_{\beta \beta}$.

Our data (Fig. 4.3.7.1) confirm the rapid pharmacokinetics of scFvs in this study. Affinity maturation has little or no effect on the clearance times in this study ($t_{\alpha \alpha}$ 7-9 min, $t_{\beta \beta}$ 175-206 min). This is to be expected since clearance of these molecules is primarily a function of molecular size via renal sieving. The increase in tumour retention demonstrated with higher affinity clones is not likely to influence the overall pharmacokinetics since the increase in the proportion of the injected scFv in the tumour is small. These figures are similar to those previously reported for other scFv molecules in vivo of $t_{\alpha \alpha}$ 2-12 min, $t_{\beta \beta}$ 1.5-4 hours (Adams, 1998). Larger whole IgG molecules typically have much longer clearance half-lives of the order of $t_{\alpha \alpha}$ 100min and $t_{\beta \beta}$ 100hours (Colcher et al., 1990a).

4.4.4.3 Biodistribution

4.4.4.3.1 Localisation to Tumour

Increasing affinity is associated with markedly higher tumour retention of the scFvs (Tables & Figs. 4.3.7.2a-d). All 3 melanoma specific scFvs showed greater tumour retention than the control anti-CD18 scFv. RAFT2-11 scFv showed a 1.6 and 2.6-fold increase in tumour %ID/g at 1 hour compared to RAFT3 scFv and RAFT2 scFv respectively. The increase in peak retention is progressive with increased affinity and proportionally greater at later timepoints due to greater tumour retention of scFv in the highest affinity clone (3 and 7-fold increase in %ID/g at 18 hours compared to RAFT3 scFv and RAFT2 scFv, respectively). This marked increase in tumour retention could dramatically increase the intensity of any tumour hotspot on gamma-camera imaging and would similarly increase the tumour dose of any therapeutic construct based on these scFvs. The absolute tumour retention of the RAFT2-11 scFv at 18 hours of 1.186%ID/g is comparable to the values reported in other similar studies. For example,
Adams et al., generating a range of affinities by site-directed mutagenesis, reported a clear relationship between increasing affinity and tumour retention (Adams et al., 1998a). The highest value achieved was 1.42%ID/g at 24 hours for a scFv with a $K_D$ of $1.0 \times 10^{-9}$M. Interestingly, in the range of scFvs with different affinities investigated in that study, the $k_{on}$ values were relatively constant and the $k_{off}$s were the major contributor to affinity. $k_{off}$ rather than $k_{on}$ has indeed been previously suggested as an important factor in affinity maturation \textit{in vivo} (Foote and Eisen, 1995).

This tendency for increased affinity to be primarily associated with reduced $k_{offs}$ has been seen in several chain-shuffling studies (Marks et al., 1992; Schier et al., 1996a; Beiboer et al., 2000). $K_{off}$s for the RAFT series scFvs were not measured, since the lack of pure HMW-MAA made Biacore analysis impractical. However, it seems likely that $K_{offs}$ were reduced in the higher affinity clones (resulting in prolonged tumour retention).

\subsection{Localisation to Kidney}

Renal accumulation of the RAFT3 series scFvs was considerable at early timepoints and consistent with the renal accumulation typically seen with molecules of this size (Maack et al., 1979; Silbernagl, 1988). No significant difference was seen between the clones. A slight increase in the retention seen with the RAFT2-11 scFv at the 1 and 3 hour timepoints was not evident at 6 and 18 hours. These results are as expected since alteration of the protein sequence (save where dramatic changes in molecular charge are produced, see Chapter 5) is much less important in renal accumulation than the size of the molecule (Maack et al., 1979). This high level of persisting renal accumulation (over 2%ID/g at 18 hours) might hinder therapeutic approaches using these molecules but would not prevent imaging studies. The problem of renal accumulation is discussed in detail in Chapter 5.
4.4.4.3.3 ScFv Localisation to Other Organs

Distribution to normal organs was very similar for all 3 RAFT scFvs studied (Tables 4.3.7.2a-c, Figs 4.3.7.2a-c). This was to be expected since it had already been demonstrated that molecules of this lineage show very little interaction with normal tissues (Fig. 4.1). As previously mentioned, possible sampling errors in the bone specimens make the figures obtained potentially suspect. Despite this potential error, bone sampling was included in the study since previous work targeting melanoma metastases in patients using radiolabelled parent monoclonal antibody LHM2 had suggested a significant tendency to bony/marrow accumulation (S. Mather, personal communication). This tendency was not evident in the RAFT series scFvs studied in this model. Whether this accumulation would be found in the clinical setting with scFv remains to be seen. The reduced interaction with normal tissues in frozen section exhibited by RAFT series scFvs in comparison to LHM2 monoclonal antibody suggests that this accumulation might not necessarily occur.

4.4.4.3.4 Radio-localisation Index

Data for radio-localisation indices were obtained using anti-CD18 as a negative control.

Calculations of the Radio-localisation Index (RI) were obtained using the formula:

\[
\frac{\% \text{ID/g of specific scFv in organ concerned}}{\% \text{ID/g of control scFv in organ concerned (anti-CD18)}}
\]

Results are shown in Figures & Tables 4.3.7.2i-k. In order to demonstrate tumour-specific localisation, one must compare data to those obtained from an irrelevant control of similar chemistry and size. In this case, anti-CD18 scFv, derived from a humanised mouse monoclonal IgG (Kupsch et al., 1995), was used. Organ-specific localisation is indicated by a radio-localisation index in excess of 1. For RAFT3 and
RAFT2-11 scFvs, clear tumour-specific localisation is demonstrated with tumour RIs consistently greater than one. This confirms that tumour localisation is a consequence of antigen-scFv interaction rather than non-specific accumulation. The tendency to RIs >1 in normal organs at the early timepoints largely resolves at the later timepoints. A RI clearly greater than one is seen in most normal organs at the 1 and 3 hour timepoints. This is possibly a consequence of the longer circulating half-lives of the two melanoma-specific scFvs in comparison to the anti-CD18 which would result in somewhat greater retention in normal organs. Although RAFT2 scFv shows tumour-specific localisation this is much less pronounced (Table & Fig. 4.3.7.2k). This is consistent with the relatively poor tumour localisation (%ID/g in tumour) which was observed.

A possible alternative control would be the inclusion of a second non-melanoma tumour xenograft (e.g. colon) in the same mice. However, it was felt that this modification would considerably complicate the animal experiments and the current model was adopted.

**4.4.4.3.5 Tumour to Normal Tissue Contrast**

Tumour to normal tissue ratios are shown in Tables 4.3.7.2e-h and Figures 4.3.7.2e-h. A striking improvement in tumour to normal tissue (T:NT) contrast is seen with improving affinity. This improvement should result in both increased imaging contrast and therapeutic efficiency. Since the normal tissue accumulation remains relatively constant, the increase in T:NT is almost entirely a function of increased tumour retention.

As noted in Section 4.3.7.2, the tumour to bone ratio for the RAFT2-11 scFv at 18 hours (of 55.236) was skewed by a very low %ID/g of muscle result in one mouse. This error, however, demonstrates the effect of 2 possible methodologies in calculating T:NT. Tumour to normal tissue ratios can be calculated either by finding the mean of the T:NTs for each subject in the study (in this case the mean of five
individual mouse T:NTs) or by finding the mean tumour and normal tissue %ID/gs for the group as a whole then dividing the two to find the overall T:NT (i.e. means are calculated at the %ID/g stage). The latter method would be less likely to be skewed by a single erroneous result in %ID/g but does not truly represent the T:NT achieved in each subject mouse. A perusal of published papers using this model revealed no detailed description of the exact methodology employed. We reasoned that the former method produced a more accurate representation of the contrast achieved mouse by mouse and, therefore, used it in all our studies. This results in the situation where, although the mean %ID/g in tumour at this timepoint is 1.186 and in muscle 0.066, the accurate T:NT is 55.236 (taken as a mean of T:NTs) rather than a value of 17.9 which one might expect (i.e. 1.186/0.066). Excluding the clearly erroneous murine value gives a tumour to muscle ratio of almost 18 in this instance. Even with the potential vulnerability of this method to experimental error, we maintain it presents a more accurate reflection of the situation in vivo.

No reduction in targeting efficacy is seen with the highest affinity clone as proposed (in the context of monoclonal antibodies) by Fujimori et al. (Fujimori et al., 1989). This is consistent with other studies that investigated scFvs targeting non-melanoma antigens and found increasing affinity resulted in increased tumour localisation (Adams et al., 1998a; Colcher et al., 1998). However, this may reflect the improved but still relatively modest affinity of the RAFT2-11 scFv and a binding site barrier might only become apparent at higher affinities. Weinstein et al. originally suggested the binding site barrier effect based on a mathematical model of IgG tumour penetration (Weinstein et al., 1987; Fujimori et al., 1989). This was subsequently supported in a murine tumour model comparing tumour-penetration of a high affinity monoclonal to a negative control (Juweid et al., 1992) and a later study suggesting a similar effect in micrometastases (Saga et al., 1995). Each of these studies focussed on relatively high affinity targeting antibodies ($K_a >10^9$/mol). Indeed, two studies have apparently supported the “binding site barrier effect” hypothesis in scFvs (Adams et al., 1998a; Jackson et al., 1998). Using scFv affinity mutants to target HER2/neu and CEA respectively, these studies suggested that increasing affinity did indeed improve
tumour retention up to a ceiling of around $K_A = 10^9 \text{M}$, beyond which no further improvement was noted.

While the major factor in the increase in tumour localisation is likely to be affinity for the antigen, other possible factors merit consideration. The studies in this chapter have attempted to standardise as many variables as possible, including target epitope, molecular size, labelling technique and immunoreactivity. However, molecular charge plays a part in the pharmacokinetics of targeting molecules and cannot be easily standardised in experiments of this type. In fact, previous studies have demonstrated that cationisation of Fab' fragments can markedly increase renal excretion without affecting tumour localisation (Tarburton et al., 1990). Conversely, increasing the isoelectric point (pI) of a whole antibody from 8.9 to >9.5 has been shown to increase cellular uptake and reduce residence time in the systemic circulation (Pardridge et al., 1995). Interaction of the charged protein molecules with negatively charged surfaces of vascular endothelial cells and proximal renal tubular cells have been proposed as the explanation of these findings (Maack et al., 1979; Sumpio and Maack, 1982).

The isoelectric points of our scFvs have been estimated at 6.5, 8.6 and 8.3 for the RAFT2, RAFT3 and RAFT2-11 scFvs respectively using Protparam™ software from ExPASy (Expert Protein Analysis System, Swiss Institute of Bioinformatics. http://expasy.cbr.nrc.ca/tools/protparam.html). In our studies, little difference in renal clearance was apparent whilst tumour scFv concentration varied markedly, suggesting that charge is unlikely to be a major factor. Indeed, the isoelectric points of RAFT3 and RAFT2-11 scFvs are similar and a significant difference in charge interactions would seem unlikely. In previous studies, the primary effect of charge modification has been on renal clearance rather than cellular uptake (Tarburton et al., 1990; Pardridge et al., 1995) and is at odds with the findings in our scFvs of varying affinity. We conclude, therefore, that the differences in biodistribution are likely to be a result of varying affinity for the antigen. It is not possible, however, to exclude absolutely a charge effect. Further studies on the influence of scFv molecular charge are needed to clarify this issue. Additionally, the stability of the radiolabel in vivo for each scFv and the
inherent resistance of the constructs to proteolysis were not studied specifically and would play a part in the overall targeting efficiency.

Nevertheless, our results are most encouraging and demonstrate the improvement that can be achieved by affinity maturation. In comparison to other published studies, the T:NT ratios achieved by the RAFT2-11 scFv are highly promising. A tumour to blood ratio of 11 at 18 hours is superior to that obtained by Verhaar et al. using an anti-CEA scFv in mice whose tumour to blood ratio had risen to only 8 at 48 hours (Verhaar et al., 1996) and which was used subsequently to produce impressive patient images (Begent et al., 1996).
4.5 Conclusion

The results of the experiments described in this chapter clearly demonstrate the potential of affinity maturation techniques for the improvement of targeting by antibody fragments. The relatively simple method used has demonstrated a clear link between targeting efficiency and affinity. The findings are particularly interesting in that a single antibody lineage has been studied, targeting a single antigenic epitope in a single animal model. These factors remove many of the confounding variables which have made comparison of results in some other studies difficult.

The improved targeting efficiency achieved with only moderate improvement in binding affinity is highly encouraging and results in clearly superior tumour localisation and tumour to normal tissue contrast. Further improvements in affinity may require the use of random or site directed mutagenesis though the benefits of further enhancement in antigen binding remain controversial and merit further study.

Future work will be aimed at the development of very high affinity clones in the hope of improving targeting still further, together with a more detailed investigation of the effect of molecular charge on scFv pharmacokinetics and biodistribution.
Chapter 5

5. Effect of Coadministration of L-lysine on Biodistribution of ScFv
5. Effect of Coadministration of L-lysine on Biodistribution of ScFv

5.1 Introduction

Renal accumulation of small tumour-targeting molecules reduces the efficacy of both imaging and therapeutic studies. Significant renal accumulation is typical with antibody fragments, especially those small enough to be freely filtered in the kidney (below approximately 60kDa). Antibody fragments conjugated to intracellularly retained isotopes such as $^{99m}$Tc and $^{111}$In used in imaging studies and $^{90}$Y, $^{186/188}$Re, $^{67}$Cu and $^{177}$Lu used in therapeutic studies show an increased propensity to localise to the kidney (Kwekkeboom et al., 1993; Baum et al., 1994). Whilst renal localisation may represent only a minor nuisance in diagnostic imaging, in therapeutic studies nephrotoxicity becomes a possibility and may represent the dose-limiting factor. Indeed, whilst a great many imaging studies using antibody fragments have now been published, relatively few in vivo studies using therapeutic constructs have been seen in the literature. Moreover, nephrotoxicity has been a serious concern in many of those published so far (Behr et al., 1997; de Jong et al., 1997; Reubi, 1997; Behr et al., 1998). Strategies to reduce renal accumulation are therefore of critical importance if the superior tumour to normal tissue ratios achieved using antibody fragments rather than whole immunoglobulins are to be exploited in cancer therapy.

5.1.1 Mechanism of Renal Accumulation

As noted above, the renal accumulation of antibodies and small peptide molecules is largely a function of the kidney's ability to filter and reabsorb peptides falling below a threshold of around 60kDa in size (Rennke et al., 1978; Silbernagl, 1988). These molecules are filtered through the glomerular basement membrane and then actively reabsorbed in the proximal convoluted tubule by pinocytosis. Under physiological conditions, little or no protein or peptide material is lost in the urine. Interestingly, F(ab)$_2$ fragments show a greater tendency to renal accumulation of radiolabel than
whole IgG despite being considerably larger (at 100kDa) than the renal threshold (Silbernagl, 1988). The exact mechanism of this phenomenon is unclear but it has been suggested that the F(ab') fragments are degraded elsewhere and breakdown products are then filtered and accumulate in the kidney (Behr et al., 1995).

Glomerular filtration of smaller molecules such as scFvs (27kDa) would be expected to be more efficient than for larger molecules such as Fab fragments (~55kDa). The glomerular basement membrane in fact carries a negative charge capable of attracting cationic peptides and therefore facilitating their filtration into the urine (Maack et al., 1979; Silbernagl, 1988). Conversely, anionic peptides are repelled by the basement membrane, thus reducing the rate of filtration. Similarly, the cells of the proximal convoluted tubule carry a negative charge on their surface (Groniowski et al., 1969), producing preferential reabsorption of cationic peptides in comparison to anionic peptides. Whilst varying the charge in individual peptides can enhance or reduce renal reabsorption, there is considerable variation in the reabsorption of different peptides, even when they carry the same charge and are of a similar mass (Christensen et al., 1983; Bianchi et al., 1988). This finding suggests that the distribution of charged residues and protein conformation may be important.

Following pinocytosis, peptides within the proximal tubular cells are transferred to lysosomes and digested by proteolytic enzymes. The breakdown products (including any radiolabelled fractions) are then primarily returned to the bloodstream though some will be excreted in urine. Similarly, iodinated tyrosine is rapidly released into circulation whilst radiometal-chelated amino acids are trapped in the lysosomes and remain in the kidney (Duncan and Welch, 1993; Duncan et al., 1997). Where radioiodine has been carbohydrate-linked to the amino acid, however, the radiolabel cannot escape the lysosome (Stein et al., 1997).

5.1.2 Reducing Renal Accumulation

Early physiological studies of renal function suggested the presence of competition between proteins or proteins and other compounds for renal reabsorption. For example,
in 1962 Strauss reported reduced renal accumulation of intravenously injected horseradish peroxidase when egg white albumen was coadministered intraperitoneally (Strauss, 1962). A similar effect was noted between myoglobin and cadmium-metallothionein in a rabbit kidney model (Foulkes, 1978). However, some proteins appeared to have little competitive effect. A previous canine study had failed to demonstrate any inhibitory effect by Ovalbumin, Bence Jones Proteins or myoglobin on the reabsorption of lysosome (Harrison and Barnes, 1970). Subsequent studies shed some light on the situation by demonstrating the powerful inhibitory effect of basic (cationic) amino acids on renal protein reabsorption in vitro and in vivo (Madsen et al., 1977; Mogensen and Solling, 1977). Two studies then localised this effect to inhibition of protein binding to brush border membranes in the renal tubules (Just and Habermann, 1977; Beyer et al., 1983). The competitive inhibition of protein reabsorption can be achieved using high enough concentrations of most proteins. Cojocel et al. indicated that whilst a wide range of proteins would inhibit lysozyme reabsorption and renal accumulation (in a sufficiently high dose), the more basic proteins and amino acids had a substantially greater effect (Cojocel et al., 1981). This phenomenon is explained in terms the interaction of peptides in the proximal renal tubule with negatively charged “receptors” on the endothelial cells which form the first point of binding prior to pinocytosis of the peptide (Just and Habermann, 1977; Mogensen and Solling, 1977). Positively charged peptides and amino acids, it has been suggested, preferentially bind to these “receptors” and are therefore reabsorbed more readily than the study peptide.

Pimm and Gribben subsequently demonstrated that the same strategy could be applied to radiolabelled antibody fragments. In their study, repeated intraperitoneal injections of L-lysine significantly reduced the renal uptake of a \(^{111}\)In-labelled Fab’ fragment in a mouse model (Pimm and Gribben, 1994). A similar effect was observed in a clinical study of \(^{111}\)In-labelled octreotide (a peptide somatostain analogue) in patients receiving a mixed amino acid solution by continuous intravenous infusion (Hammond et al., 1993).

Behr et al. studied the effect of a variety of basic amino acids administered intravenously, intraperitoneally or orally on radiolabelled antibody fragment distribution
(Behr et al., 1995; Behr et al., 1996). These studies demonstrated a reduction in renal accumulation of Fab' fragments by almost one order of magnitude in comparison to untreated controls. Furthermore, oral and intraperitoneal routes of administration were found to be equally effective, as were the D- and L-isomers of lysine.

Finally, Kobayashi et al. have investigated the effect of L-lysine on renal uptake of both $^{125}$I- and $^{99m}$Tc-labelled anti-Tac disulphide-stabilised Fv fragment (Kobayashi et al., 1996). The authors described a 95% reduction in renal accumulation of the radiolabel when L-lysine was administered with or shortly before the radioimmunoconjugate.

These preceding studies suggest that renal accumulation of peptides can be significantly reduced by the coadministration of cationic amino acids. Such a reduction would be particularly useful in the case of scFvs whose small size makes renal reabsorption a particular problem. To date, no studies of this strategy in scFv radioimmunotargeting have been published.

### 5.1.3 Rationale and Experimental Design

Studies of the in vivo targeting properties of radioiodinated RAFT series scFvs have shown an improved tumour to normal tissue ratio in comparison to the parent whole mouse monoclonal antibody LHM2 (Kang, 1998; Kang et al., 2000). However, a significant renal accumulation resulted in tumour to kidney ratios no greater than 1:1 at any timepoint up to 18 hours after injection. This kidney accumulation would be likely to hinder any radioimmunotherapeutic construct based on these scFvs due to dose-limiting renal toxicity. Since cationic amino acids and in particular lysine (either D- or L-) have shown an ability to reduce renal peptide accumulation in a competitive fashion, it was decided to investigate whether a similar approach would indeed reduce renal reabsorption of scFv. The data would then be studied in order to assess the likely impact of this strategy on the usefulness of scFvs as imaging or therapeutic targeting agents.

The effect of coadministration of L-lysine intraperitoneally together with radioiodinated RAFT3 scFv intravenously was studied in Balb/c nu mice bearing human
melanoma xenografts. RAFT3 scFv had previously been shown to target human melanoma effectively in this model (Chapter 3). Analysis of tumour and organ radioactivity at appropriate timepoints would allow assessment of the effect of L-lysine treatment in comparison to untreated controls receiving only iodinated scFv.
5.2 Materials and Methods

5.2.1 Origin of RAFT3 scFv

The genealogy and production of RAFT3 scFv is described in detail in Chapter 3 Sections 3.2.1-3.

In short, the parent LHM2 mouse monoclonal antibody was a gift from Professor I. Leigh (Queen Mary and Westfield Medical College, London). Dr. Jorg Kupsch isolated LHM2 scFv from this monoclonal by PCR techniques. RAFT2 scFv was produced by sub-cloning LHM2 scFv into pUC119 His 6 myc Xba, a gift of Dr. G. Winter (Cambridge). RAFT3 scFv was produced as an affinity-matured mutant of RAFT2 scFv by means of chain swapping using a human V-kappa library and selecting the clones produced by antibody phage display techniques with the human melanoma cell line A375M. The enriched clones were then screened in cell ELISA to select those exhibiting strongest binding to melanoma cells. One of the best binding clones was designated RAFT3 scFv. The scFv was cloned and expressed in the vector pUC119 His 6 myc Xba which incorporates a c-myc detection tag and a polyhistidine purification tag and is produced as a secreted recombinant protein in E.coli TG1.

5.2.2 Production and Purification of ScFv

One litre cultures of the E.coli TG1 incorporating the RAFT3 scFv plasmid were made and induced to produce recombinant protein as described in Sections 2.4.2&3. After dialysis of the concentrated, filtered bacterial supernatant against PBS at 4°C, the scFv was purified using Immobilised Metal Ion Affinity Chromatography (IMAC) on Hi-trap Sepharose Columns™ and a stepwise gradient of imidazole (Section 2.4.5). Fractions collected were then assayed by SDS-PAGE and Western blotting under reducing conditions to establish the fractions containing scFv and confirm its purity (Section 2.2). Analysis of the gels confirmed pure scFv was obtained in the 50 to 200 mM imidazole samples. Impure fractions were retained for reloading with the next purification run.
5.2.3 Radiolabelling

Radiolabelling was carried out using the Iodogen™ method (Pierce) and \(^{125}\text{I}\) as the radiolabel (Section 2.6).

Immuno-reactivity after labelling was assessed by comparing equivalent amounts of scFv to cold antibody in cell ELISA (Section 2.5.3).

5.2.4 In Vivo Studies

Biodistribution data were obtained in Balb/c nu mice bearing human melanoma xenografts (Section 2.8) for RAFT3 scFv in the presence or absence of L-lysine. 0.5μg of iodine-125 labelled scFv in 100μl PBS was injected via the tail vein and the mice euthanased at appropriate timepoints. Three to five mice were used for each timepoint. Anti-CD18 scFv (directed against a human white blood cell antigen not expressed in mice) was used as a negative control for all experiments. The effect of cationic amino acid administration on renal accumulation was studied by injecting 50mg L-lysine in 200μl PBS buffer intraperitoneally 10 minutes prior to and 1, 3 and 5 hours following scFv injection.

5.2.4.1 Biodistribution

Mice were sacrificed at 1, 3, 6 and 18 hours after injection. A blood sample was obtained by cardiac puncture and organs removed for weighing and gamma counting. The tumour, left quadriceps, left femur, spleen, liver, kidneys and lungs were counted.

5.2.4.2 Analysis of Biodistribution Results

Results were expressed in terms of percentage of the injected dose per gram of tissue (%ID/g) and from this tumour to normal tissue ratios (T:NT) were calculated.
5.3 Results

5.3.1 Radiolabelling

The results of radio-iodination of RAFT3 scFv and control anti-CD18scFv are shown in Table 5.3.1 and Figure 5.3.1. An incorporation of 24.7% of the radiolabel reaching a specific activity of 0.027MBq/microgram was obtained. Immunoreactivity, as assessed by cell ELISA directed against A375M melanoma cell line, demonstrated maintenance of 88.2% of melanoma binding in comparison to unlabelled RAFT3 scFv.

5.3.2 In vivo studies

5.3.2.1 Biodistribution of RAFT3 scFv

Biodistribution data for RAFT3 scFv are shown in Table 5.3.2a and Figure 5.3.2a. Control anti-CD18 scFv data are shown in Table 5.3.2b and Figure 5.3.2b. RAFT3 scFv tumour accumulation is highest at the 1 hour timepoint (2.5%ID/g) and then falls to 0.370%ID/g at the 18 hour timepoint. Renal accumulation is marked and falls from over 16%ID/g at 1 hour to 2.089%ID/g at 18hours. Tumour localisation exceeds that in all other normal organs save blood at all timepoints. Blood scFv concentration is higher than that seen in tumour at 1 and 3 hours but lower at later timepoints.

In contrast, control anti-CD18 scFv (targeting an irrelevant human white cell antigen) shows no tumour-specific accumulation. Tumour accumulation does not exceed 0.334%ID/g in the timepoints studied.

Tumour to normal tissue contrast (T:NT, the ratio of tumour %ID/g to normal organ %ID/g) for RAFT3 scFv and anti-CD18 scFv is shown in Tables 5.3.2d & e and Figures 5.3.2d & e. Clear tumour to normal tissue contrast for RAFT3 scFv is evident for all normal organs studied with the exception of the kidney. T:NT increases across the timepoints studied. No clear tumour to normal tissue contrast is demonstrated for the
### Table 5.3.2a

<table>
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<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
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<th>Mu</th>
<th>Bn</th>
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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 5.3.2b

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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 5.3.2c

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Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 5.3.2a %ID/g for \(^{125}\text{I}\) Labelled RAFT3 scFv

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 5.3.2b %ID/g for \(^{125}\text{I}\) Labelled Anti-CD18 scFv

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 5.3.2c %ID/g for \(^{125}\text{I}\) Labelled RAFT3 scFv & Lysine

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
### Table 5.3.2d

T:NT for ¹²⁵I Labelled RAFT3 scFv

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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 5.3.2e

T:NT for ¹²⁵I Labelled Anti-CD18 scFv

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<td>1.817</td>
<td>1.066</td>
<td>0.615</td>
</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 5.3.2f

T:NT for ¹²⁵I Labelled RAFT3 scFv & Lysine

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.407</td>
<td>1.458</td>
<td>1.015</td>
<td>2.941</td>
<td>2.474</td>
<td>1.441</td>
<td>1.127</td>
</tr>
<tr>
<td>3</td>
<td>0.423</td>
<td>1.723</td>
<td>1.378</td>
<td>3.750</td>
<td>2.532</td>
<td>2.036</td>
<td>1.377</td>
</tr>
<tr>
<td>6</td>
<td>0.752</td>
<td>2.675</td>
<td>3.128</td>
<td>11.299</td>
<td>7.610</td>
<td>3.110</td>
<td>3.140</td>
</tr>
<tr>
<td>18</td>
<td>0.999</td>
<td>2.864</td>
<td>4.040</td>
<td>16.499</td>
<td>4.518</td>
<td>4.796</td>
<td>5.283</td>
</tr>
</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 5.3.2d  
**T:NT for $^{125}$I Labelled RAFT3 scFv**

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

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Fig. 5.3.2e  
**T:NT for $^{125}$I Labelled Anti-CD18 scFv**

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

---

Fig. 5.3.2f  
**T:NT for $^{125}$I Labelled RAFT3 scFv & Lysine**

Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
control scFv and indeed, T:NT ratios decrease over the timecourse of the experiment. Suggesting a non-specific nature of accumulation at early time points.

5.3.2.2 Effect of L-lysine on Tumour and Normal Organ Localisation

Biodistribution results for RAFT3 scFv co-administered with L-lysine are shown in terms of %ID/g (Table and Fig. 5.3.2c) and tumour to normal tissue ratios (Table and Fig. 5.3.2f).

%ID/g data in comparison to RAFT3 scFv alone (Table and Fig. 5.3.2a) shows comparable tumour and normal tissue accumulation. Renal accumulation, however, is markedly reduced in the presence of L-lysine. (7.207%ID/g vs. 16.210%ID/g at 1 hour and 0.357%ID/g vs. 2.089%ID/g at 18 hours). These data show a reduction in renal accumulation to 44.5, 43.2, 15.9 and 17.1% of the negative control values (RAFT3 scFv without L-lysine) at 1, 3, 6 and 18 hours respectively.

Tumour to normal tissue contrast results show comparable tumour to normal tissue ratios achieved by RAFT3 scFv alone or in the presence of co-administered L-lysine. The largest differences are seen in muscle and bone. At 18 hours, T:NT ratio for RAFT3 scFv without L-lysine is 10.244 and 21.649 for muscle and bone respectively. For RAFT3 scFv with L-lysine, the figures are 16.499 and 4.518.

Tumour to normal tissue ratios for renal accumulation show values significantly less than 1 for RAFT3 scFv alone at all timepoints, the highest being 0.201 at 18 hours. However, for RAFT3 scFv with L-lysine, the tumour to kidney ratio achieved approaches 1 by 18 hours (0.999). This is a consequence of the significantly reduced renal accumulation seen in the presence of the cationic amino acid.
5.4 Discussion

5.4.1 Effect of L-lysine on Localisation

It can be seen from the RAFT3 localisation data that tumour-specific antibody fragment accumulation occurs over the course of the experiment. A high degree of renal accumulation (over 16%ID/g at 1 hour) is observed and is typical of molecules of this size, which fall below the renal filtration threshold. A rapid fall in normal organ and blood accumulation (with the exception of the kidneys) produces relative tumour accumulation in excess of all normal organs studied by the 6-hour timepoint. The rapid serum clearance is consistent with short serum half-lives previously described for scFvs (Colcher et al., 1990a; Milenic et al., 1991; Adams et al., 1993). It is this rapid clearance from normal tissues which makes scFv-mediated targeting a possibility despite the relatively low tumour accumulation achieved (in comparison to studies using whole monoclonal antibodies). However, whilst the tumour to normal tissue ratios achieved rise over the 18 hour timecourse of the experiment, the failure of the tumour to kidney ratio to increase beyond 0.201 is likely to hinder the use of any radio-therapeutic constructs based on this scFv (due to dose-limiting nephrotoxicity).

The addition of L-lysine to the experiment produces a marked reduction in renal accumulation of the scFv (83% at 18 hours) without adversely affecting the tumour or normal tissue accumulation. Indeed, tumour accumulation is marginally higher at all 4 timepoints in the lysine group. Kobayashi et al. noted a similar increase when studying disulphide-stabilised Fv (Kobayashi et al., 1996). An apparent increase in bony accumulation in the lysine group may be related to sampling difficulties rather than a true difference since the activities involved (in terms of cps) and the masses of the tissue samples were both very low and could easily compound any minor error. Indeed values for bony %ID/g within each sample group differed wildly between individual mice and would suggest a possible sampling error (data not shown).

The observed reduction in renal accumulation is comparable to that achieved in other studies. Kobayashi described a reduction of 95% in comparison to the negative control.
(Kobayashi et al., 1996). Behr et al. described a 74% reduction using intraperitoneal lysine and 80% using oral lysine when studying anti-CEA Fab’ fragments (Behr et al., 1995).

5.4.2 Timing of Lysine Administration

The choice of L-lysine for this experiment was arrived at arbitrarily. Behr et al. have demonstrated that D- and L- isomers are equally effective in suppressing renal accumulation using Fab’ and F(ab’)2 in a murine model (Behr et al., 1995). The efficacy of D-lysine is interesting in that it supports the theory that simple blockade of negatively-charged receptors in the renal tubule is responsible for its inhibitory effect. This assertion rests on the fact that there are no known luminal carriers for D-lysine in mammalian tubule cells and its effect must therefore be extracellular (Silbernagl, 1988). The reduction in renal F(ab’)2 accumulation is interesting. As expected, Behr’s study revealed intact Fab’ fragments in the urine of the lysine-treated group (Behr et al., 1995). However, only breakdown products of F(ab’)2 were identified in the lysine-treated F(ab’)2 cohort. This suggests that the F(ab’)2 fragments are catabolised elsewhere and reabsorption of breakdown products is prevented by the cationic amino acid. This in turn explains the efficacy of lysine treatment in reducing renal accumulation in this group since intact F(ab’)2 would not be expected to filter through the glomerulus.

The administration schedule for L-lysine was also chosen arbitrarily. As noted above, oral and intraperitoneal routes have been shown to be equally effective (Behr et al., 1995; Behr et al., 1997). Previous studies have suggested a maximum tolerated dose of intraperitoneal L-lysine of 10mg per gram body weight in 4 divided hourly doses in a mouse (Behr et al., 1995). Single doses in excess of 2500μg/g body weight provoked lethargy and body cavity effusions. Behr’s data suggest that single doses prior to injection of the radiotargeting agent are as effective as equivalent divided doses. However, a clear dose response relationship between L-lysine and reduction in renal accumulation would imply greater benefit from a larger total dose given in tolerable aliquots. Behr’s study initiated lysine administration 30min prior to antibody injection.
Kobayashi et al. showed the efficacy of injections 5 or even 2 minutes prior to radiotargeting agent administration (Kobayashi et al., 1996). We therefore elected to administer L-lysine in 50mg doses (approx. 2000μg/g body weight) 5min before and 1, 3 and 5 hours after injection of the labelled scFv. As noted above, this achieved comparable results to those of other investigators using whole antibodies or their fragments.

### 5.4.3 Implications for RAFT3 ScFv in Therapy and Imaging

A reduction in renal accumulation has clear implications for the renal dose of any radioimmunotherapeutic targeting agent. Other authors have observed an increase in maximum tolerated dose (MTD) of radiometal-labelled antibody fragments when coadministered with lysine (Behr et al., 1997). In this murine study using ^{90}Y-labelled Fab and F(ab)_2, a fivefold reduction in renal dose was achieved, increasing the MTD by 25% to 250μCi at which myelotoxicity became dose-limiting. When combined with bone marrow transplantation, a 100% increase in MTD (to 400μCi) could be achieved without evidence of renal toxicity. Kobayashi et al. have recently reported a 62% reduction in renal accumulation of an anti-Tac(Fv)-based immunotoxin when coadministered with lysine in a murine model (Kobayashi et al., 2000).

The reduction in renal scFv accumulation demonstrated in our studies shows the efficacy of this technique in improving absolute tumour to kidney contrast in scFvs. However, the 0.999 tumour to kidney ratio achieved at 18 hours still represents considerable renal accumulation. For straightforward radio-immunoscintigraphic imaging, this is probably not particularly important since renal melanoma metastases are rare and the renal accumulation would be constant and predictable. For radioimmunotherapeutic purposes, on the other hand, such renal accumulation would be likely still to result in dose-limiting toxicity at subtherapeutic levels. Whilst our data show the efficacy of L-lysine coadministration in reducing renal scFv accumulation, RAFT3 scFv may not be suitable for radioimmunotherapeutic use in its present form.
Other anti-melanoma scFvs produced in our department have shown superior tumour to kidney localisation ratios without co-administered cationic amino acid and may be more suitable (J. Odili, personal communication). Alternatively, modification of the RAFT3 scFv itself might be employed to improve its targeting properties and in particular its level of renal accumulation (see below).

5.4.4 Clinical Experience with Amino Acid Co-administration

No clinical studies investigating the effect of radiolabelled scFv and cationic amino acid administration have appeared in the literature. However, a number of studies have been carried out using whole antibodies and other antibody fragments. Hammond et al. studied the effect of an intravenous nutritive amino acid solution on the renal uptake of an \(^{111}\)In-labelled somatostatin analogue (octreotide) in a clinical trial (Hammond et al., 1993). A clear reduction in renal uptake was indeed demonstrated. A subsequent study confirmed these findings in five patients with gastrointestinal malignancy undergoing radioimmunodetection using \(^{99m}\)Tc-anti-CEA Fab’ fragments (Behr et al., 1996). A reduction in renal accumulation of 37.3% was observed at 24 hours in the amino acid infused patients in comparison to the negative controls. The lower reduction than that seen in animal studies was attributed to the relatively low doses of amino acid administered. The authors concluded that higher doses of amino acid would be likely to result in greater benefit but that further studies of the safety of amino acid infusions were required.

5.4.5 Safety of Amino Acid Co-administration

The safety of high-dose amino acid administration has been a contentious issue. The toxicity of L-lysine in animal studies seems to be species-dependent. (Zager, 1987). Zager et al. found evidence of renal failure in rats exposed to high doses of L-lysine (Zager et al., 1983). However, earlier clinical studies had suggested that intravenous amino acid solutions might have a beneficial effect in patients suffering acute tubular necrosis (Abel et al., 1973). Certainly in individuals suffering a genetically high lysine
level (Familial Hyperlysinaemia) there appear to be no toxic effects (Dancis et al., 1983).

It is possible that D-lysine may represent the safer choice since toxicity may be restricted to the L-isomer (Silbemagl, 1988). This suggestion rests on the facts that D-lysine is metabolically inert and unlikely to upset metabolic processes involving amino acids and that no transmembrane receptors for D-lysine are known in humans. Indeed, even in murine models, the MTD of D-lysine is 1.4 times that of L-lysine (Adams, 1998).

Nevertheless, whilst high dose D-lysine infusions may well be safe in this context, definitive human or even primate studies have yet to be performed.

5.4.6 Future Prospects

As noted above, the overall renal accumulation of RAFT3 scFv may be too high for radioimmunotherapeutic purposes even in the presence of co-administered cationic amino acid. However, the technique will be applicable to other scFvs with more favourable biodistribution properties. Such molecules are now being studied in this laboratory. These scFvs will, we hope, soon be ready for clinical testing where lysine-mediated reduction in renal accumulation of scFv might be explored in a clinical trial for the first time.

Moreover, the clear effect of competing charged molecules on the biodistribution of RAFT3 scFv raises the interesting possibility that charge modification of the scFv itself might be employed favourably to influence renal accumulation. This strategy has been previously studied in the context of whole antibodies or larger antibody fragments. However, scFvs offer new possibilities in terms of site-directed protein modification in view of their recombinant nature. Over 20 years ago it was noted that renal filtration of horseradish peroxidase varied markedly between variants of different charge (Rennke et al., 1978). Despite almost identical molecular size (~40kDa), renal filtration of the anionic variant was reduced by factors of 8.7 and 48 in comparison to neutral and
cationic variants, respectively, in the rat model. It has been demonstrated that charge modification of albumin by means of acetylation of amine groups can have a profound effect on the pharmacokinetics of the molecule. Raising the iso-electric point (pI) of albumin from 4.9 to around 7.7 has been shown to increase renal excretion 300-fold (Purtell et al., 1979).

It was subsequently revealed that similar effects could be achieved in whole antibody molecules and Fab fragments by acetylation (Tarburton et al., 1990). Conversely, increasing the pI of a whole antibody from 8.9 to >9.5 has been shown to increase cellular uptake and reduce residence time in the systemic circulation (Pardridge et al., 1995). Interaction of the charged protein molecules with negatively charged surfaces of vascular endothelial cells and proximal renal tubular cells have been proposed as the explanation for these findings (Maack et al., 1979; Sumpio and Maack, 1982).

These studies suggested that charge modification of the anti-melanoma scFv RAFT3 might be employed to improve its tumour targeting properties by altering its interaction with charged surfaces on vascular endothelium and renal tubular cells. Charge modified RAFT3 scFv-derived molecules have now been produced and the effect on biodistribution and in particular reducing renal accumulation has thus far proved encouraging (J. Odili, personal communication). One study has recently been published suggesting that reducing the pI of a scFv did not reduce renal accumulation (Pavlinkova et al., 1999b). These contradictory early results emphasise the need for further study in this area.
5.5 Conclusion

The data presented in this chapter demonstrate the clear efficacy of co-administered L-lysine in reducing the renal accumulation of the anti-melanoma RAFT3 scFv. This reduction in renal accumulation is especially important for antibody fragments of this type where renal accumulation is a particular problem. Such a reduction may enable some scFvs which show promise as radioimaging agents to be used for radioimmunotherapeutic purposes. The effect of charge in the biodistribution of scFvs merits considerable further study.
Chapter 6

6. Summary & Future Directions
6.1 Summary

The continuing clinical burden of metastatic melanoma and the typically disappointing results of current therapies (Section 1.1) clearly indicate the need for new and improved imaging and treatment modalities. Sentinel lymph node biopsy (SNB) and positron emission tomography (PET) have shown great promise in melanoma. However, SNB gives only staging information about the regional node basin itself and gives no assurance that metastases have not developed more distantly. PET is not yet universally available and remains expensive. Moreover, false positive results may confound diagnosis. Many previous studies have demonstrated the potential of tumour-specific antibodies and antibody fragments for tumour targeting (Sections 1.2&1.3). Melanoma seems particularly suited to this approach and one intention of the work in this thesis was to improve upon the production and tumour targeting properties of an anti-melanoma scFv such that it would be suitable for a clinical imaging trial. Perhaps more exciting are the therapeutic possibilities of small tumour-specific targeting molecules which might follow from a successful imaging trial.

Optimising the induction and purification conditions for our scFvs together with enhanced yield from our domain-swapped scFv resulted in a 600% increase in eventual pure protein yield without loss of affinity or antigen epitopic specificity (Section 3.4). This increased yield greatly improves the potential usefulness of these scFvs in the context of a clinical trial. By inverting the orientation of the V domains from the usual $V_H$-$V_\kappa$ orientation to the less common $V_\kappa$-$V_H$ orientation, a significant improvement in yield was obtained and the in vivo melanoma targeting properties of the original scFv retained. It has been shown that seemingly minor modifications of recombinant antibodies can have dramatic effects on tumour targeting. In a recent study Goel et al. moved the his$_6$ IMAC purification tag of an anti-tumour scFv from its usual location at the C-terminus to the N-terminus and found that antigen binding was abolished (Goel et al., 2000). Other examples include the sometimes rather unpredictable effects of antibody engineering on stability or spontaneous multimerisation (Hudson and Kortt, 1999). It is conceivable that there is a significant bias against the publication of such observations. In the case of RAFT3 scFv and its domain-swapped counterpart, no
reduction in antigen binding was noted. Some authors have reported a tendency to spontaneous dimerisation in V\textsubscript{L}-V\textsubscript{H} orientated scFvs containing 15 amino acid linkers (such as RAFT3DS scFv) (Plückthun and Pack, 1997). The same group demonstrated that longer linkers removed this tendency and postulated that the conformation of the scFv in this orientation favoured dimerisation (Krebber et al., 1997). Our domain-swapped scFv, however, did not appear to exhibit this tendency, again emphasising the considerable variation in the properties of individual scFvs. However, this potential for spontaneous, stable dimerisation after domain swapping might be found in other scFvs of the RAFT lineage and could produce clinically useful molecules.

The limited solubility seen in several molecules of the RAFT3 lineage might prove problematic in subsequent studies and the discovery that imidazole maintains the scFvs in solution is important. We have demonstrated that such storage does not adversely affect labelling and tumour targeting properties and, therefore, enhances the usefulness of these molecules.

We have shown that affinity maturation of RAFT series scFvs by chain shuffling and antibody phage display techniques has produced scFvs with enhanced tumour antigen affinity and a corresponding significant improvement in tumour targeting in vivo (Chapter 4). These experiments are especially relevant in that affinity variants from a single scFv lineage, targeting a single antigenic epitope in the same model are studied, thus removing several potentially confounding variables. Whilst these data may be at odds with the concept of an antigen "binding site barrier effect" (Section 4.1), it is possible that higher affinity mutants may begin to exhibit such a phenomenon. Recently, Adams et al. have reported that such an effect is evident in scFv affinity mutants targeting HER2 at affinities beyond 10^{-9}M (Adams et al., 2001). The improvements obtained in tumour localisation using our scFvs are striking considering the relatively modest differences in affinity (between RAFT3 and RAFT2-11 scFvs) and further enhancement in affinity might provide greater gains. Nevertheless, the improvements identified thus far in the mouse model are impressive and have allowed the selection of the RAFT2-11 scFv for a patient imaging study to commence in the near future.
Since a particular problem in the use of single chain Fvs for therapeutic constructs is the degree of renal accumulation, the confirmation that coadministration of cationic amino acids would reduce kidney scFv accumulation is encouraging. This reduction in renal accumulation (>80% at 18 hours) mirrors the effect previously described in larger antibody-based molecules and would clearly reduce the kidney dose of any therapeutic study based on these scFvs.

We have demonstrated, therefore, that clinically useful amounts of specific anti-melanoma scFv can be produced and enhanced tumour targeting achieved. A strategy for the development of bivalent dimeric scFv targeting melanoma has been explored. Whilst this strategy proved unsuccessful in the course of this thesis (Appendix Chapter 7), it provides interesting data and the basis for reproduction of the same experimental model in the future with a high chance of success. We have further shown that reduction in renal antibody fragment localisation can be achieved using coadministered cationic amino acid, making the production of a therapeutic construct based on these scFvs a more realistic possibility.


6.2 Future Directions

Enhancement of the apparently limited solubility of the RAFT series scFvs has been achieved by the addition of imidazole to the buffer. However, further improvement may be achieved by modification of the antibody molecule itself and the addition of hydrophilic residues may provide a more satisfactory solution.

Increasing the yield of our scFvs further would be achieved by the use of large fermenter-based production systems. Recently, improved yields have been reported using heat-inducible vectors which may further enhance scFv expression (Power and Hudson, 2000). More tightly regulated promoters (e.g. tet, phoA, araBAD and lambda) have also been associated with improved yield (Power and Hudson, 2000). Other authors have described the successful employment of yeast cells for markedly increased protein production (Freyre et al., 2000). However, significant further enhancement of yield is probably not required for our purposes and the possibility of inducing antigenicity in the targeting antibody due to non-human glycosylation patterns formed in some yeasts might render this method less attractive.

As discussed previously (Section 4.4), other authors have described much greater increases in antigen affinity than we obtained by chain-shuffling and antibody phage display. Further attempts could be made utilising random mutagenesis in order to increase affinity further and to determine whether a true “binding site barrier effect” is present in this model. Whilst higher affinity may lead to even greater improvement in tumour targeting, we feel other factors such as protein stability and multimerisation may prove more important. Nielsen et al.’s demonstration of a significant increase in targeting efficiency using scFv dimers which appeared unrelated to the affinity of the original monomeric scFvs or the dimers themselves is highly interesting (Nielsen et al., 2000). It is, therefore, particularly disappointing that our attempts to engineer scFv dimers proved problematic (Appendix Chapter 7). However, efforts are now focussing on production of such molecules. Several authors have suggested that larger scFv multimers such as trimers may be optimal for tumour targeting (Hudson and Kortt, 1999; Todorovska et al., 2001) and these are currently being studied in our anti-
melanoma scFvs. In particular, bringing the molecular weight of the molecule above the renal threshold would reduce the tendency to renal accumulation. This strategy might be combined with an amino acid co-infusion to minimise renal toxicity. Charge modification aimed at increasing solubility (Section 3.4) might also influence renal accumulation (Section 5.4) and is being further investigated. Pavlinkova et al. have recently published results with an anti-CEA scFv suggesting such charge manipulation did indeed reduce kidney accumulation (Pavlinkova et al., 1999a). Similarly, Onda et al. have recently reported significantly reduced dose-limiting toxicity using charge-modified (acidified) scFv-based immunotoxins targeting a variety of tumours in a murine model (Onda et al., 2001). This effect merits further study in the context of our scFvs.

Antibody-fragment based therapy is still in its infancy. Several animal studies in other tumour models have been highly promising. For example, combining scFv with immuno-active agents such as interleukin-2 (Melani et al., 1998) or radiotherapeutic nuclides such as iodine-131 (Pavlinkova et al., 1999c; Goel et al., 2001) has been encouraging. The potential of gene therapy in cancer has received much attention recently yet the problem of targeted delivery has not yet been solved. Our scFvs have been used to target retroviral vectors to melanoma cells in vitro (Martin et al., 1998; Martin et al., 1999) and may prove useful in vivo. Bispecific multimeric scFv molecules capable of recruiting T-cell or complement responses have been developed in other tumour models (Kontermann et al., 1997; Kipriyanov et al., 1998; Arndt et al., 1999; Manzke et al., 1999). These molecules are simple to design and could be applied in the setting of malignant melanoma. Finally, one group has recently reported successful tumour eradication in a colon carcinoma/murine model by the combination of an antivasculare agent and an iodine-131 labelled antibody (Pedley et al., 2001). Such combinatorial treatments may represent the way forward in antibody-based tumour therapy. It is clear that many potential therapeutic approaches are available and the versatility of scFvs is a particular strength in this regard.

This project is now focussing both on our proposed clinical imaging trial and on further attempts to improve our ability to target melanoma. Specifically, as mentioned above,
multimeric and charge-modified targeting molecules will be produced. Efforts to demonstrate the efficacy of these fragments in the clinical setting must take priority. Furthermore, whilst a reduction in renal accumulation is likely to be essential for the development of therapeutic scFvs in melanoma, therapeutic constructs are undergoing investigation in the meantime.

In conclusion, the work of this thesis has enhanced our ability to target malignant melanoma using scFvs in this animal model and brought a clinical trial significantly closer. Further improvements in targeting and reduced renal accumulation will, we hope, unlock the potential of these exciting molecules for the therapy of metastatic melanoma.
Chapter 7

7. Appendix: Dimeric scFv production
7. ScFv Dimer Production

7.1 Introduction

Foreword

This appendix describes our rationale and experiments aimed at producing and characterising a modified, dimeric scFv based on the RAFT series scFvs both \textit{in vitro} and \textit{in vivo}. The experiments were undertaken contemporaneously with those described in previous chapters. After the completion of the timecourse of this project and the preparation of this thesis it was discovered that an external error in DNA sequencing had erroneously suggested that the desired modified DNA sequence had been produced. The clone studied contained a linker peptide and was not of the intended linker-free sequence. The results of the experiments cannot, therefore, be interpreted as originally intended. However, useful data was obtained pertaining to monomeric and dimeric scFvs and is discussed in this context. In accordance with the advice of the senior supervisor of this project, the chapter is included for completeness and the data interpreted in light of the discovered error. A similar strategy aimed at producing multimeric anti-melanoma scFvs is again being investigated in this laboratory.

Introduction

Targeting solid tumours by means of antibodies has been a laboratory tool for almost thirty years. Unfortunately, much of the optimism generated for this technique following Köhler and Milstein’s development of hybridomas and monoclonal antibodies (Köhler and Milstein, 1975) has been dampened by a failure to achieve the desired degree of tumour-localisation either in animal models or clinical trials. The advent of antibody fragments has, at least in some cases, improved the tumour to normal tissue contrast (Kang \textit{et al}., 1999) and tumour penetration achieved (Yokota \textit{et al}., 1992). This is not without cost, however, since the absolute amount of antibody fragment localising
Appendix Chapter 7

to the tumour is usually significantly reduced. This is probably due to a combination of factors which contrast with intact monoclonal antibodies:

1) The shorter circulatory half-life of these smaller molecules reduces the time available for the targeting molecules to find and bind to the antigen,

2) With the exception of F(ab)\textsubscript{2} fragments, these molecules contain only one antigen-binding site. Whilst the unchanged antigen binding site has the same affinity for the antigen as those of the whole antibody, it exhibits a lower avidity for the target than the bivalent parent molecule.

Strategies to prolong the circulatory half-lives of small antibody fragments are in the early stages of development. However, reconstituting the bivalent nature of whole gammaglobulin has recently received much attention (Plückthun and Pack, 1997; Adams, 1998; Colcher et al., 1998). In essence, one would seek to increase the avidity of the scFv for the antigen to levels seen in a whole antibody, while maintaining the advantages conferred by the small size of the molecule and the absence of a constant region (Fc). This can be achieved by combining two single chain molecules in order to produce a dimeric entity with two antigen binding sites.

Two main strategies have been employed to produce such bivalent antibody fragments. Covalent dimers have been formed by chemical cross linking either hinge cysteine residues (Shalaby et al., 1992) or by including a small C-terminal peptide (typically incorporating a thiol-bearing amino acid) which promotes dimerisation (Kostelny et al., 1992; Pack and Plückthun, 1992). Such disulphide-linked scFv dimers have been studied in several non-melanoma tumours and have shown improved tumour targeting properties compared to monomeric scFv (Adams et al., 1993; McCartney et al., 1995). Alternatively, non-covalent dimers have been formed by shortening or removing the peptide linker between the two V-domains of the single chain molecule (Holliger et al., 1993) (Fig. 7.1). This strategy was developed from the observation that scFvs are sometimes secreted as a mixture of monomers and dimers (Griffiths et al., 1993). These molecules have also demonstrated enhanced tumour localisation in vivo in comparison to the parent monomer in a colonic carcinoma model (Wu et al., 1996).
Fig. 7.1

ScFv Dimer Formation

Schematic representation of proposed non-covalent scFv dimer formation
Two other strategies have been similarly employed to produce dimeric scFvs. De Kruif and Logtenberg employed a truncated murine IgG3 hinge region together with Fos or Jun leucine zippers to produce spontaneous dimerisation of scFv of around 64kDa in size (de Kruif and Logtenberg, 1996). Similarly, Hu Shi-Zhen et al. produced dimeric "minibodies" of 80kDa by fusing the gene for a human IgG1 C\textsubscript{H}3 domain to that of an scFv (Hu et al., 1996). Dimerisation occurs due to the very high affinity interaction between two C\textsubscript{H}3 domains. The latter two strategies have as yet not been extensively tested in vivo.

We chose to utilise the non-covalent approach in order to produce anti-melanoma scFv dimers with the intention of improving the tumour retention and hence tumour to normal tissue contrast achieved with our scFvs. This technique involves no modification or processing of the secreted scFv dimer which should be produced in its active state. This method seemed most likely to yield the greatest amount of functional scFv whilst minimising the potential to denature or lose scFv during subsequent processing. In comparison to leucine zipper-mediated dimers and minibodies, these non-covalent dimers remain as close as possible to the small size of the original scFv and would, therefore, hopefully maintain the favourable normal tissue clearance and rapid tumour penetration associated with scFvs.

**Rationale**

By reducing the size of the peptide linker in a scFv molecule below the typical 15 amino acid length, normal folding of the molecule and alignment of the V-domains is prevented (Fig. 7.1). This potentially allows the spontaneous formation of dimeric molecules, reconstituting Fvs at either end of the dimer and conferring the potential for bivalent antigen binding (Holliger and Winter, 1993). We therefore attempted to modify the RAFT3 scFv by removing its 15 amino acid linker entirely using polymerase chain reaction techniques and transforming the modified DNA into *E. coli* for production of the recombinant protein. Purified dimeric scFv was then characterised in vitro and its tumour-targeting properties investigated in vivo in an animal model.
7.2 Methods

7.2.1 ScFv Development

The parent LHM2 mouse monoclonal antibody was a gift from Professor I. Leigh (Queen Mary and Westfield Medical College, London). Dr. Jorg Kupsch isolated LHM2 scFv from this monoclonal antibody by PCR techniques (Kupsch et al., 1995). RAFT3 scFv was derived from LHM2 scFv by chain shuffling (Kang et al., 1999; Hamilton et al., in preparation) and subcloning into the plasmid pUC119 His 6 myc Xba, a gift of Dr. G. Winter (Cambridge). This process is described in detail in Section 3.2.3.

7.2.2 Dimeric ScFv Engineering

Removal of the peptide linker of the RAFT3 scFv was attempted by PCR techniques. Briefly, the V\textsubscript{K} and V\textsubscript{H} domains of the scFv were isolated using custom primers (Fig. 7.2.2) (Section 2.1.3) which excluded the (Gly\textsubscript{4}Ser\textsubscript{3}) linker and the two domains religated into fresh vector pUC119 His 6 myc Xba. The correct sequence was verified by DNA sequencing and the new plasmid was retransformed into E. coli TG1 for protein production. The steps are described in more detail below.

Isolation of Variable Domains

RAFT3 scFv plasmid DNA was prepared as a mini-prep of an overnight culture of E. coli bacteria containing the plasmid (Section 2.1.1). The DNA segments corresponding to the Fv V\textsubscript{H} and V\textsubscript{K} domains were amplified by PCR (Section 2.1.3) and gel purified (Section 2.1.2). TAE gel analysis was used to confirm the correct fragment size (Section 2.1.8). Splicing by overlap extension (SOE) was used to join the 2 segments as follows: One \( \mu \text{g} \) fragment of each segment were mixed and reamplified using the outer PCR primers at 0.2\( \mu \text{M} \) final concentration. The correct size fragment (~800bp) was gel purified (Section 2.1.2) and digested with HindIII and NotI (Section 2.1.4).
The $V_h$ was amplified with R165 and R494; $V_k$ with R492 and R493 using RAFT3DS as DNA template. The fragments were purified, $NheI$ digested and ligated. The ligation was used as template for a PCR with R165 and R492. The PCR product was cloned $SfiI/NotI$ into pUC119 his 6 myc Xba. The inner primers R493 and R494 encode a unique $NheI$ site absent in RAFT3DS. In addition, the outer primers R165 and R492 cannot prime contaminating RAFT3DS template in the ligation because the strategy swaps the clone back to the $V_hV_k$ orientation. The $NheI$ site is absent in all other RAFT scFvs and allowed quick screening against such contaminants.
Vector (pUC119 His 6 myc Xba) was then digested using restriction enzymes HindIII and NotI (Section 2.1.4) and the PCR fragment corresponding to the joined Fv V\textsubscript{H} and V\textsubscript{K} domains ligated with the vector (Section 2.1.5).

### 7.2.3 Bacterial Transformation and Screening of Clones

The ligated plasmid containing the desired scFv sequence was transformed into \textit{E. coli} TG1 by means of electroporation (Section 2.3.2). The bacteria were plated on Luria Agar plates containing 2% glucose and 100\mu g/ml ampicillin to allow selection of colonies resistant to ampicillin and, therefore, incorporating the plasmid. Various dilutions of bacteria were plated on separate plates to ensure that separate single colonies could be clearly identified.

The following morning, transformed colonies were identified on the plates and a random sample of 20 was picked for inoculation of overnight cultures. 2ml overnight cultures were made and scFv production was induced with IPTG (Section 2.4). The supernatants of the induced clones were then screened in cell ELISA on A375M melanoma cells (Section 2.5.3) to confirm melanoma binding. The best binding clone in this assay was selected for subsequent analysis. This clone was designated "RAFT3DB".

### 7.2.4 Production and Purification of RAFT3DB

One litre cultures of the \textit{E.coli} TG1 encoding RAFT3DB were made and induced to produce recombinant protein as described in Section 2.4.2. After dialysis of the concentrated, filtered bacterial supernatant against PBS at 4\degree C, the scFv was purified using Immobilised Metal Ion Affinity Chromatography (IMAC) on Hi-trap Sepharose Columns\textsuperscript{TM} and a stepwise gradient of imidazole (Section 2.4.5). Fractions collected were then assayed by SDS-PAGE and Western Blotting under reducing conditions to establish the fractions containing scFv and confirm its purity (Section 2.2). Analysis of the gels confirmed pure scFv was obtained in the 50 to 200 mM imidazole fractions. Impure fractions were retained for reloading with the next purification run.
7.2.5 Confirmation of Epitopic Specificity

In order to demonstrate that the RAFT3DB binds to the same epitope of the High Molecular Weight Melanoma Associated Antigen as the parent RAFT3, a competition cell ELISA was set up. The wells were initially loaded with 500ng of scFv per well and serial dilutions of LHM2 IgG (the parent mouse monoclonal antibody) were carried out across the plate from 500 to 1ng per well. The ELISA was stained and developed as described in Section 2.5.3 and the plate read at 490nm. The anti-mouse IgG•HRP Conjugate (Dako) used as the secondary antibody reacts with the whole IgG parent LHM2 but not the scFvs.

7.2.6 Characterisation of Multimeric Status

The multimeric status of the new clone was assessed by means of gel-filtration chromatography as described in Section 2.4.8. 100μg of RAFT3DB protein were loaded onto the gel filtration column and eluted with phosphate buffered saline at 0.5ml per minute. Aliquots of the eluate corresponding to monomeric, dimeric and trimeric scFv were collected and assessed by Western Blot Analysis (Section 2.2.4) using the anti-c-myc monoclonal antibody 9E10 as the primary antibody. This allowed the identification of those fractions containing scFv and therefore the elution volume of the scFv. This could then be compared to the calibration chart (Section 2.4.8) to allow the mass of the molecule to be determined.

7.2.7 Radiolabelling

Radiolabelling was carried out using the Iodogen™ method (Pierce) and $^{125}$I as the radiolabel (Section 2.6).

Immuno-reactivity after labelling was assessed by comparing iodinated scFv to equivalent amounts of cold antibody in cell ELISA (Section 2.5.3).
7.2.8 Affinity Studies

The affinity constants of the RAFT3DB were assessed in order to study the potential increase in avidity for the antigen in vitro due to the intended bivalent nature of the new protein. Saturation binding and Scatchard plots were obtained after incubating the radiolabelled antibody with A375M melanoma cells as described in Section 2.7.3. Affinity and dissociation constants were calculated using Graphpad Prism™ analysis software.

7.2.9 In Vivo Studies

Pharmacokinetic and biodistribution data were obtained in Balb/c nu mice bearing human melanoma xenografts (Section 2.8). 0.5µg of iodine-125 labelled RAFT3DB in 100µl PBS was injected via the tail vein and the mice euthanased at appropriate timepoints. Three to five mice were used for each timepoint. Anti-CD 18 scFv was used as a negative control for all experiments and RAFT3 scFv data as a monomeric control. It must be noted that anti-CD18 scFv is not the perfect negative control for the proposed dimeric RAFT3DB. Ideally, an irrelevant dimeric molecule would be used. However, none was available for use in these studies.

7.2.9.1 Pharmacokinetics

Mice were sacrificed at 1 min, 5 mins, 15 mins, 30 mins, 60 mins, 180 mins, 6 hours and 18 hours after injection. Blood samples were obtained by cardiac puncture, weighed and the radioactivity measured in a gamma-counter. Results were expressed as percentage of the injected dose per gram and the 1 min timepoint used as the 100% value. Equilibrium and clearance half-lives were calculated from the curve using Microcal Origin™ software.
7.2.9.2 Biodistribution

Mice were sacrificed at 1, 3, 6 and 18 hours after injection. A blood sample was obtained by cardiac puncture and organs removed for weighing and gamma-counting. The tumour, left quadriceps, left femur, spleen, liver, kidneys and lungs were counted.

7.2.9.3 Analysis of Biodistribution Results

Results were expressed in terms of percentage of the injected dose per gram of tissue (%ID/g) and from this tumour to normal tissue ratios were calculated (T:NT). Comparison to the negative control anti-CD18 scFv allowed calculation of the radio-localisation index (RI) which represents the tumour-specific localisation of the antibody or fragment under study.
Appendix Chapter 7

7.3 Results

7.3.1 PCR Production of Modified Clones

PCR fragments encoding the $V_h$ and $V_k$ segments of the correct size were produced as confirmed by TAE gel electrophoresis. These fragments of 405bp and 415bp, respectively, were joined by SOE (see Section 7.2.1).

7.3.2 Screening of Bacterial Clones

Transformation of electrocompetent *E. coli* TG1 after ligation of the fragment with the parent vector (pUC119 His 6 *c-myc* Xba) yielded $3 \times 10^5$ ampicillin-resistant colonies. Screening of small-scale inductions of 20 clones for melanoma binding in ELISA on dried melanoma cells confirmed clear melanoma-binding in 2 clones (Fig. 7.3.2). The binding exhibited by the best-performing clone was slightly superior to that exhibited by the parent RAFT3 scFv. This clone was chosen for subsequent analysis and designated RAFT3DB. At this stage sequencing analysis carried out by another department suggested this clone contained the desired linker-free sequence. However, it was subsequently discovered that the sequence in the linker region was identical to that of the parent scFv (which has been shown to be monomeric on gel filtration analysis, J. Odili, personal communication). Point mutations towards the C-terminus have also been identified.

7.3.3 Production and Purification of RAFT3DB

IMAC purification of scFv from bacterial supernatants revealed pure scFv in the fractions eluted using between 50 and 200mM imidazole in the elution buffer as shown by Coomassie Brilliant Blue staining of SDS-PAGE gels made from the eluted fractions. The identity of the protein band was confirmed by Western Blotting of an identical gel using anti-*c-myc* as the primary antibody.
Cell ELISA on A375-M melanoma cell line. 25μl of bacterial supernatant from a 2 ml induction was loaded. A similar sample of parental RAFT3 supernatant was used as a positive control and anti-CD18 scFv as a negative control. Results are representative of 3 cell ELISAs. * indicates clearly positive clones.
7.3.4 Gel Filtration Chromatography

Results of the Western blot of RAFT3DB after gel filtration suggested that a mixture of monomer and dimer was present. The elution volumes of the bands identified were 93 to 105 ml and 78 to 88 ml (Fig. 7.3.4a). These correspond to molecular weights around 25 and 50kDa consistent with monomeric, and dimeric scFv as demonstrated on the calibration chart (Fig. 7.3.4b). A similar study using RAFT3 scFv demonstrated only monomeric scFv (J. Odili, personal communication).

7.3.5 Confirmation of Epitopic Specificity

The epitopic specificity was confirmed by competitive inhibition of binding of LHM2 IgG, the parental mouse monoclonal of the RAFT scFv series (the results of the competition cell ELISA are shown in Figure 7.3.5). As expected, the RAFT3DB continued to inhibit binding of LHM2 to melanoma cells in vitro, confirming that the original epitopic specificity of the RAFT3 scFv had been retained.

7.3.6 Radiolabelling

The results of iodinations are summarised in Table 7.3.6. These figures demonstrate a comparable labelling efficiency in megabequerels/microgram of protein for the new RAFT3DB and the parental RAFT3 scFv. The differing percentage incorporations can be explained by differing initial concentrations of protein during labelling. Higher concentration of scFv results in a greater percentage uptake of the radiolabel as would be expected.

The effect of labelling on the immuno-reactivity was assessed in cell ELISA by comparing the melanoma-binding exhibited by equivalent amounts of labelled and unlabelled scFv. The results are shown in Figure 7.3.6 and indicate that over 90% of the immuno-reactivity was retained after radiolabelling (Table 7.3.6).
**Fig 7.3.4a**

**Gel Filtration of Modified Clone**

<table>
<thead>
<tr>
<th>Elution volume (ml)</th>
<th>130-140</th>
<th>95-105</th>
<th>78-88</th>
<th>62-72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corresponding molecule</td>
<td>Blank Control</td>
<td>Monomer</td>
<td>Dimer</td>
<td>Trimer</td>
</tr>
</tbody>
</table>

S300 gel filtration sample Western blot. 20μl samples of each 1ml concentrate were loaded.
Sample chart recorder tracing of U.V. absorbance (at 280 nm) for HiPrep™ S-300 (Pharmacia Biotech) gel filtration column. To calibrate the column, ribonuclease (250 μg), chymotrypsinogen (250 μg), ovalbumin (250 μg) and albumin (250 μg) in 1 ml PBS were loaded at 0.3 ml/min. Figures for \( V_s \) represent the average of 2 experiments. \( V_i \) for the pre-packed S-300 column = 120 ml. The void volume \( (V_v) \) = 54 ml.
Fig 7.3.5

Competitive Inhibition of LHM2 Binding - Evidence of Shared Epitopes

LHM2 at increasing dilution was incubated in the presence or absence of 500ng scFv/well. LHM2 was detected using anti-mouse IgG-HRP conjugate. Results are representative of 3 cell ELISAs.
Immunoreactivity of unlabelled and labelled scFvs assessed in cell ELISA against A375-M melanoma cells. 0.5ug of each scFv was loaded. Anti-CD18 scFv is shown as a negative control.

### Table 7.3.6 Summary of Radio-iodination Results

<table>
<thead>
<tr>
<th>scFv</th>
<th>Incorporation (%)</th>
<th>Average Specific Activity (MBq/microg)</th>
<th>Immunoreactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT3</td>
<td>26.9</td>
<td>0.038</td>
<td>92.1</td>
</tr>
<tr>
<td>RAFT3DB</td>
<td>8.9</td>
<td>0.036</td>
<td>95.3</td>
</tr>
<tr>
<td>anti-CD18</td>
<td>4.1</td>
<td>0.008</td>
<td>N/A</td>
</tr>
</tbody>
</table>
7.3.7 Affinity Analysis

Saturation binding and Scatchard plots were prepared for RAFT3DB and RAFT3 scFv and the results used for analysis of binding affinity. The plots for the RAFT3DB are shown in Figures 7.3.7a & 7.3.7b and the results for both scFvs summarised in Table 7.3.7. These figures reveal comparable affinities for the scFvs in question. Production of a “dimeric” molecule resulted in a two-fold increase in the apparent affinity of the scFv for the antigen ($K_d = 1.78\times10^{-8}$ vs. $3.13 \times 10^{-8} M^{-1}$). However, since subsequent analysis suggested a less than pure dimeric sample, these data must be reinterpreted. Assuming a molecular weight of 25 rather than 50kDa (monomer rather than dimer) halves the affinity calculated and brings the two molecules to almost identical $K_d$s. This perhaps suggests that binding is indeed monomeric and no avidity effect is evident. Alternatively, a modest increase in affinity could result represent an avidity effect in the context of a mixture of monomer and dimer.

7.3.8 In Vivo Studies

7.3.8.1 Pharmacokinetics

Blood clearance curves for RAFT3 scFv and RAFT3DB together with the control anti-CD18 scFv are shown in Figure 7.3.8.1. The $t_{1/2\alpha}$ and $t_{1/2\beta}$, representing equilibration and clearance half-lives respectively, were calculated from the curve using Microcal Origin-software and are shown in Table 7.3.8.1. Both RAFT3 and RAFT3DB exhibited rapid clearance. This was slightly longer in the case of the modified variant with $t_{1/2\beta}$ of 253 minutes for the dimer / monomer mixture and 201 minutes for the monomer. These values are in agreement with previously reported values for RAFT3 monomer (Kang, 1998; Kang et al., 2000). Th

7.3.8.2 Biodistribution

Biodistribution results for normal organs and tumour are shown as %ID/g in Tables 7.3.8.2a-c and expressed graphically in Figures 7.3.8.2a-c. No tumour-specific
Fig. 7.3.7a  RAFT3DB Saturation Plot

![Graph showing RAFT3DB saturation plot with Uninhibited and Inhibited data points.]

Fig. 7.3.7b  RAFT3DB Scatchard Plot

![Graph showing RAFT3DB Scatchard plot with a line representing the Scatchard equation.]

Table 7.3.7  Scatchard Summary

<table>
<thead>
<tr>
<th>scFv</th>
<th>$K_A$ (l/mol)</th>
<th>$K_D$ (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAFT3</td>
<td>$3.19 \times 10^7 (+/-0.74)$</td>
<td>$3.13 \times 10^{-8}$</td>
</tr>
<tr>
<td>RAFT3DB</td>
<td>$5.59 \times 10^7 (+/-0.93)$</td>
<td>$1.78 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

(Error = SEM)
Fig. 7.3.8.1  Plasma Clearance Curves

Table 7.3.8.1

<table>
<thead>
<tr>
<th>scFv</th>
<th>$t_{1/2}\alpha$ (min)</th>
<th>$t_{1/2}\beta$ (min)</th>
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<tbody>
<tr>
<td>RAFT3</td>
<td>8</td>
<td>201</td>
</tr>
<tr>
<td>RAFT3DB</td>
<td>10</td>
<td>253</td>
</tr>
<tr>
<td>aCD18</td>
<td>7</td>
<td>175</td>
</tr>
</tbody>
</table>

% ID/g of blood. Each datapoint represents the averages from 3-5 mice.
Curve fitting and statistics using Origin™ (version 4.1 Microcal™).
### Table 7.3.8.2a %ID/g for $^{125}$I Labeled RAFT3 scFv

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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<td>1</td>
<td>2.510</td>
<td>16.210</td>
<td>1.702</td>
<td>1.980</td>
<td>0.802</td>
<td>0.591</td>
<td>1.772</td>
<td>3.428</td>
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<td>3</td>
<td>1.220</td>
<td>8.820</td>
<td>0.853</td>
<td>0.933</td>
<td>0.602</td>
<td>0.407</td>
<td>0.669</td>
<td>1.267</td>
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<tr>
<td>6</td>
<td>0.750</td>
<td>6.712</td>
<td>0.307</td>
<td>0.209</td>
<td>0.134</td>
<td>0.178</td>
<td>0.297</td>
<td>0.217</td>
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<tr>
<td>18</td>
<td>0.370</td>
<td>2.089</td>
<td>0.144</td>
<td>0.070</td>
<td>0.041</td>
<td>0.019</td>
<td>0.087</td>
<td>0.073</td>
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</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 7.3.8.2b %ID/g for $^{125}$I Labeled RAFT3DB scFv

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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<tr>
<td>1</td>
<td>1.643</td>
<td>15.783</td>
<td>2.449</td>
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<td>1.164</td>
<td>1.542</td>
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<td>1.230</td>
<td>6.282</td>
<td>0.978</td>
<td>0.712</td>
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<td>0.400</td>
<td>0.503</td>
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<td>6</td>
<td>0.938</td>
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<td>0.193</td>
<td>0.379</td>
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<td>1.308</td>
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<td>0.022</td>
<td>0.054</td>
<td>0.066</td>
<td>0.068</td>
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</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 7.3.8.2c %ID/g for $^{125}$I Labeled Anti-CD18 scFv

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
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<tbody>
<tr>
<td>1</td>
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<td>3.790</td>
<td>2.230</td>
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<td>0.074</td>
<td>0.036</td>
<td>0.300</td>
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<tr>
<td>3</td>
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<td>0.727</td>
<td>0.087</td>
<td>0.037</td>
<td>0.045</td>
<td>0.150</td>
<td>0.477</td>
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<tr>
<td>6</td>
<td>0.182</td>
<td>1.192</td>
<td>0.526</td>
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<td>0.024</td>
<td>0.044</td>
<td>0.086</td>
<td>0.343</td>
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<td>18</td>
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<td>0.013</td>
<td>0.021</td>
<td>0.036</td>
<td>0.062</td>
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</tbody>
</table>

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Fig. 7.3.8.2a  %ID/g for $^{125}$I Labelled RAFT3 scFv

%ID/g

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 7.3.8.2b  %ID/g for $^{125}$I Labelled RAFT3DB scFv

%ID/g

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

Fig. 7.3.8.2c  %ID/g for $^{125}$I Labelled Anti-CD18 scFv

%ID/g

Time after injection
- 1h
- 3h
- 6h
- 18h

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
localisation is seen in the case of the irrelevant control anti-CD18 scFv. Highest tumour localisation is seen at the 1 hour timepoint for both RAFT3 scFv and RAFT3DB. Tumour localisation is greater in the case of the monomeric scFv molecule at 1 hour with 2.51%ID/g in the tumour in comparison to 1.643%ID/g for the RAFT3DB. Similarly, tumour localisation at 18 hours exceeds that of the modified clone. Localisation to normal organs is comparable with the RAFT3DB exhibiting slightly greater accumulation in the liver and bone with a tendency to slightly lower accumulation in the remainder of the organs.

The tumour to normal tissue ratios achieved are shown in Tables 7.3.8.2d-f and Figures 7.3.8.2d-f. The negative control anti-CD18 scFv shows no clear tumour-specific localisation in comparison to the anti-melanoma scFvs. Both RAFT3 scFv and RAFT3DB show increasing T:NT ratios over the timecourse of the experiment. The T:NT results are similar for both antibody fragments save for liver and blood where the monomer outperforms the dimer / monomer mixture. The only inconsistent result seems to be the 18hour T:NT for bone (RAFT3 scFv 21.648 vs. RAFT3DB 3.716) and this point is considered further in the discussion.

The radio-localisation indices indicate tumour-specific localisation for the RAFT3 scFv and RAFT3DB in comparison to the anti-CD18 scFv control (Tables 7.3.8.2g-h & Figures 7.3.8.2g-h). Tumour values range from 4.6 to 9.7 for monomer and 4.9 to 5.7 for monomer / dimer mixture. Values for normal organs tend to fall rapidly from their relatively high values at 1 hour. The persistently high radio-localisation index in the case of tumour in contrast to the falling values for normal organs for both the RAFT3 scFv and RAFT3DB indicate preferential retention in tumour in comparison to the negative control anti-CD18 scFv and suggest an antigen-specific mechanism.
### Table 7.3.8.2d

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
<th>Sp</th>
<th>Bl</th>
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<td>3.145</td>
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<td>21.649</td>
<td>4.828</td>
<td>5.753</td>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 7.3.8.2e

<table>
<thead>
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<th>Hours after injection</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood

### Table 7.3.8.2f

<table>
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<th>Hours after injection</th>
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<th>Lu</th>
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<th>Bn</th>
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Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
Each datapoint represents the average value from 3-5 mice. (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood.
### Table 7.3.8.2g

Radio-localisation Index
125I Labeled RAFT3 scFv

<table>
<thead>
<tr>
<th>Hours after injection</th>
<th>Tu</th>
<th>Ki</th>
<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
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<td>2.440</td>
<td>1.181</td>
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</table>

### Table 7.3.8.2h

Radio-localisation Index
125I Labeled RAFT3DB scFv

<table>
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<th>Hours after injection</th>
<th>Tu</th>
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<th>Li</th>
<th>Lu</th>
<th>Mu</th>
<th>Bn</th>
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</table>

Radiolocalisation index (RI) = \[
\frac{\%ID/g \text{ of specific scFv}}{\%ID/g \text{ of control scFv (anti-CD18)}}\]

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood.
Radiolocalisation index (RI) = \frac{\%ID/g of specific scFv}{\%ID/g of control scFv (anti-CD18)}

Each datapoint represents the average value from 3-5 mice. (Tu) Tumour, (Ki) Kidney, (Li) Liver, (Lu) Lung, (Mu) Muscle, (Bn) Bone, (Sp) Spleen, (Bl) Blood
7.4 Discussion

The intention of the described experiments was to produce and assess, *in vivo* and *in vitro*, a dimeric variant of the RAFT3 scFv. As indicated in the introduction (Section 7.1), several dimeric scFvs have previously been described although none of these target melanoma. A dimeric molecule, it was hoped, would increase tumour retention and hence tumour to normal tissue contrast by means of its increased avidity for the antigen and, perhaps, a longer circulatory half-life due to its increased size. The data in this chapter cannot be interpreted in these terms. The discovery that the modified clone fortuitously contained a mixture of monomer and dimer allowed some useful interpretation of the data to be made. Our data demonstrate that the RAFT3DB does not bind melanoma more effectively *in vitro* or target melanoma more efficiently *in vivo* than the monomeric parent RAFT3 scFv. These findings are discussed below.

7.4.1 Cloning

The cloning strategy described was unsuccessful in the case of the scFv studied. However, other clones produced in the same manner have now been successfully produced containing the intended sequence (J. Kupsch, personal communication).

7.4.2 Production

The lower yield of modified scFv using IMAC purification was due to lower levels of secreted scFv in the bacterial supernatant rather than less efficient purification. This is demonstrated by the fact that very little scFv was lost in the non-eluate fractions during purification and that the intensity of the scFv band upon Western blotting of neat bacterial supernatant was clearly less in the modified clone in comparison to RAFT3 scFv (blot not shown). This may represent differing bacterial synthesis of minor sequence differences along the protein chain. It has been demonstrated previously that single amino acid mutations can have a profound effect on recombinant protein yield (Dueñas *et al.*, 1995). The reduced yield could be further investigated by assessing the protein content of cell and periplasmic extracts to determine whether absolute
production was diminished or only secreted protein yield. However, previous studies in this lab have demonstrated that RAFT3 scFv isolated from periplasm is not as active as secreted protein (Kang, 1998). Furthermore, refolding of intracellular material can prove difficult. Therefore, whilst such investigations might help pinpoint the source of the problem and suggest possible solutions, they were not likely to be immediately helpful to the aims of this study and were not pursued further. In terms of diabodies, few studies have discussed the effect of non-covalent dimer production on protein yield. One group reported equivalent yields of scFv and non-covalent dimer of the same lineage (Wu et al., 1996; Wu et al., 1999).

7.4.3 Gel Filtration of Purified RAFT3DB

Gel filtration analysis of purified RAFT3DB (Figs. 7.3.4a&b) revealed a mixture of monomeric and dimeric scFv. Unfortunately, the gel filtration apparatus available for these experiments was insufficiently sensitive to produce an exact profile and allow quantification of the protein peaks. Experiments subsequent to this thesis have suggested that higher performance gel filtration columns give more accurate and reproducible profiles of scFv multimeric status.

The finding of a mixture of species would be consistent with previous studies. Despite early modelling predictions that linkers around 14 amino acids in length (15 in our scFv) should be sufficient to allow monomer formation (Bird et al., 1988), experimental work suggested that anti-TAG72, anti-fluorescein and anti-CEA scFvs incorporating such a linker all formed mixtures of monomers and dimers (Essig et al., 1993; Whitlow et al., 1993; Wu et al., 1996). This finding is not universal, however. Dolezal et al. have shown that the anti-neuraminidase scFv NC-10 produces purely monomeric scFv with a 15 amino acid linker in both the V_HV_K and V_KV_H orientations. We intended to produce dimers by removing the linker peptide (Dolezal et al., 2000). In fact, Kortt demonstrated that one anti-neuraminidase scFv formed trimers when the peptide linker was completely removed (Kortt et al., 1997). However, such polymerisation behaviour may be specific to individual scFvs. If the modified scFv is indeed a mixture of monomer and dimer in contrast to the apparently monomeric parent RAFT3 scFv, this difference
would be attributable to the small number of protein sequence differences between the two proteins. This phenomenon was not studied in more detail in the circumstances of this experiment since it was originally thought that an absent linker was the cause. Certainly the trend in the literature suggests that longer linkers favour monomers and shorter linkers polymers. Increasing the linker length to 18 or 28 amino acids eliminated dimer formation of the anti-TAG72 and anti-CEA scFvs, respectively (Whitlow et al., 1993; Wu et al., 1996). Desplancq and co-workers have studied this phenomenon systematically by characterising a series of scFvs containing one to six repeats of the sequence gly4ser (Desplancq et al., 1994) and showed that linkers of 25 or more amino acids favoured monomer formation in the B72.3 scFv. Similar results were obtained when varying the linker length in an anti-phenyloxazolone scFv (Alfthan et al., 1995). Conversely, it has been found that the recovery of good antigen binding when using scFvs with shorter linkers is associated with dimer formation (Essig et al., 1993; Desplancq et al., 1994; Alfthan et al., 1995). Interestingly, Plückthun and Pack have suggested that the V-domain orientation is also a major factor, suggesting that the Vh-linker-Vl orientation tended to favour monomers to a greater extent than the reverse orientation (Plückthun and Pack, 1997). This was explained in terms of the difficulty in folding Vl-linker-Vh constructs into a monomeric conformation. However, again, these findings seem to be specific to individual scFvs and have not been reported by other authors (Dolezal et al., 2000).

7.4.4 Radiolabelling

Relatively poor incorporation of radio-iodine was seen in the case of the RAFT3DB. This, however, was equivalent to that seen using the parent RAFT3 scFv (Table 7.2.6). The iodination was sufficient to allow adequate assessment of the in vitro and in vivo characteristics of the scFvs in question. Estimation of pre- and post-labelling immunoreactivity by cell ELISA suggested that over 90% of antigen binding was preserved. This might be expected when using a relatively gentle labelling technique such as the Iodogen method.
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7.4.5 \textit{In Vitro} Characteristics

7.4.5.1 Epitope Specificity of RAFT3DB

Inhibition of antigen binding of the parent whole antibody LHM2 in cell ELISA (Fig. 7.3.5) demonstrates that the RAFT3DB binds an epitope overlapping with the original RAFT3 scFv epitope. These results confirmed that the two antibody fragments target the same epitope of the high molecular weight antigen (the target of the LHM2 monoclonal antibody).

7.4.5.2 Affinity

Analysis of the data from the saturation binding experiments of the RAFT3 and RAFT3DB (Fig. 7.3.7a&b, Table 7.3.7) indicates that the monomer / dimer mixture has an affinity for the antigen approximately two-fold higher than that of the monomer ($K_A 5.59 \times 10^7$ vs. $3.19 \times 10^7$/mol). This may reflect a greater avidity for the antigen due to the mixed monomer and dimer in the modified clone samples. This two-fold increase in the strength of binding may also reflect the fact that the RAFT3DB is primarily monomeric and binding only in a monovalent fashion, the same apparent increase in affinity might be evident (since the molecular weight is halved). In other words, this increase in affinity is not evidence of bivalent binding \textit{per se}. Polyvalent binding of scFv multimers has been demonstrated using purified soluble antigen by sedimentation analysis (Adams \textit{et al.}, 1993) or size-exclusion HPLC (Wu \textit{et al.}, 1996). However, this is not possible in the case of the RAFT3DB since purified HMW-MAA is not available. Other studies comparing monomeric and dimeric scFvs have shown a variable effect on affinity / avidity. Adams \textit{et al.} reported an unchanged affinity constant of $0.5 \times 10^8$/mol when comparing the anti-c-erbB-2 scFv 741F8 and its dimer (Adams \textit{et al.}, 1993). Kortt \textit{et al.} noted a two-fold increase in affinity of a scFv dimer derived from monoclonal NC10 (Kortt \textit{et al.}, 1994) and Wu \textit{et al.} reported a greater than ten-fold increase in the affinity constant of an anti-CEA scFv when comparing monomer and dimer (Wu \textit{et al.}, 1996; Wu \textit{et al.}, 1999). It seems, therefore, that the effect of dimerisation on apparent affinity is related to the individual molecule concerned.
Much larger increases in apparent affinity have been described for scFv tetramers produced using scFvs fused to the tetramerisation domain of human transcription factor p53 (Rheinnecker et al., 1996) or a streptavidin moiety (Kipriyanov et al., 1996). Indeed, the latter study reported a 35-fold increase in apparent affinity for the antigen in comparison to the parent monomeric scFv. However, such an increase in avidity is obtained at the expense of a significant increase in size to equal or exceed the size of a whole IgG molecule. This increase in mass may negate many of the beneficial pharmacokinetic properties of small scFv molecules and the efficacy of these large tetrameric constructs is yet to be investigated in vivo.

7.4.6 In Vivo Studies

7.4.6.1 Overview

The intention of these experiments was to determine the effect of dimerisation on biodistribution and pharmacokinetics of the scFvs. To assess this, distribution in a variety of normal organs and tumours was assessed together with blood clearance. Any alteration in the behaviour of the molecule in vivo could be a function of the size of the molecule or its avidity for the antigen.

7.4.6.2 Pharmacokinetics

Radio-immunopharmaceuticals typically display a biphasic pattern of clearance from the circulation (Milenic et al., 1991).

The primary phase represents the development of equilibrium with bodily tissues and is influenced by a variety of factors. These are examined in the context of a dimer below. The first phase has a half-life designated $t_{90\%}$ and is usually rapid.

The secondary phase is less rapid and reflects the gradual release of intact radiopharmaceuticals dissociating from the antigen and from normal tissues and being
excreted from the body. The half-life of the secondary phase is termed \( t_{1/2} \). The mononuclear phagocyte system plays an important role in the removal of large molecules such as whole antibodies. However, in the case of small molecules such as scFvs, renal excretion is more rapid than the energy-dependent process of phagocytosis, catabolism and eventual release of the radiolabel.

Our data (Fig. 7.3.8.1) confirm the rapid pharmacokinetics of the RAFT3 scFv and RAFT3DB and are consistent with those values reported by other authors for similar molecules (Adams, 1998 for review). The \( t_{1/2} \) of the monomer, monomer / dimer mixture and control anti-CD18 scFv are all similar (8, 10 and 7 min, respectively). However, the monomer / dimer mixture shows a \( t_{1/2} \) 25% longer than the monomer (253 vs. 201 min). If the modified clone represents a mixture of different sized molecules, a tendency to prolonged clearance half lives for the larger molecules would be expected (Adams, 1998). Nevertheless, the dimeric scFv would not exceed the 65kDa or so necessary for a molecule to evade “renal sieving” (Maack et al., 1979) and thus the \( t_{1/2} \) would still be considerably shorter than the 384 minutes seen with LHM2 IgG (Kang et al., 1999). This prolonged secondary circulatory phase might be expected to enhance tumour antibody fragment concentrations and to prolong the serum retention of the molecule and is discussed below.

7.4.6.3 Biodistribution

7.4.6.3.1 ScFv Localisation to Tumour

Contrary to our expectations, tumour localisation of the RAFT3DB was in fact inferior to that of the monomer (Tables 7.3.8.2a & b, Figs. 7.3.8.2a & b). Peak %ID/g in these experiments reached only 1.643% for the dimer in comparison to 2.51% for the monomer (1 hour timepoint). Both anti-melanoma molecules clearly exceeded the localisation achieved by the negative control which reached only 0.334% ID/g in tumour at one hour. The diminished accumulation of RAFT3DB in the tumour may reflect reduced targeting efficacy of larger dimeric components. In terms of dimers, Wu et al. observed almost two-fold increase in tumour accumulation of a monomeric /
dimeric anti-CEA scFv mixture in comparison to monomer alone (10.38 vs. 6.57 %ID/g at 1 hour) which increased at later timepoints (Wu et al., 1996). Specific avidities were not discussed. Adams et al. demonstrated a similar improvement when targeting the c-erbB-2 antigen on SK-OV-3 ovarian carcinoma xenografts though the monomer and dimer exhibited equivalent avidities for the antigen in vitro (Adams et al., 1993). In the same study, scFv dimer was compared to a Fab fragment of similar mass which failed to show increased tumour localisation. The authors concluded that increased avidity in the case of the dimer was responsible for improved targeting rather than increased mass.

7.4.6.3.2 Localisation to Kidney

Renal accumulation of the RAFT3DB was marginally less than that seen with monomeric RAFT3 scFv (Tables 7.3.8.2a&b, Figs. 7.3.8.2a&b). This would be consistent with the increased molecular weight of a mixture of monomer and multimers and previously published studies (Adams et al., 1993; McCartney et al., 1995). However, the difference in renal accumulation noted is modest and probably within the scope of experimental error.

7.4.6.3.3 ScFv Localisation to Other Organs

Distribution to normal organs was very similar in RAFT3 scFv and RAFT3DB with very little difference evident (Tables 7.3.8.2a&b, Figures 7.3.8.2a&b). Again, this might be expected when comparing an scFv monomer to an scFv monomer / dimer mixture both falling below the threshold for renal filtration and clearing very rapidly from circulation.

7.4.6.3.4 Radio-localisation Index

Data for Radio-localisation Indices were obtained using anti-CD18 as a negative control. Calculations of the Radio-localisation Index (RI) were obtained using the formula:
Appendix Chapter 7

\[
\frac{\%ID/g\ of\ specific\ scFv\ in\ organ\ concerned}{\%ID/g\ of\ control\ anti-CD18\ scFv\ in\ organ\ concerned}
\]

Results are shown in Tables & Figures 7.3.8.2g&h. In order to demonstrate tumour-specific localisation, one must compare data to that obtained from an irrelevant control of similar chemistry and size. In this case anti-CD18 scFv, derived from a humanised mouse monoclonal IgG is used (Kupsch et al., 1995). Organ-specific localisation is indicated by a radio-localisation index in excess of 1. In the case of the RAFT3 scFv and RAFT3DB, clear tumour-specific localisation is evident at all timepoints. This confirms that tumour localisation is a consequence of antigen-scFv interaction rather than non-specific accumulation. A RI clearly greater than one is seen in most normal organs at the 1 and 3 hour timepoints. This is possibly a consequence of the longer circulating half-lives of the two melanoma-specific scFvs in comparison to the anti-CD18 (Section 7.3.8.2) which would result in somewhat greater retention in normal organs. This accumulation has largely resolved by the later timepoints where RIs are seen to be approaching one for normal tissues but are maintained for tumour.

7.4.6.3.5 Tumour to Normal Tissue Contrast

Tumour to normal tissue ratios are shown in Tables 7.3.8.2d-f and Figures 7.3.8.2d-f. These indicate a clear tendency to tumour localisation in excess of that seen in normal tissues, with the exception of the kidney for both RAFT3 scFv and RAFT3DB. However, the tumour to normal tissue ratios achieved by the monomer / dimer mixture are generally inferior to that seen with the monomer. Whilst somewhat less renal accumulation was seen with the RAFT3DB, the reduced tumour localisation has resulted in diminished tumour to kidney ratios. The largest disparities were in the ratios achieved in muscle and bone. In fact the RAFT3DB performed better in the tumour to muscle ratio (13.5 vs. 10.2) but much worse in the case of bone (3.7 vs. 21.6). However, this latter result may be a function of experimental error since the masses of bone samples obtained were small (10-40mg) and any small inaccuracy in weighing the sample would be amplified when extrapolating the results to theoretical masses of one gram.
7.4.6.3.6 Overview of in Vivo Studies

Despite the encouraging performance of the RAFT3DB in vitro in comparison to the monomeric RAFT3 scFv, it failed to out-perform the monomer in vivo. The accumulation in normal organs was broadly similar (with a slightly prolonged circulating time for the mixture of monomer and dimer. The failure of the RAFT3DB to localise significantly more efficiently to the tumour in contrast to other dimer studies is not surprising since the planned purely dimeric scFv was never produced. However, as noted above, Wu et al. did demonstrate enhanced tumour targeting using a monomer / dimer scFv mixture in comparison to pure monomer (Wu et al., 1996). If the RAFT3DB contains functioning dimeric moieties there are several reasons why the larger molecules might not enhance tumour localisation.

Firstly, three dimensional structural constraints in multimeric molecules might distort the antigen binding sites and their efficacy. However, the possibility that binding was largely monomeric remains and would confuse the issue. Ideally, HPLC studies using mixtures of purified multimeric species and antigen could be used to demonstrate multimeric binding. To date, no pure high molecular weight melanoma-associated antigen is available.

Secondly, the conformation and proximity of suitable epitopic binding sites would be critical in vivo for multivalent binding. Movement of HMW-MAA in the cell membrane in vivo might be limited by interactions with the extracellular matrix and render multimeric scFv crosslinking of antigen sterically impossible. Dimeric scFvss certainly do not have the “reach” which might be demonstrated by the Fabs of an intact IgG. Whilst the multimeric scFv might bind well to melanoma cells in solution, this situation does not necessarily correspond to that seen in vivo. In that case, dimeric scFvss, capable only of monomeric binding in vivo might find their larger size disadvantageous in terms of tumour targeting.
Finally, the timecourse of the experiment may be biased against the larger multimeric molecules since their pharmacokinetics are slower (especially $t_{1/2}$). It is possible that later timepoints would be more suited to the dimer. However, most of the dimer studies cited above used 6 to 24 hour timepoints and, whilst the dimeric molecules' advantage was greatest at later timepoints, improved targeting was evident prior to this. It is worth noting that, since the pharmacokinetics of scFvs seem to be significantly slower in the clinical setting (Begent et al., 1996; Larson et al., 1997), it may be that any superiority of multimeric molecules would be amplified. The actual effect of dimerisation on targeting in the clinical setting has still to be evaluated. For the results of the modified scFv to be interpreted usefully, the exact nature of the scFv mix would require to be elucidated, perhaps by size-exclusion HPLC and the individual components isolated if possible. However, since these problems were not evident during the timecourse of this thesis, these experiments could not be undertaken. The production of the intended clone and other multimeric variants will hopefully clarify the situation with regard to these anti-melanoma scFvs.
7.5 Conclusion

The failure of this particular experiment to identify the desired modified scFv clone due to circumstances beyond the control of the investigators was regrettable. However, the experimental rationale and structure remains valid and has been “test-run” successfully. Deficiencies in the proposed methods, such as the techniques used in identifying the molecular weights of the molecule produced were identified and will improve the investigation of multimeric anti-melanoma scFvs which are already being produced in this laboratory. Serendipitously, the clone studied proved to contain a mixture of monomeric and dimeric moieties and therefore yielded useful data. The failure to improve on the in vivo performance of the parent scFv of the apparent mixture of monomeric and multimeric scFv molecules is not evidence enough to discount this approach to melanoma targeting. Certainly researchers in other tumour models have demonstrated the potential of dimeric scFvs. As discussed in the introduction to this chapter, it is possible to develop multimeric scFvs by a number of different strategies including disulphide-linked scFvs, chemically linked scFv pairs, leucine zippers and minibodies. In this lab, we are actively pursuing the first two approaches at present in addition to our original proposed strategy and it may be that one of these other methods may be the correct technique for use with these scFvs in melanoma targeting. These experiments, while inconclusive in themselves, have already streamlined and focussed our approach to this strategy. The potential of dimeric and larger multimeric scFvs in melanoma has yet to be fully explored.
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8. Appendix: References


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Reed, W. B., Becker, S. W., Sr., and Becker S.W., J. (1965), Giant pigmented naevi, melanoma and leptomeningeal melanocytosis. *Arch Dermatol* 91: 100-119.


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Waibel, R., Alberto, R., Willuda, J., Finnern, R. et al. (1999), Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)-carbonyl complex [see comments]. *Nature Biotechnology* 17(9): 897-901.


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Published Material Relating to This Thesis

The following publications relate in whole or in part to the experimental work contained in this thesis:


9. Appendix: Suppliers

**Actigen, Affitech AS**
Gaustadalleen 21, N-0349, Oslo, Norway.
Tel No: 22 95 88 77

**Amersham Pharmacia Biotech**
23 Grosvenor Road, St. Albans, Hertfordshire AL1 3AW, United Kingdom.
Tel No. (01727) 814 000 Fax No: (0800) 318 354

**ATCC (American Tissue Culture Collection)**
12301 Parklawn Drive, Rockville, Maryland 20852, USA.
Tel No: (301) 881 2600 Fax No: (301) 816 4367

**BDH Laboratory Supplies**
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Tel No: (01202) 660444 Fax No: (01202) 666856

**Biometra and Cambridge Electrophoresis supplied by:**
**Prior Lab Supplies**
Unit 6, Forest Row Business Park, Station Road, Forest Row, East Sussex RH18 5DW, UK.
Tel No: (01342) 826 836 Fax No: (01342) 826 771

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Tel No: (0800) 181 134 Fax No: (01442) 259 118

**Boeringer Mannheim, Roche Diagnostics Ltd.**
Bell Lane, Lewes, East Sussex BN7 1LG, UK.
Tel No: (01273) 480444 Fax No: (01273) 480266

**DAKO Ltd.**
Denmark House, Angel Drove, Ely, Cambs. CB7 4ET.
Tel No: (01353) 669911 Fax No: (01353) 667309

**European Catalogue of Animal Cell Culture (ECACC)**
Centre for Applied Microbiology Research, Salisbury, Wiltshire SP4 0JG
Tel No: (01980) 615 212 Fax No: (01980) 611 313

**Filtron Technology Corporation**
50 Bearfoot Road, Northborough, Massachusetts, USA
Supplied by **Flowgen UK** (a division of Philip Harris Plc.)
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**Invitrogen BV**  
De Schelp 12, 9351 NV Leek, Netherlands.  
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**Janssen Pharmaceutical suppliers Vet Drug Ltd.**  
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**Jencons (Scientific) Ltd**  
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**Mini Instruments (Radiation Monitors)**  
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**SAS Institute**  
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**Sigma-Aldrich Company Ltd.**  
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3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS, UK.  
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10. Appendix: Abbreviations

AJCC  American Joint Committee on Cancer
ADCC  Antibody Directed Cell Mediated Cytotoxicity
bp    base pair
BSA   Bovine Serum Albumin
CDR   Complementarity Determining Region
CH    constant heavy region
CL    constant light region
cps   counts per second
cpm   counts per minute
dH2O  Distilled water
EDTA  ethylene diaminetetra-acetic acid
ELISA Enzyme Linked Immunosorbent Assay
F(ab')2 100 kDa Antibody Fragment
F(ab') 50 kDa antibody Fragment
HAMA  Human anti-mouse antibody
HMW-MAA High molecular weight melanoma-associated antigen
His 6  6 Histidine amino acid residues on the C-terminus of scFv
IgG1  Immunoglobulin gamma (isotype Class I)
ISG   Immunoscintigraphy
IPTG  Isopropyl-D-thio-galactopyranoside
KA    association constant for equilibrium binding of antibodies
KD    dissociation constant for equilibrium binding of antibodies
kDa   Kilodalton
keV   Kilo-electron volt
HMW-MAA High Molecular Weight Melanoma Associated Antigen proteoglycan
IMAC  Immobilised Metal-ion Affinity Chromatography
ISG   Immunoscintigraphy
MAb   Monoclonal Antibody
Appendix Chapter 10

µCi MicroCurie
PBMC Peripheral Blood Mononuclear Cell
PCR Polymerase Chain Reaction
PEG<sub>6000</sub> Poly-Ethylene Glycol 6000
PET Positron Emission Tomography
pI Iso-electric point
psi Pounds per square inch
RAFT Restoration of Appearance and Function Trust
rpm Revolutions per minute
RT Room Temperature
scFv Single-chain Antibody Fragment
(scFv)<sub>2</sub> Covalently-linked ScFv Dimer
SDS-PAGE Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
SEM Standard Error of the Mean
SPECT Single Photon Emission Computerised Tomography
Tris Tris(hydroxymethyl)aminomethane
TWEEN 20 Polyoxylsorbital monolaurate
V<sub>H</sub> Variable heavy region
V<sub>k</sub> Variable kappa region
V<sub>L</sub> Variable light region