The Role of Corrective Gene Therapy
Targeting the C-myc Oncogene in Melanoma

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Abstract

C-myc oncogene expression is known to be a highly significant prognostic factor in melanoma. Corrective gene therapy targeting this gene has been demonstrated to inhibit the growth of melanoma in-vitro and in-vivo. This project has investigated the role of the c-myc oncogene in interferon resistance, the efficacy of combining c-myc antisense with interferon and chemotherapeutic drugs and has also designed and tested a ribozyme targeting the c-myc oncogene.

The efficacy of interferon as adjuvant therapy for melanoma has recently been questioned. Overexpression of the c-myc oncogene has been shown to be associated with interferon resistance in cell lines. This project investigated the relationship between c-myc gene expression and interferon sensitivity in a series of 45 uveal melanomas using flow cytometry and the ATP chemosensitivity assay.

High c-myc expression was found to correlate with interferon resistance as measured by the maximum percentage of inhibition (p=0.05) and the interferon sensitivity index (p=0.07). These results demonstrate that tumours with high c-myc expression and poor prognosis are also associated with interferon resistance.

To assess whether corrective gene therapy targeting the c-myc gene could be used to improve the efficacy of interferon, 4 melanoma cell lines were treated with c-myc antisense and interferon, individually or in combination, and the effects on cell growth measured using the ATP-chemosensitivity assay. Combination of c-myc antisense with interferon was found to significantly increase the growth inhibitory effects of interferon in the cell lines expressing the highest levels of the c-myc gene. Super-additive effect was demonstrated in the A375m cell line. This suggests that corrective gene therapy could be used to improve the efficacy of interferon as adjuvant therapy.

The prognosis for patients with advanced melanoma is very poor as standard chemotherapy drugs are generally ineffective. Tumour c-myc overexpression is known to be associated with chemo-resistance. C-myc antisense was therefore combined with a number of chemotherapeutic drugs and the effects on the growth of the A375m melanoma cell line assessed with the ATP-chemosensitivity assay. Combination of c-myc antisense with cis-platinum resulted in synergistic effect on cell growth inhibition, suggesting that this strategy could be used to overcome chemo-resistance in advanced melanoma.

As an initial step in the development of c-myc antisense for use in patients a ribozyme targeting exon 2 of c-myc mRNA was designed, synthesised and subsequently tested in-vitro using the MTS assay. Although inhibition of melanoma cell growth was demonstrated to occur, this was found to be a non-specific transfection-related effect and not accompanied by down-regulation of c-myc expression. A second ribozyme, targeting a different part of exon 2 of the c-myc gene, has subsequently been designed which is known to be effective in down-regulating the c-myc oncogene in other cell lines.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>SSM</td>
<td>Superficial spreading melanoma</td>
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<td>NM</td>
<td>Nodular melanoma</td>
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<tr>
<td>ALM</td>
<td>Acral lentiginous melanoma</td>
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<tr>
<td>LMM</td>
<td>Lentigo maligna melanoma</td>
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<tr>
<td>DM</td>
<td>Desmoplastic melanoma</td>
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<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
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<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
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<tr>
<td>AMS</td>
<td>Atypical mole syndrome</td>
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<tr>
<td>ELND</td>
<td>Elective lymph node dissection</td>
</tr>
<tr>
<td>SLND</td>
<td>Selective lymph node dissection</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>TIL</td>
<td>Tumour infiltrating lymphocyte</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>GMD</td>
<td>Geometric mean diameter</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Td</td>
<td>Tumour doubling time</td>
</tr>
<tr>
<td>PDT</td>
<td>Potential doubling time</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<tr>
<td>MEM</td>
<td>Modified Eagle’s Medium</td>
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<tr>
<td>Opti-MEM</td>
<td>Optimised MEM</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
</tbody>
</table>

iv
## Contents

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>iv</td>
</tr>
<tr>
<td>Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## Chapter 1  General Introduction

### Section 1  Cutaneous Melanoma

1.1.1  Introduction  2
1.1.2  Epidemiology  2
1.1.2.1  Incidence  2
1.1.2.2  Mortality  3
1.1.2.3  Age  4
1.1.2.4  Sex  4
1.1.2.5  Geographical Variation  4
1.1.3  Risk factors and precursor lesions  5
1.1.3.1  Ultraviolet Radiation  5
1.1.3.2  Skin Type  6
1.1.3.3  Family History  7
1.1.3.4  Pre-existing Naevi  7
1.1.4  Clinical features of cutaneous melanoma  9
1.1.4.1  Superficial Spreading Melanoma (SSM)  9
1.1.4.2  Nodular melanoma (NM)  10
1.1.4.3  Lentigo maligna melanoma (LMM)  11
1.1.4.4  Acral Lentiginous Melanoma (ALM)  11
1.1.4.5 Desmoplastic Melanoma (DM) 11
1.1.5 Treatment of primary melanoma 16
1.1.5.1 Management of primary melanoma 16
1.1.5.2 Elective lymph node dissection 18
1.1.5.3 Sentinel lymph node biopsy 19
1.1.6 Prognostic factors used in Melanoma 20
1.1.6.1 Established prognostic factors 20
1.1.6.2 Prognostic models 25
1.1.6.3 Measurement of tumour vascularity 25
1.1.6.4 Cell Cycle Proteins 25
1.1.6.5 Immunological and other markers of prognosis 26
1.1.6.6 Detection of circulating melanoma cells in the peripheral blood 28
1.1.6.7 Serum S-100 levels 28
1.1.6.8 The role of prognostic factors in melanoma 29
1.1.7 Adjuvant treatment of melanoma 29
1.1.8 Management of disseminated melanoma 33
1.1.8.1 Management of regional lymph node metastases 33
1.1.8.2 Treatment of stage IV disease 33

Section 2 Uveal Melanoma

1.2.1 Epidemiology 34
1.2.2 Risk factors and precursor lesions 35
1.2.3 Clinical features of uveal melanoma 36
1.2.4 Determination of prognosis in uveal melanoma 36
1.2.5 Treatment of uveal melanoma 37
1.2.5.1 Management of primary disease 37
1.2.5.2 Treatment of advanced disease 40
# Section 3 Experimental Treatment Strategies in Melanoma

1.3.1 Immunotherapy 41

1.3.2 Gene therapy 43

1.3.2.1 The cell cycle 44

1.3.2.2 p16, cyclin-D1, cdk-4, the retinoblastoma gene and E2F 44

1.3.2.3 p53 46

1.3.2.4 Bcl-2 46

1.3.2.5 Ras 47

1.3.2.6 C-myc 48

1.3.2.6.1 Introduction 48

1.3.2.6.2 Anatomy of the c-myc gene 49

1.3.2.6.3 C-myc and the control of growth and differentiation 50

1.3.2.6.4 C-myc and apoptosis 52

1.3.2.6.5 C-myc and cancer 53

1.3.2.6.6 C-myc and melanoma 53

1.3.2.6.7 Manipulation of c-myc expression 54

1.3.2.6.8 Antisense Oligonucleotides 55

1.3.2.6.8.1 Mechanism of action 55

1.3.2.6.8.2 Biochemistry of antisense oligomers 56

1.3.2.6.8.3 Applications of antisense oligonucleotides 59

1.3.2.6.8.4 Antisense oligonucleotides targeting the c-myc gene 61

1.3.2.6.9 The limitations of antisense oligonucleotides as corrective gene therapy 61

1.3.2.6.10 Ribozymes 62

1.3.3 Experimental Aims 63
### Chapter 2  Materials and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Cell lines and culture conditions</td>
<td>65</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Cell culture medium</td>
<td>65</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Cell lines</td>
<td>65</td>
</tr>
<tr>
<td>2.1.2.1</td>
<td>A375m</td>
<td>66</td>
</tr>
<tr>
<td>2.1.2.2</td>
<td>MeWo</td>
<td>66</td>
</tr>
<tr>
<td>2.1.2.3</td>
<td>MM96L</td>
<td>66</td>
</tr>
<tr>
<td>2.1.2.4</td>
<td>SkMel 28</td>
<td>66</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Cell maintenance</td>
<td>66</td>
</tr>
<tr>
<td>2.1.3.1</td>
<td>Storage of cell lines in liquid nitrogen</td>
<td>66</td>
</tr>
<tr>
<td>2.1.3.2</td>
<td>Recovery of cell lines from liquid nitrogen</td>
<td>67</td>
</tr>
<tr>
<td>2.1.3.3</td>
<td>Maintenance of human melanoma cell lines</td>
<td>67</td>
</tr>
<tr>
<td>2.1.3.4</td>
<td>Mycoplasma testing</td>
<td>67</td>
</tr>
<tr>
<td>2.2</td>
<td>The ATP chemosensitivity assay</td>
<td>68</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Assessment of the effect of combination of antisense with chemotherapeutic drugs or interferon on the growth of melanoma cell lines</td>
<td>68</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Assessment of the interferon sensitivity of uveal melanoma tumours</td>
<td>69</td>
</tr>
<tr>
<td>2.3</td>
<td>Flow Cytometry</td>
<td>70</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Description of the FACscan</td>
<td>70</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Detection of c-myc oncoprotein in patient material</td>
<td>72</td>
</tr>
<tr>
<td>2.3.2.1</td>
<td>De-waxing of specimens</td>
<td>72</td>
</tr>
<tr>
<td>2.3.2.2</td>
<td>Preparation of nuclei</td>
<td>72</td>
</tr>
<tr>
<td>2.3.2.3</td>
<td>C-myc antibodies</td>
<td>72</td>
</tr>
<tr>
<td>2.3.2.4</td>
<td>Staining of c-myc oncoprotein in patient material</td>
<td>74</td>
</tr>
<tr>
<td>2.3.2.5</td>
<td>Staining of c-myc oncoprotein expression in cell lines</td>
<td>74</td>
</tr>
<tr>
<td>2.3.2.6</td>
<td>Data Analysis and measurement of oncoprotein</td>
<td>75</td>
</tr>
<tr>
<td>2.3.2.6.1</td>
<td>Use of Computer-Generated Windows (CGWs)</td>
<td>76</td>
</tr>
<tr>
<td>2.3.2.6.2</td>
<td>Calculation of Oncoprotein levels</td>
<td>76</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Fluorescence Activated Cell Sorting</td>
<td>79</td>
</tr>
<tr>
<td>2.3.3.1</td>
<td>Cell-sorting based on degree of GFP expression</td>
<td>80</td>
</tr>
<tr>
<td>2.3.3.2</td>
<td>Green Fluorescent Protein</td>
<td>81</td>
</tr>
<tr>
<td>2.4</td>
<td>Oligonucleotides</td>
<td>84</td>
</tr>
<tr>
<td>2.4.1</td>
<td>C-myc antisense and ‘4G’ quartet control sequences</td>
<td>84</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Preparation and storage of oligonucleotides</td>
<td>84</td>
</tr>
<tr>
<td>2.5</td>
<td>Targeting the c-myc gene using a ribozyme</td>
<td>85</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Target and structure of the c-myc ribozyme</td>
<td>85</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Production of a ribozyme-encoding plasmid targeting the c-myc oncogene</td>
<td>86</td>
</tr>
<tr>
<td>2.5.2.1</td>
<td>Annealing of oligonucleotides</td>
<td>86</td>
</tr>
<tr>
<td>2.5.2.2</td>
<td>Ligation</td>
<td>86</td>
</tr>
<tr>
<td>2.5.2.3</td>
<td>Transformation of DH5α e. coli bacteria</td>
<td>89</td>
</tr>
<tr>
<td>2.5.2.4</td>
<td>Assay of the completed plasmid sequence using PCR screening of the transformed colonies</td>
<td>90</td>
</tr>
<tr>
<td>2.5.2.5</td>
<td>Extraction and purification of pREVmyc17 DNA from DH5α e. coli bacteria using the mini-prep technique</td>
<td>90</td>
</tr>
<tr>
<td>2.5.2.6</td>
<td>Large-scale production of pREVmyc17 by DH5α e. coli and extraction and purification using the maxi-prep protocol</td>
<td>91</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Investigation of Optimal Transfection Strategy</td>
<td>93</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Assessment of the effect of pREVmyc17 on the growth of the A375m melanoma cell line</td>
<td>95</td>
</tr>
<tr>
<td>2.5.4.1</td>
<td>Experiment 1</td>
<td>96</td>
</tr>
<tr>
<td>2.5.4.2</td>
<td>Experiment 2</td>
<td>96</td>
</tr>
<tr>
<td>2.5.5</td>
<td>Establishment of optimal transfection conditions for A375m cells</td>
<td>97</td>
</tr>
<tr>
<td>2.5.6</td>
<td>Assessment of the effect of the pREVmyc17 ribozyme on c-myc oncogene expression in A375 cells</td>
<td>98</td>
</tr>
</tbody>
</table>
Chapter 3

An investigation of the relationship between c-myc expression and interferon sensitivity in uveal melanoma

3.1 Introduction 101
3.2 Materials and Methods 105
3.3 Clinical and Histopathological Data 109
3.4 C-myc oncoprotein expression in uveal melanoma 109
3.4.1 Patient age and c-myc expression 109
3.4.2 Patient sex and c-myc expression 109
3.4.3 Tumour Diameter and c-myc Expression 111
3.4.4 Mitotic index and c-myc expression 111
3.4.5 Predominant cell type and c-myc expression 111
3.5 Analysis of factors influencing the interferon sensitivity of uveal melanoma 111
3.5.1 Age and interferon sensitivity index 113
3.5.2 Age and interferon maximum inhibitory percentage 113
3.5.3 Sex and interferon sensitivity 113
3.5.4 Tumour diameter and interferon sensitivity index 113
3.5.5 Tumour diameter and maximum interferon inhibitory percentage 114
3.5.6 Mitotic index and interferon sensitivity 114
3.5.7 Predominant cell type and interferon sensitivity index 114
3.5.8 Predominant cell type and maximal inhibitory percentage 114
3.5.9 C-myc oncoprotein expression and interferon sensitivity index 115
3.5.10 C-myc oncoprotein expression and maximum inhibitory index 115
3.6 Discussion 118
Chapter 4
Combination of c-myc antisense and interferon in melanoma

4.1 Introduction 121
4.2 Methods 122
4.3 Results
4.3.1 Combination of c-myc antisense with interferon in the A375m melanoma cell line 122
4.3.2 Combination of c-myc antisense with interferon in the SkMel 28 melanoma cell line 123
4.3.3 Combination of c-myc antisense with interferon in the MM96L melanoma cell line 124
4.3.4 Combination of c-myc antisense with interferon in the MeWo melanoma cell line 127
4.3.5 C-myc expression levels in the A375M, SkMel 28, MeWo and MM96L melanoma cell lines 127
4.3.6 The relationship between interferon sensitivity and c-myc expression in the 4 cell lines 130
4.3.7 Additive effect produced by the interferon and c-myc antisense Combination and c-myc expression 130
4.4 Discussion 133

Chapter 5
Combination of c-myc and bcl-2 antisense with chemotherapy drugs in melanoma

5.1 Introduction 136
5.2 Methods 137
Chapter 6

Targeting the c-myc gene using a ribozyme

6.1 Introduction 149
6.2 Structure of the hammerhead ribozyme 150
6.3 Stability of ribozymes 150
6.4 Delivery of ribozymes to target cells 152
6.5 Uses of ribozymes 153
6.6 Target and structure of the c-myc ribozyme 154
6.7 Production of a ribozyme-encoding plasmid targeting the c-myc gene 156
6.7.1 Results 157
6.8 Investigation of the optimal transfection strategy 157
6.8.1 Results 161
6.9 Assessment of the effect of pREVmyc17 on the growth of the A375m melanoma cell line 161
6.9.1 Results of experiment 1 162
6.9.2 Results of experiment 2 162
Chapter 7  General Discussion

7.1  Introduction 174
7.2  The relationship between c-myc expression and interferon sensitivity in melanoma 175
7.3  Combination of c-myc antisense with interferon in melanoma 177
7.4  Combination of c-myc antisense with chemotherapeutic drugs in melanoma 178
7.5  The development of a ribozyme targeting the c-myc oncogene in melanoma 181
7.6  Conclusions: multiple therapeutic strategies for the treatment of melanoma 183

References 187
# Figures

## Chapter 1

1.1 Superficial spreading melanoma 13  
1.2 Nodular melanoma 13  
1.3 Lentigo maligna melanoma 14  
1.4 Subungual melanoma 14  
1.5 Acral lentiginous melanoma 15  
1.6 The cell cycle 45  
1.7 Chemical structure of oligonucleotide derivatives 57  

## Chapter 2

2.1 Typical contour flow cytometry profile of a melanoma 77  
2.2 Contour plot of the isotypic control antibody sample 78  
2.3 Contour plot of the c-myc antibody stained sample 79  
2.4 Cytogram dot-plots for the cell-sort experiments 83  

## Chapter 3

3.1 Postulated mechanism of action of interferon 104  
3.2 FACS profile of a uveal melanoma stained for c-myc oncoprotein 107  
3.3 IFN sensitivity data from 4 uveal melanoma specimens 108  
3.4 Distribution of c-myc positivity of the series of uveal melanomas 110  
3.5 Distribution of interferon sensitivity indices of the series of uveal melanomas 112  
3.6 Distribution of the interferon maximum inhibiton percentages of the series of uveal melanomas 112  
3.7 C-myc expression and interferon sensitivity index in uveal melanoma 116
3.8 C-myc expression and maximal percentage inhibition by interferon in uveal melanoma

Chapter 4
4.1 C-myc antisense and interferon in the A375m cell line 125
4.2 C-myc antisense and interferon in the A375m cell line: Window of theoretical additivity 125
4.3 C-myc antisense and interferon in the SkMel 28 cell line 126
4.4 C-myc antisense and interferon in the MM96L cell line 126
4.5 C-myc antisense and interferon in the MeWo cell line 128
4.6 C-myc expression in the A375m, MeWo, MM96L and SkMel 28 cell lines 129
4.7 Interferon sensitivity index and c-myc expression in the 4 melanoma cell lines 131
4.8 Additive effect of c-myc antisense in combination with interferon and c-myc expression in the 4 melanoma cell lines 132

Chapter 5
5.1 C-myc antisense and cis-platinum in A375 m cells 141
5.2 The window of theoretical additivity for the combination of c-myc antisense and cis-platinum in the A375m cell line 141
5.3 C-myc antisense + DTIC in A375m melanoma cells 142
5.4 C-myc antisense + temozolomide in A375m cells 142
5.5 Taxol and c-myc antisense in A375m cells 143

Chapter 6
6.1 Structure of hammerhead ribozymes 151
6.2 Sequence and structure of the ribozyme targeting c-myc mRNA 151
6.3 Structure of the pREV plasmid 155
6.4 Sequence and annealing of oligonucleotides c-mycRzA and c-mycRzB 155
6.5 Electrophoresis gel of DNA fragments produced following PCR of DNA suspension with CMV and SV40 primers 158
6.6 DNA sequencing analysis of pREVmyc17 base sequence 158
6.7 Establishment of the optimal transfection agent 159
6.8 Examples of histograms from transfection of the A375m cell line with the pREVmyc17 plasmid 160
6.9 Effect of pREVmyc17 on the growth of A375m cells - experiment one 163
6.10 Effect of pREVmyc17 on the growth of A375m cells - experiment two 163
6.11 Establishment of optimal transfection conditions 165
6.12 The effect on c-myc expression in A375m cells following transfection with the pREVmyc or pREV control plasmid 168
6.13 The effect on c-myc expression in A375m cells following transfection with either the pREVmyc17 or pREV control plasmid - experiment 1 histogram plots 169
6.14 The structure of the c-myc ribozyme described by Ohta et al 172

Chapter 7
7.1 Design for a targeted retrovirus encoding the c-myc ribozyme 184

Tables

Chapter 1
1.1 Classification of skin type and melanoma risk 7
1.2 Risk factors for the development of melanoma 9
1.3 Thickness of primary melanoma and 5 year survival 21
1.4 Established prognostic factors in primary melanoma 22
1.5 The modified AJCC classification of melanoma 23
1.6 Prognostic factors in stage III and IV melanoma 24
1.7 Experimental prognostic factors in melanoma 27
1.8 Properties of normal and modified oligodeoxynucleotides 58

Chapter 2
2.1 C-myc antisense and ‘4G’ ODN control sequences 84
Chapter 1  General Introduction

The incidence of melanoma has increased rapidly over the last fifty years. Several factors have contributed to make melanoma the subject of intense research in an attempt to improve patient survival. The prognosis for patients once the disease has metastasised is poor due to a lack of effective treatment for patients in this group. In addition melanoma patients are often relatively young. More recently doubts have been raised regarding the efficacy of interferon as adjuvant therapy for melanoma. Previous research undertaken by the fellows in this project have highlighted the prognostic significance of c-myc oncogene expression in melanoma and have demonstrated that corrective gene therapy targeting this gene could be used to inhibit the growth of melanoma in-vitro and in-vivo.

The experiments undertaken in this thesis were used to investigate a number of hypotheses. First, could a relationship be demonstrated between c-myc oncogene expression and interferon resistance in melanoma, indicating that tumours of poor prognosis were also resistant to the effects of interferon. This would explain the failure of interferon to produce survival benefit when used as adjuvant therapy in the majority of clinical trials. Second, could the combination of c-myc antisense therapy with interferon produce additive or synergistic effects in-vitro, suggesting that this might be a therapeutic strategy to overcome interferon resistance and increase its efficacy as an adjuvant agent. Third, could c-myc antisense therapy be combined with other chemotherapeutic drugs to produce additive or synergistic effects in-vitro, which would suggest that corrective gene therapy could be used to overcome chemoresistance in advanced melanoma. Fourth, a ribozyme targeting the c-myc oncogene was designed, synthesised and its efficacy tested in-vitro in order to amplify the effects of c-myc antisense, as a first stage in the development of c-myc antisense for use in humans.

Flow cytometry was used to measure c-myc expression in a series of uveal melanoma specimens to assess its relationship with the respective interferon sensitivities measured using the ATP-chemosensitivity assay. The results are presented in Chapter 3. The ATP-chemosensitivity assay was also used to assess the
effects of combining c-myc antisense with interferon and chemotherapeutic agents and the results are presented in Chapters 4 and 5. The ribozyme targeting the c-myc oncogene was synthesised using standard molecular biology techniques and its efficacy tested in-vitro using the MTS assay to measure the effects on cell growth. Flow cytometry was used to assess its effects on c-myc expression.

Section 1  
Cutaneous Melanoma

1.1.1 Introduction

Melanoma is a malignant tumour derived from melanocytes. These cells originate from the neural crest of the embryo before migrating to the skin, the choroid of the eye, leptomeninges, gastrointestinal and genitourinary epithelia. Melanoma is most commonly found as a cutaneous tumour but may arise at any of the above sites where melanocytes are found histologically in postnatal life.

1.1.2 Epidemiology

1.1.2.1 Incidence

The incidence of cutaneous melanoma has increased rapidly over the last fifty years. On average it has doubled every ten years, increasing more rapidly than for any other solid tumour, largely due to increases in sun exposure (Coggon et al. 1994). This increase in incidence has occurred worldwide, predominantly affecting caucasian populations, but also occurring in populations at lower risk (Cancer-Research-Campaign 1995). In 1989 the incidence of melanoma was 7.1 new cases per hundred thousand population in the UK. The lifetime risk for an individual born in the year 2000 of acquiring melanoma has been estimated at 1 in 90 in the USA (Rigel et al. 1987) having risen from 1 in 1500 for those born in 1935.
Mortality

Mortality rates for melanoma have also increased rapidly. However, more recently some evidence of stabilisation or even a small reduction in mortality has been noted for certain groups of patients in several countries. In England and Wales, melanoma mortality rates have fallen for women aged under 50 years and for men aged under 35 years between 1985-89 and 1990-91 (Cancer-Research-Campaign 1995). In Scotland the incidence of melanoma in women under 65 years has stabilised since 1986. A fall in female mortality was noted for those aged under 65 years between 1979 and 1994 but this was not statistically significant (MacKie et al. 1997). A similar effect has also been reported in studies from Australia (mortality rates have plateaued since a peak in 1985 and fallen in women under 55 years since) (Giles et al. 1996) and the USA (Scotto et al. 1991). The reason for these trends may be a reduction in ultraviolet light exposure due to changes in sunbathing behaviour and also the earlier detection and treatment of precursor lesions and primary melanomas - both resulting from public education campaigns. This may explain the increased proportion of thinner primary melanomas observed compared to thicker lesions in some studies (Armstrong et al. 1994, MacKie et al. 1997). In addition, more melanoma patients are now treated at specialised plastic surgery units. Following narrower excision guidelines, they more commonly receive local rather than general anaesthetics - producing less immunosuppression post-operatively. These developments may also have contributed to improvements in mortality.

Survival rates are better for women than men (Cancer-Research-Campaign 1995), (Balch et al. 1992), for younger compared to older patients (Balch et al. 1992) and for patients of higher compared to those from lower socio-economic groups, irrespective of tumour thickness and other prognostic factors (MacKie et al. 1996). However, the incidence of melanoma is more common in more affluent socio-economic groups, affecting sites normally covered by clothing, probably due to increased intermittent sun exposure during vacations (MacKie et al. 1996).
1.1.2.3 Age

The average age of presentation in the UK is 54, however it has a significant impact on young adults - melanoma has the highest incidence of any malignant tumour in fair skinned populations between the ages of 25-29 and in males between 35-39 years of age (Cancer-Research-Campaign 1995). Melanoma is also the second commonest cancer for women and the sixth commonest for men aged 20-34 years of age (Cancer-Research-Campaign 1995).

1.1.2.4 Sex distribution

In the UK melanoma is more common in females than males. However, in the decade to 1989 melanoma incidence increased more rapidly in males than females such that by 1989 the female : male ratio had fallen from 2:1 to 1.6:1 (Cancer-Research-Campaign 1995). Worldwide, the variation between the sexes is less marked - the ratio is approximately 1:1 in the USA and the increase in incidence has affected both sexes equally (Grin-Jorgensen et al. 1992).

1.1.2.5 Geographical variation

There is a wide variation in the incidence of melanoma worldwide. In 1989 the incidence of melanoma was 7.1 new cases per hundred thousand population in the UK – similar to other Northern European countries (Balch et al. 1992, Cancer-Research-Campaign 1995). In the USA the incidence in 1990-1994 was estimated at 15 / 100 000 population (Polednak 1997). The highest incidence of melanoma is seen where whites live in tropical climates and is thought to be due to the high UV irradiation exposure in the fair-skinned population. In Australia the incidence varies between 17.6 / 100 000 in Victoria to 43.5 / 100 000 seen in Queensland (Grin-Jorgensen et al. 1992).
1.1.3 Risk factors and precursor lesions

1.1.3.1 Ultra-violet light

Ultraviolet (UV) irradiation is the most important risk factor in the development of melanoma - up to 90% of melanomas in Australia are thought to be caused by UV exposure (Armstrong et al. 1993, Armstrong et al. 1994). Radiation in the ultraviolet B range (280 to 320nm) is thought to be the critical component (Koh et al. 1990). This fact is supported by the worldwide variation in melanoma incidence, with the highest incidences found where fair-skinned populations live in tropical climates. Melanoma incidence also varies within individual countries - an inverse correlation between melanoma incidence and latitude has been shown to exist in Sweden, the USA, the United Kingdom and also Australia (Eklund et al. 1978, Rigel et al. 1987, Grin-Jorgensen et al. 1992, Swerdlow et al. 1993).

The age at which an individual is first exposed to high UV radiation levels and also the pattern of exposure are also both important. Exposure to high levels of UV radiation early in life is a highly significant risk factor for the development of subsequent melanoma - migration to Australia below the age of ten confers a risk of developing melanoma equal to that of the indigenous population (Armstrong et al. 1982, Khat et al. 1992).

Melanoma is strongly associated with excess, intermittent UV exposure leading to episodes of burning and blistering (Koh et al. 1990, Elwood et al. 1997). In contrast, basal-cell and squamous cell carcinoma are thought to be associated with chronic, cumulative UV exposure. The reasons for these differences have recently been studied. 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. 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. It has been proposed that the small DNA fragments which are excised following episodes of UV-induced DNA damage can directly enhance DNA repair mechanisms (Gilchrest 1999). They may also slow melanocyte proliferation and stimulate melanin production. These processes further increase cell protection against UV-induced DNA damage (Pedeux et al. 1998). Additional 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. Intermittent exposure to high UV doses is therefore likely to result in melanomas in relatively young patients. Non-melanotic skin cancer is more likely to occur following multiple low dose UV exposures as high doses of UV radiation are likely to lead to cell death by apoptosis. Non-melanotic skin cancer therefore tends to occur in older patients who have acquired a sufficient number of lower-dose UV exposures.

Depletion of the earth’s ozone layer, thought to be induced by artificial chlorofluorocarbons, and the subsequent increase in the amount of ultraviolet radiation reaching the earth’s surface may lead to an increase in melanoma and other skin cancers in the future (Elwood et al. 1984).

1.1.3.2 Skin type

The degree of skin pigmentation, and therefore sensitivity to sun, has an important influence on the risk of acquiring melanoma which has been confirmed by migration studies. Those migrants to Australia of darkest skin complexion and/or who tan the easiest have the lowest risk of acquiring melanoma (Armstrong et al. 1994). Black populations have a much lower risk of melanoma than white populations. Skin type has been classified into six types by degree of pigmentation and ease of tanning (see table 1.1). Type 1 having the greatest risk of developing melanoma and type 6 the least (Cancer-Research-Campaign 1995).
<table>
<thead>
<tr>
<th>Skin Type</th>
<th>Intrinsic Pigmentation and Response to Sunlight</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>White skin, never tans, always burns</td>
</tr>
<tr>
<td>2</td>
<td>White skin, burns initially, tans with difficulty</td>
</tr>
<tr>
<td>3</td>
<td>White skin, tans easily, burns rarely</td>
</tr>
<tr>
<td>4</td>
<td>White skin, always tans, never burns (Mediterranean)</td>
</tr>
<tr>
<td>5</td>
<td>Brown skin</td>
</tr>
<tr>
<td>6</td>
<td>Black skin</td>
</tr>
</tbody>
</table>

Table 1.1 Classification of skin type and melanoma risk

1.1.3.3 Family History

One first-degree relative affected by melanoma has been reported to increase an individual’s risk of melanoma by 2.3 times normal and by 8 times in an individual with two affected relatives (Holman et al. 1984). A number of inherited genetic abnormalities are associated with familial melanoma, with loss of the p16 tumour suppressor gene being the most important (FitzGerald et al. 1996).

1.1.3.4 Pre-existing Naevi

The presence of congenital moles, a high mole-count, dysplastic moles or lentigo maligna all increase the risk of developing malignant melanoma (Holman et al. 1984).

Dysplastic naevi are both markers for an increased risk of melanoma and potential precursor lesions from which melanoma may develop (Albert et al. 1990). They are larger than common melanocytic naevi and have irregular pigmentation and borders that are qualitatively similar, but not as extreme as those seen in melanoma.
They differ in histological appearance from common melanocytic naevi in their atypical architectural, cytologic and stromal features (Clemente et al. 1991). Individuals with familial dysplastic naevus syndrome (or Atypical Naevus Syndrome, AMS) have a high risk of melanoma. Those with dysplastic naevi, and two or more first-degree relatives with dysplastic naevi and melanoma, have an estimated lifetime risk of melanoma approaching 100 percent (Kraemer et al. 1983, Greene et al. 1985). However, such families are uncommon and the risk associated with sporadic dysplastic naevi is therefore more important. The prevalence of sporadic dysplastic naevi in the general population is estimated at between 5 and 10% on average, but estimates have ranged to 50% or more, clearly reflecting different diagnostic criteria (Kraemer et al. 1983, Koh 1991). Most authorities presume that sporadic dysplastic naevi increase the risk of melanoma, but there is no agreement on the magnitude of the risk (Koh 1991).

A summary of risk factors for the development of cutaneous melanoma is shown in Table 1.2.
### Table 1.2: Risk factors for the development of cutaneous melanoma collated from different studies assessing individual risk factors

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adulthood ( &gt;15 years )</td>
<td>88</td>
</tr>
<tr>
<td>Pigmented lesions</td>
<td></td>
</tr>
<tr>
<td>Dysplastic moles (and familial melanoma)</td>
<td>148</td>
</tr>
<tr>
<td>Dysplastic moles (no familial melanoma)</td>
<td>7 - 70</td>
</tr>
<tr>
<td>Lentigo maligna</td>
<td>10</td>
</tr>
<tr>
<td>Higher than average no of benign naevi</td>
<td>2 - 64</td>
</tr>
<tr>
<td>Congenital mole</td>
<td>17 - 21</td>
</tr>
<tr>
<td>White (vs black) race</td>
<td>12</td>
</tr>
<tr>
<td>Previous cutaneous melanoma</td>
<td>5 - 9</td>
</tr>
<tr>
<td>Cutaneous melanoma in parents, children, siblings</td>
<td>2 - 8</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>2 - 8</td>
</tr>
<tr>
<td>Sun sensitivity</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Excessive sun exposure</td>
<td>3 - 5</td>
</tr>
</tbody>
</table>

1.1.4 **Clinical features of cutaneous melanoma**

There are a number of distinct morphological types of cutaneous melanoma which are used to categorise the disease. Five major growth patterns have been described each distinct from the pattern of benign lesions and exhibiting a differing prognosis.

1.1.4.1 **Superficial Spreading Melanoma (SSM)**

Approximately 70-80% of melanomas in most clinical series are of superficial spreading type (Fitzpatrick et al. 1992). They often develop from pre-existing moles
over the course of several months. The lesion is usually flat with an irregular edge and coloration. Prior to achieving the full invasive state, tumour cells may grow horizontally above the basement membrane and are classified as melanoma in situ during this stage. Invasion into the underlying dermis, after breaching the epithelial basement membrane, is initially through lateral spread and is termed the radial growth phase (Clark et al. 1984). After several months some areas within the tumour may become nodular due to an increased rate of proliferation. This constitutes the vertical growth phase and is thought to be associated with the potential of tumour cells to invade dermal capillaries and lymphatics, therefore allowing micrometastasis to occur (Clark et al. 1984). An example of a superficial spreading melanoma is shown in Figure 1.1.

### 1.1.4.2 Nodular melanoma (NM)

This is the second commonest form of primary cutaneous melanoma contributing approximately 15-30% of cases in most series (Fitzpatrick et al. 1992). Nodular melanomas are more likely than SSM to develop de novo in unaffected skin, although they can also arise from a pre-existing melanocytic naevus. They tend to have a shorter clinical history than SSM and are more common in males than females (Fitzpatrick et al. 1992). NMs are more protuberant and symmetrical in outline than SSMs. Their histological appearance is characterised by a direct vertical growth phase and high mitotic rate which is associated with aggressive clinical behaviour and the appearance of ulceration of the tumour surface. Approximately 5% of NMs are devoid of pigmentation (i.e. amelanotic) which may delay the clinical diagnosis. An example of a nodular melanoma is shown in Figure 1.2.
1.1.4.3 Lentigo maligna melanoma (LMM)

Lentigo maligna melanoma account for 4-10% of primary melanomas in large series (Fitzpatrick et al. 1992). They show a more marked relationship with sun exposure than any of the other varieties of primary disease. LMM is most often seen on the face of elderly patients developing over a period of years from a large pre-existing naevus, referred to as a lentigo maligna. The pre-malignant phase is often as large as 3cm in diameter and resembles a large freckle. Over a period of years irregularities in colour and outline develop which represent foci of radial growth phase melanoma. Later, this may progress to the development of a vertical growth phase tumour with an increased capacity for invasion. An example of a LMM is shown in Figure 1.3.

1.1.4.4 Subungual (SUM) and Acral Lentiginous Melanoma (ALM)

These variants of primary melanoma are rare in Caucasians, accounting for 1-5% of primary tumours, but up to 90% of those seen in dark skinned or oriental people (Reintgen et al. 1982). SUM arises from the nail bed, and may therefore mimic a number of benign conditions therefore delaying the diagnosis (Briggs 1985). ALM arises on the palms of the hands or soles of the feet, and also tends to present late (Sutherland et al. 1993). These tumours have a poor prognosis independent of the advanced stage at which they present (Balch et al. 1992). Examples of an SUM and a ALM are shown in Figures 1.4 and 1.5.

1.1.4.5 Desmoplastic Melanoma

Desmoplastic melanoma is extremely rare and is characterised by spindle-shaped invasive tumour cells accompanied by an increase in collagen. This subtype of
melanoma most often arises in sun-exposed skin, usually in the head and neck region, particularly on the face, where it may be associated with lentigo maligna (Jain et al. 1989). This tumour occurs in the elderly and affects males most commonly. The clinical appearance is of a dermal nodule which is not very distinctive, unless a pre-existent pigmented lesion such as lentigo-maligna is present. Desmoplastic melanoma is an aggressive neoplasm with a significantly worse prognosis than other varieties of melanoma (Mooi et al. 1992).
Figure 1.1: Superficial-spreading melanoma

Figure 1.2: Nodular melanoma
Figure 1.3: Lentigo maligna melanoma

Figure 1.4: Subungual melanoma
Figure 1.5: Acral lentiginous melanoma
1.1.5 Treatment of melanoma

1.1.5.1 Management of primary melanoma

Patients who are concerned about a change in the appearance of a mole should be reviewed by a specialist with an interest in pigmented lesions, such as a dermatologist or plastic surgeon. Many hospitals now offer a pigmented lesion clinic offering a prompt opinion on suspicious lesions by clinicians with an interest in the field.

Symptoms or signs which should raise suspicion include change in the size or shape of a mole — particularly where the size exceeds 6mm or the outline is irregular. Change in or irregular pigmentation and itching or bleeding should also alert suspicion. Loss of skin surface markings is also associated with malignant change.

Where malignant melanoma is suspected a complete examination of the patient should be performed noting particularly sites of potential metastasis such as the regional lymph nodes and liver.

Where there is suspicion or doubt regarding a mole it should be excised, initially by excision biopsy with a narrow margin. Studies, even in specialist clinics, show a variability in diagnostic accuracy for melanoma using clinical means alone. Rates of between 64% and 80% have been reported (MacKenzie Wood et al. 1998, Morton et al. 1998). Therefore histological examination is important.

If histology confirms the diagnosis of malignant melanoma baseline investigations of full blood count, urea and electrolytes, liver-function tests and chest X-ray should be performed followed by re-excision of the scar with a wider margin. This aims to remove any further local deposits of disease which may have been missed by the initial excision. The extent of this margin has been the subject of debate over many years.

Initial studies by Handley in 1907 on post-mortem specimens lead to the recommendation that skin margins for melanoma excision should be 2.5cm from the border of the tumour and should be extended to a margin of 5cm in the subcutaneous tissue down to and including the deep fascia. These guidelines
became routine surgical practice over the next fifty years. However, margins of this magnitude required skin grafts for wound closure, a general anaesthetic and several days of post operative hospital stay.

More recently a number of studies have shown that narrower margins can safely be used in the majority of cases. In 1979, the WHO commenced a randomised prospective trial comparing 1 cm and 3 cm excision margins in tumours less than 2 mm thick. 612 patients were evaluable in this trial and matched for age, sex, tumour thickness and site. At publication, the median follow up was 100 months and there was no significant difference in overall survival between the two groups (Veronesi et al. 1988). However, subsequent analysis revealed a slight difference in recurrence rate for tumours 1-2 mm thick treated by the two widths of excision. Five local recurrences were observed in the group with the narrow margin (tumours 1-2 mm thick) compared to one local recurrence in the group having the wider margin. This trial therefore showed that a 1 cm excision margin was safe for tumours below 1 mm in thickness but was inconclusive for tumours between one and two millimetres in thickness. However, the overall recurrence rate whichever margin was applied remained very low at 3% (Veronesi et al. 1991).

A subsequent multi-institutional study randomized 486 patients with intermediate-thickness lesions between 1.0 and 4.0 mm on the trunk and extremities to excisions with margins of either 2 cm or 4 cm (Balch et al. 1993). No significant difference was found in the rate of local recurrence or in-transit metastases. Recurrence rates in both groups did not correlate with surgical margins even among stratified thickness groups. This data led to a reduction in margins of excision for intermediate-thickness (1.0 - 4.0 mm) lesions to 2.0 cm.

Current guidelines for margins of excision for cutaneous malignant melanoma are (Harris et al. 1995):

1. Lesions classified as < 1.0 mm in thickness: conservative excision with margins no greater than 1.0 cm, thereby allowing a primary closure in virtually all instances.

2. Intermediate-thickness lesions (1.0 – 4.0 mm): 2.0-cm margins are appropriate.
3. Thick lesions (> 4.0 mm): margins wider than 2.0 cm may be required to optimise local control.

A study organised by The Melanoma Study Group (MSG) and the British Association of Plastic Surgeons (BAPS) is currently in progress to assess the effect of 1 cm versus 3 cm excision margins for tumours greater than 2 mm in thickness.

Where possible, after excision of the primary tumour wounds should be closed by primary closure. For larger defects split-thickness skin grafts or otherwise flaps may be necessary. The current recommendations for narrower excision margins have allowed the majority of wounds to be closed by primary closure. Balch et al noted that the need for skin grafting was reduced from 47% with 4.0-cm margins to 11% with 2.0-cm margins (Balch et al. 1993). In cosmetically important areas, such as the face, the reduction in size of excision margins has allowed significant improvement in cosmetic results.

Melanomas on the digits, such as subungual melanomas, usually require amputation of the digit following biopsy for histology. Amputation through the proximal interphalangeal joint is performed if margins allow and, in the case of the thumb, leaves a stump that is still of functional benefit. Lesions of the scalp can be closed with advancement flaps or skin grafts depending on the cosmetic needs of the individual patient.

1.1.5.2 Elective Lymph Node Biopsy

Patients with thick primary tumours are at significant risk of recurrence. Those with tumours greater than 4 mm thickness have a 5-year survival rate of 47% (Koh 1991). There has therefore been considerable debate as to whether further surgical or medical intervention before recurrent disease is evident would be of benefit to patients.

A number of studies first looked at elective lymph node dissection (ELND) to assess if excision of the regional lymph nodes, removing any potential micrometastatic deposits before they became clinically evident, gave patients
survival advantage. Of the four randomised prospective studies performed (Veronesi et al. 1977, Veronesi et al. 1982, Cascinelli et al. 1993, Balch et al. 1996), none have shown any benefit in patient survival except for two patient sub-populations in the Intergroup study (Balch et al. 1996) - patients over 60 years old and those with thick tumours 1-2mm thickness (Balch et al. 1996). Since the procedure is accompanied by significant morbidity ELND has ceased to be part of normal practice.

1.1.5.3 Sentinel Lymph Node Biopsy

Sentinel lymph node biopsy (SLNB) has been shown to be an accurate method for detecting the overall status of the regional lymph nodes for patients with malignant melanoma (Morton et al. 1999). The use of pre-operative lymphoscintigraphy and the injection of blue-dye and radioisotopes adjacent to the primary lesion are all necessary to provide accurate identification of the sentinel node (SLN). Direct visualisation of the blue dye and the use of a hand-held gamma camera identify the correct node at operation.

Some studies have also shown the status of the SLN to be a highly accurate prognostic marker (Gershenwald et al. 1999). However it is an expensive and time-consuming procedure and is associated with complications. The use of SLNB has not yet been shown to produce any survival advantage for melanoma patients – the Sunbelt and the Multicentre Selective Lymphadenectomy studies are currently underway to investigate this.

There are two potential ways in which SLNB could produce a survival advantage to melanoma patients. Either detection of occult lymph node metastases leading to a subsequent lymph node dissection would improve patient survival, or secondly, detection of nodal micrometastasis would lead to treatment of a patient with adjuvant therapy who otherwise would not have received it. However, the results of ELND as discussed above do not appear to show a survival benefit from this procedure which negates the first argument in favour of SLND. Furthermore, at present the use of interferon, the only licensed adjuvant therapy for melanoma, is
controversial as will be discussed later in this chapter, as the published trials do not agree on whether it produces survival benefit. The role of sentinel node biopsy in melanoma therefore remains controversial.

1.1.6 Prognostic factors in melanoma

1.1.6.1 Established prognostic factors

The most important prognostic factor used in melanoma to date has been tumour thickness. In 1953, Allen and Spitz, suggested that thicker primary tumours carried a worse prognosis, a conclusion also reached by Peterson (Peterson 1962). This relationship was confirmed by Clark in 1969 who classified primary melanoma on the basis of microscopic dermal invasion (Clark et al. 1969). Five levels of invasion were defined as follows: tumour confined to the epidermis (level I), invasion of the papillary dermis (level II), tumour cells at the junction of papillary and reticular dermis (level III), invasion of the reticular dermis (level IV) and invasion of the subcutaneous tissue (level V). Clark observed that clinical outcome correlated with the depth of invasion and was worst in tumours which had invaded beyond the dermis, closer to the lymphatic and blood vessels.

Breslow simplified the prognostic assessment of microscopic tumour examination by utilising the maximum vertical height of the tumour alone (Breslow 1970). Five year survival rates correlated with tumour thickness are illustrated in Table 1.3 (Koh 1991).

The Breslow thickness has been shown to be more accurate than Clarke’s levels and to be the most accurate prognostic variable by a number of studies (Balch et al. 1992), (Van Der Esch et al. 1981). Survival is influenced by Breslow thickness within each of levels III, IV and V but is not influenced by Clarke’s levels of invasion within each Breslow thickness subset.

A number of other factors have also been shown to provide useful prognostic information. Patient’s age is known to be a significant factor. Although older patients tend to present with thicker melanomas, they are known to have a poorer
prognosis when compared to younger patients, even after controlling for tumour thickness (Balch et al. 1992).

<table>
<thead>
<tr>
<th>Tumour thickness (mm)</th>
<th>Five year survival (%)</th>
</tr>
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<tbody>
<tr>
<td>&lt;0.75</td>
<td>96</td>
</tr>
<tr>
<td>0.75-1.49</td>
<td>87</td>
</tr>
<tr>
<td>1.5-2.49</td>
<td>75</td>
</tr>
<tr>
<td>2.5-4.0</td>
<td>66</td>
</tr>
<tr>
<td>&gt;4.0</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 1.3: The thickness of primary melanoma and five-year survival.

Patient's sex is also significant. Male patients with melanoma are known to have a poorer prognosis than females, even when controlling for their higher incidence of thicker, ulcerated lesions and lesions present on the trunk (Balch et al. 1992).

Although the incidence of melanoma is greater in higher socio-economic groups, the prognosis is worse for patients of lower social class (MacKie et al. 1996).

The site of the primary lesion also provides important prognostic information. Lesions on the trunk and head and neck have a significantly worse prognosis than those on the limbs (Balch et al. 1992). Those on the hands and feet have a poorer prognosis compared to those on other parts of the limbs (Day et al. 1981, Day et al. 1981). In the head and neck region, melanomas on the scalp are associated with a poorer prognosis compared to those on the face and ear (Urist et al. 1984).

The influence of tumour growth pattern has also been of great interest. Although nodular melanomas were thought to carry a poorer prognosis than the superficial spreading type, after allowing for tumour thickness, the 10-year survival rates are the same (Balch et al. 1992). Lentigo maligna melanomas have a better prognosis even when allowing for tumour thickness – 10-year survival rates of 80% have been reported for lesions as thick as 3mm (McGovern et al. 1980). Acral lentiginous melanomas have been shown to be associated with a worse prognosis even when accounting for tumour thickness (Balch et al. 1992).
Tumour ulceration is known to be associated with more aggressive tumour behaviour, even when controlling for tumour thickness and other prognostic factors such as male sex which are both associated with tumour ulceration (Balch et al. 1992).

Tumour mitotic rate is an independent prognostic factor (Clark et al. 1989). However, the presence of microscopic tumour satellites and vascular invasion have been shown to provide prognostic significance by some, but not all, studies when assessed by multivariate analysis (Day et al. 1982, Clark et al. 1989).

Survival analysis of the large number of patients on the University of Alabama and Sydney melanoma database has confirmed Breslow thickness to be the most significant prognostic factor for predicting survival in melanoma patients. Tumour ulceration and the site of the primary were the second and third most significant prognostic factors respectively followed by patient sex (Balch et al. 1992).

<table>
<thead>
<tr>
<th>Table 1.4: Established Prognostic Factors in Primary Melanoma</th>
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<tbody>
<tr>
<td>Breslow thickness</td>
</tr>
<tr>
<td>Ulceration</td>
</tr>
<tr>
<td>Site</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Histogenic Type</td>
</tr>
<tr>
<td>Others: Clarke’s levels</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Mitotic rate</td>
</tr>
<tr>
<td>Lymphocyte response</td>
</tr>
<tr>
<td>Socio-economic group</td>
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</table>
The Breslow thickness of the primary tumour directly effects the management of the primary lesion by determining the margin of surgical excision that should be performed. A number of studies have shown that tumours less than 1mm in vertical height should be excised with a minimum margin of 1cm of normal tissue. Those 1-4mm in total height should be excised with a minimum of 2cm margin (Veronesi et al. 1991, Balch et al. 1993, Harris et al. 1995).

Staging of the patient provides useful prognostic information for more advanced disease. A number of classifications systems have been described. Currently the most widely used system is the Modified American Joint Committee on Cancer (AJCC) Staging System which classifies melanoma into four stages as shown in Table 1.5. Survival rates can be calculated according to these stages (Ketchem et al. 1992).

Table 1.5: The Modified AJCC Classification of Melanoma

<table>
<thead>
<tr>
<th>Stage</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Primary melanoma &lt;= 0.75 mm thick and/or Clark Level II</td>
</tr>
<tr>
<td>IB</td>
<td>Primary melanoma 0.76 - 1.5 mm thick and/or Clark Level III</td>
</tr>
<tr>
<td>IIA</td>
<td>Primary melanoma 1.51 - 4.0 mm thick and/or Clark Level IV</td>
</tr>
<tr>
<td>IIB</td>
<td>Primary melanoma &gt; 4.0 mm thick or Clark level V</td>
</tr>
<tr>
<td>III</td>
<td>Regional lymph node or in-transit metastasis</td>
</tr>
<tr>
<td>IV</td>
<td>Systemic metastasis including subcutaneous or lymph node metastasis beyond the regional nodes</td>
</tr>
</tbody>
</table>

For stage III patients with lymph node metastasis, the number of positive lymph nodes and extra-capsular extension are prognostic (Ahmed 1997). Ulceration and the thickness of the primary tumour have also been shown to have prognostic significance (Ahmed 1997), as has the number of regional lymph node groups involved (Barth et al. 1991). For stage IV disease visceral metastases have a worse prognosis than other sites (Ahmed 1997) and the number of sites involved is also
prognostic. More recently studies have demonstrated that Albumin and LDH levels may be more accurate than site in predicting prognosis for patients with stage IV (Eton et al. 1998). The prognostic factors used in stage III and IV patients are shown in Table 1.6.

<table>
<thead>
<tr>
<th>Table 1.6: Prognostic Factors in Stage III and IV Melanoma</th>
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</thead>
<tbody>
<tr>
<td>Stage III: Number of positive lymph nodes</td>
</tr>
<tr>
<td>Extra-capsular spread</td>
</tr>
<tr>
<td>Breslow thickness</td>
</tr>
<tr>
<td>Ulceration</td>
</tr>
<tr>
<td>Number of regional lymph node groups involved</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage IV: Visceral metastases have a worse prognosis than other sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sites involved</td>
</tr>
<tr>
<td>Serum [LDH]</td>
</tr>
<tr>
<td>Serum [albumin]</td>
</tr>
</tbody>
</table>

Although the Breslow thickness of the primary tumour is the most accurate prognostic indicator for stage I and II disease, it may fail to accurately predict patient outcome in a significant number of patients, particularly those with thin and intermediate thickness melanomas (Slingluff et al. 1988, Andersson et al. 1996). Late recurrence has been shown to occur in up to 5% of patients with primary melanomas less than 0.76mm (Slingluff et al. 1988) – increased risk of recurrence was found to be associated with male sex, level IV lesions and lesions on the trunk. Therefore a number of other methods for assessing prognosis in melanoma patients have been investigated in an attempt to improve on the accuracy provided by the Breslow thickness.
1.1.6.2 Prognostic models

Models combining some of the other prognostic factors, such as anatomic site, age, and sex, with Breslow thickness have been reported to be more accurate than the Breslow thickness alone in predicting prognosis (Clark et al. 1989, Halpern et al. 1997).

1.1.6.3 Measurement of tumour vascularity

The degree of tumour vascularity also gives prognostic information (as measured by microscopy and CD31 expression (Vlaykova et al. 1997). Expression of cell-surface molecules is required to maintain adherence to the endothelial basement membrane. High expression levels of the integrin VLA-4 (present on tumour cells) and the selectins ELAM-1 and CD62 (present in tumour-associated blood vessels) are associated with significantly shorter survival (Schadendorf et al. 1993, Schadendorf et al. 1995).

1.1.6.4 Cell Cycle Proteins

More recently the underlying tumour biology of melanoma has been studied in more detail and some of the changes in oncogene expression which give rise to the development and progression of the primary tumour, and any subsequent metastasis, have been described. This pattern of oncogene expression, and other associated changes in phenotype of the tumour (such as changes in cell surface molecule expression) which result, directly affects the biological behaviour of the tumour. They therefore determine the ability of the tumour to invade adjacent tissues, metastasise, responses to various treatment modalities and therefore, ultimately, the duration of patient survival. They can therefore provide very useful prognostic information regarding patient survival.
Oncogenes encode a number of proteins which control the cell cycle and therefore are able to influence cell growth rates and other types of cell behaviour as discussed previously.

Expression levels of the c-myc oncogene have recently been shown to be an independent prognostic factor, more accurate than other clinico-pathological factors including Breslow thickness, for a range of different types of melanoma (Grover et al. 1997, Grover et al. 1997, Chana et al. 1998, Chana et al. 1998, Ross et al. 1998).

The oncogene bcl-2 blocks apoptosis, or programmed cell-death. Over-expression of this oncogene has been shown to be significantly associated with poorer survival in patients with metastatic melanoma (Grover et al. 1996).

Loss of the tumour suppressor gene p16 has been shown to correlate with the development of primary melanoma and progression to metastasis, although it was not shown to provide prognostic information in the study performed by Grover et al using flow cytometry (Grover et al. 1998). A study performed by Straume et al using immunohistochemistry however did show an association between loss of p16 and disease recurrence (Straume et al. 1997).

Expression of mutant p53 has been shown to be associated with tumour thickness, ulceration and lymph node metastasis (Yamamoto et al. 1995). Overexpression of wild-type p53 has been shown to be associated with improved survival in some but not all studies (Florenes et al. 1995, Ross et al. 1997).

### 1.1.6.5 Immunological and Other Markers of Prognosis

The numbers of tumour infiltrating lymphocytes (TIL) present in a melanoma has been shown to be significantly associated with length of survival (Clemente et al. 1996) – those patients with higher numbers of TIL’s present (termed a “brisk response”) survive for longer.

Expression of particular cell surface molecules by melanomas may provide information about prognosis and potential responsiveness to immunotherapy. Cell surface expression of the class II HLA antigen DQB *0301 by primary melanomas is known to be associated with a poorer prognosis (Lee et al. 1996). However,
Table 1.7: Experimental Prognostic Factors in Melanoma

<table>
<thead>
<tr>
<th>Prognostic models</th>
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<tbody>
<tr>
<td>Measures of tumour vascularity:</td>
</tr>
<tr>
<td>VLA-4, CD31, ELAM-1, avβ3</td>
</tr>
<tr>
<td>Cell cycle proteins:</td>
</tr>
<tr>
<td>p53, c-myc, p16, p53</td>
</tr>
<tr>
<td>Immunological factors:</td>
</tr>
<tr>
<td>TIL’s</td>
</tr>
<tr>
<td>HLA DQB*031</td>
</tr>
<tr>
<td>IL-2, IL-6</td>
</tr>
<tr>
<td>Gp-100, MART-1, MAGE</td>
</tr>
<tr>
<td>GM-2 antibody</td>
</tr>
<tr>
<td>RT-PTCR detecting circulating</td>
</tr>
<tr>
<td>melanoma cells</td>
</tr>
<tr>
<td>Serum S-100 levels</td>
</tr>
<tr>
<td>Sentinel lymph node biopsy</td>
</tr>
</tbody>
</table>

Expression of the antigens gp100, MART-1 and MAGE is thought to be associated with increased responsiveness to immunotherapy (Slominski et al. 1995). Patients with high IL-6 levels have been shown to obtain less survival benefit from IL-2 therapy as IL-6 is thought to act as a growth factor for tumour cells. High tumour expression of receptors for the growth factors EGF, TGF-β2, IGF-1 (Slominski et al. 1995) and IL-2 (Boyano et al. 1997) are also thought to be associated with a poorer prognosis for the same reason.

The presence of GM2 antibodies in patient’s serum following vaccination with ganglioside vaccines has been shown to be associated with improved survival (Livingston et al. 1989).

Other postulated prognostic factors for melanoma under investigation include levels of melanin metabolites in the serum, such as 6H5MI2C (Slominski et al. 1995).
1.1.6.6 Detection of circulating melanoma cells in the peripheral blood

The ability to detect metastatic cells circulating in the peripheral blood at concentrations as low as 1 cell in 2ml of blood has been demonstrated using the reverse-transcriptase polymerase chain reaction (RT-PCR) technique (Smith et al. 1991) and may prove useful for detecting patients at high risk of metastasis. This technique detects messenger RNA encoding the enzyme tyrosinase, the enzyme responsible for the catalysis of tyrosinase to dopaquinone in melanin synthesis, and is therefore present in melanoma cells. Some studies have shown RT-PCR positivity to provide useful prognostic information (Ghossein et al. 1998, Curry et al. 1999). It can also identify circulating melanoma cells at an early stage to be clinically useful. However there is wide variation in results between the published studies (Brossart et al. 1993, Hoon et al. 1995, Kunter et al. 1996). RT-PCR detecting tyrosinase in peripheral blood failed to identify up to a third of patients who went on to develop recurrence and also showed significant false positives in some studies (Curry et al. 1998). This may be because tumour cells are only shed intermittently into the circulation. However, it is likely that not all the tumour cells that are shed necessarily have metastatic potential, which may lead to false positive results; this has been shown to occur in as many as 16% of patients (Curry et al. 1999). These high false negative and significant false positive rates have limited the clinical usefulness of RT-PCR detection of melanoma cells in the peripheral blood at present.

1.1.6.7 Serum S-100 levels

S-100 is an acidic-calcium-binding protein composed of alpha and beta subunits. It is known to be present in cells of neuroendocrine origin, including melanoma cells, and is detected in the serum of melanoma patients. Increasing serum S-100 levels are associated with increasing stage of disease (Banfalvi et al. 1999) and have been shown to provide prognostic information (von Schoultz et al. 1996). However, the
sensitivity of S-100 levels for detecting recurrence has been shown to be less than RT-PCR in some studies (Curry et al. 1999).

1.1.6.8 The role of prognostic factors in melanoma

The importance of prognostic factors is that they indicate which patients have the most biologically aggressive tumours and therefore influences the width of primary tumour excision to obtain optimal local control. They also predict those patients at highest risk of distant metastasis and, therefore, those who might benefit most from adjuvant treatment. The newer biological factors such as oncogene expression may offer more important information as they indicate which biological pathways have played the most significant role in the pathogenesis of the tumour and therefore which represent potential targets for new therapeutic strategies such as gene therapy. In addition, the use of markers to assess the tumour and patient immunological status may aid patient selection for treatment with tumour vaccines. Ultimately, a combination of some of these newer prognostic factors with the more established clinico-pathological parameters such as the Breslow thickness may allow clinicians to predict more accurately those patients at greatest risk of recurrence and those likely to obtain the maximum benefit from adjuvant therapy.

1.1.7 Adjuvant treatment of melanoma

A significant number of patients who are potentially rendered disease-free by surgery remain at high-risk from recurrent disease. These include patients with deep primary melanomas (stage II disease) and those with regional lymph node metastasis (stage III disease) who, following resection of macroscopic disease, may harbour micrometastatic deposits of melanoma. Patients with primary lesions greater than 4mm thickness have a 5-year survival rate of 47% (Koh 1991) and those with lymph node metastases 10-46% - dependent on the number of nodes
involved and other prognostic factors (Buzaid et al. 1997). These groups of patients may therefore benefit from adjuvant treatment - ie treatment to eliminate any micrometastatic deposits that may exist elsewhere in the body at the time of surgery for the primary tumour or lymph node metastasis.

A number of strategies for adjuvant therapy have been proposed. Chemotherapeutic agents such as Dacarbazine, immune stimulants such as BCG, and combinations of immunotherapy and chemotherapy have all failed to show significant benefit in randomised trials (Johnson et al. 1995, Demierre et al. 1997). Hyperthermic isolated limb perfusion with the combination of melphalan, tumour necrosis factor and interferon showed benefit as adjuvant therapy for resected stage II and III disease patients in an early trial (Ghussen et al. 1989). However, these benefits were not demonstrated by a subsequent phase III trial performed by the World Health Organisation (Lejeune et al. 1999).

More recently attention has focused on the use of interferons (IFN) as adjuvant agents. These naturally occurring substances can now be produced synthetically. In addition to direct anti-tumour activity they may also up-regulate the expression of HLA and tumour-specific antigens on melanoma cells and stimulate the activity of anti-tumour natural-killer cells and lymphocytes.

A number of trials have investigated the role of IFNα2b as adjuvant therapy. The Eastern Co-operative Oncology Group Trial 1684 studied 287 melanoma patients randomised to receive high-dose IFN or observation between 1984 and 1990 (Kirkwood et al. 1996). 28 patients had deep primary melanoma (>4mm - AJCC stage IIB disease) and 249 had regional lymph node metastasis (stage III disease). All patients in the study had undergone lymph node dissection. The initial phase of treatment consisted of IFNα2b at 20 MU/m²/day IV, five days a week for 4 weeks followed by 10 MU/m²/day subcutaneously for 48 weeks. The results of this trial showed relapse-free survival to be increased in the IFN treated group from 1.0 to 1.7 years (p = 0.002). Median survival was also increased in the IFN-treated group from 2.8 to 3.8 years at 7 years of follow-up (p = 0.02). This effect was only seen in the larger group of patients with lymph-node metastases and not in those with deep primary melanoma who comprised only 11% of the patients. IFN was subsequently
licensed as adjuvant therapy for high-risk melanoma patients following publication of this study.

To date only IFN administered by the high-dose, one-year regime used in ECOG 1684 has been shown to offer survival benefit. Other trials (Cascinelli 1995), (Creagen et al. 1995) (Grob et al. 1998, Pehamberger et al. 1998) at lower doses have not demonstrated survival benefit. However, at high doses, significant side-effects from interferon occur. Dosage reductions were required in 50% of patients in the ECOG 1684 trial, although this was not shown to effect the efficacy of the treatment in those patients where this was necessary (Kirkwood et al. 1997).

A number of other trials investigating the use of IFN as adjuvant treatment for high-risk melanoma patients are currently in progress. The ECOG 1690 trial is comparing the results of conventional high-dose IFN therapy (as in ECOG 1684) with a lower-dose strategy of IFNα2b of 3MU subcutaneously three times a week. In addition, the number of patients with thicker primary lesions is higher (24% of all patients) than in the ECOG 1684 trial and this may clarify the effect of adjuvant IFN for patients in this group. Preliminary results have reported an increased length of relapse-free survival with the high-dose compared with the low-dose interferon regime but no effect on overall survival has yet been demonstrated (Kirkwood 1999).

The preliminary results of this trial are controversial as it has failed to repeat the overall survival benefit shown by the ECOG 1684 trial. The organisers of the trial argue that this is because the control, untreated group demonstrated improved survival rates compared with the control group in ECOG 1684. They speculate that this is because they received interferon treatment following relapse and therefore survived for longer. This makes the interpretation of the results of this trial difficult at this stage. The final results will therefore be of great interest.

A further trial, ECOG 1697, is investigating the effect of a month course of high-dose IFN alone with observation to determine if the high plasma doses of IFN produced in the induction phase of ECOG 1684 are responsible for the adjuvant effect. This dosage strategy avoids the side-effects, and cost, of the subsequent weekly sub-cutaneous doses over the following 48 weeks.
At present, although there is evidence provided by the ECOG 1684 trial of some survival benefit in patients treated with high-dose IFN, this remains to be confirmed by other studies. The treatment exposes patients to significant side-effects and is very costly. The use of adjuvant IFN therapy for melanoma is therefore controversial at present.

If IFN proves to offer benefit to a subgroup of melanoma patients as adjuvant therapy the development of methods to predict which patients are sensitive to it, and might therefore benefit from its use, will be important. This would avoid exposing resistant patients to the side-effects of its use.

In view of these considerations the role of other possible adjuvant strategies is of great interest. Vaccines have shown potential for use as adjuvant agents for high-risk melanoma patients and their development will be discussed in more detail later. They offer the prospect of producing adjuvant effect without the degree of toxicity accompanying the use of IFN. The combination of the ganglioside vaccine GM2 with BCG was shown to provide disease-free and overall survival benefit in those patients who were GM-2 antibody positive in a trial for stage III patients (Livingston et al. 1994). The Intergroup phase III trial E1694 therefore is currently assessing the effectiveness of the GM2 ganglioside vaccine, in combination with KLH and QS-21 adjuvant agents, compared with the high-dose IFN regimen used in the ECOG 1684 trial. In Europe, this vaccine is being compared with placebo in a phase III trial. An additional Intergroup phase II trial comparing concurrent with sequential use of IFN and vaccines for patients with resectable melanoma is also underway (Kirkwood 1998). Previous trials of autologous vaccines used as adjuvant agents have not shown benefit (McIlmurray et al. 1977). A vaccinia virus oncolysate has been used as an adjuvant tumour vaccine in a phase III trial performed by the AJCC and was shown to produce survival benefit but this was not sufficient to demonstrate statistical significance (Wallack et al. 1996).

Gene therapy is another potential treatment strategy which may prove useful as an adjuvant treatment in the future but as yet has not reached a sufficient stage of development to be investigated for this purpose in patients.
1.1.8 Treatment of metastatic melanoma

1.1.8.1 Management of regional lymph node metastases

Surgical excision by block dissection remains the treatment of choice for clinically involved regional lymph nodes. Morbidity is greater after inguinal than after axillary dissection. The frequency of wound complications can be reduced by trimming the skin flaps before closure and transposing the sartorius muscle to cover the femoral vessels. In a report of 168 patients undergoing inguinal dissections at the MD Anderson Cancer Centre, wound complications occurred in 20% of cases and were related to age (over 50 years) and smoking (Beitsch et al. 1992). Besides anecdotal reports, there is no convincing evidence that survival is altered after radical ilio-obturator lymph node dissection (RID) relative to superficial femoral dissection (SFD). However, in a retrospective study of 133 patients at the Royal Marsden Hospital, groin recurrence was significantly more frequent after SFD (57.1%) than after RID (23.3%) (Kissin et al. 1987).

1.1.8.2 Treatment of stage IV disease

The prognosis for patients with stage IV disease (spread beyond the regional lymph nodes) remains poor, 5-year survival rates are approximately 5% (Balch et al. 1992). This is largely because distant melanoma metastases respond poorly to conventional therapeutic options such as surgery, chemotherapy or radiotherapy. The most effective single chemotherapeutic drug to date has been Dacarbazine (DTIC) with overall response rates of 10-20%. However, these responses are generally of short duration only, with long-term complete responses only seen in 1-2% of patients (Hill et al. 1984). In addition the impact on visceral compared with skin and subcutaneous metastases is poor. Phase II studies have shown that Temozolomide is more effective against brain metastases (Bleehen et al. 1995).
A range of combination chemotherapy regimens have also been tested but few have shown advantages over DTIC alone. Cisplatinum, Vinblastine and DTIC (CVD) has been reported to yield overall response-rates of between 24% and 45% (Legha et al. 1989, Lee et al. 1995) in early trials but the results of randomised studies comparing this regimen with DTIC alone are not yet known. The Dartmouth regimen (Cisplatinum, Carmustine, DTIC and Tamoxifen) showed early promise in metastatic melanoma patients with overall response rates of 55% reported by early trials (Del Prete et al. 1984). However, later phase II trials by the Southwest Oncology Group showed overall response rates of only 15% (Margolin et al. 1998). The taxanes paclitaxel and docataxel have been shown to produce response rates of 15-19% in metastatic melanoma patients (Bedikian et al. 1995, Einzig et al. 1998). These rates are considerably lower than those produced in patients with metastatic ovarian or breast cancer (Foà et al. 1994) and are no better than those produced by DTIC alone in metastatic melanoma patients.

More recently the use of IFN and Interleukin-2 (IL-2) alone, and in combination with multiple chemotherapeutic drugs (biochemotherapy), has been investigated. Initial studies of IFN and IL-2 alone reported overall response rates of 16% and 15-20% respectively with complete responses in 4-6% of patients (Kirkwood 1995, Agarwala et al. 1996). However, biochemotherapy has yielded more promising results - phase II trials of IFN and IL-2 in combination with CVD have shown overall response rates of 62% with long-term durable complete remissions observed in up to 10% of patients (Legha et al. 1997). The results of phase III trials are awaited.

Section 2 Uveal Melanoma

1.2.1 Epidemiology

Melanoma is the commonest primary intraocular tumour. The uveal tract, comprising the iris, ciliary body and choroid is the most frequent site of origin.
The incidence of uveal malignant melanoma in the general population is estimated to be 7 per million per year (Egan et al. 1988). It can affect any age or racial group but white, caucasians are most commonly affected, with a peak incidence in late middle age.

Both naevi and malignant melanomas of the uvea are extremely rare in blacks and appear to be relatively rare in Asians (Shields et al. 1992).

1.2.2 Risk factors and precursor lesions

Ultraviolet light is not thought to play a part in the aetiology of uveal melanoma. A Danish study demonstrated no overall increase in the incidence of uveal melanoma during a period in which the incidence of cutaneous melanoma increased 5 to 6 fold (Osterlind 1987). These tumours therefore do not appear to be related to sun exposure.

Most uveal melanomas are thought to arise from pre-existing benign uveal naevi. Benign naevi have been reported in up to 2% of eyes on clinical examination and 6.5% at autopsy, however the chance of malignant change has been estimated to be less than 1 in 500 during a 10 year period (Ganley et al. 1973). In rare cases, melanomas have been observed to arise de novo, apparently from areas of the choroid that were previously examined and found to be clinically normal (Shields et al. 1992).

Ocular melanocytosis and oculodermal melanocytosis (naevus of Ota) are both strongly associated with uveal melanoma (Velazquez et al. 1983). Ocular melanocytosis is characterised by hyperpigmentation of the episclera and uveal tract and is estimated to occur in 0.04% of the white population. There is a dense concentration of heavily pigmented melanocytes in the iris, ciliary body, and choroid. Uveal melanoma associated with ocular melanocytosis usually occurs in the choroid, ciliary body or both.

Oculodermal melanocytosis (neavus of Ota) differs from ocular melanocytosis by the presence of hyperpigmentation of the periocular skin as well as the episcleral tissues and varies from a small, well-circumscribed naevus to widespread
involvement of both eyelids. Malignant melanoma has been known to occur in the skin, iris, ciliary body, choroid and orbit, meninges and brain in patients with this condition (Shields et al. 1992).

Finally, it has been reported that there is an increased incidence of uveal melanoma in individuals with Atypical Mole Syndrome (AMS), which is known to be associated with cutaneous melanoma (Albert et al. 1985).

1.2.3 Clinical features of uveal melanoma

Approximately 80% of uveal tract melanomas arise in the choroid where small asymptomatic lesions are frequently detected early during routine sight testing. The typical uveal melanoma presents as a brown, elevated, dome-shaped, sub-retinal mass. The degree of pigmentation ranges from dark brown to totally amelanotic. With time many tumours erupt through Bruch’s membrane to assume a mushroom-like appearance. Larger lesions may present with reduced visual acuity or a field defect caused either by the tumour itself, or from an associated retinal detachment or vitreous haemorrhage. If extensive retinal detachment occurs, anterior displacement of the lens-iris diaphragm and secondary angle-closure may result. Neovascularisation may also appear, and spontaneous haemorrhage into the sub-retinal space can occur. Vitreous haemorrhage is usually only seen in those cases where the melanoma has ruptured through Bruch’s membrane. Occasionally, the tumour exhibits a diffuse, spreading pattern of growth with a pronounced tendency to extrascleral extension (Hungerford 1993).

1.2.4 Determination of prognosis in uveal melanoma

In contrast to cutaneous melanoma, uveal melanoma does not spread to regional lymph nodes and the time to development of systemic metastases is relatively long. Therefore, staging based on the presence of regional disease is inapplicable. Many
uveal melanomas are treated primarily with radiotherapy and therefore information based on histological parameters of prognosis cannot always be obtained. However, a number of clinicopathological factors have been correlated with prognosis in patients with posterior uveal melanoma. These include the tumour cell type (McLean et al. 1977, Shammas et al. 1977), tumour diameter (Flocks et al. 1955), patient age (McLean et al. 1977), and the presence or absence of extrascleral extension (Shammas et al. 1977).

The study performed by Mclean et al concluded that the four most accurate factors for predicting prognosis in uveal melanoma were cell type, the largest tumour diameter, extrascleral extension and mitotic activity (McLean et al. 1977). Shammas et al found nine factors which significantly influenced prognosis following enucleation: age, tumour location, location of the anterior border of the tumour, the largest tumour diameter, height of the tumour, integrity of Bruch’s membrane, cell type, pigmentation, and scleral infiltration (Shammas et al. 1977). In general, older patients with larger tumours that contain epithelioid cells, or demonstrate extrascleral extension, have a worse prognosis following enucleation.

1.2.5 Treatment of uveal melanoma

1.2.5.1 Management of primary disease

The maximum size of a uveal melanoma tumour that is curable is not known. Many uveal melanomas are more than 5mm thick when first detected. Most of these have probably disseminated already, although the long lead time to the appearance of metastases may give the impression of a cure. Early detection of small melanomas is difficult because they are hard to distinguish clinically from choroidal naevi, which are thought to be the precursors of most of the malignant lesions. Unlike atypical cutaneous naevi, choroidal naevi are not amenable to diagnostic excision or biopsy. Fluoroscein angiography may be used to detect intrinsic vasculature but this has only been found to correctly predict malignancy in less than 50% of cases (Char et al. 1980). Thickness greater than 1.5mm, presence of orange lipofuscin pigment on
the surface of the tumour, and fluid leakage are all predictive of malignancy (Augsburger et al. 1989). Small peripheral lesions demonstrating all three features are treated immediately as small melanomas without waiting for documentary evidence of enlargement. Treatment of such tumours near the macula and optic disc is associated with substantial loss of vision and therefore it is recommended to watch these lesions with serial photographs and withhold treatment until sustained growth is documented (Hungerford 1993).

The controversy over whether enucleation provides the best chance of cure (Zimmerman et al. 1979, Manschot et al. 1980) has led to a trend away from this procedure and towards the increasing use of more conservative therapeutic methods (Shields et al. 1991). In specialist centres, most small melanomas are treated without removal of the eye. The conservative method chosen depends on the size of the tumour and its location within the uveal tract. Currently, radiotherapy is the most widely used treatment modality for most posterior uveal melanomas (Shields 1993, Hungerford 1995). The majority of tumours are treated by radiotherapy, with episcleral radioactive plaques sutured temporarily to the sclera over the base of the tumour (Hungerford 1995). Melanomas are relatively radioresistant and this method of treatment is used because it can deliver a high dose to the tumour whilst limiting the dose to the radiosensitive retina, choroid and optic nerve circulation to the maximum tolerated dose of below 50Gy (Hungerford 1995). Brachytherapy by radioactive plaque achieves these aims because of the rapid attenuation of radiation away from the source. Using radioactive plaques, a dose of between 80 and 100 Gy is delivered to the apex of the tumour at a dose rate of at least 40 cGy/h. The size of melanoma which can be treated by plaque radiotherapy without serious side effects depends on the energy of the source and the location of the tumour. Choroidal melanomas up to 5mm have been treated with β-ray emitting ruthenium-106/rhodium-106 plaques (Hungerford 1995). In some studies tumours up to 8mm thickness have been found to regress after treatment by iodine-125 plaques which emit X-rays (Bosworth et al. 1988).

When appropriately selected for radiotherapy, more than 90% of eyes can be successfully retained, often with good acuity or useful field of vision episodically (Hungerford 1995). Regression is slow and often continues episodically over two
years or more. Melanomas less than 5mm in thickness often regress to a flat scar, although larger tumours will have some residual thickness when regression ceases. On the basis of current information, 5-year survival rates for patients who receive radiotherapy are similar to those treated by enucleation (Shields et al. 1993). However, studies have shown that between 5 and 10% of patients will ultimately require enucleation of the affected eye because of recurrent tumour or radiation induced complications (Shields et al. 1989).

Enucleation is currently recommended for advanced melanomas with a volume of greater than 1.5cm³, for diffuse melanomas occupying more than a quarter of the choroid, for melanomas which have produced a secondary neovascular glaucoma or which show massive extrascleral extension, and for melanomas which have failed to respond to conservative therapy (Hungerford 1993). Another relative indication for enucleation is the rare instance in which the melanoma invades the optic nerve (Hungerford 1993). Although pre-enucleation radiotherapy has been advocated, non-randomised studies have suggested that it offers no advantages over enucleation alone (Char et al. 1988).

Direct xenon arc photocoagulation has been shown to be effective in the treatment of small choroidal melanomas 3 mm or less in thickness which are located too close to the optic disc to avoid the ischaemic optic neuropathy following irradiation (Hungerford 1993). The standard surgical technique for removal of selected melanomas of the iris and ciliary body is local resection by iridocyclectomy. Recently modern microsurgical techniques have also allowed local resection of some larger choroidal melanomas. Local resection of a posterior uveal melanoma offers several theoretical advantages over enucleation and radiotherapy. In contrast to enucleation, it preserves vision and maintains a cosmetically normal eye. In contrast to radiotherapy, it is associated with fewer long-term complications if the initial operation is successful. Current evidence shows no difference in patient survival between local resection of posterior uveal melanomas and enucleation or radiotherapy (Shields et al. 1993). Smaller, more anteriorly located tumours are associated with fewer complications and better visual results (Shields et al. 1993). However, profound hypotensive anaesthesia is required, and this limits its
applicability to young, fit patients. In the future, as the surgical technique is refined, local resection of posterior uveal melanoma will probably be used more often. Orbital exenteration is rarely necessary except for neglected melanomas with gross orbital extensions and for orbital recurrence. Orbital radiotherapy reduces the orbital recurrence rate after enucleation of an eye with extrascleral extension (Hykin, 1990).

### 1.2.5.2 Treatment of advanced disease

Metastasis to the liver or other distant organs is associated with an extremely poor prognosis and treatment is palliative. The role of chemotherapy and immunotherapy is unproven. Regression of ocular melanoma has been noted after hepatic arterial chemoembolisation with cisplatin and polyvinyl sponge (Mavligit et al. 1988). Such treatment may prolong survival for a few months but is unlikely to be curative.

There is no current evidence indicating that adjuvant therapy is effective in the management of uveal melanomas. Most affected patients have no detectable evidence of metastatic involvement at the time of diagnosis of the uveal melanoma. Consequently, clinicians have been disinclined to use such treatment.

### Section 3 Experimental treatment strategies

The poor response of metastatic melanoma to conservative therapies such as surgery, chemotherapy and radiotherapy has led to the investigation of new treatment strategies such as immunotherapy and gene therapy for use as both adjuvant therapy and as treatment for metastatic disease.
Effective immune responses leading to spontaneous disease remissions have been observed in small numbers of patients with both melanoma and renal carcinoma (Sumner et al. 1960). This suggested that the immune system could be harnessed therapeutically to provide effective treatment for melanoma as both adjuvant therapy and also for the treatment of metastatic disease.

A number of approaches to immune therapy have been proposed. Adoptive immunotherapy consists of the administration to patients of activated immune cells. Lymphocytes, non-specific for the tumour, but activated by IL-2 in culture (lymphokine-activated killer cells - LAK), have been administered to patients in combination with IL-2. However, results of clinical trials have not shown any improvement in response rate over that of IL-2 alone (Rosenberg et al. 1987). Tumour-infiltrating lymphocytes (TIL’s), which are lymphocytes isolated from tumour material and selected for their tumour-specificity, have been re-administered to patients in combination with IL-2 but this strategy also appears unlikely to offer an improvement over IL-2 alone (Rosenberg 1995).

An alternative approach is the use of tumour vaccines. Early studies demonstrated that injection of tumour nodules with Bacillus Calmette-Guerin (BCG) lead to tumour regression locally and at distant sites (Morton et al. 1970), presumably by non-specific stimulation of the immune system.

Other methods of tumour vaccination have included the use of allogeneic, irradiated whole cell vaccines with BCG. This, however, failed to show efficacy as adjuvant therapy (Morton et al. 1977). Allogeneic tumour cells lysed by viruses, such as vaccinia, have been demonstrated to induce immune responses in patients and increase survival in stage III patients (Wallack et al. 1996). A trial using vaccinia melanoma cell oncolysates for stage IIb and III patients has reported an increase in median survival from 79.6 to 96 months at interim analysis (Hersey et al. 1999).

The use of autologous patient tumour cells, following enzymic dissociation and irradiation has shown some promising results with 10 out of 40 patients showing tumour regression in one study, of which 4 were complete remissions (Berd et al. 1991).
However, a significant amount of tumour must be easily accessible for removal (e.g., a lymph node or a large skin metastasis) in order to prepare the vaccine and it is labour intensive.

Another approach has been to immunise patients with melanoma-specific antigens which, in theory, should lead to anti-tumour activity by the immune system. The most important antigens used to date have been the ganglioside cell surface molecules GM2, GD2 and GM3 (Livingston 1992). Combination of the ganglioside vaccine GM2 with BCG was shown to provide disease-free and overall survival benefit in those patients who were GM-2 antibody positive in a trial for stage III patients (Livingston et al. 1994). Other melanoma antigens which are known to produce immune responses include MAGE-1 and -3, MELAN-A/MART-1 and gp100 (Livingston et al. 1998).

However, although some vaccines have been shown to produce immune responses in patients, the challenge remains to convert this into an anti-tumour effect. This is because tumours have developed a number of strategies to evade the immune system. Antigens may be shed by the tumour, or down-regulation of cell-surface costimulatory molecules may prevent an effective immune response being produced. Tumour heterogeneity may mean that surface antigen expression may vary throughout the tumour. To produce an effective immune response therefore these defence mechanisms must be bypassed.

This has lead to the development of gene therapy strategies to manipulate melanoma cells in order to make them more easily recognisable by the immune system and therefore induce a more effective anti-tumour immune response. Autologous, inactivated melanoma cells, genetically modified ex-vivo to either secrete cytokines such as IL-2, interferon or GM-CSF or to express T-cell costimulatory molecules, such as B7-1, when re-injected into patients have been shown to elicit a more effective immune response (Dranoff et al. 1993, Parmiani et al. 1996).

Other strategies include the use of vaccines containing bacterial DNA, which is known to elicit a powerful immune response, and this, in combination with melanoma antigens, may lead to improved melanoma vaccine efficacy (Raz et al. 1996).
1.3.2 Gene Therapy

Gene therapy is defined as a treatment involving the introduction of DNA or RNA into cancer cells. Currently there is a wide range of research underway investigating this therapeutic strategy. There are a number of different approaches.

Genetic modification of tumour cells ex-vivo can be used to augment the host immune response as described previously.

Gene therapy can also be utilised to sensitise cancer cells to chemotherapy. Genes encoding enzymes, which metabolise a non-toxic pro-drug to a toxic metabolite, can be administered to cancer cells. For example uptake of the gene encoding thymidine-kinase by cancer cells will convert Gancyclovir to a toxic metabolite killing the cancer cells (Vile et al. 1993). This method is also termed 'suicide gene therapy'. The advantage of this method is that not all cancer cells have to be transfected with the gene as the toxic drug metabolite also kills adjacent non-transfected cancer cells. This is termed a bystander effect.

Corrective gene therapy aims to reverse the primary genetic abnormality that has lead to the production of the tumour. The targets for corrective gene therapy are those genes which have an important role in controlling the rate of cell growth. They encode either growth factors, such as the sis oncogene, their receptors eg the erb-b2 gene, second messengers eg the ras oncogene or nuclear proteins such as c-myc (Hall et al. 1992). These genes are mainly involved in the control of the cell cycle and when damaged they are termed oncogenes. Expression of oncogenes is normally very tightly controlled to prevent over-proliferation of a particular tissue or organ and subsequent tumour development. However, damage to these genes, for example by chemical carcinogens or ultra-violet light, can lead to loss of this control, abnormal over-expression of these genes and eventual tumour production.

Correction or control of the effects of the genetic damage can either be achieved by blocking abnormal oncogene overexpression (for example using an antisense mechanism) or alternatively by the replacement of a wild-type tumour suppressor gene (such as p53 or p16) when it has been deleted, mutated or suffered loss of function in tumour cells.
1.3.2.1 The cell cycle

In a multicellular organism, there is an important need to control the number of cells that are proliferating in relation to those that are terminally differentiated, growth arrested or those that are lost by apoptosis. This ensures that the numbers of any particular cell type in each tissue is controlled and maintains their spatial and kinetic organisation.

At any particular time, different populations of cells in each tissue are in different phases of cell growth. Alternatively, their growth has been arrested and they may have reached full differentiation. These cell populations are called growth fractions. The series of stages the cells pass through in their growth and progression to cell division is called the cell cycle.

Following mitosis, a period of cell growth (Gl) is followed by DNA replication (S phase). Further cell growth subsequently occurs in the G2 phase prior to cell division. A proportion of the cells in each tissue are resting and are not actively replicating depending on the tissue type, ie they are in G0 phase.

The cell cycle is very tightly controlled, with regulation occurring primarily in Gl phase. Therefore, the length of the Gl phase varies considerably more than the lengths of the G2, S and M phases with variation in the cell proliferation rate. A number of different proteins encoded by oncogenes play important roles in regulating the cell cycle and their respective functions are described below.

The cell cycle is represented in Figure 1.6.

1.3.2.2 p16, Cyclin-D1, cdk-4, the retinoblastoma gene and E2F

The retinoblastoma gene acts as a tumour suppressor gene. It is known to block Gl to S phase progression in the cell cycle via its action on the E2F gene. The cyclin-D1/cdk-4 gene complex inhibits the suppressor function of the retinoblastoma gene by inactivating it via phosphorylation. P16 functions as a tumour suppressor gene
Figure 1.6: The Cell Cycle

DNA Damage

-ve

mdm2

+ve

-tve

 ras

+ve

 Bcl-2

-tve

 Bax

+ve

 P53

 Signals from Growth and Differentiation Factors

-ve

 p16

+ve

 WAF/p21/cip1

-ve

 Cdk2

-ve

 Cdk4

+ve

 Cyclin-D1

-ve

 Rb

+ve

 Rb

+ve

 C-Myc

-ve

 Bax

+ve

 APOPTOSIS

-ve

-ve

 GO

+ve

 G1 phase

-ve

 MITOSIS

+ve

 S phase

+ve

 G2 phase
by blocking the inhibitory action of the Cyclin-D1/cdk4 complex on the retinoblastoma gene. Loss of p16 has been shown to correlate with the development of primary melanoma and progression to metastasis, as has been discussed previously, and is also known to be associated with familial melanoma (FitzGerald et al. 1996, Grover et al. 1998).

1.3.2.3 p53

The p53 oncogene also functions as a tumour-suppressor gene. Following DNA damage it induces apoptosis by inhibiting the action of the bcl-2 gene and through up-regulation of the bax gene (Sherr 1996). It also blocks G1 to S phase progression in the cell cycle by inhibiting c-myc and up-regulating the retinoblastoma gene, via its action on the p21 and WAF genes (Wahl et al. 1997). Several studies have highlighted its potential prognostic significance in melanoma (Florenes et al. 1995, Ross et al. 1997, Essner et al. 1998, Yamamoto et al. 1995). Corrective gene therapy to replace wild-type p53 has been shown to be effective in a number of cancers in-vitro and in-vivo (Fujiwara et al. 1994). Early trials administering wild-type p53 by adenovirus vector to patients with unresectable non-small cell carcinoma of the bronchus bearing p53 mutations have shown tumour regression.

1.3.2.4 Bcl-2

The oncogene bcl-2 blocks apoptosis, or programmed cell-death. Increased bcl-2 oncogene expression therefore leads to increased cell survival and tumour growth. The role of the bcl-2 gene in the control of apoptosis has been of great interest due to its importance in mechanisms of oncogenesis. A number of other oncogenes are related to bcl-2 in structure to varying degrees. Their inter-relationships are important for the control of apoptosis and are conserved throughout evolution. They share homology within four conserved regions, termed bcl-2 homology regions 1-4 which control the ability of these proteins to dimerize and function. The members
of this family of genes include bax, the action of which counteracts that of bcl-2, and bad, bid and bcl-X_s which are also pro-apoptotic. Bcl-X_l is anti-apoptotic.

Bcl-2 and Bax can either form homo-dimers or hetero-dimers with each other. The ratio of bax:bcl-2 is of critical importance in controlling apoptosis (Korsmeyer 1999). Bax is located in the cell membrane and cytosol. In response to death signals such as dexamethasone, IL-3 withdrawal or gamma irradiation bax is thought to translocate to the mitochondrial membrane and induce mitochondrial dysfunction. The mechanisms by which it does this may include forming ion-conduction pores, altering the membrane potential and altering the mitochondrial volume (Korsmeyer 1999). Bax also induces caspase activity, cleaving nuclear and cytoplasmic substrates. Caspases are cysteine proteases which cleave after aspartic acid residues. Bcl-2 is located in the mitochondrial membrane. Increased bcl-2 concentrations protect the cell from the pro-apoptotic activity of bax by forming hetero-dimers with bax and inhibiting its activity (Korsmeyer 1999).

Alterations in the activity of this family of genes are important in the development of a number of tumours. Bax mutations have been observed in up to 20% of haematopoietic malignancies (Korsmeyer 1999) and in 50% of colonic adenocarcinomas (Rampino et al. 1997). Over-expression of the bcl-2 oncogene has been shown to be significantly associated with poorer survival in patients with metastatic melanoma (Grover et al. 1996). In contrast, bcl-2 over-expression is known to be associated with improved survival in breast cancer (Le et al. 1999) and colon cancer (Buglioni et al. 1999).

1.3.2.5 Ras

The ras oncogene encodes a protein which functions as a second messenger in cells, transducing signals involved in the control of cell growth. The protein itself encodes a GTPase enzyme. Ras is a member of a family of small GTPase enzymes which includes ras, rap1, R-ras, and ral. The family is characterised by similarities in the effector domain. Rap1 is thought to antagonise ras by interfering in Ras effector function (Zwartkruis et al. 1999).
Ras mutations are associated with melanomas in chronically-sun exposed sites and also nodular melanoma (van Elsas et al. 1996). They have been shown to be associated with increasing tumour depth and metastatic progression (Ball et al. 1994). Overexpression of ras in melanoma cell lines in-vitro is associated with increased proliferation, invasiveness and anchorage-independent growth (Fujita et al. 1999) and has been shown to be associated with chemo-resistance in human melanoma xenografts grown in SCID mice (Jansen et al. 1997). S-trans, trans-farnesylthiosalicylic acid (FTS), a ras antagonist that dislodges ras from its membrane-anchoring sites, reduces the amounts of activated N-ras and wild-type ras isoforms in human melanoma cells and reverses their transformed phenotype. FTS also causes a profound inhibition of 518A2 and 607B human melanoma xenograft growth in SCID mice (Jansen et al. 1999).

1.3.2.6 The c-myc gene

1.3.2.6.1 Introduction

The c-myc oncogene encodes a nucleoprotein which regulates DNA synthesis in response to signals from growth factors by facilitating the transition from G1 into S phase. C-myc expression levels are tightly controlled in normal cells, probably by autoregulation (Evan et al. 1994). A defect in autoregulation is thought to be the mechanism producing c-myc overexpression in melanoma cells. Overexpression leads to an increase in cellular proliferation, a reduction in differentiation (Evan et al. 1994) and down-regulation of cell-surface molecule expression, thereby reducing immune responses to the melanoma cells (Versteeg et al. 1988). It is also thought to mediate resistance to platinum-based cytotoxic agents (Kashani-Sabet et al. 1990, Sklar et al. 1991). Overexpression of c-myc has previously been shown to be associated with a poor prognosis in a number of tumours including breast, colonic and cervical carcinoma (Garte 1993). C-myc overexpression has subsequently been found to be highly-significantly associated with prognosis, both for patients with a wide range of primary melanomas and also those with regional
metastases by the previous RAFT fellows in this project (Grover et al. 1997, Grover et al. 1997, Chana et al. 1998, Chana et al. 1998, Ross et al. 1998). These findings have highlighted this gene as a potentially important target for corrective gene therapy in melanoma and has lead to extensive research by this group and others in this area.

1.3.2.6.2 Anatomy of the c-myc gene

The c-myc gene is a member of the myc family of proto-oncogenes that also includes N-myc and L-myc. Both N-myc and L-myc show considerable tissue and tumour specificity in their expression. However, c-myc is expressed in almost all normal tissues and deregulated in many human cancers. The c-myc gene is located on chromosome 8(q24) and encodes two proteins of between 62 and 64kDa molecular weight.

The structure of the c-myc gene has been highly conserved throughout evolution, particularly in certain regions or ‘myc homology boxes’ thought to encode important functional domains of the c-myc oncoprotein (Evan et al. 1993). The gene contains three exons, the first of which is essentially non-coding along with four promoter regions, P0 to P3 (Evan et al. 1993).

Deletion of three highly conserved areas in exon two has shown that the amino acid sequences between aa44-65 and aa128-144 greatly reduce the transforming activity of the gene (Sarid et al. 1987).

DNA sequence-specific binding is mediated by a carboxyl terminal basic region helix-loop-helix-leucine zipper domain (bHLH-LZ), common to many DNA binding transcription factors. This region is essential for all c-myc functions (Evan et al. 1992, Evan et al. 1993) as it facilitates dimerisation to Max, another bHLH-LZ protein.
1.3.2.6.3  C-myc and the control of growth and differentiation

The response of normally quiescent cells to growth factors is accompanied by the rapid induction of a number of genes including c-myc, c-jun and c-fos, which appear to facilitate the early growth response. C-myc mRNA levels have been shown to increase 10-30 fold in fibroblasts when stimulated by external growth factors. This may promote passage of quiescent cells from the G0 phase into G1 phase in the cell cycle. C-myc continues to be expressed subsequently throughout proliferation. Measurement of the number of molecules of c-myc in individual, untransformed fibroblasts before and after mitogenic stimulation (Moore et al. 1987) has shown that in G0, each fibroblast contained approximately 300 molecules of c-myc. This increased rapidly to over 10,000 in the early part of the G1 phase of the cell cycle and fell to around 4,000 molecules thereafter, persisting at this level throughout the remainder of the cell-cycle as long as the cell continued to divide. The importance of c-myc in promoting the G0 to G1 transition has been demonstrated by the fact that it is able to stimulate proliferation of quiescent fibroblasts independently of other immediate early response genes (Ning et al. 1996).

Antisense oligonucleotides to c-myc mRNA have been shown to inhibit progression throughout the cell cycle, interestingly not at the G0-G1 phase transition, but in G1 to S-phase (Heikkila et al. 1987). Extrinsic c-myc relieves this inhibition, to allow continued progression through the cell cycle (Roussel et al. 1991). C-myc therefore has an important effect in promoting progression from G1 into S phase. C-myc may also influence cell-cycle checkpoints at the S-G2 phase (Evan, 1993) and regulate passage through the cell cycle at different stages in different cell types.

Continued cell proliferation is dependent on maintenance of steady state c-myc expression and continued mitogenic stimulation. Continued expression of the gene is only seen in exponentially growing cells. Withdrawal of mitogens produces a rapid down regulation of both c-myc mRNA and protein, consistent with a system of sensitive and rapid degradation. The mechanism by which this is thought to occur is via autoregulation which appears to involve control at several sites including
regulation of DNA transcription and mRNA turnover and translation (Hirvonen et al. 1991). Failure of autoregulation is thought to be one of the most important mechanisms by which c-myc over-expression leading to malignancy occurs. This concept is supported by the observation that many transformed cells appear to have lost the ability to autoregulate c-myc expression and may in part be due to the abnormal expression of other oncogenes (Hirvonen et al. 1991).

A fall in c-myc levels is associated with growth arrest and cell differentiation. Contact inhibition of confluent cells in culture is accompanied by a fall in c-myc mRNA and protein levels (Moore, 1987). Chemical induction of differentiation of cells in vitro produces a rapid fall in c-myc protein levels (Campisi et al. 1984). However, if c-myc levels are artificially elevated, cells do not differentiate, presumably because they are under continual stimulation to proliferate and unable to exit the cell cycle. C-myc is therefore intimately involved in those processes which select cells to proliferate, differentiate or apoptose.

Dimerization of c-myc with the product of the max gene is necessary for c-myc function as it allows binding to CACGTG, a core DNA sequence (Blackwood et al. 1992), and is a pre-requisite for transcriptional activity. Overexpression of Max results in formation of Max-Max homodimers which may play a part in regulating c-myc activity (Kretzner et al. 1992). Max has a long half-life, and intra-cellular levels remain stable throughout proliferation. During proliferation, c-myc is up-regulated and Max is likely to be present as part of the c-myc-Max heterodimer once more. Another protein belonging to the bHLH-LZ family of transcription factors termed Mad competes with myc for dimerization with Max (Hurlin et al. 1994). In contrast to myc-Max heterodimers, which activate gene expression, Mad-Max heterodimers suppress transcription from promoter constructs containing the myc target sequence CACGTG (Hurlin et al. 1994). The relative abundance of Mad and myc could therefore determine the activity of myc target genes involved in the control of growth and differentiation.

Specific DNA binding allows both activation and suppression of a number of genes including p53 and the HLA-B gene, as well as those involved with growth and apoptosis (Blackwood et al. 1992).
1.3.2.6.4 C-myc and apoptosis

Programmed cell death, or apoptosis, is a major process involved in tissue homeostasis and tumour growth. It differs from necrosis both morphologically and in being a genetically controlled process. It is central to embryogenesis and neuronal development and forms an integral part of those processes that regulate cell numbers in a given tissue. C-myc also plays a role in physiological apoptosis, as elevated c-myc protein levels accompany involution of thymocytes and breast tissue. Deregulation of apoptosis has come to be increasingly recognised as a potential mechanism for tumour formation. Apoptosis is an active process that requires the induction of certain genes and their encoded proteins, such as endonucleolytic enzymes for digestion of DNA. Evan et al. (1992) showed that apoptosis in rat fibroblasts was c-myc dependent, though this only occurred in cells whose cell cycle had slowed. C-myc has been shown to induce apoptosis in serum-deprived fibroblasts or in cells where proliferation has been suppressed by anti-proliferative cytokines (Evan, 1993). Conversely, apoptosis may be inhibited by cytokines such as IL-3. Withdrawal of IL-3 from cell medium results in rapid c-myc-induced apoptosis (Askew et al. 1991).

Other oncogenes are known to regulate susceptibility to apoptosis. Fibroblasts transfected with both c-myc and bcl-2 show a significant reduction in their apoptotic potential compared to cells which express c-myc alone. Cells expressing both genes proliferate normally, suggesting that bcl-2 is only interacting with processes leading to death of the cell (Evan et al. 1994). Consequently, this represents a dynamic relationship where one gene prevents the lethal effects of another and in which both genes may be under the modulating influence of growth factors and cytokines. In uniting these findings to form a conceptual model of c-myc function in tumour formation, Evan and Littlewood (1993) suggest deregulated c-myc activity may not only stimulate inappropriate cell proliferation, but also allow survival of cells (with genetic abnormalities) that would otherwise die.
1.3.2.6.5 C-myc and cancer

A number of studies have shown an association between c-myc and malignancy. Overexpression of c-myc produces immortilisation and reduced growth factor requirements in cultured fibroblasts (Minks et al. 1992). In avian bursal lymphoma, a chronic infection produced by avian leukosis virus, the viral promoter/enhancer sequence is inserted near the c-myc gene, leading to c-myc over-expression (Battey et al. 1983). A similar mechanism is involved in the formation of Burkitt's lymphoma in humans. Chromosomal translocation results in relocation of the c-myc gene from chromosome 8(q24) to a transcriptionally-active region adjacent to the immunoglobulin heavy chain locus on chromosome 14(q32) and the \( \kappa \) and \( \lambda \) light chain loci on chromosomes 2(p12) and 22(q11) (Taub et al. 1984). Loss of transcriptional regulation of c-myc occurs with overexpression of the oncoprotein. However, translocation and amplification has only been found to explain c-myc overexpression in a small proportion of cancers studied, the remainder occur by unknown mechanisms but may include loss of control of c-myc levels by autoregulation.

C-myc overexpression has been observed in a wide range of haematopoietic and solid cancers including those of the gastro-intestinal tract, urogenital tract, lung and breast (Prins et al. 1993). In several cancers the level of c-myc expression has been found to be of prognostic significance including cervical carcinoma (Bourhis, 1990), breast cancer (Garte, 1993) and head and neck squamous carcinomas (Field, 1989).

1.3.2.6.6 C-myc and melanoma

C-myc mRNA levels have been shown to be elevated 9-14 times in some metastatic melanoma cell lines, compared with melanocytes, and c-myc overexpression has also been shown to be associated with anchorage-independent growth (Husain et al. 1990). A previous RAFT Research Fellow showed an association between oncoprotein positivity and the potential doubling time of tumours (Ross 1994, MD Thesis). In this series, an analysis of both primary and secondary tumours together also
suggested that c-myc oncoprotein expression may be of prognostic value (Ross, 1998). These studies were continued by the next RAFT Research Fellow who used multivariate analysis to establish a significant relationship between c-myc oncoprotein expression and clinical outcome for intermediate thickness tumours (Grover 1996, MD Thesis). C-myc oncoprotein expression was found to be an independent prognostic parameter which was more accurate than all other clinicopathological markers including the Breslow thickness. A similar association was found in primary acral lentiginous and subungual melanomas (Grover 1996, MD Thesis). C-myc oncoprotein expression also maintained an independent association with outcome and proved to be the most accurate prognostic marker for regional disease (Grover, 1997).

Continuing studies by Jagdeep Chana at RAFT also demonstrated prognostic significance for c-myc expression in melanomas of the head and neck and also for uveal melanoma. Again multivariate analysis demonstrated that this was more accurate than all other conventional clinico-pathological factors (Jagdeep Chana MD thesis 1998).

1.3.2.7 Manipulation of c-myc expression

The poor prognosis for patients with metastatic melanoma and the ineffectiveness of current modalities of treatment has lead to the investigation of new treatment strategies to treat this condition.

The use of antisense oligonucleotides to block gene expression has been widely demonstrated and their development for use in a wide range of conditions including cancer, inherited conditions, cardiovascular disease and Acquired Immunodeficiency continues.

The prognostic importance of the c-myc oncogene in melanoma suggests that overexpression of this oncogene may have a vitally important role in the development of melanoma. This oncogene may therefore provide the best target for corrective gene therapy in melanoma in order to produce an effective clinical response.

Studies by the previous two RAFT research fellows and another group have demonstrated that c-myc antisense oligonucleotides can be used to inhibit the proliferation and invasive potential of melanoma cells in vitro and also to delay the growth of melanoma xenografts in immunodeficient mice (Grover 1996, Chana 98 MD Theses, Leonetti, 1996; Citro, 1998).

1.3.2.8 Antisense Oligonucleotides

1.3.2.8.1 Mechanism of action

Antisense oligodeoxynucleotide (ODN) are short sequences of synthetic ODNs complementary to the target RNA sequence. They are known to be actively taken up by cells and hybridise to the mRNA sense sequence of interest, according to the principle of Watson-Crick base pairing. Binding of the antisense to the target RNA blocks protein translation and therefore blocks the expression and function of the target gene. Antisense ODNs may impair translation after hybridising to its corresponding mRNA sequence through a number of mechanisms (Kregnow, 1995):

1. hybridisation to pre-mRNA in the nucleus may impair subsequent splicing and processing to mRNA.
2. hybridisation can impair the translocation of mRNA from the nucleus to the cytoplasm
3. hybridisation can prevent the binding of ribosomes to the mRNA molecule
4. hybridisation can impair the translation process itself
5. the hybridised antisense oligomer-mRNA duplex acts as a substrate for intracellular digestion by RNase H
1.3.2.8.2 Biochemistry of antisense oligomers

Antisense ODNs are short stretches of synthetic RNA, typically 10 to 30 bases long. Sequences of this length are likely to be unique to the target gene and are known to be taken up by cells. Shorter sequences may lack sequence specificity while longer sequences may not be taken up by cells so easily and also suffer from specificity breakdown due to looping out of non-hybridising sequences (Kregnow et al. 1995).

The ideal antisense drug should be stable in the intracellular and extracellular environment, be able to cross cell membranes, demonstrate hybridisation specificity, and have a low non-sequence related toxicity (Stein et al. 1993). The first two of these properties are related to the biochemical properties of the ODN and can be biochemically modified. The last two are a function of the antisense sequence and its ability to interact specifically with intracellular molecules.

Unmodified ODNs contain phosphodiester bonds which are readily digested by 5' or 3' exonucleases as well as endonucleases (Tidd 1990). These enzymes are present in both extracellular and intracellular fluids restricting their use in-vivo, although they can be used for in-vitro experiments utilising serum free media. A number of strategies may be used to increase their resistance to degradation by nucleases. The first generation of modified ODNs contained modified phosphodiester bridges between nucleotides. These were created either by utilising the methylphosphonate or phosphorothioate derivatives (Figure 1.9), changing the glycosidic linkage from the β to the α anomeric form or capping or altering the 3' or 5' ends of the oligomer molecule. Second generation modifications have included the synthesis of phosphorothioate-phosphodiester co-polymers possessing some of the properties of both modified and unmodified ODNs (Ghosh et al. 1993).

Alternatively, the peptide nucleic acid analogue (PNA) has the entire deoxyribose phosphate backbone exchanged for a chemically different, but structurally homorphous, polyamide backbone composed of (2-aminoethyl) glycine units (Frank-Kamenetskij 1991). These derivatives can bind to single-stranded genomic
Figure 1.7: Chemical structure of oligonucleotide derivatives
DNA by Watson-Crick base pairing after displacing the other DNA strand, and to
double stranded genomic DNA by forming a triple helix in the major groove.
However, these compounds do not have the ability to enter living cells.

Some of the properties of unmodified phosphodiester and unmodified
methylphosphonate and phosphorothioate ODN are shown in Table 1.5. The
phosphorothioate derivative has the best combination of properties and has been the
most commonly used type of ODN for experimental purposes. This derivative has
therefore been utilised for the experiments presented in this thesis and in those of
the previous RAFT fellows in this project.

<table>
<thead>
<tr>
<th>Property</th>
<th>Phosphodiester</th>
<th>Methylphosphonate</th>
<th>Phosphorothioate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biologic stability</td>
<td>-</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Cellular Uptake</td>
<td>+</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>Hybridisation</td>
<td>+ + +</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>RNase H activation</td>
<td>+ + +</td>
<td>-</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

Table 1.8  Properties of normal and modified oligodeoxynucleotides
Note: - poor, + fair, ++ good, +++ excellent.

ODNs do not readily enter cells as, with the exception of methylphosphonate
derivatives, ODNs are polyanionic (Kregnow et al. 1995). They are therefore
similar to most antineoplastic agents which are of low molecular weight,
hydrophilic, and incapable of passively diffusing across cell membranes. Cellular
uptake of ODNs has been found to be dependent upon time, concentration, energy
and temperature which are characteristic of an active process (Crooke 1991). These
findings led to the discovery that ODNs are taken up by cells by two active
processes: fluid phase endocytosis and receptor-mediated endocytosis. Once across
the membrane, the mechanisms by which they are distributed throughout the cell are
controversial. Fluorescent-labelled ODN microinjected into the cytoplasm of cells
are found to rapidly accumulate in the nucleus (Leonetti et al. 1991). However,
when fluorescent-labelled ODNs are placed in tissue culture media, the labelled molecules accumulate in vacuoles, presumably in endosomes and lysosomes, within the cytoplasm forming a punctate, perinuclear pattern (Cerruzi et al. 1990). In contrast to ODN localisation when the compounds are introduced by microinjection, there is no visible fluorescence in the nucleus itself, suggesting that the release of ODNs from these vacuoles is an inefficient process. Methylphosphonate derivatives, however, are uncharged particles that have been reported to enter cells via passive diffusion (Miller 1991).

Since ODN uptake is inefficient, several different strategies have been employed to augment this process in order to increase efficiency. ODNs have been conjugated with synthetic polypeptides such as poly-L-lysine, cholesterol, and transferrin (Leonetti et al. 1993). In addition, liposomes containing ODNs can be targeted to reach cell surface determinants with appropriate monoclonal antibodies (Leonetti et al. 1993). The poly-L-lysine modification may mask the negative charge on the ODN, therefore destabilizing the endosomal membranes and permitting ODN escape from the endosomal compartment. Conjugation with cholesterol permits the binding of ODN to apolipoprotein E and to low-density lipoprotein (LDL) which allows internalisation of the ODN via the LDL receptor (Leonetti et al. 1993). Cationic lipids such as N-[1(-2,3-dioeyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA, Lipofectin®) and some antifungal drugs such as amphotericin B have also been shown to increase oligomer uptake by cells (Bennett et al. 1992). Lipofectin® not only increases the cellular uptake of ODN, but also increases the uptake of ODN into the nucleus.

1.3.2.8.3 Applications of antisense oligonucleotides

Antisense oligonucleotides have been used to block the expression of a number of different genes, both in-vitro and in-vivo, and also in early human trials.

The bcl-2 oncogene has been targeted by antisense in a number of studies. It has been shown to block the growth of melanoma in-vitro and in-vivo and also to
chemo-sensitise melanoma cells to cis-platinum (Jansen et al. 1998). Bcl-2, mdm2
and Bcl-Xs antisense have also been shown to inhibit the proliferation of cells from
a number of different haematological malignancies in-vitro and in-vivo (Cotter
1999). A recent trial of bcl-2 antisense administered by daily intra-venous infusion
for 14 days in 9 patients with relapsing non-Hodgkins lymphoma has been reported.
Computed tomography scans demonstrated a reduction in tumour size in two
patients (one minor, one complete response). Bcl-2 protein expression was
measured in the samples of five patients by flow cytometry and down-regulation of
the Bcl-2 protein was demonstrated in two patients. The number of circulating
lymphoma cells decreased during treatment in two patients and in four patients
serum concentrations of lactate dehydrogenase fell. Two of these patients had an
improvement in symptoms. No treatment-related toxic effects occurred, apart from
local inflammation at the infusion site. A trial of intra-venous bcl-2 antisense
therapy for metastatic melanoma patients is currently underway (Jansen 1999).
Antisense targeting the BRCA1 oncogene has been shown to increase sensitivity to
cisplatinum, decrease the proficiency of DNA repair, and enhance apoptosis in
breast cancer cells in-vitro (Husain et al. 1998). Antisense targeting the EGF-
receptor mRNA decreases the expression of the EGF receptor and partially reverses
the malignant phenotype of the MDA-MB-231 breast cancer cell line in-vitro (Fan
et al. 1998). Antisense oligonucleotides targeting protein kinase A have been shown
to produce a co-operative inhibitory effect in combination with docetaxel and anti-
epidermal growth factor-receptor antibody on the growth of human breast cancer
cells in-vitro. A phase I trial in patients is now underway (Tortora et al. 1999).
Gag antisense in combination with AZT, ddC or indinavir has been demonstrated to
inhibit HIV-1 replication 10-fold more effectively than that produced by the single
antiviral drug used alone in human T cell lines (Junker et al. 1997). In addition, the
Gag antisense showed antiviral efficacy against several reverse-transcriptase
inhibitor-resistant HIV-1 isolates.
Antisense targeting the angiotensin-II receptor has been shown to lead to
permanent protection from hypertension in the spontaneously hypertensive rat
model of human hypertension (Reaves et al. 1999). The antisense was delivered by
a retrovirus-mediated delivery system. Downregulation of intimal cell bcl-xL
expression using antisense oligonucleotides has been shown to induce apoptosis and acute regression of vascular lesions in animal models (Pollman et al. 1998).

1.3.2.8.4 Antisense oligonucleotides targeting the c-myc gene

The use of antisense oligonucleotide to inhibit c-myc gene expression was first reported by Heikkila et al. in 1987. They studied the effect of a pentadecadeoxyribonucleotide (15-mer) on the c-myc gene expression of human T-lymphocytes grown in serum-free culture. Using western blotting they demonstrated a consistent reduction in c-myc oncoprotein levels after treatment with medium containing 30μm concentration of oligonucleotide. The sequence used by Heikkila consisted of 15 bases complementary to the initiation codon and four downstream codons of exon two of the c-myc mRNA molecule (5' ACGTTGAGGGGCAT-3'). Since this initial study, a number of other groups have used this technique to reduce c-myc gene expression in a number of cancers. C-myc antisense has been utilised to inhibit the growth of melanoma in-vitro and in-vivo and also to increase melanoma sensitivity to cisplatinum using the same ODN sequence (Leonetti et al. 1996, Citro et al. 1998, Leonetti et al. 1999). Similar effects have also been demonstrated against bladder carcinoma cells in-vitro (Mizutani et al. 1994). The previous fellows in this project have also used this antisense sequence to show inhibition of melanoma growth in-vitro and in-vivo (Rajiv Grover MD thesis 1996; Jagdeep Chana MD thesis 1998).

1.3.2.9 The limitations of antisense oligonucleotides as corrective gene therapy

The demonstration that antisense ODN’s can specifically block the expression of a target gene has highlighted this mechanism as a potential corrective gene therapy strategy for use in the treatment of a wide range of diseases, including cancer.
However, for antisense to work effectively in cancer patients a number of hurdles need to be overcome. The antisense needs to be effectively targeted to the tumour. Once it has reached the tumour cells it needs to be expressed at high enough concentrations for a sufficient length of time to effectively block expression of the target gene, and death of the tumour cell to occur. In addition, targeting of multiple genes may be required to prevent tumour resistance to the gene therapy from developing and also to kill all the tumour cells which may comprise more than one genotype. Use of the ‘bystander effect’ may be required in which transfection of one tumour cell results in the death of other, neighbouring, untransfected cells, either using a biochemical or immunological bystander effect. This therefore means that not all of the tumour cells have to be transfected by the gene therapy for death of all the tumour cells to occur. Combination of gene therapy with other more conventional treatment modalities, such as chemotherapy and interferon, may lead to additive or synergistic effects.

1.3.2.10 Ribozymes

As a first stage in the development of c-myc antisense for use in cancer patients the incorporation of c-myc antisense into a ribozyme system has been investigated in this thesis. Ribozymes are naturally-occurring forms of RNA that possess the ability to cleave other RNA sequences by acting as enzymes, independent of the presence of protein. Alteration of the ribozyme structure to target a specific RNA sequence therefore produces a molecule which can act in the same manner as antisense but is much more efficient. One molecule of antisense can only block the effects of one molecule of RNA before being consumed in the process and inactivated. However one ribozyme molecule targeting a specific RNA sequence can cleave multiple mRNA molecules. For example a ribozyme directed against the tobacco-mosaic virus RNA-dependent polymerase, an important protein for viral replication, was shown to reduce virus production by 90%, compared to 20% for antisense directed against the same target, in tobacco plant protoplasts (Edington 1992).
The existence of ribozymes was first discovered in viroids, which are helper viruses found in plants, and in bacteria (Zaug et al. 1986). Their role is to cleave large RNA precursor molecules, some containing multiple RNA copies, into unit-length progeny.

Five types of ribozymes have been described: group I introns, ribonuclease P, hammerhead, hairpin and hepatitis delta virus ribozymes. Group I introns process ribosomal RNA in a two-step process. Ribonuclease P was first described in bacteria and comprises a small subunit protein and catalytic RNA. It acts as an endonuclease to process precursor tRNA molecules into mature molecules. Hammerhead ribozymes were first described in plant viroids and serve to process large, circular RNA precursor molecules into individual transcripts (Sullivan 1994). Hairpin ribozymes are also found in plant viroids and have a similar role to hammerhead ribozymes. Hepatitis delta virus is a helper virus and its RNA also serves to process RNA in a similar fashion to hairpin and hammerhead ribozymes (Sullivan 1994).

The hammerhead ribozyme is the smallest ribozyme and its structure has been the best characterised. Its target sequences have the least constraints compared with the other types and potential target sites are more common than for the other types. This type of ribozyme has been the most suitable for development for therapeutic use and has been the most studied. Its basic structure has therefore been utilised and modified in this project to target the c-myc oncogene and amplify the effect of c-myc antisense.

A more detailed description of the structure, synthesis and use of ribozymes is presented in Chapter 6.

1.3.3 Experimental Aims

The main areas of interest and controversy in melanoma at present are the use of adjuvant therapy, and interferon in particular, and the treatment of metastatic disease for which the outlook in melanoma is very poor. This project has therefore
investigated the mechanism of action of interferon in melanoma and investigated ways to improve its efficacy.

In addition, the project has sought to build on the previous work performed by the RAFT fellows in this project who have demonstrated the efficacy of c-myc antisense for inhibiting the growth of melanoma in-vitro and in-vivo. We have therefore assessed the benefits of combining c-myc antisense therapy with other chemotherapeutic drugs, a strategy which may prove beneficial for the treatment of metastatic melanoma.

Finally we have designed a ribozyme targeting the c-myc gene in order to amplify the antisense effect as a first step in developing c-myc antisense for use in humans.

The aims of the project presented in this thesis therefore are:

1. To assess the relationship between c-myc oncogene expression and interferon sensitivity in melanoma.
2. Investigate the use of c-myc antisense therapy in combination with interferon in a series of melanoma cell lines.
3. Evaluate the use of c-myc antisense in combination with a range of chemotherapy agents in the A375m human melanoma cell line.
4. Design and assess the efficacy of a ribozyme targeting c-myc mRNA in order to amplify the effects of c-myc antisense.
Chapter 2

Materials and Methods

2.1 Cell lines and culture conditions

2.1.1 Cell culture medium

All melanoma cell lines were grown in cell culture medium which was constituted every two weeks and stored at 4°C. The cell lines were grown as monolayer cultures in Modified Eagle's Medium (MEM, Gibco, UK) which had been supplemented with:

- 10% heat inactivated fetal calf serum (heated to 65°C for 20 minutes)
- L-Glutamine (200mM)
- Non essential amino acids (Sigma, UK)
- Penicillin 200μg/ml
- Streptomycin 0.1mg/ml
- Amphotericin B 1μg/ml

2.1.2 Cell lines

Established melanoma cell lines were obtained from the European Catalogue of Animal Cell Culture (ECACC) and included the A375m, MeWo, MM 96L, and SkMel 28.
2.1.2.1 A375M

Human amelanotic melanoma derived from a 54-year old patient with metastatic melanoma. Culture produces a confluent monolayer with a doubling time of 29 hours.

2.1.2.1 MeWo

Human melanoma derived from a 65-year old patient with metastatic melanoma. Culture produces a confluent monolayer with a doubling time of 32 hours.

2.1.2.2 MM96L

Human melanoma derived from a 73-year old patient with metastatic melanoma. Culture produces a confluent monolayer with a doubling time of 23 hours.

2.1.2.4 SkMel28

Human melanoma derived from a 51-year old patient with metastatic melanoma. Culture produces a confluent monolayer with a doubling time of 34 hours.

2.1.3 Cell maintenance

2.1.3.1 Storage of cell lines in liquid nitrogen

Cells were stored frozen in liquid nitrogen at a concentration of $1 \times 10^6$ cells /ml in 2ml cryovials in a mixture of MEM supplemented medium and 10% dimethysulphoxide (DMSO). The vials were frozen slowly overnight, to avoid
excess physical damage, by placing above liquid nitrogen and then stored in liquid nitrogen until required.

2.1.3.2 Recovery of cell lines from liquid nitrogen

Frozen aliquots of cells were removed from liquid nitrogen and thawed rapidly in a 37°C water bath. The cells were then added to a T-75 flask with 20ml of MEM supplemented medium and allowed to attach for at least twelve hours. The medium was then replaced with 30 ml of fresh medium to remove DMSO derived from the frozen aliquot.

2.1.3.3 Maintenance of human melanoma cell lines

Monolayer tumour cells were kept in T-75 tissue culture flasks in 20ml of MEM supplemented medium in a 37°C incubator, equilibrated with 5% CO₂ in air. Cells were trypsinized twice a week and re-seeded at a density of 2 x 10⁵ cells/flask.

2.1.3.4 Mycoplasma testing

All cell lines were tested for mycoplasma contamination before use and at six month intervals thereafter. To test for mycoplasma, cells were sub-cultured into flasks containing antibiotic-free medium in which they were maintained for 3 days prior to testing. They were then removed from the growth surface using EDTA/trypsin and approximately 2 x 10² cells were inoculated onto a covered slide (‘Flaskette’ chamber slide 177453 Nunc) with 5ml of EMEM and placed in an incubator for 72 hours. The medium was then poured off and the cell layer was washed with fixative (3 washes with 3:1 methanol:acetic acid at 5 minute intervals) and left to air dry. The cells were then stained with a 0.05 µm³l⁻¹ solution of Hoechst stain (Benzimidazole 33258 at 0.05 µgml⁻¹ ) which was left in contact for 10 minutes. The stain was washed off with double-distilled water and the preparation was then inspected using a Nikon
UFX-II microscope under UV illumination. The preparation was carefully inspected for bright cytoplasmic staining in discrete dots which represents Mycoplasma contamination.

2.2 The ATP chemosensitivity assay

The ATP chemosensitivity assay was used both to assess the effect of combinations of antisense and interferon or chemotherapeutic drugs on the growth of melanoma cells in-vitro and also to assess the interferon sensitivity of uveal melanoma tumours obtained from patients.

2.2.1 Assessment of the effect of combination of antisense with chemotherapeutic drugs or interferon on the growth of melanoma cell lines

A375m, MeWo, MM96L or SkMel 28 cells were added to each well of a 96-well plate at a concentration of 5 000 cells/well in 100µl of Complete Assay Media (CAM - DCS Innovative Diagnostik Systeme, Hamburg, Germany). The cells were incubated for 6 hours to allow them to attach to the plate. Chemotherapeutic drugs or c-myc antisense were subsequently added alone or in combination to the wells. The c-myc antisense was added at a concentration of 25µm. The 4G-quartet oligonucleotide was used at the same concentration as a control for the c-myc antisense. The oligonucleotides were added to the cells 24 hours prior to the chemotherapeutic drugs to allow sufficient time for any effect on c-myc oncogene expression to become apparent prior to the addition of the chemotherapy drugs. The concentrations of chemotherapeutic drugs used corresponded to 6.25%, 12.5%, 25%, 50%, 100% or 200% of the standard therapeutic dose. The drugs tested were Cisplatinum, DTIC, Temozolamide, Interferon and Taxol. Each dose of each drug combination was tested in triplicate. One row of twelve wells received CAM only, and no drugs, and was termed M0 as the negative control. One further row of twelve wells in each plate acted as the positive control (M1) and received a maximal
inhibitor of cell survival (MI; DCS Innovative Diagnostik Systeme – 0.02% Triton X100, a detergent).

The plate was incubated for 6-7 days at 37° C in 5% CO₂ and high humidity. The cells were checked every 2-3 days for infection or overgrowth by microscopy. Following the incubation ATP was extracted from the cells using 50µl of TCER (DCS Innovative Diagnostik Systeme). 50ul from each well was transferred to another 96-well plate and 55µl of luciferin-luciferase (DCS Innovative Diagnostik Systeme) added to each well to hydrolyse the ATP. The plate was loaded into a Dynatech ML1100 luminometer and the light emitted from each well measured and expressed as relative light units (RLU). This was used as a measure of total ATP levels and therefore cell growth. Mean percent inhibition of cell growth / survival for the triplicate wells was calculated using the formula:

\[
\text{Mean percent inhibition of cell growth} = 1 - \frac{(\text{Test} - \text{M1}) \times 100}{(\text{MO} - \text{M1})}
\]

The data was analysed using an Excel 5.0 spreadsheet (Microsoft) and graphs of percentage growth inhibition versus test drug concentration plotted.

2.2.2 Assessment of the interferon sensitivity of uveal melanoma tumours

A portion of each fresh uveal melanoma specimen for interferon sensitivity testing was removed by consultant histopathologist IAC under sterile conditions. The sample were then cut into small cubes, 10ml of collagenase blend H (0.375mg/ml Sigma) was added and the mixture incubated overnight to allow digestion to occur. The digest was washed with 10ml of PDS, centrifuged at 1100rpm for 5 minutes and the pellet resuspended in 10ml of CAM. The cells were then counted using the trypan blue exclusion method. 20µl of trypan blue was added to 20µl of cells. The dead cells appeared blue and the live cells, which appeared clear, were counted using a haemocytometer.
5000 cells were subsequently added to each well of a 96-well plate in 100μl of CAM. The cells were incubated for 6 hours to allow them to attach to the plate.

Intron-A (Interferon - Schering Plough) was then added to each well at a number of concentrations corresponding to 6.25%, 12.5%, 25%, 50%, 100% or 200% of the standard therapeutic dose (100% = 1000 IU/ml). Each combination was tested in triplicate. One row of twelve wells received CAM only, and no interferon, and was termed M0 as the negative control. One further row of twelve wells in each plate acted as the positive control (M1) and received the maximal inhibitor of cell survival (MI; DCS Innovative Diagnostik Systeme).

The plate was incubated for 6-7 days and the rest of the assay performed as in section 2.2.1. Mean percent inhibition of cell growth for the triplicate wells was calculated using the formula:

\[
\text{Mean percent inhibition of cell growth} = 1 - \frac{(\text{Test} - \text{M1}) \times 100}{(\text{MO} - \text{M1})}
\]

The interferon sensitivity index was calculated by adding the mean percentage growth inhibition for all 6 drug concentrations together and subtracting this figure from 600. A sensitivity index of less than 600 corresponds to tumour sensitivity to interferon and above 600 to resistance.

The maximum percentage inhibition of interferon for each tumour was the maximum inhibitory percentage produced on the dose-response curve at any dose. Four examples of uveal melanoma interferon dose-response curves are shown in Figure 3.2 in Chapter 3.

2.3 Flow Cytometry

2.3.1 Description of the FACscant

The FACScan system is an automated cell analyser developed for both research and clinical applications. It is used to analyse cell populations based on the principles of
flow cytometry in which cells and cell sub-populations are recognised based on their light scattering and reflecting properties when they are illuminated by laser light. Unstained cell populations can be recognised based on cell size (forward scatter of laser light) and granularity (side scatter). Cellular stains which produce characteristic fluorescence can also be used to differentiate sub-populations.

The FACScan was used in this project to assess c-myc oncoprotein levels in uveal melanoma surgical specimens and also in melanoma cell lines. Following staining of the cells with antibodies targeting the c-myc protein, the level of c-myc expression was measured based on the degree of fluorescence emitted from the secondary antibody.

The FACScan consists of a bench top sensor module coupled with a computer module which controls both acquisition and analysis of data. The cells to be analysed are enclosed in a pressurised saline solution and pass through a flow cell where they are illuminated and generate up to 5 signal pulses simultaneously. They are irradiated by a 15mW, 488nm air-cooled argon-ion laser. Dichroic mirrors spectrally filter emitted light, separating and deflecting longer wavelengths whilst transmitting shorter wavelengths. Longer wavelengths are detected on two light scatter channels (front and side). These shorter wavelengths are further separated by other mirrors into the path of one of three photomultiplier detectors, FL1, FL2 and FL3, following which the signals are amplified, digitised, processed and stored on computer for further analysis.

At 530nm the FL1 detector is optimised for FITC detection (green fluorescence) whilst the FL3 detector detects wavelengths in excess of 650nm, suitable for detection of red light emitted by propidium iodide. The FL2 channel detects intermediate wavelengths (585nm), emitted by phycoerythrin in the red/orange band (this channel was not used in the present investigations). Dual parameter collection of data on FL1 and FL3 allows bivariate dot plots to be formulated, recording expression of FITC-labelled data on the FL1 channel against DNA content on the FL3 channel (examples are shown in Figures 2.1, 2.2 and 2.3).
2.3.2 Detection of c-myc oncoprotein in patient material

2.3.2.1 De-waxing of specimens

Four 35\(\mu\)m sections were cut from each paraffin-embedded block, applied to Sellotape and then onto a plain glass slide. Slides were stored at 4°C until rehydration. Attempts were made to limit analysis to tumour cells by excising the appropriate area from each slide, with reference to a haematoxylin and eosin stained serial section. Samples were dewaxed in conical glass tubes, and the Sellotape removed, using 5ml of xylene (BDH, Poole UK) for 10 min and the process repeated. Specimens were then rehydrated through successive increasingly hydrated alcohols, commencing with 100% ethanol (Hayman Ltd, UK), 90%, 70% and finally 50% solutions. In each case the sample was kept in 5ml of the alcohol for 10 min, prior to centrifugation at 1100rpm for 5 min. Samples could be stored in plastic universal containers with 50% ethanol at 4°C.

2.3.2.2 Preparation of nuclei

Pepsin\textsuperscript{\textregistered} inoculation was achieved using a concentration of 4mg/ml in 0.1M hydrochloric acid and incubated for a period of 45 minutes at 37°C. The resulting suspension was filtered through a 35\(\mu\)m nylon mesh in a Swinnex holder. Nuclear concentration was estimated using a haemocytometer (Weber, UK) and adjusted to achieve a concentration of \(10^6\) nuclei/mm\(^3\). The suspension was then aliquoted into two samples and washed twice in phosphate buffered saline (PBS). Centrifugation at 2000rpm, for 5 minutes was used between washes.
2.3.2.3 C-myc antibodies

The c-myc protein was identified using a rabbit polyclonal antibody raised against myc oncoproteins (Cambridge Research Biochemicals Ltd.). Both N-myc and L-myc are highly tissue specific (Garte, 1993) and the oncoprotein products of these genes have not been described in melanoma. Furthermore, no evidence of either the mRNA or protein product of N-myc or L-myc were found in a study of 21 different melanoma cell lines of varying metastatic capability (Chevenix-Trench et al., 1990). Consequently, it was assumed that neither of these genes would be expressed in benign or malignant melanocytes and that the polyclonal myc antibody would only label the c-myc oncoprotein. Immunohistochemical detection of c-myc oncoprotein using the c-myc monoclonal antibody, 6E10, has been described in several papers (Sikora et al., 1987; Jack et al., 1986, Watson, 1986). However, the experience of previous RAFT fellows suggested that for c-myc estimation in paraffin embedded tissue samples the polyclonal antibody was superior (Ross, 1998). This was also the finding of other workers (Lincoln & Bauer, 1989). The monoclonal antibody, 6E10, was found to be slightly more specific than the polyclonal antibody in the estimation of c-myc oncoprotein in whole cells (Ross, 1998) and was therefore used for measurement in the experiments involving c-myc manipulation in vitro.

Rabbit polyclonal antibody to myc oncoproteins: (Cambridge Research Biochemical Ltd) is raised against a synthetic oligopeptide, Ala-Pro-Ser-Glu-Asp-Ile-Tip-Lys-Lys-Phe-Glu-Leu-Cys, which is common to c-, L- and N-myc and between several species. The IgG is isolated directly from rabbit sera and affinity purified prior to stabilisation and lyophilisation.

6E10: (Cambridge Research Biochemical Ltd) is a mouse monoclonal antibody raised against a synthetic peptide sequence, which recognises an epitope between residues 171 and 188 on the human c-myc oncoprotein (Evan et al., 1985). The immunoglobulin was obtained from a cell culture supernatant.
2.3.2.4  Staining of c-myc oncoprotein in patient material

One pair of samples was allocated for the c-myc analysis; sample and a control. In the case of test samples (as opposed to control samples), each nuclear pellet was incubated with the pan-myc (Cambridge Research Biochemicals Ltd, Cheshire UK) antibody for 1 hour at room temperature. The antibody was diluted 1:25 in 100μl of a solution of PBS containing 0.5% normal goat serum (NGS) and 0.5% of a detergent, Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma Chemical Co. UK). The control tubes were incubated with a solution of rabbit immunoglobulin fraction (Sigma Immunochemicals, Poole UK) added at the same concentration as the pan-myc antibody and diluted 1:25 in a solution of PBS containing 0.5% NGS and 0.5% Tween 20. The suspension was then washed in PBS and incubated with a secondary antibody; goat anti-rabbit IgG-FITC conjugate (Sigma Immunochemicals, Poole UK). This was added at a dilution of 1:50 in 100μl of the PBS/NGS/Tween 20 staining solution for 30 minutes at room temperature.

The mixture was then washed in PBS and re-suspended in a 1ml solution of PBS containing 1mg/ml ribonuclease A (Sigma Chemical Co., Poole UK), prior to addition of 20μl of propidium iodide (Sigma Chemical Co., Poole UK) at a concentration of 1mg/ml in PBS. Ribonuclease was used to minimise labelling of extrachromosomal oligonucleotides and RNA which would otherwise interfere with measurement of DNA content. Flow cytometric analysis was usually performed on the day of processing and always carried out within 24 hours to minimise leakage and other potential artefacts.

2.3.2.5  Staining of c-myc oncoprotein expression in cell lines

1. 250,000 fixed melanoma cells were aliquoted into conical bottomed test tubes.
2. The same number of cells were added to the control tube.
3. The cells were washed with 2ml of PBS and centrifuged at 2200rpm for 5 minutes.
4. The cellular pellet of the test sample was incubated with monoclonal anti-human c-myc antibody 6E10 (Cambridge Research Biochemicals Ltd.) at a dilution of 1:25 in 50μl of phosphate buffered saline (PBS) containing 0.5% normal goat serum (NGS) and 0.5% detergent (Tween 20) for one hour at room temperature. The control sample was incubated in the absence of the myc antibody, but with mouse IgG class specific antibody (Sigma Ltd, UK).

5. A further wash with PBS was performed as above to remove the primary antibody.

6. The secondary antibody (rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate - Sigma Immunochemicals, UK) was added at a dilution of 1:20 in phosphate buffered saline containing 0.5% NGS and 0.5% Tween 20 and the cells incubated for 45 minutes at room temperature.

7. The secondary antibody was removed by washing with PBS as above.

8. The mixture was re-suspended in 1ml of PBS containing 1mg/ml of Ribonuclease A (Sigma Immunochemicals, UK). DNA staining was performed by the addition of 20μl of propidium iodide at a concentration of 1mg/ml (Sigma Immunochemicals, UK).

9. The stained samples were analysed on a FACScan flow cytometer (Becton Dickenson, San Jose CA).

Data was analysed on at least 10,000 events (cells) for each sample. The fluorescence ratio was calculated by dividing the mean fluorescence of the sample (i.e. with primary antibody) by that of the corresponding control (i.e. without primary antibody) for each specimen.

2.3.2.6 Data Analysis and measurement of oncoprotein

Data was analysed using a computer acquisition/analysis programme, Lysys II (Becton Dickinson, San Jose Calif). Events were quantified by the imposition of computer generated windows (CGW) to define specific regions on the histogram or two-dimensional dot plot.
2.3.2.6.1 Use of Computer-Generated Windows (CGWs)

CGWs allow definition of specific populations of nuclei or cells and required phases of the cell cycle. Regions can be set around populations to omit extraneous interference from debris, or from populations of cells whose data is not required, as shown in figure 2.1. These regions are retained and superimposed on the control sample, to allow comparison of the number of nuclei and fluorescence in identical regions.

2.3.2.6.2 Calculation of Oncoprotein levels

Using Lysys II, oncoprotein positivity was calculated from comparison of the number of events within regions applied to the dot plots of both the control and test sample. Initially, a region was set around the control dot plot to demarcate and differentiate labelled from unlabelled nuclei. However the secondary antibody (FITC) adheres non-specifically to a small proportion of nuclei and cellular debris, estimated to be less than 2% of the whole population of labelled material. To exclude this fluorescence, a region is set around those 2% of nuclei exhibiting the highest fluorescence values (Region 1 as shown in Figure 2.2). Using computer analysis this same region was superimposed on the antibody-labelled dot plot of the same specimen (Region 1 as shown in Figure 2.3) and subtraction of the event count of the antibody sample from the control gives the overall number of nuclei showing specific labelling due to oncoprotein expression. This is represented as a percentage of all nuclei, to give the % positivity. Further regions can be set around populations of nuclei that lie within different phases of the cell-cycle, to allow analysis of oncoprotein expression within each phase as illustrated in figure 2.1. The mean fluorescence of the FITC labelled-nuclei gives an estimate of the relative amount of protein contained within each cell cycle phase and this can be represented numerically as a ratio of mean green fluorescence of positive cells compared to control (unlabelled) cells.
This illustrates the regions used to define specific populations within phases of the cell cycle. The number of labelled nuclei in each phase of the cell cycle in diploid tumours was calculated by application of computer generated windows to the control and test contour plots (see fig 2.2 and 2.3). The contour plot above shows c-myc expression in a diploid primary melanoma.

- R1 Labelled nuclei in G1
- R2 Labelled nuclei in S phase
- R3 Labelled nuclei in G2
- R4 Unlabelled nuclei in G1
- R5 Unlabelled nuclei in S phase
- R6 Unlabelled nuclei in G2
Figure 2.2  An example of a contour plot of an isotypic control antibody sample
Figure 2.3  
An example of a contour plot of the c-myc antibody stained sample  
(Region 1 defines the % positivity of the sample )

2.3.3  
Fluorescence Activated Cell Sorting

The Fluorescence Activated Cell Sorter (FACScan) is an automated cell analyser attached to a computer. Cell analysis is based on the principles of flow cytometry. In the experiments described here a Becton-Dickinson FACScan was used for flow cytometry studies. This FACScan is capable of measuring 5 optical parameters simultaneously, forward and side scatter and 3 different spectral regions of fluorescence. It has an air-cooled 15 miliamp Argon-ion laser with an excitation wavelength of 488nm. The sensors consist of 3 high performance photo multipliers with band pass filters of 530, 585 and >650 nm. The computer system displays the optical information as either a frequency histogram, which shows the fluorescence intensity plotted against the number of events, or as a dual parameter correlated plot (dot plot) which displays one parameter plotted against another. These computer-
generated cytograms can be further analysed by setting polygons around the populations of interest for which the computer will generate statistical information.

The Cell Sorter (CS) is a flow cytometer with an additional sort facility. In addition to the recognition of cell populations based on optical parameters, the cell sorter enables sub-populations of cells to be selected and individual cells from the selected population to be sorted and collected. It utilises a droplet sorting method in which the cells exit the flow chamber in a jet which breaks up into regular droplets. The droplets contain the cells of choice, having been selected using the computer system described above by gating a subset of cells on a cytogram. The cells of choice are charged and can therefore be deflected as they pass through a high voltage electrostatic field into a 96-well plate or falcon tube. The cells are sorted individually and the exact number sorted can be displayed during the sorting process.

2.3.3.1 Cell-sorting based on degree of GFP expression

In the cell sort experiments described in this thesis, a Becton-Dickinson FACSVantage cell sorter was used to sort and count live A375m cells based on their degree of green-fluorescence (as a measure of their transfection with either the pREV or the pREV myc17 plasmid which express GFP).

A sterile cell suspension was introduced into the flow cell and single live cells were selected from the cell population by analysing the cytogram dot-plot of forward scatter against green fluorescence. This displays cell doublets, debris and single cells as separate regions. The settings used were a flow rate of 3000 cells/second, drop-delay frequency of 11.0, a drive frequency of 22.6kHz, log amplification and a 3-drop envelope. The count mode was set to normal, the sheath pressure at 11psi and the sample differential at 1psi.

The single, green-fluorescent live cells were selected for sorting using the computer system by generating a polygonal area around this population only. Two examples of the dot-plots obtained are displayed in Figure 2.4. The upper dot-plot shows the GFP positive cells in region 1. They have FL1 values for green fluorescence greater than $10^5$. Those cells in region 2 demonstrate no green fluorescence, with FL1 values below $10^1$. The lower dot-plot illustrates the regions used to sort the cells for the
second experiment assessing the effect of the pREVmyc17 and pREV plasmids on the growth of A375m cells. Region 1 contains those cells demonstrating high levels of green fluorescence, region 2 contains those cells weakly green fluorescent and region 3 contains cells exhibiting no fluorescence. In this experiment cells from each region were sorted into different plates to assess the difference between high and low expression of the pREVmyc17 plasmid on the growth of A375m cells.

As the cells were sorted, they were simultaneously counted and the count was displayed on the vantage console. An exact pre-determined number of live cells could therefore be sorted into either a 96-well plate, for the growth effect experiments, or into a falcon tube for the assessment of A375m c-myc expression.

2.3.3.2 Green Fluorescent Protein

Green Fluorescent Protein (GFP) is a naturally-occurring, spontaneously fluorescent protein found in coelenterates, such as the Pacific jellyfish, Aequoria victoria (Chalfie et al. 1994). Its role is to transduce, by energy transfer, the blue chemiluminescence of the protein aequorin into green fluorescent light. GFP can be used as a protein tag by fusion to a wide variety of proteins, many of which have been shown to retain their function. When expressed in mammalian cells GFP fluorescence is distributed throughout the cytoplasm and nucleus. It therefore has a wide range of applications as a measure of protein-protein interactions and as a reporter of gene expression.

GFP is a cylindrical protein, composed of 238 amino acids. Its absorbance/excitation peak is at 395nm, with a minor peak at 475nm. The emission peak is at 508nm. However, wild type GFP is sub-optimal for many applications due to its low fluorescence intensity when excited by blue light (488 nm), a significant lag in the development of fluorescence after protein synthesis, and poor expression in many higher eukaryotes. To improve upon these qualities, a mutant of GFP with a significantly larger extinction coefficient for excitation at 488nm has been combined with a re-engineered GFP gene sequence containing codons preferentially found in highly expressed human proteins (Yang 1996). The combination of improved fluorescence intensity and higher expression levels yield an enhanced GFP (EGFP)
which provides greater sensitivity in most systems. EGFP can be detected at much lower concentrations than GFP, 100nM as compared with 1μM for GFP. This is equivalent to 10 000 molecules per cell. The excitation peak for EGFP is 380nm and the emission peak is 440nm.

For the experiments performed in this thesis EGFP was therefore used (Clontech).
Figure 2.4: Cytogram dot-plots for the cell-sort experiments
2.4 Oligonucleotides

All oligonucleotides used were synthesised by Genosys Biotechnologies Ltd. (Cambridge), with a phosphorothioate backbone to aid stability in-vitro.

2.4.1 C-myc antisense and ‘4G’ quartet control sequences

The c-myc antisense sequence was a 15-mer ODN complementary to the translation-initiation codon of exon 2 of the c-myc gene. The ‘4G’ quartet control was also a 15-mer ODN. This was not complementary to the c-myc gene sequence but contained 4 guanosine residues which are known to produce non-specific effects on cell growth in some experiments. This was therefore the most important control sequence to use. Neither sense nor nonsense ODNs had previously been shown to effect the growth of the A375m cell line in-vitro (Jagdeep Chana thesis 1998) and were therefore not used in these experiments. The sequences of the c-myc antisense and the ‘4G’ control are shown in Table 2.1.

<table>
<thead>
<tr>
<th>Phosphorothioate Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-myc antisense</td>
<td>5’-AACGTTGAGGGGCAT-3’</td>
</tr>
<tr>
<td>‘4G’ Quartet Control</td>
<td>5’-AAGCATACGGGTTG-3’</td>
</tr>
</tbody>
</table>

Table 2.1 C-myc antisense and ‘4G’ quartet ODN control sequences

2.4.2 Preparation and storage of oligonucleotides

Oligonucleotides were purified by high-pressure liquid chromatography (Genosys Biotechnologies Ltd). The products were supplied lyophilised and were dissolved at a concentration of 0.5nm/µl in sterile phosphate buffered saline containing 20mM/l
of HEPES buffer. Prepared aliquots containing 20µl of dissolved oligonucleotide were stored at -20°C in eppendorf tubes until required.

2.5 Targeting the c-myc gene using a ribozyme

2.5.1 Target and structure of the c-myc ribozyme

A ribozyme was designed according to standard principles to target the same translation-initiation codon of exon 2 of the c-myc mRNA which is targeted by the 15-mer c-myc ODN (base pairs 296-311). The efficacy of this c-myc antisense ODN has been demonstrated in melanoma by Jagdeep Chana (MD thesis 1998) and by Citro and Leonetti both in-vitro and in-vivo (Leonetti et al. 1996, Citro et al. 1998). A -GUU- triplet is present at the 3’ end of the translation-initiation codon of exon 2 (base pairs 303-305) of the c-myc mRNA. This triplet sequence is known to be a cleavage site for ribozymes and the structure of a hammerhead ribozyme was therefore designed around this site (figure 6.2). The standard -AAAG- and -CUGAUGA- sequences, along with a sequence high in guanine and cytosine residues, were selected to construct stem II of the ribozyme. A sequence of 5’-GAAGCU-3’ was complementary to the base sequence on the 3’ side of the -GUU-triplet and formed stem I. A 5’-GUUGAGG-3’ sequence was complementary to the base sequence on the 5’ side of the -GUU- triplet and formed stem II.

The plasmid pREV, previously constructed by Spencer Collis at the Paterson Institute of Cancer Research in Manchester, was selected as a vector to express the ribozyme targeting c-myc. The structure of this plasmid is shown in figure 6.3. The plasmid contains Green Flourescent Protein (GFP) as a green fluorescent marker whose expression is promoted by a CMV I.E. promoter. It also has a SV40 polyadenylation site at the 3’ end to stabilize the RNA transcript in mammalian cells. A ribozyme-cloning site is present, with a CMV promoter and SV40 polyadenylation site at the 3’ end, to promote ribozyme expression. The plasmid also contains genes encoding ampicillin and neomycin resistance whose expression is promoted by SV40 promoters.
2.5.2 Production of the ribozyme-encoding plasmid targeting the c-myc oncogene

2.5.2.1 Annealing of oligonucelotides

The first stage in the production of the ribozyme was annealing of the two oligonucleotides MycRzA, encoding the ribozyme, and a complementary oligonucleotide MycRzB. Both oligonucleotides were 41 bases in length and their sequences are represented in figure 6.4. Annealing of the oligonucleotides occurred with a 4-base overhang at each end such that a double-stranded base sequence resulted with two single-stranded ends (see figure 6.4). The single-stranded terminae were complementary to the Xba-I and Mlu-I cloning sites in the vector.

The two ODN’s were mixed together at a ratio of 1:1 at two different concentrations (100pmol and 250pmol) in 10µl of distilled water. The mixture was heated at 55°C for 5 minutes and then cooled to allow annealing to occur, and then left overnight.

2.5.2.2 Ligation

Before the annealed nucleotide sequences could be ligated into the pREV plasmid vector, the pREV circular plasmid DNA was digested to yield a linear, double-stranded DNA sequence to which the annealed nucleotides could be ligated and thus reform the circular loop. After digesting the plasmid to form the linear sequence, each end of the plasmid DNA was digested to form ‘sticky’ ends to which the annealed nucleotides would bind.

Linearisation of the pREV plasmid

The pREV plasmid has a Xba-I cleavage site which is the ribozyme cloning site. To cleave the circular plasmid DNA, 10 units of Xba-I enzyme (MBI Fermentas) was therefore added to 4µg of pREV DNA (in 5µl of distilled water), 1ul of buffer Y
(MBI Fermentas) and 3ul of distilled water and the solution incubated at 37°C for 2 hours. This yielded a linear plasmid DNA sequence with 2 Xba-1 terminae.

The mixture then underwent a clean-up stage:

1. 100µl of PN buffer (QIAgen) was added to the DNA digest to allow binding to the resin in the QIAquick centrifuge columns (QIAgen) and the mixture added to the QIAquick columns in centrifuge tubes.
2. The tubes were centrifuged at 6500rpm for 1 minute to allow filtration. The plasmid fragments remained in the resin.
3. 750µl of buffer PE (QIAgen) was added and the tubes spun again at 13000rpm for 1 minute to wash the final salts off the DNA fragments. A second spin at 13000rpm for 1 minute was used to wash off the alcohol.
4. 100µl of elution buffer (QIAgen) was then added to the column for 1 minute and the column centrifuged at 13000rpm for a further minute to remove the plasmid DNA fragments from the resin.
5. One end of the linearised DNA sequence was then digested by the Mlu-1 enzyme. 2µl (20 units) of Mlu-1 enzyme solution (Fermentas) was added to 5µg of linearised pREV DNA (in 5µl of distilled water), with 2µl of buffer R (Fermentas) and 11µl of distilled water. This yielded linearised pREV DNA with a Xba-1 binding site at one end and a Mlu-1 binding site at the other. These were complementary with the Xba-1 and Mlu-1 binding sites which were present at either end of the annealed nucleotide sequences.
6. A second clean-up stage was then followed for the Mlu-1 digested plasmid DNA using the same method as described above.
7. A 10µl portion of the elute was then run on an electrophoresis gel to establish the concentration of plasmid DNA in the solution. The elute contained linearised plasmid DNA at a concentration of 50ng/µl. The size of the linearised DNA fragment was 7.8kb.

Ligation reaction

Addition of the annealed nucleotide sequence to the linearised plasmid sequence, with the Xba-1 and Mlu-1 ends, resulted in ligation of the nucleotide sequence into the pREV plasmid and the reformation of the circular plasmid DNA, incorporating
the ribozyme-encoding nucleotide sequence. The digested plasmid was then mixed with the annealed ODN’s in the presence of ligase enzyme and ligase buffer to allow ligation to occur. A range of concentrations of both DNA reagents was used to ensure at least one of the ligation reactions was efficient. Either 200ng of linearised pREV plasmid in 4μl of distilled water or 350 ng of linearised pREV plasmid in 7μl of distilled water was mixed with the annealed ODNs. 2μl of ligase buffer, 1μl of ligase enzyme and 3μl of distilled water were used in the first case to make a total of 10μl, however no water was added for the second, higher concentration. The two concentrations of ODNs used were either 0.1nM of each ODN or 0.25nM. There were therefore 4 different mixtures resulting. The solutions were incubated overnight at 22°C to allow ligation to occur.

In addition, 4 control reactions, containing the single-chain ODN c-mycRzA instead of the annealed ODN’s, were set up paralleling each of the 4 combinations as described above. These were used to assess which reaction was the most efficient – ie to assess which reagent concentration combination produced the most efficient ligase enzyme effect. After bacterial transformation, plating and incubation, the concentration combination that produced the best ratio of colonies on the positive plate in comparison to the negative one, was the combination at which the enzyme had worked best. Isolation and PCR screening of bacterial colonies from this positive plate was therefore most likely to yield the highest chance of obtaining successfully transformed clones.

2.5.2.3 Transformation of DH5α e. coli bacteria

1. DH5α e. coli competent bacteria were slowly thawed on ice from −70°C storage in eppendorf tubes.
2. After the overnight incubation 10μl of each of the 4 ligation mixtures and 4 controls were added to 100μl of the competent DH5α bacteria and incubated on ice for 30 minutes to allow DNA binding to the bacterial surface.
3. The bacteria were then heat-shocked at 37°C for 45 seconds to allow transformation to occur and then put back on ice for 2 minutes.
4. The bacterial suspensions were then added to 950μl of L-broth (Luria broth) for 1 hour at 37°C with shaking to allow expression of the plasmid containing the gene for ampicillin resistance.

5. After 1 hour, 100μl of each bacterial suspension were plated out onto ampicillin-containing LB agar plates and incubated overnight at 37°C. Only those bacteria which had been successfully transformed by the plasmid, containing the ampicillin resistance gene, should therefore have grown.

### 2.5.2.4 Assay of the completed plasmid sequence using PCR screening of the transformed colonies

The positive plate selected for colony PCR screening was that which had produced the best ratio of colonies in comparison to its negative control. A number of colonies from the positive plate were screened for the presence of the ribozyme-containing plasmid using PCR. CMV and SV40 primers were utilised in order to screen for the presence of both the GFP and the ribozyme sequences.

1. A small amount of bacterial material from each colony to be screened was removed from the plate using a loop and placed into 100μl of distilled water in an eppendorf tube.

2. The eppendorfs containing bacterial material from each colony were then heated to 95°C for 3 minutes using a programmable thermostat (Biometra Trio Thermoblock) in order to lyse the cells and release the genetic material.

3. 10μl of each lysed cell suspension was then added to 40μl of PCR solution. The PCR solution consisted of 1μl of SV40 primer, 1μl of CMV primer and 25μl of Ready Load (containing nucleotides, Magnesium, tak polymerase, and buffer) and 13μl of distilled water.

4. The tubes were then placed into the programmable thermostat and an initial 2 minute 94°C warm-up cycle was followed by a 1 minute 94°C cycle in order to split the two DNA strands.

5. Subsequently the mixtures were subjected to a series of 40 cycles, each heating the mixture to 50°C for 3 minutes to allow primer binding and followed by a 5-minute 72°C cycle to allow polymerisation to occur.
6. Following PCR, 100µl of each PCR mixture was run on a 2% agarose electrophoresis gel at 100 volts for 45 minutes to establish the number and size of amplified DNA fragments. The ataxia telangectasia gene, which at 450 base-pairs is the same size as the ribozyme DNA fragment, was used as a positive control. A 100b ladder marker (Fermentas) was used for measurement of the band sizes. A polaroid photograph of the gel was obtained for records.

The gel was prepared from a 2% agarose mixture containing 2g of agarose dissolved in 100ml of a solution containing 10ml of tris-EDTA ten-times concentrate, 5µl of ethydium bromide and 90ml of distilled water.

The clone selected for further experiments was grown up initially at a small scale to produce enough DNA for DNA sequence analysis. It was therefore added to a flask containing 4ml of Lennox L broth (Sigma) and incubated overnight to grow up more bacteria containing the ribozyme-encoding plasmid. This was termed pREVmyc17 in reference to the bacterial colony which had been successfully transfected with the ribozyme-encoding plasmid.

2.5.2.5 Extraction and purification of pREVmyc17 DNA from DH5α e. coli bacteria using the mini-prep technique

The pREVmyc17 plasmid DNA was extracted and purified from the small-scale bacterial suspension using the mini-prep (QIAgen) technique.

1. 1ml of the bacterial suspension was transferred into an eppendorf and the bacterial pellet obtained by centrifugation at 6500rpm for 2 minutes.

2. The pellet was then vortexed and resuspended in 250µl of resuspension buffer P1. This contained RNAase enzyme at a concentration of 100µg/ml to remove any traces of RNA which might interfere with DNA sequencing.

3. 250µl of buffer P2 was added, containing sodium hydroxide and SDS detergent to lyse the bacteria and release the DNA. The tubes were inverted 4-6 times to mix them rather than vortexing them which would shear the DNA.

4. 350µl of buffer N3 was then added, containing potassium acetate to neutralise the acid. The tubes were inverted 4-6 times gently to allow mixing which was
followed by the appearance of a cloudy precipitate containing protein, DNA and other large molecules.

5. The tubes were centrifuged for 10 minutes at 13000rpm to produce a pellet containing large DNA molecules and cell membrane fragments. The supernatant, containing the plasmid DNA and cytosol components, was transferred into a QIA prep spin column (QIagen) placed in a 2ml collection tube.

6. The column with supernatant was centrifuged at 13000rpm for 30-60 seconds and the flow-through discarded.

7. The spin column was then washed with 0.75ml of buffer PE, containing ethanol, and centrifuged at 13000rpm for 30-60 seconds to remove small fragments of cell membrane.

8. The flow-through was discarded and the tube centrifuged again at 13000rpm for 1 minute to remove the remaining ethanol buffer PE.

9. The spin column was placed in a clean 1.5ml microfuge tube and the plasmid DNA eluted from the column by the addition of 50µl of buffer EB, containing 10mM Tris-Cl at pH 8.5, and centrifuged at 13000rpm for 1 minute.

10. The base sequence of the DNA plasmid pREVmyc17 was subsequently analysed using DNA sequence analysis to ensure the correct sequence was present before further production and testing of its efficacy.

2.5.2.6 Large-scale production of pREVmyc17 by DH5α e. coli and extraction and purification using the maxi-prep protocol

Following confirmation of the correct DNA sequence of the pREVmyc 17 plasmid, the DH5α e. coli bacteria producing the plasmid were grown up at a larger scale. This yielded larger quantities of the plasmid DNA to enable testing of its efficacy. The DNA was subsequently extracted and purified from the cultures using the QIagen maxi-prep protocol.

1. A starter culture of bacteria was initially produced by innoculating 5ml of L-broth containing ampicillin with the clone 17 bacteria in a universal and incubating it at 37°C for 8 hours with vigorous shaking (300rpm).
2. This starter culture was then added to 100ml L-broth (Sigma) with ampicillin in a flask and incubating overnight at 37°C with vigorous shaking (300rpm).

3. The following morning the bacterial suspension was centrifuged at 6000rpm for 15 minutes at 4°C to obtain a pellet containing approximately 100mg of bacteria.

4. The supernatant was removed and the bacterial pellet resuspended in 10ml of buffer P1 (containing 50mM of Tris-Cl, 10mM EDTA and 100μg/ml of RNAaseA).

5. 10ml of buffer P2 (containing 200mM of sodium hydroxide and 1% SDS) was then added to lyse the cells and the tube inverted 4-6 times to mix.

6. The tube was left to stand for a maximum of 5 minutes at room temperature and 10ml of cold buffer P3 (containing 3.0mM potassium acetate) was then added to neutralise the pH and the tube again mixed by inverting it 4-6 times. This was followed by the appearance of a white precipitate containing genomic DNA, proteins, cell debris and SDS.

7. The mixture was then poured into the barrel of a Maxi QIAfilter (Qiagen) and incubated for 10 minutes at room temperature to allow the precipitate to float to the top and form a layer on top of the solution.

8. 10ml of equilibration buffer QBT (containing 750mM sodium chloride, 50mM MOPS at pH 7.0 and 15% isopropanol) was added to the QIA cartridge to reduce surface tension. The cap was then removed from the base of the QIAfilter and the plunger inserted into the filter. Gentle pressure was applied to the plunger to filter the lysate through the QIA filter cartridge.

9. Approximately 25ml of lysate was recovered after filtration. The lysate was then allowed to enter the resin within the QIA cartridge by gravity.

10. The cartridge was subsequently washed twice with 30ml of buffer QC (containing 1.0M sodium chloride, 50mM MOPS at pH 7.0 and 15% isopropanol) to remove contaminants, particularly carbohydrates.

11. The plasmid DNA was eluted from the filter by the addition of 15ml of buffer QF (containing 1.25M sodium chloride, 50mM Tris-Cl at pH 8.5 and 15% isopropanolol) to the filter and the eluate collected in a 30ml tube.

12. The DNA was precipitated by the addition of 10.5ml of isopropanol at room temperature and the mixture immediately centrifuged at 9500rpm for 30 minutes at 4°C.
13. The supernatant was removed and the DNA pellet washed by the addition of 5ml of 70% ethanol at room temperature and centrifugation at 9500rpm for 10 minutes.

14. The pellet was air-dried for 5-10 minutes and redissolved in 1ml of buffer (10mM Tris-Cl at pH 8.5).

15. The quantity and purity of the DNA was measured using a spectrophotometer. The concentration of DNA was measured at 260nm and the content of RNA measured at 280nm. The purity of the DNA was assessed by the ratio of the 260nm to the 280nm measurements.

2.5.3 Investigation of Optimal Transfection Strategy

Transfection of A375m cells with either the ribozyme-encoding plasmid pREVmycl7 or pREV plasmid was used to assess the transfection efficiency of two different transfection reagents: Lipofectamine (Gibco) and Escort (Sigma). A375m cells treated with either transfection reagent, but no plasmid DNA, were used as controls.

Lipofectamine is a 3:1 (weight for weight) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOPSA) and the neutral lipid dioleoyl phosphatidyl ethanolamine (DOPE) supplied at a concentration of 2mg/ml. Escort is a liposome formulation of the cationic lipid N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and DOPE present at a ratio of 1:1 (w/w) at a concentration of 2mg/ml in 20mM MES, 150mM NaCl.

1. $5 \times 10^5$ A375m cells were added to each well of two 6-well plates and grown to 70% confluence. One plate was used to assess Lipofectamine and the other for Escort.

7. Two wells in each plate were each treated with either the pREVmycl7 or pREV plasmids complexed with one of the two transfection agents, or with distilled water together with the transfection agent to act as the negative control.
8. The DNA-transfection agent complexes were prepared prior to addition to the wells of the 6-well plates. In the case of the Lipofectamine plate, for each 2 wells 30μl of Lipofectamine was added to 20μl of DNA (containing 10μg of DNA) and 350μl of serum-free medium at 37°C added. The mixture was incubated at room temperature for 45 minutes to allow the DNA-lipofectamine complexes to form. Either the pREVmyc17 plasmid, the pREV plasmid, or distilled water (as a control) were used to represent the DNA.

9. For each two wells of the Escort plate, 30μl of Escort was added to 20μl of DNA (containing 10μg of DNA) and 460μl of serum-free medium at 37°C added. The mixture was incubated at room temperature for 15 minutes to allow the DNA-escort complexes to form. Again, either the pREVmyc17 plasmid, the pREV plasmid, or distilled water (as a control) were used to represent the DNA.

10. Following the incubation periods to allow complexing, the mixtures were added to the respective wells on the plates.

11. For the escort plate, 2ml of 10% medium was added to each well and for the Lipofectamine plate 2ml of 5% medium was added to each well.

12. The plates were then incubated in a 37°C incubator for 5 hours to allow transfection of the A375m cells to occur.

13. After the incubation period the transfection mixtures were removed, the cells washed with 0.5ml of PBS and 2ml of 10% medium replaced.

14. The plates were then incubated for 2 days at 37°C incubator to allow expression of the plasmids to occur.

15. After the two-day incubation each well was washed with 0.5ml of PBS, the cells were trypsinised with 0.5ml of trypsin-EDTA, neutralised with 1ml of 10% medium and transferred to universals.

16. After centrifugation at 1000rpm for 3 minutes, the cells were washed with 2ml of PBS and centrifuged again at 1000rpm for 3 minutes.

17. Finally they were resuspended in 0.3ml of PBS.

18. The transfection rate for the cells from each well was assessed by FACS analysis. The percentage of cells displaying green-fluorescence, which represented the GFP of the pREV and pREVmyc17 plasmids, was assessed using the FL1 detector of the FACS machine (Becton Dickinson).
2.5.4 Assessment of the effect of pREVmycl7 on the growth of the A375m melanoma cell line

The effect of transfection with the pREVmycl7 plasmid on the growth of the A375m cell line was compared with the growth rates of A375m cells transfected with the pREV plasmid alone or untreated A375m cells to act as controls.

1. A375m cells were grown to confluence in three T75 flasks – one for each of the groups as above.
2. One of the flasks was transfected with the pREVmycl7 plasmid and another with the pREV plasmid. The third flask received no treatment.
3. Each of the pREVmycl7 and pREV flasks were transfected with the appropriate DNA solution which had been pre-mixed with lipofectamine in serum-free medium.
4. 60μl of DNA solution, containing 30μg of DNA, was mixed with 90μl of lipofectamine and 1050μl of serum-free medium at 37°C and left for 45 minutes at room temperature.
5. The 10% cell medium was then removed from the flasks and the appropriate DNA / lipofectamine mixture was added together with 2.4ml of 5% medium.
6. The cells were then incubated with the DNA for 5 hours in a 5% CO₂ incubator at 37°C.
7. After 5 hours the medium and DNA were removed, the cells were washed with PBS and 15ml of 10% medium was added to each flask. The cells were then incubated in a 5% CO₂ incubator for 24 hours to allow enough time for expression of the relevant plasmid, as demonstrated by green fluorescence resulting from expression of the GFP.
8. After 24 hours the cells were sorted using a FACS machine into 96 well plates, 1500 cells/well. Each 96-well plate had 150μl of 10% medium added to each well and was incubated for at least 6 hours before the cells were added to allow the pH to equilibrate.
2.5.4.1 Experiment 1

In the first experiment the two transfected flasks were sorted into cells either positive or negative for green fluorescence, ie those cells expressing or not expressing the plasmid. The green fluorescent cells which had been exposed to the pREV plasmid were termed pREV +ve. The green fluorescent cells which had been exposed to the pREVmyc17 plasmid were termed myc +ve. The non-fluorescing cells were termed pREV -ve and myc -ve respectively. There were therefore five groups of cells from the first experiment (control cells, pREV +ve, pREV -ve, myc +ve and myc -ve).

2.5.4.2 Experiment 2

In the second experiment the two transfected flasks were both sorted into cells strongly and weakly positive for green fluorescence or negative cells, ie those cells expressing the plasmid to a high degree (termed pREV high +ve and myc high +ve), those expressing it more weakly (termed pREV low +ve and myc low +ve) and those not expressing it at all (termed pREV -ve and myc -ve). There were seven groups of cells from the second experiment (control cells, pREV high +, myc high +ve, pREV low +ve, myc low +ve, pREV -ve and myc -ve).

Figure 2.4 shows the cytogram dot-plots obtained from the two experiments and the regions selected for sorting of the cell populations.

The cells from the unexposed control flask in both experiments were also passed through the cell sorter to control for the effects of the FACS machine on the subsequent growth of the unexposed cells.

The plates were then incubated for five days to allow adequate cell growth for any differences in growth rate to be detectable using the MTS assay (Promega) in the last three days. Cell number in each well was measured indirectly by measuring the degree of conversion of MTS (Owen’s reagent) into a coloured, water-soluble formazan that absorbs light at 490nm. MTS is 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium:
1. 250mg of MTS reagent 1 was dissolved in 125ml PBS and filter-sterilised. For each plate, 10ml of this mixture was added to 900μl PBS and 100μl PMS (MTS reagent 2).

2. After two days incubation of the 96-well plates, 40μl of this mixture was added to each well and the plates incubated for four hours. The colour change was then measured using a luminometer (Berthold BL536) linked to a PC and the data analysed using Microsoft Excel.

3. Growth measurements were repeated on the next two days.

2.5.5 Establishment of optimal transfection conditions for A375m melanoma cells

To enable the effect of the pREVmyc17 plasmid on the c-myc expression of A375m cells to be assessed, a large number of transfected cells were required. To ensure an adequate number of transfected cells could be obtained, a large number of A375m cells had to be exposed to the DNA-Lipofectamine mixture and their transfection rates needed to be optimised.

To establish the optimal transfection strategy, A375m cells were transfected at a range of different cell densities and DNA-Lipofectamine concentrations.

1. Cells were plated out into 6-well plates at densities of either $2 \times 10^5$, $5 \times 10^5$ or $8 \times 10^5$ /well in 2ml of 10% medium and incubated overnight to allow attachment to the plates.

2. The following morning the wells were then transfected with pREVmyc17 DNA-lipofectamine complexes of varying quantities. Either 5μg of DNA (at 0.5μg/μl) with 15μl of Lipofectamine, 7.5μg of DNA with 22.5μl of Lipofectamine or 10μg of DNA with 30μl of Lipofectamine were added to each well of 6-well plates. There were therefore 9 different combinations of cell densities and DNA-lipofectamine quantity. 2 wells were used for each combination.

3. The DNA-Lipofectamine complexes were prepared as described previously in section 6.3.7 and added to the cells.
4. The cells were placed in a 37° C incubator for 5 hours to allow transfection to occur, after which the DNA was removed, each well was washed with 2ml of PBS and 2ml of 10% medium added to each well.

5. The cells were then incubated at 37° C for 24 hours to allow expression of the plasmid to occur.

6. After 24 hours, the cells were washed in 2ml of PBS, trypsinised with 0.5ml of trypsin-EDTA, centrifuged at 1000rpm for 5 minutes and resuspended in 1ml of PBS.

7. The percentage of green fluorescent cells (transfected with the plasmid) was assessed using a FACS machine (Becton Dickinson) as described in section 2.8.1.

8. The optimal transfection conditions could therefore be determined by assessing the percentages of cells transfected by each combination of DNA concentration and cell density.

2.5.6 Assessment of the effect of the pREVmyc17 ribozyme on c-myc oncogene expression in A375m melanoma cells

1. 9x10^6 A375m melanoma cells were seeded into each of three T150 flasks. The flasks were incubated overnight in a humidified, 5% CO₂ incubator at 37°C to allow them to attach.

2. The following day the cells were checked for the presence of infection, an even cell distribution in the flask and the correct density for optimal transfection. One flask was designated as the control flask and received no treatment. The cells in the second flask were designated for transfection with the pREVmyc17 ribozyme and those in the third flask to receive the pREV plasmid control sequence.

3. The transfection mixtures containing either the ribozyme or plasmid control were prepared in the following manner. 260μl of DNA solution containing 130μg of DNA mixed with 390μl of Lipofectamine (Gibco) reagent. 4.2ml of
serum-free medium was then added at 37°C. The universal was mixed and left to stand for 45 minutes at room temperature to allow DNA-lipofectamine complexes to form.

4. After 45 minutes the medium was removed from the A375m cells in the T150 flasks and the cells washed with 20ml of serum-free medium. The transfection mixtures were then added to the A375m cells and 5ml of medium containing 5% FCS added.

5. The flasks were then placed back in the incubators (level to ensure even coverage of the cells with the DNA) for 5 hours to allow transfection to occur.

6. After 5 hours the DNA was removed from the flasks and the cells washed with 20ml of PBS. 50ml of culture medium containing 10% FCS was added to each flask and the cultures incubated for a further 48 hours.

7. After 48 hours the cells were harvested from each flask in the normal manner by trypsinisation and centrifugation at 1000rpm for 5 minutes and resuspended in 3ml of 10% medium.

8. The cell suspension from each flask was then sorted as described in section 2.3.4. Cells were sorted from both the pREVmyc17 and pREV flasks by the assessment of their green fluorescence into GFP positive and negative populations. The cell suspension from the control flask was also passed through the cell sorter to control for the effect of the sorting process.

9. At least 5x10^5 cells were sorted for each group except for the control cells, where at least 1 million were sorted, 5x10^5 for each of the positive and negative controls.

10. The sorted cell suspensions were then transferred into conical tubes and centrifuged at 1500 rpm for 5 minutes to form a cellular pellet. The cells were then resuspended in 1ml of ice cold 70% ethanol to fix them whilst being agitated on a vortex machine.

11. Each cell suspension was then counted using a haemocytometer and 5x10^5 cells from each suspension transferred into conical tubes for staining of c-myc protein.

12. 5x10^5 cells from the control group were transferred into each of two conical tubes, one to act as the positive control and one to act as the negative control.

13. The cells were then washed once with 1ml of PBS and centrifuged at 2200rpm for 5 minutes.
14. The mouse anti-human c-myc primary antibody 6E10 (Genosys Cambridge) was then added and incubated for 1 hour.

15. The cells were washed again as previously, the secondary antibody (goat anti-mouse-FITC) added and they were incubated for 45 minutes.

16. After a further wash in PBS, as above, the cells were resuspended in 1ml of PBS containing Ribonuclease A and propidium iodide. The c-myc oncoprotein level was assessed by FACS analysis.

A full description of the methods used for the staining of c-myc protein described in steps 11-16 is present in section 2.3.2.5.
Chapter 3

An investigation of the relationship between c-myc expression and interferon sensitivity in uveal melanoma

3.1 Introduction

A significant number of patients who are potentially rendered disease-free by surgery remain at high-risk from recurrent melanoma. These include patients with deep primary melanomas (> 4mm in thickness) and those with lymph node metastasis who, following resection of macroscopic disease, may harbour micrometastatic deposits of melanoma in other sites. These groups of patients have 5-year survival rates of 47% and 10-46% (dependent on the number of nodes involved and other prognostic factors) respectively and may therefore potentially benefit from adjuvant treatment (Koh 1991).

To date the only adjuvant treatment licensed for use in melanoma patients is interferon following the publication of the ECOG 1684 trial (Kirkwood 1996). This trial demonstrated that patients with stage III melanoma, when treated with high-dose interferon, had an increase in relapse-free survival from 1.0 to 1.7 years and overall survival from 2.8 to 3.8 years at 7 years of follow-up. However, the survival benefit shown by the high-dose interferon regimen used in this trial have not been demonstrated by the preliminary results of the subsequent ECOG 1690 trial or by the lower dose strategies used in other studies (Cascinelli 1995), (Creagen et al. 1995) (Grob et al. 1998, Pehamberger et al. 1998, Kirkwood 1999). In addition, interferon is expensive and its use is associated with a significant incidence and degree of toxicity. Therefore, its use as adjuvant therapy in melanoma is now controversial.

The mechanisms by which interferon exerts its anti-tumour effect have not been fully established. Administration of interferon to tumours has been shown to lead to down-regulation of c-myc oncogene expression and up-regulation of p21/WAF gene expression, together leading to G1/S phase delay in the cell cycle and a reduction in tumour proliferation (Dron et al. 1986, Subramaniam et al. 1998).
expression was shown to increase following the administration of interferon α to
Daudi human Burkitt lymphoma cells in-vitro. A large reduction in c-myc expression
preceeded the increase in p21/WAF1 expression. This was accompanied by their
growth arrest in G1 phase resulting in terminal differentiation. Cell death by
apoptosis followed, accompanied by the induction of the apoptotic ICE-family
protease CPP32. However, this was not observed when the cells were exposed to
interferon gamma to which they are resistant (Subramaniam et al. 1998). C-myc
down-regulation and p21/WAF1 up-regulation occur by independent mechanisms
after exposure of cells to interferon, but both are thought to be required for
interferon-induced G0/G1 arrest to occur (Resnitzky et al. 1992).

Further evidence of the role of c-myc down-regulation has been provided by
experiments conducted with Balb/c 3T3 fibroblasts transfected with activated c-myc.
The untransfected cells were sensitive to interferon α and β and the cell cycle was
blocked in G1 phase following exposure of these cells to either agent. However in
the c-myc transfected line, a 3-10 fold increase in c-myc mRNA levels was noted and
the cells were resistant to the actions of the two agents. The transfected cells passed
freely from G1 into S phase even in the presence of interferon α or β (Einat et
al. 1988). A similar effect was noted in M1 myeloblastic cells transfected with an
SV-40 promoted c-myc plasmid (Resnitzky et al. 1991). Over-expression of the c-
myc gene, and the subsequent failure to down-regulate its expression after cell
exposure to interferon, is therefore associated with interferon resistance. This has
been demonstrated in experiments conducted with variant hairy cell leukaemia cell
lines in-vitro (Lehn et al. 1986).

Down-regulation of c-myc is thought to occur via a reduction in the half-life of its
mRNA, which was observed in Daudi cells treated with interferon. No effect on the
c-myc transcription rate in isolated nuclei of the Daudi cells following exposure to
interferon has been observed (Dani et al. 1985). Activation of the 2′,5′-
oligoadenylate synthetase/RNAase L enzyme has been observed in the DLD-1 Clone
A human colonic carcinoma and Daudi cell lines, associated with a reduction in c-
myc expression, following their exposure to interferon-β (Knight et al. 1985,
Chatterjee et al. 1992). Activation of this enzyme by interferon may therefore be the
mechanism by which c-myc mRNA is degraded and the reduction in activity of the
c-myc gene produced by interferon occurs.
Interferon is also known to increase surface expression of HLA molecules either directly, or via down-regulation of c-myc oncogene expression, therefore leading to increased cell killing by cytotoxic T-cells. Stimulation of HLA class I mRNA transcription has been observed in Daudi lymphoma cells following their exposure to interferon α and β (Dron, 1986) (Dani, 1985). Reduced tumour c-myc oncogene expression is known to be associated with increased HLA expression on the surface of tumour cells (Grover et al. 1996), increasing their antigenicity.

It can therefore be postulated that c-myc down-regulation plays a key role in the anti-tumour effect of interferon, leading to both inhibition of the cell cycle in the G1 phase, and up-regulation of HLA class I molecules on the cell surface. Cell death follows either by apoptosis or by killing from cytotoxic T cells. A postulated mechanism of action for interferon is shown in figure 3.1.

For interferon to be clinically useful as an adjuvant agent it must be effective against all melanoma tumours, including those with the worst prognosis as these are the group of patients most likely to experience disease recurrence. However this group of patients has been shown to have tumours which express the highest levels of the c-myc oncogene by a number of studies (Grover et al. 1997, Chana et al. 1998, Ross et al. 1998). As one of the mechanisms that interferon uses to block tumour growth is to down-regulate c-myc expression, the anti-tumour effect of interferon may be least effective in the patients who have the tumours of worst prognosis. However, this is the group of patients who need to obtain maximum benefit from interferon. This may potentially be a reason why interferon is of doubtful value as an adjuvant agent in melanoma.

The relationship between melanoma c-myc expression and tumour interferon sensitivity has therefore been investigated in this chapter using a series of uveal melanomas.
Figure 3.1: Postulated Mechanism of Action of Interferon

2',5'-OA = 2',5'-Oligoadenylate Synthetase / RNAse
3.2 Materials and methods

The surgical specimens from 45 patients who had undergone enucleation for uveal melanoma were obtained from Moorfields Eye Hospital and St. Bartholomew’s Hospital. This combined centre is the main specialist referral unit in the UK for patients diagnosed with ocular melanomas.

Uveal melanomas were detected in patients presenting with reduced visual acuity, a field defect or by an associated retinal detachment or vitreous haemorrhage. All patients were treated by surgical enucleation of the affected eye performed as an inpatient procedure under general anaesthetic. All patients presented with only primary disease as confirmed by a preoperative metastatic evaluation consisting of clinical examination, routine liver function tests and a liver ultrasound. Patients with tumours demonstrating extrascleral extension at the time of resection and histopathological diagnosis were treated subsequently with orbital radiotherapy. Those developing distant metastases at the time of this study were treated with chemotherapy as for metastatic cutaneous melanoma.

Each uveal melanoma specimen was divided into 2 portions under sterile conditions. One underwent conventional histopathological examination, which was performed by consultant histopathologist (IAC), and the other was used to assess its sensitivity to interferon. Measurement of the c-myc oncogene expression in each tumour was subsequently performed from material obtained from the paraffin blocks as described in section 2.3.2. Examples of the FACS profiles obtained following staining of a uveal melanoma specimen for c-myc oncoprotein are displayed in figure 3.2.

The methods used to assess the interferon sensitivity levels of each tumour are described in section 2.2. Two end-points of interferon activity were used; the interferon sensitivity index and the percentage of maximal inhibition as described in section 2.2. Examples of the interferon dose-response curves for 4 uveal tumours are displayed in Figure 3.3. The specimen 98M055a was relatively resistant to the action of interferon. It had a maximum percentage of inhibition of 5.5% and an interferon sensitivity index of 593.8. The specimen 98M059a was sensitive to interferon with a maximum percentage of inhibition of 64.1% and an interferon sensitivity index of 296.8.
The portion of the specimen for conventional histopathological examination was fixed in 10% formal saline prior to embedding in paraffin and sectioning. 4μm thick sections of the paraffin-embedded specimen were subsequently stained with haematoxylin and eosin (H & E).

In order to evaluate which factors influenced interferon sensitivity, the age, sex, and histological parameters of primary tumour were obtained from each patient's records. The histological parameters of the tumours available were the largest tumour diameter, the mitotic index and the predominant cell type (spindle cell, epithelial or mixed).

The tumour c-myc expression, interferon sensitivity values and corresponding clinical and histopathological data for each patient were entered into a JMP table and the statistical relationships between them assessed using the Annova t-test.

The relationships between the c-myc gene expression, tumour diameter and mitotic index were compared with the results of a previous series of c-myc oncogene expression measurements in uveal melanoma performed by Jagdeep Ghana (J.S. Chana MD Thesis 1998).

Interferon sensitivity measurements and clinicopathological details were not made available until after flow cytometric data had been analysed.
Figure 3.2: FACS profile of a uveal melanoma stained for c-myc oncoprotein

a: negative control

b: positive sample
Figure 3.3: IFN sensitivity data from 4 uveal melanoma specimens
3.3 Clinical and histopathological data

The median age at presentation was 59.5 years (range 25 – 85 years). 26 patients were male and 19 female. All patients in the study group presented with only primary disease at the time of enucleation. The mean largest tumour diameter, measured from the histological section, was 13.8mm (range 8-22mm). 16 tumours were of spindle cell morphology, 19 tumours were of mixed cell type and 9 tumours exhibited epithelioid cell type. 3 tumours were necrotic and therefore unclassifiable.

3.4 C-myc oncoprotein expression in uveal melanoma

All 45 uveal melanomas analysed in this study showed some staining for the c-myc oncoprotein. The median positivity for the series was 92.0% and the mean 85.1% (range 11.8 - 100%). The distribution of c-myc positivity in the series of tumours is shown in figure 3.4.

3.4.1 Patient age and c-myc expression

There was no significant difference between oncoprotein positivity in the younger patients (mean = 84.8%) compared to the older patients (mean = 80.5%) when the patients were stratified according to age, above and below the median for the group as a whole (61 years).

3.4.2 Patient sex and c-myc expression

There was a trend (p = 0.074) towards higher c-myc positivity in uveal melanomas from male patients (mean 88.5%) compared with those from females (mean 76.3%).
Figure 3.4: Distribution of c-myc positivity of the series of uveal melanomas

Median 92.00%
Mean  85.05%
Range 11.80 - 100.00%
Std Dev 21.20%
Std Error 3.13%
3.4.3  **Tumour diameter and c-myc expression**

A trend \( (p = 0.073) \) was also observed towards higher oncoprotein positivity levels (mean = 89.7%) in the larger tumours compared to the smaller tumours (mean = 75.7%) using a stratification above and below the median diameter for the group as a whole (13mm).

3.4.4  **Mitotic index and c-myc expression**

There was no difference in c-myc positivity between those tumours with a high mitotic index compared to those with a low index.

3.4.5  **Predominant cell type and c-myc expression**

There was no significant difference in c-myc positivity between tumours demonstrating a predominantly spindle cell, mixed or epithelial cell type.

3.5  **Analysis of factors influencing the interferon sensitivity of uveal melanoma**

The distributions of maximum percentages of inhibition and interferon sensitivity indices for the series of tumours are shown in Figures 3.4 and 3.5. The mean maximum percentage of inhibition by interferon was 17.3%. The mean interferon sensitivity index for the series of tumours was 617.5.
Figure 3.5: Distribution of interferon sensitivity indices of the series of uveal melanomas

Figure 3.6: Distribution of interferon maximum inhibition percentages of the series of uveal melanomas
3.5.1 **Age and interferon sensitivity index**

There was a trend ($p = 0.058$) towards a lower interferon sensitivity index in the younger patients (mean = 527.7) compared with the older patients (mean = 643.5) when the patients were stratified according to age, above and below the median for the whole group (61 years).

3.5.2 **Age and interferon maximum inhibitory percentage**

There was a statistically significant difference ($p = 0.048$) in maximum inhibitory index between the younger patients (mean = 30.8%) compared to the older patients (mean = 11.3%) when the patients were stratified according to age, above and below the median for the whole group (61 years).

3.5.3 **Sex and interferon sensitivity**

There was no difference in the interferon sensitivities of the tumours between the male and female patients when analysed by either their interferon sensitivity indices or maximum inhibitory percentage.

3.5.4 **Tumour diameter and interferon sensitivity index**

The larger tumours were found to have higher interferon sensitivity indices (mean = 582.9) compared to the smaller tumours (mean = 546.9) but this was not statistically significant - ie the larger tumours were less sensitive to interferon.
3.5.5 Tumour diameter and maximum interferon inhibitory percentage

The larger tumours were observed to have lower maximum interferon inhibitory percentages (mean = 16.9%) compared to the smaller tumours (mean = 28.6%) but this was not statistically significant – ie the larger tumours were found to be less sensitive to interferon by this measure of interferon sensitivity also.

3.5.6 Mitotic index and interferon sensitivity

There was no difference in interferon sensitivity when comparing the tumours with high or low mitotic indices using either the interferon sensitivity index or the maximum inhibitory level.

3.5.7 Predominant cell type and interferon sensitivity index

Tumours of mixed cell type were found to have higher interferon sensitivity indices (mean = 630.8) than the epithelial (mean = 567.6) and spindle tumours (mean = 574.5) but this was not statistically significant.

3.5.8 Predominant cell type and maximal inhibitory percentage

Tumours of mixed cell type were found to have lower maximal inhibitory percentages (mean = 8.5%) than the epithelial (mean = 24.2%) and spindle tumours (mean = 28.6%) but this was not statistically significant (p=0.11).
3.5.9 C-myc oncoprotein expression and interferon sensitivity index

The mean interferon sensitivity index for the whole group of tumours was 617.5 (range 133-1269). The series of tumours were divided into two groups above and below the median c-myc positivity value (92%). The mean interferon sensitivity index for the lower c-myc expression group was 553.4 (in the sensitive range). The mean interferon sensitivity index for the high c-myc expression group was 673.5 (in the resistant range) - an interferon sensitivity index above 600 implies resistance to interferon. The p-value for the Annova t-test used to compare the means of the two groups was 0.0728. Therefore there was a trend for the tumours expressing high levels of c-myc to be resistant to interferon. This data is represented are in figure 3.7.

3.5.10 C-myc oncoprotein expression and maximum inhibitory index

The mean maximal inhibitory percentage for the whole group of tumours was 17.3% (range -99.9% to 99.8%). The mean maximal inhibitory percentage for the low c-myc expression group was 28.1%. The mean maximal inhibitory percentage for the high c-myc expression group was 7.0%. The tumours expressing lower levels of c-myc were again found to be more sensitive to interferon. When the differences in interferon maximal inhibitory percentages between the high and low c-myc expressing groups was compared using the Annova t-test a p-value of 0.0499 resulted which was marginally statistically significant. The range and mean values for the maximal inhibitory percentage of the two groups are represented in figure 3.8.
Figure 3.7: C-myc expression and interferon sensitivity index in uveal melanoma

![Graph showing C-myc expression and interferon sensitivity index]

<table>
<thead>
<tr>
<th>Tumour C-myc Expression</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>21</td>
<td>553.38</td>
<td>47.72</td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>673.54</td>
<td>44.64</td>
</tr>
</tbody>
</table>

P = 0.0728
Figure 3.8: C-myc expression and maximal percentage inhibition by interferon in uveal melanoma

<table>
<thead>
<tr>
<th>Tumour C-myc Expression</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>22</td>
<td>28.1218</td>
<td>7.4849</td>
</tr>
<tr>
<td>High</td>
<td>23</td>
<td>7.0000</td>
<td>7.3204</td>
</tr>
</tbody>
</table>
3.6 Discussion

There is a clear need for an effective adjuvant therapy for patients at high risk of recurrence from melanoma. To date the only treatment licensed for use as adjuvant therapy in melanoma has been interferon α2b which followed the publication of the results of the ECOG 1684 trial. However interferon is a costly and toxic drug and the survival benefits demonstrated by the ECOG 1684 trial have not been replicated by any other clinical trials. The use of interferon as adjuvant therapy in melanoma is therefore now controversial.

The mechanisms by which interferon exerts its effects are not fully established. A number of studies have demonstrated that the growth inhibitory effects of interferon are associated with c-myc down-regulation. Furthermore, interferon is also known to up-regulate cell surface HLA expression, either directly or via c-myc down-regulation. This can be expected to lead to more effective anti-tumour activity by cell-mediated immunity. It can therefore be postulated that c-myc down-regulation plays a pivotal role in the anti-tumour activity of interferon. However the worst prognosis primary and metastatic melanoma tumours are known to express the highest levels of the c-myc oncogene (Grover et al. 1997, Ghana et al. 1998, Ross et al. 1998). This may potentially render them relatively resistant to the anti-tumour effects of interferon. To investigate the relationship between tumour c-myc expression and interferon sensitivity, these two parameters were measured in a series of 45 uveal melanomas.

The overall c-myc expression of the whole series of tumours was uniformly high – the mean c-myc positivity was 85.1% and the median value was 92%. This was significantly higher than the mean c-myc positivity of 70.2% which was demonstrated in a series of 71 uveal melanomas by the previous RAFT fellow (Jagdeep Chana MD thesis 1998).

A trend towards higher tumour c-myc positivity was observed in tumours of male patients. Tumours of larger diameter and those of mixed cell type demonstrated higher c-myc positivity levels, although this was not statistically significant. No relationship was shown between c-myc positivity and these factors in the previous study. The relationship between tumour c-myc expression and prognosis could not be assessed in this series of uveal melanoma tumours due to the short period of patient
follow-up. The future analysis of this relationship in this tumour series will be of interest as the previous series of uveal melanomas analysed by Jagdeep Chana showed high c-myc expression to be associated with an improved prognosis (Jagdeep Chana MD thesis 1998). This is in contrast to all other studies performed in other types of melanoma and other cancers. The reasons for this inverse relationship were not clear but may be related to the failure of conventional antibodies to detect abnormal tumour c-myc molecules in the poorest prognosis uveal melanoma tumours.

When the factors affecting tumour interferon sensitivity were assessed, age was found to be a statistically significant factor in predicting sensitivity to interferon as measured by the maximum inhibitory percentage. A trend towards increased sensitivity in younger patients was observed when the interferon sensitivity index was used to measure the interferon sensitivity. Both the larger tumours and those of mixed tumour cell type were associated with decreased interferon sensitivity by both measures of assessing interferon sensitivity although this didn’t reach statistical significance. Older melanoma patients are known to have a poorer prognosis when compared to younger patients with tumours of the same size. Tumour diameter and cell type have also been shown to be prognostic factors for uveal melanoma. These factors were related to interferon sensitivity in this series but without statistical significance, although it is epithelial rather than mixed cell type tumours that are thought to have the worst prognosis.

When the c-myc positivity was correlated with the maximum inhibitory percentage a marginally statistically significant relationship was demonstrated at the 5% degree of significance. The tumours expressing the highest levels of c-myc were the least sensitive to interferon. A similar relationship was found when the interferon sensitivity index was used to assess interferon sensitivity, although this did not reach statistical significance. In addition, the observation that the group of tumours as a whole expressed high levels of the c-myc oncogene (mean = 80.5%) and were resistant to interferon (interferon sensitivity index = 617.5) lends further weight to the c-myc-interferon sensitivity link. Furthermore, the larger tumours and those of mixed cell type were associated with both higher c-myc positivity and interferon resistance.

These results support the concept that c-myc down-regulation is associated with the cell growth inhibition produced by interferon. They also demonstrate that interferon
resistance is associated with tumour c-myc overexpression. Therefore not only is c-myc over-expression associated with poor prognosis in the majority of melanoma types, in addition to a number of other cancers, but this study appears to show that it is also associated with resistance to interferon. This has important implications for the use of interferon as adjuvant therapy for melanoma. The patient group which would gain most from effective adjuvant therapy, that is those with the worst prognosis and the highest c-myc expression levels, are the least sensitive to interferon. This may explain why the efficacy of interferon as an adjuvant agent in melanoma has been questioned. It suggests that the use of interferon alone as adjuvant therapy in melanoma is likely to be of no benefit as has been demonstrated by all the clinical trials except one - ECOG 1684.

More importantly, perhaps, the link between c-myc over-expression and interferon resistance suggests that the use of therapeutic strategies to block the effects of the c-myc gene, such as by the use of antisense gene therapy, may potentially overcome tumour resistance to interferon. The combination of these two therapies may therefore offer a potential method to increase the efficacy of interferon and offer the prospect of more successful adjuvant therapy in the future. This is therefore the subject of the next chapter in this thesis.
Chapter 4

Combination of c-myc antisense and interferon in melanoma

4.1 Introduction

Interferon is currently the only adjuvant therapy available for use in melanoma. However, interferon is a toxic and expensive drug and the survival benefit demonstrated by its use as adjuvant therapy in the ECOG 1684 trial has not been shown by any other trials (Kirkwood 1996) (Cascinelli 1995) (Creagen et al. 1995) (Grob et al. 1998, Pehamberger et al. 1998, Kirkwood 1999).

The evidence that interferon down-regulates c-myc expression in order to block cell growth has been described in detail in the previous chapter. It is known that melanomas of the poorest prognosis express the highest levels of the c-myc oncogene (Grover et al. 1997, Chana et al. 1998, Ross et al. 1998). We have therefore hypothesised that it is more difficult for interferon to down-regulate c-myc expression in this group of tumours and they are therefore more resistant to its mode of action. This may explain why interferon is of doubtful value as an adjuvant agent in melanoma as the most aggressive tumours may be interferon-resistant.

Our study has demonstrated an association between c-myc over-expression and interferon resistance in uveal melanoma which correlates well with observations made in a number of tumour cell lines (Einat et al. 1988) (Lehn et al. 1986, Resnitzky et al. 1991). We therefore postulate that blocking c-myc expression by an antisense strategy may increase the growth inhibitory effects of interferon and potentially increase its efficacy as an adjuvant therapy.

To test this hypothesis we have treated a series of melanoma cell lines with a combination of interferon and c-myc antisense to assess whether any additive or synergistic effects result. We have also measured the c-myc expression levels of each cell line in order to correlate the degree of benefit produced by the addition of c-myc antisense to interferon with the c-myc expression level of each cell line.
4.2 Experimental Protocol

Four cell lines were used in this investigation – the A375m, MeWo, MM96L and SkMel 28 human melanoma cell lines. All were obtained from Dr David Jackson, of the Imperial Cancer Research Fund, Leeds, except for the A375m, which was held at the Gray Laboratory.

The effect of combining c-myc antisense with interferon on the growth rates of each cell line was assessed using the ATP-chemosensitivity assay as described in section 2.2.1. The interferon sensitivity index of each cell line was calculated from this information as described in section 2.2.2. The c-myc expression levels of each cell line were subsequently measured using flow cytometry, as described in section 2.3.2.5, and correlated with both the interferon sensitivity index and the degree of additive effect produced by the combination of c-myc antisense with interferon at the 25% test drug concentration. This was calculated using the formula:

\[
\text{Degree of additive effect} = \\
(\% \text{ inhibition produced by ifn + antisense}) - (\% \text{ inhibition produced by ifn}) - \\
(\% \text{ inhibition produced by ifn + ODN control} - \% \text{ inhibition produced by ifn})
\]

4.3 Results

4.3.1 Combination of c-myc antisense with interferon in the A375m melanoma cell line

A dose-response curve was produced by the use of interferon alone. This ranged from 10.8% at 6.25% TDC (standard error = 8.7%) to 46.4% at 200% TDC (standard error = 20.8%). C-myc antisense and the control ODN inhibited cell growth by 28.6% (standard error = 4.8%) and 17.1% (standard error = 5.6%) respectively at a constant 25μm concentration.
Combination of the control ODN with interferon resulted in no significant increase in growth inhibition above that produced by interferon alone. However, combination of c-myc antisense with interferon significantly increased the inhibition of cell growth produced by interferon. This was most evident at the low and medium interferon doses used. At 6.25 % TDC, growth inhibition was increased from 10.8% at 6.25% TDC with interferon alone (standard error = 8.7%) to 54.2% by the combination (standard error = 4.7%). At 50 % TDC, growth inhibition was increased from 27.6% with interferon alone (standard error = 13.9%) to 57.4% by the combination (standard error = 6.7%). These results are demonstrated in figure 4.1.

The magnitude of the additive effect observed by the combination of c-myc antisense with interferon was analysed using the methods described by Steel and Peckham (Steel et al. 1979). The additive effect window was calculated, demonstrating the growth inhibitory effect that would be expected by the combination of interferon with c-myc antisense if the effects were additive only. At each dose of interferon, the sum of the growth inhibitory effects produced by each drug acting alone was calculated. The error of the window was obtained using the formula:

$$\sqrt{(\text{standard error of c-myc antisense effect})^2 + (\text{standard error of interferon})^2}$$

The window of additivity for the combination of interferon and c-myc antisense is represented in figure 4.2. It demonstrates that at the lower doses of interferon, the actual growth inhibitory effect produced by the combination of the two drugs is greater than would be expected by the addition of the effects produced by the two drugs acting individually. This suggests a synergistic mechanism.

### 4.3.2 Combination of c-myc antisense with interferon in the SkMel 28 melanoma cell line

Interferon alone inhibited cell growth by -2.2% at the 6.25% TDC (standard error = 1.2%) up to 46.5% at the 200% TDC (standard error = 2.4%). C-myc antisense
inhibited cell growth by 45.7% at a constant 25μm concentration (standard error = 1.5%). The control ODN produced 48.4% inhibition.

Combination of c-myc antisense with interferon increased the inhibition of cell growth produced by interferon alone at all the doses of interferon assessed. At the 6.25% TDC, growth inhibition was increased from -2.2% with interferon alone (standard error = 1.2%) to 27.7% by the combination (standard error = 3.6%) and from 46.5% (standard error = 2.4%) to 97.8% (standard error = 0.9%) at the 200% TDC. However, the control ODN also increased the growth inhibition produced when combined with interferon, although the effect was not as great as that produced with the c-myc antisense. Growth inhibition was increased from -2.2% (standard error = 1.2%) to 12.2% (standard error = 1.9%) at the 6.25% TDC and from 47.2% to 99.4% at the 200% TDC. These results are demonstrated in figure 4.3.

4.3.3 Combination of c-myc antisense with interferon in the MM96L melanoma cell line

The growth inhibition produced by both the c-myc antisense and the ODN control were greater in the MM96L cell line, although that produced by the antisense (64.0% - standard error = 6.4%) was less than that produced by the control (72.5% - standard error = 4.3%).

Interferon alone inhibited cell growth by 3.1% at the 6.25% TDC (standard error = 1.8%) up to 69.0% at the 200% TDC (standard error = 12.7%). Combination of c-myc antisense with interferon significantly increased the inhibition produced. At the 6.25% TDC, growth inhibition was increased from 3.1% with interferon (standard error = 1.8%) to 63.6% (standard error = 3.9%) by the combination. At the 200% TDC growth inhibition was increased from 69.0% (standard error = 12.7%) by interferon to 89.9% (standard error = 2.4%) by the combination. However, the increase in growth inhibition was larger when the control ODN was added to interferon, 74.6% (standard error = 3.3%) growth inhibition was produced at the 6.25% TDC and 94.8% at the 25% TDC. These results are demonstrated in figure 4.4.
Figure 4.1: C-myc antisense and interferon in the A375m cell line

Figure 4.2: C-myc antisense and interferon in the A375m cell line: Window of theoretical additivity
Figure 4.3: C-myc antisense and interferon in the SkMel 28 cell line

![Graph showing the effect of C-myc antisense and interferon on SkMel 28 cell line growth inhibition.]

Figure 4.4: C-myc antisense and interferon in the MM96L cell line

![Graph showing the effect of C-myc antisense and interferon on MM96L cell line growth inhibition.]

126
4.3.4 Combination of c-myc antisense with interferon in the MeWo melanoma cell line

The growth inhibition produced by the c-myc antisense (11.1% - standard error = 1.3%) and control ODN (11.4% - standard error = 2.7%) at constant 25μm concentrations were of similar magnitudes, although they both had less effect than in the other cell lines. Interferon inhibited cell growth by 6.1% (standard error = 1.4%) at the 6.25% TDC up to 44.6% at the 200% TDC (standard error = 2.0%). Combination of c-myc antisense with interferon significantly increased the inhibition of cell growth produced by interferon alone at the lower and medium doses of interferon used. Growth inhibition was increased from 6.1% (standard error = 1.4%) with interferon alone to 36.7% (standard error = 3.1%) by the combination at the 6.25 % TDC and from 21.3% (standard error = 2.3%) to 29.45 (standard error = 1.8%) at the 25 % TDC. However, there was also a significant increase in growth inhibition produced when the control ODN was added to interferon. Combination of the control ODN with interferon increased the growth inhibition produced by interferon alone from 6.1% (standard error = 1.4%) to 36.7% % (standard error = 3.1%) at the 6.25% TDC and from 21.3% (standard error = 2.3%) to 34.5% at the 25% TDC. These results are demonstrated in figure 4.5.

4.3.5 Measurement of c-myc expression levels in the A375M, SkMel 28, MeWo and MM96L melanoma cell lines

The results of the measurements of c-myc expression in the 4 melanoma cell lines are shown in figure 4.6. The c-myc expression levels are displayed as mean fluorescence ratios. C-myc expression was found to be highest in the A375m cell line which had a mean fluorescence ratio of 17.9. The SkMel 28 cell line had a c-myc mean fluorescence ratio of 11.6, although the standard error was relatively large at 4.5. The MeWo and MM96L cell lines had lower c-myc expression levels with mean fluorescence ratios of 9.0 and 8.2 respectively.
Figure 4.5: C-myc antisense and interferon in the MeWo cell line
Figure 4.6: C-myc expression in the A375m, MeWo, MM96L and SkMel 28 cell lines
4.3.6 The relationship between interferon sensitivity and c-myc expression in the 4 cell lines

The interferon sensitivity index for the A375m and SkMel 28 cell lines were 475.6 (standard error = 52.6) and 480.1 (standard error = 9.7) respectively. The MeWo and MM961 cell lines had sensitivity indices of 461.0 (standard error = 7.6) and 448.2 (standard error = 26.7). No statistical relationship was found between the interferon sensitivities for the cell lines and their c-myc expression levels, as is shown in figure 4.7.

4.3.7 The relationship between the additive effect produced by c-myc antisense with interferon, at the 25% test drug concentration, and c-myc expression in the 4 melanoma cell lines

The A375m cell line produced the greatest additive effect with the c-myc antisense and interferon (28.8%) and also had the highest c-myc expression level with a mean fluorescence ratio of 17.9. The SkMel 28 also showed significant additive effect (23%) and had a c-myc mean fluorescence ratio of 11.1. The MeWo cell line produced a lower degree of additivity (4.9%). Antagonism of interferon’s effect occurred in the MM96L cell line (-14.1% growth inhibition). Both the MeWo and MM96L cell lines expressed lower levels of the c-myc oncogene. These results are shown in figure 4.8. A relationship between the degree of additive effect produced by the combination of c-myc antisense with interferon and the c-myc expression of the 4 cell lines was therefore demonstrated, although the correlation coefficient of 0.66 did not reach statistical significance (p = 0.19).
Figure 4.7: Interferon sensitivity index and c-myc expression in the 4 melanoma cell lines
Figure 4.8: Additive effect produced by c-myc antisense with interferon and c-myc expression in the 4 melanoma cell lines.

Linear Fit

Correlation coefficient = 0.66

\( p = 0.19 \)
4.4 Discussion

Patients with thick primary melanomas (> 4mm) and those with regional lymph node metastasis are at high-risk from recurrent melanoma following resection of macroscopic disease. These groups of patients have 5-year survival rates of 47% and 10-46% (dependent on the number of nodes involved and other prognostic factors) respectively (Koh 1991). This is because a significant number of patients in these groups harbour micrometastatic deposits of melanoma at distant sites and they therefore would benefit from adjuvant treatment.

Although interferon has been used as adjuvant treatment in melanoma patients following the publication of the ECOG 1684 trial (Kirkwood 1996), its efficacy has now been questioned. The survival benefits shown by the high-dose interferon regimen used in the ECOG 1684 trial have not been demonstrated by the preliminary results of the subsequent ECOG 1690 trial, or by the lower dose strategies used in other studies (Cascinelli 1995), (Creagen et al. 1995) (Grob et al. 1998, Pehamberger et al. 1998, Kirkwood 1999).

The mechanisms by which interferon exerts its anti-tumour effect are not fully established. However, it is known that administration of interferon to a number of cell lines leads to down-regulation of c-myc oncogene expression, accompanied by growth arrest in the G1 phase of the cell cycle, terminal differentiation and cell death via apoptosis (Subramaniam et al. 1998) (Resnitzky et al. 1992). Furthermore, overexpression of the c-myc gene, and a subsequent failure to down-regulate its expression after cell exposure to interferon, has been associated with interferon resistance in a number of cell lines (Einat et al. 1988) (Resnitzky et al.1991). Overexpression of the c-myc gene, which is known to be associated with melanomas of worst prognosis (Grover et al. 1997, Chana et al. 1998, Ross et al. 1998), would therefore also seem to be associated with resistance to interferon. The previous chapter demonstrated a relationship between c-myc overexpression and interferon resistance in uveal melanoma which would also appear to confirm this hypothesis. Combining this information, it would appear that interferon resistance is most likely to be prevalent in tumours which stand to gain the most benefit from adjuvant therapy. This might provide an explanation for the failure of interferon to provide

If the failure of interferon as adjuvant therapy in melanoma is related to the overexpression of the c-myc gene in the worst prognosis tumours, it could then be postulated that blocking c-myc overexpression using antisense ODNs might offer a means of overcoming resistance in this group of tumours. This would potentially increase the anti-tumour efficacy of interferon and lead to an effective adjuvant therapy strategy for use in melanoma. By increasing the efficacy of interferon, combination with c-myc antisense might also allow the use of interferon at lower doses, reducing the significant toxicity associated with its use.

To test this hypothesis a number of human melanoma cell lines were treated with a combination of interferon and c-myc antisense and the effects on cell growth compared with cells treated with either agent alone or with a non-specific ODN control.

When the interferon and c-myc antisense were combined together in the A375m cell line an additive effect was observed which was most obvious at the lower and medium doses of interferon used (Figure 4.1). These results were analysed using the methods described by Steel and Peckham (Steel et al. 1979) (Figure 5.2). This demonstrated that the actual inhibition of cell growth produced by the combination was greater than that produced by the sum of the effects of the two drugs used individually at the 6.25%, 12.5% and 25% test drug concentrations. This suggests that the interferon and c-myc antisense may act by a synergistic mechanism, such as via down-regulation of the c-myc oncogene.

In the SkMel 28 cell line an additive effect was also observed by the combination of c-myc antisense and interferon (Figure 5.3). Growth inhibition was increased from 13.6% produced by interferon alone (standard error = 2.0%) to 63.1% (standard error = 8.9%) by the combination. However, an additive effect was also observed with the non-specific ODN control, although this was not as great as with the c-myc antisense. Growth inhibition was increased from 13.6% (standard error = 2.0%) to 40.1% (standard error = 6.0%) by addition of the control ODN.

Although the MeWo and MM96L cell lines did show increased growth inhibition by addition of c-myc antisense to interferon, this effect was also observed with the ODN control and was therefore likely to have been due to a non-specific toxicity-related effect caused by both ODNs.
Analysis of the c-myc expression levels of the 4 cell lines showed that the A375m and SkMel 28 cell lines expressed the highest levels of the c-myc oncogene, with mean fluorescence ratios of 17.9 and 11.1 respectively. The MM96L and MeWo cell lines were found to have lower levels of c-myc oncogene expression with mean fluorescence ratios of 8.2 and 9.0 (Figure 4.6).

No association was demonstrated between the c-myc expression levels and interferon sensitivity indices of the cell lines (Figure 4.7). However, a relationship was evident between the degree of additive effect produced by the combination of c-myc antisense with interferon and the c-myc expression of the 4 cell lines, although the correlation co-efficient of 0.66 did not reach statistical significance (Figure 4.8). The greatest additive effect was observed in the A375m and SkMel 28 cell lines which also expressed the highest levels of the c-myc oncogene. A low level of additive effect was observed in the MeWo cell line, and antagonism of the action of interferon was seen in the MM96L cell line, both of which expressed low levels of the c-myc oncogene.

These observations are consistent with an antisense additive effect only occurring in cell lines that over-express the c-myc oncogene. This may be because these cell lines have a degree of interferon resistance due to their overexpression of the c-myc gene. Although the c-myc expression of the cell lines did not correlate with their interferon sensitivity, a number of other factors may also influence interferon sensitivity in addition to c-myc expression in these cell lines, such as p21/WAF gene expression or variations in the JAK/STAT cell signalling pathway.

In summary we have observed additive effect when combining c-myc antisense with interferon in melanoma cell lines which overexpress the c-myc oncogene. This was most obvious in the A375m cell line, which expresses the highest levels of the c-myc oncogene. This suggests that the combination of c-myc antisense with interferon may increase the anti-tumour efficacy of interferon. The use of corrective gene therapy targeting the c-myc oncogene, or other factors producing interferon resistance, may therefore be a potential strategy to overcome the resistance of the worst prognosis melanoma tumours to interferon, and increase its efficacy as adjuvant therapy in melanoma.
Chapter 5

Combination of c-myc antisense with chemotherapy drugs in melanoma

5.1 Introduction

The prognosis for patients with metastatic melanoma is very poor - only 5% of patients with stage IV disease are expected to survive for 5 years (Balch et al. 1992). Conventional forms of treatment, such as surgery, chemotherapy and radiotherapy, are generally ineffective.

Of the chemotherapy drugs currently in use Dacarbazine (DTIC) is thought to be the most effective single agent, with response rates of 10-20%. However, these responses are generally of short duration only, with long-term complete responses only seen in 1-2% of patients (Hill et al. 1984). In addition, the impact on visceral metastases compared with skin and subcutaneous is poor. A number of combination chemotherapy regimens have also been tested but few have shown advantages over DTIC alone. Cis-platinum, Vinblastine and DTIC (CVD) has been reported to yield overall response-rates of between 24% and 45% in early trials (Legha et al. 1989, Lee et al. 1995) but the results of randomized studies compared with DTIC alone are not yet known. The Dartmouth regimen (Cis-platinum, Carmustine, DTIC and Tamoxifen) showed early promise with overall response rates of 55% reported by early trials (Del Prete et al. 1984), however later phase II trials by the Southwest Oncology Group showed overall response rates of only 15% (Margolin et al. 1998).

The low efficacy of standard forms of therapy in melanoma has led to the investigation of new treatment strategies such as corrective gene therapy. A number of groups have demonstrated the efficacy of antisense gene therapy against melanoma in-vitro and in-vivo (Leonetti et al. 1996, Citro et al. 1998, Jansen et al. 1998, Rieber et al. 1999). As abnormal over-expression of the c-myc oncogene is thought to play a key role in the development of melanoma, this project has investigated the use of c-myc antisense therapy for melanoma and has demonstrated its efficacy in a number of cell lines and also in-vivo (Rajiv Grover MD thesis 1996,
Jagdeep Chana MD thesis 1998). Inhibition of cell growth was shown in-vitro and this was correlated with down-regulation of c-myc oncogene expression by the melanoma cells. The effect on cell growth was greatest in the cell lines which expressed the highest levels of the c-myc oncogene.

The purpose of this study was to establish whether the combination of c-myc antisense with conventional chemotherapeutic drugs would produce additive effects which might be of benefit to metastatic melanoma patients.

The importance of combining antisense therapy with conventional chemotherapeutic drugs is two-fold. First, additive anti-proliferative effects may be produced. Second, the development of tumour resistance to chemotherapeutic drugs is thought to be associated with abnormal oncogene expression. Targeting these oncogene defects with antisense ODN’s and blocking their expression may not only lead to inhibitory effects on tumour growth, but may also overcome tumour chemo-resistance. This approach may lead to the more effective treatment of metastatic disease in cancer patients in the future.

Over-expression of the c-myc oncogene is known to be associated with cisplatinum resistance (Kashani-Sabet et al. 1990, Sklar et al. 1991, Walker et al. 1996) and combination of c-myc antisense with cis-platinum may offer a potential mechanism to overcome this. The A375m human melanoma cell line was therefore used in these experiments as it is known to express a high level of the c-myc oncogene which can be down-regulated by exposure to c-myc antisense (Jagdeep Chana MD thesis 1998).

A number of other chemotherapeutic agents were also assessed. Dacarbazine is at present the best single agent used for the treatment of metastatic melanoma and was tested in combination with the c-myc antisense. Temozolomide, which is the activated form of DTIC and Taxol were also assessed. Taxol has been demonstrated to be a highly effective anti-tumour drug by a number of studies (Foa et al. 1994) although it is thought to be less effective in the treatment of metastatic melanoma.

5.2 Methods

A375m cells were grown in 96-well plates at a concentration of 5x10^3 cells/well in antibiotic-containing serum-free medium. The chemotherapeutic drugs, at a range of concentrations, c-myc antisense or a combination were added to the wells and the
effects on cell growth measured using the ATP-chemosensitivity assay. A more
detailed account of the methods is provided in section 2.2.1.

5.3 Results

5.3.1 Combination of cis-platinum and c-myc antisense in the A375m cell line

The effects of the combination of cis-platinum and c-myc antisense therapy on the
growth of the A375m cell line are shown in figure 5.1. Cis-platinum alone produced
a dose response relationship ranging from 17.6% growth inhibition at 6.25% TDC
(standard error 3.0%) up to 93.7% at 200% TDC (standard error 7.1%). The c-myc
antisense and control ODN at constant 25μm concentrations produced 19.3% growth
inhibition (standard error 2.8%) and 7.5% inhibition (standard error 2.6%)
respectively. Addition of the control ODN to cis-platinum produced no significant
increase in growth inhibition above that of either agent alone. However, combination
of the c-myc antisense with cis-platinum significantly increased growth inhibition at
the low and medium cis-platinum doses. At the 6.25% TDC growth inhibition was
increased from 17.6% produced by cis-paltinum (standard error 3.0%) to 57.6% by
the combination (standard error 10.1%). The increases were of similar magnitude at
the 12.5% and 25% TDC's.

The magnitude of the additive effect observed by the combination of c-myc
antisense with cis-platinum was analysed using the methods described by Steel and
Peckham (Steel et al. 1979) as described in Chapter 5. The additive effect window
was calculated demonstrating the growth inhibitory effect that would be expected by
the combination of cis-platinum with c-myc antisense if the effects were additive
only. This window of additive effect is demonstrated in Figure 5.2. When the curve
representing the actual growth inhibition produced by the combination of c-myc
antisense with cis-platinum is added, a difference between the additive effect
window and the actual effects produced by the combination can be seen. This effect
is most obvious at the lower doses of cis-platinum and demonstrates a synergistic
effect between the two drugs.
5.3.2 Combination of DTIC and c-myc antisense in the A375m cell line

The results of the combination of DTIC and c-myc antisense therapy in the A375m cell line are shown in figure 5.3. The dose response relationship produced by DTIC alone ranged from -4.4% growth inhibition at the 6.25% TDC (standard error = 4.4%) to 94.0% at the 200% TDC (standard error = 7.3%). C-myc antisense at a constant concentration of 25 µm produced 38.0% growth inhibition (standard error = 3.0%). The control ODN produced 27.3% inhibition (standard error = 3.3%). Addition of the ODN control to DTIC produced no further increase in growth inhibition above that caused by either agent alone. Combination of c-myc antisense and DTIC produced a small increase in growth inhibition at the 25% and 50% TDC’s of 10.3% and 11.4% respectively.

5.3.3 Combination of Temozolomide and c-myc antisense in the A375m cell line

The effects of the combination of Temozolomide with c-myc antisense on the growth of the A375m cell line is shown in figure 5.4. Temozolomide produced a dose response inhibition of growth inhibition ranging from 12.0% (standard error = 3.8%) at 6.25% TDC up to 99% at the 200% TDC (standard error = 0.3%). C-myc antisense and the 4-G control ODN, at constant 25 µm concentrations, inhibited cell growth by 38% (standard error = 3.3%) and 27.6% (standard error = 3.3%) respectively. Combination of the ODN control and Temozolomide again produced no increase in growth inhibition above that of either agent alone except at the 12.5% and 25% TDC’s where 10% and 6% increases in growth inhibition were produced respectively. However, combination of c-myc antisense and Temozolomide significantly increased growth inhibition at the low and medium doses of Temozolomide by between 22% and 30% above that produced by c-myc antisense alone. This increase was reduced to a 14% increase at 100% TDC.
5.3.4 Combination of Taxol and c-myc antisense in the A375m cell line

The effects of the combination of Taxol with c-myc antisense in the A375m cell line is shown in figure 5.5. Taxol highly effectively inhibited the growth of A375m melanoma cells alone even at the lowest dose. At the 6.25% TDC, 94% growth inhibition was produced (standard error 2.3%) which increased to 100% at 50% TDC (standard error 0%). Addition of c-myc antisense or the ODN control produced maximal growth inhibition at the lowest TDC of Taxol.
Figure 5.1: C-myc antisense and cis-platinum in A375 m cells

Figure 5.2: The window of theoretical additivity for the combination of c-myc antisense and cis-platinum in the A375m cell line
Figure 5.3: C-myc antisense and DTIC in A375m melanoma cells

Figure 5.4: C-myc antisense and Temozolomide in A375m cells
Figure 5.5: Taxol and c-myc antisense in A375m cells
5.4 Discussion

The prognosis for patients with metastatic melanoma remains poor with the majority of chemotherapeutic agents offering little long-term survival benefit. A number of novel therapeutic strategies, including gene therapy, are therefore under investigation for use in the treatment of metastatic melanoma. The efficacy of c-myc antisense at inhibiting the growth of A375m melanoma cells in-vitro and in-vivo, in association with down-regulation of c-myc oncogene expression, has been demonstrated by the previous RAFT melanoma fellow (Jagdeep Chana MD thesis 1998) and also by Leonetti et al. (Leonetti et al. 1996). The effects of combining this treatment strategy with a number of conventional chemotherapeutic agents on the growth of human melanoma cells was studied in this chapter using the ATP-chemosensitivity assay.

DTIC was found to be the least effective chemotherapy drug when used alone in the A375m cell line. Combination with c-myc antisense produced little significant additional benefit in cell growth inhibition.

Temozolomide was a more effective drug when used alone in the A375m cells and combination with c-myc antisense significantly increased the growth inhibition produced. This effect was most obvious at the lower and medium concentrations of Temozolomide. Dacarbazine and Temozolomide, its active metabolite, exert their anti-tumour effect via methylation of DNA. The enzyme O-6-alkylguanine-DNA alkyltransferase is known to repair methylated DNA lesions but no link has been demonstrated between repair of DNA methylation and altered oncogene expression. The additive effect of the combination was therefore unlikely to have occurred via synergy between the two agents.

Taxol when used alone was highly effective at inhibiting the growth of A375m cells. Almost 100% growth inhibition was produced even at the lowest concentrations of Taxol used. Therefore no additive effect could be demonstrated by the addition of c-myc antisense in this assay at the concentrations of Taxol used. No further supplies of the drug could be obtained from the manufacturer to continue the experiments using lower Taxol doses.

A number of trials have assessed the use of Taxol in different cancers. Phase II trials have shown Taxol to be highly effective for the treatment of disseminated ovarian and breast cancer. Significant activity has also been reported in lung cancer and head
and neck cancer, however Taxol has been reported to be less effective for the treatment of metastatic melanoma (Foa et al. 1994). Overall response rates of up to 17% have been reported in phase II trials in metastatic melanoma patients (Aamdal, 1994) although combination with Tamoxifen was shown to increase the response rate to 24% in one trial (Nathan et al. 2000).

The reason why Taxol is less effective in melanoma is not known. Taxol is thought to exert its anti-tumour effects by inhibiting spindle formation in tumour cells, preventing cell division, but also by phosphorylation of the bcl-2 oncogene thereby blocking its inhibitory action on apoptosis. The bcl-2 gene is overexpressed in metastatic melanoma and its expression level is an accurate prognostic marker for advanced disease (Grover et al. 1996). Strategies to block the activity of the bcl-2 oncogene, such as the use of bcl-2 antisense, may potentially increase the efficacy of Taxol. Overexpression of the bcl-Xs gene, an inhibitor of the bcl-2 gene, has been demonstrated to significantly increase the activity of Taxol in MCF-7 human breast cancer cells (Sumantran et al. 1995). Antisense oligonucleotides targeting protein kinase A have been shown to produce a co-operative inhibitory effect in combination with docetaxel and anti-epidermal growth factor receptor antibody on the growth of human breast cancer cells in-vitro. Each single agent was demonstrated to induce bcl-2 phosphorylation. The three agents, used in combination at suboptimal doses, produced a higher degree of bcl-2 phosphorylation and apoptosis of ZR-75-1 breast cancer cells. A phase I trial in patients is now underway (Tortora et al. 1999). This may offer an alternative or additive strategy for increasing the efficacy of Taxol in addition to bcl-2 antisense.

Inhibition of the cell cycle at multiple check points, ie by combining taxol (which produces arrest at the G2/M stage) with a drug acting at another site in the cell cycle, has been shown to be highly effective in inducing apoptosis of tumour cells (Li et al. 1996). This suggests that, although no additive effect was shown in our study, there may be potential for additive effect when combining taxol with c-myc antisense, which blocks the cell cycle at the G1/S stage, or with other drugs acting at different points in the cell cycle. Future in-vitro studies investigating the combination of Taxol, used at a lower concentration producing lower cell kill rates, with c-myc and bcl-2 antisense will therefore be of great interest.

The greatest additive effect was seen when c-myc antisense was combined with cis-platinum – this was seen at the lower and medium doses of cis-platinum used and
was not seen with the 4-G quartet control oligonucleotide. Percentage growth inhibition was increased from 18.9% to 58.3% at a TDC of 12.5% for cis-platinum and from 26.9% to 59.9% at a TDC of 25%. Calculation of the window of theoretical additivity for the combination of the two agents as displayed in figure 5.2 demonstrates that the growth inhibitory effect produced by the combination of the two agents is greater than the sum of the effects produced by the two agents acting individually. This therefore suggests a synergistic mechanism. Cis-platinum alone effectively inhibited the growth of the A375m cells at medium and high doses, therefore this assay may have been unable to show the full additive effect of the c-myc antisense at the medium and high TDC’s of cis-platinum.

A potential mechanism to explain this synergy is that the c-myc antisense may reverse a degree of cis-platinum resistance in the A375m cell line. Over-expression of the c-myc oncogene by tumours has been shown to be associated with resistance to cis-platinum by a number of studies (Kashani-Sabet et al. 1990, Sklar et al. 1991, Walker et al. 1996). The A375m melanoma cell line expresses high levels of the c-myc gene and exposure to c-myc antisense is known to result in significant down-regulation of c-myc expression in these cells (Jagdeep Chana MD thesis 1998). Down-regulation of c-myc gene expression produced by the c-myc antisense may therefore have reversed a degree of cis-platinum resistance in the A375m cell line and resulted in the significant increase in efficacy in growth inhibition produced by the combination treatment.

The subject of tumour resistance to chemotherapeutic agents is of great interest in oncology and the study of the mechanisms involved may potentially lead to significant benefits for the treatment of disseminated malignancy in the future. A number of potential mechanisms by which tumour chemo-resistance develops have been postulated. Amplification or up-regulation of membrane transporters, such as members of the P-glycoprotein family eg MDR-1, may lead to transport of chemotherapy drugs out of tumour cells and reduced intra-cellular concentrations (Schadendorf et al. 1995). Increases in the activity of enzymes which detoxify chemotherapy agents in tumour cells, such as glutathione S-transferase-\( \pi \), may lead to increased drug inactivation (Ozols et al. 1990). Up-regulation of DNA-repair mechanisms, eg topoisomerase II, may lead to the DNA damage produced by the
chemotherapeutic drug being repaired and tumour resistance to the drug (Potmesil et al. 1987).

Perhaps the most important subject in the study of the chemo-resistance is abnormal oncogene expression. Not only does abnormal oncogene expression affect patterns of tumour growth, differentiation, invasion and metastasis, but it also effects tumour sensitivity to chemotherapeutic agents; perhaps via some of the mechanisms described above. A number of oncogene expression abnormalities have been described which are known to be associated with chemo-resistance. Overexpression of bcl-2 has been shown to be associated with chemo-resistance in a number of cancers including ovarian and breast (Kamesaki et al. 1993, Eliopoulos et al. 1995) and is thought to result from bcl-2 overexpression blocking apoptosis. The tumour suppressor gene p53 is known to induce apoptosis following DNA damage by chemotherapeutic drugs. Mutation of p53, resulting in loss of function, is known to be associated with chemo-resistance (Ruley 1996). The MDM-2 gene acts as a negative feedback loop to inhibit p53 activity. Overexpression of MDM-2 has been shown to be associated with cis-platinum resistance in glioblastoma (Kondo et al. 1995). The c-jun and c-fos genes are thought to regulate GST-\(\pi\) expression and their overexpression is known to be associated with chemoresistance. N-ras mutations are known to be present in up to 15% of melanoma and are known to be associated with chemoresistance by inhibiting apoptosis (Jansen et al. 1997). This has been shown to be via up-regulation of bcl-2 (Borner et al. 1999).

The mechanism by which over-expression of the c-myc oncogene leads to cis-platinum resistance has been postulated to be via upregulation of transport of cis-platinum out of the tumour cell, therefore reducing its effect. It is also suggested that c-myc over-expression induces DNA-repair mechanisms in the tumour, allowing the tumour cell to repair some of the DNA strand cross-linkages produced by the cis-platinum thereby reducing its efficacy (Sklar et al. 1991, Schadendorf et al. 1995).

Addition of c-myc antisense has been shown to reverse cis-platinum resistance in both resistant bladder cancer and resistant lung cancer cell lines (Mizutani et al. 1994, Van Waardenburg et al. 1997). This has also been shown in melanoma (Leonetti et al. 1999). These studies would support our observations.

Corrective gene therapy targeting other oncogene abnormalities implicated in chemo-resistance has demonstrated efficacy in reversing chemo-resistance in a
number of studies. Antisense or ribozymes have been utilised to block the effects of overexpression of bcl-2 (Kitada et al. 1994, Jansen et al. 1998), c-fos (Scanlon et al. 1994), and ras (Ohta et al. 1996). Replacement of p53 has also been shown to reverse chemo-resistance (Fujiwara et al. 1994). Antisense has also been combined with immunotherapy and chemotherapy to produce highly effective anti-tumour effects in some studies (Nieborowska Skorska et al. 1994).

In summary, combination of c-myc antisense gene therapy with conventional chemotherapeutic agents resulted in additive effect against melanoma cells in-vitro. Synergistic effect was demonstrated when c-myc antisense was combined with cis-platinum. These effects were observed in a cell line where the c-myc oncogene is over-expressed. Blocking abnormal oncogene expression using corrective gene therapy may therefore not only lead to inhibition of tumour growth, but may also increase tumour sensitivity to chemotherapeutic agents such as cis-platinum. In addition, the potent effect of Taxol against the A375m melanoma cell line highlights the potential efficacy of this drug for the treatment of metastatic melanoma patients. Combination of this drug with bcl-2 antisense may in theory increase its efficacy.

At present there is much evidence to suggest that it will be possible in the future to increase the sensitivity of tumours to chemotherapeutic agents using corrective gene therapy and therefore lead to much more effective treatment of metastatic disease. Analysis of tumour oncogene profile may provide important information regarding the degree and cause of chemoresistance and direct corrective therapy. Ultimately, combinations of conventional chemotherapeutic agents with corrective gene therapy, biological response modifiers such as IFN and IL-2 and immunotherapy may offer greatly improved survival rates in metastatic melanoma. These developments are likely to be of great benefit to metastatic melanoma patients in the future whose prognosis at present is very poor.
Chapter 6

Targeting the c-myc gene using a ribozyme

6.1 Introduction

The demonstration that antisense ODN’s can specifically block the expression of a target gene has highlighted this mechanism as a potential corrective gene therapy strategy for use in the treatment of a wide range of diseases, including cancer. However, for corrective gene therapy to work effectively in cancer patients a number of hurdles need to be overcome. The gene therapy needs to be effectively targeted to the tumour cells. Once it has reached the tumour, sufficient amounts need to be expressed for an adequate length of time to effectively block expression of the target gene and death of the tumour cell to occur.

As a first stage in the development of c-myc antisense for use in cancer patients the incorporation of c-myc antisense into a ribozyme system has been investigated. Ribozymes are naturally-occuring forms of RNA that possess the ability to cleave other RNA sequences by acting as enzymes. They were first discovered in viroids and bacteria (Zaug et al. 1986) Their role is to cleave large RNA precursor molecules, some containing multiple RNA copies, into unit-length progeny.

Ribozymes can be designed to target a specific RNA sequence therefore producing a molecule which acts in the same manner as an antisense but much more efficiently. One molecule of antisense can only block the effects of one molecule of RNA before being consumed in the process and inactivated. However, one ribozyme molecule targeting a specific RNA sequence can cleave multiple RNA molecules.

The hammerhead ribozyme is the smallest and best-characterized type of ribozyme. The target sequences of the hammerhead ribozyme have the least constraints compared with the other forms resulting in more common potential target sites. This type of ribozyme has been the most studied and is currently the most suitable for development for therapeutic use. In view of these considerations, this approach was studied as a method of corrective gene therapy targeting the c-myc oncogene.
6.2 Structure of the hammerhead ribozyme

The hammerhead ribozyme is composed of three parts: stem I, II and III (figure 6.1). Stems I and III bind to the target RNA sequence as determined by the Watson and Crick base-pairing principles. Stem II forms the catalytic core and can vary in length. It is formed of a loop composed of two non-base paired RNA sequences on either side. The composition of these two non-base paired sequences are highly conserved throughout most hammerhead ribozymes and consist of a CUGAUGA sequence on the 5' end of stem II and a GAAA sequence on the 3' end. The GAAA sequence and the high proportion of Guanine and Cytosine residues in the remainder of stem II stabilises the loop (see figure 6.2).

Stems I and III hybridise with the target RNA sequence aligning a specific series of nucleotides over the catalytic core sequence. This nucleotide sequence can vary to a degree and still allow cleavage but it must consist of a -UH- sequence where the H can be a uridine, cytosine or adenine but not a guanine (Sullivan 1994). The -GUC- sequence has been identified as the triplet which results in the most efficient cleavage rates (Ruffner et al.).

The length and composition of the nucleotide sequences of stems I and III determines the targeting specificity and rates of hybridisation to, and dissociation from, the substrate. Either very short substrates (< 20 nucleotides in length) or very long substrates (> 500 nucleotides) are thought to result in the most efficient ribozyme function (Sullivan 1994).

The three-dimensional structure of a potential ribozyme sequence can be predicted using computer simulations to check that the potential cleavage site is accessible to the target RNA sequence to allow cleavage to occur (Sullivan 1994).

6.3 Stability of Ribozymes

Ribozymes are susceptible to attack from nuclease enzymes in common with other forms of RNA and therefore their modification to render them resistant to attack by these enzymes is crucial in the development of ribozymes for therapeutic use. Modifying the phosphodiester backbone to a phosphorothioate derivative and
Figure 6.1: Structure of hammerhead ribozymes

Figure 6.2: Sequence and structure of the ribozyme targeting c-myc mRNA
changing the 2’-OH of the ribose to either 2’-0-methyl/allyl or 2’-deoxy derivatives is known to stabilise the ribozyme to nuclease attack (Paolella et al. 1992, Sullivan 1994). However this can result in some loss of RNA cleavage efficacy and a balance is required.

### 6.4 Delivery of ribozymes to target cells

Ribozymes can potentially be utilised in a number of ways as gene therapy. Direct administration to tumour cells without any form of delivery system is feasible but their cellular uptake is known to be low. Cationic lipids or liposomes can be utilised to increase uptake rates and are useful for in-vitro experiments (Felgner et al. 1987). Liposomes enter cells by endocytosis and once inside the cells they are degraded, releasing the ribozymes. This process varies between cell type. Polyacrylic acid, a controlled release polymer, has been used for delivering ribozymes, topically, in-vivo (Sullivan 1994).

Alternatively, ribozymes can be incorporated into a plasmid or viral vector which can be administered to the tumour. Uptake of the ribozyme-encoding vector by the tumour leads to multiple copies of the ribozyme being produced and a two-stage amplification of the antisense effect. Cationic lipid-mediated transfection of ribozyme-encoding plasmids has been demonstrated to yield high transfection rates both in-vitro and in-vivo (Sullivan 1994). Examples of viral vectors under investigation include retroviruses, adeno-associated viruses and adenoviruses. The advantage of the former two is that efficient entry into the target cell and integration into the target genome is achieved and stable, long-term transfection with continuous expression of the ribozyme occurs. However, in the case of retroviruses, there is a limit to the amount of genetic material that can be delivered and the target cell needs to be dividing; this is a major disadvantage. There is also a possibility of genetic damage due to random integration into the host genome.

Transfection with adenovirus is very efficient compared to that of retroviruses and results in high levels of gene expression. The target cell does not need to be dividing for transfection to occur. However transfection is only transient, lasting only 1-3
weeks in vivo, and significant immune responses to the virus, accompanied by inflammatory reactions, may occur.

Transfection with the adeno-associated virus requires the target cell to be dividing and is also disadvantaged by the limited amount of genetic material that can be delivered. It also requires the presence of replicating adenovirus to grow.

All the viruses used are rendered incapable of replication. In the case of retroviruses this is achieved by removal of the gene sequences necessary for the packaging of the genome into the virus during replication. They are replaced by the ribozyme-encoding genes, which are flanked by the viral long-term repeats necessary for incorporation of the genetic material into the target cells.

Targeting strategies are required to achieve tumour-specific delivery of the ribozyme-encoding vector. An example utilised in melanoma is the use of the tyrosinase promoter/enhancer element to drive production of the gene therapy (Vile et al. 1993).

Having entered the target cell, efficient ribozyme transcription must occur for effective function. If it is transcribed by the same RNA polymerase as the target RNA ie RNA polymerase II, the ribozyme must have a stronger promoter driving its production than the target gene. Alternatively, a different promoter can be used which is transcribed by RNA polymerase I or III and has a higher transcription rate than the target gene.

### 6.5 Uses of ribozymes

To date, ribozymes have been developed for use in a number of diseases. Ribozymes targeting the H-ras oncogene have been shown to be effective in reducing proliferation and increasing the cellular differentiation of a number of cancer cell lines in-vitro, including melanoma and bladder cancer (Kashani Sabet et al., Ohta et al. 1996). In addition H-ras ribozymes have reduced tumour burden in nude mice bearing bladder cancer xenografts (Kashani Sabet et al.). Ohta et al described the construction of anti-fos and anti-c-myc ribozymes and demonstrated growth inhibition in-vitro in melanoma cells (Ohta et al. 1996). A ribozyme targeting the
abl-bcr fusion RNA sequence in Chronic Myeloid Leukaemia has also been shown to inhibit cell proliferation in-vitro (Shore et al. 1993).

Currently, a phase I clinical trial is investigating the use of a hairpin ribozyme targeting the 5’ leader sequence of the LTR of the HIV virus which has been shown to be successful in preventing HIV I and III3b infection in-vitro (Yu et al. 1993). Other potential targets for ribozymes for the treatment of HIV include the RNA encoding the retroviral gag protein (Ramezani et al. 1996).

6.6 Target and structure of the c-myc ribozyme.

A ribozyme was designed according to standard principles to target the same translation-initiation codon of exon 2 of the c-myc mRNA as the 15-mer c-myc ODN (base pairs 296-311). The efficacy of this c-myc antisense ODN has been demonstrated in melanoma by Rajiv Grover (MD thesis 1996), Jagdeep Chana (MD thesis 1998) and by Citro and Leonetti both in-vitro and in-vivo (Leonetti et al. 1996, Citro et al. 1998).

A -GUU- triplet is present at the 3’ end of the translation-initiation codon of exon 2 (base pairs 303-305) of the c-myc mRNA. This triplet sequence is known to be a cleavage site for ribozymes and the structure of a hammerhead ribozyme was therefore designed around this site (figure 6.2). The standard -AAAG- and -CUGAUGA- sequences along with a sequence high in guanine and cytosine residues were selected to construct stem II of the ribozyme. A sequence of 5’-GAAGCU-3’ was complementary to the base sequence on the 3’ side of the -GUU- triplet and formed stem I. A 5’-GUUGAGG-3’ sequence was complementary to the base sequence on the 5’ side of the -GUU- triplet and formed stem II.

The plasmid pREV, previously constructed by Spencer Collis at the Paterson Institute of Cancer Research in Manchester, was selected as a vector to express the ribozyme targeting c-myc. The structure of this plasmid is shown in figure 6.3. The plasmid contains Green Fluorescent Protein (GFP) as a marker whose expression is promoted by a CMV I.E. promoter. It also has a SV40 polyadenylation site at the 3’ end to stabilize the RNA transcript in mammalian cells. A ribozyme cloning site is present also with a CMV promoter and SV40 polyadenylation site at the 3’ end to
Figure 6.3: Structure of the pREV plasmid

Figure 6.4: Sequence and annealing of oligonucleotides c-mycRzA and c-mycRzB

**C-mycRzA (5' - 3')**

CGCG GAA GCT CTG ATG CCG TGA GGA CGA AAC GTT GAG G

**C-mycRzB (3' - 5')**

CTT CGA GAC TAC GGC ACT CCT GCT TTG CAA CTC CGA TC

(mlu 1)

CGCG GAA GCT CTG ATG CCG TGA GGACGA AAC GTT GAGG (xba 1)

CTT CGA GAC TAC GGC ACT CCT GCT TTG CAA CTC CGA TC

155
promote ribozyme expression. The plasmid also contains genes encoding ampicillin and neomycin resistance whose expression is promoted by SV40 promoters.

6.7 Production of a ribozyme-encoding plasmid targeting the c-myc oncogene

The full description of the synthesis of the ribozyme-encoding plasmid is described in Chapter 2.

Annealing of the two oligonucleotides, one encoding the ribozyme, and the other encoding its complementary sequence occurred such that a double-stranded base sequence resulted, with two single-stranded ends (see figure 6.4). The single-stranded terminae were complementary to the Xba-1 and Mlu-1 cloning sites in the vector.

The pREV circular plasmid DNA was digested to yield a linear, double-stranded DNA sequence. Each end of the plasmid DNA was digested to form 'sticky' ends to which the annealed nucleotides could be ligated, and thus reform the circular loop.

The pREV plasmid was linearised by enzymic cleavage at the Xba-1 cleavage site, which is the ribozyme cloning site. One end of the linearised DNA sequence was then digested by the Mlu-1 enzyme to yield linearised pREV DNA with a Xba-1 binding site at one end and a Mlu-1 binding site at the other. These were complementary with the Xba-1 and Mlu-1 binding sites present on the ends of the annealed nucleotide sequences.

Addition of the annealed nucleotides to the linearised plasmid DNA bearing the Xba-1 and Mlu-1 ends, resulted in ligation of the nucleotide sequence into the pREV plasmid. This reformed the circular plasmid DNA and incorporated the ribozyme-encoding nucleotide sequence.

Following transformation and incubation of DH5α e. coli bacteria a number of colonies were screened for the presence of the ribozyme-containing plasmid using PCR. A portion of each PCR mixture was run on an electrophoresis gel to establish the number and size of amplified DNA fragments.
6.7.1 Results

A polaroid photograph of the gel (figure 6.5) shows that both clone 10 and clone 17 contain 450 base pair DNA fragments, representing the ribozyme DNA fragment, in addition to the 1300 base-pair fragment representing GFP. The ataxia telangectasia gene, which at 450 base-pairs is the same size as the ribozyme DNA fragment, represents the positive control. The other clones showed a band of 1300 base pairs in size only, representing the DNA encoding GFP. They therefore contained only DNA encoding the pREV plasmid and not the ribozyme. The other lanes therefore represent clones containing the plasmid in which the original ligation reactions had failed to take up the ribozyme sequence.

Clone 17 was therefore selected for further experiments. The ribozyme-encoding plasmid was subsequently termed pREVmycl7.

The pREVmyc17 plasmid DNA was extracted and purified and the base sequence of the ribozyme-encoding plasmid assessed by DNA sequence analysis. The sequence was found to be correct and is demonstrated in figure 6.6.

Following confirmation of the correct DNA sequence of the pREVmyc17 plasmid, larger amounts of the plasmid DNA were produced to test its efficacy.

The quantity and purity of the DNA produced was measured using a spectrophotometer. The DNA concentration of the pREVmyc17 plasmid was found to be 1684ug/ml and that of the pREV plasmid 1863ug/ml. The purity of the DNA was assessed by the ratio of the 260nm to the 280nm measurements and a measurement of 1.88 was obtained for the pREVmyc17 plasmid. This represents a high purity with minimal RNA contamination.

6.8 Investigation of the optimal transfection strategy for A375m cells

Transfection of A375m cells with the ribozyme-containing plasmid pREVmyc17, and the pREV plasmid was used to assess the transfection efficiencies of two different transfection reagents: Lipofectamine (Gibco) and Escort (Gibco). The full description of the methods used is described in section 2.5.3 of Chapter 2.
Figure 6.5: Electrophoresis Gel of DNA Fragments produced following PCR of DNA suspension with CMV and SV40 primers

Clone 10

GFP 1300bp

Ribozyme DNA 450bp

Clone 17

Positive Control

GFP 1300bp

Ribozyme DNA 450bp

Figure 6.6: DNA Sequencing Analysis of pREVmyc17 Base Sequence

1  DttKTTKgKg  TgGRAGGTCT ATtaAACAGC GCTTTGGTTTA GTGGAACCCY
51  CAGATACGTA GAGCCTTAT GCCTGATTT TATAGAGTT AAGATGCTA
101  CGCAGTCGCT GTCCGCTACA CAAAGCTCTC GAACTTAAAG TGCAAGAGTT
151  GCTCTGAGCC CACTGCGCAG GTAGATAGCA AGTTACAAG AGAGGCTTAA
201  GAGAAAACTT ACAGATAGGG CTTGCGAGA CAGAGAAAGAC TCTTGCGTTT
251  CGTATAGCCA CCCTTTGTC ATCAGACAT CCACTTGGCC TTCCCTCCTCA
301  CAGGTCGCTCA CTCCTCGTTC ATTACAGCT GTTAAAGCTTA CAGCTACCA
351  TAGAAGGCTG TAAGAAACAT GCTGTAGACA GCTTCAGAC TCTTCAGATA
401  GTCCTGAGGC AGAGACGTTG AGCTTTATGC CGACCGGGC QCCGCTTTCG
451  AGCGAGCTATG ATAGATACA TGGAGACGTG TGGAGAGACC ACGAGCAGA
501  TGAGTGAAA AAAATGCTTT ATTAGTGAA TTTGTGATGC TATTGTCTTA
551  TTGTGACCCA TTATAGCTCG CAAATAAACA GATAAAGAC ACAATGGCAT
601  TACATATTAT TTTCCGGCTTC AGGCGGGAGT GTGCGAGGTT TTTAAGCAAA
651  GTAAAACCTGC TACAAAGTGG GTAATAATCGA TAAAGCTCG CTTATAGGCG
701  ACTCTGAAGT CAATCCGGTC TGGAGCCCGA TATTTAGGCC ASGCCCGACA
751  aCGCGCAAAA CGCGCGTGACG CGCGCGTTCG GCTCGCCATC eYCCCGCAYC
801  GGCATTACGAc AacgtgKAGS gCTCCGGRS TGCAAGTG
Figure 6.7: Establishment of the Optimal Transfection Agent

**Escort**

![Graph showing Transfection Rate for Escort](image)

**Lipofectamine**

![Graph showing Transfection Rate for Lipofectamine](image)
Figure 6.8: Examples of histograms from transfection of the A375m cell line with the pREVmyc17 plasmid using:

a: Escort

![Histogram for Escort](image)

b: Lipofectamine

![Histogram for Lipofectamine](image)
6.8.1 Results

Figure 6.7 shows the data obtained. The transfection rates of the A375m cells using Escort were poor, only 0.64% of cells were transfected with the pREV plasmid and 0.68% with the pREVmyc17 plasmid. However, the transfection rates when using Lipofectamine were superior, 21.2% for the pREV plasmid and 18.4% for pREVmyc17 plasmid. There was no significant green fluorescence in the untransfected controls. Lipofectamine was therefore used as the transfection agent in all subsequent experiments. Two examples of the FACS profiles obtained using Lipofectamine and Escort with the pREVmyc17 plasmid are shown in figure 6.8. Region M1 shows the transfected cells emitting green fluorescence.

6.9 Assessment of the effect of pREVmyc17 on the growth of the A375m melanoma cell line

The effect on the growth of the A375m cell line transfected with pREVmyc17 was compared with the growth rates of the A375m cells transfected with the pREV plasmid alone or untreated A375m cells to act as controls.

A375m cells were grown in three T75 flasks – one for each of the groups. One of the flasks was transfected with the pREVmyc17 plasmid and another with the pREV plasmid. The third flask received no treatment.

After 24 hours the cells were sorted using a FACS machine into 96 well plates. Figure 6.9 shows the cytogram dot-plots obtained. In the first experiment the two transfected flasks were sorted into cells either positive or negative for green fluorescence (those cells expressing or not expressing the plasmid). The positive cells selected were those with FL1 green fluorescence values above $10^2$. The negative cells that were sorted were those with green fluorescence values below $10^1$.

In the second experiment the two transfected flasks were both sorted into cells strongly and weakly positive for green fluorescence or negative cells, i.e., those cells expressing the plasmid to a high degree, those expressing it more weakly and those not expressing it at all. The strongly positive cells selected were those with green fluorescence values above $10^2$. Weakly positive cells were those with green...
fluorescence values between $10^1$ and $10^2$ and the negative cells were those below $10^1$.

The plates were then incubated for five days to allow adequate cell growth for any differences in growth rate to be detectable using the MTS assay over the subsequent three days.

The full description of the methods used is in section 2.5.4.

6.9.1 Experiment 1 results

Figure 6.9 shows the results obtained from sorting GFP positive or negative cells from each of the plasmid transfections and untreated control cells. Error bars have been omitted for clarity. It can be observed that the cells which failed to be transfected (ie the GFP-negative cells) showed similar growth characteristics to the untreated controls. In those cells that were successfully transfected with the pREVmyc17 plasmid, there was a reduction of 36% in growth over the 120 hour experiment period. However, a similar reduction in growth (37%) was also observed in the cells containing the pREV control vector. This suggests that there was no specific effect of the c-myc ribozyme. The error bars all overlapped at all time points (Fig. 6.9).

6.9.2 Experiment 2 results

Figure 6.10 shows the results obtained from sorting strongly or weakly positive GFP cells from negative cells for each of the plasmid transfections. The results for the untreated control cells are also displayed. Error bars have again been omitted for clarity. It can be observed that the cells which failed to be transfected by either plasmid (ie the GFP-negative cells) and the weakly-positive GFP cells (pREV low +ve and pREVmyc17 low +ve) showed similar growth characteristics to the untreated controls.

Those cells that were most efficiently transfected with the pREVmyc17 plasmid (myc high +ve) demonstrated a non-significant reduction in growth of 12% over the 120 hour experiment period. However, a reduction in growth of 24% was also
Figure 6.9: Effect of pREVmyc17 on the growth of A375m cells
- experiment one

Figure 6.10: Effect of pREVmyc17 on the growth of A375m cells
- experiment two
observed in the cells that were most efficiently transfected by the pREV control vector (pREV high +ve). This again suggested that there was no specific effect of the c-myc ribozyme. The error bars all overlapped at all time points.

6.10 Establishment of the optimal transfection conditions for A375m cells for flow cytometric analysis of c-myc protein down-regulation

6.10.1 Method

The analysis of the effect of the pREVmyc17 plasmid on c-myc expression in A375m required a large number of transfected cells for antibody staining and FACS analysis, at least $5 \times 10^5$. This entailed exposing a larger number of A375m cells to the DNA-lipofectamine mixture to ensure an adequate proportion of transfected cells. This required a different, optimized transfection strategy to that used for the MTS assay.

A375m cells were transfected at a range of different cell densities and DNA-Lipofectamine concentrations in 6-well plates. After incubation for 24 hours to allow expression of the plasmid to occur, the percentage of green fluorescent cells (transfected with the plasmid) was assessed using a FACS machine (Becton Dickinson). The optimal transfection conditions could therefore be determined by assessing the percentages of cells transfected by each combination of DNA concentration and cell density. These methods are described in more detail in section 2.5.5.

6.10.2 Results

The transfection rates of A375m cells following their exposure to the pREVmyc17 plasmid at the 3 different cell densities and DNA-Lipofectamine concentrations are shown in figure 6.11. For each cell density, exposure to the greatest quantity of DNA
Figure 6.11: Establishment of Optimal Transfection Conditions

a: Cell density = $2 \times 10^5$ / well

![Graph](image)

b: Cell density = $5 \times 10^5$ / well

![Graph](image)

c: Cell density = $8 \times 10^5$ / well

![Graph](image)
and Lipofectamine (10μg and 30μl respectively) yielded the highest transfection rates.

There was little variation in transfection rate with cell density. Exposure of 2x10^6 cells / well to the highest quantity of DNA and Lipofectamine yielded a transfection rate of 33%. When the cell densities were increased to 5 x10^5 and 8 x10^5 cells / well and the cells were exposed to the highest amount of DNA and Lipofectamine used, the transfection rates were 26% and 27% respectively.

The highest concentration of DNA (10μg) and Lipofectamine (30μl) were therefore selected for use in the subsequent experiments to measure the effect of the pREVmyc17 plasmid on c-myc expression.

6.11 Assessment of the effect of the pREVmyc17 ribozyme-encoding plasmid on c-myc oncogene expression in A375m melanoma cells

6.11.1 Methods

9 x10^6 A375m melanoma cells were seeded into each of three T150 flasks. One flask was designated as the control flask and received no treatment. The cells in the second flask were designated for transfection with the pREVmyc17 ribozyme and those in the third flask to receive the pREV plasmid control sequence.

The following day the cells were transfected with the respective DNA-lipofectamine complexes. After a 48-hour incubation to allow expression of the vectors to occur the cells were sorted into GFP positive and negative populations.

The sorted cell suspensions were fixed in ethanol, counted and 5x10^5 cells from each group transferred into conical tubes for staining of c-myc protein. 5x10^5 cells from the control group were transferred into each of two tubes to act as the positive and negative controls. The c-myc oncoprotein levels were assessed by FACS analysis. A more detailed description of these methods is presented in section 2.5.6.
6.11.2 Results

The flow cytometry data obtained from the transfections is shown in Figure 6.12 and the histograms obtained displayed in Figure 6.13. The data shown in Figure 6.12 is expressed as mean fluorescence ratios compared to the negative control. No significant reduction was observed in c-myc oncoprotein level between the pREVmyc17-transfected cells, the GFP positive pREV cells and the positive control group. The positive control had a mean fluorescence ratio of 21.7, the pREVmyc17-transfected cells a ratio of 22.7 and the pREV transfected control cells a ratio of 20.9. Furthermore, there was no significant difference between GFP positive and negative cells in either of the transfection groups, emphasising that the vectors were ineffective in down-regulating c-myc protein.
Figure 6.12: The effect on c-myc expression in A375m cells following transfection with the pREVmyc or pREV control plasmid.
Figure 6.13: The effect on c-myc expression in A375m cells following transfection with either the pREVmyc17 or pREV control plasmid - experiment 1 histogram plots.
6.12 Discussion

Corrective gene therapy offers a new strategy for the treatment of metastatic melanoma for which current treatments are ineffective. Antisense targeting the c-myc, bcl-2 and cyclin-D1 genes has been demonstrated to be effective against melanoma in-vitro and in-vivo in a number of studies (Becker et al. 1989, Miele et al. 1994, Resnicoff et al. 1994, Durko et al. 1997, Glukhov et al. 1998, Jansen et al. 1998, Oku et al. 1998, Sharma et al. 1998).

However, for antisense to be of use in patients it requires substantial development, including targeting and amplification. The synthesis of a ribozyme to target c-myc mRNA was therefore undertaken as an initial step in the development of c-myc antisense for use in patients.

The ribozyme was designed to target the same target sequence as the antisense previously used by Jagdeep Chana (MD thesis 1998) and Leonetti and Citro (Leonetti et al. 1996, Citro et al. 1998), the translation-initiation sequence of exon 2. The ribozyme was successfully constructed and incorporated into the pREV plasmid as demonstrated by PCR. The correct sequence was confirmed by DNA sequencing. High transfection rates of the A375m melanoma cell line were also demonstrated. However, although transfection of the A375m cell line with the pREVmycl7 ribozyme was shown to significantly reduce cell growth rates over 6 days, this effect was also shown with the pREV plasmid alone. This therefore implies that the effect was non-specific and most likely due to a transfection-related effect. Transfection of cells by a vector leading to expression of vector DNA is known to lead to a demand on the metabolic requirements of the cell which can adversely affect the cellular growth rate.

This effect was confirmed to be non-specific by the demonstration that transfection of A375m cells by the pREVmyc17 ribozyme did not result in a significant down-regulation in c-myc gene expression as shown by the similar c-myc oncprotein levels in the pREVmyc17, pREV-transfected cells and the untransfected controls.

There are a number of potential reasons to explain the failure of the pREVmyc17 ribozyme in blocking c-myc expression or inhibiting the growth of the A375m melanoma cell line. First, the ribozyme targeted a -GUU- sequence in exon 2 of the c-myc mRNA. Although ribozymes are known to be effective against nucleotide sequences containing a variation on the -UH- sequence (where the H can be a
uridine, cytosine or adenine, but not a guanine), the -GUC- sequence is known to be the best triplet to target in order to achieve the highest ribozyme cleavage efficiency (Sullivan 1994).

Second, although the ribozyme may have the primary nucleotide sequence to potentially bind to the translation-initiation site in exon 2, the 3-dimensional structure may not have allowed the target nucleotide sequence to bind to the cleavage site. Analysis of the 3-dimensional structure of the pREVmyc17 ribozyme may reveal if access to the cleavage site is obstructed by the tertiary structure of the ribozyme. This may aid the planning of a revised ribozyme structure targeting the same sequence of the c-myc mRNA.

Alternatively, a different base sequence of the c-myc mRNA could be targeted, containing a different triplet sequence such as -GUC- which is known to be cleaved more efficiently by ribozymes.

Ohta et al published the results of an experiment in 1996 demonstrating the successful use of three ribozymes targeting the ras, fos and c-myc oncogenes in a melanoma cell line transfected with the ras oncogene. All three ribozymes were shown to function as demonstrated by specific down-regulation of protein expression, a reduction in cell growth rates and an increase in differentiation. Their conclusions were that the ras ribozyme was the most effective as it caused the greatest inhibition of cell growth. However, as the ribozymes had been tested in a ras over-expressing cell line, this observation was not unexpected. The effect of the c-myc ribozyme was inferior to that of the ras ribozyme in terms of reducing cell growth. Although there is likely to have been some secondary increase in c-myc expression in this cell line as a result of the increased replication rate, the c-myc level is likely to have been much lower than that in the A375m cell line, which is a high c-myc expressor. Had the c-myc ribozyme been tested in a melanoma cell line expressing high levels of c-myc its effects may have been greater and their conclusions might have been different.

C-myc expression has been shown to be a highly significant prognostic factor for a number of different types of primary and also metastatic melanoma (Grover et al. 1997, Grover et al. 1997, Ross et al. 1998, Chana et al 1998). This has not been demonstrated for either the ras or fos oncogenes. Therefore it is likely that c-myc is a more useful target for corrective gene therapy for metastatic melanoma patients than
Figure 6.14: The structure of the c-myc ribozyme described by Ohta et al
either ras or fos, and the successful demonstration of the efficacy of a ribozyme targeting the c-myc oncogene by Ohta et al is therefore important.

The ribozyme targeting the c-myc oncogene described by Ohta et al targets a different part of exon 2 of the c-myc gene than the pREVmyc17 ribozyme (bases 1405-1420). The sequence and structure of this ribozyme is shown in figure 6.14.

The testing of this ribozyme in a high c-myc-expressing melanoma cell line, such as A375m, which is perhaps more representative of human metastatic melanoma, is therefore important and is the next stage of this project.

If this ribozyme is successful in blocking c-myc expression and cell growth in the A375m cell line, then the addition of a targeting sequence such as the tyrosinase promoter might allow tumour-specific delivery to melanoma. The tyrosinase promoter has been successfully used to target other forms of gene therapy to melanoma, such as the use of herpes simplex virus thymidine kinase to sensitise melanoma to killing by gancyclovir (Vile et al. 1993). In addition this same strategy has been used to target a ras ribozyme to melanoma (Ohta et al. 1996).

In summary, this chapter has demonstrated the principles used in designing a ribozyme to target a specific mRNA sequence and has shown that this can be successfully synthesised. However, the ribozyme produced was not successful in targeting the c-myc mRNA sequence as shown by its failure to down-regulate c-myc oncoprotein levels in transfected A375m melanoma cells. Although a degree of growth inhibition was produced when the pREVmyc17 ribozyme transfected the A375m cell line, this was also shown by the pREV plasmid control. This growth inhibition effect was therefore non-specific and likely to have resulted from the effects of the transfection process on the melanoma cell. The transfection process and the subsequent transcription and translation of the foreign DNA and mRNA can alter cell metabolism, leading to a reduction in growth rate independently of the effects of the ribozyme itself.

The demonstration of a successful ribozyme design targeting a different region of the c-myc mRNA sequence by Ohta et al is of great interest and is currently being developed for use in the A375m cells to assess its effect in a c-myc overexpressing cell line.
Chapter 7 General Discussion

7.1 Introduction

The incidence of melanoma has increased rapidly over the last 40 years. Although the prognosis for patients who present early with thin primary tumours is generally good, the outlook for patients with thicker tumours or metastatic disease is much poorer. This project has studied the use of corrective gene therapy as a novel therapeutic strategy for use in melanoma as both adjuvant therapy and also for the treatment of metastatic disease.

The only adjuvant therapy currently licensed for use in melanoma is interferon α2b. However the efficacy of interferon for use as an adjuvant agent in melanoma is now in doubt. This project has therefore investigated the mechanism of action of interferon and assessed the results of combining it with c-myc antisense therapy in an attempt to improve its efficacy against melanoma. The results have been presented in Chapters 3 and 4 of this thesis.

The prognosis for patients with stage IV metastatic melanoma is very poor as conventional treatments are generally ineffective. Research by the previous RAFT fellows in this project (Rajiv Grover MD thesis 1996, Jagdeep Chana MD thesis 1998) has highlighted the role of the c-myc oncogene in the development of melanoma and as a target for corrective gene therapy. Targeting the c-myc gene with antisense therapy was demonstrated to inhibit the growth of melanoma both in-vitro and in-vivo. The potential benefit of combining c-myc antisense with conventional chemotherapeutic drugs in melanoma has therefore been assessed in-vitro and the results presented in Chapter 5.

For corrective gene therapy to be of practical use in humans, extensive development needs to be undertaken. Accurate tumour targeting and sufficient expression of the gene therapy need to take place to allow efficient anti-tumour effect, without excessive side-effects. As a first stage in the development of corrective gene therapy targeting the c-myc gene for use in humans, a ribozyme targeting the c-myc mRNA sequence was designed and tested in-vitro and the results presented in Chapter 6.
The relationship between c-myc expression and interferon sensitivity in melanoma

The prognosis for patients with thick primary melanomas is poor, those with tumours thicker than 4mm have a 5-year survival rate of 47%. Once spread to the regional lymph nodes has occurred, 5-year survival rates of between 10% and 46% can be expected after block dissection (Koh 1991). This has lead to the investigation of various adjuvant therapeutic strategies in an attempt to reduce recurrence and increase survival in melanoma (Demierre et al. 1997). To date only interferon has been licensed for use in patients. However, the failure of the majority of interferon adjuvant trials to show survival benefit in melanoma (Cascinelli 1995), (Creagen et al. 1995) (Grob et al. 1998, Pehamberger et al. 1998, Kirkwood 1999) has cast doubt on the use of interferon in melanoma.

A number of studies have investigated the possible mechanisms by which interferon exerts its effects. Administration of interferon to a number of cell lines leads to down-regulation of c-myc expression and up-regulation of p21/WAF 1 expression. This is accompanied by growth arrest in the G1 phase of the cell cycle, terminal differentiation and cell death via apoptosis (Subramaniam et al. 1998) (Resnitzky et al. 1992). C-myc over-expression, and failure to down-regulate c-myc after exposure to interferon, are both associated with interferon resistance in cell lines (Einat et al. 1988) (Resnitzky et al.1991). The c-myc gene is therefore thought to play an important role in the mechanism of action of interferon. This section of the thesis investigated the relationship between c-myc expression and interferon sensitivity in a series of uveal melanoma specimens to establish if c-myc over-expression was associated with interferon resistance in a series of human melanoma tumours.

The c-myc expression levels and the interferon sensitivities of 45 uveal melanomas were measured and the results correlated. Patient age was found to be significantly associated with tumour interferon sensitivity as measured by the maximum inhibitory percentage. Age has previously been shown to be a prognostic factor for uveal melanoma (Jagdeep Chana MD thesis 1998) and has now been shown to be associated with interferon sensitivity also.
Although the majority of tumours were resistant to interferon, differences were found when their interferon sensitivities were related to their c-myc expression levels. The tumours expressing the highest levels of c-myc were found to be the least sensitive to interferon. Tumour c-myc positivity was significantly associated with interferon sensitivity as measured by the maximum inhibitory percentage, although this was marginal at the 5% degree of significance. A similar relationship was found when the interferon sensitivity index was used to assess interferon sensitivity, although this did not demonstrate statistical significance. In addition, the observation that the series of tumours as a whole expressed high levels of the c-myc oncogene and were relatively resistant to interferon adds further weight to the c-myc-interferon sensitivity link. Furthermore, the larger tumours and those of mixed cell type were associated with both higher c-myc positivity and interferon resistance, although these relationships did not show statistical significance.

These results therefore support the theory that c-myc down-regulation is associated with the cell growth inhibition produced by interferon previously demonstrated in cell lines. More importantly, however, they also demonstrate that interferon resistance is associated with tumour c-myc overexpression. Therefore, not only is c-myc over-expression associated with poor prognosis in the majority of melanoma types (Grover et al. 1997, Chana et al. 1998, Ross et al. 1998), but this study shows that it is also associated with resistance to interferon.

This has important implications for the use of interferon as adjuvant therapy for melanoma. It suggests that the patients with the worst prognosis tumours for which adjuvant therapy is most important have tumours which are resistant to interferon. This would explain the failure of interferon to provide survival benefit in the majority of clinical trials (Cascinelli 1995), (Creagen et al. 1995) (Grob et al. 1998, Pehamberger et al. 1998, Kirkwood 1999). It suggests that the use of interferon alone as adjuvant therapy in melanoma is unlikely to be of no benefit as has been demonstrated by all the clinical trials, except the ECOG 1684 study.

More importantly, the link between c-myc over-expression and interferon resistance suggests that the use of corrective gene therapy strategies to block c-myc expression, such as antisense therapy, could be used to overcome interferon resistance. The combination of interferon and corrective gene therapy may therefore offer a potential method to increase the efficacy of interferon, and offer the prospect of more successful adjuvant therapy in the future. The effect of the combination of c-myc
antisense and interferon on the growth of melanoma cell lines was therefore investigated in chapter 4 of this thesis.

7.3 Combination of c-myc antisense with interferon in melanoma

The observations that c-myc over-expression is associated with interferon resistance in cell lines (Einat et al. 1988) (Resnitzky et al. 1991), and the demonstration of the association between c-myc overexpression and interferon resistance in uveal melanoma in the Chapter 3 of this thesis, suggested that blocking c-myc overexpression by antisense ODNs might be a potential mechanism for increasing the efficacy of interferon as an adjuvant agent.

To test this hypothesis a series of melanoma cell lines were treated with a combination of interferon and c-myc antisense to assess if additive or synergistic effects could be produced. The c-myc expression levels of the cell lines were measured in order to correlate the degree of additive effect produced with the c-myc expression level of each cell line.

A significant additive effect was observed when interferon and c-myc antisense were combined in the A375m cell line which was most obvious at the lower and medium doses of interferon used. When the size of the additive effect was assessed by the calculation of the theoretical window of additivity for the two agents, super-additive or synergistic effect was demonstrated at the lowest three doses of interferon used. This suggests that the two drugs act in a synergistic manner, such as via down-regulation of the c-myc oncogene. Smaller additive effects was also observed in the SkMel 28 and MeWo cell lines.

A relationship was demonstrated between the size of the additive effect produced by the interferon and c-myc antisense combination and the c-myc expression of the 4 cell lines. The greatest additive effect was produced in the cell lines that expressed the highest levels of the c-myc oncogene. Although this relationship did not reach statistical significance, only 4 cell lines were assessed. Further analysis of a larger number of melanoma cell lines may confirm the statistical significance of this
relationship. We can therefore infer that c-myc antisense only yields additive effect with interferon when it is used in cell lines that over-express the c-myc oncogene. These cell lines may have a degree of interferon resistance due to their c-myc overexpression. This has important implications for the use of c-myc antisense with interferon in patients as it suggests that the greatest benefit will be obtained by patients whose tumours express the highest levels of the c-myc oncogene. These are the patients who have the most aggressive tumours, are therefore most in need of effective adjuvant therapy but may have tumours that are relatively interferon resistant.

In summary, additive effect was observed when combining c-myc antisense with interferon in the melanoma cell lines which overexpressed the c-myc oncogene. This was most obvious in the A375m cell line which had the highest level of c-myc oncogene expression. The combination of c-myc antisense with interferon may increase the anti-tumour efficacy of interferon and be a potential strategy to overcome the resistance of the worst prognosis melanoma tumours to this drug. This may therefore provide an effective adjuvant strategy for patients with high-risk melanoma in the future.

7.4 Combination of c-myc antisense with chemotherapeutic drugs in melanoma

The prognosis for patients with stage IV melanoma (spread beyond the regional lymph nodes) is very poor. Only 5% of patients can be expected to survive 5 years (Koh 1991) as metastatic melanoma responds poorly to conventional treatments such as surgery, chemotherapy and radiotherapy.

Dacarbazine (DTIC) is the most effective chemotherapy drug used in metastatic melanoma patients, producing response rates of 10-20%. However, these responses are generally of short duration only, with long-term complete responses only seen in 1-2% of patients (Hill et al. 1984). In addition the impact on visceral compared with skin and subcutaneous metastases is poor. A number of combination chemotherapy regimens have also been tested but few have shown advantages over DTIC alone (Legha et al. 1989, Lee et al. 1995), (Margolin et al. 1998). The use of interferon and
interleukin-2 in combination with multiple chemotherapeutic drugs (biochemotherapy) has been demonstrated to produce overall response rates of 62% with long-term durable complete remissions observed in up to 10% of patients (Legha et al. 1997).

There is therefore a clear need to develop more effective forms of therapy for patients with metastatic melanoma. This project has focussed on the use of corrective gene therapy in melanoma. Previous fellows have demonstrated the efficacy of antisense ODNs targeting the c-myc oncogene at inhibiting the growth of melanoma in-vitro and in-vivo (Rajiv Grover MD thesis 1996, Jagdeep Chana MD thesis 1998). This gene was targeted due to its strong prognostic significance in melanoma and therefore its likely important role in the development and progression of melanoma in patients. As the next stage in this project, the use of c-myc antisense in combination with other chemotherapeutic drugs was investigated in-vitro to assess if additive effects could be produced which might be of benefit to patients with metastatic disease.

The combination of c-myc antisense with cis-platinum, DTIC, Temozolomide and Taxol was investigated in the A375m human melanoma cell line. A large additive effect was demonstrated when c-myc antisense was combined with cis-platinum in the A375m melanoma cell line, which is known to over-express the c-myc oncogene. Calculation of the window of theoretical additivity showed that the actual growth inhibitory effect produced by the combination was greater than would be expected by the addition of the effects produced by the two agents acting individually. This suggests a synergistic mechanism.

In addition to the direct promotion of tumour growth, c-myc overexpression is known to alter the behaviour of tumour cells in other ways which promote disease progression in melanoma, and other cancers (Evan et al. 1993). Overexpression of c-myc down-regulates surface MHC molecule expression, thus reducing the anti-tumour immune response (Grover et al. 1996) and is associated with resistance to a number of chemotherapy drugs, including cis-platinum (Kashani-Sabet et al. 1990, Sklar et al. 1991, Walker et al. 1996). This is the likely reason to explain the super-additive effects observed in this experiment. Exposure of the A375m cells to c-myc antisense may have reversed a degree of cis-platinum resistance, therefore resulting in a much larger growth-inhibitory effect when the cells were subsequently treated with this drug.
The use of corrective gene therapy to block abnormal oncogene expression may therefore not only lead to inhibition of tumour growth, but may also increase tumour sensitivity to chemotherapeutic agents such as cis-platinum. This may offer important benefits to patients with metastatic melanoma in the future.

The other interesting result from this study was the potent effect of Taxol against the A375m melanoma cell line. This highlights the potential efficacy of this drug for the treatment of metastatic melanoma patients. At present, although Taxol has demonstrated good efficacy against metastatic disease in ovarian and breast cancer patients, the response rates in metastatic melanoma have been relatively disappointing. The response rates produced by Taxol have been no better than those for DTIC (Aamdal et al. 1994). Taxol is thought to exert its anti-tumour effects in part by phosphorylation of the bcl-2 oncogene, thereby blocking its inhibitory action on apoptosis. However the bcl-2 gene is overexpressed in metastatic melanoma and its expression level has been shown to be an accurate prognostic marker in this condition (Grover et al. 1996). The efficacy of Taxol has been shown to be greatly increased by overexpression of the bcl-Xs gene, which is an inhibitor of bcl-2 activity (Sumantran et al. 1995). Therefore, inhibition of bcl-2 expression by other methods, such as by the use of antisense, may potentially increase the efficacy of Taxol. Combination of Taxol and bcl-2 antisense therapy may therefore offer potential benefits for the treatment of metastatic melanoma.

In summary, the results in this chapter, along with those published by other groups (Mizutani et al. 1994, Nieborowska Skorska et al. 1994, Jansen et al. 1998), suggest that it is possible to increase the sensitivity of tumours to chemotherapeutic agents using corrective gene therapy. This offers obvious benefits to patients with advanced malignancy as their tumours become resistant to standard chemotherapeutic drugs, shortening their survival. The use of corrective gene therapy to reverse chemoresistance, in combination with chemotherapeutic drugs, may therefore lead to the more effective treatment of metastatic disease in the future.
7.5 The development of a ribozyme targeting the c-myc oncogene in melanoma

The demonstration that antisense ODN's can specifically block the expression of a target gene has highlighted this mechanism as a potential form of corrective gene therapy for use in melanoma, and a wide range of other diseases. However, there are limitations which prevent the effective use of antisense ODNs in patients. Effective tumour-targeting is required and adequate expression levels must be achieved at the target site, for a sufficient period of time, to effectively block expression of the target gene and produce therapeutic effect.

As a first stage in the development of c-myc antisense for use in melanoma patients a hammerhead ribozyme targeting the c-myc oncogene was designed, synthesised and its efficacy assessed. Ribozymes are naturally-occurring forms of RNA that possess the ability to cleave other RNA sequences by acting in an enzymic manner. Alteration of the ribozyme structure to target specific RNA sequences produces a molecule which can act in the same manner as antisense but is much more efficient. One molecule of antisense can only block the effects of one molecule of mRNA before being consumed in the process. However one ribozyme molecule targeting a specific RNA sequence can cleave multiple RNA molecules.

A ribozyme was designed and synthesized, according to standard principles, to target the same translation-initiation codon of exon 2 of c-myc mRNA which has been targeted by the 15-mer c-myc ODN used by previous RAFT fellows and other groups (Leonetti et al. 1996)(base pairs 296-311). Its efficacy in-vitro was subsequently tested in the A375m melanoma cell line.

Although transfection of the A375m cell line with the pREVmyc17 ribozyme was shown to significantly reduce cell growth rates over a 6-day period, this effect was also shown by the pREV plasmid alone. This therefore implied that the effect was non-specific and most likely due to a transfection-related effect reducing the cellular growth rate.

This effect was confirmed to be non-specific by the demonstration that transfection of A375m cells by the pREVmyc17 ribozyme did not result in significant down-regulation of c-myc gene expression.
There are a number of reasons why the pREVmyc17 ribozyme may not have been effective against the c-myc oncogene. The ribozyme targeted a -GUU- sequence in exon 2 of the c-myc mRNA. Although this sequence is known to be an effective target for ribozymes, the -GUC- sequence is known to be the best triplet to achieve the highest ribozyme cleavage efficiency. Alternatively, although the ribozyme may have had the primary nucleotide sequence to potentially bind to the translation-initiation site in exon 2, the 3-dimensional structure may have prevented the target nucleotide sequence from binding to the cleavage site.

There are therefore a number of different options available to produce an effective ribozyme which targets c-myc mRNA. Analysis of the 3-dimensional structure of the pREVmyc17 ribozyme may reveal if access to the cleavage site is blocked by the tertiary structure of the ribozyme and aid the planning of a revised ribozyme structure targeting the same c-myc mRNA sequence.

Alternatively, a different base sequence of the c-myc mRNA could be targeted, containing the triplet sequence -GUC-. Ohta et al demonstrated the successful use of three ribozymes targeting the ras, fos and c-myc oncogenes in a melanoma cell line transfected with the ras oncogene (Ohta et al. 1996). Their conclusions were that the ras ribozyme was the most effective as it reduced the cell growth rate the most. However, the ribozymes had been tested in a ras over-expressing cell line so this observation was not unexpected. Although there is likely to have been some secondary increase in c-myc expression in this cell line due to the increased replication rate, the c-myc level is likely to have been much lower than that of the A375m cell line, which is a high c-myc expressor. Had the c-myc ribozyme been tested in a melanoma cell line expressing high levels of c-myc its effects may have been greater and the conclusions might therefore have been different.

C-myc expression has been shown to be a highly significant prognostic factor for a number of different types of primary and also metastatic melanoma (Grover et al. 1997, Grover et al. 1997, Chana et al. 1998, Ross et al. 1998). This has not been demonstrated for either the ras or the fos oncogenes. Therefore, it is likely that the c-myc oncogene is a more useful target for corrective gene therapy in metastatic melanoma patients than either ras or fos, and the successful demonstration of the efficacy of a ribozyme targeting the c-myc oncogene by Ohta et al is therefore important. The testing of this ribozyme in a high c-myc-expressing melanoma cell
line, such as A375m, which is perhaps more representative of human metastatic melanoma, is therefore important and is the next stage of this project. If this ribozyme is successful in blocking c-myc expression and cell growth in the A375m cell line, then the design of a targeting strategy, such as the use of a retrovirus containing the tyrosinase promoter sequence, may allow tumour-specific delivery of the ribozyme to melanoma. Retroviruses containing the tyrosinase promoter sequence have been successfully used to target other forms of gene therapy to melanoma, such as the use of herpes simplex virus thymidine kinase to sensitize melanoma to killing by gancyclovir (Vile et al. 1993). In addition this same strategy has been used to target a ras ribozyme to melanoma (Ohta et al. 1996). A potential design for a retrovirus incorporating the tyrosinase promoter and containing the base sequence for the c-myc ribozyme is shown in Figure 7.1. This may represent a route for the development of an effective corrective gene therapy targeting the c-myc oncogene.

7.6 Conclusions: Multiple therapeutic strategies for the treatment of melanoma

Ultimately, a combination of a variety of different therapeutic strategies is likely to be required to lead to the successful treatment of metastatic melanoma patients. Gene therapy and immunotherapy, in addition to more conventional treatments such as chemotherapy and interferon, when used simultaneously might yield different anti-tumour effects but act in synergy.

Considering corrective gene therapy first, the obstacles preventing its successful use against tumours in humans are considerable. Each individual tumour cell carries a number of different oncogene defects. A number of different tumour clones, each with varying oncogene profiles, may be present in one particular patient at any given time. The tumour is therefore heterogeneous. This profile changes with time as the tumour grows and acquires new oncogene abnormalities, which influence its behaviour. The pattern of abnormal oncogene expression is therefore complex and varies both between patients, and within an individual patient with time. The composition of the corrective gene therapy that is required to reverse or control the
Figure 7.1: Design for a targeted retrovirus encoding the c-myc ribozyme

pLNCX retroviral vector

CMV promoter removed and replaced with tyrosinase promoter
genetic defects in all the tumour cells at any particular time is therefore also complex. The exact genetic profile of the tumour burden in an individual patient will therefore need to be established first before a cocktail of different gene therapy treatments can be designed and administered to that patient. Multiple oncogene defects will need to be targeted to adequately block the growth of all the tumour cells and prevent resistant clones being selected for. In addition effective delivery systems need to be used to achieve high enough doses of the gene therapy in the tumour and prevent side-effects in normal tissues.

The use of immunotherapy avoids the need to develop a targeting strategy. Once suitably primed, the immune system can produce a specific immune response targeted to the tumour cells. The problem remains how to produce an effective anti-tumour immune response. Tumours have strategies to allow them to remain hidden from the immune system or prevent the induction of an effective immune response. The use of gene therapy strategies to increase the recognition of tumour vaccines by the immune system and produce a more effective immune response may yield more effective tumour vaccines for use in melanoma patients.

In theory combining corrective gene therapy with tumour vaccines may produce synergistic anti-tumour effect. Blocking abnormal oncogene over-expression in tumour cells with antisense, such as the c-myc gene, may lead to increased levels of surface immune-stimulatory molecules such as MHC class I on tumour cells in addition to a growth inhibitory effect. This may aid the recognition of tumour cells by the immune system leading to more effective tumour killing and increase the efficacy of tumour vaccines. The added benefit of this is that a tumour immune bystander effect is produced. Not all the tumour cells have to be transfected with the gene therapy for the immune system to destroy them. The immune system destroys the transfected cell in addition to a number of neighbouring untransfected tumour cells.

The use of gene therapy with chemotherapy agents and interferon is also likely to lead to synergistic effect as already been discussed in Chapters 3-5. Treatment of tumour cells with gene therapy may potentially increase their sensitivity to conventional chemotherapeutic drugs and interferon, leading to synergistic anti-tumour effects and the reversal of tumour resistance to these agents.

Ultimately, the use of corrective gene therapy in melanoma and other cancers may not only lead to inhibition of tumour growth, but may also alter the behaviour of
tumours, increasing their sensitivity to immunotherapy, conventional chemotherapeutic drugs and biological response modifiers such as interferon and IL-2. This may therefore lead to additive or synergistic anti-tumour effects and is likely to offer great benefits to patients with metastatic melanoma and other cancers in the future.

In summary, a large number of hurdles need to be overcome in the development of successful gene therapies for use in humans and this is likely to take many years. However, the Human Genome Project is due to publish the sequence of the entire human genome in 2001, and an acceleration in the rate of development in this field is therefore likely as knowledge regarding a whole range of new genes becomes available.
References


blood by means of reverse transcriptase and polymerase chain reaction. Lancet

Steel G. G. and Peckham M. J. (1979). Exploitable mechanisms in combined
Phys. 5: 85-91.

oligodeoxynucleotides: a question of mechanism [editorial; comment]. J Natl Cancer
Inst 88(7): 391-3.

is the bullet really magical? Science 261(5124): 1004-12.


of the Cdk-inhibitor p21WAF1 is accompanied by ordered G1 arrest, differentiation and

103(5 Suppl): 85S-89S.

sensitizes MCF-7 cells to chemotherapy-induced apoptosis. Cancer Res 55(12): 2507-
10.


Supplementary References

Chenevix-Trench, G., Martin, N.R., Ellem, K.A (1990). Gene expression in melanoma cell lines and cultured melanocytes: correlation between levels of c-src-1, c-myc and p 53
Oncogene 5(8) 1187-93.

Mol Cell Biol 5(12): 3610-6

Br J Cancer 53(6):713-9

Lincoln, S.T., Bauer, K.D. (1989). Limitations in the measurement of c-myc oncoprotein and other nuclear antigens by flow cytometry
Cytometry 10(4): 456-62

Br J Surg 85:46-51

Cancer 59(7): 1289-95


Br J Cancer 53: 331-7