AN IMMUNOHISTOPATHOLOGICAL AND FUNCTIONAL INVESTIGATION
OF β3 INTEGRIN ANTAGONISM AS A THERAPEUTIC STRATEGY IN
CANCER

F O F ALSHAMMARI

PhD

2013
AN IMMUNOHISTOPATHOLOGICAL AND FUNCTIONAL INVESTIGATION
OF β3 INTEGRIN ANTAGONISM AS A THERAPEUTIC STRATEGY IN
CANCER

Characterisation, development, and utilisation of preclinical cancer models to investigate novel β3 integrin antagonists

FATEMAH O F O ALSHAMMARI

Submitted for the degree of Doctor of Philosophy

Institute of Cancer Therapeutics

University of Bradford

2013
FATEMAH O F O ALSHAMMARI
Title: An immunohistopathological and functional investigation of β3 integrin antagonism as a therapeutic strategy in cancer

Key words: integrins, α\textsubscript{IIb}β\textsubscript{3}, α\textsubscript{V}β\textsubscript{3}, integrin antagonists, tumour cell migration, histopathology of integrin expression, cancer therapy.

Abstract
Tumour cell dissemination is a major issue with the treatment of cancer, thus new therapeutic strategies which can control this process are needed. Antagonism of integrins highly expressed in tumours is one potential strategy.

The integrins are transmembrane glycoprotein adhesive receptors. Two of the integrins, α\textsubscript{V}β\textsubscript{3} and α\textsubscript{IIb}β\textsubscript{3}, are highly expressed in a number of tumours and induce bi-directional signalling through their interaction with extracellular matrix proteins, and growth factor receptors. Through this signalling they play an important role in a number of cellular processes that are involved in tumour dissemination such as tumour growth, migration, invasion, metastasis and angiogenesis. Dual α\textsubscript{IIb}β\textsubscript{3} and α\textsubscript{V}β\textsubscript{3} integrin antagonism will have a direct effect on β\textsubscript{3}-expressing tumour cells that leads to the inhibition of cell migration and dissemination. Furthermore, through targeting tumour cell interaction with endothelial cells and platelets, this will also lead to inhibition of angiogenesis and metastasis.

The aim of this project was to characterise the expression of α\textsubscript{V}β\textsubscript{3} and α\textsubscript{IIb}β\textsubscript{3} integrin in a panel of tumour cell lines and in human tumour xenograft samples, and to develop and utilise cell-based models to investigate potential novel β\textsubscript{3} antagonists.

The expression of α\textsubscript{V} and β\textsubscript{3} subunits was detected in xenograft tissue using immunoblotting techniques. A panel of cell lines of different tumour types including melanoma, prostate, breast, colon and non small cell lung carcinoma was then characterised for α\textsubscript{V}β\textsubscript{3} and α\textsubscript{IIb}β\textsubscript{3} integrin expression using immunoblotting and immunocytochemistry. Melanoma cell lines demonstrated the strongest α\textsubscript{V}β\textsubscript{3} expression. No α\textsubscript{IIb}β\textsubscript{3} integrin expression was seen in any of the cell lines evaluated. A selection of cell lines with varying α\textsubscript{V}β\textsubscript{3} expression were then used to develop a functional test for cell migration, the scratch wound healing assay. Migration of tumour cells that expressed α\textsubscript{V}β\textsubscript{3} integrin was inhibited by the known β\textsubscript{3} antagonists, cRGDFV peptide and LM609 antibody. A panel of 12 potential novel β\textsubscript{3} integrin antagonists was screened for cytotoxicity and activity in the validated scratch assay. ICT9055 was the most effective antagonist in inhibition of M14 cell migration as determined by the scratch assay, with an IC\textsubscript{50} of < 0.1 µM.

Therefore the work presented in this thesis has established models and tools for evaluating potential novel β\textsubscript{3} integrin antagonists, and identified a promising molecule to progress for further preclinical evaluation.
Contents

Abstract ...................................................................................................................................... i

Contents ...................................................................................................................................... ii

List of Figures .............................................................................................................................. viii

List of Tables ................................................................................................................................. xi

Acknowledgement ......................................................................................................................... xii

Abbreviations ................................................................................................................................. xiii

1 Chapter 1: Introduction ............................................................................................................... 1

1.1 Cancer background ..................................................................................................................... 1
  1.1.1 Definition and aetiology of cancer ........................................................................................ 1
  1.1.2 Incidence of cancer .............................................................................................................. 2
  1.1.3 Treatment of cancer ............................................................................................................ 3
    1.1.3.1 Surgery .......................................................................................................................... 3
    1.1.3.2 Radiotherapy ................................................................................................................. 3
    1.1.3.3 Biological therapy ......................................................................................................... 4
      1.1.3.3.1 Endocrine therapy ................................................................................................. 4
      1.1.3.3.2 Gene therapy .......................................................................................................... 4
      1.1.3.3.3 Antibodies .............................................................................................................. 5
    1.1.3.4 Chemotherapy .............................................................................................................. 6
      1.1.3.4.1 Conventional chemotherapy ................................................................................. 6
  1.1.4 The general pathobiology of cancer ..................................................................................... 9

1.2 The Integrins ............................................................................................................................. 15
  1.2.1 Introduction ....................................................................................................................... 15
  1.2.2 Structure of integrins ......................................................................................................... 15
  1.2.3 Types of integrins ............................................................................................................... 19
  1.2.4 Function of integrins ......................................................................................................... 24
    1.2.4.1 Function of αvβ3 integrin .......................................................................................... 27
      1.2.4.1.1 Role of αvβ3 integrin in angiogenesis ................................................................. 28
    1.2.4.2 Function of αIIbβ3 integrin ....................................................................................... 30
  1.2.5 Role of αvβ3 and αIIbβ3 integrins in cancer development ................................................. 31
    1.2.5.1 Role of αvβ3 integrin in cancer development ............................................................. 31
      1.2.5.1.1 Role of αvβ3 integrin in breast cancer ................................................................. 33
      1.2.5.1.2 Role of αvβ3 integrin in prostate cancer .............................................................. 35
      1.2.5.1.3 Role of αvβ3 integrin in melanoma ....................................................................... 36
      1.2.5.1.4 αvβ3 integrin in glioma ....................................................................................... 38
1.2.5.2  Role of $\alpha_{\text{IIb}}\beta_3$ integrin in cancer development ........................................ 38
  1.2.5.2.1  Role of $\alpha_{\text{IIb}}\beta_3$ integrin in melanoma ........................................ 38
  1.2.5.2.2  Role of $\alpha_{\text{IIb}}\beta_3$ integrin in prostate cancer .......................... 40
  1.2.5.3  $\alpha_V\beta_3$ and $\alpha_{\text{IIb}}\beta_3$ integrin antagonism in cancer therapeutics .... 41
   1.2.5.3.1  Anti-$\alpha_V\beta_3$ antibodies as cancer therapeutics .......................... 41
   1.2.5.3.2  Integrin binding peptides as cancer therapeutics .......................... 42
   1.2.5.3.3  Small molecule integrin antagonists as cancer therapeutics.............. 43
   1.2.5.3.4  Dual $\alpha_V\beta_3/\alpha_V\beta_5$ integrin antagonism in cancer therapy .... 45
   1.2.5.3.4.1  Cilengitide, a dual $\alpha_V\beta_3/\alpha_V\beta_5$ integrin antagonist, in cancer therapy .............................................................. 47
   1.2.5.3.5  Dual $\alpha_V\beta_3/\alpha_{\text{IIb}}\beta_3$ integrin antagonism .......................... 49

1.3  Aims and objectives .................................................................................. 57

2  Chapter 2: Characterisation of integrin $\alpha_{\text{IIb}}\beta_3$ and $\alpha_V\beta_3$ expression in human xenograft tissue in mice using immunohistochemical and immunoblotting techniques ......................................................... 59

2.1  Introduction ............................................................................................... 60
  2.1.1  Aims and objectives ........................................................................... 63

2.2  Materials and methods ............................................................................ 64
  2.2.1  Materials .......................................................................................... 64
  2.2.2  Cell lines ......................................................................................... 64
  2.2.3  Methods .......................................................................................... 66
   2.2.3.1  Collection of xenograft materials .............................................. 66
   2.2.3.2  Slide coating .............................................................................. 66
   2.2.3.3  Tissue fixation, processing, embedding and paraffin sectioning .... 66
   2.2.3.4  Haematoxylin and Eosin staining .............................................. 68
   2.2.3.5  Immunohistochemistry ............................................................... 69
   2.2.3.5.1  Immunodetection of $\alpha_{\text{IIb}}$ and $\alpha_V\beta_3$ integrins expression in FFPE human tumour xenograft sections ................................. 69
   2.2.3.5.1.1  Sample preparation ............................................................ 69
   2.2.3.5.1.2  Antigen retrieval ................................................................. 69

A. Citrate buffer antigen retrieval ................................................................. 69

B. Pre-warmed pepsin digestion method antigen retrieval ...................... 70

C. Proteinase K method antigen retrieval .................................................. 70
   2.2.3.5.1.3  Antibody incubation ........................................................... 70
   2.2.3.5.2  Immunodetection of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_V\beta_3$ integrins expression in frozen human xenograft tumour ........................................ 71
   2.2.3.5.2.1  Sample preparation ............................................................ 71
Chapter 3: Characterisation of integrin α_{1IB}β_{3} and α_{V}β_{3} expression in a panel of human tumour cell lines

3.1 Introduction ............................................................................................................. 109
3.1.1 Aims and objectives .......................................................................................... 110

3.2 Materials and Methods ......................................................................................... 112
3.2.1 Materials .......................................................................................................... 112

2.3 Results .................................................................................................................. 80
2.3.1 Detection of the expression of α_{1IB}β_{3} and α_{V}β_{3} integrin in human tumour xenograft tissue by IHC ................................................................. 80
2.3.1.1 Tumour xenograft ......................................................................................... 80
2.3.1.2 Studies on FFPE tissue samples ................................................................. 81
2.3.1.3 IHC studies on Frozen Sections ................................................................. 84
2.3.2 Immunodetection of β_{3} and α_{V}β_{3} integrin subunits using an M.O.M. kit ......................................................................................................................... 87
2.3.2.1 M.O.M. Immunodetection of β_{3} and α_{V}β_{3} integrins in FFPE sections ......................................................................................................................... 87
2.3.2.2 M.O.M. Immunodetection of β_{3} and α_{V}β_{3} integrins in frozen sections ......................................................................................................................... 92
2.3.3 Detection of the expression of α_{1IB}, α_{V}, and β_{3} subunits in homogenised tumour xenograft mouse tissue by immunoblotting ........................ 95
2.3.3.1 Expression of α_{V} and β_{3} integrin in homogenised human xenograft mouse tissue compared to negative control tissue and human tumour cells .......................................................................................................................... 98

2.4 Discussion .............................................................................................................. 100

2.5 Conclusion ............................................................................................................. 107
3.2.2 Methods ........................................................................................................ 112
3.2.2.1 Cell maintenance .............................................................................. 112
3.2.2.2 Tumour cell line growth kinetics ......................................................... 115
3.2.2.3 Immunoblotting ............................................................................... 115
3.2.2.3.1 Cell Lysis ...................................................................................... 116
3.2.2.3.2 IMB of nitrocellulose membrane with different anti α₃, α₃β₃ and β₃ integrin antibodies ................................................................. 116
3.2.2.4 Immunocytochemistry (ICC) .............................................................. 118
3.2.2.4.1 Detection of α₃, α₃β₃, and β₃ subunits, and α₃β₃ integrin expression in different tumour cell lines by ICC ........................................... 118
3.2.2.4.1.1 Materials .............................................................................. 118
3.2.2.4.1.2 Cell preparation ...................................................................... 118
3.2.2.4.1.3 Confocal microscopy ................................................................. 119
3.2.2.4.1.4 ICC analysis ........................................................................... 119

3.3 Results ............................................................................................................. 121
3.3.1 Characterisation of cellular growth kinetics .............................................. 121
3.3.2 Detection of α₃, α₃β₃, and β₃ integrins using IMB .................................. 123
3.3.2.1 Optimisation of IMB ......................................................................... 123
3.3.2.2 Screening different tumour cell lines for expression by IMB technique using the optimised conditions for anti-α₃, Q20, and anti-β₃, B7, antibodies ................................................................. 126
3.3.3 Detection of α₃, α₃β₃, β₃ and α₃β₃ integrins using ICC ...................... 129
3.3.3.1 Optimisation of ICC .......................................................................... 129
3.3.3.2 Evaluation of the effect of method of cell harvesting on integrin expression ...................................................................................... 132
3.3.3.3 Screening different cell lines for expression of α₃, β₃, α₃β₃ integrins by ICC technique ................................................................. 133
3.3.3.4 Analysis of the expression of α₃, α₃β₃, β₃ and α₃β₃ integrin in different human tumour cell lines detected by ICC ......................... 136
3.3.3.5 Confirmation of sub-cellular immunolocalisation of α₃β₃, β₃ and α₃β₃ integrin using confocal microscopy ........................................... 137
3.3.4 Comparison of result of IMB and ICC ..................................................... 139

3.4 Discussion ...................................................................................................... 140

3.5 Conclusion ..................................................................................................... 148

4 Chapter 4: Investigation of the effect of α₃β₃ integrin inhibition on tumour cell migration ................................................................. 149

4.1 Introduction .................................................................................................. 150
4.1.1 Aims and objectives ...................................................................... 152

4.2 Materials and methods ............................................................................. 154
4.2.1 Materials ......................................................................................... 154
4.2.2 Methods ......................................................................................... 154
4.2.2.1 Wound healing migration assay .................................................. 154
4.2.2.1.1 Characterisation of the phenotype of the migrated cells in terms of proliferation and integrin expression........................................ 156
4.2.2.2 Evaluation of the cytotoxicity of cRGDFV and LM609 using the MTT assay.................................................................................. 157
4.2.2.3 Validation of the scratch assay using the integrin antagonists cRGDFV and LM609........................................................................ 158
4.2.2.4 Statistical analysis........................................................................... 160

4.3 Results .................................................................................................... 161
  4.3.1 Development of the wound healing migration assay..................... 161
    4.3.1.1 Seeding density...................................................................... 161
    4.3.1.2 Healing time ........................................................................ 163
  4.3.2 Evaluation of the cytotoxicity of cRGDFV and LM609 using the MTT assay................................................................. 167
  4.3.3 The effect of cRGDFV on the migration of human tumour cell lines ....................................................................................... 168
  4.3.4 The effect of LM609 on human tumour cell migration ............... 171

4.4 Discussion .............................................................................................. 173

4.5 Conclusion .................................................................................................. 177

5 Chapter 5: Investigation of the cytotoxicity of potential novel β3 integrin antagonists and their effect on tumour cell migration ........ 178
  5.1 Introduction ............................................................................................. 179
    5.1.1 Aims and objectives .................................................................. 180
  5.2 Materials and methods .......................................................................... 181
    5.2.1 Materials ...................................................................................... 181
  Table 5.1 Compounds used ....................................................................... 181
    5.2.2 Methods ....................................................................................... 182
      5.2.2.1 Evaluation of the cytotoxicity of potential novel β3 integrin antagonists using the MTT assay ........................................ 182
      5.2.2.2 Wound healing migration assay .............................................. 182
      5.2.2.3 Statistical analysis ................................................................ 182
  5.3 Results .................................................................................................... 183
    5.3.1 Evaluation of the cytotoxicity of potential novel β3 integrin antagonists ........................................................................ 183
    5.3.2 Characterisation of the anti-migratory effect of potential novel β3 integrin antagonists using the scratch assay ......................... 185
  5.4 Discussion .............................................................................................. 192
  5.5 Conclusion .................................................................................................. 194
Chapter 6: Discussion and future work .................................. 195

6.1 General discussion and future perspective ...................... 196

6.2 Conclusion ........................................................................... 206

Chapter 7: References and Appendixes .............................. 207

7.1 References ........................................................................... 208

7.2 Appendices ........................................................................ 280
  7.2.1 Appendix 1 ................................................................. 280
  7.2.2 Appendix 2 ................................................................. 281
List of Figures

Figure 1.1 The basic steps of the cancer metastatic pathway (Bellahcene et al. 2008).......................... 11
Figure 1.2 Role of integrins in cancer development................................................................. 14
Figure 1.3 The structure of αIIbβ3 integrin in its active and inactive state .... 18
Figure 1.4 The integrin family.................................................................................................... 19
Figure 1.5 inside-out-signalling pathways for integrins (Askari et al. 2009). 26
Figure 1.6 Interaction of active integrin with signalling molecules regulates cellular function (Hynes 2002).............................................................................................................. 27
Figure 1.7 Interaction of αvβ3 integrin and VEGFR-2 (Somanath et al. 2009). .......................................................... 30
Figure 1.8 Active αIIbβ3 integrin in platelet activation and thrombus formation (Millard, Odde & Neamati 2011). .......................................................... 31
Figure 1.9 Anti-integrin antagonists ............................................................................................... 52
Figure 2.1 Representative calibration curve using Bradford assay. ............... 76
Figure 2.2 Confirmation of successful protein transfer ............................................................. 78
Figure 2.3 Haematoxylin and Eosin stain in M14 human melanoma xenograft paraffin embedded tissues.............................................................................................. 80
Figure 2.4 Optimisation of IHC to detect αIIbβ3 and αvβ3 integrin expression in FFPE human xenografts, using different methods .............................................................. 82
Figure 2.5 Optimisation of IHC to detect αv and β3 integrin expression in FFPE M14 human melanoma xenografts using Q20 and B7.......................... 83
Figure 2.6 Optimisation of the protocol used to characterise expression of αIIbβ3 and αvβ3 integrin in frozen human xenograft mouse tissue by IHC..... 85
Figure 2.7 IHC of αv integrin expression in human xenograft mouse frozen tissue using Q20 .................................................................................................................. 86
Figure 2.8 IHC detection of αvβ3 and β3 integrin in human FFPE tumours with an M.O.M. kit................................................................................................................. 89
Figure 2.9 IHC detection of β3 integrin expression using B7 with M.O.M. kit in FFPE M14 melanoma human tumour xenografts in mouse.............. 90
Figure 2.10 IHC detection of β3 integrin expression in FFPE human tumour xenografts in mouse using B7 with M.O.M. kit .................................................. 91
Figure 2.11: IHC optimisation for αvβ3 and β3 integrin expression in frozen sections using the M.O.M. detection kit............................................... 93
Figure 2.12 Expression of β3 integrin in human tumour xenograft frozen tissues immunolabelled with BV4 (anti-β3) using M.O.M. detection kit...... 94
Figure 2.13 Optimisation steps for extraction of protein from human xenograft mouse tissue ............................................................ 96
Figure 2.14 Expression of β3 subunit in M14 human xenograft mouse tissue using B7 by IMB ................................................................. 97
Figure 2.15 Expression of αv and β3 integrin in homogenised tissue ........ 99
Figure 3.1 Growth parameters of human tumour cell lines ......................... 122
Figure 3.2 Optimisation of IMB protocol .................................................. 124
Figure 3.3 Expression of αv and β3 integrin in a panel of human tumour cell lines from a supernatant .................................................. 127
Figure 3.4 Expression of αv and β3 integrin protein in a panel of human tumour cell lines from a pellet resuspended in lysis buffer .............. 128
Figure 3.5 Detection of αv, αllb, β3 and αvβ3 integrin in PC-3 using ICC ....... 130
Figure 3.6 Comparison of cell harvesting techniques ................................. 132
Figure 3.7 Screening of the first part of the cell line panel with anti-αV (Q20), anti-αllb (C20), anti-β3 (B7), and anti-αVβ3 (LM609) using ICC .... 134
Figure 3.8 Screening of the second part of the cell line panel with anti-αV (Q20), anti-αllb (C20), anti-β3 (B7), and anti-αvβ3 (LM609) using ICC ...... 135
Figure 3.9 The expression of αllb, β3, and αvβ3 integrin in human tumour cell lines by confocal microscopy ........................................ 138
Figure 4.1 Wound healing assay steps (Hulkower 2011) .......................... 156
Figure 4.2 Scratch assay analysis ............................................................. 159
Figure 4.3 Formation of confluent monolayers for the cell line panel (cell density per ml and time in hours) ............................................. 162
Figure 4.4 Migration of different tumour cell lines as assessed by the scratch assay ................................................................. 164
Figure 4.5 Labelling M14 cells at initial wound perimeter (T=0) and after 24 hours of wound healing with LM609 and Ki-67 using ICC .......... 166
Figure 4.6 Evaluation of cRGDfV and LM609 cytotoxicity against human tumour cell lines M14 and HT-29 ........................................... 167
Figure 4.7 Effects of cRGDfV on tumour cell migration in the cell panel using the scratch assay .......................................................... 170
Figure 4.8 The effect of LM609 on tumour cell migration ............................ 172
Figure 5.1 Evaluation of the cytotoxicity of the potential novel β3 integrin antagonists in the tumour cell line panel ................................ 184
Figure 5.2 Effect of potential β3 integrin antagonists on M14 cell migration 186
Figure 5.3 Effect of 10 μM β3 antagonists on the inhibition of M14 cell migration by the scratch assay .................................................. 188
Figure 5.4 Effect of potential $\beta_3$ integrin antagonists in inhibition M14 cell. 190
List of Tables

Table 1.1 Drugs currently used for targeted therapy ........................................... 8
Table 1.2 Functions and ligands of different types of integrins ...................... 20
Table 1.3 Further anti-integrin agents in clinical use and advanced development ................................................................. 53
Table 2.1 Primary antibodies and related secondary antibodies used in immunohistochemistry (IHC) and immunoblotting (IMB) ................. 65
Table 3.1 Primary antibodies and related secondary antibodies used in immunocytochemistry (ICC) and IMB techniques ......................... 113
Table 3.2 Cell lines .......................................................................................... 115
Table 3.3 Conditions investigated while optimising detection of expression of $\alpha_v$, $\alpha_{\text{lib}}$, $\beta_3$ and $\alpha_v\beta_3$ integrin in human tumour cell lines using ICC. ........ 120
Table 3.4 The optimised protocol for all antibodies used to detect the expression of $\alpha_v$, $\alpha_{\text{lib}}$, and $\beta_3$ subunit and $\beta$-actin using IMB technique. .... 125
Table 3.5 Optimised protocols for all antibodies used to detect the expression of $\alpha_v$, $\alpha_{\text{lib}}$, $\beta_3$ subunits, and $\alpha_v\beta_3$ integrin in cell membrane using ICC .... 131
Table 3.6 Screening different tumour cell lines for the membranous expression of $\alpha_v$, $\beta_3$, $\alpha_{\text{lib}}$ and $\alpha_v\beta_3$ by ICC .................................................. 136
Table 3.7 Comparison of integrin expression in different tumour cell lines using specific antibodies for $\alpha_v$ and $\beta_3$ integrin subunits using ICC and IMB. .................................................................................. 139
Table 4.1 Comparison of cell migration assays (Kramer et al. 2013) ........... 153
Table 5.1 Compounds used ............................................................................. 181
Table 5.2 IC_{50} values for the potential novel $\beta_3$ integrin antagonists in the tumour cell line panel ......................................................................................... 185
Table 5.3 A summary of effect of the potential novel $\beta_3$ integrin antagonists in inhibition of M14 cell migration after 24 hours treatment with the scratch assay ..................................................... 191
Acknowledgement

Firstly, I would like to express my profound gratitude to my supervisors Dr. Steve Shnyder, Dr. Helen Sheldrake and Professor Laurence Patterson for providing me with an exciting and challenging research project. I would like to thank them for help and guidance and illustrious advice which they given me, throughout my PhD studies.

I am extremely thankful to the staff of Institute of Cancer Therapeutics who had helped in this research either by their support or encouragement. I would like also to thank the staff in the lab: in the histology lab I would like to thank Beryl Cronin and Patricia Cooper, in cell culture I would like to thank Dave Healey and in the molecular lab I would like to thank Dr. Mark Sutherland and Andrew Gordon (who provided PCR data shown in the appendix).

I would also like to thank the Public Authority for Applied Education and Training (PAAET) for their funding.

My special thanks must also go to my dear parents (Khairyah Al Shammar and Owayed Al Shammar), my husband Abdul Mohsen Al Shammar and for my four daughters Sarah, Nourah, Noof and Shaikha.
Abbreviations

ABC: Avidin biotin complex
ADMIDAS: Adjacent to metal-ion-dependent adhesion site
ADP: Adenosine diphosphate
Akt/PKB: Protein kinase B
Angpt13: Angiopoietin-like 3
APES: 3-aminopropyltriethoxysilane
ARMD: Age-related macular degeneration
ATCC: American Type Culture Collection
bFGF: basic fibroblast growth factor
BME: Basal membrane extract
BSA: Bovine serum albumin
BSP: Bone sialoprotein
CAMs: cell adhesion molecules
CD3: Cluster of differentiation 3
CMRIT: Combined radioimmunotherapy with cilengitide
cRGDfV : Arg-Gly-Asp-phe-Val
CTGF: Connective tissue growth factor
CTP: Cyclotetrapeptide
CYR61: Cysteine-rich angiogenic inducer 61
2D: Two dimensional
3D: Three dimensional
DAB: 3,3-diaminobenzidine tetrahydrochloride
DAPI: 4,6-Diamidino-2-phenylindole
Db: Double bands
dH₂O: Distilled water
D/I: Dilution/incubation
DMSO: Dimethyl sulphoxide
DNA: Deoxyribonucleic acid
DPX: Distyrene/plasticiser/xylene
ECM: Extracellular matrix
EDTA: Ethylenediamine tetraacetic acid
EGF: Epidermal growth factor
EGFR: Epidermal Growth Factor Receptor
EMT: Epithelial mesenchymal transmission
EP: Endogenous peroxidase
ERK: Extracellular Signal-regulated Kinase
FAK: Focal adhesion kinase
FBS: Foetal bovine serum
FFPE: Formalin fixed paraffin embedded
FGFR: Fibroblast growth factor receptor
FITC: Fluorescein isothiocyanate
g: Gram
GAR: Goat anti rabbit

GIST: Gastrointestinal stromal tumour

GTPase: Guanosine triphosphate

H$_2$O$_2$: Hydrogen peroxide

HBSS: Hanks’s balanced salt solution

H&E: Haematoxylin and Eosin

HER2: Human Epidermal Growth Factor Receptor 2

HPV: Human papilloma virus

HRP: Horse-radish peroxidase

hrs: hours

HTS: High throughput screening

HUVECs: Human umbilical vein endothelial cells

IARC: International Agency for Research on Cancer

iC3b: Inactivated C3b

IC$_{50}$: Inhibitory concentration

ICA: Ice pre-cooled acetone

ICAM: Interacellular Adhesion Molecule

ICC: Immunocytochemistry

ICM: Ice pre-cooled methanol

IF: Immunofluorescence

Ig: Immunoglobulin

IgG: Immunoglobulin g
IHC: Immunohistochemistry
IMB: Immunoblotting
ISH: In-situ hybridisation
isoDGR: iso-Aspartic acid-Glycine-Arginine
kDa: Kilodalton
kg: Kilogram
LAP-TGF- β: Latency associated peptide-Transforming growth factor B
LIMBS: Ligand induced metal binding site
mA: Milliampere
MAAdCAM-1: Mucosal vascular addressin cell adhesion molecule 1
MAPK: Mitogen-activated protein kinase
Mb: Multiple bands
MIDAS: Metal-Ion-Dependent Adhesion Site
ml: Milliliter
mM: Millimolar
MMAb: Monoclonal mouse antibody
MMPs: Matrix metalloproteinases
MMP-9: Matrix metalloproteinase-9
M.O.M.: Mouse on mouse
mTOR: mammalian Target Of Rapamycin
MTT: 3-(4,5-Dimethylthiazole-2-y1)-2,5-diphenyl tetrazolium bromide
NaCl: Sodium chloride
Nc: No clear band

NCI: National Cancer research

n.d.: Not done

NF-Kb: Nuclear factor kappa-light chain enhancer of activated B cells

NGS: Normal goat serum

NHS: Normal horse serum

nM: Nanomolar

NRS: Normal rabbit serum

NS: Normal serum

NSCLC: Non small cell lung cancer

NSP: Non specific binding

OPN: Osteopontin

P13k/Akt: Phosphotidylinositol 3-kinase/protein kinase B

PAb: Primary antibody

PBS: Phosphate buffered saline

PC: Polycarbonate

PCI: Percutaneous coronary intervention

PDGFR: Platelet Derived Growth Factor Receptor

PET: Polyethylene terephthalate

PFA: Paraformaldehyde

PGAb: Polyclonal goat antibody

PGAR: Polyclonal goat anti rabbit
TEM: Transendothelial migration
TGF-β1: Tissue growth factor β1
THN: Tetramethylethylenediamine
TNF: Tumour necrosis factor
TRITC: Tetramethylrhodamin isothiocyanate
µg: Microgram
µl: Microliter
µM: Micromolar
VCAM-1: Vascular cell adhesion protein 1
VEGF: Vascular Endothelial Growth Factor
VEGFR-2: Vascular Endothelial Growth Factor receptor 2
VEGFR-A: Vascular Endothelial Growth Factor receptor A
VGP: Vertical growth phase
vWF: von Willebrand factor
1 Chapter 1: Introduction

1.1 Cancer background

1.1.1 Definition and aetiology of cancer

Cancer is a complex family of diseases characterised by uncontrolled growth of cells and spread from the primary tumour site to other sites (Varmus 2006). The formation of cancer is a multistep process that originates from a single cell in which vital regulatory pathways have been disturbed. Cancer has multiple causes, with a wide range of genetic, physiological and histological features. However, genetic and environmental factors such as chemical exposure, ionizing radiation exposure, and certain viruses (such as human papilloma virus; HPV) are the main causes (Fearon 1997).

Clinically, tumours include two main types: benign and malignant. A benign tumour grows locally, with a gradual increase in size, and causes local pressure or obstruction within the surrounding tissue. In contrast, a malignant tumour grows rapidly, metastasises via the lymph or blood vessels to distant sites, and is dangerous due to its potential for invasion and destruction of normal tissues (van Slooten et al. 1985).
1.1.2 Incidence of cancer

A wide variation is reported for the incidence and prevalence of cancer at different anatomical sites, and for patients of different ages, sex and geographical locations. The overall trend is for an increase in cancer incidence and death annually. Statistics obtained from the International Agency for Research on Cancer (IARC), showed that the incidence of cancer in the world reached 12.7 million cases in 2008 with a mortality rate of 7.6 million. The incidence of cancer was estimated to increase to reach 13.2 million cases for both sexes in 2011 (Jemal et al. 2011). Europe estimated 1.3 million deaths from cancer in 2013 (Malvezzi et al. 2013). In the United Kingdom, the cancer incidence reported in 2008 and cancer mortality in 2009 was 408,381 new cancer cases and 156,090 cancer deaths (CRUK 2012). Moller indicated that the incidence rate of all cancers in the English population will rise from 224,000 in 2001 to around 299,000 in 2020, or an increase of 33 per cent (Moller et al. 2007). In the United States, cancer is the second most common cause of death after heart disease and it is estimated that one in four people will die from cancer. The American Cancer Society estimates that in 2013 there will be 1.7 million new cancer cases diagnosed and about 580,350 Americans are expected to die of cancer (Siegel et al. 2013). These statistics support the need for continued research into the causes of cancer and the development of effective preventive methods and effective treatments.
1.1.3 Treatment of cancer

Treatment of cancer is a challenge. The choice of treatment depends on tumour type, histological grade and stage of the tumour at the time of diagnosis. The overall aim of the treatment is to remove all cancerous cells without affecting normal tissue (Rosenberg 2001). The major types of treatment for cancer are surgery, radiotherapy, biological therapy and chemotherapy.

1.1.3.1 Surgery

In most cases, surgery is the first line of cancer treatment. However, before diagnosis, the cancer may have already metastasised to another area of the body; therefore, chemotherapy and/or radiotherapy must be used in conjunction with surgical removal in order to slow tumour growth and prevent further metastasis. In addition, some primary tumours, such as brain tumours, are not easily removed by surgery. Therefore, neoadjuvant chemotherapy (the use of chemotherapy prior to surgery or radiation) can also play an important role. This treatment can reduce the tumour load thereby making surgery or radiotherapy easier. The removal of residual tumour after surgery can also be done by adjuvant treatment (Rosenberg 2001).

1.1.3.2 Radiotherapy

Radiotherapy is used to reduce the size of tumour before surgery (Camporeale 2008), ensures that all cancer cells are destroyed following
surgery and slows down the progression of the disease. For example, radiotherapy can be used in conjunction with surgery for treatment of carcinomas of the pancreas (Alfieri et al. 2001), larynx, other head and neck sites, cervix, breast, bladder and prostate. However, several studies have shown that prostate cancer patients who undergo radiotherapy treatment can have recurrence of the disease after a few months (Tefilli et al. 1998; Tefilli et al. 1998; Tefilli et al. 1998). Furthermore, radiotherapy is not efficient at curing metastasis.

1.1.3.3 Biological therapy

1.1.3.3.1 Endocrine therapy

The success of endocrine therapy depends on its ability to suppress further growth of the tumour rather than its ability to kill cells. It can be used to treat hormone dependent tumours such as breast and prostate cancer (Labrie 2004). The treatment depends on lowering the plasma concentration of a particular hormone (e.g. by removing the producing organ such as by orchidectomy (removal of the testes) (Suzuki et al. 2010) or by antagonising its action at its receptor (e.g. tamoxifen, which binds to oestrogen receptors on the mammary epithelium and thereby blocks the proliferative actions of oestrogen) (Criscitiello et al. 2011).

1.1.3.3.2 Gene therapy

The principle of gene therapy involves changing the actual gene responsible for the neoplastic processes. This therapy requires that the gene must
access to the cell via a vector. The vector may be a virus that is defective in replication and into which the therapeutic gene has been inserted, along with the promoter that will activate the gene once inside the cell. Gene therapy may have one of the following aims: restoring the function of a defective tumour-suppressor gene such as p53 to inhibit tumour growth; blocking the action of a mutated or over-expressed oncogene or insertion of a gene that activates a prodrug or produces other cell-killing effects thereby eliminating cancer cells near the cell into which the gene has penetrated (Brown & Lillicrap 2002; Edelstein et al. 2007). However, one type of gene therapy, ganciclovir/Hstk, reached phase III clinical trials but did not show any significant effect on malignant brain tumours due to low transduction of the vector and immune response to vector-producing cells (Rainov 2000). Gene therapy can cause damage to the immune system (Brown & Lillicrap 2002).

1.1.3.3.3 Antibodies

Monoclonal antibodies can disrupt cancer cells through a variety of mechanisms (Strome et al. 2007; Trikha et al. 2002). Monoclonal antibodies can trigger the immune system to attack cancer cells (e.g. Rituximab and Alemtuzumab) (Murakami et al. 2011; Schweighofer & Wendtner 2010), prevent the cancer cells from taking up proteins (e.g. Transtuzumab), block angiogenesis through inhibition of VEGF-A (e.g. Bevacizumab) or carry cancer drugs or radiation to cancer cells (e.g. Zevalin, Bexxar and Mylotarg). However, a recent study reported that Bevacizumab administration led to increased treatment-related mortality (Ranpura et al. 2011). Several
limitations also make antibodies unattractive for use as therapeutics, including production costs, pharmacokinetics versus tissue penetration and modes of action (Chames et al. 2009).

1.1.3.4 Chemotherapy

1.1.3.4.1 Conventional chemotherapy

Cancer chemotherapy is a systemic treatment and its selectivity depends on the ability of the drugs to target the tumour. However, cancer chemotherapy drugs also affect normal cells that replicate rapidly, such as hair follicles (Botchkarev 2003), bone marrow, intestinal mucosa and gonads (Meirow & Nugent 2001; Vijayalaxmi 2011). Chemotherapy works mainly by disrupting cell proliferation, especially targeting DNA synthesis and cell division. Traditional chemotherapy drugs include: anti metabolites such as 5-fluorouracil (inhibits DNA synthesis), alkylating agents such as cisplatin (blocks DNA replication), anti microtubule agents such as taxanes (blocks microtubule depolymerisation) and anthracyclin antibiotics like doxorubicin (interacts with topoisomerase II, intercalates between DNA nucleotides and blocks DNA synthesis) (Kaye 1998).

Tumour cells may be resistant to cytotoxic drugs because of extracellular circumstances that limit drug access to the tumour or due to defects in the p53 gene that render the tumour cell resistant to the cytotoxic drug. Tumour cell resistance to chemotherapy can also arise due to DNA damage, decreased uptake of drug by the cell and increased drug efflux by a p-
glycoprotein that acts as a drug efflux pump and reduces the intracellular concentrations of some cytotoxic agents. Another problem with chemotherapy is that the drug may reach the target cells at insufficient concentrations as a result of inactivation or removal of the drug from the circulatory system (Moxley & McMeekin 2010).

1.1.3.5 New strategies for cancer therapy

While the increased incidence and mortality of cancer and the inadequacy of the different kinds of therapy already mentioned, a clear need thus exists for the development of treatments with greater effectiveness. The development of targeted therapies has the aim of improving differentiation between normal and neoplastic cells. The discoveries in molecular biology about the control of cell cycle events highlighted that certain signalling pathways that control the growth and differentiation were inappropriately activated in various tumours (Hersey et al. 2009). Targeted agents can be used against cell adhesion molecules, growth factors, intracellular signalling and angiogenesis (Gerber 2008; Korpanty et al. 2010; Lord & Ashworth 2010). Table 1.1 summarises the targeted therapy drugs currently in clinical use (Collins & Workman 2006; Malinowsky et al. 2010).
### Table 1.1 Drugs currently used for targeted therapy

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tumour type</th>
<th>Target</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trastuzumab</td>
<td>Metastatic breast cancer, gastric cancer</td>
<td>HER2</td>
<td>(Farolfi et al. 2013)</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Metastatic colorectal cancer</td>
<td>EGFR</td>
<td>(Stintzing et al. 2013)</td>
</tr>
<tr>
<td>Imatinib Mesylate</td>
<td>Chronic myloid leukkaemia and Gastrointestinal stromal tumours with activated</td>
<td>Bcr/abl, c-kit, PDGFR,</td>
<td>(Spiers et al. 2011)</td>
</tr>
<tr>
<td>(Gleevec)</td>
<td>c-kit receptor tyrosine kinase, other sarcomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Colorectal cancer</td>
<td>VEGF</td>
<td>(Bennouna et al. 2013)</td>
</tr>
<tr>
<td>Gefitinib (Iressa)</td>
<td>Non-small-cell lung cancer</td>
<td>mutant EGFR</td>
<td>(Sugiura et al. 2013)</td>
</tr>
<tr>
<td>Erlotinib (Tarveca)</td>
<td>Non-small-cell lung cancer</td>
<td>mutant EGFR</td>
<td>(Stevenson &amp; El-Modir 2011)</td>
</tr>
<tr>
<td>Rapamycin RAD001</td>
<td>Breast, prostate and renal cancer</td>
<td>mTOR</td>
<td>(Armstrong et al. 2010)</td>
</tr>
<tr>
<td>Sorafenib BAY 43-9006</td>
<td>Melanoma, Renal carcinoma</td>
<td>RAF kinase</td>
<td>(Panka et al. 2006; Procopio et al. 2013)</td>
</tr>
<tr>
<td>Vemurafinib</td>
<td>Melanoma</td>
<td>BRAF mutation</td>
<td>(Gonzalez et al. 2013)</td>
</tr>
<tr>
<td>Dasatinib BMS354825</td>
<td>Gastrointestinal stromal tumour, Chronic myloid leukemia</td>
<td>Kit, PDGFR, Dual Src/Abl kinase</td>
<td>(Caenepeel et al. 2010; Quintas-Cardama et al. 2007)</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>Breast cancer</td>
<td>EGFR, HER2</td>
<td>(Bachelot et al. 2013)</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>Renal cell cancer</td>
<td>VEGFR, PDGFR, cKit, Flt-3</td>
<td>(Blagoev et al. 2013; Huang et al. 2010)</td>
</tr>
<tr>
<td>Pertuzumab</td>
<td>Breast cancer</td>
<td>HER2</td>
<td>(O’Sullivan &amp; Swain 2013)</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Breast cancer</td>
<td>Bcr/abl</td>
<td>(Somlo et al. 2013)</td>
</tr>
</tbody>
</table>

1.1.4 The general pathobiology of cancer

Cell growth is usually well organised and controlled to meet the needs of the body. In contrast, cancer cell division is uncontrolled. Growth factors such as tumour necrosis factor (TNF) and extracellular matrix (ECM) components that interact with cell surface receptors can lead to changes in cell division which activate different protein pathways inside the cell (Jinka et al. 2012). With the help of signal transducers inside the cell, these pathways will lead to changes in gene expression and induce cell proliferation (Radeff-Huang et al. 2007).

Apoptosis is the process of programmed cell death. Apoptosis involves biochemical events that lead to cell morphology changes and ultimately cell death. These changes include blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Tumour cells can survive and proliferate if they can escape from undergoing apoptosis (Fulda 2009). Apoptosis can be mediated by both extrinsic and intrinsic pathways. The extrinsic pathway is initiated by extracellular death ligand such as tumour necrosis factor (TNF) and tumour necrosis factor-related apoptosis inducing ligand (TRAIL), which leads to the recruitment of caspase-8 to form a death-inducing signal complex. The extrinsic pathway can activate caspase-3 and cleave BID to tBID which facilitates the release of the cytochrome c from the mitochondria. The intrinsic pathway is mediated by BAX, BAK and BID proapoptotic proteins which promote release of cytochrome c from the
mitochondria leading to activation of caspase-9 ultimately leading to apoptosis.

The tumour suppressor gene p53 can bind to DNA and activates genes that control the cell cycle and programmed cell death. If the cell has damaged DNA, p53 can either repair the damage or induce apoptosis to prevent proliferation of the defective cell and potential cancer development. Therefore, inactivation of p53 can also play a role in the survival of tumour cells. Cell adhesion molecules can also control cell apoptosis via anoikis (apoptosis resulting from loss of interactions with the extracellular matrix), direct induction of caspases and increased bcl-2 expression, p53 inactivation and several other pathways related to survival, such as Extracellular Signal-regulated Kinase (ERK) and protein kinase B/Akt (Guadamillas et al. 2011; Stiewe 2007; Tan et al. 2004; Vachon 2011; Zhong & Rescorla 2012).

As a malignant tumour progresses it will metastasise (Bozzuto et al. 2010). Metastasis is the process whereby the cancer cells migrate from the primary tumour site to other parts of the body (Geho et al. 2005). In essence, it is the fundamental difference between benign and malignant tumours. The ability of cancer cells to metastasise depends on the interaction of their cell surface molecules with the microenvironment including the ECM and neighbouring cells. Several steps are involved in metastasis (Figure 1.1) including migration, intravasation, transportation, extravasation and metastatic colonisation (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011; Talmadge & Fidler 2010; Williams 1974).
Figure 1.1 The basic steps of the cancer metastatic pathway (Bellahcene et al. 2008)
Normal cells are transformed into cancer cells (a). Cancer cells proliferate (b), detach from each other, penetrate the basement membrane, migrate and invade the surrounding tissues (c). Cells intravasate into blood vessels, travel in the circulation to distant sites. They then extravasate through the vessel wall (d) and migrate (e) to nearby tissue, where they may form distant metastases (f).

The tools of cell migration are the cell adhesion molecules (CAMs) (Hood & Cheresh 2002) and protease enzymes. Cell adhesion molecules such as cadherins and integrins play a role in the interactions of similar and different cells together. Cell adhesion molecules can also induce gene expression in the nucleus by binding with transcription factors. Integrins can interact with actin binding proteins and specific kinases such as focal adhesion kinase (FAK) (Jockusch et al. 1995). FAK mediates cell motility through Src (Cary et
al. 1996) and activation of the RAS pathway (Figure 1.2) (Schlaepfer et al. 1994).

Further, integrins can promote the movement of metastatic cells by allowing the cell to adhere to different ECM components. Cell migration starts with cell polarization in the direction of movement and the formation of a protrusion. Integrins serve as points of contact with the ECM and stabilise the protrusion via connections to actin filaments. At the leading edge adhesion of the protrusion to the substratum allows migration through enhancement of focal adhesion formation (Gawecka et al. 2010; Wozniak et al. 2005). Finally, the detachment of the cell from the previous attachment happens through contraction of the cell from the edge toward the nucleus and the adhesion receptor released from the cytoskeleton. This detachment is associated with the release of cell vesicles and fragments (Lock et al. 2008; Sahai 2007).

The last factor that has a role in cell migration is the protease. Proteases degrade a path through the ECM and stroma. The cancer cells in a tumour can synthesise matrix metalloproteinases (MMPs); these MMPs also have an association with integrins (Brooks et al. 1996; Jin et al. 2011), since integrins induce the surrounding stromal cells to produce MMPs (Brooks et al. 1996). Different integrin signalling pathways control MMP expression, thereby controlling tumour cell invasion (Kubota et al. 1997; Westermarck & Kahari 1999). MMPs are involved in integrin processing and integrins can activate MMPs in the cell. MMP activity can lead to proteolytic cleavage of the
extracellular domain of E-cadherin, which can lead to disruption of the ability of E-cadherin to control cellular interactions (David & Rajasekaran 2012).

Migrating tumour cells will intravasate into the blood and lymphatic vessels. During this process, tumour cells attach to the stromal face of the blood vessel and degrade its basement membrane, passing between the endothelial cells into the bloodstream. The tumour cells are then transported with platelets in the direction of blood flow (Felding-Habermann et al. 2001; Weis & Cheresh 2011). The role of integrins in this step will be discussed in section 1.2.4.1.1.

When a tumour cell reaches the metastatic site, it will escape from the blood or lymph vessel (extravasation) by attaching to endothelial cells lining the vessel and passing through them into the surrounding stroma. In this step, another type of cell adhesion molecule can also play a role. Selectins, which are expressed on endothelial cells, also help the attachment of cancer cells to the endothelium (Barthel et al. 2013).

The last stage of metastasis is the colonisation of the tumour at a distant site. With the help of angiogenesis (formation of new blood vessels), the tumour can grow (Weis & Cheresh 2011). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induce and regulate angiogenesis (Hanahan & Weinberg 2011; Weis & Cheresh 2011).
Figure 1.2 Role of integrins in cancer development
1.2 The Integrins

1.2.1 Introduction

Adhesion receptor molecules on the cell surface became attractive targets for cancer therapy due to their important role in the control of tumour cell dissemination (Okegawa et al. 2004; Simmons 2005). Many types of adhesion cellular receptors (Shimaoka et al. 2002) are recognised including cadherins, selectins, immunoglobulins and integrins (Mousa 2002). The integrins are transmembrane glycoprotein adhesive receptors found on the cell surface; they bind to the extracellular matrix components at the outer membrane surface and interact with the cytoskeletal components at the inner membrane surface. Integrins induce signals (Dedhar & Hannigan 1996; Huveneers et al. 2007; Qin et al. 2004; Stupack 2005; Stupack & Cheresh 2002) through interaction with the extracellular matrix proteins (Brakebusch et al. 2002) and growth factor receptors (Hynes 2002); these are important in cell migration (Felding-Habermann 2003), cell cycle regulation (Fahraeus & Lane 1999), gene expression and apoptosis (Jinka et al. 2012).

1.2.2 Structure of integrins

The integrins are heterodimers consisting of α and β chains that are non covalently associated (Hynes 2002). The two chains each have a large extracellular portion comprising multiple domains, a single transmembrane domain and a short cytoplasmic tail (Figure 1.3) (Shimaoka et al. 2002; Srichai 2010; Ulmer 2010).
The N-terminus of the α subunit consists of seven segments folded into a single compact domain, forming a structure called the β-propeller. The α subunit may also contain another domain that is inserted between blades 2 and 3 of the β propeller; this is known as the I domain, or von Willebrand factor type A domain (Shimaoka et al. 2002). The I-domain is the key ligand-binding domain in those integrins where it is present. The β subunit has a highly conserved N-terminus region, which is similar to the I domain and therefore named the β I-like domain; this domain is responsible for ligand binding in integrins that do not contain an I domain (Figure 1.3) (Humphries 2000; Humphries 2004; Humphries et al. 2004; Takada et al. 2007; Takagi 2007).

The N-terminus of the integrin has a divalent cation binding site known as MIDAS (Metal-Ion-Dependent Adhesion Site) located in each I and I-like domain. Divalent cations bind here, as well as at other sites such as the ligand induced metal binding site (LIMBS) and ADMIDAS, and serve a central function in ligand recognition by the I domain and I-like domain (Humphries 2002; Lee et al. 1995; Shimaoka & Springer 2003).

The C-terminus of the α subunit includes three β sandwich domains as a thigh, and two calf regions (calf 1, calf 2). In the β subunit, the C-terminus is composed of the PSI (Plexin-Semaphorin-Integrin) domain and four epidermal growth factor domains (EGF1, EGF2, EGF3 and EGF4). The region where the integrin is bent in the resting state is termed the knee region. In the β subunit, it forms a junction between the hybrid domain and
two EGF and PSI, while in the α subunit, it is found between the thigh and the two calf regions (Figure 1.3). The terminal end of the C-terminus of both subunits spans the plasma membrane to interact with the intracellular cytoskeleton. During inside-out signalling (the regulation of integrin function from within the cell) (Hynes & Lander 1992), the integrin undergoes a structural change from the bent, closed or inactive conformation (Kalli et al. 2011), which has a low affinity for ligand binding, to an extended, open or active form that has a high affinity for ligand binding (Chen et al. 2011; Hynes 2002). After activation, the signals are transmitted to the extracellular domains of the integrin, which then alters the conformational of the ligand binding site and hence the affinity for the ligand (Figure 1.3) (Hynes 2002; Kim et al. 2011).
Figure 1.3 The structure of αIIbβ3 integrin in its active and inactive state
The resting inactive integrin (left), has a closed bent structure. The active form (right) is open. The N-terminus includes the β-propeller and βA-domain in αIIb and β3 subunits respectively. It has an activation-dependent ligand-binding domain (indicated by the red arrow) for ECM proteins, macromolecules or receptors on the surface of opposing cells. The C terminus includes the thigh domain and two calf regions in αIIb and the hybrid domain, PSI domain and β-tail domain in β3 region. The tails of both subunits span the plasma membrane to receive signals from inside the cell (Shattil et al. 2010).
1.2.3 Types of integrins

Humans have 24 integrins made up of a combination of 18 α and 8 β subunits. As shown in Figure 1.4, the different types of integrins can be classified according to specificity of ligand binding, and to the presence or absence of the I domain. Each integrin has a different function (Table 1.2), although a number of integrins can bind the same ECM proteins, providing redundancy in cell signalling and adhesion pathways.

**Figure 1.4 The integrin family**

Different kinds of integrin subunits can bind together to form an integrin sub family. There are 5 subfamilies: Red indicates the RGD receptors, yellow the laminin the receptors, blue the collagen receptors and green the leukocyte receptors. The black frame indicates an I-domain integrin.
<table>
<thead>
<tr>
<th>Integrin</th>
<th>Function</th>
<th>Ligands</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1\beta_1$</td>
<td>Regulates cell adhesion, migration and controls matrix accumulation. Regulates epidermal growth factor receptors.</td>
<td>Laminin E1 fragment, Collagen</td>
<td>(Bodary &amp; McLean 1990; Henry et al. 2001; Rossino et al. 1991; Tomaselli et al. 1993)</td>
</tr>
<tr>
<td>$\alpha_2\beta_1$</td>
<td>Mediates the adhesion of platelets to collagen. Used by fibroblasts for tissue collagen remodelling during wound repair. Involved in tumour progression.</td>
<td>Collagen I-IV, Laminin, Echovirus-1 Thrombospondin</td>
<td>(Klein et al. 1991; Schiro et al. 1991; Staatz et al. 1991)</td>
</tr>
<tr>
<td>$\alpha_4\beta_1$</td>
<td>Maintains the structural integrity of the placenta and heart during embryogenesis. Mediates adhesion of inflamed vascular endothelial cells to T cells.</td>
<td>VCAM-1, Fibronectin, Thrombospondin, Osteopontin, Mad CAM-1</td>
<td>(Hsia et al. 2005; Moyano et al. 1997; Springer et al. 1994; Wu et al. 1995)</td>
</tr>
<tr>
<td>α₄β₇</td>
<td>Mediates lymphocyte adhesion to Mad CAM-1 on endothelial specific Peyer’s patches in the gut.</td>
<td>Mad CAM-1, VCAM-1, Fibronectin, Osteopontin</td>
<td>(Erle et al. 1994)</td>
</tr>
<tr>
<td>α₆β₁</td>
<td>Platelet interaction with sub endothelial basement membrane laminin exposed on blood vessel damage. Activation of T-cells. Angiogenesis</td>
<td>Laminin</td>
<td>(Leu et al. 2003; Sonnenberg et al. 1990)</td>
</tr>
<tr>
<td>α₆β₄</td>
<td>Adhesion to basement membranes and matrix protein</td>
<td>Laminin 5</td>
<td>Sonnenberg et al. 1990</td>
</tr>
<tr>
<td>α₇β₁</td>
<td>Involved in muscle development, linkage between muscle fibre and extracellular matrix.</td>
<td>Laminin 1,2,4, E8 region</td>
<td>(Song et al. 1992)</td>
</tr>
<tr>
<td>α₈β₁</td>
<td>Kidney morphogenesis. Expressed in neural tissues</td>
<td>Osteopontin, Fibronectin, Vitronectin, Tenascin</td>
<td>(Bossy et al. 1991; Muller et al. 1995)</td>
</tr>
<tr>
<td>α₉β₁</td>
<td>Involved in osteoclast formation and function. Cell migration and adhesion via direct binding of VEGF.</td>
<td>Osteopontin, Tenascin-c VCAM-1 VEGF A,C,D</td>
<td>(Desloges et al. 1998; Oommen et al. 2010; Rao et al. 2006)</td>
</tr>
<tr>
<td>α₁₀β₁</td>
<td>Expressed on chondrocytes, deficiency leads to chondrodysplasia.</td>
<td>Collagen II Laminin</td>
<td>(Bengtsson et al. 2005)</td>
</tr>
<tr>
<td>$\alpha_1\beta_1$</td>
<td>Required on periodontal ligament fibroblasts for cell migration and collagen reorganisation to help generate the forces needed for axial tooth movement.</td>
<td>Collagen I</td>
<td>(Carracedo et al.; Popova et al. 2007)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>$\alpha_E\beta_7$</td>
<td>Intestinal targeting of lymphocyte subpopulations.</td>
<td>E-cadherin</td>
<td>(Cepek et al. 1994)</td>
</tr>
<tr>
<td>$\alpha_\nu\beta_1$</td>
<td>Cell matrix and intracellular interaction.</td>
<td>Fibronectin, Osteopontin LAP-TGF-β</td>
<td>(Brockbank et al. 2005; Hu et al. 1995; Marshall et al. 1995; Vogel et al. 1990)</td>
</tr>
<tr>
<td>$\alpha_\nu\beta_5$</td>
<td>Vitronectin endocytosis and angiogenesis.</td>
<td>Vitronectin, Osteopontin, BSP</td>
<td>(Gladson et al. 1997)</td>
</tr>
<tr>
<td>$\alpha_\nu\beta_6$</td>
<td>Response of epithelium to inflammatory stimuli and injury such as in asthma. Regulation of epithelial proliferation in vitro and wound healing. Activation of LAP-TGF-β</td>
<td>Fibronectin, Osteopontin, LAP-TGF-β</td>
<td>(Breuss et al. 1995; Erikson et al. 2009; Munger et al. 1999)</td>
</tr>
<tr>
<td>$\alpha_\nu\beta_8$</td>
<td>Neural function. Receptor for foot and mouth disease virus.</td>
<td>LAP-TGF-β, Vitronectin</td>
<td>(Chernousov &amp; Carey 2003; Jackson et al. 2004; Nishimura et al. 1994; Pozzi &amp; Zent 2011)</td>
</tr>
<tr>
<td>αLβ2</td>
<td>Involved in immune function, cell-cell interactions during lymphocyte transit through endothelium to sites of inflammation and during recirculation through the lymph node.</td>
<td>ICAM 1,2,3</td>
<td>(Springer 1990; Springer 1990)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>αMβ2</td>
<td>Has a role in inflammation due to its expression on many leukocytes involved in immune system. Mediates adhesion to and transmigration through vascular endothelium of monocytes and granulocytes. Participates in leucocyte aggregation. Involved in chemotaxis, apoptosis and other phagocytic activities.</td>
<td>Complement fragment iC3b, ICAM 1,2,4, Fibrinogen, Factor X</td>
<td>(Springer 1990)</td>
</tr>
<tr>
<td>αXβ2</td>
<td>Cytotoxic T cell killing and monocyte and granulocyte adhesion to endothelium.</td>
<td>Complement fragment iC3b, Fibrinogen, ICAM-1, Collagen</td>
<td>(Keizer et al. 1987; Vorup-Jensen et al. 2003)</td>
</tr>
<tr>
<td>αDβ2</td>
<td>Has role in phagocytosis by macrophages.</td>
<td>ICAM-3, VCAM-1</td>
<td>(Grayson et al. 1998)</td>
</tr>
<tr>
<td>αIIIβ3</td>
<td>Essential for normal platelet adhesion to damaged vascular endothelium and normal platelet aggregation.</td>
<td>Fibrinogen, Fibronectin, Vitronectin, Thrombospondin, VWF</td>
<td>(Buensusceto et al. 2005; Muller et al. 1993; Podolnikova et al. 2003)</td>
</tr>
<tr>
<td>Integra</td>
<td>Function</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>------------</td>
<td></td>
</tr>
</tbody>
</table>

### 1.2.4 Function of integrins

As can be seen in Table 1.2, the integrins have multiple functions, including the adhesion between the cell and the surrounding tissue (Hynes 1999; Niland & Eble 2012). The integrins help the membrane to adhere to the extracellular sites and create an attractive force that adheres the cells to their surroundings (Schwartz 2010). Integrons are important in the regulation of cell shape, migration (Schneider et al. 2011) and cell cycle regulation. They are also involved in signalling pathways (Scales & Parsons 2011) that regulate cell growth, proliferation, survival, division, differentiation and apoptosis (Guo & Giancotti 2004).
The regulation of integrin function can occur internally within the cell through signalling complexes pathways (Hynes 2002), where the activation of integrins can result from binding to certain ligands or by inside-out-signalling (Figure 1.5). Cell-matrix contact induces a wide range of signalling pathways mediated by adaptor proteins and enzymes (Takada, Ye & Simon 2007). For example, integrins can be activated intracellularly by signals from G-protein coupled receptors that lead to phosphorylation of the cytoplasmic domain of the β subunit. The cytoskeletal adaptor proteins such as talin, skelemin, filamin, α-actinin, myosin and tensin can regulate integrin affinity, changing the integrin into its active conformation. The adaptor protein head binds to the β tail and induces conformational changes that are accompanied by dissociation of α and β subunits and increased affinity for the ligand binding, so that inside-out signals regulate the cell adhesion. When the integrins bind to their ligands in the ECM, they cluster to form a focal adhesion, which affects the organisation of the cytoskeleton underlying the plasma membrane (Humphries 2000).

In the extracellular region, ligand binding transfers a signal to the interior of the cell (outside-in signalling) and can induce conformational changes, including separation of the α and β legs (Humphries 2000; Humphries et al. 2003; Legate et al. 2009). This separation allows the interaction of the cytoplasmic tail with intracellular signalling molecules such as FAK/c-Src (Huveneers et al. 2007), the small GTPases Ras & Rho and adaptors like cas/crk and paxillin (Guo & Giancotti 2004; Takada, Ye & Simon 2007;
Yamada et al. 2003). These signals then regulate cell function (Figure 1.6) (Millard et al. 2011).

This project will concentrate on the study of the $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins ($\beta_3$ subfamily). The subsequent sections will describe their role in cancer development, survival, metastasis and invasion.

**Figure 1.5** inside-out-signalling pathways for integrins (Askari et al. 2009)

Inside-out signals occur through binding of $\beta$ integrin tail with cellular proteins (B). The inside signals leads to integrin changing from inactive (A and B) to active form, which is able to bind to ECM proteins (C). Binding ECM proteins like fibrinogen, vitronectin, fibronectin, thrombospondin, von Willebrand factor and osteopontin, and interaction with other proteins and receptors induce various outside-in signals (D) which begin signalling cascades controlling cellular processes (E).
**1.2.4.1 Function of $\alpha_V\beta_3$ integrin**

The $\alpha_V\beta_3$ integrin can recognise several ligands such as vitronectin, fibronectin, fibrinogen, proteolysed collagen and other proteins. It does this by recognising the tripeptide sequence Arginine-Glycine-Aspartic acid (RGD) which is common to the above ligands. The $\alpha_V\beta_3$ integrin can also recognise WGD, and isoDGR (iso-Aspartic acid-Glycine-Arginine) (Spitaleri et al. 2008) and contains another non-RGD binding site for MMP-2 and MMP-9 (Brooks et al. 1996).

$\alpha_V\beta_3$ is widely expressed on the endothelial cells (Takada et al. 2007), epithelial cells, smooth muscle cells and monocytes. It is expressed on activated endothelial cells and new vessels but it is absent in resting endothelial cells and normal organ systems (Rusnati et al. 1997). It regulates
both angiogenesis in endothelial cells and cell adhesion to the extracellular matrix (Soldi et al. 1999). It also has a role in osteoclast activity where it mediates osteoclast adhesion and regulates the cytoskeletal organisation required for cell migration (Duong et al. 2000; Nakamura et al. 1999; Srivatsa et al. 1997; Tolar et al. 2004).

1.2.4.1.1 Role of αvβ3 integrin in angiogenesis

Angiogenesis is the physiological process involving the growth of new blood vessels. Angiogenic blood vessels express αvβ3 at elevated level, whereas αvβ3 expression is low in quiescent endothelial cells (Leu et al. 2002; Weis & Cheresh 2011). Interaction between αvβ3 and the ECM can control angiogenesis through pro-angiogenic and anti-angiogenic molecules that originate from ECM (Robinson & Hodivala-Dilke 2011). The pro-angiogenic function of αvβ3 is promoted through its co-operation with factors that enhance angiogenesis such as VEGFR-2, vitronectin, fibronectin, Del1, Angptl3, CYR61, bone sialoprotein (BSP) and thrombin. In contrast, the anti-angiogenic effect occurs through its interaction with thrombospondin, angiotatin and tumstatin (Hodivala-Dilke et al. 2003).

For example, adhesion of endothelial cells (expressing αvβ3) to vitronectin leads to tyrosine phosphorylation of VEGFR-2, indicating an involvement of αvβ3 in VEGFR-2 activation (Soldi et al. 1999). The αvβ3 integrin binds directly to VEGFR-2, activating VEGFR-2 and leading to β3 subunit phosphorylation. In turn, the phosphorylated αvβ3 will phosphorylate VEGFR-2 in the presence of VEGF, indicating that the association of αvβ3 integrin
with VEGFR-2 promotes the activation of each receptor. Experiments using mutant integrin have shown that the cytoplasmic tyrosine residues of β3 integrin (Y747 and Y759) are necessary for successful functional communication between β3 integrin and VEGFR-2 (Figure 1.7) (Robinson & Hodivala-Dilke 2011; Robinson et al. 2004). Studies using the same model found that a mutant β3 (model had mutant β3 with two tyrosine residue Tyr747 and Tyr759 which were unable to induce signalling) integrin is expressed on the cell surface but it is defective in signalling (Mahabeleshwar et al. 2006). However, a study using a β3 knockout mouse model showed an induction of angiogenesis and tumour growth, indicating role for the αv integrin as well as in angiogenesis such as αvβ3 integrin (Reynolds et al. 2002).

The ability of αvβ3 integrin antagonists to block in vivo angiogenesis supports a role for αvβ3 integrin in angiogenesis. LM609 anti αvβ3 integrin, a specific antibody that targets αv β3 integrin, has been used but LM609 cannot react with mouse αvβ3 integrin. Thus, the effect of LM609 on angiogenesis was studied by Brook et al., who used human breast carcinoma cells injected into chimeric human/mouse model (this is a mouse model in which a piece of skin is removed and replaced with a sutured piece of human skin). When MCF-7PB cells were injected into the human skin, LM609 was able to block tumour growth and angiogenesis in the human skin microenvironment (Brooks et al. 1995).
1.2.4.2 Function of αIIbβ3 integrin

The αIIbβ3 integrin (also known as GPIIb/IIIa) is expressed on the surface of platelets, megakaryocytes, monocytes, granulocytes and lymphocytes. It is inactive in resting platelets but is rapidly activated by thrombogenic stimuli and promotes binding to fibrinogen (Huang et al. 1993; O'Toole et al. 1990), von Willebrand factor and fibronectin. This integrin is necessary for platelet aggregation (Bennett 2005).

Platelet aggregation occurs through the activation of platelets by physiological molecules such as thrombin (Hodivala-Dilke et al. 1999), collagen or ADP (Bhavaraju et al. 2011; Gay & Felding-Habermann 2011). The activation of αIIbβ3 is mediated by a G-protein that acts through protein kinase C (PKC) to cause a conformational change. Active αIIbβ3 binds to fibrinogen, a multivalent ligand, through RGD and KQAGDV recognition motifs, resulting in clustering of the integrin-ligand complex and formation of adhesive patches on the surface of platelets, thereby allowing platelet cross-
linking and thrombus formation (Figure 1.8) (Geiger et al. 2009; Giuliano et al. 2003; Quinn et al. 2003; Rocco et al. 1993; Ruoslahti 1996; Springer et al. 2008).

![Diagram of platelet activation and thrombus formation](image)

**Figure 1.8 Active α<sub>IIb</sub>β<sub>3</sub> integrin in platelet activation and thrombus formation (Millard, Odde & Neamati 2011)**

### 1.2.5 Role of α<sub>V</sub>β<sub>3</sub> and α<sub>IIb</sub>β<sub>3</sub> integrins in cancer development

#### 1.2.5.1 Role of α<sub>V</sub>β<sub>3</sub> integrin in cancer development

Alteration in the pattern of the α<sub>V</sub>β<sub>3</sub> integrin receptor is observed in tumour cells (Arosio et al. 2009; Gasparini et al. 1998; Gladson & Cheresh 1991; Landen et al. 2008; Max et al. 1997). The expression of α<sub>V</sub>β<sub>3</sub> integrin has been detected in many cancer cell lines such as breast (Verbisck et al. 2009), ovarian (Landen et al. 2008) cervical carcinoma (Shen et al. 2006), glioblastoma (Gladson & Cheresh 1991), melanoma (Petitclerc et al. 1999; Seftor et al. 1992), prostate carcinoma (Chatterjee et al. 2001), kidney cancer cells (Arosio et al. 2009) and lung cancer cells (Tsai et al. 2008). The β<sub>3</sub> and α<sub>V</sub>β<sub>3</sub> integrin receptor are also detected in clinical samples of melanoma.
(Albelda et al. 1990; Neto et al. 2007) and ovarian carcinoma (Partheen et al. 2009). Breast cancer (Gasprini et al. 1998) and colon cancer show expression of αvβ3 integrin (Reinmuth et al. 2003).

The αvβ3 integrin plays an important role in a number of cellular process that lead to cancer development including malignant transformation, tumour growth and progression, invasion, metastasis (Byzova et al. 2000), apoptosis (Desgrozoller & Cheresh 2010; Jin & Varner 2004; Li et al. 2001; Lu et al. 2008; Mizejewski 1999; Mousa 2002) cell surface localisation of metalloproteinases and angiogenesis (Jin & Varner 2004; Varner & Cheresh 1996). Consequently, it plays a key role in the regulation of tumour cell survival and apoptosis. Signalling by αvβ3 integrin regulates both the expression and activity of bcl-2 and suppresses the expression of the pro-apoptotic protein Bax by down regulation of p53. αvβ3 increases the bcl-2: BAX ratio, which leads to increased tumour cell survival by preventing the mitochondrial apoptotic pathway that involves the release of cytochrome c and caspase activation (Martin & Vuori 2004; Uhm et al. 1999). Crosstalk between αvβ3 and fibroblast growth factor receptor (FGFR) also prevents apoptosis through the mitogen-activated protein kinase (MAPK) pathway. αvβ3 also enhances tumour progression by activation of the tyrosine kinase SRC, which leads to activation of the FAK-independent survival pathway and promotion of tumour growth (Desgrozoller & Cheresh 2010).

Increased expression of αvβ3 allows tumour cells to bind to extracellular matrix proteins such as fibronectin, fibrinogen, vitronectin, von Willebrand
factor and osteopontin that are present in the tumour microenvironment. These adhesive interactions provide survival signals for invading endothelial cells (Desgrosellies & Cheresh 2010). MMP-2 binds to αvβ3 on the cell surface and this interaction leads to activation of MMP-2 and localizes its proteolytic activity to the invasive front of the cell (Varner & Cheresh 1996).

Breast, prostate, lung and thyroid tumour cell lines became more invasive when αvβ3 forms a link with bone sialoprotein and MMP-2 (Karadag et al. 2004). The αvβ3-ERK1/2 pathway is essential for connective tissue growth factor (CTGF) induced ERK1/2 activation and cellular migration in the MCF-7 breast carcinoma cell line (Chen et al. 2007).

1.2.5.1.1 Role of αvβ3 integrin in breast cancer

αvβ3 is expressed in human breast cancer tissue (Zhao et al. 2007), as well as in breast cancer cell lines such as MDA-MB-231 (Cai et al. 2006; Liu et al. 2009; Manzoni et al. 2009; Takayama et al. 2005). αvβ3 expression correlates with the metastasis of MDA-MB-435 human breast cancer cell line, which interacts with platelets to promote arrest of tumour cells during blood flow and leads to haematogenous metastasis (Felding-Habermann 2003). Expression of αvβ3 has been shown on cancer cells in the active conformation and it activates platelets, leading to increased metastatic activity to the lung. The activated state of αvβ3 in breast cancer cells may be responsible for the tumour cell-platelet interaction that leads to arrest MDA-MB-435 tumour cell during blood flow, resulting in increased binding of a ligand mimetic antibody and increased cell migration toward vitronectin. The
metastasis of breast cancer cells was seen to be strongly inhibited by LM609 anti-αvβ3 antibody (Felding-Habermann et al. 2001).

Sloan et al. detected expression of αvβ3 on the cell surface of the 66c14 mammary cell line, which led to metastasis of breast tumour from the mammary gland to the bone. The increase in metastasis resulted from the ability of αvβ3 to adhere to vitronectin and invade the basement membrane and the surrounding stroma, which depended on protease and MMP activity. αvβ3 expression can induce haptotactic migration towards osteopontin (Sloan et al. 2006).

Other studies have shown that αvβ3 is important for the development of bone metastasis; expression of β3 was detected in human breast carcinoma with bone metastasis and was stronger in tissue with bone metastasis than in primary tissue. Tumour cells expressing αvβ3 integrin stimulate bone destruction and osteoclast-mediated bone resorption once they are located in the bone marrow (Zhao et al. 2007). Transfection of breast carcinoma cells MDA-MB-231 with αvβ3 integrin or using cells from primary site of bone metastasis led to increased number and area of osteolytic bone metastases in vivo, due to expression of active αvβ3 integrin on the cell surface. Inhibition of αvβ3 function by LM609 inhibited tumour cell invasion and adhesion to cortical bone (Pecheur et al. 2002).
1.2.5.1.2 Role of $\alpha_V\beta_3$ integrin in prostate cancer

Prostate cancer cells express both $\alpha_V\beta_3$ and $\alpha_V$ (Cooper et al. 2002; van der Horst et al. 2011; Wang et al. 2005). The expression of $\alpha_V\beta_3$ may differ between different prostate carcinoma cell lines and between labs (Saxena et al. 2012; Zheng et al. 1999). A recent study that examined the expression of $\alpha_V$ and $\beta_3$ integrin under different conditions reported the expression of $\alpha_V$ and $\beta_3$ in the prostate carcinoma cell line, PC-3 and in human xenograft mouse tissue. The expression differed in the *in vitro* and *in vivo* environments (Taylor et al. 2011).

The expression of $\alpha_V\beta_3$ was correlated with the ability for adherence and migration of the cells on vitronectin. The PC-3 cells expressing $\alpha_V$ and $\alpha_V\beta_3$ integrin migrated on vitronectin and fibronectin whereas LNCaP cells, which do not express $\alpha_V\beta_3$, migrated only on fibronectin. Transfection of LNCaP cells with the $\beta_3$ subunit resulted in adherence to vitronectin, which was inhibited by LM609 (Zheng et al. 1999). In contrast, an immunocytochemistry study using LM609 found higher expression of $\alpha_V\beta_3$ in LNCaP than PC-3 cells. After expression was characterised, the cells were treated with cRGDfV (cyclic peptide: Arg-Gly-Asp-phe-Val) which was more effective in LNCaP and had no effect on PC-3 cells. Blocking of $\alpha_V\beta_3$ integrin on LNCaP membrane by cRGDfV treatment caused FAK cleavage, and reduced Akt/PKB phosphorylation, which led to activation of caspase-9 and caspase-3, indicating that cRGDfV induced programmed cell death in the LNCaP cells (Chatterjee et al. 2001).
αvβ3 plays an important role in migration of prostate cancer to the bone; expression of αvβ3 in PC-3 promoted cancer migration to the bone matrix (McCabe et al. 2007). Barthel et al. found that prostate cancer extravasation into bone marrow endothelium under physiological blood flow conditions depended on the presence of E-selectin, αvβ3 integrin, β1, and Rac1/Rap1 GTPase activity (Barthel et al. 2012).

1.2.5.1.3 Role of αvβ3 integrin in melanoma

Melanoma cells and tissue express αvβ3, with the highest expression in metastatic melanoma tissue (Hofmann et al. 2000). Using frozen melanoma sections, Albelda et al. found that the expression of β3 integrin was more common in invasive melanoma than in primary melanoma: vertical growth phase (VGP) showed immunoreactivity with β3 integrin while radial growth phase (RGP) failed to show any reactivity. The expression of β3 integrin was detected only in metastatic melanoma cells and not in benign melanocytes. These results suggested that the expression of β3 integrin could be important for the development of tumour invasiveness and could be useful as a marker for melanoma cells entering the aggressive phase of the malignancy process (Albelda et al. 1990).

Li et al. reported that high expression of αvβ3 integrin in vitro and in vivo can increase metastasis of melanoma cells by mediating adhesion to vitronectin (Li et al. 2001). The adhesion of αvβ3-expressing A375M melanoma cells to vitronectin was inhibited by LM609, whereas the same antibody had no effect on melanoma cell adhesion to fibronectin and laminin. However, an increase
in melanoma cells invasion through the basement membrane was not upon exposure to either anti \( \alpha_v \beta_3 \) antibody or vitronectin. Exposure of melanoma cells to vitronectin was associated with an increased production of type IV collagenase as a result of \( \alpha_v \beta_3 \)-initiated signalling indicated that \( \alpha_v \beta_3 \) integrin had a role in melanoma cell invasion through modulation of protease levels (Seftor et al. 1992).

Denatured collagen colocalised with \( \alpha_v \beta_3 \) integrin in melanoma cells and attachment of M21 cells to vitronectin and denatured collagen led to an increase in the bcl-2: BAX ratio. The \( \alpha_v \beta_3 \) antagonist LM609 could inhibit M21 cell growth in vivo. This antagonism inhibits tumour growth by inducing apoptosis through the regulation of bcl-2 and BAX expression, indicating that the role of \( \alpha_v \beta_3 \) integrin in tumour survival involves interaction with the ECM (Petitclerc et al. 1999).

Voura et al. localised \( \alpha_v \beta_3 \) integrin expression on the cell surface of WM293 melanoma cells using immunofluorescence. The expression was localised in cell-to-cell contact regions when the WM293 cells were co-cultured with HUVECs and was diffuse before the cells started extravasation, increasing to be strongest during extravasation. The study reported a 40% inhibition of melanoma cell transmigration in response to the linear RGD peptide at concentration 100 µM, whereas a cyclic RGD peptide (c-RGDfV) showed 50% inhibition of melanoma cell transmigration at concentration 5 µM and had its greatest effect at 5 hours co-culture. Treatment with LM609 at concentration 40 µg/ml led to 40-50% inhibition, indicating that this
melanoma extravasation is at least partially mediated by $\alpha_\text{v}\beta_3$ integrin (Voura et al. 2001).

1.2.5.1.4 $\alpha_\text{v}\beta_3$ integrin in glioma

$\alpha_\text{v}\beta_3$ is overexpressed in glioblastoma tumour cells such as U-87MG (Cheng et al. 2005), U-373MG and U-251MG (Mattern et al. 2005) and in endothelial cells of tumour-associated proliferating microvessels. $\alpha_\text{v}\beta_3$ activation was related to tumour cell invasion through an enhancement of angiogenesis that prevents development of hypoxia in brain lesions (Lorger et al. 2009). Glioblastoma cell line U-251MG also showed expression of $\alpha_\text{v}\beta_3$, which was involved in cell adhesion to vitronectin (Gladson & Cheresh 1991).

$\alpha_\text{v}\beta_3$ is expressed on clinical glioma tumour samples and its expression correlates with histological grade (Bello et al. 2001). Different glioblastoma samples showed heterogenous expression of $\alpha_\text{v}\beta_3$ integrin and most of the expression of $\alpha_\text{v}\beta_3$ was derived from glial tumour cells (Schnell et al. 2008). A higher expression of $\alpha_\text{v}\beta_3$ was seen in grade III tumours than in grade I&II tumours for glioblastoma.

1.2.5.2 Role of $\alpha_\text{IIb}\beta_3$ integrin in cancer development

1.2.5.2.1 Role of $\alpha_\text{IIb}\beta_3$ integrin in melanoma

The expression of $\alpha_\text{IIb}\beta_3$ integrin has been detected in tumour cells or tissues (Chen et al. 1997; Trikha et al. 1998; Trikha et al. 1997). $\alpha_\text{IIb}$ expression was first seen in malignant melanoma by Puerschel et al. by immunohistochemistry using the anti-$\alpha_\text{IIb}$ monoclonal antibody sz22 (Santa
Cruz). In contrast, none of the tested tissue samples from patients with non-
metastatic melanoma showed $\alpha_{\text{IIb}}$ immunoreactivity (Puerschel et al. 1996).

Chen et al. detected the expression of $\alpha_{\text{IIb}}\beta_3$ in melanoma cell line WM35 by
reverse transcription-polymerase chain reaction (RT-PCR) (Chen et al.
1997). Trikha et al. showed melanoma cells and tissue expressed high
affinity $\alpha_{\text{IIb}}\beta_3$ (Trikha et al. 1997).

The expression of $\alpha_{\text{IIb}}\beta_3$ was low in thin melanoma tissue (early stage
tumours) but increased in thick melanoma tissue (later stage disease),
whereas the expression of $\alpha_v\beta_3$ integrin was intense in thin melanoma. The
importance of $\alpha_{\text{IIb}}\beta_3$ integrin in human melanoma cell invasion, spreading and
migration was demonstrated using isogenic cell lines. The $\alpha_{\text{IIb}}\beta_3$ expressing
cells adhered to fibrinogen but could not adhere to vitronectin, whereas $\alpha_{\text{IIb}}\beta_3$
negative cells (expressing only $\alpha_v\beta_3$ integrin) adhered to vitronectin but
could not adhere to fibrinogen, suggesting that expression of $\alpha_{\text{IIb}}\beta_3$ integrin
suppresses the function of $\alpha_v\beta_3$ integrin. No difference was observed in the in
vitro growth between $\alpha_{\text{IIb}}\beta_3$ expressing cells and mock-transfected cells, but
$\alpha_{\text{IIb}}\beta_3$ expressing cells grew larger tumours in vivo due to a reduction in the in
vivo rate of apoptosis (Trikha et al. 2002). Dome et al. found that expression
of $\alpha_{\text{IIb}}\beta_3$ integrin in the vertical growth phase upregulate bFGF expression,
thereby providing an angiogenic growth factor for melanoma cells and
promoting tumour growth (Dome et al. 2005).
1.2.5.2.2 Role of α_{IIb}\β_{3} integrin in prostate cancer

The expression of α_{IIb} has been reported in human prostate tumour tissue (Trikha et al. 1996), as well as in cell lines such as PC-3 and DU-145 (Chen et al. 1997).

Trikha et al. detected the expression of α_{IIb} DNA/RNA in prostate carcinoma tissue using an in situ hybridisation (ISH) technique using an antisense riboprobe to α_{IIb} mRNA. Prostate cancer tissues produced both α_{IIb} and β_{3} integrin proteins. The presence of intact α_{IIb}β_{3} integrin was confirmed by flow cytometry, dot blotting and cell adhesion assays. α_{IIb}β_{3} was stored intracellularly and translocated to the cell surface in response to PKC activation. Monoclonal antibodies directed to α_{IIb}β_{3} integrin blocked DU-145 cell invasion (Trikha et al. 1996).

Expression of α_{IIb}β_{3} differed between PC-3 and DU-145 cell lines. Although both expressed α_{IIb}β_{3}, the expression was intracellular in PC-3 whereas α_{IIb}β_{3} was localised on the cell surface of DU-145 cells and was involved in the focal adhesion sites. DU-145 cells were also more invasive than PC-3. Mice injected with PC-3 cells developed intraprostatic tumours whereas mice injected with DU-145 developed tumours that infiltrated the surrounding tissue and metastasised to the lymph nodes. The function blocking antibodies, 10E5 and PAC-1 directed to α_{IIb}β_{3}, inhibited lung colonization in a tail vein injection model. These antibodies can bind to platelet α_{IIb}β_{3} to inhibit platelet function and therefore platelet tumour interaction. Antibodies can also block the interaction of DU-145 cells with host tissues by binding to the α_{IIb}β_{3}
integrin expressed by tumour cells. Consequently, combined blockage of
\( \alpha_{IIb}\beta_3 \) integrins on multiple cell types can lead to a blockade of lung
colonisation by DU-145 cells (Trikha et al. 1998).

1.2.5.3 \( \alpha_v\beta_3 \) and \( \alpha_{IIb}\beta_3 \) integrin antagonism in cancer therapeutics

The role of integrins in cancer development, metastasis and angiogenesis
discussed in the previous sections suggests that the \( \beta_3 \) subfamily may
represent attractive therapeutic targets (Cox et al. 2010; Desgrosellier &
Cheresh 2010; Eble & Haier 2006; Millard, Odde & Neamati 2011; Niu &
Chen 2011; Rust et al. 2002; Shimaoka & Springer 2003). Therapies based
on integrin antagonism that include antibodies, peptides, small molecules
and small interfering RNA (siRNA) are summarised in Figure 1.8 and Table
1.3 (Bisanz et al. 2005; Goodman & Picard 2012; Liu et al. 2008).

1.2.5.3.1 Anti-\( \alpha_v\beta_3 \) antibodies as cancer therapeutics

Abegrin (etaracizumab, previously known as vitaxin or MEDI-522) is an IgG1
humanized monoclonal antibody engineered from the murine monoclonal
antibody LM609. LM609 targets \( \alpha_v\beta_3 \) and has the ability to reduce tumour
growth \textit{in vivo} and \textit{in vitro} by inhibiting the tumour cells from interacting with
underlying extracellular matrix proteins such as fibrinogen. LM609 can block
human angiogenesis and reduce invasion; thus, it may be useful as an anti-
angiogenic approach for treatment of any cancer (Brooks et al. 1995).

Abegrin can inhibit the growth of ovarian cancer \textit{in vitro} and \textit{in vivo} (Landen
et al. 2008), although at high doses it can show impaired anti-tumour activity.
This is possibly because the high serum level might cause aggregation of the antibody, limiting its availability (Mulgrew et al. 2006).

A phase I clinical trial conducted on advanced stage metastatic cancer found Abegrin to have no significant toxicity (McNeel et al. 2005). Abegrin was then used in a phase II clinical trial on stage IV melanoma, either as a single agent or in combination with dacarbazine. However, Abegrin showed no tumour response if used alone and the combination of Abegrin with dacarbazine had only a low percentage of tumour response. Consequently, Abegrin is no longer being investigated (Hersey et al. 2010).

1.2.5.3.2 Integrin binding peptides as cancer therapeutics

In addition to antibodies, peptides have been used as tool compounds in cancer studies (Danhier et al. 2012; Millard, Odde & Neamati 2011; Temming et al. 2005). Bello et al. examined the effects of the RGD peptide IS201 as an inhibitor of \( \alpha_\beta_3 \) integrin \textit{in vitro} and \textit{in vivo} on malignant glioma cells U87-MG, 4373-MG, U118-MG and endothelial cells. The IS201 peptide inhibited glioma growth \textit{in vivo}, and its effects were associated with a decrease in angiogenesis and cell proliferation and an increase in apoptosis (Bello et al. 2003).

Another peptide used by Chatterjee et al. who reported the effects of cyclic cRGDFIV and linear RGD peptides in glioma cells. They found that cRGDFIV blocked cell-to-cell adhesion and inhibited either survival or proliferation in human glioma cells, whereas RGD had no inhibitory effect (Chatterjee et al. 2003).
The same group also examined the effects of cRGDfV on prostate cancer and found that it induced apoptosis in LNCaP prostate carcinoma cells, whereas the linear RGD peptide caused only a partial reduction in the number of prostate cancer cells. However, cRGDfV had a significant cytotoxic effect on prostate cancer cells expressing αVβ3 integrin (Chatterjee et al. 2001).

Another study by Allman et al. showed that cRGDfV inhibited melanoma cell adhesion in vitro and prevented development of tumours in vivo. However, the peptide was unable to induce apoptosis in malignant melanoma that expressed high levels of bcl-2 (Allman et al. 2000). Furthermore, although cRGDfV apparently induced melanoma cell detachment, the peptide did not remain bound to the cell surface (Castel et al. 2001).

At present, the most advanced peptide antagonist undergoing clinical development is Cilengitide which an N-methylated cRGDfV derivative c(RGDf(NMe)V) that shows high affinity for αVβ3 and αVβ5 integrins. For further details see section 1.2.5.3.4.1.

**1.2.5.3.3 Small molecule integrin antagonists as cancer therapeutics**

The small molecule antagonist IH0162 can induce anoikis in vitro through inhibition of αVβ3 expressing M21 melanoma cells binding to vitronectin. It was able to inhibit pulmonary metastasis of M21 cell in vivo. IH0162 activates caspases 3, 8, and 9; both caspase 8 and 9 affect the intrinsic and extrinsic
apoptosis pathways. IH0162 can also decrease bcl-2 and survivin, extending its effects further to the FAK pathway (Zhang et al. 2011).

The antagonism of $\alpha_v\beta_3$ can be exploited as a therapeutic target to prevent skeletal metastasis. MDA-MB-435 breast carcinoma cells were transfected with $\alpha_v\beta_3$ integrin and injected into tail vein of animal to determine the role of $\alpha_v\beta_3$ integrin in development of bone metastasis in vivo. A lower bone volume to tissue volume ratio, indicating higher bone destruction, was observed when cells were injected directly into the tibial bone marrow cavity, and a larger number of $\alpha_v\beta_3$ integrin expressing tumour cells were found residing in the bone marrow; these stimulated osteoclast mediated bone resorption. For example, long-term treatment with the $\alpha_v\beta_3$ integrin antagonist PSK1404 resulted in multiple inhibitory effects on cancer cells, endothelial cells, and osteoclasts and enhanced the anticancer efficiency while short term therapy inhibited tumour cell invasion in vivo (Zhao et al. 2007).

The effect of oral administration of XV454 (a specific $\alpha_{IIb}\beta_3$ antagonist) on platelet aggregation, tumour induced thrombocytopenia and metastasis has been investigated by Amirkhosravi et al. Oral administration of 5 mg/kg XV545 led to complete inhibition of platelet aggregation within ten minutes. Tumour cell induced thrombocytopenia was inhibited significantly by both oral and intravenous administration of XV454 and it had anti-metastatic activity with a reduced number of metastatic lung nodules seen (Amirkhosravi et al. 2003).
Bakewell et al. hypothesised that tumour cells require β3 integrin for metastasis to bone and for induction of bone osteolysis. ML464 (a pro-drug antagonist of αIIbβ3) protected mice from metastasis in vivo but it did not disrupt platelet/tumour interactions in vitro because the pro-drug requires activation in vivo (Bakewell et al. 2003).

Tirofiban, an αIIbβ3 antagonist, can block the interaction of αIIbβ3 integrin with the col15 cell adhesion domain of collagen XVII, thereby blocking the transmigration and invasion of the HSC-3 cell line (squamous cell carcinoma) normally induced by col15. This transmigration is mediated by factors other than αIIb. Since the blockade of α5 and αV reduced migration of HSC-3 in response to col15 by 53%, whereas a 35% reduction in migration was caused by tirofiban (Parikka et al. 2006).

1.2.5.3.4 Dual αVβ3/αVβ5 integrin antagonism in cancer therapy

Targeting more than one integrin with one compound is becoming an attractive concept (Sheldrake & Patterson 2009). Dual antagonists can show important antiangiogenic and antitumour effects, and may represent a targeted approach for the inhibition of tumour angiogenesis and growth. One pseudopeptide, ST1646, targets αVβ3/αVβ5 with antiangiogenic effects both in vivo and in vitro, and has shown inhibitory effects on the growth of human ovarian carcinoma A2780 cells injected into xenograft tissue in nude mice (Belvisi et al. 2005).
S247 is a small molecule $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonist that has been investigated in both *in vitro* and *in vivo* models. Both continuous treatment and short-term treatment with S247 were highly effective in reducing metastasis, either by blocking early events such as stable attachment of tumour cells to vasculature of distant organs or by blocking their extravasation from vessels into nearby tissues evaluated by measurements of gross lung weight, flow cytometric analysis, and histology. The effect of S247 in inhibition of metastasis at the early stage (during stable attachment of tumour cell to the vasculature of distant organ) was examined in another tumour cell line because the 435/HAL cells did not colonise the lung after i.v. injection. GFP-expressing B16-F10 murine melanoma cells were injected into the tail vein of mice, following S247 injection just prior to tumour injection. After first day of injection, 68% decrease in final tumour burden and an 86% decrease in the tumour-associated gain in lung weight were observed. Therefore, the study indicated that the use of $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonists shows promise for treatment of both early and late steps of metastasis (Shannon *et al.* 2004).

Treatment with S247 prevented the development of colon cancer liver metastasis *in vivo* but had no growth inhibitory effect on the primary tumour. *In vitro*, S247 affected both endothelial cells and some types of tumour cell, causing decreased cell proliferation, adhesion and migration and also induced apoptosis (Reinmuth *et al.* 2003). A further study showed the effect of S247 is increased if it is combined with radiotherapy (Abdollahi *et al.* 2005).
Kumar et al. found that a dual antagonist of $\alpha_v\beta_3/\alpha_v\beta_5$ may be useful in blocking tumour induced angiogenesis by using the nonpeptide small molecule SCH221153, which inhibits the binding of radiolabelled echistatin to $\alpha_v\beta_3/\alpha_v\beta_5$ with an IC$_{50}$ equal to 3.2 and 1.7 nM, respectively. SCH221153 has the ability to block adhesion of foetal bovine aortic endothelial GM7373 cells to vitronectin and FGF2. In addition, it inhibits HUVECs proliferation and the mitogenic activity of FGF2 and VEGF. Testing in the human melanoma Lox cell line (which expresses low levels of $\alpha_v\beta_5$) showed SCH221153 to be ineffective in inhibiting cell proliferation in vitro, but it inhibited tumour growth in vivo by decreasing the number of blood vessels surrounding a xenograft tumour (Kumar et al. 2001).

1.2.5.3.4.1 Cilengitide, a dual $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonist, in cancer therapy

Cilengitide is another dual $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonist. It is a cyclic pentapeptide that binds to integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ with high affinity (Alghisi et al. 2009). Cilengitide has antiangiogenic and anti-proliferative effect in glioma cells and xenografts (MacDonald et al. 2001; Mas-Moruno et al. 2010; Mikkelsen et al. 2009; Tabatabai et al. 2010; Taga et al. 2002).

In phase I of a clinical trial, Cilengitide was well tolerated and showed limited side effects, although the maximum tolerated dose in phase I was not identified (MacDonald et al. 2008; Nabors et al. 2007). Hariharan et al. carried out a clinical study using different doses of this peptide (600 or 1200
mg/ml) against different solid tumours, and concluded that Cilengitide has a mild toxicity and can be used as antiangiogenic agent (Hariharan et al. 2007).

In a randomized phase II clinical trial by Reardon et al., patients were assigned to receive either 500 mg or 2000 mg Cilengitide to determine whether a higher dose would give a greater response. They recommended Cilengitide as a safe and non-toxic agent and suggested that it could be used in combination regimens for glioblastoma, because it had a high anti-tumour activity (Reardon et al. 2008). Combination of Cilengitide with standard chemotherapy such as temozolomide was safe and well tolerated (Stupp et al. 2010).

A recent preclinical study by Mikkelsen et al. reported that treatment with Cilengitide could lead to prolonged survival if it were given prior to radiotherapy. Cilengitide increased the sensitivity of U-251MG cells and HUVECs to radiation, leading to suppression of U-251MG growth and death of the HUVECs in vitro. Cilengitide dramatically prolonged survival in vivo if it was used with radiation rather than as a single agent. Further, Cilengitide decreased the expression of p13k/pAkt, a gene in the NF-kB signalling network with a recognised role in cell resistance to radiation in vivo (Mikkelsen et al. 2009).

Cilengitide alone had no effect on breast tumour growth, whereas combined radioimmunotherapy with Cilengitide (CMRIT) substantially increased the cure rate (44% cure rate for CMRIT, 20% cure rate for RIT alone). CMRIT
increased apoptosis and decreased proliferation more than Cilengitide alone (Burke et al. 2002).

Reynolds et al. found that nanomolar concentrations of Cilengitide enhanced tumour growth and tumour vascularisation. When Cilengitide was administered as a bolus at 200 mg/kg, its concentration in the plasma fell rapidly from micromolar to nanomolar levels, which was then sustained for 16 to 24 hours after administration. This led to promotion of tumour growth by direct stimulation of angiogenesis in vitro and in vivo. In $\beta_2/\beta_5$ deficient mice, no increases in tumour growth or angiogenesis were observed. Cilengitide therefore did not promote tumour growth directly but it acted in the cells that expressed $\alpha_v\beta_3/\alpha_v\beta_5$ by enhancing cell migration in response to VEGF and increased VEGFR-2/$\alpha_v\beta_3$ receptor recycling (Hodivala-Dilke 2008; Reynolds et al. 2009).

**1.2.5.3.5 Dual $\alpha_v\beta_3/\alpha_{IIb}\beta_3$ integrin antagonism**

Initial studies showed the antibody m7E3 (a monoclonal antibody created by joining the Fab fragments of the mouse monoclonal antibody 7E3 to the FC fragment of human Ig; it has high affinity and specificity for $\alpha_{IIb}\beta_3/\alpha_v\beta_3$) reduced tumour growth and angiogenesis by blocking host $\alpha_v\beta_3/\alpha_{IIb}\beta_3$ in vivo in $\beta_3$ negative tumour xenografts in rat bone. m7E3 increased tumour-free survival and decreased tumour volume and weight, but did not have any effect on tumour metastasis (Engebraaten et al. 2009).
The c7E3 Fab fragment binds with equivalent affinity to α<sub>IIbβ3</sub> and α<sub>vβ3</sub>, and c7E3 can redistribute between α<sub>IIbβ3</sub> and α<sub>vβ3</sub> integrins. c7E3 completely inhibited α<sub>vβ3</sub>-mediated cell adhesion of HUVECs and A375S2 cells to fibrinogen and gelatin, and partially inhibited adhesion to fibrin and vitronectin. c7E3 inhibited bFGF-mediated migration of HUVECs and A375S2 cells and also inhibited cell invasion through fibrin. c7E3 reduced proliferation by inducing apoptosis of endothelial cells and completely inhibited angiogenesis. Inhibition of both endothelial α<sub>vβ3</sub> and the platelet α<sub>IIbβ3</sub> receptor inhibited endothelial sprouting stimulated either by bFGF or by platelets. c7E3 inhibited growth of human melanoma tumours in nude mice, but blocking of expression of human melanoma cell-expressed α<sub>vβ3</sub> integrin without inhibition of mouse β<sub>3</sub> integrin failed to completely inhibit tumour growth in vivo. Taken together, these results suggested that a combined blockade of both tumour cell expressed α<sub>vβ3</sub> and host β<sub>3</sub> integrin (endothelial α<sub>vβ3</sub> and platelets α<sub>IIbβ3</sub>) could enhance inhibition of tumour growth in vivo (Trikha et al. 2002).

Another study investigated the contribution of α<sub>vβ3</sub> and α<sub>IIbβ3</sub> to MDA-MB-231 breast adenocarcinoma cells adhesion to endothelial cell ECM under flow conditions. The α<sub>vβ3</sub> antagonist (SB-273005) had no effect on tumour cell adhesion to the ECM while α<sub>IIbβ3</sub> antagonist lamifiban (Ro 44-9883) decreased adhesion of tumour cell to ECM. However, the combination of two antagonists (Ro 44-9883 and SB-273005) resulted in synergistic effect on adhesion when compared to the effects of single antagonist. Further, the previous finding of combining two antagonists was confirmed using c7E3
Fab, which targets both \( \alpha_V\beta_3 \) and \( \alpha_{IIb}\beta_3 \) integrins with the same affinity as lamifiban Ro 44-9883 and SB-273005. The result was similar, indicating a cooperation between \( \alpha_V\beta_3 \) and \( \alpha_{IIb}\beta_3 \) in the adhesion of MDA-MB-231 cells to the ECM (Gomes et al. 2004).

Activation of platelets by thrombin leads to an increased capability for migration by HeLa cells exposed to platelets. Migration and invasion of HeLa cells decreased after inhibition of \( \alpha_{IIb}\beta_3 \) and \( \alpha_V\beta_3 \) integrin by eptifibatide and RGDWE peptides respectively. HeLa cell migration depended on both platelets and endothelial cells. Blocking \( \alpha_{IIb}\beta_3 \) on platelets and \( \alpha_V\beta_3 \) on HeLa cells led to decreased adhesion of HeLa cells on monolayer HUVECs. The study concluded that both \( \alpha_V\beta_3 \) and \( \alpha_{IIb}\beta_3 \) participated in the adhesion interactions between cancer cells and endothelial cells (Liu et al. 2009).
Figure 1.9 Anti-integrin antagonists
<table>
<thead>
<tr>
<th>Agent</th>
<th>Integrin Target</th>
<th>Type of agent</th>
<th>Disease indication</th>
<th>Stage of development</th>
<th>Comments</th>
<th>Ref</th>
</tr>
</thead>
</table>
| Abciximab  | α\textsubscript{Iib}β\textsubscript{3}  
      α\textsubscript{V}β\textsubscript{3}  
      α\textsubscript{M}β\textsubscript{2} | Antibody      | Acute myocardial infarction  
      Unstable angina  
      Percutaneous coronary intervention (PCI) | Clinical use         | Adverse effect include bleeding, thrombocytopenia  
      Lead to double increase the need for red blood cells and platelets transfusion | (Derer et al. 2009; Jones et al. 2008; Kim et al. 2006; Schwarz et al. 2002) |
| Eptifibatide | α\textsubscript{Iib}β\textsubscript{3} | Peptide      | Percutaneous coronary intervention (PCI)  
      Unstable angina  
      Myocardial infarction | Clinical use         | Adverse effect include bleeding, thrombocytopenia and severe hypotension | (Giugliano et al. 2009; Pathak et al. 2008) |
| Tirofiban  | α\textsubscript{Iib}β\textsubscript{3} | Small Molecule | Percutaneous coronary intervention (PCI)  
      Unstable angina  
      Myocardial infarction | Clinical use         | Adverse effect includes intracranial retroperitoneal bleeding, pulmonary hemorrhage, spinal-epidural hematoma. | (Valgimigli et al. 2010) |
| Tysabri    | α\textsubscript{4}       | Antibody     | Multiple sclerosis  
      Crohn’s disease | Suspended from clinical use | Inhibits immune cells from crossing blood vessels to reach the affected organ. Several patients with MS in a long-term clinical trial of Tysabri recently died from progressive multifocal leukoencephalopathy (PML). Treatment possibly associated with melanoma development. | (Gani et al. 2008; Ismail et al. 2009) |
<table>
<thead>
<tr>
<th>Cilengitide</th>
<th>αvβ3/αvβ5</th>
<th>Peptide</th>
<th>Glioblastoma</th>
<th>Phase III</th>
<th>(Reynolds et al. 2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNTO-95</td>
<td>αv</td>
<td>Antibody</td>
<td>Solid tumours</td>
<td>Phase I</td>
<td>Safe, partial activity against angiosarcoma (Mullamitha et al. 2007; Ning et al. 2008)</td>
</tr>
<tr>
<td>Vitaxin</td>
<td>αvβ3</td>
<td>Antibody</td>
<td>Lymphoma, Leukemia, Rheumatoid arthritis, solid tumours</td>
<td>Phase II</td>
<td>(Brooks et al. 1995; Cai et al. 2006)</td>
</tr>
<tr>
<td>Volociximab</td>
<td>α5β1</td>
<td>Antibody</td>
<td>Renal cell carcinoma, Melanoma, ARMD</td>
<td>Phase II</td>
<td>Inhibits tumour angiogenesis by blocking binding of activated endothelial cells to fibronectin present in the extracellular matrix, thereby inducing apoptosis and inhibiting tumour growth. It has efficacy against kidney cancer but showed poor clinical activity as a single agent to treat melanoma. (Bell-McGuinn et al. 2011; Ricart et al. 2008)</td>
</tr>
<tr>
<td>PF-04605412</td>
<td>α5β1</td>
<td>Antibody</td>
<td>Solid tumours</td>
<td>Phase I</td>
<td>Inhibits endothelial cell-cell interaction. It has anti-angiogenic and anti-metastatic effects. (Li et al. 2010)</td>
</tr>
<tr>
<td>Compound</td>
<td>Target(s)</td>
<td>Category</td>
<td>Indication</td>
<td>Phase</td>
<td>Notes</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------------------</td>
<td>-------------------</td>
<td>-----------------------------------</td>
<td>-------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Ro 27-2441</td>
<td>α₄β₁, α₄β₇</td>
<td>Small molecule</td>
<td>Asthma</td>
<td>Phase II completed</td>
<td>(<a href="http://clinicaltrials.gov/show/NCT00048022">http://clinicaltrials.gov/show/NCT00048022</a>)</td>
</tr>
<tr>
<td>EMD525797</td>
<td>αᵥ</td>
<td>Antibody</td>
<td>Colorectal and ovarian carcinoma with liver metastasis</td>
<td>Phase I</td>
<td>(Manfred Wirth 2013)</td>
</tr>
<tr>
<td>IMGN388</td>
<td>No target revealed by company</td>
<td>Antibody conjugate</td>
<td>Solid tumours</td>
<td>Phase I</td>
<td>(Vater 2008)</td>
</tr>
<tr>
<td>GLPG0187</td>
<td>αᵥβ₁, αᵥβ₃, αᵥβ₅, αᵥβ₆, α₅β₁</td>
<td>Small molecule</td>
<td>Anti-angiogenic</td>
<td>Phase I</td>
<td>Inhibits osteoclast bone resorption and angiogenesis <em>in vitro</em> and <em>in vivo</em>. Inhibits the progression of bone metastasis and the formation of new bone metastasis during the treatment. (van der Horst <em>et al.</em> 2010)</td>
</tr>
<tr>
<td>JSM6427</td>
<td>α₅β₁</td>
<td>Small molecule</td>
<td>Proliferative vitreoretinopathy</td>
<td>Phase 1 completed</td>
<td>Inhibits proliferation of retinal cells. (Zahn <em>et al.</em> 2009)</td>
</tr>
<tr>
<td><strong>ATN-161</strong></td>
<td><strong>α_5β_1</strong>/α_5β_3</td>
<td>Peptide (linear)</td>
<td>Lung cancer/Renal carcinoma</td>
<td>Phase II</td>
<td>PHSCN derived from PHSRN of human fibronectin in which a cysteine residue replaces the arginine in the original sequence. PHSRN of fibronectin interacts with α5β1 integrin and increases its avidity for RGD. PHSRN- increases tumour growth. ANT-161 inhibits angiogenesis and tumour progression. It inhibits tumour growth and metastasis.</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Efalizumab</strong></td>
<td>αLβ2</td>
<td>Antibody</td>
<td>Psoriasis</td>
<td>Suspended from clinical use</td>
<td>Associated with PML fatal side effect through its immune suppressive properties</td>
</tr>
<tr>
<td><strong>E7820</strong></td>
<td>α_2</td>
<td>Small molecule</td>
<td>Solid tumours</td>
<td>Phase II</td>
<td>It can be used for treatment of advanced solid tumour in combination with cetuximab</td>
</tr>
</tbody>
</table>

(Donate et al. 2008)  
(Frampton & Plosker 2009)  
(Funahashi et al. 2002; Mita et al. 2011; Semba et al. 2004)
1.3 Aims and objectives

New drugs for the treatment of cancer that can control the tumour dissemination process are required. As has been reviewed here; the β₃ integrin subfamily (α₁IIBβ₃ and αᵥβ₃) plays an important role in this process.

The hypothesis underlying this work is that dual α₁IIBβ₃ and αᵥβ₃ integrin antagonism will have an enhanced effect by having a direct effect on β₃-expressing tumour cells (compared to antagonism of α₁IIBβ₃ and αᵥβ₃ separately), inhibiting cell migration and dissemination. Furthermore, through targeting tumour cell interaction with endothelial cells and platelets, this will also lead to inhibition of angiogenesis and metastasis.

The main aims of the work described in this thesis are to assemble a panel of human tumour cell lines characterised for their integrin expression which will be utilised to evaluate potential novel β₃ integrin antagonists.

These aims will be achieved through the following objectives:

- The characterisation of α₁IIBβ₃ and αᵥβ₃ integrin expression in human tumour xenograft tissue.

- Evaluation of the expression of α₁IIBβ₃ and αᵥβ₃ integrins in a panel of human tumour cell lines with different integrin expression to use in cell based assays to investigate the effects of β₃ integrin antagonists.

- Development and validation of cell based assays to investigate the effect of β₃ inhibition on tumour cell migration.
• Evaluation of the effect of potential novel β₃ integrin antagonists in inhibition of human tumour cell migration.
Chapter 2: Characterisation of integrin $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ expression in human xenograft tissue in mice using immunohistochemical and immunoblotting techniques
Chapter 2

2.1 Introduction

The development of effective targeted therapies for cancer relies on the availability of in vivo models that have been characterised for expression of the target (Teicher 2006). Preparation of an in vivo model can involve subcutaneous implantation of a tumour cell line or orthotopic grafting at a site where the tumour commonly metastasises; for example, into the lungs.

Subcutaneous transplantation models are considered effective for investigating integrins. A study done by Gouon et al. used a subcutaneous model to determine the difference in $\alpha_V\beta_3$ integrin expression in subcutaneous vs. in vitro melanoma cells using HT-144 (weak expression of $\alpha_V\beta_3$ integrin) and SK-MEL-2 (high expression of $\alpha_V\beta_3$ integrin) cell lines. The HT-144 human melanoma cells showed up-regulation of $\beta_3$ integrin, which was correlated with MMP-9 expression, when the tumour was grown in nude mice (Gouon et al. 1996). Trikha et al. found that subcutaneous implantation of PC-3 and DU-145 cells, which express $\alpha_{IIb}\beta_3$ integrin, was tumorigenic but not metastatic while an orthotopic in vivo model showed that DU-145 cells were both tumorigenic and invasive (Trikha et al. 1998).

Tumour implants at a specific site in the model (orthotopic xenograft) produce tumours with metastasis; these models permit study of compounds which affect the tumour dissemination process in vivo (Bibby 2004). Such models have been useful in identifying new therapeutics (Kerbel 2003; Popkov et al.)
2006) and in anti-integrin drug evaluation. For example, Landen et al. used an orthotopic model to evaluate the effects of the etaracizumab (anti-αvβ3) antibody on ovarian carcinoma cell lines such as SKOV-3, A2780, and HeyA. Etaracizumab was effective in inhibiting tumour growth and invasion (Landen et al. 2008). Allman et al. used an in vivo model to determine the effect of cRGDfV peptide on melanoma cells, with A375 administered subcutaneously into nude mice. cRGDfV was able to decrease melanoma growth and invasion (Allman et al. 2000). Furthermore, Zhao et al. used an in vivo model to appraise the effect of the small molecule PSK1404 (anti-αvβ3) peptide on breast and prostate carcinoma (Zhao et al. 2007). An orthotopic model was also used to reveal the functional state of αIIbβ3 integrin and its role in tumour metastasis in vivo. Injection of DU-145 cells had membranous expression of αIIbβ3 integrin in mouse led to development of tumour which infiltrated in the surrounding tissue, and metastasised to lymph node. Inhibition of αIIbβ3 integrin with 10E5 antibody (specific for αIIbβ3 integrin) led to block interaction of DU-145 cells with host tissue and therefore led to blockade of colonisation of DU-145 cells in the lung (Trikha et al. 1998).

Human prostate carcinoma cell lines with a high expression of αvβ3 integrin are more tumorigenic in vivo; for example, PC-3 cells with a high expression of αvβ3 integrin are more tumorigenic when xenografted, and grow more rapidly when implanted in nude mice than do LNCaP cells which have a low expression of αvβ3 integrin (Taylor et al. 2012). Trikha et al. reported that a tumour cell line with expressed αIIbβ3 integrin can develop a large tumour in
vivo due to the decreased apoptotic rate in the cells expressing $\alpha_{\text{IIb}}\beta_3$ integrin (Trikha et al. 2002).

Several studies have reported a lack of expression of $\alpha_\text{v}\beta_3/\alpha_{\text{IIb}}\beta_3$ integrin in normal tissues. For example, using immunoblotting, Zheng et al. found no expression of $\beta_3$ integrin in normal human prostate tissue, whereas human prostate adenocarcinoma tissue expressed this protein (Zheng et al. 1999). Immunohistochemistry of FFPE tissue samples revealed no expression of $\beta_3$ integrin in normal breast tissue, while breast carcinoma tissue clearly expressed $\beta_3$ integrin (Pontes-Junior et al. 2009; Wang et al. 2010). Havaki et al. found no expression of $\beta_3$ integrin in normal breast tissue by immunoblotting, whereas breast carcinoma tissue expressed $\beta_3$ integrin (Havaki et al. 2007).

In the present study, immunohistochemistry and immunoblotting were used to characterise the expression of $\alpha_\text{v}\beta_3/\alpha_{\text{IIb}}\beta_3$ integrin in human tumour xenograft mouse tissue generated in house. Previous studies have detected integrin expression using immunohistochemistry on either fresh frozen tissue sections (Brooks et al. 1995; Li et al. 2001) or paraffin embedded tissue sections (Bisanz et al. 2005; Brooks et al. 1995).

The mouse LM609 monoclonal antibody that targets the $\alpha_\text{v}\beta_3$ integrin can be used to detect expression of $\alpha_\text{v}\beta_3$ in angiogenic vessels and in the tumour cells of many human cancers such as colorectal carcinoma, pancreatic carcinoma, breast and glioma (Vonlaufen et al. 2001). LM609 has been reported to successfully detect integrins in breast xenograft mouse tissue,
formalin-fixed, paraffin embedded tissue (Brooks et al. 1995) as well as in frozen tissue sections. LM609 was also used in an in vivo model to detect the expression of αvβ3 integrin in ovarian carcinoma (Landen et al. 2008). Thus this antibody was chosen for use in this part of the study to characterise the expression of αvβ3 integrin in human xenograft mouse tissue.

2.1.1 Aims and objectives

The overall aim of the work described in this chapter is to evaluate αIIb and αvβ3 integrin expression in human tumour xenografts, using immunohistochemistry and immunoblotting. The information obtained will be of great use in guiding the selection of cell lines to be characterised for inclusion in the in vitro screening panel and for model selection, once novel antagonists eventually progress to in vivo screening (the latter is beyond the scope of this thesis).

The aims will be addressed by pursuing the following objectives:

1- Characterisation of the expression of αIIb and αvβ3 integrin in formalin fixed paraffin embedded human tumour xenograft tissue using immunohistochemistry.

2- Characterisation of the expression of αIIb and αvβ3 integrin in fresh frozen human tumour xenograft tissue using immunohistochemistry.

3- Characterisation of the expression of αIIb, αv and β3 integrin in fresh frozen human tumour xenograft tissue using immunoblotting.
2.2 Materials and methods

2.2.1 Materials

All general chemicals were obtained from Sigma-Aldrich (Poole, UK), unless otherwise specified. Primary antibodies and related secondary antibodies were used in this part of the study to detect expression of αV, αIIb, β3, αVβ3 and β actin as summarised in Table 2.1. The blocking reagents were bovine serum albumin (BSA) (Sigma-Aldrich, Poole, UK), normal rabbit serum (NRS), normal goat serum (NGS), and normal horse serum (NHS) obtained from Vector Laboratories Ltd (Peterborough, UK). Vectastain Avidin Biotin Complex (ABC) and the M.O.M. immunodetection basic kit were from Vector Laboratories Ltd (Peterborough, UK). 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen (Sigma-Aldrich, Poole, UK).

2.2.2 Cell lines

The cell lines used to generate the tumour xenograft material: PC-3 prostate carcinoma, DLD-1 and HCT-116 colon adenocarcinoma, M14 and UACC-62 melanoma, SKOV-3 and A2780 ovarian carcinoma, A549 and H460 human non small cell lung carcinoma, and PANC-1 pancreatic carcinoma. These were all obtained from American Type Culture Collection (ATCC, Middlesex, UK) except for M14 and UACC-62, which was from the US National Cancer Institute (NCI, USA). One xenograft was analysed per cell line.
Table 2.1 Primary antibodies and related secondary antibodies used in immunohistochemistry (IHC) and immunoblotting (IMB)

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Receptor</th>
<th>Type</th>
<th>Source</th>
<th>Secondary antibody (IHC)</th>
<th>Source</th>
<th>Secondary antibody (IMB)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV4</td>
<td>Anti-β₃</td>
<td>MMAb</td>
<td>BV4. ab 7167, Abcam (Cambridge, UK)</td>
<td>biotinylated horse anti-mouse</td>
<td>BA2000, Vector Laboratories Ltd (Peterborough, UK)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B7</td>
<td>Anti-β₃</td>
<td>MMAb</td>
<td>Clone B-7, SC-46655, Santa Cruz Biotechnology (Santa Cruz, USA)</td>
<td>biotinylated horse anti-mouse</td>
<td>BA2000, Vector Laboratories Ltd (Peterborough, UK)</td>
<td>Polyclonal rabbit anti mouse immunoglobulins horseradish peroxidase</td>
<td>P0260, Dako Cytomation, Glostrup, Denmark</td>
</tr>
<tr>
<td>C20</td>
<td>Anti-α₁₁b</td>
<td>PGab</td>
<td>SC-6602, Santa Cruz Biotechnology (Santa Cruz, USA)</td>
<td>biotinylated rabbit anti-goat</td>
<td>BA500, Vector Laboratories Ltd (Peterborough, UK)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q20</td>
<td>Anti-αᵥ</td>
<td>PRAb</td>
<td>SC-6617-R, Santa Cruz Biotechnology (Santa Cruz, USA)</td>
<td>biotinylated goat anti-rabbit</td>
<td>BA1000, Vector Laboratories Ltd (Peterborough, UK)</td>
<td>Polyclonal goat rabbit immunoglobulins horseradish peroxidase</td>
<td>P0448, Dako Cytomation, Glostrup, Denmark</td>
</tr>
<tr>
<td>LM609</td>
<td>Anti-αᵥβ₃</td>
<td>MMAb</td>
<td>Clone LM609, MAB 1976, Chemicon-Millipore, Watford, UK</td>
<td>biotinylated horse anti-mouse</td>
<td>BA2000, Vector Laboratories Ltd (Peterborough, UK)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A1978</td>
<td>β-actin</td>
<td>MMAb</td>
<td>Sigma-Aldrich, Poole, UK</td>
<td>-</td>
<td>-</td>
<td>Polyclonal rabbit anti mouse immunoglobulins horseradish peroxidase</td>
<td>P0260, DAKO Cytomation, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

Key: MMAb: monoclonal mouse antibody, PGab: polyclonal goat antibody, PRAb: polyclonal rabbit antibody.
2.2.3 Methods

2.2.3.1 Collection of xenograft materials

Creation of in vivo tumour models was carried out in-house under a project licence issued by the UK Home Office and in accordance with the UK National Cancer Research Institute Guidelines for the Welfare of Animals (Workman et al. 2010). Human tumour cell lines were xenografted in immunodeficient mice (Harlan, Loughborough, UK) aged six to eight weeks under general inhalation anaesthesia with recovery. Animal welfare and tumour growth was monitored frequently and when the tumours were approximately 500 mm$^3$ in volume, the mice were sacrificed and the tumours removed and processed as indicated below.

2.2.3.2 Slide coating

Adherence of tissue sections to microscope slides during immunohistochemical procedures was improved by coating all slides with 3-aminopropyltriethoxysilane (APES). Slides were washed for two minutes with acetone, then immersed for two minutes in a freshly prepared solution of 2% APES in acetone. The slides were washed for two minutes in running tap water and left to dry at room temperature and then stored in a dust free environment.

2.2.3.3 Tissue fixation, processing, embedding and paraffin sectioning

Harvested tissue was placed for 24 to 48 hours in 10% neutral buffered formalin (VWR International Ltd. Poole, UK) before transferring to 70%
ethanol. Then tissue was placed in plastic cassettes (Raymond A. Lamb Ltd, UK) along with labels giving histology numbers. The cassettes were processed using an automatic tissue processor (Leica TP 1020, Leica Microsystems, Nussloch, Germany). The tissue processing steps were achieved by incubating the tissues in 70% ethanol (Fisher Scientific, UK) for one hour, 80% ethanol for one hour, 95% ethanol for one hour, 100% ethanol for one hour (twice, in different tanks), 100% ethanol for two hours, Histoclear for one hour (twice, in different tanks), Histoclear for two hours and paraffin wax for two hours and thirty minutes (twice, in different tanks).

Finally, the tissue was placed in labelled plastic moulds (Polysciences, Warrington, USA) which were filled with molten paraffin wax and left to set on a refrigerated surface. Wax blocks were stored at room temperature until required for sectioning.

The wax rigidity was increased before sectioning by placing the wax blocks containing tumour tissues at -20 °C for at least 30 minutes. Then, 5 µm sections were cut from each wax block using a Leitz rotary microtome (Leica RM2155, Leica Microsystems, Nussloch, Germany). Sections were collected, placed in a warm water bath and collected onto APES coated slides. Slides were placed on a hot plate for thirty minutes to one hour to increase adhesion of tissue to the slides and then stored at room temperature.
2.2.3.4 Haematoxylin and Eosin staining

All steps were carried out at room temperature. Sections were de-paraffinised prior to staining by passing the sections through xylene and different grades of ethanol. Sections were immersed in two different changes of 100% xylene, 50% xylene/ethanol, two changes of 100% ethanol, 90% ethanol and 70% ethanol for five minutes in each solution.

Deparaffinised sections were stained with Harris’s Haematoxylin solution for ten minutes. Slides were washed with running tap water, and immersed in acidic alcohol (0.25% hydrochloric acid in 70% ethanol) for five seconds to differentiate staining. Differentiated sections were washed in tap water and placed for two minutes in Scott’s Tap Water (3.5 g sodium bicarbonate and 20 g magnesium sulphate dissolved in 1000 ml H₂O). Sections were counterstained with 1% aqueous Eosin for one minute and washed briefly with running tap water. Sections were then drained to allow the excess water to run off.

Stained sections were dehydrated through different grades of ethanol and xylene, sections were immersed sequentially for two minutes in absolute ethanol, three minutes in 50% xylene/ethanol and three minutes in 100% xylene (twice, in different tanks). Finally, sections were mounted with Distyrene-plasticiser-xylene (DPX) medium (VWR International Ltd. Poole, UK), a coverslip was applied, and the sections were left to dry.
2.2.3.5 Immunohistochemistry

2.2.3.5.1 Immunodetection of αⅡb and αvβ3 integrins expression in FFPE human tumour xenograft sections

2.2.3.5.1.1 Sample preparation

Five µm thick FFPE tissue sections were mounted on APES coated slides. Sections were deparaffinised and rehydrated by sequential incubation in 100% xylene for ten minutes (twice, in different tanks), 50% xylene/ethanol for five minutes, absolute ethanol for five minutes (twice, in different tanks), 90% ethanol for five minutes, 70% ethanol for five minutes and distilled water for five minutes. Sections were incubated with 1% or 3% hydrogen peroxide (H₂O₂) for thirty minutes to block the endogenous peroxidase activity. Sections were washed in PBS, or 0.15 M TBS, pH 7.35 for 3 washes for 5 minutes each.

2.2.3.5.1.2 Antigen retrieval

Using antigen retrieval, attempts were made to expose antigens masked by fixation. In this part of the study, three types of antigen retrieval methods were evaluated (citrate buffer, pepsin digestion and proteinase K).

A. Citrate buffer antigen retrieval

Slides were placed in a plastic microwavable container filled with citrate buffer (10 mM citrate buffer, pH 6). The container was covered with clingfilm and heated in a microwave (NN-E201W, Panasonic) at 600 W for 3-6 × 5
minutes each time. The level of the citrate buffer was checked and topped up if needed each time. Microwaved slides were allowed to cool at room temperature for thirty minutes then washed in PBS for 3 washes of 5 minutes each.

B. Pre-warmed pepsin digestion method antigen retrieval

Sections were incubated at 40 °C with pre-warmed pepsin (0.65 mg/ml) in PBS at for five minutes then washed with PBS for 3 washes of 3 minutes each.

C. Proteinase K method antigen retrieval

Sections were incubated with 0.1 µg/µl Proteinase K (MB2005, Melford Lab, Ltd, Suffolk) in distilled water (dH₂O) for five minutes at room temperature then washed in 1X Tris buffered saline (TBS, 1.2 g Tris-HCl, 0.28 g Tris base, 4.4 g NaCl dissolved in 450 ml dH₂O, adjusted to pH 7.4 then volume adjusted to 500 ml with dH₂O), for five minutes.

2.2.3.5.1.3 Antibody incubation

Sections were incubated with normal blocking serum diluted with PBS or TBS (1.5%) for twenty minutes at room temperature in order to avoid non-specific binding of the antibody. The blocking serum was selected according to the species in which the secondary antibody was raised. The excess blocking serum was carefully removed by blotting with a tissue and primary antibodies applied (see text for antibody dilution and incubation time). Sections were then washed in PBS (3 washes for 3 minutes each). Secondary antibody;
horse anti mouse, goat anti rabbit, or rabbit anti goat (dilution, 1:200) was applied for thirty minutes at room temperature, washed as described for the primary antibody, incubated with peroxidase-labelled streptavidin for thirty minutes at room temperature and then washed in 3 changes of PBS for 3 minutes in each change. The immunoreactivity was visualised by incubating sections with DAB chromogen solution for two to five minutes. Sections were then washed in dH₂O for five minutes and counterstained with Harris’s Haematoxylin solution for twenty seconds. Sections were washed well in running tap water, and then incubated in Scott’s Tap Water substitute for two minutes.

Sections were dehydrated and cleared by incubation in 70%, 90%, absolute ethanol (twice, in different tanks), 50% xylene/ethanol for three minutes each, xylene (two different tanks for five minutes each). Finally, coverslips were mounted with DPX medium and left to dry at room temperature. The slides were examined by light microscope (Leica DMRB, Leica Microsystems, Wetzlar, Germany).

2.2.3.5.2 Immunodetection of αⅡbβ₃ and αVβ₃ integrins expression in frozen human xenograft tumour

2.2.3.5.2.1 Sample preparation

Fresh frozen human tumour xenografts (section 2.2.2) were sectioned (5 µm) using a Cryostat (Leica CM1100, Leica Microsystems, Nussloch Germany)
and mounted on APES coated slides. Slides were air-dried at room temperature then stored at -20 °C.

2.2.3.5.2.2 Labelling procedure

Slides were removed from the freezer and allowed to equilibrate at room temperature for about twenty minutes. Sections were fixed with acetone, methanol or 4% paraformaldehyde (PFA) for ten minutes, air-dried for ten minutes then rehydrated in PBS for ten minutes. Sections were placed in a humidified chamber and the non-specific binding of antibodies was blocked by incubating with either normal serum (NS) diluted in PBS (1.5%) or 5% BSA for twenty minutes at room temperature. The excess blocking solution was removed and the primary antibody was applied (Table 2.1). Sections were then washed in PBS (3 washes of 3 minutes each), incubated with 1:200 secondary antibody (Table 2.1) and washed as described for primary antibody. Sections were incubated for thirty minutes with ABC peroxidase labelled streptavidin at room temperature. The immunoreactivity was visualised with DAB, counterstained with Harris’s Haematoxylin and mounted with DPX as in section 2.2.3.5.1.3.

2.2.3.5.3 Immunodetection of β3 subunits and αVβ3 integrin expression in FFPE and frozen human tumour xenograft sections by using a mouse on mouse (M.O.M.) kit

The M.O.M. kit is specifically designed to localise mouse primary antibodies on mouse tissues and should eliminate background staining in mouse tissue.
The M.O.M. kit was used during immunolocalisation for mouse primary antibodies using FFPE and fresh frozen sections.

2.2.3.5.3.1 Sample preparation

FFPE tissue sections 5 µm-thick were de-waxed and rehydrated as stated in section 2.2.3.5.1.1. Following rehydration and antigen retrieval sections were treated with 3% hydrogen peroxide (H₂O₂) in order to block endogenous peroxidase activity and then washed twice in PBS for two minutes.

Frozen sections were prepared as in section 2.2.3.5.2.1.

2.2.3.5.3.2 Preparation of M.O.M. kit working solutions

The mouse Ig blocking reagent was prepared by adding two drops of stock solution to 2.5 ml of PBS. The diluent solution was prepared by diluting 600 µl of protein stock solution with 7.5 ml of PBS. Biotinylated anti-mouse IgG reagent was prepared by mixing 10 µl of stock solution with 2.5 ml of diluent solution prepared as above.

2.2.3.5.3.3 Antibody incubations

Blocking reagent was applied to the sections before the application of the primary antibody in order to avoid non-specific binding, and incubated for one to two hours followed by washing with PBS (2 washes for 3 minutes each). Sections were incubated with diluent for five minutes, the excess diluent removed, and the primary antibody directly applied. All incubations were undertaken in a humid chamber at room temperature for all the
immunolabelling steps, except for primary antibody, which was either at room temperature or at 4 °C depending on the optimised conditions for each antibody. The negative control was incubated with M.O.M. mouse blocking solution for the same period of time. Sections were then washed in PBS (2 washes for 3 minutes each). M.O.M. biotinylated anti-mouse IgG reagent was applied for ten minutes then sections were washed in PBS (2 washes for 3 minutes each). Sections were incubated with the peroxidase-labelled streptavidin for thirty minutes at room temperature and then washed in PBS (2 washes for 3 minutes each). Sections were counterstained with Harris’s haematoxylin, differentiated in Scott’s Tap Water, dehydrated and mounted as described in section 2.2.3.5.1.3.

2.2.3.6 Immunoblotting (Western Blotting)

2.2.3.6.1 Materials

Primary and secondary antibodies used in this part of the study are summarised in Table 2.1 and section 2.2.1.

2.2.3.6.2 Tissue homogenisation

Human xenograft mouse frozen tissue samples (-80°C) were weighed and cut into approximately 2 mm³ pieces. Each piece was placed in a 1.5 ml microfuge tube with lysis buffer (1:3 w/v compared to the weight of tissue to be lysed). The lysis buffer was either PBS buffer alone, or was assembled as follows: 2x lysis buffer [100 mM Tris pH 8.8, 5 mM EDTA, 300 mM NaCl, 2% Triton X-100], protease inhibitor cocktail (Roche, Mannheim, Germany) and
water. The samples were incubated on ice for thirty minutes, and then homogenised using an Ultra Turax homogeniser (IKA Labortechnik, Germany), for 3 pulses of 5 seconds with cooling on ice between each homogenisation to avoid overheating. The homogenate was centrifuged for different speeds and times, and both the supernatant and pellet collected. The samples were aliquoted and stored at -80 °C for later use.

2.2.3.6.2.1 Bradford assay

Protein concentration was measured using the Bradford Assay (Bradford 1976). Serial dilutions for six protein standards (0.0625 - 1.0 mg/ml) were prepared using 2 mg/ml of BSA dissolved in dH2O. After preparing serial dilutions, 50 µl from each BSA dilution was added to 1.5 ml of Bradford reagent. All tubes were vortexed and left to stand at room temperature for ten minutes to allow Coomassie Brilliant Blue dye to bind to protein. Dilutions of tissue lysates were prepared in dH2O (50 µl in total) and added to 1.5 ml of Bradford reagent. 1 ml from each BSA dilution (standard) and lysate were vortexed and transferred to cuvettes. Absorbance readings were recorded at 595 nm using a spectrophotometer (Thermo Scientific Multiskan Spectrum, 1500, Thermo Fisher Scientific, Finland) and a standard curve plotted. If the sample absorbance was outside of the range of the standard curve, then appropriate dilutions were carried out to bring the reading within the range of standard curve (Figure 2.1).
Figure 2.1 Representative calibration curve using Bradford assay.
Each data point represents the mean +/- SD of 3 readings at 595 nm. Linear regression analysis gives a straight line with $r^2 = 0.99$.

2.2.3.6.2.2 SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated using pre-cast 8% polyacrylamide SDS gels (Bio-Rad, Hertfordshire, UK). The gel was immersed in running buffer (5X Tris-glycine electrophoresis buffer: 25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). 20-75 µg of protein was diluted in 20 µl SDS loading buffer (50 mM Tris Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated at 70 °C for ten minutes followed by rapid incubation on ice to denature proteins. 20 µl of sample was loaded into each well. PageRuler™ Plus Prestained Protein Ladder marker (SM1811, Thermo Fisher Scientific Inc. Rockford, USA) was loaded into first lane. The marker
had a range of size from 10 kDa to 250 kDa. The electrophoresis was run at 20 mA for 90 minutes to separate the proteins.

2.2.3.6.2.3 Western Blot for $\alpha_\nu$ and $\beta_3$ Integrins

Proteins were transferred from the SDS-polyacrylamide gel to nitrocellulose membrane (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK) using transfer buffer (2.9 g glycine, 5.8 g Tris base, 0.37 g SDS, 200 ml methanol, 800 ml water). Protein transfer was run at 42 mA for 120 minutes. After transfer, membranes were immersed in Ponceau S solution to verify protein transfer, and the SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue to confirm that all proteins were transferred to the membrane.

2.2.3.6.2.4 Ponceau S Stain

Protein blotted onto the nitrocellulose membrane was stained with Ponceau S (1 g Ponceau S plus 50 ml acetic acid, made up to 1 l with distilled water) for one to three minutes. The membrane was scanned (HP scan jet 2400) then washed with distilled water three times in order to destain the membrane. Figure 2.2A shows a representative Ponceau S membrane where successful transfer has taken place.

2.2.3.6.2.5 Coomassie Brilliant Blue Staining

Complete protein transfer was confirmed by immersing SDS-polyacrylamide gels in Coomassie Brilliant Blue solution (dissolved in methanol: $H_2O$ [1:1 v/v] with 10% acetic acid) and fixing and staining them for one hour. The gels
were then destained in a solution of 40% methanol plus 10% acetic acid and 50% water. Gels were scanned with HP scan jet 2400. Figure 2.2B shows a representative Coomassie Brilliant Blue stain gel where successful transfer of most of the proteins has taken place.

![Image](image_url)

**Figure 2.2 Confirmation of successful protein transfer**

(A) Sample membrane stained with Ponceau S indicating that protein has been transferred to the membrane from (B) SDS stained with Coomassie Brilliant Blue and as can be seen most of the protein transferred to the gel.

2.2.3.6.2.6 Immunoblotting of nitrocellulose membrane with anti-β₃ and anti-αᵥ integrin subunits

The nitrocellulose membranes were blocked with either 5% non-fat dried milk in PBS with 0.05% Brij-35 solution (B4184) or 5% BSA in PBS- with 0.05% Brij-35 solution for one hour at room temperature, followed by application of primary antibody. The membrane was then washed with PBS with 0.05% Brij-35 solution (3 washes for 10 minutes each) followed by application of secondary antibody as specified in Table 2.1. The ECL plus Western blotting detection system (NEL104, Perkin Elmer LAS) reagents A and B were mixed
in a 1:1 ratio and applied to the membrane for one minute after washing the membrane with PBS with 0.05% Brij-35 solution (3 minutes for 5 minutes each). The membrane was exposed to Kodak Scientific imaging film (Amersham Biosciences) in the dark for five minutes. Films were developed using Ilford Rapid Multigrade developer (1/10 dilution) for three minutes, rinsed with water and fixed in Ilford rapid film and paper fixer solution (1/10 dilution) for two minutes. Expression of the integrin subunits was semi-quantified by using Molecular Imager FX System (Bio-Rad Laboratories, Inc., UK) by dividing the signal intensity of the expression of αv or β3 protein by the signal intensity of the expression of internal control β-actin.
2.3 Results

2.3.1 Detection of the expression of α_{\text{IIb}}β_3 and α_\text{V}β_3 integrin in human tumour xenograft tissue by IHC

2.3.1.1 Tumour xenograft

Haematoxylin and Eosin (H & E) staining was carried out on M14 human xenograft tissue sections. Microscopic examination revealed the tumours to be poorly differentiated due to lack of organisational structure (Figure 2.3, A and B).

![Figure 2.3 Haematoxylin and Eosin stain in M14 human melanoma xenograft paraffin embedded tissues](Image)

Haematoxylin and Eosin stain shows histology of melanoma tissue. The black arrows indicate tumour cells. Bar length = 200 µm (A) and 60 µm (B).
2.3.1.2 Studies on FFPE tissue samples

Detection of the expression of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin was attempted to optimise by evaluating several antibodies in FFPE sections, and investigating the effect of changing the antigen retrieval method, blocking procedure, antibody incubation time and concentration.

Primary antibodies Q20 (anti-$\alpha_v$), C20 (anti-$\alpha_{\text{IIb}}$), BV4 (anti-$\beta_3$), B7 (anti-$\beta_3$) and LM609 (anti-$\alpha_v\beta_3$) (Table 2.1) were used to characterise integrin expression in FFPE tissue. However, assessment of all variables (as summarised in Figure 2.4) revealed that all methods were unsuccessful for immunodetection of $\alpha_v$, $\alpha_{\text{IIb}}$, $\beta_3$ and $\alpha_v\beta_3$ (as shown in Figure 2.5). All antibodies except Q20 (anti-$\alpha_v$) showed non-specific immunolabelling in both sections with and without primary antibody. Q20 (anti-$\alpha_v$) had no immunolabelling in the section without primary antibody whereas the section with primary antibody had non-membranous immunolabelling.
Studies of FFPE tissue samples such as M14, UACC-62, PC-3, DLD-1 & HCT-116

1- Antigen retrieval

- A-Proteinase K
- B-Pepsin digestion
- C-Citrate buffer

Damage tissue

All primary antibodies: BV4 & B7 (anti-β3), Q20 (anti-αv), C20 (anti-αIIb) & LM609 (anti-αvβ3) had non-specific immunolabelling in sections without primary antibody. Q20 had non membranous immunolabelling.

2- Blocking

- A-Normal serum
  - Figure 2.5, E - H
- B- 1-5 % BSA
  - Figure 2.5, I - L

No change in the results with all antibodies

3- Primary antibody dilution: 1:50 – 1:400

- 1 hour room temperature
  - Figure 2.5, M & N
- Overnight 4 °C

4- Secondary antibody dilution: 1:200-1:800. Figure 2.5, O & P

All attempts tried gave same result: BV4, B7, C20 & LM609 gave non specific immunolabelling in sections without primary antibody. Q20 had non membranous immunolabelling.

Figure 2.4 Optimisation of IHC to detect αIIbβ3 and αvβ3 integrin expression in FFPE human xenografts, using different methods
Figure 2.5 Optimisation of IHC to detect αV and β3 integrin expression in FFPE M14 human melanoma xenografts using Q20 and B7

M14 tissue (A-H & M-P), blocked with normal serum and immunolabelled with primary antibodies: B7 (A-F) or Q20 (G&H) at dilution 1:50, overnight incubation 4 °C. Proteinase K damaged M14 tissue immunolabelled with B7 (A) and without B7 (B). Pepsin digestion damaged M14 tissue with B7 (C) and without B7 (D). A 30 minute citrate buffer antigen retrieval duration was effective in sections immunolabelled with B7 (E) but non-specific immunolabelling developed in sections without B7 (F). A 30 minute citrate buffer antigen retrieval used in a M14 section immunolabelled with Q20 (G) showed non-membranous immunolabelling whereas a section without Q20 (H) did not develop any non-specific binding. Under the same conditions with blocking changed to 5% BSA, M14 immunolabelled with B7 (I) had membranous immunolabelling but a section without B7 (J) developed non-specific binding. Under the same conditions, non-membranous immunolabelling developed in a section with Q20 (K) and a clean section without Q20 (L). Dilution of B7 to 1:400 and incubation for 1 hour at room temperature gave the same pattern of sections with (M) and without B7 (N). Increasing the dilution of primary antibody to 1:400 and the secondary antibody to 1:800 did not change the result in sections immunolabelled with Q20 (O) and without Q20 (P). Sections shown are a representative example of three independent experiments done using different primary antibodies on different human tumour FFPE tissue. B7 in M14 tissue was chosen as a representative example with the same results being seen for the other primary antibodies used. Bar length = 60 µm.
2.3.1.3 IHC studies on Frozen Sections.

In order to avoid adverse effects of fixation and processing issues associated with FFPE tissue, frozen sections were also evaluated. Frozen sections were used with the same antibodies as used for the paraffin sections. Figure 2.6 summarises the steps of optimisation for different primary antibodies used to characterise expression of $\alpha_\mathrm{V}$, $\alpha_\mathrm{IIb}$, $\beta_3$ and $\alpha_\mathrm{V}\beta_3$ integrins by IHC. The results were similar to those obtained with FFPE tissues (Figure 2.7).
Studies of frozen tissue samples such as M14, UACC-62, SKOV-3, A2780, A549, H460, PANC-1, DLD-1, HCT-116 & PC-3

1. Different fixatives tried
   - A- Acetone
   - B- Methanol
   - C- 4% PFA

   Damaged the tissue

   C20 (anti-αv) had diffuse immunolabelling and clean section without primary antibody. Other antibodies such as BV4 & B7 (anti-β3), C20 (anti-αv) & LM609 (anti-αIIbβ3) had immunoreactivity in both sections, with and without PAb.

2. Block endogenous peroxidase with 1% H2O2 for 1, 5 & 20 minutes

3. Block non specific binding of primary antibody
   - A- Normal serum
   - B- 5% BSA

4. Primary antibody at dilution 1:50-1:400 incubated: 1 hour at room temperature or at overnight 4° C.

5. Secondary antibody: 1:200-1:800, 30 minutes at room temperature then wash 3 x 5 minutes with PBS.

6. Avidin biotin complex (ABC) incubation time decreased from 30 to 15 minutes

7. DAB time decreased from 5 to 2 minutes.

Figure 2.7 Optimisation of the protocol used to characterise expression of αIIbβ3 and αvβ3 integrin in frozen human xenograft mouse tissue by IHC
Figure 2.7 IHC of αv integrin expression in human xenograft mouse frozen tissue using Q20

IHC of M14 with anti αv antibody Q20: non-membranous immunolabelling developed further to tissue damage in tissue fixed with acetone (A & B), methanol (C & D) and diffuse immunolabelling in tissue fixed with 4% PFA, blocked with NGS and incubated with 1:50 Q20 for overnight incubation at 4 °C (E & F). The immunolabelling of M14 tissue blocked with 1% H2O2 for twenty minutes (G & H) did not change when blocking was changed to 5% BSA (I & J) or when the Q20 incubation time was reduced to one hour at room temperature and was diluted to 1:400 (K & L). EP: endogenous peroxidise, NSB: non-specific binding, D/I: dilution/incubation. Bar length = 60 µm.

One reason why the use of antibodies for αv, αIIb, β3, and αvβ3 integrin was unsuccessful for immunodetection in FFPE human tumour xenograft and frozen tissues that is the use of mouse antibodies to detect the expression in
mouse tissue led to non-specific binding developing in sections without primary antibody. Therefore, the next step was to address this issue using a kit specifically designed to allow the use of mouse antibodies on mouse tissues.

2.3.2 Immunodetection of β3 and αvβ3 integrin subunits using an M.O.M. kit.

2.3.2.1 M.O.M. Immunodetection of β3 and αvβ3 integrins in FFPE sections.

The kit was first used on FFPE sections, where M14 sections were incubated with B7 (anti-β3), BV4 (anti-β3) or LM609 (anti-αvβ3), (changing variables as shown in Figure 2.8). Different conditions were tested in order to optimise final conditions of the above primary antibodies, such as dilution and incubation: B7 gave concentration-dependent membranous immunoreactivity at dilutions of 1:50 and 1:200. However, non-specific binding developed in the blood vessels in the sections incubated with the blocking serum instead of the B7 primary antibody, whereas sections without primary antibody and without a secondary antibody did not develop any non-specific binding. This result indicates that the non-specific binding that developed in the negative control (without primary antibody) without using the M.O.M. kit was due to the reaction of mouse secondary antibody on mouse tissue (Figure, 2.9 A - F).

The results of experiments on M14 xenograft sections immunolabelled with B7 were confirmed by immunolabelling other human xenograft tissue that
supposedly have different expressions of $\beta_3$ integrin; PC-3, DLD-1, HCT-116 and H460. As shown in Figure 2.10, low expression of $\beta_3$ was found in PC-3 (A and B), weak expression in DLD-1 (C and D) and H460 (G and H), and no expression was observed in HCT-116 (E and F).
Studies of FFPE tissue samples such as M14, using M.O.M kit

- Citrate buffer antigen retrieval
  - 3 × 5 minutes cycle
  - 3 × 6 minutes cycle
- Block endogenous peroxidase
  - 1 % H₂O₂
  - 3 % H₂O₂
- Primary antibodies B7 and BV4 (anti-β3) & LM609 (anti-αVβ3) used at dilution 1:50
  - 1 hour room temperature
  - Overnight 4 °C
  - Secondary antibody 1:250 for 10 minutes

BV4 and LM609 developed non-specific binding in sections without primary antibody. B7 developed non-specific binding in section without primary antibody mainly in blood vessels.

1. Section had 30 minutes antigen retrieval, 3% H₂O₂ treatment, immunolabelled with B7, 1:50, 1 hour at room temperature. Secondary antibody 1:250 for 10 minutes.

2. Same treatment as section 1 except using PBS instead of primary antibody.

3. Same treatment as section 1 except using PBS instead of primary antibody and secondary antibody.

M14 tissue had membranous expression of β3 integrin.

M14 tissue had non-specific binding in blood vessels only.

M14 tissue clean no reactivity observed.

Figure 2.8 IHC detection of α₅β₃ and β₃ integrin in human FFPE tumours with an M.O.M. kit
Membranous expression was observed in M14 sections treated for 30 minutes with citrate buffer as a method of antigen retrieval, blocked with 3% H₂O₂, immunolabelled with 1:50 B7 for one hour at room temperature (C) with clean section without primary antibody (B) just the blood vessels that showed non-specific binding. The non-specific binding disappeared when the same tissue (used in B & C) had no primary and no secondary antibody (A). 1:50 dilution of B7 overnight incubation at 4 °C, gave a high background signal (E) while a section without B7 had non-specific binding in blood vessels (D). Dilution of B7 to 1:200 and incubation at room temperature (F) changed the intensity of signal in comparison to Figure C. Bar length = 60 µm.
Figure 2.10 IHC detection of β3 integrin expression in FFPE human tumour xenografts in mouse using B7 with M.O.M. kit

All tumour tissues were treated with citrate buffer as a method of antigen retrieval for 30 minutes, blocked with 3% H$_2$O$_2$ and immunolabelled with B7 1:50 for 1 hour at room temperature. PC-3 (A & B), DLD-1 (C & D), HCT-116 (E & F) and H460 (G & H) were used as comparison with the expression of β3 seen in M14 tissue in Figure 2.9, C. Low expression of β3 was detected in PC-3 (A), very weak in DLD-1 (C) and H460 (G) and no expression in HCT-116 (E). Sections without B7 (B, D, F, H) developed non-specific binding in blood vessels in a similar manner to M14 (Figure 2.9, B). Bar length = 60 µm.
2.3.2.2 M.O.M. Immunodetection of $\beta_3$ and $\alpha_V\beta_3$ integrins in frozen sections.

It was hoped that the use of the M.O.M. kit would enable the characterisation of the $\beta_3$ expression in FFPE xenograft tissue. However, sections without primary antibody still showed non-specific binding. The same antibodies used on FFPE sections were used to characterise the integrin expression in frozen sections using the M.O.M. kit and optimisation of conditions described in Figure 2.11. The expression of $\beta_3$ was detected in PC-3 using BV4 (anti-$\beta_3$) (Figure 2.12). B7 (anti-$\beta_3$) and LM609 (anti-$\alpha_V\beta_3$) showed non-specific immunolabelling in both controls. BV4 (anti-$\beta_3$) was further used to investigate the expression of $\beta_3$ in other human tumour xenograft mouse tissues such as UACC-62, A549, H460, PANC-1, A2780 and SKOV-3, but non-specific positive immunolabelling resulted. The same non-specific immunolabelling was obtained with B7 (anti-$\beta_3$) and LM609 (anti-$\alpha_V\beta_3$).
Studies of frozen tissue samples using M.O.M

Fixation

Ice cooled acetone

4% PFA

Block with 0.1% H₂O₂

Block with 1% H₂O₂

BV4 (anti-β₃)

B7 (anti-β₃)

LM609 (anti-αᵦβ₃)

Primary antibody 1:50-1:400, 1 hour room temperature.

Non-specific immunolabelling developed in both controls

Increase blocking time from 1 hour to 2 hours

BV4: high membraneous expression with clean section without primary antibody in PC-3.

B-7 & LM609: non-specific immunolabelling developed in both controls.

Screen other tumours such as A549, H460, SKOV-3, A2780, PANC-1 & UACC-62.

Not all tumours screened developed the same membranous expression found in PC-3, further to non-specific binding developed in section without primary antibody.

Figure 2.11: IHC optimisation for αᵦβ₃ and β₃ integrin expression in frozen sections using the M.O.M. detection kit.
Figure 2.12 Expression of β₃ integrin in human tumour xenograft frozen tissues immunolabelled with BV4 (anti-β₃) using M.O.M. detection kit

Tissue was fixed with 4% PFA, treated with 1% H₂O₂, blocked for 2 hours and immunolabelled with 1:50 BV4 for one hour at room temperature. PC-3 had a membranous immunolabelling and no non-specific binding developed in section without primary antibody. Screening a panel of human tumour cell line xenografts in mouse did not give similar results. Non-specific immunolabelling developed in both sections with and without BV4. Bar length = 60 µm.
2.3.3 Detection of the expression of $\alpha_{\text{IIb}}$, $\alpha_V$, and $\beta_3$ subunits in homogenised tumour xenograft mouse tissue by immunoblotting

Integrin expression was also evaluated using immunoblotting (IMB) to characterise the expression of $\alpha_{\text{IIb}}$, $\alpha_V$, and $\beta_3$ integrin in human xenograft mouse tissue. IMB was optimised for each antibody, taking into consideration the type of lysis buffer, centrifugation speed and time, and protein concentration loaded in the gel. The optimisation steps are summarised in Figure 2.13. After the conditions for protein extraction had been optimised, the optimised method was used for detecting expression of $\alpha_V$ and $\beta_3$ integrin. No expression of $\alpha_{\text{IIb}}$ was detected, probably because the cell lines tested expressed $\alpha_{\text{IIb}}$ at negligible levels (in-house data). Once again, non-specific binding developed in protein from xenograft tissues with B7 (anti-$\beta_3$) whereas human tumour cell lines (Chapter 3) did not develop any non-specific binding under the same conditions (Figure 2.14).
Optimise protocol for protein extraction from human xenograft mouse tissue

1- Buffer

PBS

Lysis buffer

2- Centrifugation for different speed & time

- 9000 rcf, 20 minutes
- 20000 rcf, 20 minutes
- 100000 rcf, 1 hour

Supernatant collected

Pellet resuspended either in lysis buffer or PBS

PBS excluded

Lysis buffer selected at 9000 rcf, 20 minutes

Protein concentration

- 7.5 µg
- 25 µg
- 50 µg

It was selected as an optimum extraction of protein using lysis buffer and centrifuged using 9000 rcf for 20 minutes.

B7 (anti-β3), mouse antibody had non-specific binding with mouse tissue. Confirmed by incubation of the membrane without primary antibody (B7) and another membrane without primary antibody and without secondary antibody in presence of human tumour cell line that did not develop any non-specific binding.
β₃ expression was detected on the blot at 125 kDa. B7 mouse antibody had non-specific binding with M14 human xenograft tissue (Lane 2). Non-specific binding to mouse protein was confirmed by incubation the membrane without B7 (Lane 4) and another membrane without B7 and without secondary antibody (Lane 6). Protein extracted from M14 cells grown in vitro did not demonstrate any non-specific binding (Lane 3, 5 and 7).
2.3.3.1 Expression of α\textsubscript{v} and β\textsubscript{3} integrin in homogenised human xenograft mouse tissue compared to negative control tissue and human tumour cells

Once blotting conditions were optimised for each antibody, the expression level of α\textsubscript{v} and β\textsubscript{3} integrin subunits was evaluated in a panel of human tumours. Figure 2.15 shows a representative blot and analysis using the Molecular Imager FX System.
Expression of αV and β3 integrin related to β-actin control

In all membranes, 20 µg of M14 and HT-29 cell protein were loaded and 50 µg of homogenised tissue. A: Expression of αV detected using Q20 antibody, B: membrane treated same as A, but with PBS instead of Q20. C: Expression of β3 detected using B7 antibody bordered with red rectangle, D: membrane treated with PBS instead of B7; anti-mouse secondary antibody shows non-specific binding of mouse antibody on mouse tissue. Films were semiquantitatively analysed by Bio-Rad, Molecular Imager FX System. While αV was similarly expressed in all human xenograft tissues, β3 showed a range of expression with M14 and UACC-62 showed the highest expression followed by PC-3 and weaker expression was seen in DLD-1 and HCT-116 cells. Normal mouse liver tissue was negative for human integrins and used as a control here since it had non-specific binding with B7. Human tumour cell lines M14 and HT-29 used as a positive and negative control for β3 respectively. Results in the graph calculated from three independent experiments.
2.4 Discussion

The overall aim of this part of the study was to characterise $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin expression in human tumour xenograft tissue in mice. This is because the ultimate goals of the drug discovery project of which this thesis is a part require *in vivo* models that can be used to test the efficacy of novel integrin antagonists as drug targets in cancer therapy, and also to guide selection of cell lines for *in vitro* studies.

The expression of $\alpha_{\text{IIb}}$ and $\alpha_v\beta_3$ integrin in tissue can be evaluated by immunohistochemistry and immunoblotting (only $\alpha_{\text{IIb}}$ subunit was investigated because it hetrodimer with $\beta_3$ subunit only so expression of $\alpha_{\text{IIb}}$ subunit and $\beta_3$ indicated the expression of $\alpha_{\text{IIb}}\beta_3$). However, immunohistochemistry has been the more widely used of the two techniques reported in the literature because it gives information about specific location of integrin in tissue. In a study on clinical human tumours, archival FFPE tissue was utilized and immunohistochemistry was able to characterise the expression of $\alpha_v\beta_3$ integrin in glioma tissue, where it was associated with poor prognosis (Schittenhelm *et al.* 2013). Bisanz *et al.* used immunohistochemistry to detect the expression of $\alpha_v$ subunit. The study treated PC-3 cells with anti-$\alpha_v$ siRNA inhibitor and indicated role of $\alpha_v$ subunit mediated interaction of prostate cancer cells with bone stroma when the tumour cells were implanted in tumour as xenografts (Bisanz *et al.* 2005).

The expression of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin was initially investigated in FFPE human xenograft tissues using immunohistochemistry. However,
immunohistochemistry was unsuccessful due to non-specific immunolabelling that developed in the controls.

The problems experienced with immunohistochemistry may have been due to masking antigenic epitopes; thus, different antigen retrieval methods were tried. The use of citrate buffer as a method for antigen retrieval in human xenograft tissue was unsuccessful. As shown in the results section, the use of different primary antibodies did not reveal any that worked with the citrate buffer microwave method of antigen retrieval. Therefore, pepsin and proteinase K digestion were also tried.

Initially, LM609 was used to immunolabel different human xenograft mouse tissue treated with pepsin, but pepsin digestion was unsuccessful with LM609 and also with other primary antibodies tried, such as B7 and BV4 (anti-β3), C20 (anti-αIIb), and Q20 (anti-αv). Proteinase K antigen retrieval methods led to tissue damage. All three different methods of antigen retrieval were ineffective, due to the non-specific immunolabelling that developed in both controls. The problem with antigen retrieval seen in this study has been reported previously, where the embedding of tumour tissue in paraffin after fixation can block integrin detection, requiring the use of an antigen retrieval method to expose the integrin receptor to facilitate immunolabelling of the integrin receptor with the primary antibody (Liapis & Hutton 1997). The failure of the citrate buffer method in the present study was similar to that reported in another study where the citrate buffer antigen retrieval method was
unsuccessful for integrin detection in formalin-fixed tissue (Cattoretti et al. 1993).

The non-specific binding observed in this study likely resulted from cross-reactivity between the secondary antibody and endogenous mouse immunoglobulins. Both sections (with primary antibody and without primary antibody) developed immunoreactivity. These findings were confirmed by using different fresh frozen human xenograft tumour tissue, which also developed the same non-specific binding.

The detection of $\alpha_V\beta_3$ integrin in FFPE tissue remains a challenge. Goodman et al. have summarised the problems with currently available antibodies, the non specific staining developed in formalin fixed paraffin embedded tissue, and have developed new rabbit monoclonal antibodies for characterisation of integrin expression in archival FFPE human tumour tissue. Goodman’s results reflected the problem faced in the present study with currently available antibodies; LM609, BV4, B7 (mouse monoclonal antibodies) which failed to detect specific expression of $\alpha_V\beta_3$ and $\beta_3$ integrin in FFPE tissues (Boger et al. 2013).

The use of the M.O.M. immunodetection kit has been proposed as a solution for these issues (Jin et al. 2007). Using a mouse primary antibody in a mouse xenograft tissue produced background labelling. The M.O.M. kit is specifically designed to localize mouse primary antibodies in mouse tissues and to eliminate background immunolabelling. The M.O.M. kit has been used successfully with LM609 to detect the expression of $\alpha_V\beta_3$ integrin in the
frozen tissue of human IGROV-1 ovarian carcinoma nodules disseminated in the peritoneal cavity of a mouse (Garanger et al. 2005). Here, $\beta_3$ expression was detected using BV4 (anti-$\beta_3$) in cryo-sections, but not in paraffin sections, probably because the integrin epitopes are affected by fixation. BV4 (anti-$\beta_3$) showed $\beta_3$ subunit membranous immunolabelling, which was observed in a PC-3 frozen section with a M.O.M. kit, but the resulting expression was not reproducible. Furthermore, no expression was detected in other fresh frozen human tumour xenografts using the same protocol, although they were expected to express $\beta_3$; the heavy background obscured the results further to non-specific immunolabelling developed in sections without BV4 (anti-$\beta_3$) as a primary antibody.

The search for the $\beta_3$ subunit integrin in tissue was continued using another antibody B7 (anti-$\beta_3$), aiming to result in membranous immunolabelling with a clean negative control with a lack of non-specific binding. B7 gave a membranous immunolabelling, but without a clean negative control. Although a M.O.M. kit was used, the outcome was not changed: the same results were obtained with the use of mouse monoclonal antibodies BV4, B7, and LM609. This indicated that either non-specific immunolabelling developed in both controls was due to the use of mouse antibody on mouse tissue, or it was due to the inability of the primary antibodies used here to detect the expression of $\alpha_{\text{IIb}}\beta_3/\alpha_{\text{V}}\beta_3$ in FFPE and frozen tissue using immunohistochemistry.
The results obtained in the present study indicate an inability to detect the expression of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrin in xenograft tissue using immunohistochemistry. Liapis and Hutton used a panel of primary antibodies to detect the expression of $\alpha_2\beta_1$, $\alpha_V\beta_3$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, $\beta_1$, and $\beta_5$ integrin in FFPE human ovarian carcinoma, and reported that not all antibodies work with the microwave citrate buffer method of antigen retrieval, although they used clinical tissues (Laurens et al. 2009; Liapis & Hutton 1997; Singh et al. 2000).

The second reason for the failure of antibodies used in this study may be due to difficulties in characterising the expression of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrin in tumours grown in mouse tissue using a mouse antibody. The observation that indirect immunohistochemical detection with a primary antibody homologous with the test tissues usually results in non-specific background, has been mentioned in the literature; for example, Lu et al. reported that binding of a Fc fragment of secondary antibody to a mouse tissue component was the cause of background immunolabelling (Lu & Partridge 1998).

Due to the issues of non-specificity seen in the immunohistochemistry experiments, immunoblotting was also utilised to detect the expression of human $\alpha_V\beta_3$ integrin, in the presence of normal mouse tissue as a negative control. Expression of $\alpha_V$ and $\beta_3$ subunits was successfully detected using the immunoblotting technique in a panel of human tumour xenograft tissue; the xenograft tissues had varying levels of expression of $\beta_3$ whereas $\alpha_V$ was expressed in all human tumour xenograft tissues tested. Expression of $\beta_3$
was highest in melanoma xenograft tissue (UACC-62 and M14), followed by prostate adenocarcinoma (PC-3), which had moderate expression, with very weak expression detected in colonic carcinoma (DLD-1 and HCT-116). These results were confirmed by comparing them with normal mouse liver tissue that has no expression of human $\alpha_V$ and $\beta_3$ integrin subunits (Taylor et al. 2012).

The B7 antibody was able to reveal the expression of $\beta_3$ but gave an additional band just below the expected position of a band for $\beta_3$ expression, indicating non-specific binding. In order to confirm the non-specific binding developed with B7, several variables were tried such as incubating the membrane without B7 as a primary antibody and incubating another membrane without a primary antibody and without a secondary antibody. It was obvious that normal mouse liver tissue and human xenograft mouse tissue developed non-specific binding in a membrane incubated with anti-mouse secondary antibody only, due to the reaction of anti-mouse on mouse tissue.

Further confirmation came from using human tumour cell lines M14 and HT-29, which did not develop any non-specific binding in the blot membrane without B7 as a primary antibody. The lack of non-specific binding in cell lines indicates it is a problem with proteins present in mouse tissue, as shown in Figure 2.15.

In addition, rabbit antibody Q20 (anti-$\alpha_V$) did not show any non-specific binding in membrane without Q20 as a primary antibody, either in normal
mouse liver tissue or in human xenograft mouse tissue. This further confirms the results obtained with B7 (anti-β3).

The results convincingly revealed that M14, UACC-62, PC-3, HCT-116 and DLD-1 expressed αV. The expression of the αV integrin subunit has been detected by immunoblotting in other types of human tumour cells however, homogenised human tissue is rarely utilised to detect the expression of integrin using immunoblotting. A study done by Schnell et al. detected the αV and β3 integrin subunits in homogenised glioma tumour tissue using immunoblotting (Schnell et al. 2008). The non-specific binding observed in this study was also noted by Gouon et al. when immunoblotting was utilised to detect the expression of β3 integrin in HT-144 melanoma homogenised human xenograft mouse tissues (Gouon et al. 1996).

The expression of β3 varies among the different tissues, with the highest levels seen in melanoma cells such as UACC-62 and M14. Studies found strong β3 integrin expression was associated with the presence of tumour necrosis, increased tumour thickness, tumour ulceration, vascular invasion, tumour cell proliferation and high level of cytoplasmic p-cadherin. Significant association of both αV and β3 integrin with tumour necrosis was also associated with reduced overall survival in nodular melanoma (Bachmann et al. 2008; Hieken et al. 1999; Si & Hersey 1994). The expression of β3 is significant in malignancy, and αVβ3 integrin expression could be an important step with in situ melanoma development and invasion.
The expression of $\alpha_V$, $\beta_3$ subunits and $\alpha_{IIb}\beta_3$ integrin had previously been detected by other groups in human melanoma tissue embedded in paraffin (Trikha et al. 1997). Goodman has confirmed earlier work that found no $\beta_3$ integrin expression in colon carcinoma tissue (Goodman et al. 2012). This supports this study result where very weak expression of $\beta_3$ integrin in colon carcinoma xenograft tissue DLD-1 and HCT-116, was observed using immunoblotting, whilst the $\alpha_V$ subunit was detected in the same tissue through immunohistochemistry labelling. In addition, this previous study supports the finding of the present study where the $\alpha_V$ subunit was detected in all human xenograft tissues. Since no expression of $\beta_3$ integrin was observed, the $\alpha_V$ subunit must be present as a heterodimer with another $\beta$ subunit such as $\beta_1$, $\beta_5$ and $\beta_6$ (Agrez et al. 1996).

2.5 Conclusion

In conclusion, $\alpha_{IIb}$, $\alpha_V$, $\beta_3$, and $\alpha_V\beta_3$ integrin expression could not be detected in human xenograft tissue grown in mice using immunohistochemistry. However, using the immunoblotting technique, $\alpha_V$ and $\beta_3$ integrin expression could be successfully characterised in the panel of xenograft mouse tissues. Differential $\beta_3$ expression was seen over the panel of tumours, while $\alpha_V$ levels were similar across the panel whereas $\alpha_{IIb}$ could not be detected due to in ability of the primary antibody to work with IMB.
Chapter 3: Characterisation of integrin αIIbβ3 and αVβ3 expression in a panel of human tumour cell lines
Chapter 3

3.1 Introduction

Evaluation of any novel targeted therapy requires careful selection of appropriate models. In this thesis, the target is the β₃ integrins. A panel of cell lines with a range of expression levels of the different β₃ integrins is therefore required for the evaluation of novel integrin antagonists.

The expression of the β₃ integrins is widely reported in the literature in various human tumour cell lines and tissues, as reviewed in section 1.2.5. Whilst the findings of the previous chapter may give some guidance for the cell lines worthy of further investigation, the expression is not likely to be the same *in vitro* as *in vivo* for all cell lines evaluated (Taylor et al. 2012). Thus, this part of the study focused on characterisation of the expression in human tumour cell lines grown *in vitro*.

Ideally, a panel for novel therapeutic evaluation should include: 1) cell lines that express both αᵥβ₃ and αᵢᵢᵢβ₃ integrin; 2) cell lines that are negative for both αᵥβ₃ and αᵢᵢᵢβ₃ integrin; 3) cell lines that express αᵥβ₃ but are negative for αᵢᵢᵢβ₃; 4) cell lines that are negative for αᵥβ₃ but express αᵢᵢᵢβ₃ integrin.

The cell lines selected as models should ideally grow as a monolayer in cell culture flasks whilst maintaining the specific integrin expression. They must also have a reproducible expression of αᵢᵢᵢβ₃ and αᵥβ₃ integrins.

Optimisation of the protocols for the antibodies to be used in this part of the study first requires a cell line known to express αᵥβ₃ and αᵢᵢᵢβ₃ integrin as a
positive control. A number of researchers have investigated the expression of αVβ3 and αIIbβ3 in PC-3 cells, using different techniques for protein detection, such as flow cytometry and immunoblotting (Cooper et al. 2002; Trikha et al. 1998; Wang et al. 2005). αVβ3 integrin plays an important role in prostate cancer development and progression (Cooper et al. 2002; McCabe et al. 2007) and the expression of αVβ3 integrin has been detected in PC-3 cells using the LM609 antibody (Arosio et al. 2009; Chatterjee et al. 2001). In addition, Trikha et al. detected the expression of αIIb in PC-3 using the MAB 1990 antibody (Trikha et al. 1998).

3.1.1 Aims and objectives

The principle aim of this chapter will be to assemble a panel of cell lines that can be used for screening novel antagonists. This will be done through characterising integrin expression in human tumour cell lines.

The aims will be addressed using the following objectives:

1- Characterisation of the growth patterns of selected cell lines in order to optimise seeding densities for further experiments.

2- Optimisation of the immunoblotting protocols for the antibodies used to characterise the expression of αV, αIIb and β3 integrins.

3- Optimisation of the immunocytochemistry protocols for the antibodies used to characterise the expression of αV, αIIb, β3, and αVβ3 integrins.
4- Evaluation of the expression of $\alpha_V$, $\alpha_{IIb}$, $\beta_3$, integrin subunits in a panel of cell lines using the immunoblotting and immunocytochemistry protocols optimised in 2 and 3.
3.2 Materials and Methods

3.2.1 Materials

All general chemicals, media and media supplements were from Sigma-Aldrich (Poole, UK) unless otherwise specified. Primary antibodies and related secondary antibodies were used in this part of the study to detect expression of αV, αIIb, β3 and αVβ3 as summarised in Table 3.1. The blocking reagents (BSA, NRS, and NHS), and Vectashield hard-set mounting medium with DAPI were all from Vector Laboratories Ltd (Peterborough, UK). All cell lines used as summarised in Table 3.2 were stored in liquid nitrogen prior to use.

3.2.2 Methods

3.2.2.1 Cell maintenance

The cell lines were selected to represent a range of tumour types (Table 3.2). Cell culture procedures were performed in a sterile Micro-flow class II cabinet (NUAIRE, biological safety cabinet, Plymouth, UK).

Cells were routinely maintained as monolayer cultures in 75 cm² cell culture treated flasks (T75 flask) (Corning, Amsterdam, Netherlands) in 10 ml complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (FBS) 200 mM L-glutamine and 100 mM sodium pyruvate.
Table 3.1 Primary antibodies and related secondary antibodies used in immunocytochemistry (ICC) and IMB techniques.

<table>
<thead>
<tr>
<th>PAb</th>
<th>R</th>
<th>Type</th>
<th>Source</th>
<th>2\textsuperscript{nd} Ab (IMB)</th>
<th>Source</th>
<th>2\textsuperscript{nd} Ab (ICC)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV4</td>
<td>Anti-β\textsubscript{3}</td>
<td>MMAb</td>
<td>BV4 ab 7167, Abcam, Cambridge, UK</td>
<td>PRAM, Ig HRP</td>
<td>P0260, Dako Cytomation, Glostrup, Denmark</td>
<td>PRAM Igs, TRITC</td>
<td>R0270, Dako Cytomation Glostrup, Denmark</td>
</tr>
<tr>
<td>B7</td>
<td>Anti-β\textsubscript{3}</td>
<td>MMAb</td>
<td>Clone B7, SC-46655, Santa Cruz Biotechnology, Santa Cruz, USA</td>
<td>PRAM, Ig HRP</td>
<td>P0260, Dako Cytomation, Glostrup, Denmark</td>
<td>PRAM Igs, TRITC</td>
<td>R0270, Dako Cytomation Glostrup, Denmark</td>
</tr>
<tr>
<td>C20</td>
<td>Anti-α\textsubscript{IIb}</td>
<td>PGAb</td>
<td>SC-6602, Santa Cruz Biotechnology, Santa Cruz, USA</td>
<td>Goat Igs</td>
<td>P0449, Dako Cytomation, Glostrup, Denmark</td>
<td>PRAG Igs, FITC</td>
<td>F0250, Dako Cytomation Glostrup, Denmark</td>
</tr>
<tr>
<td>P2W7</td>
<td>Anti-α\textsubscript{v}</td>
<td>MMAb</td>
<td>SC-9969, Santa Cruz Biotechnology, Santa Cruz, USA</td>
<td>PRAM, Ig HRP</td>
<td>P0260, Dako Cytomation, Glostrup, Denmark</td>
<td>PRAM Igs, TRITC</td>
<td>R0270, Dako Cytomation Glostrup, Denmark</td>
</tr>
<tr>
<td>Q20</td>
<td>Anti-α\textsubscript{v}</td>
<td>PRAb</td>
<td>SC-6617-R, Santa Cruz Biotechnology, Santa Cruz, USA</td>
<td>PGAR, Ig HRP</td>
<td>P0448, Dako Cytomation, Glostrup, Denmark</td>
<td>GAR, Alexa fluor 546</td>
<td>A-11010, Invitrogen, California, CA, USA</td>
</tr>
<tr>
<td>LM609</td>
<td>Anti-α\textsubscript{v}β\textsubscript{3}</td>
<td>MMAb</td>
<td>Clone LM609, MAB 1976, Chemicon-Millipore, Watford, UK</td>
<td>-</td>
<td>-</td>
<td>PRAM Igs, TRITC</td>
<td>R0270, Dako Cytomation Glostrup, Denmark</td>
</tr>
<tr>
<td>A1978</td>
<td>Anti-β-actin</td>
<td>MMAb</td>
<td>Sigma-Aldrich, Poole, UK</td>
<td>PRAM, Ig HRP</td>
<td>P0260, DAKO Cytomation, Glostrup, Denmark</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The cells were maintained at 37 °C in humidified conditions and a 5% CO$_2$ atmosphere. When the cells attained 75 to 80% confluence, they were passaged by discarding the medium and rinsing the contents of the flask twice with 10 ml of Hank’s Balanced Salt Solution (HBSS), prior to trypsinisation using 3 ml of a 0.25% trypsin-EDTA solution, and incubating at 37 °C for three to five minutes. When cells became detached from the flask, 10 ml of medium was added to deactivate the action of trypsin/EDTA. The trypsinised cells were then centrifuged for five minutes at 1000 rcf at room temperature (Heraeus Megafuge 1.0 centrifuge, DJB Labcare Ltd. UK). The resulting supernatant was discarded and cell pellet was resuspended in 10 ml of fresh complete culture medium. Cells were then counted with 10 µl of cell suspension placed in each chamber of a Neubauer Haemocytometer (7201004, VWR International Ltd. Poole, UK). An inverted microscope (Olympus, CK2, x-20 objective lens magnification) was used to count cells in ten 1 mm$^2$ areas of the haemocytometer and the mean count was calculated. Cell counts were then expressed as (mean cell count) $\times 10^4$ cells/ml medium, and the required amount of cell suspension was seeded into new flasks containing the appropriate amount of complete medium.
Table 3.2 Cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Identification code</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>Human non small cell lung carcinoma (NSCLC)</td>
<td>ATCC CCL-185</td>
</tr>
<tr>
<td>DLD-1</td>
<td>Human colorectal adenocarcinoma</td>
<td>ATCC CCL-221</td>
</tr>
<tr>
<td>DU-145</td>
<td>Prostate adenocarcinoma</td>
<td>ATCC HTB-81</td>
</tr>
<tr>
<td>H460</td>
<td>Human NSCLC</td>
<td>ATCC HTB-177</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Human colorectal carcinoma</td>
<td>ATCC CCL-247</td>
</tr>
<tr>
<td>HT-29</td>
<td>Human colorectal adenocarcinoma</td>
<td>ATCC HTB-38</td>
</tr>
<tr>
<td>M14</td>
<td>Human melanoma</td>
<td>NCI-0507466</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast adenocarcinoma cell line from</td>
<td>ATCC HTB-22</td>
</tr>
<tr>
<td></td>
<td>metastatic site: pleural effusion</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>Human prostate adenocarcinoma derived from</td>
<td>ATCC CRL-1435</td>
</tr>
<tr>
<td></td>
<td>grade IV cancer metastasised to bone</td>
<td></td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>Human malignant melanoma</td>
<td>ATCC HTB-68</td>
</tr>
<tr>
<td>UACC-62</td>
<td>Human malignant melanoma</td>
<td>NCI-0507381</td>
</tr>
</tbody>
</table>

Key: ATCC: American Type Culture Collection. NCI: National Cancer Institute

3.2.2.2 Tumour cell line growth kinetics

The growth kinetics of all the tumour cell lines used in this study were characterised to determine when cells would be in a log phase and hence be functionally active. Cells were seeded at $1 \times 10^4$ cells/ml in 5 ml fresh complete medium in T25 flasks, and were maintained at 37 °C, 5% CO₂ as described above. Cell counts were performed over 7 days and growth curves plotted. Growth curves were carried out in this study using cells from different passages. Readings are reported as the average (± SD) recorded from three different flasks per day.

3.2.2.3 Immunoblotting

Immunoblotting (IMB) was carried out using whole cell lysates in order to characterise the expression of $\alpha_v$, $\alpha_{IIb}$, and $\beta_3$ integrin in a panel of human tumour cell lines.
3.2.2.3.1 Cell Lysis

Once the cell lines reached 70 to 80% confluence, cells were scraped from the flask surface after a gentle wash with HBSS. Cells were centrifuged (Eppendorf centrifuge, G417R, Natheler-Hizz, Hamburg, Germany) for five minutes at 1000 rcf, the cell pellet was washed three times with PBS for four minutes with centrifuging (4600 rcf) after each wash. Lysis buffer (2X lysis buffer [100 mM Tris pH 8.8, 5 mM EDTA, 300 mM NaCl, 2% Triton X-100], protease inhibitor (Complete mini EDTA-free (Roche, Mannheim, Germany) and water) was applied for one hour on ice, after which sonication (Philip Harries Scientific Sonicator, Scientific Laboratory) was carried out for two pulses of five seconds each. Sonicated cells were centrifuged at 9000 rcf for ten minutes at -20 °C, after which the cytosolic supernatant of the lysed cells was collected and the cellular pellet resuspended in fresh lysis buffer. Since functional integrin receptors span the cell membrane, but can also be endocytosed or present in a storage pool within the cell both the plasma membrane (cell pellet) and cytosolic content were collected here for further confirmation of their integrin expression.

3.2.2.3.2 IMB of nitrocellulose membrane with different anti αV, αIIb and β3 integrin antibodies

The Bradford assay, SDS-polyacrylamide gel electrophoresis and immunoblotting were carried out using the same conditions as described in Chapter 2. In this part of the study, the primary antibodies were optimised to characterise expression of αIIb, αV and β3 integrin in human tumour cell lines.
With the initial optimisation protocol for each antibody, the nitrocellulose membranes were blocked with either 5% non-fat dried milk in PBS-buffered saline (with 0.05% Brij, 35 solution), or 5% BSA/PBS, for one hour at room temperature. Then, the primary antibody as summarised in Table 3.1 was applied at different dilutions ranging from 1:200 to 1:5000 for either one hour at room temperature or overnight at 4 °C; each antibody had its own optimum dilution and incubation condition (Table 3.4). The membrane was washed with PBS (0.05% Brij) (3 washes of 10 minutes each). The recommended secondary antibody (Table 3.1) was applied in the dilution range from 1:1000 to 1:5000 for one hour at room temperature followed by washing with PBS (0.05% Brij) (3 washes for 5 minutes). The ECL Plus Western blotting detection system (NEL104, Perkin Elmer LAS) reagents A and B were mixed in 1:1 ratio and applied to membrane for one minute. The membrane was exposed to Kodak Scientific Imaging film in the dark for five to thirty minutes. Films were developed using Ilford Rapid Multigrade developer (1/10 dilution) for three minutes, rinsing with water and fixing in Ilford Rapid Film and Paper fixer solution (1/10 dilution) for two minutes. The immunoblotting was repeated three times to confirm the reproducibility of the optimised protocol.
3.2.2.4 Immunocytochemistry (ICC)

3.2.2.4.1 Detection of αV, αIIb and β3 subunits, and αVβ3 integrin expression in different tumour cell lines by ICC

3.2.2.4.1.1 Materials

All materials used here were the same as described in section 3.2.1 with the addition to the primary antibody panel of LM609, anti-αVβ3 integrin.

3.2.2.4.1.2 Cell preparation

In order to detect the expression of αV, αIIb and β3 subunits and αVβ3 integrin, 1 × 10^4 to 1 × 10^5 cells/ml were seeded on autoclaved 22mm × 22mm cover slips in a six-well plate and incubated at 5% CO₂, 37 °C until cells were seen adhering to the cover slip when viewed under the microscope no more than 12 hours. The medium was removed and the cells washed for two washes of two minutes with 1 ml HBSS.

Cells were fixed with either ice pre-cooled methanol for twenty minutes at -20 °C, 1% PFA or 4% PFA for ten minutes at room temperature, or four to five dips with ice pre-cooled acetone. The fixative was removed and the cells allowed to air dry. Fixed cells were either used for immunolabelling directly or stored at -20 °C. Following rehydration, blocking was followed by direct application of primary antibody (Table 3.1) at different dilutions and incubation conditions followed by washing for 3 washes for 5 minutes each (Table 3.3). The secondary antibody (Table 3.1) was then applied at different incubations times after labelling with secondary antibody, and cells washed
for 3 washes for 5 minutes each as shown in Table 3.3. Cells were mounted with Vectashield with DAPI and kept at 4 °C until dry and examined by fluorescence microscope (Leica DMRB, Leica Microsystems, Wetzlar, Germany).

3.2.2.4.1.3 Confocal microscopy

Confocal microscopy was used in order to confirm the expression observed by fluorescent microscope. Cells were prepared as in section 3.2.2.4.1.2 and were analysed and images captured with a Zeiss LSM510 confocal system attached to an Axiovert 200 M inverted microscope using LSM510 software (all from Zwiss, Welwyn Garden City, UK).

3.2.2.4.1.4 ICC analysis

Semi-quantitative analysis of integrin expression in the tumour cells was done by counting cells in 10 fields at 40 magnification, and giving a score to the labelling of each cell dependent upon its intensity. The expression intensity scoring scheme was confirmed by an independent observer. The high expression was designated as ++++, moderate ++, low + and weak ±. The mean intensity was calculated for each sample.
Table 3.3 Conditions investigated while optimising detection of expression of αV, αIIb, β3 and αVβ3 integrin in human tumour cell lines using ICC.

<table>
<thead>
<tr>
<th>PAb</th>
<th>Anti-β3 subunit, BV4</th>
<th>Anti-β3 subunit, B7</th>
<th>Anti-αV subunit, Q20</th>
<th>Anti-αIIb subunit, C20</th>
<th>Anti-αVβ3 integrin, LM609</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehydration</td>
<td>PBS 5 minutes, R.T.</td>
<td>PBS 5 minutes, R.T.</td>
<td>PBS 5 minutes, R.T.</td>
<td>PBS 5 minutes, R.T.</td>
<td>PBS 5 minutes, R.T.</td>
</tr>
<tr>
<td>Blocking</td>
<td>2 &amp; 15% NRS/PBS, 10 minutes, R.T.</td>
<td>2% NRS/PBS, 10 minutes, R.T., or 5% BSA/PBS, 1 hour, R.T.</td>
<td>2% NGS/PBS, 10 minutes, R.T., or 5% BSA/PBS, 1 hour, R.T.</td>
<td>2% NRS/PBS, 10 minutes, R.T., or 1-5% BSA/PBS, 1 hour, R.T.</td>
<td>2% NRS/PBS, 10 minutes, R.T., or 5% BSA/PBS, 1 hour, R.T.</td>
</tr>
<tr>
<td>Primary antibody dilution</td>
<td>1:50 - 1:800</td>
<td>1:20 - 1:400</td>
<td>1:25 - 1:400</td>
<td>1:20 - 600</td>
<td>1:50 – 1:400</td>
</tr>
<tr>
<td>Primary antibody incubation</td>
<td>30 minutes &amp; 1 hour at R.T., or 1 hour at 37 °C, or overnight at 4 °C</td>
<td>30 minutes &amp; 1 hour at R.T., or 1 hour at 37 °C, or overnight at 4 °C</td>
<td>30 minutes at R.T., or 1 hour at 37 °C, or overnight at 4 °C</td>
<td>30 minutes &amp; 1 hour at R.T., or 1 hour at 37 °C, or overnight at 4 °C</td>
<td>30 minutes &amp; 1 hour at R.T., or 1 hour at 37 °C, or overnight at 4 °C</td>
</tr>
<tr>
<td>Secondary antibody dilution</td>
<td>PRAM, TRITC 1:50/PBS</td>
<td>PRAM, TRITC 1:50/PBS</td>
<td>GAR IgG, Alexa fluor 546, 1:50 in PBS, or 1:50 in 5% BSA/PBS</td>
<td>PRAG, FITC, 1:50 in PBS, or 1:50 in 5% BSA/PBS, or 1:50 in 1% BSA/PBS</td>
<td>PRAM, TRITC 1:50 in PBS, or 1:50 in 5% BSA/PBS</td>
</tr>
<tr>
<td>Secondary antibody incubation</td>
<td>30 minutes to 1 hour in dark</td>
<td>30 minutes to 1 hour in dark</td>
<td>30 minutes to 1 hour in dark</td>
<td>30 minutes to 1 hour in dark</td>
<td>30 minutes to 1 hour in dark</td>
</tr>
<tr>
<td>Washing after primary and secondary antibody</td>
<td>PBS (3 × 5 minutes)</td>
<td>PBS or 5% BSA/PBS (3 × 5 minutes)</td>
<td>PBS or 5% BSA/PBS (3 × 5 minutes)</td>
<td>PBS or 5% BSA/PBS or 1% BSA/PBS (3 × 5 minutes)</td>
<td>PBS or 5% BSA/PBS (3 × 5 minutes)</td>
</tr>
</tbody>
</table>

3.3 Results

3.3.1 Characterisation of cellular growth kinetics

Growth rates varied between tumour cell lines as demonstrated by differences in their growth curves and the duration of lag, log and plateau phases.

Most of the cell lines had a lag phase, except DU-145 and DLD-1, which were started in log phase and showed exponential growth from the first day. Cell lines were in plateau phase once the cells became confluent except DLD-1 and PC-3 which continued in log phase until day 7. Figure 3.1 shows the growth curves obtained for the cell panel.
Figure 3.1 Growth parameters of human tumour cell lines
Growth parameters were obtained as described in section 3.3.1.
Values correspond to the mean ± SD of 3 independent experiments.
3.3.2 Detection of $\alpha_v$, $\alpha_{\text{IIb}}$, and $\beta_3$ integrins using IMB

3.3.2.1 Optimisation of IMB

In order to optimise the IMB protocols for each antibody used here, different variables were tried. These variables as shown in the flowchart in Figure 3.2 included: protein concentrations, blocking the non-specific binding of primary antibody, primary antibody dilution and incubation time, and film exposure time. All primary antibodies detected expression of integrin at a protein concentration of 20 µg except C20 (anti-$\alpha_{\text{IIb}}$). C20 did not detect expression even when using a protein concentration of 100 µg. B7 (anti-$\beta_3$) and Q20 (anti-$\alpha_v$) were able to detect expression in membrane blocked with 5% BSA/PBS whereas the rest of the antibodies worked only with 5% non-fat milk. The different dilutions and incubations tried for primary antibodies clearly showed that 1:2000 dilution, overnight incubation at 4 °C was optimum for B7 (anti-$\beta_3$) and Q20 (anti-$\alpha_v$) whereas 1:500 at room temperature for two hours was optimum for the rest of the antibodies. All antibodies detected expression after five minutes exposure time except C20 (anti-$\alpha_{\text{IIb}}$) which did not detect clear expression even if exposed for thirty minutes. Thus no further work was carried out for this antibody and hence attempts at detecting $\alpha_{\text{IIb}}$. Figure 3.2 shows an example of a representative blot (three independent experiments were done for each antibody using optimum conditions for each antibody as summarised in Table 3.4). From the above results B7 (anti-$\beta_3$) and Q20 (anti-$\alpha_v$) were identified as specific antibodies that can be used to screen the expression of $\alpha_v$ and $\beta_3$ integrin in
a panel of different tumour cell lines using the optimised protocol as summarised in Table 3.4.

Figure 3.2 Optimisation of IMB protocol.
Expression of αV, αIIb and β3 were detected in PC-3 cells using different antibodies. Q20 and P2W7 were anti-αV, C20 anti- αIIb, and BV4 and B7 were anti-β3. Protein concentrations, blocking conditions, PAAb dilutions and incubations and film exposing time were optimised for each antibody. Key: Nc: no clear band. Mb: multiple bands. Db: double bands. Sb: single band.
Table 3.4 The optimised protocol for all antibodies used to detect the expression of αV, αⅡb, and β3 subunit and β-actin using IMB technique.

<table>
<thead>
<tr>
<th>Immunolabelling conditions</th>
<th>BV4 anti-β3</th>
<th>B7 anti-β3</th>
<th>P2W7 anti-αV</th>
<th>Q20 anti-αV</th>
<th>C20 anti-αⅡb</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration</td>
<td>20 µg</td>
<td>20 µg</td>
<td>20 µg</td>
<td>20 µg</td>
<td>100 µg</td>
<td>20 µg</td>
</tr>
<tr>
<td>Blocking</td>
<td>5% non-fat milk, 1 hour R.T.</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
<td>5% non-fat milk, 1 hour R.T.</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
<td>5% non-fat milk, 1 hour R.T.</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
</tr>
<tr>
<td>Primary antibody Dilution/Incubation</td>
<td>1:500 in 5% non-fat milk, 2 hour R.T.</td>
<td>1:2000 in 5% BSA/PBS, overnight 4 °C</td>
<td>1:500 in 5% non-fat milk, 2 hour R.T.</td>
<td>1:2000 in 5% BSA/PBS, overnight 4 °C</td>
<td>1:500 in 5% non-fat milk, 2 hour R.T.</td>
<td>1:5000 in 5% BSA/PBS, overnight 4 °C</td>
</tr>
<tr>
<td>Washing</td>
<td>PBS (0.05 % Brij), (3 × 5 minutes)</td>
<td>PBS (0.05 % Brij), (3 × 10 minutes)</td>
<td>PBS (0.05 % Brij), (3 × 5 minutes)</td>
<td>PBS (0.05 % Brij), (3 × 10 minutes)</td>
<td>PBS (0.05 % Brij), (3 × 5 minutes)</td>
<td>PBS (0.05 % Brij), (3 × 10 minutes)</td>
</tr>
<tr>
<td>Washing</td>
<td>PBS (0.05% Brij), (3 × 5 minutes)</td>
<td>PBS (0.05% Brij), (3 × 5 minutes)</td>
<td>PBS (0.05% Brij), (3 × 5 minutes)</td>
<td>PBS (0.05% Brij), (3 × 5 minutes)</td>
<td>PBS (0.05% Brij), (3 × 5 minutes)</td>
<td>PBS (0.05% Brij), (3 × 5 minutes)</td>
</tr>
<tr>
<td>Exposing</td>
<td>5 minutes</td>
<td>5 minutes</td>
<td>5 minutes</td>
<td>5 minutes</td>
<td>30 minutes</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Result</td>
<td>Double bands</td>
<td>Single band size 125 KDa</td>
<td>Multiple bands</td>
<td>Single band size 132 KDa</td>
<td>No detectable band</td>
<td>Single band size 42 KDa</td>
</tr>
</tbody>
</table>

3.3.2.2 Screening different tumour cell lines for $\alpha_V$ and $\beta_3$ integrin expression by IMB technique using the optimised conditions for anti-$\alpha_V$, Q20, and anti-$\beta_3$, B7, antibodies

In the preceding section, two antibodies were identified as giving reliable and specific detection of integrin subunits, Q20, anti-$\alpha_V$ and B7, anti-$\beta_3$. These were therefore used to screen the extended cell line panel. The expression of $\alpha_V$ and $\beta_3$ was detected in the supernatant (Figure 3.3), as well as in the pellet resuspended in lysis buffer (Figure 3.4) indicating expression of the integrin subunits in both the cell membrane and cytosol. $\alpha_V$ was expressed in all cell lines with HCT-116 demonstrating a notably lower expression, whereas $\beta_3$ expression levels showed more variation between cell lines. The melanoma cell lines UACC-62, M14 and SK-MEL-2 had the highest expression of $\beta_3$ subunit. Moderate expression of $\beta_3$ was seen for the prostate lines PC-3 and DU-145, whilst weak expression was seen in the NSCLC lines, A549 and H460 followed by the colon lines DLD-1 and HCT-116 and very weak in breast line MCF-7. As would be expected from earlier studies (section 2.3.3.1), no expression was seen in the HT-29 colon line which used here as a negative control.
Figure 3.3 Expression of α\text{V} and β\text{3} integrin in a panel of human tumour cell lines from a supernatant

A. Expression of α\text{V} and β\text{3} integrin subunits in a panel of human tumour cell lines. All cell lines expressed the α\text{V} integrin subunit. Expression of β\text{3} integrin was highest in M14 followed by UACC-62 and SK-MEL-2. Moderate expression of β\text{3} integrin was seen in the PC-3 and DU-145. A549, H460, DLD-1 and HCT-116 and MCF-7 showed weak expression of β\text{3} integrin. HT-29 was negative with no clear band observed. α\text{V} integrin was expressed at molecular weight 132 kDa and β\text{3} at 125 kDa. Data shown are representative of at least three independent experiments. Lane 1 is Page Ruler Plus Prestained Protein Ladder marker range from 10 to 250 kDa but because of a short exposure time of five minutes it is not clearly seen on the film. B. Results are presented as mean ± SD of three independent experiments. Films analysed using Bio Rad Molecular Imager FX. The expression of α\text{V} and β\text{3} band density and β-actin band density were subtracted from the film background density. α\text{V} and β\text{3} signal intensity were divided by β-actin signal intensity to obtain relative expression levels.
Figure 3.4 Expression of $\alpha_V$ and $\beta_3$ integrin protein in a panel of human tumour cell lines from a pellet resuspended in lysis buffer

A. Expression of $\alpha_V$ and $\beta_3$ integrin subunit in the panel of human tumour cell lines pellets resuspended in lysis buffer. Expression in the cell pellet showed that $\alpha_V$ and $\beta_3$ subunit levels correlated with the expression found in cell supernatant (Figure 3.3) except UACC-62 and PC-3 cell lines where the expression was higher in their supernatant. B. Results are presented as mean ± SD of three independent experiments. Films were analysed as described in Figure 3.3.
3.3.3 Detection of $\alpha_v$, $\alpha_{IIb}$, $\beta_3$ and $\alpha_v\beta_3$ integrins using ICC

3.3.3.1 Optimisation of ICC

Whilst IMB identifies the expression of a specific integrin subunit within a cell line, it does not give any information as to the localisation of the integrin expression within the cell and further cannot detect intact heterodimers. Therefore, the ICC technique was also used to evaluate the cell panel. In order to optimise the detection of expression of $\alpha_v$, $\alpha_{IIb}$, $\beta_3$ and $\alpha_v\beta_3$ by ICC, several variables were evaluated as shown in Figure 3.5. The optimised protocol for each antibody is summarised in Table 3.5.
Figure 3.5 Detection of αv, αIIbβ3 and αvβ3 integrin in PC-3 using ICC. Different antibodies: Q20 and P2W7 anti-αv, BV-4, and B7 anti-β3, C20, anti-αIIb, and LM609, anti-αvβ3 integrin were used to immunolabel PC-3 cells using the optimum conditions for each antibody as summarised in Table 3.5. Bar length = 60 µm.
KEY: ICM: ice-cooled methanol, ICA: ice-cooled acetone, PFA: paraformaldehyde.
Table 3.5 Optimised protocols for all antibodies used to detect the expression of α\textsubscript{v}, α\textsubscript{IIb}, β\textsubscript{3} subunits, and α\textsubscript{v}β\textsubscript{3} integrin in cell membrane using ICC

<table>
<thead>
<tr>
<th>Labelling Conditions</th>
<th>P2W7 (anti-α\textsubscript{v})</th>
<th>Q20 (anti-α\textsubscript{v})</th>
<th>C20 (anti-α\textsubscript{IIb})</th>
<th>BV4 (anti-β\textsubscript{3})</th>
<th>B7 (anti-β\textsubscript{3})</th>
<th>LM609 (anti-α\textsubscript{v}β\textsubscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>4% PFA, 10 minutes R.T.</td>
<td>4% PFA, 10 minutes R.T.</td>
<td>1 or 4% PFA, 10 minutes R.T. or ICM 20 minutes at -20 °C</td>
<td>ICM, 20 minutes at -20 °C</td>
<td>4% PFA, 10 minutes at R.T.</td>
<td>4% PFA, 10 minutes at R.T.</td>
</tr>
<tr>
<td>Blocking</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
<td>1% BSA/PBS, 1 hour R.T.</td>
<td>2% NRS/PBS, 10 minutes R.T.</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
<td>5% BSA/PBS, 1 hour R.T.</td>
</tr>
<tr>
<td>PAb D/I</td>
<td>1:50 in 5% BSA/PBS, overnight 4 °C</td>
<td>1:50 in 5% BSA/PBS, overnight 4 °C</td>
<td>1:50 in 1% BSA/PBS, overnight 4 °C</td>
<td>1:50 in 2% NRS/PBS, 30 minutes R.T.</td>
<td>1:50 in 5% BSA/PBS, overnight 4 °C</td>
<td>1:50 in 5% BSA/PBS, overnight 4 °C</td>
</tr>
<tr>
<td>2\textsuperscript{nd} Ab D/I</td>
<td>TRITC, 1:50 in PBS, 30 minutes in dark</td>
<td>TRITC, 1:50 in 5% BSA/PBS, 1 hour in dark</td>
<td>TRITC, 1:50 in 5% BSA/PBS, 1 hour in dark</td>
<td>TRITC, 1:50 in 5% BSA/PBS, 1 hour in dark</td>
<td>TRITC, 1:50 in 5% BSA/PBS, 1 hour in dark</td>
<td></td>
</tr>
<tr>
<td>Washing</td>
<td>PBS (3 × 5 minutes)</td>
<td>5% BSA/PBS (3 × 5 minutes)</td>
<td>1% BSA/PBS (3 × 5 minutes)</td>
<td>5% BSA/PBS (3 × 5 minutes)</td>
<td>5% BSA/PBS (3 × 5 minutes)</td>
<td>5% BSA/PBS (3 × 5 minutes)</td>
</tr>
</tbody>
</table>

Key: ICM: ice pre-cooled methanol, PAb: primary antibody, 2\textsuperscript{nd} Ab: secondary antibody, D/I: dilution/ incubation.
3.3.3.2 Evaluation of the effect of method of cell harvesting on integrin expression

Trypsinisation of cells can affect integrin expression (Brown et al. 2007). Since cells were harvested by scraping for IMB and trypsinisation for ICC, any effect on the integrin expression was investigated to ensure correlation between expression of integrins by both techniques. Both scraped and trypsinised PC-3 cells were immunolabelled with LM609 (anti-\(\alpha_V\beta_3\)) following the optimum ICC protocol (Table 3.5). As shown in Figure 3.6, there was no obvious difference in \(\alpha_V\beta_3\) integrin expression between scraped and trypsinised PC-3 cells using ICC.

Figure 3.6 Comparison of cell harvesting techniques
No difference was seen in \(\alpha_V\beta_3\) integrin expression on the membrane of PC-3 cells which had been scraped (A) and trypsinised (B). Cells were immunolabelled with LM609 using ICC. Bar length = 60 \(\mu\)m.
3.3.3.3 Screening different cell lines for expression of $\alpha_v$, $\beta_3$, $\alpha_{llb}$ and $\alpha_v\beta_3$ integrins by ICC technique.

A panel of tumour cell lines was tested for the expression of $\alpha_v$, $\beta_3$, $\alpha_{llb}$ subunits and $\alpha_v\beta_3$ integrin immunoreactivity according to the optimised protocols (Table 3.5). Following optimisation of the antibodies for ICC, the panel of cell lines was screened for expression of $\alpha_v$, $\beta_3$, $\alpha_{llb}$ subunits and $\alpha_v\beta_3$ integrin (Figure 3.7 and 3.8).
The antibodies were used for immunodetection of αV, αIIb, β3 subunits and αVβ3 integrin in the panel of human tumour cell lines (A549, H460, DU-145, PC-3, M14 and SK-MEL-2) according to protocols optimised above. Apart from C20, all samples were counterlabelled with DAPI (blue) to visualize cell nuclei. Bar length = 60 µm
Figure 3.8 Screening of the second part of the cell line panel with anti-α\text{\textsubscript{v}} (Q20), anti-α\text{\textsubscript{\ll}} (C20), anti-β\text{\textsubscript{3}} (B7), and anti-α\text{\textsubscript{v}}β\text{\textsubscript{3}} (LM609) using ICC. The antibodies were used for immunodetection of α\text{\textsubscript{v}}, α\text{\textsubscript{\ll}}, β\text{\textsubscript{3}} subunits and α\text{\textsubscript{v}}β\text{\textsubscript{3}} integrin in the panel of human tumour cell lines (UACC-62, MCF-7, DLD-1, HCT-116 and HT-29) according to protocols optimised above. Apart from C20, all samples were counterlabelled with DAPI (blue) to visualize cell nuclei. Bar length = 60 µm.
3.3.3.4 Analysis of the expression of $\alpha_V$, $\alpha_{\text{IIb}}$, $\beta_3$ and $\alpha_V\beta_3$ integrin in different human tumour cell lines detected by ICC.

After screening a panel of human tumour cell lines for the expression of $\alpha_V$, $\alpha_{\text{IIb}}$, $\beta_3$ and $\alpha_V\beta_3$ integrin using ICC technique, the expression was as either: high expression, moderate expression, low expression, weak expression or no expression. Table 3.6 summarises the expression seen in all cell lines based on three independent experiments for each cell line.

**Table 3.6 Screening different tumour cell lines for the membranous expression of $\alpha_V$, $\beta_3$, $\alpha_{\text{IIb}}$ and $\alpha_V\beta_3$ by ICC**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>BV4 anti-(\beta_3)</th>
<th>B7 anti-(\beta_3)</th>
<th>Q20 anti-(\alpha_V)</th>
<th>LM609 anti-(\alpha_V\beta_3)</th>
<th>C20 anti-(\alpha_{\text{IIb}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>+</td>
<td>±</td>
<td>+++</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>H460</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Colon cancer cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLD-1</td>
<td>++</td>
<td>±</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>HCT-116</td>
<td>++</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>HT-29</td>
<td>±</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Prostate adenocarcinoma cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU-145</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PC-3</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Melanoma cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M14</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>UACC-62</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Breast carcinoma cell line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>+</td>
<td>±</td>
<td>+++</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

3.3.3.5 Confirmation of sub-cellular immunolocalisation of $\alpha_{IIb}$, $\beta_3$ and $\alpha_V\beta_3$ integrin using confocal microscopy.

The sub-cellular localisation of expression of $\alpha_{IIb}$, $\beta_3$ and $\alpha_V\beta_3$ integrins were confirmed using ICC by observing the expression using confocal microscopy. As shown in Figure 3.9, membranous expression of $\alpha_V\beta_3$ integrin and the $\beta_3$ subunit were observed in human tumour cells with high, moderate, and weak expression of $\beta_3$, $\alpha_V\beta_3$, and $\alpha_{IIb}$ integrin according to IMB and ICC result for M14, PC-3, and MCF-7.
Figure 3.9 The expression of $\alpha_{\text{IIb}}$, $\beta_3$, and $\alpha V \beta_3$ integrin in human tumour cell lines by confocal microscopy.

The human melanoma tumour cell line, M14, the human prostate carcinoma cell line, PC-3, and the human breast carcinoma, MCF-7, were selected as examples of high, moderate, and weak expression of $\beta_3$ and $\alpha V \beta_3$ respectively, and were examined using confocal microscopy. $\beta_3$ and $\alpha V \beta_3$ integrin immunoreactivity was detected in the cell membrane using B7 and LM609, respectively. Weak expression of $\alpha_{\text{IIb}}$ was detected in M14, and low expression was detected in PC-3 and MCF-7 cell membrane using C20 under the optimum conditions summarised in Table 3.5. The white arrow indicates the different expression of $\beta_3$ and $\alpha V \beta_3$ integrin in human tumour cell line and weak and low expression of $\alpha_{\text{IIb}}$. Bar length for C20 images = 30 µm and for B7 and LM609 = 150 µm.
3.3.4 Comparison of result of IMB and ICC

The inability to detect $\alpha_{IIb}$ by IMB and the possibility that expression obtained by ICC might represent false positive expression (since no expression was detected by IMB), meant that this was excluded from comparison of integrin expression between the two detection techniques, as shown in Table 3.7. The expression of $\alpha_V$ and $\beta_3$ integrin was similar in all the human tumour cell lines included the panel.

Table 3.7 Comparison of integrin expression in different tumour cell lines using specific antibodies for $\alpha_V$ and $\beta_3$ integrin subunits using ICC and IMB.

<table>
<thead>
<tr>
<th>Human tumour cell lines</th>
<th>Q20 (anti-$\alpha_V$)</th>
<th>B7 (anti-$\beta_3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IMB</td>
<td>ICC</td>
</tr>
<tr>
<td>A549</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>H460</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>DLD-1</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>HCT-116</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HT-29</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DU-145</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PC-3</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M14</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UACC-62</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>MCF-7</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Key for IMB and ICC: +++: High expression, ++: Moderate expression, +: Low expression, ±: Weak expression, -: no expression.
The expression of $\alpha_V$ and $\beta_3$ integrin subunits was quantified visually by giving the highest cell expression +++ and correlated to the rest of the cell lines expression.
3.4 Discussion

The main aim of this chapter was to characterise $\alpha_{\text{IIb}}$ and $\alpha_v\beta_3$ integrin expression in a panel of human tumour cell lines, in order to develop a set of models to be use in screening novel $\beta_3$ integrin antagonists.

Initially, growth curves were recorded to identify the log phase where cells functionally active in the days where cells reach 70 to 80% confluence. Cells were collected in this period of growth to detect expression of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin because, according to previous reports, integrin expression is reduced when cells are highly confluent (Haywood-Reid et al. 1997). In the present study the expression was detected only in the log phase of the tumour cells, to know how the expression of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin changed during different growth phase, further study needs to be done.

The expression of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin was investigated by immunoblotting, thereby confirming integrin protein expression in human tumour cell lines and the specificity of the primary antibodies used. Immunocytochemistry was then used to localise the site of expression as either membranous or intracellular, using the same antibodies.

The expression of $\alpha_v$ and $\beta_3$ integrin subunits in tumour cells lines was successfully detected using the immunoblotting technique but $\alpha_{\text{IIb}}$ expression was not. Different antibodies were used comprising anti-$\beta_3$ BV4 and B7 and anti-$\alpha_v$ P2W7 and Q20 and anti-$\alpha_{\text{IIb}}$ C20. Looking at this range of antibodies, some seemed to be non-specific; for example, BV4 (anti-$\beta_3$) and P2W7 (anti-
αv) showed double and multiple bands in immunoblotting. A specific antibody should give a single band on the exposed film and any non-specificity that develops is due to binding of the primary antibody to other non-target proteins. C20 (anti-αIIb) did not reveal any obvious expression, which is most likely due to the presence of the target protein at negligible levels in the cell lines investigated. Further investigations involving this antibody should use known expressors (e.g. human platelet lysates) (Lau et al. 2004) or inducible samples (PMA treatment of K562) (Sevinsky et al. 2004) as a positive controls to optimise the antibody reaction. For these reasons, P2W7, C20 and BV4 were excluded from further study. Q20 and B7 successfully detected the expression of integrin subunits in tumour cell lines showing a single band in the immunoblotting indicating the specificity in detecting the expression. B7 binds to amino acids 635-730, mapping near the C-terminus of the β3 integrin and Q20 recognises a peptide mapping to the C-terminus of integrin αv indicating the chance of αv and β3 subunits to form αvβ3 integrin heterodimer. Q20 and B7 were then used to screen a panel of cell lines for the expression of αv and β3 integrin using immunoblotting. Q20 had been used previously for immunoblotting in human 1321N1 astrocytoma (Liao et al. 2007).

After optimising the protocol for each antibody, specific antibodies were utilised to screen a panel of human tumour cell lines. All the human tumour cell lines expressed αv integrin, whereas the expression of β3 integrin differed between different tumour cell lines. High expression of β3 integrin was
observed in melanoma human tumour cell lines which agrees with the literature (Li et al. 2001; Voura et al. 2001).

In order to support the expression seen by immunoblotting, immunocytochemistry was utilised using the same antibodies in addition to an anti-αvβ3 integrin antibody LM609. This antibody binds to a conformational epitope resulting from the post-translational association of the αv and β3 subunits and detects the αvβ3 complex, so it could not be used with the immunoblotting technique because cell lysis causes dissociation of the dimer. LM609 has been used in several studies to detect the expression of αvβ3 integrin in different cell membranes (Arosio et al. 2009; Hofmann et al. 2000; Seftor et al. 1992). The protocol of Arosio et al. (Arosio et al. 2009) was followed for immunodetection of αvβ3 by immunocytochemistry using LM609. Optimised protocols for other antibodies were developed by altering variables until optimisation was reached, indicating that each antibody had different protocol requirements.

Moderate expression of αv and β3 and αvβ3 integrin was observed in PC-3 using Q20, B7, and LM609, respectively, by immunocytochemistry and immunoblotting. The human prostate carcinoma cell line PC-3 was selected to optimise the methodologies because earlier studies have documented high expression of αv, αIIb, β3 and αvβ3 in this prostate adenocarcinoma cell line (Arosio et al. 2009; Trikha et al. 1998; Trikha et al. 1996; Zheng et al. 1999). Zheng et al. detected high expression of αvβ3 integrin in a PC-3 cell line using flow cytometry analysis and immunoblotting techniques, and αv was
shown to complex with β₃ using an immunoprecipitation technique (Zheng et al. 1999). Trikha et al. detected intracellular expression of αᵢᵢβ₃ in PC-3 and DU-145 cells, and showed that αᵢᵢβ₃ can translocate to the cell surface following the activation of PKC (Trikha et al. 1996). They had previously detected membranous expression of αᵢᵢβ₃ in DU-145, using immunocytochemistry using with the anti-αᵢᵢβ₃ integrin antibody, MAB 1990 (Trikha et al. 1998). However, very little expression was reported by some others; for example, Arosio et al. found little expression of αᵥβ₃ integrin in PC-3 using LM609 by immunofluorescence technique (Arosio et al. 2009). Chatterjee et al. also reported little expression of αᵥβ₃ integrin in PC-3 in comparison to LNCaP (Chatterjee et al. 2001), and Goodman et al. found no expression of αᵥβ₃ and αᵢᵢβ₃ integrin in PC-3 (Goodman, Grote & Wilm 2012). This variation in αᵥβ₃ expression in the PC-3 cell line may be due to different culture conditions, as proposed by Nemeth et al. who suggested that expression of αᵥβ₃ integrin in PC-3 can be lost after a long period of culturing (Nemeth et al. 2003). Here in the present study, the cells were not used after passage 10 to avoid loss of the expression which can occur if late-passage cells are used. Variation in the expression reported between laboratories may be due to different culture conditions, and the use of fetal bovine serum from different batches (Haywood-Reid, Zipf & Springer 1997).

After successful detection of integrin expression in PC-3 cells, a panel of tumour cell lines was screened for the expression of αᵥ, αᵢᵢ, β₃ and αᵥβ₃ integrins. To address the objectives of the immunodetection of αᵢᵢ, αᵥ and β₃ subunits and αᵥβ₃ integrins in melanoma, three cell lines were used; M14,
UACC-62 and SK-MEL-2. The $\alpha_v$, $\beta_3$ subunits and $\alpha_v\beta_3$ integrin complex were expressed in all cell line membranes whereas $\alpha_{\text{IIb}}$ subunit expression was weak. Melanoma cell lines had the highest expression of $\alpha_v$, $\beta_3$ subunits and $\alpha_v\beta_3$ integrin. This is the first study to investigate the expression of $\alpha_v$, $\beta_3$ and $\alpha_v\beta_3$ integrin in the UACC-62 melanoma cell line using immunoblotting and immunocytochemistry techniques.

Expression of $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ and their respective subunits have been reported in melanoma cell lines. For example, Seftor et al. found that $\alpha_v\beta_3$ integrin was expressed in the melanoma cell line A375M using LM609 with immunofluorescence (Seftor et al. 1992; Trikha et al. 1997). Petitclerc et al. found the expression of $\alpha_v\beta_3$ integrin in the M21 melanoma cell line and used it as a model to explore $\alpha_v\beta_3$ integrin expression on melanoma growth in vivo (Petitclerc et al. 1999). Del Bufalo et al. found that M14 expresses $\alpha_v$ and $\beta_3$ integrin subunits but did not show any expression of $\alpha_{\text{IIb}}$ indicating that M14 expressed $\alpha_v\beta_3$ integrin as the only $\beta_3$ containing receptor complex (Del Bufalo et al. 1998). Seoane et al. reported the expression of $\alpha_v$ and $\beta_3$ integrin and no expression of $\alpha_{\text{IIb}}$ in another melanoma cell line (SK-MEL-28) using PCR and flow cytometry techniques (Seoane et al. 2010). Trikha et al. reported that the expression of $\alpha_{\text{IIb}}\beta_3$ integrin was not limited to only one single cell line; all melanoma cell lines expressed $\alpha_{\text{IIb}}\beta_3$ integrin, furthermore, translocation of $\alpha_{\text{IIb}}\beta_3$ integrin from the intracellular pool to the cell surface can be mediated under the effect of an agonist (Trikha et al. 1997).
Both positive and negative control cell lines needed to be investigated for each integrin subunit. The presence of positive and negative controls in the study confirms the expression detected in other cell lines is true expression rather than a false negative/positive.

In this part of the study, MCF-7 was utilised and as indicated by immunoblotting very weak expression of β3 integrin. Previous studies have detected the expression of αV, αIIb, β3 subunits and αVβ3 integrin in breast carcinoma cell lines, such as MCF-7, using different techniques such as immunoblotting and immunocytochemistry (Beauvais et al. 2004). The weak β3 subunit expression in MCF-7 was also seen by Wang et al. who found that MCF-7 had a lower expression than another breast cell line, MDA-MB-231 (Wang et al. 2010).

The A549 human NSCLC cell line was used as a possible negative control for use in the functional assay for αVβ3. Goodman reported no expression of αVβ3 integrin in A549 (Goodman, Grote & Wilm 2012) however, in the present study αVβ3 expression was detected, by immunoblotting and immunocytochemistry, in human NSCLC cell lines such as A549 and H460, with both cell lines showing weak expression of the β3 subunit and αVβ3. This difference may be due to different culture conditions between labs.

The expression of αV and β3 subunits and αVβ3 integrin was also detected in the colon carcinoma cell lines, DLD-1 and HCT-116, by immunoblotting and immunocytochemistry, but no expression of β3 was noted in HT-29. The immunodetection of β3 integrin by immunocytochemistry indicated that colon
carcinoma cell lines DLD-1 and HCT-116 had weak expression of the β3 subunit; the expression of the αvβ3 integrin was lower than the β3 subunit. Few reports describe the expression of αv, β3 and αvβ3 integrin in colon cancer. Most of the previous reports concentrate on β1 integrins rather than β3 (Gong et al. 1997; O'Brien et al. 1996). Immunocytochemistry and immunoprecipitation detected different integrins expression in HT-29, i.e. α2, α3, α6, β1 and β5 but weak expression of αv and no expression of β3 integrin (Haier et al. 1999). Burbridge et al. detected the expression of αvβ3 integrin in HCT-116 and no expression in HT-29 by immunoblotting and immunocytochemistry (Burbridge et al. 2003). This result agrees with Trikha et al. who used LM609 and reported that HT-29 was negative for αvβ3 integrin (Trikha et al. 2002), and other studies which also found no expression of αvβ3 integrin in HT-29 cell (Caltabiano et al. 1999) using the immunofluorescence technique (Liu et al. 2011).

αv was expressed at a high level in all screened cell lines, which is to be expected as αv does not bind exclusively to β3 but can also bind to β1, β5, β6, and β8. The expression obtained from immunoblotting and immunocytochemistry in this chapter was similar to previous reports (Trikha et al. 1998; Trikha et al. 2002; Zheng et al. 1999; Wang et al. 2005). β3 integrin expression differed between cell lines; β3 expression was highest in the melanoma cell lines M14, SK-MEL-2 and UACC-62, followed by the prostate carcinoma cell lines: PC-3 and DU-145. Lung carcinoma cell lines A549 and H460, colon carcinoma cell line DLD-1 and HCT-116 cells and
breast carcinoma cell line MCF-7 showed weak expression of $\beta_3$ integrin. The HT-29 colon carcinoma cell line did not express $\beta_3$ integrin.

A weak expression of the $\alpha_{IIb}$ subunit was noted using immunocytochemistry technique. However, although there was evidence among a panel of tumour cell lines not all of them had an expression, it was not convincing. The result obtained by immunocytochemistry did not agree with immunoblotting technique which suggests the expression of $\alpha_{IIb}$ observed by immunocytochemistry is may be false positive result or the conditions in western blot did not optimised properly. In addition the serum added to the media may also play an important role in the expression because the growth factors and the serum can be differs between labs.

$\alpha_v\beta_3$ is believed to be present over the entire surface of the cell (Wang et al. 2005). Confocal microscopy was used to localise expression as intracellular or on the cell surface. $\alpha_v\beta_3$ integrin was found to be membranous in all cell lines tested. Immunolabelling was done on cells adhering to a coverslip, so reducing the chance for cell to cell contact resulting in increasing membranous expression, since the expression of $\alpha_v\beta_3$ was known to be more prominent when cells start to adhere and migrate (Carreiras et al. 1999).
3.5 Conclusion

Immunoblotting and immunocytochemistry were used to characterise the expression of $\alpha_V$ and $\beta_3$ integrins in a panel of cell lines, whose growth characteristics have been determined. Expression of $\alpha_V\beta_3$ was successfully detected and found to be highest in M14, UACC-62 and SK-MEL-2, moderate in PC-3 and DU-145, weak in A549, H460, DLD-1, HCT-116 and MCF-7, negative in HT-29. The $\alpha_{IIb}$ subunit was expressed in the human tumour cell lines by ICC but not with IMB. The $\alpha_V$ subunit was expressed in all human tumour cell lines while expression levels of the $\beta_3$ subunit were more variable.
Chapter 4: Investigation of the effect of $\alpha_\nu \beta_3$ integrin inhibition on tumour cell migration
Chapter 4

4.1 Introduction

There is strong evidence that \( \alpha_v\beta_3 \) integrin plays a role in tumour dissemination, and therefore developing a therapeutic strategy to antagonise this integrin should be of value in reducing dissemination.

One of the key stages of the tumour dissemination process is cell migration. Tumour cell migration is initiated by cell polarization and the formation of membrane protrusions at the leading edge of the cell (Wang et al. 2005; Brakebusch et al. 2002). There are two types of tumour cell migration: single cell migration and collective cell migration. When a single cell migrates, it needs to polarize and protrude at the leading edge, then attach the leading edge to the substratum over which it migrates, followed by proteolytic degradation of the tissue component, actomyosin contraction, and finally, forward sliding of the cell rear. Collective cell migration occurs when cells maintain cell-cell junctions at the leading edge and lateral regions inside the moving cell group via cadherins (Vicente-Manzanares et al. 2005).

As previously discussed, research supports the view that the \( \beta_3 \) integrins, particularly \( \alpha_v\beta_3 \), play an important role in various stages of tumour metastasis including migration (Seftor et al. 1992; McCabe et al. 2007; Takayama et al. 2005). Voura et al. indicated that the strongest expression of the \( \alpha_v\beta_3 \) integrin was seen in the protrusions and pseudopods of migrating cells, and also in cells spreading on matrigel. This indicates a role for \( \alpha_v\beta_3 \)
integrin in extravasation and in cell-extracellular matrix interactions (Voura et al. 2001). Blocking β₃ integrin can inhibit interaction of tumour cells with the ECM and block tumour cell migration.

Several experimental assays (summarised in Table 4.1) are available for measuring tumour cell migration in vitro, such as the Boyden chamber assay, ring assay and scratch assay (Kramer et al. 2013). The scratch assay involves scratching a confluent monolayer of the cells and then evaluating the extent of tumour cell migration into the scratch. This assay has several advantages that make it an attractive assay; it is simple, inexpensive and can record the cell movement in real time (Liang et al. 2007). Furthermore, the scratch assay can be used to study cell migration by cell-cell interaction and cell interaction with ECM when cells are seeded on coated plate as a monolayer, rather than held in suspension, which disrupts cell-to-cell and cell-to-ECM interactions (Goetsch & Niesler 2011; Liang, Park & Guan 2007).

The scratch assay has been used to investigate the effect of anti-integrin antagonists in inhibition of tumour cell migration in vitro and to study the functional activity of tumour cells. For example, it has been used to evaluate the anticancer effects of the disintegrin contortrostatin on the PC-3 prostate cancer cell line (Lin et al. 2010), and to investigate the role of receptor glycosylation on αᵥβ₃ function (Kremser et al. 2008).

In order to utilise this assay in the evaluation of novel β₃ antagonists, the assay must be initially validated using known anti-αᵥβ₃ integrin antagonists. Cyclic RGD peptides such as (cRGDFV) (section 1.2.5.3.2) (Manzoni et al.
and the LM609 antibody are known α₃β₃ integrin antagonists (section 1.2.5.3.1). Whilst LM609 is specific to the α₃β₃ integrin (Trikha et al. 2002; Voura et al. 2001; Zheng et al. 1999), cRGDFV may also antagonise the α₅β₅ integrin (Manzoni et al. 2009).

4.1.1 Aims and objectives

The overall aim of the work described in this chapter is to develop and validate a functional assay of cell migration, the scratch assay, for use in the evaluation of β₃ integrin antagonists.

This aim was achieved by addressing the following objectives:

1- Setting up the scratch assay using the cell line models characterised in Chapter 3.

2- Determination of a safe dose of the known α₃β₃ integrin antagonists, cRGDFV and LM609.

3- Validation of the assay using cRGDFV and LM609.
<table>
<thead>
<tr>
<th>Trans-well migration assay</th>
<th>Wound healing assay (Scratch assay)</th>
<th>Cell exclusion zone assay</th>
<th>Fence assay</th>
<th>Micro-carrier bead assay</th>
<th>Spheroid migration assay</th>
<th>Capillary chamber assays</th>
<th>Capillary tube assays</th>
<th>Leukocyte migration agarose technique assay</th>
<th>Colloidal particle assay</th>
<th>Time-lapse cell tracking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensionality</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
<td>2D (3D)</td>
<td>2D (3D)</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
<td>2D</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Stable chemokine gradient</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Assay dependent</td>
</tr>
<tr>
<td>Measurement</td>
<td>Cell count fluorescence</td>
<td>Migration area</td>
<td>Migration area</td>
<td>Migration area</td>
<td>Migration area</td>
<td>Migration area</td>
<td>Cell count migration area</td>
<td>Migration distance</td>
<td>Migration distance</td>
<td>Cell migration path</td>
</tr>
<tr>
<td>Live imaging</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IF, IHC</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Substrate</td>
<td>PC, PET membrane or coated</td>
<td>Plastic, glass, or coated</td>
<td>Plastic, glass, or coated</td>
<td>Plastic, glass, or coated</td>
<td>Plastic, or coated</td>
<td>Plastic, or coated</td>
<td>Glass, or coated</td>
<td>Glass</td>
<td>Plastic, or coated</td>
<td>Plastic, or coated</td>
</tr>
<tr>
<td>Direction of movement</td>
<td>Horizontal then vertical</td>
<td>Horizontal</td>
<td>Horizontal</td>
<td>Vertical then horizontal</td>
<td>Mixed then horizontal</td>
<td>Horizontal</td>
<td>Horizontal</td>
<td>Horizontal</td>
<td>Horizontal</td>
<td>Horizontal</td>
</tr>
<tr>
<td>HTS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Type of analysis</td>
<td>End point</td>
<td>Kinetic</td>
<td>kinetic</td>
<td>Kinetic</td>
<td>kinetic</td>
<td>kinetic</td>
<td>Endpoint (kinetic)</td>
<td>Endpoint (kinetic)</td>
<td>kinetic</td>
<td>kinetic</td>
</tr>
<tr>
<td>Recapitulated in vivo migration mode</td>
<td>Single cell migration of epithelial sheets, EMT</td>
<td>Collective migration of epithelial sheets, EMT</td>
<td>Collective migration of epithelial sheets, EMT</td>
<td>Attachment and migration</td>
<td>Migration from cell cluster, established cell-cell interaction</td>
<td>Single cell migration, Chemotaxis</td>
<td>Leukocyte migration, single cell migration</td>
<td>Leukocyte migration</td>
<td>Single cell migration</td>
<td>Assay dependent</td>
</tr>
</tbody>
</table>

4.2 Materials and methods

4.2.1 Materials

Human tumour cell lines (Table 3.2) were maintained as described in section 3.2.2.1. All general chemicals, media and media supplements were purchased from Sigma-Aldrich (Poole, UK), unless otherwise specified. cRGDfV (ENZO Life Sciences, Farmingdale, NY) 4.3 Mm was dissolved in PBS, aliquoted and stored at -20 °C, and LM609 (see Table 2.1) was diluted in RPMI medium and used fresh. Ki-67 rabbit polyclonal primary antibody (AB9260, Chemicon Millipore Watford, UK), LM609 (see Table 2.1) and the Alexa fluor 546, anti rabbit, IgG (A11010, Invitrogen, USA) were used for immunocytochemical studies.

4.2.2 Methods

4.2.2.1 Wound healing migration assay

Cells were seeded into six-well plates (culture area: 9.6 cm²) at different concentrations in 2 ml of RPMI 1640 medium and then incubated at 37 °C in 5% CO₂ humidified atmosphere for three different durations (24, 48 and 72 hours) to determine the concentration and time at which a confluent monolayer was formed. Once a confluent monolayer of cells had formed, the cell monolayer was scratched in a straight line with a sterile P200 pipette tip to create a gap (approximately 2 cm in length and 650 µm in width) in the centre of the well. After scratching, the medium was slowly aspirated and discarded, and the cells were washed with 1 ml of HBSS to remove the
debris and to smooth the edge of the scratch. After washing, fresh medium was added to the cells, and the plate was incubated for 3, 6, 12, 24, 48 or 72 hours in order to investigate the time of wound healing for each cell line (Figure 4.1). During incubation, the plates were periodically monitored using phase-contrast microscopy (Olympus, Model: CK2). The real-time tracking of the scratch perimeter was done by measuring the average between the leading cells at five points along the scratch field using an eyepiece graticule. After determining the time of wound healing for each cell line, the cells were washed twice with HBSS and fixed with ice pre-cooled methanol for thirty minutes at -20 °C. After fixation, the cells were hydrated by two washes in PBS and counterstained with Harris’s Haematoxylin solution for two minutes. The cells were then washed in tap water for one minute and left to dry at room temperature.

The resulting plates were observed using an inverted microscope and images were taken (Nikon camera, model: C-D 55230, Japan) at ten positions throughout the scratch area. Scratch width was calculated using the grid and percentage migration was calculated as follows:

\[
\text{Migration at time } x \text{ } (\%) = \frac{\text{average scratch width at } T_x}{\text{average scratch width at } T_0} \times 100.
\]

The data were expressed as means ± SD of three independent experiments.
Cells were seeded as a confluent monolayer in six-well plates (A). The monolayer was scratched using a sterile P200 pipette tip (B). The initial perimeter was washed twice with HBSS and incubated with 1 ml of RPMI media for 24 hours, the initial scratch measured at five points as indicated by blue arrows (C). Cells that migrated toward the wound area were indicated by narrowing of the scratch area after cell migration as indicated by blue arrows (D).

4.2.2.1 Characterisation of the phenotype of the migrated cells in terms of proliferation and integrin expression

ICC was carried out with LM609 to confirm that cells migrating into the wound area expressed αvβ3 integrin, and with Ki-67, a marker for proliferation (Gasparini et al. 1998), to confirm that wound healing was due to cell migration rather than proliferation. M14 cells were seeded on autoclaved coverslips in six-well plates using the optimum seeding concentration and the required time for each cell line to grow to give a confluent monolayer. The confluent monolayer was scratched and left to heal for 24 hours. Both healed and initial perimeter scratches were immunolabelled by ICC technique as previously described in Chapter 3. For Ki-67 immunolabelling, M14 cells were fixed with 4% PFA, and blocked with 5% BSA/PBS for one hour at room temperature. Cells were immunolabelled with Ki-67, 1:50 at 4 °C overnight.
then the secondary antibody (Alexa fluor 546, anti rabbit) was applied, 1:50 for one hour in the dark at room temperature (for optimisation see Figure 3.5).

4.2.2.2 Evaluation of the cytotoxicity of cRGDfV and LM609 using the MTT assay

The cytotoxicity of cRGDfV and LM609 was measured in M14 and HT-29 human tumour cell lines using the MTT assay (Mosmann 1983). The assay is based on the ability of viable cells to reduce a yellow water-soluble tetrazolium salt (MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) to a purple water-insoluble formazan product by mitochondrial succinate dehydrogenase, with the amount of formazan produced being proportional to the number of viable cells present. The formazan products are dissolved in DMSO and the absorbance of the resulting solution is determined spectrophotometrically. In the first lane (8 wells per lane) of a 96-well plate, 200 µl of RPMI medium was added to serve as a blank. To subsequent lanes, $1 \times 10^4$/ml tumour cells were plated in a final volume of 180 µl of medium and incubated for 24 hours. 20 µl of the test compounds: 0.01, 0.05, 0.5, 5.0 and 50 µM cRGDfV and 2.5, 5.0, 7.5 and 10 µg/ml LM609 were then added to all wells except the RPMI blank control and untreated control for 96 hours of continuous drug exposure at 37 °C in a humidified 5% CO$_2$ atmosphere.

Following incubation, the medium was removed and 200 µl of MTT solution (5.0 mg/ml) was added per well. The plate was incubated for four hours in the
dark at 37 °C, 5% CO₂ humidified atmosphere. The plate was centrifuged at 1000 rcf for five minutes, the supernatant was removed and 150 µl/well DMSO was added with vigorous mixing in the pipettor to dissolve the formazan crystals. The absorbance of the resulting solution was determined at 550 nm using a Lab system Multiscan Plus spectrophotometer (Lab-systems Group, UK). The adjusted mean absorbance for each compound concentration was calculated by subtracting the mean background absorbance (average of the blank well) from the mean absorbance of the test wells. A dose response curve of adjusted mean absorbance against concentration was constructed, and the IC₅₀ values for each compound against each cell line were calculated using least squares method in Microsoft Excel.

4.2.2.3 Validation of the scratch assay using the integrin antagonists cRGDfV and LM609

The scratch assay was carried out as described in section 4.2.2.1. cRGDfV used on A549, DLD-1, DU-145, M14, PC-3 and UACC-62. LM609 used on A549, DLD-1, M14 and PC-3. cRGDfV (0.05, 0.5, 5.0 and 50 µM) and LM609 (0.5 and 2.5 µg/ml) were diluted in a final volume of 1 ml RPMI 1640 medium, added to the scratched monolayer after the washing step, and incubated for 24 hours. In each six-well plate, two wells were used as controls, one for the initial perimeter, and the other for 24 hours of wound healing. The specific positions were photographed as described in section 4.2.2.1 and analysed using a superimposed grid (Figure 4.2, A & B).
Migration compared to untreated control (%) was calculated as \( \% \text{ migration} = 100 \times \frac{(S_{T0} - S_{T24})}{(S_{U0} - S_{U24})} \times 100 \).

Where \( S_{T0} \) is scratch at initial perimeter, \( S_{U24} \) is untreated scratch after 24 hours and \( S_{T24} \) is treated scratch after 24 hours. The data were expressed as the means ± SD of three independent experiments.

**Figure 4.2 Scratch assay analysis**

Figure A shows a black line drawn at the back of each well to separate it into two to ensure the images were captured at the same position each time. After image capture, the original image was analysed by superimposing a grid over the image, as shown in B.
4.2.2.4 Statistical analysis

The student’s t-test and Kruskall wallis test were used for statistical analysis of the scratch assays, with results considered statistically significant and highly significant for $p < 0.05$ and $p < 0.01$ respectively. Data are presented as mean ± standard deviation, with each experiment repeated at least three times.
4.3 Results

4.3.1 Development of the wound healing migration assay

A scratch wound healing assay was performed using cell lines that showed high, low, weak or no expression of α₅β₃ integrin. Developing this assay required: optimisation of seeding density required for each cell line to form a confluent monolayer, working on the scratching technique to produce a consistent scratch width, determination of the wound healing times for the different cell lines, and confirmation that cells occupying the wound area had migrated there and were not just the result of proliferation by cells at the wound edges.

4.3.1.1 Seeding density

As the scratch assay method is dependent on the cell density per well leading to a confluent monolayer before scratching, the different tumour cell lines were seeded into six-well plates at different concentrations for 24, 48 and 72 hours in order to determine the optimum seeding concentration for each cell line.

Initially different concentrations of cells were seeded into six-well plates, ranging from $1 \times 10^3$ to $1 \times 10^6$ cells /ml and incubated at 37 °C, 5% CO₂ for 24 hours. After 24 hours, the appearance of the monolayer was analysed visually, and if a confluent monolayer was reproducibly formed, then the concentration was deemed suitable for seeding. Cell lines that did not form a confluent monolayer after 24 hours were incubated for longer times. Different
cell lines required different seeding concentrations and incubation times to form a confluent monolayer (Figure 4.3).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Density (x10^5)</th>
<th>Incubation Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>H460</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>DLD-1</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>HT-29</td>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td>HCT-116</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>UACC-62</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>M14</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td>UACC-62</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>PC-3</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>DU-145</td>
<td>3</td>
<td>24</td>
</tr>
</tbody>
</table>

Figure 4.3 Formation of confluent monolayers for the cell line panel (cell density per ml and time in hours)
Bar length = 200 µm.
4.3.1.2 Healing time

The time required for wound closure in each cell line was evaluated. After 24 hours, only M14 cells showed a high percentage (85%) of wound closure. PC-3, DLD-1, UACC-62, DU-145 and A549 showed a moderate wound closure in comparison to the initial perimeter after 24 hours; PC-3, DLD-1 and UACC-62 monolayers were respectively 94%, 90%, and 78% healed after 48 hours and DU-145 100% healed after 72 hours. H460, HT-29, SK-MEL-2, MCF-7 and HCT-116 had slow wound closure and scratch was not healed after 72 hours (Figure 4.4).
Figure 4.4 Migration of different tumour cell lines as assessed by the scratch assay

A: A confluent monolayer was scratched and incubated with complete medium and wound closure was measured using the grid. Images are representative of three independent experiments. Bar length = 200 µm.

B: Results were analysed and the mean percentage of wound closure shown ±SD based on three independent experiments. ** indicated p < 0.01 and * indicated p <0.05.
As a part of assay development, the phenotype of the cells seen in the wound area was characterised using ICC. The $\alpha_{V}\beta_{3}$ integrin expression of M14 cells migrating into the wound area was evaluated by labelling cells with LM609. As shown in Figure 4.5, all cells found in the wound area expressed $\alpha_{V}\beta_{3}$ integrin (Figure 4.5 A).

M14 cells were immunolabelled with Ki-67 to determine whether cells moving into the wound area had migrated and not proliferated. Nuclear labelling was obtained in cells which had moved toward the wound area, however there did not seem to be a higher rate of proliferation in the cells found in the wound area (Figure 4.5 B).
Figure 4.5 Labelling M14 cells at initial wound perimeter (T=0) and after 24 hours of wound healing with LM609 and Ki-67 using ICC

A. Expression of ανβ3 integrin at the initial wound perimeter and after 24 hours of wound healing using LM609, anti-ανβ3 integrin antibody. All M14 cells were labelled with LM609 at the initial wound perimeter and after 24 hours. Cells migrating into the wound area showed ανβ3 integrin expression as indicated by arrow. B. The immunolabelling with Ki-67 confirmed that M14 cells moving into the wound area had migrated. Examining M14 cells under higher magnification showed that Ki-67 is a nuclear immunolabelling and it did not seem that M14 had a high rate of proliferation. Bar length (A) = 60 µm and (B) = 200 µm.
4.3.2 Evaluation of the cytotoxicity of cRGDfV and LM609 using the MTT assay

The MTT assay was used to assess the cytotoxicity of cRGDfV against M14 and HT-29 cells and LM609 against M14 cells over a range of different concentrations for 96 continuous hours of drug exposure. As shown in Figure 4.6, neither antagonist demonstrated cytotoxicity in the cells at the concentration tested, and was it not possible to obtain an IC$_{50}$ for either compound. Thus, both antagonists are non-toxic within this concentration range for these cell lines and could be used in the scratch assay.

![Figure 4.6 Evaluation of cRGDfV and LM609 cytotoxicity against human tumour cell lines M14 and HT-29](image_url)

Cells were exposed to the antagonists for 96 hours. There was a negligible effect on cell survival by either compound. The results was statistically significant $p< 0.05$. 

167
4.3.3 The effect of cRGDFV on the migration of human tumour cell lines

Cell lines with varying αvβ3 integrin expression that demonstrated definite wound healing above were used to validate the wound healing assay because HT-29 (no αvβ3 integrin expression) can not be used as a model in scratch assay due to slow wound healing. Initially the M14 cell line was used due to its high expression of αvβ3 integrin in addition to its good growth and rapid wound healing. All the concentrations of cRGDFV tested led to inhibition of M14 migration. UACC-62 also showed high expression of αvβ3 integrin, and again all cRGDFV concentrations led to inhibition of UACC-62 migration, although with less of an effect than on M14 except at 0.05 µM, which showed the same percentage of inhibition in both cell lines with other concentrations tried. 50 µM cRGDFV was not toxic to M14 and UACC-62 cells. The effect of all cRGDFV concentrations was statistically significant p < 0.01 except with DLD-1 the significant reached at 50 and 5 µM only.

50 µM cRGDFV was toxic to PC-3 and A549 cells (Figure 4.7). Thus, the effect of cRGDFV on the migration of PC-3 and A549 cells was tested at 5.0, 0.5 and 0.05 µM and it was statistically significant p < 0.01 except for A549 at 0.05 µM, p < 0.05 (Figure 4.7).

DLD-1, used as tumour model of weak expression of αvβ3 integrin, did not show a dose dependent response to cRGDFV. The inhibition of DLD-1 migration seen with high concentrations of cRGDFV may be due to inhibition of αvβ5 integrin in DLD-1 (Appendix 1). The effect of 50 and 5 µM was
statistically significant with $p < 0.01$. There was no significant effect of cRGDFV at 0.5 and 0.05 µM on DLD-1 cell migration (Figure 4.7).

cRGDFV was effective in inhibition of all tumour cell migration at 5.0, 0.5, and 0.05 µM except DLD-1 which had an effect at 50 and 5.0 µM only. It was vital to know why cell lines with moderate and weak expression of $\alpha_v\beta_3$ integrin were affected by cRGDFV. These cell lines may also express $\alpha_v\beta_5$ integrin (Appendix 1) which can also be antagonised by cRGDFV. To investigate this possibility, LM609, which is reported to be specific to $\alpha_v\beta_3$ integrin, was also used in the scratch assay.
Figure 4.7 Effects of cRGDfV on tumour cell migration in the cell panel using the scratch assay
A: Migration of different human tumour cell lines toward wound area was inhibited using different concentrations of cRGDfV. Images shown are representative of three independent experiments. Bar length = 200 µm. B: Toxicity was observed with PC-3 and A549 at 50 µM as indicated by (T) in the chart. ** indicated p < 0.01 and * indicated p <0.05.
4.3.4 The effect of LM609 on human tumour cell migration

LM609 was used first with M14 cell line at two different concentrations, 0.5 and 2.5 µg/ml. LM609 at both of concentrations inhibited M14 cell migration, but had little effect on migration of the PC-3 and DLD-1 cells and a negligible effect on the migration of A549 cells, which showed cytotoxicity with cRGDFV (Figure 4.8). The effect of LM609 on tumour cells migration was statistically significant with p < 0.01. M14, PC-3, DLD-1 and A549 (as one cell line from each type of tumour) were selected as a model of high, moderate and weak expression to validate the scratch assay.
Figure 4.8 The effect of LM609 on tumour cell migration
A: Migration of tumour cells toward the scratch area was inhibited by LM609. Bar length = 200 µm. B: Images were analysed and average result shown as representative of three independent experiments. ** indicated $P < 0.01$. 
4.4 Discussion

The main aim of the work described in this part of the study was to develop a scratch assay that can be used to measure one of functional aspects of antagonising integrin expression, migration, for assessing novel integrin antagonists.

The scratch assay has some key advantages that led to its use in this study. The assay is easy to set up and does not require any specific equipment, and all the required materials are available in any cell culture laboratory (Liang, Park & Guan 2007). Nevertheless, the in vitro scratch assay presented some technical challenges: the first was the difficulty of creating scratched areas of equal sizes. This variation in wound size between wells of the same plate was resolved by practicing to achieve scratches of the same width (Appendix 2). Furthermore, there was difficulty achieving a wound without any cells. This problem was overcome by tilting the plate and washing the wound from the corner of the plate, which resulted in the removal of all unwanted cells from the wound area.

The intention was to use cell lines with different levels of αvβ3 integrin expression to investigate the effect of αvβ3 antagonism. Thus, tumour cell lines with different αvβ3 integrin expression which demonstrated clear wound healing were identified for use. The screening of a panel of tumour cell lines for wound healing (Figure 4.4) offered a chance to select the best model for testing β3 antagonists. Not all cell lines showed rapid wound healing due to the differing properties of the various cells in growing cultures. Some cells
grow in clumps rather than in flat spreading way. Cells growing in clumps are associated with difficulty in defining the starting point of cell migration which complicates image analysis.

Melanoma cell lines that exhibited high expression of $\alpha_v\beta_3$ integrin were screened for wound healing in order to select the best model. The only cell line that showed rapid wound healing (scratch nearly closed after 24 hours) was M14, followed by UACC-62, which exhibited moderate wound healing whereas SK-MEL-2 showed slow wound healing that did not close even after 72 hours. SK-MEL-2 has previously been shown to migrate very slowly, and to lack a response to TGF-\(\beta_1\), which caused a change in phenotype and signalling promoting cell migration in other melanoma cell lines (Janji et al. 1999).

PC-3 and DU-145 cell lines exhibited moderate wound healing after 24 hours. PC-3 has been reported to migrate rapidly, with a scratch being completely healed after 12 hours (Lin et al. 2010). The slower wound healing of PC-3 seen here may be due to different culture conditions or due to the original study seeding PC-3 cells on matrigel coated plates which promote integrin-mediated adhesion, whereas in the present study the PC-3 cells were seeded on uncoated plates which may have led to a slower migration of PC-3. Wang et al. found that wound healing was faster in PC-3 than in DU-145 (Wang et al. 2005), which supported the present study result where both cell types showed moderate wound healing after 24 hours, but PC-3 had wound closure after 48 hours, whereas DU-145 did not.
Colon carcinoma cell lines were screened for wound healing to be used as a \( \alpha_\text{v}\beta_3 \) weak or negative control models. However, only DLD-1 had moderate wound healing after 24 hours whereas HCT-116 and HT-29 did not show wound closure even after 72 hours. Thus, both HCT-116 and HT-29 were excluded from the rest of the scratch assay study, and DLD-1 was selected for further scratch assays as a model of weak expression of \( \alpha_\text{v}\beta_3 \) integrin.

Other cell lines with low \( \alpha_\text{v}\beta_3 \) integrin expression were also investigated. MCF-7 (weak \( \alpha_\text{v}\beta_3 \)) did not achieve wound closure after 72 hours. Lung carcinoma cell lines had weak \( \alpha_\text{v}\beta_3 \) integrin expression; moderate wound healing was observed for A549 while H460 did not have wound closure even after 72 hours. Cell lines that showed very slow wound healing (HCT-116, HT-29, H460 and MCF-7) in the scratch migration assay were excluded from the rest of the study.

The scratch assay provides valuable results regarding the expression of the \( \alpha_\text{v}\beta_3 \) integrin in tumour cell migration. Immunolabelling with LM609 of M14 cells in the scratch assay has never been done before, although this technique has been demonstrated by Carreiras et al. who showed that IGROV-1 ovarian carcinoma cells expressing \( \alpha_\text{v}\beta_3 \) integrin migrated toward a scratch area by immunolabelling migrating cells with LM609 (Carreiras et al. 1999).

The assay was validated before utilising it to evaluate novel anti-\( \beta_3 \) integrin antagonists by using well-characterised commercially available anti-\( \alpha_\text{v}\beta_3 \) integrin antagonists as positive controls. Before using the antagonists on
tumour cells, the cytotoxicity of cRGDFV and LM609 was tested using the MTT assay, which showed that cRGDFV had no toxic effects on the M14 and HT-29 human tumour cell lines. LM609 also did not show any toxicity to M14 cells. The only toxicity observed to other cell lines (did not tested by MTT assay) was with 50 µM cRGDFV to PC-3 and A549 only.

An anti-migratory effect was obtained with cRGDFV on cells with high, moderate and low αvβ3 integrin expression (Figure 4.7). PC-3 cells showed moderate expression of the αvβ3 integrin with high response to cRGDFV at 5.0, 0.5 and 0.05 µM. This may be due to expression of αvβ5, which is also antagonised by cRGDFV. Bisanz et al. have previously shown that the expression of β3 was lower than for αvβ1 and αvβ5 in PC-3 cells (Bisanz et al. 2005).

The LM609 antibody has been used to detect the expression of the αvβ3 integrin and as an inhibitor (Leavesley et al. 1992; Seftor et al. 1992; Trikha et al. 2002). A previous study used 20 µg/ml LM609 to study endothelial cell migration (Li et al. 2006). However, use of 20 µg/ml on M14 cells led to loss of M14 adhesion to the plate. Thus, lower concentrations (2.5 and 0.5 µg/ml) were used to study the effect on migration.

LM609 is specific for αvβ3, and does not antagonise related integrins such as αvβ5. The observed inhibition of cell migration suggests that αvβ3 plays a significant role in the migration of M14, and PC-3 cells and may also be involved in migration of DLD-1. αvβ3 integrin has been shown to play an important role in migration of other cell lines; for example, a study done by
Leavesley et al. reported that LM609 inhibited migration of pancreatic FG cells transfected with αVβ3 integrin in the Boyden chamber migration assay (Leavesley et al. 1992).

4.5 Conclusion

This work has developed and validated an assay for evaluating integrin-mediated cell migration, which can be used to evaluate novel αVβ3 antagonists in the following chapter.
Chapter 5: Investigation of the cytotoxicity of potential novel $\beta_3$ integrin antagonists and their effect on tumour cell migration
Chapter 5

5.1 Introduction

The previous chapter described the development and validation of the scratch assay to evaluate novel \( \beta_3 \) integrin antagonists. M14 cells were chosen as the optimal cell line to use in the scratch assay due to their high \( \alpha_v \beta_3 \) integrin expression and rapid wound healing. \( \alpha_v \beta_3 \) integrin is required for melanoma cell motility and it has been reported that blocking \( \alpha_v \beta_3 \) integrin leads to blocking of melanoma cell migration (Li et al. 2001).

In this chapter, the optimised scratch assay was utilised to evaluate potential novel \( \beta_3 \) integrin antagonists synthesized at the Institute of Cancer Therapeutics, using M14 as the model for testing for inhibition of tumour cell migration due to its high \( \alpha_v \beta_3 \) integrin expression.

In the present study the cytotoxic effect of potential novel \( \beta_3 \) integrin antagonists will be tested on tumour cells before they are evaluated in the functional scratch assay, in order to know that the effect of the potential novel \( \beta_3 \) integrin antagonists was due to inhibition \( \beta_3 \) integrin function in mediating tumour cell migration rather than cytotoxicity. Dayam et al. identified highly selective \( \alpha_v \beta_3 \) integrin antagonists through screening a panel of potential \( \alpha_v \beta_3 \) integrin antagonists they had synthesized. Six compounds were tested in breast tumour cells, MDA-MB-435 and MCF-7 as high and low \( \alpha_v \beta_3 \) models respectively. They found one of anti \( \alpha_v \beta_3 \) small molecules was toxic whereas the others showed no toxicity. This study indicated the importance
of testing the toxicity of small molecules before evaluating their effects on cancer cell migration and attachment (Dayam et al. 2006).

5.1.1 Aims and objectives

The overall aim of this chapter was to evaluate potential novel β₃ integrin antagonists in terms of their cytotoxicity and effect on tumour cell migration.

This aim was achieved through the following objectives:

1- Evaluation of the cytotoxicity of potential novel β₃ integrin antagonists using the MTT assay for cytotoxicity.

2- Determination of the effects of potential novel β₃ integrin antagonists on M14 cell migration using the scratch assay.
5.2 Materials and methods

5.2.1 Materials

Human tumour cell lines M14, MCF-7, and HT-29 (see Table 3.2 for details) were used in this chapter and maintained as described in section 3.2.2.1. All general chemicals, media and media supplements were purchased from Sigma-Aldrich (Poole, UK), unless otherwise specified. cRGDFV, was sourced as described in section 4.2.1. A series of potential novel β₃ integrin antagonists was synthesized at the Institute of Cancer Therapeutics (see Table 5.1 for details). All antagonists dissolved readily in 0.1% DMSO and a stock of 100 mM was prepared. For compound structures see attached file. LM609 details are summarised in Table 2.1.

Table 5.1 Compounds used

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ICT9003</td>
<td>320</td>
</tr>
<tr>
<td>2</td>
<td>ICT9018</td>
<td>531</td>
</tr>
<tr>
<td>3</td>
<td>ICT9019</td>
<td>503</td>
</tr>
<tr>
<td>4</td>
<td>ICT9020</td>
<td>517</td>
</tr>
<tr>
<td>5</td>
<td>ICT9053</td>
<td>538</td>
</tr>
<tr>
<td>6</td>
<td>ICT9055</td>
<td>542</td>
</tr>
<tr>
<td>7</td>
<td>ICT9057</td>
<td>500</td>
</tr>
<tr>
<td>8</td>
<td>ICT9062</td>
<td>542</td>
</tr>
<tr>
<td>9</td>
<td>ICT9064</td>
<td>528</td>
</tr>
<tr>
<td>10</td>
<td>ICT9068</td>
<td>421</td>
</tr>
<tr>
<td>11</td>
<td>ICT9072</td>
<td>474</td>
</tr>
<tr>
<td>12</td>
<td>ICT9073</td>
<td>516</td>
</tr>
</tbody>
</table>
5.2.2 Methods

5.2.2.1 Evaluation of the cytotoxicity of potential novel β₃ integrin antagonists using the MTT assay

The cytotoxicity of the potential novel β₃ integrin antagonists was evaluated in M14 (high expression of αᵥβ₃ integrin), MCF-7 (weak expression of αᵥβ₃ integrin), and HT-29 (no expression of αᵥβ₃ integrin) human tumour cell lines using the MTT assay as described in section 4.2.2. The β₃ integrin antagonists were used at the following concentrations 0.01, 0.1, 1.0, 10 and 100 µM.

5.2.2.2 Wound healing migration assay

The anti-migratory effect of the potential novel β₃ integrin antagonists on M14 cell migration was evaluated using the scratch assay as described in section 4.2.3. Briefly, wounded cultures of cells were exposed to 0.01, 0.1, 1.0, 10 and 100 µM of the potential novel β₃ integrin antagonists for 24 hours, and then the cultures were processed as described in Chapter 4. cRGDFV at 0.5 and 5.0 µM was included as a positive control.

5.2.2.3 Statistical analysis

The student’s t-test (parametric) and kruskall wallis (non parametric) test were used for statistical analysis of the scratch assays, with results considered statistically significant and highly significant for p < 0.05 and p < 0.01 respectively. Data are presented as mean ± standard deviation, with each experiment repeated at least three times.
5.3 Results

5.3.1 Evaluation of the cytotoxicity of potential novel β₃ integrin antagonists

The MTT assay was used to assess the cytotoxicity of potential novel β₃ integrin antagonists against M14, HT-29 and MCF-7 cells over a range of different concentrations for 96 continuous hours of drug exposure. As shown in Figure 5.1 and Table 5.2, the majority of compounds demonstrated minimal toxicity, with it not being possible to obtain IC₅₀ at the highest dose assayed, 100 µM.

As can be seen from Table 5.2, ICT9062, ICT9072, and ICT9073 were toxic to all cell lines with IC₅₀ calculated from three independent experiments.
Figure 5.1 Evaluation of the cytotoxicity of the potential novel \( \beta_3 \) integrin antagonists in the tumour cell line panel.
Table 5.2 IC<sub>50</sub> values for the potential novel β<sub>3</sub> integrin antagonists in the tumour cell line panel

<table>
<thead>
<tr>
<th>Compound</th>
<th>M14 IC&lt;sub&gt;50&lt;/sub&gt; ± SD (µM)</th>
<th>MCF-7 IC&lt;sub&gt;50&lt;/sub&gt; ± SD (µM)</th>
<th>HT-29 IC&lt;sub&gt;50&lt;/sub&gt; ± SD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICT9003</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9018</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9019</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9020</td>
<td>36 ± 18.6</td>
<td>&gt;100</td>
<td>80 ± 5.8</td>
</tr>
<tr>
<td>ICT9053</td>
<td>72.7 ± 9.8</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9055</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>25 ± 5.8</td>
</tr>
<tr>
<td>ICT9057</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9062</td>
<td>3.3 ± 0.8</td>
<td>1.4 ± 0.5</td>
<td>21.2 ± 30</td>
</tr>
<tr>
<td>ICT9064</td>
<td>&gt; 100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9068</td>
<td>&gt; 100</td>
<td>9.5 ± 9</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ICT9072</td>
<td>50 ± 47</td>
<td>14 ± 8.5</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>ICT9073</td>
<td>61 ± 32.9</td>
<td>6.3 ± 6.9</td>
<td>36.7 ± 42.2</td>
</tr>
</tbody>
</table>

5.3.2 Characterisation of the anti-migratory effect of potential novel β<sub>3</sub> integrin antagonists using the scratch assay

The potential novel β<sub>3</sub> integrin antagonists were tested in two batches; with information obtained from the first batch informing the concentrations used in testing the second batch. In the first batch, ICT9003, ICT9055, ICT9057 and ICT9064 were screened at four concentrations 0.1, 1.0, 10 and 100 µM. At the lowest concentration, ICT9055 showed the highest effect of inhibiting M14 cell migration; whilst the other potential novel β<sub>3</sub> integrin antagonists had lower effects at the same concentrations (Figure 5.2). The results were seen to be highly statistically significant p < 0.01 for all novel β<sub>3</sub> integrin antagonists. IC<sub>50</sub> values were calculated as 4.8 ± 0.2 µM for ICT9003, 1.0 ± 0.09 µM for ICT9057 and 0.2 ± 0.06 µM for ICT9064 (Table 5.3).
Figure 5.2 Effect of potential β3 integrin antagonists on M14 cell migration

A: M14 cells were wounded and then treated with different concentrations of different potential novel β3 integrin antagonists for 24 hours. Bar length = 200 µm. 

B: The migrated cells were counted and related to 100% untreated cells. Results were statistically significant for all antagonists with p <0.01.
A concentration of 10 µM was selected as an initial screening concentration for the second batch of compounds, which were ICT9018, ICT9019, ICT9020, ICT9053, ICT9062, ICT9068, ICT9072 and ICT9073. As seen in the cytotoxicity assay, some of the antagonists (ICT9062, ICT9072 and ICT9073) were observed to be toxic. In the MTT assay cells were treated for 96 hours, whereas in the scratch assay the M14 cells were treated for only 24 hours, thus as the exposure time was shorter it was assumed that these concentrations would not have a cytotoxic effect, however this was not the case and the toxicity was still observed (Figure 5.3). Therefore, the β3 integrin antagonists were tried at lower concentrations which were 1.0, 0.1 and 0.01 µM.
Figure 5.3 Effect of 10 µM β3 antagonists on the inhibition of M14 cell migration by the scratch assay.
A confluent monolayer of M14 cells was scratched, and then treated with 10 µM of the second batch of novel anti-β3 integrin antagonists for 24 hours. Bar length = 200 µm.
Further screening was carried out for the β3 integrin antagonists that showed an inhibitory effect on M14 cell migration using a more extensive concentration range, from 0.01 µM up to 10 µM (Figure 5.4). The compounds that were toxic at 10 µM were also evaluated, and these demonstrated a high percentage of inhibition of M14 cell migration at lower concentrations, with ICT9073 having the highest percentage of inhibition at 0.01 µM. IC₅₀s were 9.5 ± 0.9 µM for ICT9019, 0.15 ± 0.03 µM for ICT9062 and 0.15 ± 0.04 µM for ICT9072 (Table 5.3).
Figure 5.4 Effect of potential β₃ integrin antagonists in inhibition M14 cell migration by scratch assay

A: M14 cells were wounded then treated with different concentrations of different potential novel β₃ integrin antagonists for 24 hours. Bar length = 200 µm.

B: The migrated cells were counted and related to 100% untreated cells. Result was statistically significant p <0.01. ICT9062, ICT9072 and ICT9073 were toxic at 10 µM as indicated by T.
Table 5.3 A summary of effect of the potential novel β₃ integrin antagonists in inhibition of M14 cell migration after 24 hours treatment with the scratch assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICT9003</td>
<td>4.8 ± 0.2 µM</td>
</tr>
<tr>
<td>ICT9018</td>
<td>-</td>
</tr>
<tr>
<td>ICT9019</td>
<td>9.5 ± 0.9 µM</td>
</tr>
<tr>
<td>ICT9020</td>
<td>-</td>
</tr>
<tr>
<td>ICT9053</td>
<td>-</td>
</tr>
<tr>
<td>ICT9055</td>
<td>&lt; 0.1 µM</td>
</tr>
<tr>
<td>ICT9057</td>
<td>1.0 ± 0.09 µM</td>
</tr>
<tr>
<td>ICT9062</td>
<td>0.15 ± 0.03 µM</td>
</tr>
<tr>
<td>ICT9064</td>
<td>0.2 ± 0.06 µM</td>
</tr>
<tr>
<td>ICT9068</td>
<td>&gt; 50 µM</td>
</tr>
<tr>
<td>ICT9072</td>
<td>0.15 ± 0.04 µM</td>
</tr>
<tr>
<td>ICT9073</td>
<td>&lt; 0.01 µM</td>
</tr>
</tbody>
</table>
5.4 Discussion

In the previous chapters characterised cell lines were selected for their integrin expression patterns and then used to set up and validate a functional assay of integrin antagonism, the scratch assay. In this chapter the validated assay was used to evaluate novel antagonists in inhibition of migration of a cell line expressing β₃ integrin, M14.

Initially β₃ integrin antagonists were evaluated for their relative cytotoxicity. As shown in Table 5.2, different anti β₃ integrin antagonists had different cytotoxic effects. ICT9003, ICT9018, ICT9019 and ICT9057 were not toxic to M14, MCF-7 and HT-29 cells. ICT9053 had a cytotoxic effect on M14 only, and ICT9055 was cytotoxic to HT-29 only. ICT9064, the free acid of ICT9055 showed no obvious cytotoxicity with all tested cell lines. ICT9062 showed a cytotoxic effect with all tested cell lines, which is surprising since it differs from ICT9055 only in the stereochemical arrangement of bonds around the central linker. ICT9068 showed cytotoxic effect with MCF-7 only, and ICT9020 was cytotoxic with both M14 and HT-29.

The cytotoxic effect was evaluated using the MTT assay, to ensure that the results observed were due to blockade of integrin function (reduction in cell migration in the scratch assay), rather than general toxicity.

There was no obvious pattern in cytotoxicity across compounds and cell lines. Since compounds that were shown to be active in the scratch assay (and other work in-house) such as ICT9055, are not toxic in the high β₃
expressing M14 cells, the toxicities observed are unlikely to be β₃-mediated. However, cell line specific cytotoxicity may be due to effects mediated by other integrins: for example, αᵥβ₆ is known to be important in colon cancer survival, so the toxicity observed with HT-29 treated with ICT9055 may result from antagonism of β₆ (Peng et al. 2009). Future work on these compounds should involve assessment of their selectivity for similar integrins.

ICT9072 and ICT9073 had a cytotoxic effect with all tested cell lines, which may be due to the presence of the amino pyrimidine group at the start of the carbon chain, which has previously been demonstrated to cause toxicity (Gorneva 2005). Cilengitide induced cytotoxicity in glioma cells U-251MG and U-87MG (Lomonaco et al. 2011) whereas cRGDiV did not show any cytotoxic effect to same cells (Chatterjee et al. 2000).

The effect of the potential novel β₃ integrin antagonists was investigated by the scratch assay. The results indicated that different antagonists had different effects on inhibition of M14 cell migration. ICT9003 had the lowest effect, which may be due to the absence of both tetrahydronaphthyridine (THN) at the beginning of the carbon chain and the NHSO₂Mes exosite binding group at the end of the carbon chain.

As shown in Figure 5.3, ICT9018, ICT9019, ICT9020 and ICT9003 (pyrimidine) (Figure 5.4) at 10 μM were not effective in inhibiting M14 cell migration. In contrast, ICT9055, ICT9057 and ICT9064 which all contain the THN group, were all effective inhibitors of M14 cell migration. This consistent effect of ICT9057, ICT9062 and ICT9064 indicated that the presence of the
exosite binding NHSO$_2$Mes group is also required for activity. The ICT9062 had the same structure as ICT9055, but with different stereochemistry which led to it being cytotoxic to all tested cell lines suggesting that stereochemistry is a major consideration in antagonist design. ICT9055 was thus the most effective functional antagonist with no cytotoxic effect on M14 cells, among the compounds screened.

5.5 Conclusion

A total of 12 potential $\beta_3$ integrin antagonists were screened in the scratch assay, with some of the compounds demonstrating significant antagonistic effects. ICT9055 was the most effective of the compounds screened and would warrant further investigation.
Chapter 6: Discussion and future work
6.1 General discussion and future perspective

The current strategy for cancer therapy is still inadequate; chemotherapy continues to be associated with long-term toxicities and drug resistance, while surgery and radiotherapy are not suitable for treating the whole body after cancer metastasis. Thus, research has begun to focus on new strategies for cancer therapy based on targeted therapy. The development of targeted therapies improves differentiation between normal and neoplastic cells.

Integrins have become an attractive receptor target for cancer therapy (Goodman & Picard 2012; Sheldrake & Patterson 2009). However, among the panel of integrin antagonists summarised in Table 1.3, none of them specifically targets tumour cell integrins and their interaction with platelet and endothelial cells to inhibit angiogenesis and tumour metastasis. Furthermore, a large number of the available antagonists available are either antibodies or peptides and small molecules are preferred as antagonists as they are less costly to manufacture than antibodies, have better stability and pharmacokinetic/pharmacodynamic (PKPD) properties, and can be administered orally (Alig et al. 1992; Millard, Odde & Neamati 2011).

Blockade of α_{IIb}β_{3} and α_{V}β_{3} integrins has a critical role in preventing cancer metastasis and angiogenesis (Liu et al. 2009). Blockade of both tumour cell α_{V}β_{3} and host cell β_{3} integrins (endothelial α_{V}β_{3} and platelets α_{IIb}β_{3}) can enhance inhibition of tumour growth in vivo (Trikha et al. 2002). At the Institute of Cancer Therapeutics we hypothesise that dual α_{IIb}β_{3} and α_{V}β_{3}
antagonism will have a direct effect on $\beta_3$-expressing tumour cells and it will also through targeting tumour cell interaction with endothelial cells and platelets lead to inhibition of angiogenesis and metastasis.

In this thesis, the aim was to evaluate the effect of potential novel $\beta_3$ integrin antagonists in inhibition of human tumour cell migration using cell based assays. The main objectives of the work were to detect $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrin expression in human tumour cell lines and human xenograft tissue to develop models that can be used for both \textit{in vitro} and \textit{in vivo} evaluation of novel $\beta_3$ integrin antagonists.

After characterisation of $\alpha_{IIb}\beta_3/\alpha_V\beta_3$ integrin expression in a panel of human tumour cell lines, M14 was selected as a suitable cell line to be used as a model in the scratch assay. This cell line allows the study of migration mediated by $\alpha_V\beta_3$ integrin due to negligible expression of $\alpha_{IIb}\beta_3$ integrin in the cell line. Using this cell line the potential novel $\beta_3$ integrin antagonists ICT9055 and ICT9073 were identified as providing blockade of cell migration at non-toxic concentrations, with IC$_{50}$ < 0.1 and < 0.01 $\mu$M respectively. However, the other antagonists that had cytotoxic effect need to be confirmed that it non toxic to control cells in other functional assay.

Initially, the expression of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ was investigated in human xenograft mouse tissue using immunohistochemistry in order to obtain information that would guide the selection of cell lines to be used when the potential novel $\beta_3$ integrin antagonists progress to \textit{in vivo} screening. Detection of integrin expression in xenograft mouse tissue was really
important in preparing an *in vivo* model since it is known that integrin expression varies in response to extracellular environment (Zhao *et al.* 2007).

After different methodologies were followed, all the primary antibodies evaluated in these studies gave non-specific immunolabelling in sections both with and without primary antibodies, except B7 (anti-β3) using the M.O.M. kit which gave a clean negative control with non-specific immunolabelling of blood vessels only. Furthermore, non-membranous immunolabelling developed with Q20 (anti-αV). These results were confirmed by immunoblotting which showed that mouse antibody (B7) on mouse tissue developed non-specific binding while rabbit antibody Q20 did not develop any non-specific binding.

According to tissue homogenisation and protein extraction in this study, it can be said that the human xenograft tissues used in this study expressed αV and β3 integrin subunits. The problem faced with immunohistochemistry was due to the inability of the mouse primary antibodies used to selectively detect the expression of human integrin subunits in the presence of mouse tissue. In normal mouse tissue there was non-specific binding whereas there was no non-specific binding in samples from human tumour cell lines grown *in vitro* when the membrane was incubated without primary antibody. In order to investigate integrin expression in an *in vivo* model using either immunohistochemistry or immunoblotting it seems to be vital to avoid using mouse antibodies on mouse tissue. For example anti-αV (Q20) did not
develop any non-specific binding with immunohistochemistry or with immunoblotting.

The results from immunoblotting encouraged evaluation of the expression of \( \alpha_v\beta_3 \) and \( \alpha_{IIb}\beta_3 \) integrin in other human tumour cell lines. After optimisation of a panel of primary antibodies, the expression of \( \alpha_v, \beta_3 \) and \( \alpha_v\beta_3 \) integrin was detected on the surface of a panel of tumour cell lines using immunocytochemistry, and also the expression of \( \alpha_v \) and \( \beta_3 \) integrin subunits was detected in different cell lines using immunoblotting. However, the overall aim of this study can be best investigated \textit{in vitro} using a model expressing both \( \alpha_{IIb}\beta_3 \) and \( \alpha_v\beta_3 \) integrin and I did not detect expression of \( \alpha_{IIb} \) integrin in this study. Thus, to achieve a model of \( \alpha_{IIb}\beta_3/\alpha_v\beta_3 \) integrin dual expression, it will be vital to transfect the cells chosen for the study with the \( \alpha_{IIb} \) integrin subunit (O'Toole \textit{et al.} 1989). The transfection of \( \alpha_{IIb}\beta_3 \) integrin has been achieved previously; for example, \( \alpha_{IIb}\beta_3 \) integrin was transfected into the melanoma cell line WM 983B which lead to increased tumour cell adhesion, spreading and invasion \textit{in vitro} and decreased apoptotic rate \textit{in vivo} (Trikha \textit{et al.} 2002).

Here, \( \alpha_v \) protein was expressed in all tumour cell lines evaluated, and was observed to be localised to the cell surface using immunocytochemistry. This result was expected since \( \alpha_v \) can bind to other \( \beta \) subunits besides \( \beta_3 \). Goodman \textit{et al.} have also shown that \( \alpha_v \) is expressed in all human tumour cell lines (Goodman, Grote & Wilm 2012). \( \alpha_v \) has been shown to play an important role in prostate cancer angiogenesis and bone metastasis through
validation of $\alpha_v$ as a target by knockdown, and also pharmacological inhibition by GLPG0187 (Table 1.3), using in vivo and in vitro models. GLPG0187 was effective in inhibiting angiogenesis and bone metastasis through its action on prostate cancer stem cells (van der Host et al. 2011). In addition, $\alpha_v$ blocking with GLPG0187 in PC-3 cells prevented the growth of the tumour in vivo (van der Host et al. 2010). The anti-$\alpha_v$ antibody CNTO95 (Table 1.3) inhibits HUVECs and melanoma cell (A375.S2) adhesion, migration, and invasion in vitro. CNTO95 also leads to inhibition of tumour proliferation and angiogenesis in vivo (Trikha et al. 2004). Bisanz et al. treated PC-3 cells with $\alpha_v$ si-RNA which lead to decreased growth of PC-3 xenografts in bone, suggesting that $\alpha_v$ mediated communication between tumour cell and bone microenvironment is required for bone metastasis development (Bisanz et al. 2005). The above indicate that $\alpha_v$ has an important role in cancer. In the present study, it was not possible to use a model with no $\alpha_v\beta_3$ integrin because the only line explored that had no $\alpha_v\beta_3$ integrin expression, HT-29, could not be used in the scratch assay due to its low rate of migration/wound closure.

The present study developed a semi-quantitative method for the detection of $\alpha_v\beta_3$ integrin expression in human tumour cell lines and xenografts. A more quantitative measure would be to use flow cytometry, it was the advantage over the immunocytochemistry is being able to detect even weak expression (Goodman, Grote & Wilm 2012). LM609 has been used previously to characterise the expression of $\alpha_v\beta_3$ integrin in human tumour cell lines using flow cytometry (Landen et al. 2008). However, since flow cytometry does not
assess morphological expression, it must still be run in parallel with immunohistochemistry or immunocytochemistry.

The overall aims of this study were to characterise $\alpha_V\beta_3/\alpha_{IIb}\beta_3$ integrin expression in tumour cell lines and tissues, and to evaluate the pharmacological effect of novel small molecule, anti $\alpha_V\beta_3$ and/or $\alpha_{IIb}\beta_3$ antagonists. Using different tumour cell lines with different $\alpha_V\beta_3$ integrin expression, a range of wound-healing responses was seen in the scratch assay for migration. LM609 and cRGDfV were used as positive controls to demonstrate that M14 migration was mediated by $\alpha_V\beta_3$ integrin. Albelda et al. showed increased expression of $\alpha_V\beta_3$ integrin correlates with increased melanoma cell migration (Albelda et al. 1990). Although both inhibitors were effective in inhibition of M14 cell migration, cRGDfV (target $\alpha_V\beta_3/\alpha_V\beta_5$ integrin) and LM609 (target $\alpha_V\beta_3$ integrin) this indicated that $\alpha_V\beta_5$ integrin may also had a role in M14 cell migration.

After optimising the scratch assay conditions, the potential novel $\beta_3$ integrin antagonists were screened. The effect of potential novel $\beta_3$ integrin antagonists in inhibition of M14 cell migration was evaluated. From screening a panel of 12 novel $\beta_3$ integrin antagonists, ICT9055 had the highest effect in inhibition of M14 cell migration. This small molecule did not exert any cytotoxic effect even at high concentrations on M14 cells but was toxic to HT-29. ICT9053 was toxic to M14 cells only, ICT9020 was toxic to M14 and HT-29 but not to MCF-7 and ICT9068 was toxic to MCF-7 not to M14 and HT-29. Different human tumour cell lines may have different survival pathways and
this can be explained by the variation in cytotoxic effect of some potential novel β₃ integrin antagonists on some tumour cells and not others. For example, while cRGDfV was non-toxic to LNCaP prostate carcinoma cells (Chatterjee et al. 2001), and U-87MG and U-373MG glioma cells (Chatterjee et al. 2000), Cilengitide was toxic to glioma cells U-251MG and U-87MG plated onto vitronectin coated dishes. It has been shown Cilengitide can induce autophagy leading to cell death (Lomonaco et al. 2011) whilst cRGDfV can induce apoptosis through blocking FAK which mediates the signal transduction pathway necessary for cell survival (Chatterjee et al. 2001).

ICT9055 was toxic to HT-29 and this cytotoxicity may be due to expression of β₆ integrin (in house data, Andrew Gordon) which has been shown to have a role in colon cancer cell survival through ERK signalling inducing tumour cell proliferation (Peng et al. 2009). 10D5 (IgG2a, monoclonal antibody target αᵥβ₆) induced apoptosis of HT-29 cells through increase in caspase-3 and caspase-9 expression and release of cytochrome c from the mitochondria to the cytoplasm (Zhao-Yang et al. 2008). The above result suggests that the effects of the novel β₃ antagonists tested in the present study on HT-29 maybe were via antagonism of αᵥβ₆ integrin, and further work needs to be done to prove this by using anti αᵥβ₆ integrin inhibitor.

As shown in the scratch assay done here, not all cell lines were adherent or had rapid cell migration toward wound area, therefore another assay should be evaluated that may work with a broader range of cell type. Another assay
that could be used to investigate integrin antagonism is the Boyden chamber transwell migration assay. The transwell migration assay has been used to test the effect of compounds on the inhibition of cell migration in vitro, by using a transwell migration assay chamber coated with ligands such as laminin, vitronectin or fibronectin (Trusolino et al. 2000). Using the transwell migration assay coated with substrates (Byzova et al. 2000) can give an idea about the effect of potential novel β₃ integrin antagonists in inhibition of tumour cell migration in vivo. Further, the use of the transwell assay will give a chance to detect the effect of integrin antagonists on adherent and non adherent cells. The Boyden chamber assay permits migration of cells toward the ECM protein. Cell contact allows cells to exchange information that can be used to control the cell migration process (Carreiras et al. 1999).

Here, the wound healing assay was done using uncoated plates. Coating plates with substrate would provide a more physiologically relevant model to help to identify integrin antagonists. The choice of extracellular matrix ligand can be used to determine the selectivity of the antagonists or the relative contribution of related integrin to cell migration. PC-3 cells which have no expression of αᵥβ₃ integrin can adhere and migrate on vitronectin mediated by αᵥβ₁ and αᵥβ₅ integrin (Bisanz et al. 2005). PC-3 cell expressing αᵥβ₅ and αᵥβ₁ and α₅β₁ integrin have been shown to migrate on fibronectin and vitronectin, since these cells had little expression of αᵥβ₃ a selective αᵥβ₃ antagonist did not have an effect on PC-3 plated on vitronectin (Lin et al. 2010). Thus to fully understand our scratch assay results, the expression of other integrins must be detected; for example, other studies have reported
expression of α₅β₁ integrin in melanoma cell lines (Leavesley et al. 1992; Seftor et al. 1992). Eventually, targeting multiple integrins in tumour cells and the microenvironment may be investigated as a therapeutic strategy (Mark Sutherland 2012).

It will be vital to test the effects of novel integrin antagonists on the ability of tumour cell lines to adhere to ECM protein such as vitronectin or fibronectin. The adhesion assay can be used to identify novel integrin antagonists and to characterise their selectivity, using antibody controls and cell lines with fully characterised integrin expression (Caltabiano et al. 1999; Del Bufalo et al. 1998). Gentilucci, et al. used an adhesion assay to test the selectivity of cyclotetrapeptides as αᵥβ₃ and/or α₅β₁ antagonists. Selectivity was determined by studying the effects of the peptides on the inhibition of adhesion of K562 (human erythroleukemic cells) expressing α₅β₁ integrin, or SK-MEL-24 (human melanoma cells) expressing αᵥβ₃ integrin to fibronectin, and HUVECs expressing αᵥβ₃ integrin to vitronectin (Gentilucci et al. 2010).

Wang et al. investigated the role of β₃ integrins in transendothelial migration in vitro by evaluating the migratory capacity of PC-3 cells through a monolayer of HUVECs. The membranous expression of β₃ integrin in PC-3 was associated with active motility from its location at the edges of pseudopodial extensions, and the study concluded that PC-3 played an important role in TEM through its interaction with the matrix underneath the endothelium. Blocking β₃ integrin led to inhibition of the interaction of PC-3 cell with the ECM (Wang et al. 2005). Voura et al. found that cRGDFIV (5 μM)
was able to inhibit transmigration of melanoma cell WM 239 through HUVECs by 70% and LM609 (40 µg/ml) led to 40-50% inhibition indicating that transmigration was partly mediated by αvβ5. These assays will be useful in identifying compounds that would be effective against early stages of metastasis (Voura et al. 2001).

The effect of potential novel β3 integrin antagonists on downstream cell signalling needs to be tested since as has been described earlier, integrins function through inside-out and out-sidein signalling. The RAS/RAF/MEK/ERK signalling pathway is commonly activated in melanoma (Meier et al. 2005). Ligation of αvβ3 integrin with ECM has been shown to lead to focal contact formation and autophosphorylation of FAK at Y397 which provides a recognition site for c-src SH2 domain, and recruitment of Src that lead to phosphorylation of FAK at Y925 and provides an additional site to SH2 (adaptor protein) of Grb-2 that recruits SOS to activate Ras then Raf then MEK then ERK (Chen et al. 2007). For example, cRGDFV blocking FAK activation led to cleavage of FAK and reduced expression of Akt/PKB further to activation of caspase 3 and caspase 9 which induced programmed cell death in LNCaP, prostate carcinoma cells (Chatterjee et al. 2001). Cilengitide has been used with HUVECs and glioma cells where it induced cell detachment and apoptosis in cells grown on uncoated plates. The effect of Cilengitide was associated with reduction in FAK and Src phosphorylation in both endothelial and glioma cells. Akt phosphorylation was reduced in glioma cells, but there was no effect on ERK in HUVECs (Oliveira-Ferrer et al. 2008).
After successfully identifying potential novel β₃ integrin antagonists that inhibit tumour cell adhesion, migration and invasion using the functional assays *in vitro*, the effect of novel antagonists can be evaluated in term of inhibition of tumour cell angiogenesis, migration and invasion *in vivo*.

The results presented in this thesis indicated a very good start for the development of potential novel β₃ integrin antagonists. However, much work still needs to be done to evaluate the selectivity of novel integrin antagonists. This will be a big challenge since the cell line model prepared to use with the scratch assay may not work with the other assays such as adhesion assay and invasion assays. Therefore, other models will need to be investigated.

### 6.2 Conclusion

In conclusion, tumour dissemination is a major issue with the treatment of cancer, and there is a pressing need for therapies which can control this process. As has been discussed here, the integrins represent a good potential target for therapeutic intervention to control tumour dissemination. Using characterised cell lines in a validated functional assay for migration, promising potential novel β₃ integrin antagonists have been identified. Further work is required to confirm their activity as anti-cancer agents.
Chapter 7: References and Appendixes
7.1 References


with platinum-resistant advanced epithelial ovarian or primary peritoneal cancer. *Gynecol Oncol* 121: 273-9.


216


Carracedo, S., N. Lu, S. N. Popova, R. Jonsson, B. Eckes & D. Gullberg The fibroblast integrin \(\alpha 1\beta 1\) is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. *J Biol Chem*


in early breast cancer patients: a retrospective study of possible risk and protective factors. *Heart*


240


Oommen, S., S. K. Gupta & N. E. Vlahakis 2010. Vascular endothelial growth factor - A (VEGF-A) induces endothelial and cancer cell migration through direct binding to integrin \(\alpha_9\beta_1\): identification of a specific \(\alpha_9\beta_1\) binding site. *J Biol Chem*


Integrin alpha(v)beta3 expression confers on tumor cells a greater propensity to metastasize to bone. *FASEB J* 16: 1266-8.


Expression of integrin alphavbeta3 in gliomas correlates with tumor grade and is not restricted to tumor vasculature. *Brain Pathol* 18: 378-86.


7.2 Appendices

7.2.1 Appendix 1

The expression of integrins subunits in tumour cell lines: PC-3, DLD-1, DU-145 and HT-29 by PCR.
7.2.2 Appendix 2

Analysis of scratch width for each cell line in the panel.

<table>
<thead>
<tr>
<th>Tumour cell lines</th>
<th>Width of initial scratch</th>
<th>Width of wound at To</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H460</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLD-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT-116</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU-145</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-MEL-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UACC62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tumour cell lines