A thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy of the University of London.

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August 1998
Abstract
The prognosis of malignant astrocytomas in adults is very poor. The use of surgery, radiotherapy and chemotherapy can only modestly, yet significantly, prolong survival. However, whilst adjuvant chemotherapy has the potential to increase patient survival this is limited by the marked resistance to cytotoxic drugs which these tumours display, the mechanisms of which are unknown. Drug resistance has been examined using two sources of cellular material, short-term cell cultures derived from biopsy material and archival paraffin embedded material taken at the time of diagnosis and again at the time of eventual tumour recurrence. Although cultures displayed wide variation in sensitivity to two cytotoxic drugs, vincristine and doxorubicin, this did not correlate with expression of cell surface glycoproteins associated with drug resistance like P-glycoprotein and MRP (multi-drug resistance associated protein). However, overexpression of p53 protein did correlate with marked sensitivity to vincristine and an unrelated drug CCNU, but not with doxorubicin. Clinical follow-up of the patients from whom the cultures were derived suggested that overexpression of p53 correlated with short survival times, and p53 expression appeared to be a better prognostic factor than chemosensitivity in vitro or administration of adjuvant chemotherapy. The cells that repopulate the recurrent tumours often differ in their chromosomal number and chemosensitivity. In paraffin wax embedded material derived from paired samples taken at diagnosis and again at recurrence for the same patient, the cells derived from recurrent tumour appeared to contain more copies of chromosome 7, but not chromosome 22, as evidenced by FISH.

ERRATA
Page 14 1st paragraph
'Primary brain tumours' - this includes both intrinsic brain tumours and meningiomas.

Page 14 3rd paragraph
Choroid plexus tumours are neuroepithelial in origin but are not gliomas.
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<td>ATP binding cassette</td>
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<td>ACNU</td>
<td>3(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(chloroethyl)-1-nitrosourea hydrochloride</td>
</tr>
<tr>
<td>Act D</td>
<td>actinomycin D</td>
</tr>
<tr>
<td>ADR</td>
<td>Adriamycin</td>
</tr>
<tr>
<td>ANLL</td>
<td>lymphoblastic leukaemia</td>
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<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>AT</td>
<td>O6-alkylguanine transferase (see MGMT)</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
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<td>β-pol</td>
<td>DNA polymerase-β</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<td>CFTR</td>
<td>cystic fibrosis transmembrane regulator</td>
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<td>CGH</td>
<td>comparative genomic hybridisation</td>
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<td>chromosome</td>
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</tr>
<tr>
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<td>cerebro-spinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>computerised tomography</td>
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<tr>
<td>Cyclic AMP</td>
<td>cyclic adenosine mono-phosphate</td>
</tr>
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<td>C-Terminal</td>
<td>carboxy terminal</td>
</tr>
<tr>
<td>DM</td>
<td>double minute</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<td>DT</td>
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<td>DTIC</td>
<td>Dacarbazine</td>
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<td>fibroblast growth factor</td>
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<tr>
<td>FISH</td>
<td>fluorescence in situ hybridisation</td>
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<td>5-FU</td>
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</tr>
<tr>
<td>GBM</td>
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<td>glial fibrillary acidic protein</td>
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4-HC  4-hydroperoxycyclophosphamide
HBSS  Hank's balanced salt solution
HGF  hepatocyte growth factor
HPV  human papilloma virus
HSR  homogeneous staining region
ID50  50% inhibitory dose
ISH  in situ hybridisation
K562  human myeloid leukaemia
kD  kilo Daltons
LFS  Li-Fraumeni syndrome
LOH  loss of heterozygosity
LRP  lung resistance associated protein
MAb  monoclonal antibody
MCF7  human breast carcinoma
MDM2  mouse double minute protein
MDR  multidrug resistance
MDRA  MDR associated
MeCCNU  methyl-CCNU
MGMT  methylguanine methyl transferase (see AT)
MRC  Medical Research Council
MRI  magnetic resonance imaging
mRNA  messenger RNA
MRP  MDR associated protein
MTT  (3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl-tetrazolium bromide)
N-terminal  amino terminal
P  passage level
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCB  Procarbazine
PCNA  proliferating cell nuclear antigen (cyclin)
PCR  polymerase chain reaction
PCV  Procarbazine-CCNU-Vincristine
PDGF  platelet derived growth factor
PDGFR  platelet derived growth factor receptor
PGP  P-glycoprotein
PKC  protein kinase-C
RFI  relapse free interval
RNA  ribonucleic acid
RT  room temperature
RT-PCR  reverse transcription PCR
S-phase  DNA synthesis phase in cell cycle
SCLS  small cell lung cancer
SDS  sodium dodecyl sulphate
SF2  surviving fraction at 2 Gray
SPGP  sister of PGP
SSCP  single strand conformational polymorphism
SV40LT  large T antigen of SV40 oncogene product
TCNU  Tauromustine
TM  transmembrane region
<table>
<thead>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TTP</td>
<td>time to progression</td>
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<tr>
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INTRODUCTION

Astrocytic tumours

Incidence

Primary brain tumours are the 8th most common cancer in adult females and the 6th in adult males and account for 2% of all cancer deaths (Kaye and Laws 1995). The most common are the gliomas accounting for 38% of all primary brain tumours (Giles and Gonzales 1995). The gliomas are tumours of neuroepithelial origin and are subdivided into 6 groups.

Despite extensive research and novel approaches to the treatment of the malignant astrocytomas, they remain incurable tumours. The use of surgery, radiotherapy and chemotherapy can only modestly, yet significantly, prolong survival.

Classification and pathology

According to the 1993 World Health Organisation (WHO) system of classification (Kleihues et al 1993), the gliomas are divided into 6 groups, astrocytic tumours, oligodendrogliomas, ependymomas, choroid plexus tumours, mixed gliomas and other gliomas. The astrocytic tumours are further divided as shown in Figure 1.1. This study will concentrate on the anaplastic astrocytomas (Grade III) and the GBMs (Grade IV). The most malignant form of astrocytoma is the glioblastoma multiforme (GBM) and accounts for 50% of all adult gliomas. Cerebral astrocytomas occur commonly in the frontal lobes followed by the temporal and parietal lobes (McKeran and Thomas 1980). Astrocytomas are most common in patients in their 30s and 40s, whereas GBMs more often occur in the 50s and 60s. In general cerebral astrocytomas are slightly more common in males, however GBM is twice as common in males compared to females.

Using the 1993 WHO classification system, astrocytomas are divided into four grades based on increasing malignancy (Kleihues et al 1993). This grading introduces artificial divisions in the progression to increased malignancy, in this system, Grade I solely describes the pilocytic astrocytomas, which are biologically and prognostically distinct from the fibrillar astrocytomas, Grade II = low grade astrocytomas, Grade III = anaplastic astrocytomas and Grade IV = glioblastoma multiforme (GBM). The histological grading system by Daumas-Duport was specifically designed for the fibrillar or diffuse astrocytomas, it is similar to the WHO system and is based on four features indicating increasing anaplasia. These features are cellular/nuclear atypia, mitoses, vascular proliferation and necrosis, these features usually appear sequentially in the progression to higher malignancy (Daumas-Duport et al 1988).
The presence of any single feature increases the Daumas-Duport grade by one, as follows: No features = Grade I, 1 feature = Grade II, 2 features = Grade III and 3 or 4 features = Grade IV. In fact the grade I tumours are so rare it is often considered to be a three tier system (Burger and Scheithauer 1994). A comparison of these two systems is shown in Table 1.1. The Daumas-Duport grading strongly correlates with survival and clearly distinguishes 4 grades of astrocytoma on the basis of survival times, compared to the older Kernohan system which only distinguishes 2 groups, Grades 1 and 2 and Grades 3 and 4 (Kleihues et al 1993).

**Figure 1.1** Astrocytic tumour divisions according to the 1993 WHO Classification.

| Astrocytic tumours |  
|-------------------|---
| **Fibrillary/Diffuse** | **Others** |
| Astrocytoma | Pilocytic astrocytoma |
| Anaplastic astrocytoma | Pleomorphic Xanthoastrocytoma |
| Glioblastoma multiforme | Subependymal giant cell astrocytoma |
| Gliosarcoma | Infantile desmoplastic astrocytoma |
| Protoplasmic astrocytoma | Gliofibroma |
| Granular cell astrocytoma |  |
| Gliomatosis cerebri |  |
| Meningeal gliomatosis |  |

Adapted from Kleihues et al (1993).

The terms GBM and Grade IV astrocytomas are used interchangeably, these are the least differentiated and most malignant of the astrocytic tumours. They often have all of the Daumas-Duport features, including bizarre nuclei, hyperchromatic and multinucleate cells, frequent mitoses and marked endothelial proliferation.

Astrocytomas can progress through the grades to anaplastic astrocytomas and GBMs, although all grades can arise *de novo*, in one study 39 of 79 patients (49%) who had low grade astrocytomas were found to have grade III or IV astrocytomas at subsequent surgery or post mortem (Laws et al 1984).
Table 1.1 Comparison of the WHO and Daumas-Duport grading system for astrocytomas.

<table>
<thead>
<tr>
<th>WHO Grade</th>
<th>WHO Designation</th>
<th>Daumas-Duport Designation</th>
<th>Daumas-Duport Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Pilocytic astrocytoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Astrocytoma (low grade)</td>
<td>Astrocytoma Grade I</td>
<td>No criteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Astrocytoma Grade II</td>
<td>One criterion, usually nuclear atypia</td>
</tr>
<tr>
<td>III</td>
<td>Anaplastic astrocytoma</td>
<td>Astrocytoma Grade III</td>
<td>Two criteria, usually nuclear atypia and mitotic activity</td>
</tr>
<tr>
<td>IV</td>
<td>Glioblastoma multiforme</td>
<td>Astrocytoma Grade IV</td>
<td>Three criteria, usually nuclear atypia, mitoses, endothelial proliferation and/or necrosis</td>
</tr>
</tbody>
</table>

Adapted from Kleihues et al (1993)

Treatment
Surgery
Astrocytomas hardly ever metastasise outside of the central nervous system (CNS), and often appear to be discreet masses using diagnostic imaging. However, in practice it is usually impossible to resect the whole tumour because of the high degree of infiltration into the surrounding normal brain and the possibility of damage to important parts of the brain near to the tumour.

However, surgery is invaluable to the treatment of astrocytomas in five ways; first, to the definitive diagnosis of astrocytomas so that appropriate further treatment can be planned; secondly, the tumour is debulked so the number of cancer cells that need to be killed by further treatments is reduced; thirdly some symptoms are relieved by decreasing the intracranial pressure; and fourthly surgery in itself prolongs survival giving time for other therapies to be tried. Lastly, the debulking may encourage active growth of previously arrested cancer cells and so increase their susceptibility to radiation and chemotherapy (Shapiro 1982a).
Surgical intervention is common, if only for diagnostic purposes, however, patients with high grade astrocytomas (grades III or IV) who have extensive surgery tend to live longer than those having only a biopsy. Figures from the MRC Brain Tumour Working Party (Stenning 1990) comparing extent of surgery, as described by the neurosurgeon, and survival rates are shown in Table 1.2. All patients received radiotherapy and chemotherapy, (misonidazole) after surgery. Extensive surgery doubled the 2 year survival rate compared to biopsy. However other authors have not found this correlation (Coffey et al 1988). The apparent benefit of extensive surgery may really be an effect of the grade of tumour because more patients with grade III tumours may undergo more extensive surgery than those with grade IV tumours.

By measuring tumour size on CT scans no relationship between pre-operative tumour size and ultimate survival was seen, but there was a significant relationship with post-operative size (Shapiro et al 1989).

**Table 1.2** Extent of surgery and survival rates.

<table>
<thead>
<tr>
<th>Extent of neurosurgery</th>
<th>Number of patients</th>
<th>Median survival (weeks)</th>
<th>Survival rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy</td>
<td>178</td>
<td>23</td>
<td>14 4</td>
</tr>
<tr>
<td>Partial resection</td>
<td>168</td>
<td>38</td>
<td>25 7</td>
</tr>
<tr>
<td>Complete resection</td>
<td>71</td>
<td>45</td>
<td>38 9</td>
</tr>
</tbody>
</table>

(Data from MRC Brain Tumour Working Party, Stenning, 1990)

**Radiotherapy**

X-rays from a linear accelerator or cobalt-60 source are most often used for brain tumour radiation therapy. Energy from radiation causes the dissociation of water molecules within cells, forming free radicals which damage intracellular molecules, most importantly nuclear DNA. In the presence of oxygen the damage caused is irreversible, although DNA repair mechanisms can sometimes excise small lengths of damaged DNA and synthesise new DNA in its place. Radiation is usually only lethal if the cells try to undergo mitosis before any repair has occurred, hence the actively dividing cells, i.e. cancer cells, are more likely to be killed than the normal non-dividing brain.
Both normal and tumour cells differ considerably in their in vitro radiosensitivities, to quantify this a parameter termed the surviving fraction at 2 Gray (SF2) is used. The SF2 value is interpolated from a dose response curve of a clonogenic assay. Clinical radiosensitivity correlates to in vitro SF2 values, for example three neuroblastoma cell lines had SF2 values of 0.08, 0.11 and 0.13, compared to SF2 values of 0.43 and 0.47 for two melanoma cell lines (Steel et al 1989). High grade astrocytoma cells are also highly resistant to radiation with mean SF2 values of 0.5 for GBM cells and 0.34 for anaplastic astrocytoma cells (Taghian et al 1993). This observed resistance is probably not due to an increased ability to repair DNA damage, because at low doses the initially induced damage in both resistant and sensitive cells shows almost complete recovery (Steel et al 1989). It may be that lesions are further apart giving less probability of interaction leading to fixation of the damage. It is now thought more likely that in radiosensitive cells a given radiation dose induces more lethal and potentially lethal DNA lesions i.e. DNA double strand breaks, than in resistant cells. In radioresistant cells the degree of supercoiling and the association of DNA to the nuclear matrix may restrict access of free radicals (Steel et al 1989).

Radiotherapy following surgery has been shown to prolong survival (Table 1.3). In a prospective randomised study to compare treatments for anaplastic gliomas, including GBMs and anaplastic astrocytomas (Kernohan grade 3 and 4), radiotherapy increased median survival times from 17 to 38 weeks. Improvement in survival of BCNU alone over supportive care was only marginally significant (p=0.119). However radiotherapy alone or with BCNU gave clearly significant improvement (p=0.001) (Walker et al 1978). In a similar study the median survival following surgery and radiotherapy was 40-50 weeks (Chang et al 1983).

Table 1.3 Increased survival rates after radiotherapy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of patients</th>
<th>Median survival (weeks)</th>
<th>Survival rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supportive care</td>
<td>42</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>BCNU</td>
<td>68</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>93</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>Radiotherapy + BCNU</td>
<td>100</td>
<td>41</td>
<td>32</td>
</tr>
</tbody>
</table>

Radiotherapy = 5000-6000rads, BCNU = 80mg/m²/day, on 3 successive days/7 weeks. Supportive care = no radiotherapy or chemotherapy. (Adapted from Walker et al 1978)
Increasing the amount of radiation given to cells *in vitro* does increase the cell kill, but in patients there is a limit because of damage to normal brain. However, in a study to compare two post-operative radiotherapy doses of 45Gy or 60Gy, median survival times of 9 months with the lower dose and 12 months with the higher dose were found (Bleehen et al 1991). Three types of radiation reaction are recognised (Karim et al 1995): Early acute reactions may occur during the course of radiotherapy including skin problems and brain oedema. Early delayed reactions may occur weeks or months after irradiation and include nausea, vomiting, endothelial proliferation and oedema. These reactions may be treated with steroids. Late delayed reactions can develop 2-3 years after irradiation, with a dose dependent incidence of 5% in patients who received 45Gy or more (Marks et al 1981), they include vascular malformations and radionecrosis.

**Chemotherapy**

Many drugs have been used to treat malignant astrocytomas the most common of these are shown in Table 1.4. Of these drugs only the nitrosoureas and procarbazine are effective alone. The other agents are used in combination (Flowers and Levin 1995).

Before chemotherapeutic agents are used routinely for treatment, they must be assessed through a series of trials. The first of these must be a phase I toxicity trial to find the optimal working dose, this value is then used in all subsequent treatment. A prospective phase II study may then be undertaken and designed to show the activity of the agent. An objective response is used as a measure of activity, usually shrinkage of the tumour mass. Therefore, presence of measurable disease is necessary before the patient can enter a phase II trial. Agents that show antitumour activity are then tested against the best conventional therapy in a randomised phase III prospective trial. If the new agent proves more effective than the control, conventional therapy then it becomes the standard treatment (Fine et al 1994).

In early studies of chemotherapy, 16 of 30 recurrent GBMs (53%), and 7 of 12 recurrent astrocytomas (58%) responded to BCNU (Fewer et al 1972) A response was defined as an obvious, objective clinical improvement and although response was seen in over 50% of patients the average length of response time was only 14 weeks for GBMs and 29 weeks for anaplastic astrocytomas. In a later study only 8 of 40 GBMs (20%), and 16 of 36 anaplastic astrocytomas (45%) responded to BCNU (Levin et al 1985).
<table>
<thead>
<tr>
<th>Drug class and type</th>
<th>Name</th>
<th>mode of action</th>
<th>Ad.</th>
<th>Pc</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>BCNU (Carmustine)</td>
<td>alkylates DNA and causes cross links</td>
<td>IV</td>
<td>high</td>
<td>Walker 1978</td>
</tr>
<tr>
<td>Nitrosoureas</td>
<td>CCNU (Lomustine)</td>
<td></td>
<td>O</td>
<td>&quot;</td>
<td>Levin 1990</td>
</tr>
<tr>
<td></td>
<td>PCNU</td>
<td></td>
<td>IV</td>
<td>&quot;</td>
<td>Walker 1980</td>
</tr>
<tr>
<td></td>
<td>MeCCNU (Semustine)</td>
<td></td>
<td>O</td>
<td>&quot;</td>
<td>Chang 1983</td>
</tr>
<tr>
<td></td>
<td>ACNU</td>
<td></td>
<td>&quot;</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MePCNU</td>
<td></td>
<td>&quot;</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Metal salts</td>
<td>Cisplatin (CDDP)</td>
<td>Inhibits DNA precursors and binds to DNA</td>
<td>IV</td>
<td>low</td>
<td>Boiardi 1992</td>
</tr>
<tr>
<td></td>
<td>Carboplatin</td>
<td>&quot;</td>
<td>IV</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>Cyclo-phosphamide</td>
<td>alkylates DNA and causes cross links</td>
<td>IV/O</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ifosamide</td>
<td>&quot;</td>
<td>IV</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melphalan</td>
<td>&quot;</td>
<td>O</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AZQ</td>
<td>&quot;</td>
<td>IV</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Natural products</td>
<td>Vincristine (Onocin)</td>
<td>binds tubulin causes mitotic arrest</td>
<td>IV</td>
<td>low</td>
<td>Levin 1990</td>
</tr>
<tr>
<td>Vinca alkaloid</td>
<td>VP16 (Etoposide)</td>
<td>inhibits DNA synthesis via topoisomerase and binds to tubulin</td>
<td>IV/O</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>&quot;</td>
<td>&quot;</td>
<td>IV</td>
<td>low</td>
<td>Shapiro 1989</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
<td>&quot;</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Cytotoxic Antibiotics</td>
<td>Bleomycin</td>
<td>causes DNA strand breaks</td>
<td>IV</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Anthracycline</td>
<td>Doxorubicin (Adriamycin)</td>
<td>intercalates DNA and interferes with synthesis of nucleic acids</td>
<td>IV</td>
<td>low</td>
<td>Levin 1990</td>
</tr>
<tr>
<td></td>
<td>Daunorubicin (Daunomycin)</td>
<td>&quot;</td>
<td>IV</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>5-Fluorouracil</td>
<td>Inhibits DNA and RNA synthesis</td>
<td>IV</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td>Pyrimidine analogues</td>
<td>Cytosine arabinoside</td>
<td></td>
<td>IV</td>
<td>medium</td>
<td>Boiardi 1992</td>
</tr>
<tr>
<td>Purine analogues</td>
<td>Thioguanine</td>
<td>&quot;</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folic acid analogue</td>
<td>Methotrexate</td>
<td>Inhibits dihydrofolate reductase preventing purine and pyrimidine synthesis</td>
<td>O/IV</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>DEB (Dibromodulcitol)</td>
<td>alkylating agent, affects RNA and DNA</td>
<td>O</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td>Halogenated hexitol</td>
<td>Hydroxyurea</td>
<td>DNA reactive inhibitors DNA precursors</td>
<td>O</td>
<td>high</td>
<td>Levin 1990</td>
</tr>
<tr>
<td>Urea analogue</td>
<td>Procarbazine (Natulan)</td>
<td>affects DNA and RNA synthesis</td>
<td>O</td>
<td>high</td>
<td>Levin 1990</td>
</tr>
<tr>
<td>Methyl hydrazine derivative</td>
<td>Methotrexate</td>
<td>Inhibits dihydrofolate reductase preventing purine and pyrimidine synthesis</td>
<td>O/IV</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>Imidazole</td>
<td>DTC</td>
<td>forms diazo compounds</td>
<td>IV</td>
<td></td>
<td>Boiardi 1992</td>
</tr>
<tr>
<td>Carboxamides</td>
<td>(Dacarbazine)</td>
<td>may act as alkylating agent</td>
<td>IV</td>
<td></td>
<td>Chang 1983</td>
</tr>
<tr>
<td></td>
<td>Temozolomide</td>
<td></td>
<td>IV</td>
<td></td>
<td>Newlands 1992</td>
</tr>
</tbody>
</table>

Ad. = Administration, O = orally, IV = Intravenously, Pc = Brain capillary permeability (ability to cross blood brain barrier). Adapted from Flowers and Levin 1995, Reynolds 1993.
Phase III trials have shown that radiotherapy used alone or with a nitrosourea significantly improved survival of patients with anaplastic astrocytoma and GBM compared to MeCCNU alone, Table 1.5. (Walker et al 1980). In the trial by Chang the results for all age groups are shown in Table 1.6. (Chang et al 1983). However, they concluded that only those patients with anaplastic astrocytomas in the 40-60 year age group, using the single agent BCNU, had a significantly increased survival over radiotherapy alone.

**Table 1.5** Increased survival rates after radiotherapy alone or with a nitrosourea, compared to nitrosourea alone, in high grade astrocytomas including GBMs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of patients</th>
<th>Median survival (weeks)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 year</td>
</tr>
<tr>
<td>MeCCNU</td>
<td>81</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>94</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>Radiotherapy + BCNU</td>
<td>92</td>
<td>51</td>
<td>50</td>
</tr>
<tr>
<td>Radiotherapy + MeCCNU</td>
<td>91</td>
<td>42</td>
<td>37</td>
</tr>
</tbody>
</table>

Radiotherapy = 6000 rads, MeCCNU = 220 mg/m²/7 weeks, BCNU = 3 x 80 mg/m²/7 weeks. (Adapted from Walker et al 1980)

**Table 1.6** Increased survival rates after radiotherapy and nitrosourea in patients with anaplastic astrocytomas.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival (months)</th>
<th>18 month survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>GBM</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Radiotherapy + boost</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Radiotherapy + BCNU</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>Radiotherapy + MeCCNU + DTIC</td>
<td>22</td>
<td>9</td>
</tr>
</tbody>
</table>

Radiotherapy = 6000 rads/7 weeks. Boost = extra 1000 rads over 2 weeks. BCNU = 3 x 80 mg/m²/8 weeks, MeCCNU = 125 mg/m²/8 weeks, DTIC = 150 mg/m²/4 weeks. (Adapted from Chang et al 1983).
Although single agent nitrosourea when used as an adjuvant does appear to improve survival in patients with malignant astrocytoma, combination chemotherapy may provide additional benefit (Levin et al 1985). In this study 76 patients with GBM and 72 with anaplastic astrocytomas (grades III and IV) were randomised to receive radiotherapy with hydroxyurea, followed by either BCNU alone or a combination of procarbazine (PCB), CCNU and Vincristine (VCR), (PCV). With no adjustment for prognostic variables there were no significant differences between the two arms of the study, but for good risk patients with anaplastic astrocytoma there was a trend towards longer survival with PCV than with BCNU, the median times to progression were 123 and 77 weeks respectively. In 1990 the data from this study was reanalysed to include only those patients with Karnofsky scores of 70-100 at randomisation and who were adequately treated (Levin et al 1990). This analysis showed that PCV was significantly more effective than the single agent BCNU in patients with high grade astrocytomas (grade III) but again there was little benefit to patients with GBMs.

Often when various studies are compared, significantly better, or worse, survival can be attributed to the trial design and data interpretation. There are also few new agents being used in these trials. Of 13 studies, 10 used only single agent nitrosoureas (Stenning et al 1987). However, as a result of reviewing the major trials that led to the standard treatments in the early 1990s the following post-surgical treatments for malignant astrocytoma have been recommended (Fine et al 1994): Optimal external beam radiation of 6000cGy total dose over 6-7 weeks, to the entire tumour area, i.e. either MRI T2 weighted signal plus 1-2 cm margin, or CT scan plus 3-4 cm margin. Followed by PCV chemotherapy for high grade astrocytomas. There is no similar recommendation for chemotherapy for GBMs.

**Blood Brain Barrier**

There are specific problems associated with the chemotherapy of brain tumours, the main one being the presence of the blood brain barrier, (BBB). Unlike systemic capillaries, the endothelial cells of the BBB can prevent soluble polar compounds and large molecules like proteins from passively crossing from the blood to the brain tissue or CSF. These cellular differences are shown in Figure 1.2.
Figure 1.2 Comparison of brain capillary and systemic capillary.

(Adapted from Thapar et al 1995)
The ability of drugs to cross the BBB is not solely dependent on molecular weight, lipophilicity is also involved. For those compounds with a molecular weight below 400, increasing lipophilicity will improve capillary permeability. For example, CCNU and PCB have similar molecular weights (234 and 221 respectively), but CCNU is more lipophilic and has a 5-fold higher capillary permeability (Levin 1980). Although the presence of the BBB implies that transport is restricted there are many energy dependent transport mechanisms which correlate with the 3-5 times more mitochondria found in BBB endothelial cells compared to systemic endothelial cells (Thapar et al 1995).

Although the presence of the BBB would appear to preclude chemotherapy from astrocytoma treatment, significant amounts of drug may be able to enter at least part of the tumour. For example, the BBB in the area of the tumour is often damaged in some way, especially in areas of necrosis, the drug may therefore reach the necrotic centre of the tumour, but not the growing edge (Shapiro et al 1982b). Even in the absence of necrosis, disruption of the BBB is commonly found with brain tumours including opening of tight junctions and the presence of fenestrations, gap junctions and pinocytic vesicles, this causes the BBB to become 'leaky' (Thapar et al 1995). However, the extent of BBB disruptions in individual tumours is unpredictable, making the outcome of treatment difficult to foresee.

**Prognostic factors**
Prognostic factors can influence patient survival, even in the absence of treatment. Good prognostic factors include; age less than 40, good functional status indicated by a high Karnofsky score and lower histological grade of tumour (Chang et al 1983, Stenning 1990, Walker et al 1980, Shapiro et al 1982a). Long duration of symptoms and presence of seizures are also good prognostic indicators. Presence of seizures also correlated with longer survival, because patients sought medical attention earlier (Shapiro et al 1982a). The MRC working party (Stenning 1990) also found that a long history of fits and extensive surgical removal of the tumour indicated good prognoses. These factors must be addressed when comparing treatment outcomes.
Drug Resistance in Cancer
Clinical observations

Some cancers, for example some leukaemias, lymphomas and testicular teratomas, can be cured by chemotherapy, but often an initial response to a cytotoxic drug is followed by relapse and ultimately, death (Morrow and Cowan 1988). Clinically, three responses to cancer chemotherapy are seen: 1. the cancer cells are inherently sensitive to chemotherapy, e.g. some leukaemias, 2. the cancer cells are inherently resistant to chemotherapy, e.g. malignant astrocytomas, and 3. initially sensitive cancer cells acquire resistance to the drugs used in previous chemotherapy courses. Models for these three responses are shown in Figure 1.3.

A cancer that is inherently sensitive to chemotherapy will grow until chemotherapy is given. The sensitive cancer cells will be killed (Figure 1.3 Panel A), this model is applicable to all cancers that can be cured by chemotherapy. An inherently resistant cancer like malignant astrocytoma will also grow over a number of months or years until diagnosed. Taking brain tumours as an example, patients presenting with neurological symptoms usually have tumours of at least 30gm equivalent to $3 \times 10^{10}$ cells (Shapiro et al 1982a). Treatment will probably include surgery, a subtotal resection may remove 90% of the tumour, leaving in this case $3 \times 10^9$ cells. Radiotherapy may produce 2 logs of cell kill and chemotherapy at best may produce one more log of cell kill. The remaining tumour cells will continue to divide and death will ultimately occur (Figure 1.3, Panel B).

The phenomenon of acquired resistance is a major cause of death in small cell lung cancer, breast cancer and ovarian cancer (Kaye 1988). It may also be one explanation for the recurrence of malignant astrocytomas. The model in Figure 1.3, Panel C shows how the first course of chemotherapy is apparently successful, most drug sensitive cells are killed. However, newly resistant cells continue to grow. The remaining drug sensitive population also grows. A second course of chemotherapy is given, again the number of drug sensitive cells are reduced, but now the tumour is predominantly composed of drug resistant cells. The third course of chemotherapy has no apparent effect and the resistant cells continue to multiply.
Figure 1.3 Models of responses to chemotherapy.

A  **Sensitive**

![Diagram of sensitive cell response to chemotherapy.]

- Light shading = sensitive cells
- Dark shading = resistant cells
- For full explanation see text.

B  **Resistant**

![Diagram of resistant cell response to chemotherapy.]

- Light shading = sensitive cells
- Dark shading = resistant cells
- For full explanation see text.

C  **Acquired Resistance**

![Diagram of acquired resistance to chemotherapy.]

- Light shading = sensitive cells
- Dark shading = resistant cells
- For full explanation see text.

Adapted from Kartner and Ling 1989. Light shading = sensitive cells, Dark shading = resistant cells. For full explanation see text.
The model for acquired resistance assumes an existing cellular heterogeneity in the tumour, with both sensitive and resistant cells present prior to chemotherapy. It is known that in a given cell population mutations conferring resistance to a given drug occur once in $10^5 - 10^8$ cells, therefore at the time of diagnosis tumours probably contain a small fraction of resistant cells (Kartner and Ling 1989). Acquired resistance occurs only after exposure to chemotherapeutic agents and may be due to the apparent selection of the existing resistant population (Figure 1.3. Panel C) although, the chemotherapeutic drug may itself cause mutations conferring resistance.

A model for the development of drug resistance by growing populations of cells was proposed by Goldie and Coldman (Goldie and Coldman 1983). Three assumptions were made: 1. resistant cells arise from random mutations, 2. resistant and sensitive cells have the same growth kinetics, and 3. all tumour cells can proliferate. This model is shown simplistically in Figure 1.4A, where 3 divisions from one sensitive cell gives rise to 2 resistant cells and 6 sensitive cells. Figure 1.4B shows how resistance may be enhanced if some of the cells differentiate or are unable to divide due to lethal mutations. Here, after 3 divisions half of the cells are resistant, after further divisions the resistant cells will predominate. This indicates how at the time of diagnosis of a tumour, all the cells could be resistant and the tumour described as inherently resistant. In this simple model showing development of resistance by random mutations, the resistant cells are even more likely to predominate if they also have a growth advantage over the sensitive cells.

Drug resistance to single agents is a major problem in cancer chemotherapy, and many different mechanisms are involved. In theory if a number of cytotoxic drugs were used at the start of treatment, combination chemotherapy, cells within a heterogeneous tumour that were resistant to one particular drug would be killed by different drugs. This theory was based on the idea that the probability that two or more different drug resistances would arise spontaneously in the same cell was slight. Combination drug protocols became popular and had some success, for example the treatment of some childhood leukaemias and Hodgkin's disease. However some cancers for example of the lung, breast, gastrointestinal tract and brain remain refractory even to combination chemotherapy.
Figure 1.4 Goldie-Coldman model for development of drug resistance.

Circles = tumour cells, each division forms 2 new tumour cells. Squares = differentiated non-proliferating cells. Filled circles or squares = resistant cells
**Multiple drug resistance in vitro**

**Patterns of cross resistance**

The first evidence that drug resistance might be modulated by alterations in the cell membrane came from studies by Goldstein et al (1966) who produced sublines of HeLa which were made resistant by long-term chronic exposure to Actinomycin D (Act D). These cells appeared to not only exclude Act D from the nucleus but also from the cytoplasm strongly suggesting that membrane permeability had been altered. Similar experiments by Biedler and Riehm (1970) which used Chinese hamster (CH) lung fibroblasts selected for resistance to either Act D or Daunomycin (DNM), found that these cells were collaterally resistant to a selection of other drugs with apparently little mechanistic or structural similarity, (Table 1.7). The cells were cross resistant to several drugs, including mithramycin, VBL, VCR, puromycin, DNM, Demecolcine (colcemid) and mitomycin C. Responses to the other drugs were not significantly different to the parent cells (Biedler and Riehm 1970). It was noted that all these drugs were hydrophobic and derived from natural products. However, they differed both structurally and functionally. This resistance pattern was termed 'pleiotropic cross resistance' (Juliano and Ling 1976) but is now referred to as multidrug resistance (MDR) (Kartner et al 1983).

Clonal lines with wide ranging cross resistance patterns were later obtained by single step selection methods. Chinese hamster ovary (CHO) epithelial cells selected for resistance to colchicine (COL), were found to be cross resistant to Act D and vinblastine (VBL) (Ling and Thompson 1974). In these experiments stable mutants were seen at a frequency of $10^{-7}$-10$^{-5}$, this indicated that the MDR phenotype was likely to arise from the expression of a single gene (Gerlach et al 1986). This offered a plausible explanation for the failure of combination chemotherapy, since it would be highly unlikely that multiple mutations causing independent resistance to individual drugs would occur in the same cell, and more likely that a single mutation could cause resistance to many drugs (Kartner and Ling 1989).

By comparing parental and resistant sublines, the degree of resistance to DNM and VCR was found to be proportional to the concentration of the selecting drug Act D (Biedler and Riehm 1970). For example the parental cell line was relatively sensitive to DNM, VCR and Act D. The subline selected with 0.1µg/ml Act D had relative resistance values of 81 (Act D), 51 (VCR) and 9.4 (DNM) and the subline selected with 10µg/ml Act D had relative resistance values of 2450 (Act D), 556 (VCR) and 76 (DNM). This implied that a common cellular property was controlling the response to all the different agents.
Table 1.7  Resistance patterns for Act D selected CH lung cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>M(r)</th>
<th>ID50 for parental cells (µg/ml)</th>
<th>ID50 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mithramycin</td>
<td>1089</td>
<td>0.068</td>
<td>670.0</td>
</tr>
<tr>
<td>Act D*</td>
<td>1255.5</td>
<td>0.0024</td>
<td>376.0</td>
</tr>
<tr>
<td>Vinblastine sulphate</td>
<td>929.9</td>
<td>0.0043</td>
<td>239.0</td>
</tr>
<tr>
<td>Vincristine sulphate</td>
<td>923.0</td>
<td>0.02</td>
<td>189.0</td>
</tr>
<tr>
<td>Puromycin</td>
<td>471.5</td>
<td>1.47</td>
<td>84.0</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>527.5</td>
<td>0.016</td>
<td>29.0</td>
</tr>
<tr>
<td>Demecolcine</td>
<td>371.4</td>
<td>0.013</td>
<td>18.0</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>334.3</td>
<td>0.019</td>
<td>3.1</td>
</tr>
<tr>
<td>Proflavine sulphate</td>
<td>325.3</td>
<td>0.14</td>
<td>2.9</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>612.7</td>
<td>91.8</td>
<td>1.9</td>
</tr>
<tr>
<td>BrdU</td>
<td>307.1</td>
<td>18.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Nitroquinoline</td>
<td>190.2</td>
<td>0.023</td>
<td>1.1</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>454.5</td>
<td>0.012</td>
<td>1.1</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>152.2</td>
<td>0.44</td>
<td>0.5</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>486.4</td>
<td>219.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Nitrogen Mustard</td>
<td>192.5</td>
<td>0.35</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Selecting agent. ID50 ratio = ID50 for resistant, selected cells /ID50 for parental cells.
(Adapted from Biedler and Rhiem, 1970)

Following the tritiated drug uptake studies of Goldstein et al (1966), further work on drug uptake and retention was undertaken. Resistant cells showed diminished uptake of $^3$H-Act D (Biedler and Riehm 1970), and reductions in the amount of $^3$H-Colchicine entering resistant cells was also found (Ling and Thompson 1974). Reduced permeability of the membrane was suggested to explain the MDR phenomenon. However, the observation that this was due to enhanced drug efflux rather than impaired drug uptake, at either the level of the cell membrane or the nuclear membrane, came from studies on resistant Ehrlich ascites tumour cells, derived from mouse mammary carcinoma, which were selected in vivo using DNM. Isolated nuclei were shown to accumulate more $^3$H-daunomycin than resistant whole cells. Similarly, accumulation of DNM in resistant cells was enhanced by structural analogues N-Acetyl DNM and DNR, and the drugs VCR and VBL, and by the metabolic inhibitors 2-deoxyglucose and iodoacetate (Danø 1973). These findings suggested that cells were actively expelling drug and the mechanism might be located at the plasma membrane. The competition experiments also implied that the same mechanism was being used to expel all three drugs.
Drugs involved in MDR
Many drugs are implicated in the MDR phenomenon, they have no common structure or common intracellular targets. However, they are all hydrophobic natural products being derived from plants or micro-organisms, semi-synthetic analogues of these products or synthetic organic compounds. Chemically they are a diverse group of compounds with both hydrophobic and hydrophilic elements (amphipathic) and are lipophilic (Gottesman and Pastan 1993). A list of these drugs is shown in Table 1.8. Most alkylating agents, anti-metabolites and heavy metals are not implicated in the MDR phenotype.

Table 1.8 Drugs implicated in MDR.

<table>
<thead>
<tr>
<th>Class of drug</th>
<th>Name</th>
<th>M(r)</th>
<th>mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Vincristine (Oncovin)</td>
<td>825</td>
<td>Binds tubulin causes mitotic arrest</td>
</tr>
<tr>
<td></td>
<td>Vinblastine</td>
<td>930</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colchicine</td>
<td>399</td>
<td>Antimitotic</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>VP16 (Etoposide)</td>
<td>589</td>
<td>Inhibits DNA synthesis via topoisomerase and binds to tubulin</td>
</tr>
<tr>
<td></td>
<td>VM26 (Teniposide)</td>
<td>657</td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Actinomycin D (Dactinomycin)</td>
<td>1256</td>
<td>Intercalates DNA</td>
</tr>
<tr>
<td></td>
<td>Mithramycin</td>
<td>1089</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
<td>472</td>
<td></td>
</tr>
<tr>
<td>Anthracycline</td>
<td>Doxorubicin (Adriamycin)</td>
<td>543</td>
<td>Intercalates DNA</td>
</tr>
<tr>
<td></td>
<td>Daunorubicin (Daunomycin)</td>
<td>528</td>
<td>Intercalates DNA</td>
</tr>
<tr>
<td></td>
<td>Mitoxantrone</td>
<td>444</td>
<td>Intercalates DNA, causes DNA strand breaks and inhibits topoisomerase II</td>
</tr>
<tr>
<td></td>
<td>Dihydroxyanthracenedione</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Taxenes</td>
<td>Paclitaxel</td>
<td>854</td>
<td>Induces microtubule formation and disrupts cell division</td>
</tr>
<tr>
<td></td>
<td>Docetaxel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>Emetine</td>
<td>481</td>
<td>Emetic</td>
</tr>
<tr>
<td></td>
<td>Ethidium Bromide</td>
<td>314</td>
<td>Binds to DNA</td>
</tr>
<tr>
<td></td>
<td>Cytochalasin B</td>
<td>479</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
<td>334.3</td>
<td>Alkylating agent and inhibits synthesis of nucleic acids</td>
</tr>
<tr>
<td></td>
<td>Gramicidin D</td>
<td></td>
<td>Antibacterial agent</td>
</tr>
</tbody>
</table>

(Adapted from Morrow and Cowan 1988)
P-glycoprotein mediated MDR

Identification of P-glycoprotein

A membrane protein which was overexpressed in MDR cells was first discovered in Chinese Hamster Ovary (CHO) cells selected with COL (Juliano and Ling 1976). These cells showed a reduced permeability to the selecting drug, which correlated with the level of resistance, and were also cross resistant to other MDR implicated drugs. There were no major differences between the resistant cells and the sensitive parent line in terms of lipid composition. Preliminary studies by the authors had suggested surface glycoproteins may be found in resistant lines, so the surface galactose and galactosamine residues of intact resistant and sensitive cells were radiolabelled with tritiated borohydride. The plasma membranes were prepared and isolated then separated using SDS PAGE followed by auto radiography. Amongst the many bands on the gel they found a unique, large glycoprotein present in large quantities only in resistant cell lines. This glycoprotein had a molecular weight of approximately 170kD and was named P-glycoprotein (PGP) for its apparent action as a Permeability barrier to drugs. Other groups found homologous glycoproteins in different species including mouse (Danø 1973) and human (Beck and Cirtain 1982), using wide variety of selecting drugs including adriamycin (ADR), COL, Daunorubicin (DNR), VBL and VCR.

Specific antibodies have been prepared to further characterise this glycoprotein. Antiserum was raised by injecting purified plasma membrane vesicles from highly MDR CHO cells into mice. The resulting antiserum was then further purified by absorption with plasma membrane proteins from the drug sensitive parent cell line. Immunoblotting showed high levels of a 170kD protein in resistant cells and only very low levels in sensitive cells proving the antiserum's specificity for PGP. Similar results were found using plasma membranes prepared from MDR cell lines from other species with different selecting drugs, including COL or DNR selected CHO cells, COL selected mouse fibroblasts, VBL selected human lymphoid cells and Act D selected Syrian hamster fibroblasts, this indicated that PGP was a conserved molecule between species and therefore probably had an important functional role (Kartner et al 1983).

Direct proof that increased expression of PGP conferred MDR upon otherwise sensitive cells was found by isolating PGP complementary DNA (cDNA) from drug sensitive mouse cells, and transfecting this into drug sensitive hamster cells. Resulting cells were resistant to ADR, COL and VCR. cDNA and RNA encoding PGP were both over expressed (Gros et al 1986a).
The term classical, or typical, MDR is specifically used to describe cells with the following four phenotypic features: 1. resistance to a range of functionally and structurally unrelated drugs, 2. decreased cellular drug accumulation, 3. increased efflux of drug, and 4. overexpression of P-glycoprotein.

**P-Glycoprotein**  
**Structure**

The functional form of PGP in its native state is unknown, although by using radiation target size analysis and immunoblotting it was found that PGP molecules occur as dimers in the membrane (Boscoboinik et al 1990), similarly dimers and tetramers were found by freeze fracture electron microscopy (Weinstein et al 1990). Sucrose gradient velocity sedimentation showed that monomers and dimers were more phosphorylated than larger oligomers implying that they may be functionally distinct (Poruchynsky and Ling 1994). The monomeric structure of purified PGP has been determined by electron microscopy and image analysis (Rosenberg et al 1997), viewed from above PGP is toroidal with a 6-fold symmetry and a diameter of about 10nm. It has a 5nm diameter central pore which is closed at the cytoplasmic face of the membrane forming an aqueous chamber. This chamber has a putative opening to the lipid phase (Higgins et al 1997). PGP projects vertically into the membrane to a depth of 8nm, with two 3nm lobes exposed at the cytoplasmic face of the membrane corresponding to the nucleotide (specifically ATP) binding sites.

The molecular weight of mature PGP ranges from 130 to 200kD depending on the type of cell and species (Greenberger et al 1987). These differences are due to post-translational modifications including glycosylation and phosphorylation. The carbohydrate moiety may be 30-40kD of the mature PGP, but interestingly glycosylation is not necessary for the MDR phenotype (Beck and Cirtain 1982). Pulse chase labelling experiments using MDR KB cells from human oral epithelial carcinoma transfected with MDR-1 cDNA showed that PGP was synthesised as a 140kD precursor, this was converted over 2-4 hours to a 170kD glycoprotein. PGP is a very stable protein with a half life of 48-72 hours (Richert et al 1988).

The amino acid sequence homology between PGP and the HlyB protein in E. coli has been shown (Gerlach et al 1986). HlyB is a 66kD membrane protein which transports haemolysin. PGP is a tandem duplication of this protein with sequences homologous to nucleotide binding sites of bacterial ATP binding proteins. In the light of this they proposed that PGP could act as an energy dependent export pump.
Further work with a full length PGP cDNA verified PGP as a 1280 amino acid protein with tandem duplication of six pairs of transmembrane domains and a cluster of N-linked glycosylation sites near the amino terminus. The tandem duplications of approximately 500 amino acids have homology to 5 bacterial transport systems including the greatest homology (>70%) with H1yB (Gros et al 1986b). The predicted transmembrane orientation and 3D structure of PGP are shown in Figure 1.5. Each duplicate region comprises a hydrophobic sequence each with 6 transmembrane segments plus a hydrophilic region with nucleotide binding sites (Chen et al 1986).

**Function**

**Cellular level**

Although the normal physiological role of PGP remains unknown, its presence in secretory epithelia suggests a role in secretion. However, understandably most studies have concentrated on the role of PGP in multidrug resistant cells.

Based on the inherent fluorescence of DNM, drug uptake, retention and efflux, in both resistant and sensitive KB cells, was compared using light microscopy. Intracellular accumulation was rapid in sensitive KB cells but greatly reduced in MDR mutants. Accumulation of DNM was increased in lysosomes and the perinuclear Golgi region in the MDR cells, but only in sensitive cells did it accumulate in the nuclei, implying that the membrane efflux pump was present in lysosomes and Golgi of resistant cells but in a reverse orientation. Efflux of drug from MDR cells in the presence of azide-2-deoxyglucose but no glucose was very slow, suggesting that the uptake of DNM is not energy dependent but that efflux is (Willingham et al 1986)

Other more specific studies using inside out plasma membrane vesicles with partially purified PGP, showed energy dependent accumulation of drugs against a concentration gradient. Accumulation of $^3$H-Vinblatine using PGP from human KB carcinoma MDR cells (Horio et al 1988), and accumulation of $^3$H-colchicine using PGP from MDR CHO cells (Sharom et al 1993) was demonstrated. No accumulation was seen in vesicles prepared from sensitive cells.
Figure 1.5 A. Proposed membrane orientation of P-glycoprotein, and B. 3D structure viewed from above.

Adapted from Chen et al (1986). Numbers 1-12 = Transmembrane regions.

(Adapted from Higgins et al 1997)
Molecular level
The function of PGP was described by Safa et al (1986 and 1987), after studying
photoaffinity labelling of PGP using photoactive and radioactive drug analogues.
Whole cell fractions of CH lung cells, resistant to VCR and Act D, were photo
labelled then analysed by SDS PAGE. A labelled doublet, two bands of 150-180kD,
was found in resistant cells. The drug-protein complex was recognised by antibodies
to PGP. The binding was competitively inhibited by a dihydropyridine calcium
channel blocker and other drugs including VBL, Act D, Doxorubicin (DOX) and COL.

PGP is considered to be a transmembrane, pore forming protein acting as an efflux
pump, however, the removal of drugs from the cytoplasm through this pore to the
extracellular space is likely to be more complicated than it first appears. It is known
that PGP binds different drugs to specific domains. With the use of specifically
altered PGPs transfected into sensitive human melanoma cells, a PGP with a non
functioning last transmembrane region (TM12) was shown to have impaired
resistance to Act D, VCR and DOX, but not to COL. When the transmembrane loop
between TM11 and TM12 was non functional there was a more efficient efflux of Act
D, COL and DOX, but not VCR (Zhang et al 1995). Other drug binding sites have been
found using photoaffinity labelling including TM5, TM6, TM12 and the linking
regions following TM6 and TM12 (Gottesman et al 1993). These transmembrane
regions have a high percentage of amino acids with hydrogen bonding donor side
chains and it is thought drugs which interact with PGP have well defined spatial
recognition elements including two or three electron donor groups with fixed
spatial separation (Seelig 1998).

Drug efflux experiments in MDR cells show very little DOX present in the
cytoplasm, with most of the drug outside the cell or at low concentrations in
lysosomes or Golgi (Willingham et al 1986). If it is assumed that drugs simply enter
the cytoplasm by passive diffusion then are effluxed via PGP, then more drug would
initially be present in the cytoplasm. However, the drug may passively enter the
membrane but be expelled via PGP before it reaches the cytoplasm. A model to
explain this phenomenon has been suggested, at first PGP was termed a membrane
"vacuum cleaner" simply sucking drugs out of the membrane (Raviv et al 1990) but
then more specifically termed a "flippase" whereby the inner leaflet of membrane
phospholipids are flipped to the outer leaflet along with any associated drug
(Higgins and Gottesman 1992). This model involves drugs interacting with the lipid
bilayer during their entry into the cell, this links to the fact that many drugs
implicated in MDR are hydrophobic, PGP would then remove the drugs from the
membrane before they reach the cytoplasm. Presence of DOX in the membranes of sensitive cells can be shown, however, in MDR cells DOX is only found in the membrane closely associated with PGP molecules, implying that DOX has been removed from the rest of the membrane (Raviv et al 1990), support for the removal of drugs directly from the membrane was given using the fluorescent Hoechst dye and PGP in liposomes (Shapiro and Ling 1995). DOX is found in both the inner and outer leaflets of sensitive membranes during passive diffusion into erythrocytes, DOX remains comparatively longer in the inner leaflet, which may allow PGP a greater opportunity to remove the drug (Regev and Eytan 1997). The model implies that increased efflux of drug from PGP expressing cells should also be associated with an apparent decreased influx. No concomitant decreased influx of rhodamine 123 could be found in MDR CH lung fibroblasts (Altenberg et al 1994), although Stein suggests that PGP mediates both influx and efflux of its substrates in a MDR transfected mouse cell model (Stein et al 1994). PGP as "vacuum cleaner" or "flippase" is an interesting idea, however depending on the amount of PGP in the plasma membranes (30% of total protein in some synthetic proteoliposomes (Ambudkar et al 1992), drugs entering the cell by passive diffusion may not be next to a PGP molecule and so enter the cytoplasm. Efflux of drugs from cytoplasm is seen in PGP expressing cells, and drug binding sites on the cytoplasmic side of PGP may be present. It is possible therefore that PGP acts primarily as a "flippase" expelling drug from the lipid phase, and secondarily as a pump-like transport protein expelling drug from the cytoplasmic aqueous phase through the central pore (Higgins and Gottesman 1992). "Flippase" molecules may well occur in biological membranes, for example a potential phosphatidylcholine translocator in liver canalicular plasma membrane transporting biliary phospholipids has been found (Berr et al 1993), and more intriguingly a second Human PGP, with no MDR implications, located in bile canalicular membranes of hepatocytes, which when genetically disrupted in mice prevents secretion of phospholipids into the bile by the liver (Smit et al 1993).

All the evidence points towards one or more PGP molecules forming a 3D structure within the membrane with a transport channel, different drugs may have different binding regions but all would be transported through the channel, either directly from the membrane ("flippase") and/or from the cytoplasm (pump). These two models of drug transport are shown in Figure 1.6.
Figure 1.6 Two models of drug transport by P-glycoprotein.

(Adapted from Higgins and Gottesman 1992)
ATPase activity
From mutation analyses both ATP binding sites are necessary for PGP function. These two sites are different and are not interchangeable (Gottesman et al 1995). Functional PGP has a high rate of Mg$^{2+}$ dependent ATP hydrolysis, for example hydrolysis of 50 ATP molecules were required for the transport of each molecule of Hoechst dye (Shapiro and Ling 1995).

ATP hydrolysis is a requirement for drug transport and this hydrolysis may be stimulated by drug binding (Ambudkar et al 1992). However, it is not known how this energy brings about drug movement, a 'water wheel' effect of continuous ATP hydrolysis moving drugs through the pore or chamber has been suggested, also ATP hydrolysis has been linked to transport of protons and/or chloride ions into the PGP molecule (Gottesman and Pastan 1993). In the latter case water from the plasma membrane will be drawn into the transporter along with any amphipathic drugs. This does not account for the specific substrate binding often seen, but it does explain how a wide range of structurally dissimilar drugs can be effluxed. Very hydrophobic drugs like camptothecin are not at all soluble in water and are not substrates for PGP, indicating that some degree of water solubility may be required for efflux to take place (Chen et al 1991). Although the exact mechanism of action is unknown, ATP hydrolysis is known to induce conformational changes in PGP (Sonveaux et al 1996).

Phosphorylation
In its natural state PGP is phosphorylated and this may regulate the drug efflux mechanism. With MDR K562 cells (human myeloid leukaemia lymphoblasts) phosphorylation of PGP serine residues was increased in the presence of phorbol esters and verapamil, with different residues being phosphorylated in each case, indicating that a number of different phosphorylating kinases may be involved (Hamada et al 1987). In a MDR human glioma cell line (GB1), treated with protein kinase C (PKC) activators and inhibitors, it was found that VCR efflux was closely associated with PGP phosphorylation and this efflux was decreased by the PKC inhibitor calphostin C (Matsumoto et al 1995).

The major phosphorylation sites are in the central cytosolic linker region connecting the two homologous halves of PGP. This region contains charged serine residues that could be phosphorylated by protein kinases. Two transfection experiments were undertaken using human cDNA, in the first, serine residues in the linker region were replaced by non-phosphorylatable alanine residues, and in
the second they were replaced with permanently phosphorylated residues. Both of the resulting PGPs conferred MDR to previously sensitive cells, implying that phosphorylation/dephosphorylation mechanisms do not have a role in PGP mediated MDR (Germann et al 1996). However, more recently phosphorylation was shown to modulate the binding of some drugs with PGP, especially at low concentrations (Szabo et al 1997).

**ATP binding cassette family of transporters.**

It is now known that PGP belongs to a super family of ATP binding cassette (ABC) transporters, these are involved in transport of many substrates including peptides, amino acids and inorganic ions. There are over 50 members in this family including the cystic fibrosis transmembrane regulator (CFTR) (Riordan et al 1989), located on chromosome 7q31 (Callen et al 1987). The CFTR is associated with a cyclic AMP-regulated chloride channel which is found in secretory epithelial cells, and PGP may itself have chloride channel activity (Valverde et al 1992). Other members of this ABC transporter family include bacterial nutrient transporters, pigment transporters in *Drosophila melanogaster*, a chloroquine efflux pump in *Plasmodium falciparum* (*pfmdr*), yeast transporter STE6 and the MDR associated protein MRP (Bellamy 1996). Another human PGP has recently been found exclusively in liver and named Sister of PGP (SPGP) (Childs et al 1995), its function is currently unknown (Muller and Jansen 1997).

**Reversal of PGP function**

Interference with PGP function might be of clinical importance. This reversal of PGP function, with consequent chemosensitization, can be effected *in vitro* by many agents which are not themselves cytotoxic, see Table 1.9. Investigations into the use of calcium antagonists and calmodulin inhibitors *in vivo* are widespread because these agents are already in clinical use for treatment of other disorders. For many of the chemosensitizers the inhibition of PGP function appears to be due to competitive binding, although some of the agents, for example the surfactants, probably work indirectly by disrupting the membrane.

Preliminary studies with these chemosensitizing agents *in vitro*, showed that unattainable or toxic doses would be required for reversal of MDR *in vivo*. The main toxicity of the calcium channel blockers for example is on the heart, high concentrations impair atrioventricular node conduction. Another potential problem with the use of these agents is sensitisation of the bone marrow, although blast cells from ANLL relapsed post-chemotherapy patients exposed to DNR showed
no increased cell kill with the addition of verapamil (Ross et al 1986). Similarly, no increase in toxicity in normal bone marrow cells from either normal or cancer patients was observed with a combination of verapamil or nitrendipine and either DOX, VBL or VCR (Fine et al 1987).

Table 1.9 Agents that reverse PGP mediated MDR

<table>
<thead>
<tr>
<th>Class</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR implicated drug analogues</td>
<td>Cyanomorpholino-Doxorubicin, N-Acetyl daunorubicin</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Cepharanthidine, Cefoperazone, Erythromycin</td>
</tr>
<tr>
<td>Antimalarials</td>
<td>Chloroquine, Quinidine, Quinine</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>Verapamil, Nicardapine, Diltiazem, Nifedipine, Azidopine</td>
</tr>
<tr>
<td>Calmodulin inhibitors</td>
<td>Chlorpromazine, Trifluoroperazine</td>
</tr>
<tr>
<td>Cardiovascular drugs</td>
<td>Propanolol, Amiodarone, Dipyridamole</td>
</tr>
<tr>
<td>Immunosuppressants</td>
<td>Cyclosporin A, FK 506, PSC 833, Rapamycin</td>
</tr>
<tr>
<td>Steroids</td>
<td>Progesterone, Tamoxifen, tirilazad</td>
</tr>
<tr>
<td>Antihypertensives</td>
<td>Reserpine</td>
</tr>
<tr>
<td>Antihistamines</td>
<td>Terfenadine</td>
</tr>
<tr>
<td>Surfactants</td>
<td>Tween 80, Solutol HS-15</td>
</tr>
</tbody>
</table>

Adapted from Bellamy (1996), and Gottesman and Pastan (1993).

The less toxic analogue of DNR, N-acetyl-DNR, inhibits active efflux of DNR in drug resistant Ehrlich ascites tumour cell lines, with therapeutic doses the addition of N-acetyl-DNR increased the anti-tumour activity of DNR, probably by competition at PGP drug binding sites (Skovsgaard 1980). In resistant CEM cells (human leukaemic lymphoblasts), 10μM Verapamil alone often caused 20% cell death and only partially reversed MDR (Beck et al 1986). There is a continuing search for new or improved modulators of PGP, for example etoposide activity could be increased by up to 80% when given in combination with the chemosensitizing agent cyclosporin (Lum et al 1992).

Reversal of MDR using chemosensitizers is now attempted clinically, however, difficulties arise from toxicities and the need to prove that the MDR is due to PGP in each individual patient (Ozols 1995). In one study antibodies against PGP were used to detect MDR cells in blood from 18 lymphoma patients who relapsed within 3 months of DOX/VCR chemotherapy. These patients were treated with verapamil, cyclophosphamide, DOX and VCR, and 72% responded to treatment (Miller et al
1991). However, this apparent reversal of MDR could be due to the use of cyclophosphamide which is not implicated in MDR.

Genetics
Cytogenetic changes
Cytogenetic abnormalities associated with gene amplification, such as homogeneously staining regions (HSRs) and double minutes (DMs) were first noted in cells resistant to antifolates (Biedler and Spengler 1976), similar aberrations were later seen in highly multidrug resistant cell lines (Baskin et al 1981 and Roninson et al 1986). Cells selected for resistance in vitro by increasing drug concentration during culture often have many DMs and HSRs, and these are often lost in cells that have reverted back to drug sensitivity (Bradley et al 1988). Amplified PGP sequences have been shown to be localised to HSRs in CH cells (Riordan et al 1985). However, no cytogenetic or molecular evidence of gene amplification associated with MDR has been found in human tumours (Biedler 1992).

Gene families
Early studies using relatively non-specific cDNA probes found multiple bands in Southern blots of restriction enzyme digested DNA from MDR CHO cells, indicating the presence of an MDR PGP multigene family. Two different MDR CH cell lines had common amplified regions of DNA containing a transcription unit named mdr, this encoded an mRNA which when transfected into sensitive hamster cells conferred MDR (Gros et al 1986a). Amplification of two different DNA sequences in human MDR KB cells were found that were homologous to the hamster mdr, these were named MDR1 and MDR2. MDR1 sequences were amplified in highly resistant sublines and transcribed as 4.5kb mRNA but MDR2 sequences were not detected. However, both MDR1 and MDR2 were coamplified in other MDR sublines, but MDR2 mRNA expression was not detected. This implied that MDR1 is involved in drug resistance whereas MDR2 is not essential to the MDR state (Roninson et al 1986).

A family of 3 MDR genes have also been found in mouse and hamster, and due to the timing of the discoveries the nomenclature is confusing. The three genes in hamster are pgp1, pgp2 and pgp3. The pgp1 and pgp2 genes correspond to the Human MDR1 gene, pgp2 is similar to Human MDR2. In mouse the three genes are mdr1, mdr2 and mdr3. Both mdr1 and mdr3 correspond to human MDR1, mouse mdr2 is similar to human MDR2, see Table 1.10. The fact that there are two rodent genes and only one human gene coding for PGP can be explained in evolutionary
terms by a relatively recent duplication after the divergence of mammalian orders. This study will concentrate on human MDR1 only.

Table 1.10 Comparison of MDR gene families.

<table>
<thead>
<tr>
<th>Species</th>
<th>Comparable genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>MDR1</td>
</tr>
<tr>
<td></td>
<td>MDR2</td>
</tr>
<tr>
<td>Hamster</td>
<td>pgp1 and pgp2</td>
</tr>
<tr>
<td>Mouse</td>
<td>mdr1 and mdr3</td>
</tr>
<tr>
<td></td>
<td>mdr2</td>
</tr>
</tbody>
</table>

Gene localisation

The human MDR1 gene has been localised to chromosome 7, using flow sorted chromosomes probed with MDR1 (Fojo et al 1986). Using in situ hybridisation MDR1 was initially sublocalised to 7q34-36 (Bell et al 1987). Trent and Witkowski (1987) used two mutant fibroblast cell lines with deletions of 7q33-ter and 7q32-ter, to show that this did not lead to loss of MDR1 sequences. In situ hybridisation using more stringent conditions and visualising with silver grains has finally localised MDR1 to 7q21.1 (Callen et al 1987). Both the human MDR genes (MDR1 and MDR2) are located on chromosome 7q and linked within 300 kilo bases (Chin et al 1989).

Regulation of P-glycoprotein expression

P-glycoprotein is overexpressed in cells exhibiting classical MDR, but only the highly drug selected cell lines have amplification of the MDR1 gene itself. Therefore overexpression of PGP must be due to overtranscription of the MDR1 gene or overtranslation. The expression of MDR1 mRNA was studied during the development of MDR in COL selected KB carcinoma cells. A good correlation was found between drug resistance and levels of the 4.5 kb MDR1 mRNA. In the early stages of MDR development Southern hybridisation showed no MDR1 gene amplification. However, at higher levels of drug resistance, both increased mRNA levels and gene amplification were found. This implied that activation of the MDR1 gene precedes gene amplification (Shen et al 1986a). In highly resistant and revertant KB cell lines, a good correlation between gene amplification and PGP expression was found (Ueda et al 1986). There is no evidence that overexpression of PGP is due to increased translation.

Most of the studies into PGP have understandably used highly selected cell lines obtained by culturing cells in increasing concentrations of drugs, and the patterns of cross resistance are extremely variable among independently selected cell lines. Do
normal PGPs account for all of these observed phenotypes or are other factors involved? Devine and Melera showed that drug resistant CH lung cells selected by short drug exposure, less than 8 weeks, over expressed endogenous PGPs. Longer drug exposures at higher concentrations altered the cross resistance properties. Some had acquired point mutations in the PGP1 gene in codons 338 and 339, indicating that selective pressure leads to additional genetic alterations (Devine and Melera 1994). Point mutations have been shown to alter the MDR phenotype. The \textit{MDR1} cDNA sequence from VBL selected KB cells and the corresponding sequence from COL selected KB cells were compared, the sequences differed at three points, leading to a single amino acid change of glycine to valine at position 185. Further investigations into this mutant PGP showed it to be the reason behind the observed preferential resistance to COL in MDR KB cells (Choi et al 1988). Evidence for polymorphism in human \textit{MDR1} gene does exist, 20 of 31 cell lines were heterozygous for \textit{MDR1} and under normal cell culture conditions both alleles were expressed at similar levels. However, drug selection \textit{in vitro} resulted in increased expression of one allele or increased expression of both alleles. When both alleles were overexpressed this was followed by increased expression of one of the alleles together with amplification (Mickley et al 1993).

In transfection studies, full length cloned \textit{MDR1} gene cDNA sequences from MDR human KB cells were inserted by retrovirus expression vector into sensitive mouse NIH 3T3 fibroblasts and human KB cells. MDR was conferred indicating that a functional PGP was produced. Following the transfection into NIH 3T3 cells, five colonies from a single experiment were selected in media containing 60ng/ml COL. Although these colonies had undergone identical treatment there was still considerable variation in their relative drug resistances. This strongly suggests that other factors, like the expression of other proteins and post-translational modification of the PGP modulate the MDR phenotype (Ueda et al 1987).

Results from artificially resistant cell lines show that P-glycoprotein in these stressed cells can be structurally, and possibly functionally, very different from the naturally occurring molecule. The chemotherapeutic agents might themselves directly activate transcription of the \textit{MDR1} gene (Nooter and Herweijer 1991) Therefore, when undertaking these studies, and interpreting others’ results, it is important to remember that any selection pressure will not only be altering the expression of PGP, the one molecule of interest, but may change many others.
Other factors

At present there is little known about how PGP expression is regulated in human cancers or normal human tissue. However, it is known that PGP expression can be altered by many different stimuli including metabolic or toxic stress. Both retinoic acid and sodium butyrate increased \textit{MDR1} mRNA and PGP expression in human neuroblastoma cells, suggesting that expression is a function of differentiated tissue and tumours (Bates et al 1989). Also, heat shock changed the expression of a variety of cellular proteins including PGP (Watowich and Morimoto 1988), and it has now been shown that the \textit{MDR1} gene promoter has a heat shock consensus element (Chin et al 1990). A change in translational activity was seen when CHO-AuxB cells were X-irradiated, there was an increase in the PGP protein but no change in the levels of mRNA (Hill et al 1990).

At the genetic level, experiments have shown that c-Ha-ras and mutant p53 can activate the promoter region of the \textit{MDR1} gene itself. This implies that genes associated with oncogenic development may at the same time activate PGP eliciting multidrug resistance. This theory would also explain why some untreated advanced cancers show elevated levels of PGP (Benchimol and Ling 1994).

Cellular changes related to PGP-MDR

Other genes appear to be co-amplified with PGP including the gene for sorcin. Sorcin is a small, acidic, cytosolic protein of 20kDa which binds calcium and is homologous to calpain. It has been found in many MDR cell lines but not in all, suggesting that is not obligatory for the development of MDR, but it could be a resistance modulator (Meyers et al 1987). The sorcin gene itself is found very close to the \textit{MDR1} gene, and over expression of sorcin appears to be the result of its co-amplification with \textit{MDR1}. In cells which overexpress sorcin, this protein can represent the major calcium binding protein, so may affect calcium metabolism. Other protein changes have been noted in multidrug resistant human KB carcinoma cells, including decreased levels of a family of 70-80kD proteins together with over-expression of sorcin (Shen et al 1986b).

A 'mini-P-Glycoprotein' has been discovered in PGP expressing drug resistant mouse leukaemia cells (Kawai et al 1994). The levels of expression of this 65 kD protein correlated with the degree of resistance. It shares an antigenic epitope with PGP and is recognised by the monoclonal antibody C219, but is neither phosphorylated nor glycosylated. It may prove to be a truncated product of an alternatively spliced PGP transcript.
Changes in glutathione-S-transferase (GST) activity have also been noted in cells which overexpress PGP. This is of interest because of the role GST plays in protecting cells against free-radical damage. This could protect cells from anthracyclines such as DOX, as this is thought to be one of its modes of cytotoxic action. The GSTπ gene was often co-activated with MDR1 when cells were selected with certain drugs. This simultaneous emergence of two different resistance mechanisms was thought not to be the result of independent events, but the result of a single process (Hanania et al 1991). Changes in cytochrome P-450 enzymes have also been associated with MDR1 expression, for example in an MDR breast cancer cell line (DoxR MCF7), there was a decreased hydroxyl radical formation compared to the sensitive parental cell line. This was associated with decreased cytochrome P1-450 gene transcription and increased glutathione peroxidase activity and GST isozymes (Cowan et al 1986). However, no clear cut relationship has been found between the P-450 enzyme system and MDR1 expression (Pfeil et al 1994).

Another phenomenon associated with highly resistant MDR cell lines is 'reverse transformation' (Meyers et al 1987). CH lung cells, mouse sarcoma cells and human neuroblastoma cells selected with COL or ADR had altered differentiation and decreased tumourigenicity compared to the sensitive parental cell lines, and all three systems overexpressed epidermal growth factor receptor (EGFR) (Meyers et al 1986, Biedler and Spengler 1994). However, levels of EGFR in these cells do not quantitatively correspond to levels of resistance or expression of PGP (Meyers et al 1986) indicating that overexpression of PGP and EGFR are not genetically linked. It has also been suggested that expression of EGFR during neoplastic transformation is dependent on tissue type (Meyers and Biedler 1991). EGF activated phosphatases have also been found in MDR CH lung cells where the PGP was dephosphorylated (Meyers et al 1993). So there may be a link between growth advantage and expression of PGP. Elevated levels of PGP can elicit a growth advantage during chemotherapy, so PGP may be transporting other substances, for example the excretion of autocrine growth factors (Benchimol and Ling 1994), this could confer a selective growth advantage during drug exposure, to cells also overexpressing EGFR, effectively increasing drug resistance (Dickstein et al 1995).

Physical changes in MDR cells include increased susceptibility to mechanical disruption, plasma membrane vesicles from MDR CHO cells were isolated and stressed using a cell disrupter which uses pressure to rupture the cells, they found that in comparison to the sensitive parent cells much less pressure was needed to
rupture the MDR cells (Riordan and Ling 1979). Electron spin resonance spectroscopy on sarcoma cells resistant to ADR, showed that as the degree of resistance increased so too did the membrane fluidity (Siegfried et al 1983). In both of these cases it is possible that large amounts of PGP somehow disrupts normal membrane function.

Methods for detecting MDR1 expression

Immunocytochemistry

A number of antibodies have been raised which recognise different epitopes of P-glycoprotein. These have been used to detect and localise the protein in fixed tissues or cell culture, or to interfere with PGP function or used in functional studies to locate potential drug binding sites (Schinkel et al 1993). Antibodies which recognise PGP are shown in Table 1.11, the most commonly used are C219, MRK16 and JSB-1. The location and distribution of PGP molecules within single cells or in tissue can easily be visualised using immunocytochemistry. Antibodies (usually raised in mice) bind to a PGP molecule and these can then be detected by using sandwich layers of anti-mouse antibodies to amplify the signal and a final layer to visualise the signal. Different antibodies recognise different PGP epitopes so not surprisingly different staining patterns are often seen. However, there are also some known cross reactivity for these antibodies for example C219 also binds to the heavy chain of cardiac and skeletal muscle myosin, and also cross reacts with MDR2 PGP (Thiebaut et al 1989). It is therefore essential that a panel of two or more antibodies is used in any immunocytochemical study. Immunocytochemistry is at least as specific as the RNase protection assay and more sensitive than Western blotting (van der Heyden et al 1995), but precisely because of this sensitivity the staining patterns are often very difficult to quantify objectively.
Table 1.11 Antibodies which recognise P-glycoprotein.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Production</th>
<th>Specificity</th>
<th>Epitope</th>
<th>Mono- or polyclonal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C219</td>
<td>In mice, with CHO MDR membranes</td>
<td>All known PGPs</td>
<td>Cytoplasmic c-terminal</td>
<td>Mono</td>
<td>Kartner et al 1985</td>
</tr>
<tr>
<td>C494</td>
<td></td>
<td>Human and hamster PGP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C32</td>
<td></td>
<td>Hamster PGP1 and PGP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>265/F4</td>
<td></td>
<td></td>
<td></td>
<td>Mono</td>
<td>Lathan et al 1985</td>
</tr>
<tr>
<td>MRK-16</td>
<td>In mice, with MDR human leukaemia cells</td>
<td>Human PGP1</td>
<td></td>
<td>Mono</td>
<td>Hamada et al 1986</td>
</tr>
<tr>
<td>MRK-17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>In rabbits, with synthetic peptide</td>
<td>Human PGP1</td>
<td>n-terminal</td>
<td>Poly</td>
<td>Richert et al 1988</td>
</tr>
<tr>
<td>JSB-1</td>
<td>In mice, with MDR Chinese Hamster cells</td>
<td>Hamster and Human PGP1</td>
<td>cytoplasmic</td>
<td>Mono</td>
<td>Scheper et al 1988</td>
</tr>
<tr>
<td>HYB 241</td>
<td>In mice, with MDR human neuroblastoma cells</td>
<td>Hamster and Human PGP1</td>
<td>external</td>
<td>Mono</td>
<td>Meyers et al 1989</td>
</tr>
<tr>
<td>HYB 612</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HYB 195</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAb 57</td>
<td>In mice</td>
<td>Human PGP1</td>
<td></td>
<td>Mono</td>
<td>Cenciarelli et al 1991</td>
</tr>
<tr>
<td>17F9</td>
<td></td>
<td></td>
<td></td>
<td>Mono</td>
<td>Aihara et al 1991</td>
</tr>
<tr>
<td>UIC-2</td>
<td>In mice</td>
<td>Human PGP1</td>
<td>external</td>
<td>Mono</td>
<td>Mechetner et al 1992</td>
</tr>
<tr>
<td>MAb 6/1C</td>
<td>In mice, with synthetic peptide</td>
<td>Human PGP1</td>
<td></td>
<td>Mono</td>
<td>Clynes et al 1992</td>
</tr>
<tr>
<td>4E3.16</td>
<td></td>
<td>Human PGP1</td>
<td>external</td>
<td>Mono</td>
<td>Arceci et al 1993</td>
</tr>
<tr>
<td>MAb F4</td>
<td>In mice, with MDR human and Chinese hamster cells</td>
<td></td>
<td></td>
<td>Mono</td>
<td>Chu et al 1993</td>
</tr>
</tbody>
</table>

**Western blotting**

This method involves the electrophoretic separation, based on size, of all denatured membrane proteins extracted from cell cultures or tissue, followed by immunoblotting with antibodies to locate the band containing PGP. This method does not allow for the heterogeneity of expression between cells in the same tissue sample nor can it show the spatial distribution of PGP molecules. However, PGP expression is more easily quantified than with immunocytochemistry, by measuring the density of the resulting band.

**Northern blotting**

Single strand RNA isolated from cells or tissue is separated by electrophoresis then hybridised with a radiolabelled MDR1 DNA probe. The size and amount of MDR1 RNA can be found, but this again will only give an average of tumour and normal tissue and will not reflect any heterogeneity. This method may need a large amount
of tissue to isolate sufficient RNA, however, elevated levels of MDR1 mRNA have been detected in cell lines that were negative using immunohistochemistry (Fojo et al 1987a). Another potential problem is the rapid degradation of single stranded RNA by ubiquitous RNases, and general degradation of RNA in archival specimens.

**RNase protection assay**
This assay is based on the hybridisation of a radiolabelled single stranded MDR1 DNA probe to total cellular RNA, thus forming DNA/RNA duplexes, the remaining non-hybridised single stranded RNA is digested away with RNases, and the duplexes are analysed by electrophoresis. Not only does this method show the size and amount of MDR1 RNA, it can also pick up single base mutations which may alter PGP function (Ueda et al 1989).

**Expression of MDR1 mRNA and PGP**

**Normal Tissues**
Immunocytochemistry using MRK-16, JSB-1 and C219 has shown high expression of PGP in many normal tissues, including liver, gall bladder, pancreas, lung, kidney, adrenal gland and blood vessels (the implications of the presence of PGP in blood vessels of the brain will be discussed later), see Table 1.12 (van der Valk et al 1990). This confirms results of studies which detected high levels of MDR1 mRNA in the same tissue (Pastan et al 1988 and Fojo et al 1987b), PGP expression within these tissues is often specifically localised, often on the apical membranes of cells facing an excretory compartment. For example PGP was polarised in the liver in the bile canaliculi and the apical surface of biliary ductules, in colon and jejunum on apical surface of columnar epithelial cells, in kidney on the apical surface of epithelial cells of the proximal tubules, and also in pancreas on the apical surfaces of ductules. The adrenal however had high levels of PGP throughout the cortex and medulla. This pattern of expression implies that PGP's natural function could be secretion. The lower digestive tract has a large fraction of the total PGP-containing cells in the body therefore this may be a route for detoxification, in fact MDR implicated drugs are often found secreted in the bile and found in the digestive tract (Thiebaut et al 1987).
Table 1.12  P-glycoprotein expression in normal tissues, detected using a panel of three antibodies, JSB-1, C219 and MRK-16.

<table>
<thead>
<tr>
<th>NORMAL TISSUE</th>
<th>PGP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulatory tract</td>
<td>heart vessel endothelium*</td>
</tr>
<tr>
<td>Digestive tract</td>
<td>Oesophagus stomach intestinal parenchyma bile canaliculi and ductules epithelium muscle layer</td>
</tr>
<tr>
<td>Liver</td>
<td>Gall bladder epithelium</td>
</tr>
<tr>
<td>Prosac</td>
<td>Salivary glands</td>
</tr>
<tr>
<td>Respiratory tract</td>
<td>Lung bronchioli alveolar macrophage</td>
</tr>
<tr>
<td>Urogenital tract</td>
<td>Kidney glomeruli tubules epithelium and smooth muscle</td>
</tr>
<tr>
<td>Testis</td>
<td>Ovary</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>Epidermis capillaries sweat glands</td>
</tr>
<tr>
<td>Brain</td>
<td>Vessels</td>
</tr>
<tr>
<td>Skin</td>
<td>Adrenal cortex medulla</td>
</tr>
<tr>
<td>Endocrine glands</td>
<td>Endocrine islets</td>
</tr>
</tbody>
</table>

*endothelium of vessels in lung, prostate, stomach, intestine, brain, glomeruli. high=most cells strongly positive with all 3 antibodies, medium=some cells positive with more than one antibody, low=a few cells weakly positive with more than one antibody. (Adapted from van der Valk et al 1990).

Although there is general agreement on those normal tissues with high or no detectable expression of PGP, the staining patterns with any of the antibodies make it difficult to quantitate. For example weak and strong staining can be seen in any number of cells in one individual tissue, there may be weak staining in 60% of cells with one antibody and strong staining in 15% with another. There may also be varying levels of PGP and MDR1 mRNA in the same tissues from different individuals, in one study 2 of 7 lung samples had high levels of MDR1 mRNA and 5 of 7 had low levels (Pastan et al 1988).
Tumours

High levels of MDR1 mRNA and PGP are usually found in cancers derived from normal tissues which themselves have high MDR1 expression, for example cancers of the colon, kidney, liver, and adrenal gland (Pastan et al 1988). Untreated tumours can be divided into three groups (van der Heyden et al 1995): Group 1 tumours are those which express high levels of MDR1, usually derived from normal tissue with high PGP expression and are clinically drug resistant. Group 2 tumours with intermediate expression of PGP respond better to initial chemotherapy than those in group 1, but often acquire resistance. Group 3 tumours have little or no detectable PGP expression but may develop acquired resistance (Table 1.13).

Table 1.13 Correlation between MDR1 expression and clinical outcome in solid tumours.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Correlation with response to chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1</td>
<td></td>
</tr>
<tr>
<td>Tumours with high MDR1 expression levels at high frequency</td>
<td></td>
</tr>
<tr>
<td>Renal cell cancer</td>
<td>PGP expression and degree of differentiation prognostically significant</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>Increased MDR1 mRNA expression following initial chemotherapy. Expression may be important in prognosis.</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>PGP expression was significantly associated with non-responders</td>
</tr>
<tr>
<td>Phaeochromocytoma</td>
<td>Higher MDR1 mRNA corresponds to acquired resistance</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>not known</td>
</tr>
<tr>
<td>GROUP 2</td>
<td></td>
</tr>
<tr>
<td>Tumours with intermediate MDR1 expression levels at a lower frequency</td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>PGP expression correlates with shorter relapse free survival and overall survival</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>PGP expression correlates with treatment failure</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Induced PGP expression after chemotherapy. PGP expression is associated with a poor response to chemotherapy and poor prognosis</td>
</tr>
<tr>
<td>GROUP 3</td>
<td></td>
</tr>
<tr>
<td>Tumours with undetectable or low MDR1 expression levels</td>
<td></td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Occasional PGP positivity may be associated with no response to chemotherapy</td>
</tr>
<tr>
<td>Testicular cancer</td>
<td>PGP expression found in advanced stage and associated with non-responders.</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Correlation between absence of PGP and initial response to chemotherapy</td>
</tr>
</tbody>
</table>

P-glycoprotein mediated MDR in the brain

Normal brain

PGP is expressed in endothelial cells of normal brain whilst parenchymal cells show no expression (Table 1.14). Endothelial cells form the BBB and the expression of PGP implies that they may remove large molecular weight molecules including cytotoxic drugs from the brain, or prevent drugs carried in the blood from reaching the brain. However, the first of these functions would depend on reverse orientation of PGP on the brain side of the endothelial cells, so drugs would be taken up into the endothelial cells then effluxed through normally oriented PGP into the blood. There is no evidence of this reverse orientation. The second function would have normally oriented PGP expressed on the lumen side of the endothelial cells only, and this has proved to be the case in rat brain capillaries (Beaulieu et al 1997) where 400-500 fold overexpression of PGP was found in luminal membranes compared to whole brain membranes. This specific spatial expression has only been found in whole capillaries, indeed cultured endothelial cells express PGP throughout the membrane (Hegmann et al 1992).

Table 1.14  PGP expression in normal brain

<table>
<thead>
<tr>
<th>Tissue/Cells</th>
<th>MAbs</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C219</td>
<td>1 of 4, positive with MRK16.</td>
<td></td>
</tr>
<tr>
<td>Formalin fixed, paraffin embedded, normal human brain surrounding astrocytoma (2 specimens)</td>
<td>MRK16</td>
<td>1 of 2, macrophages and endothelial cells strongly positive in &gt;75% of cells</td>
<td>Schlaifer (1990)</td>
</tr>
<tr>
<td></td>
<td>C219</td>
<td>1 of 2, endothelial cells negative, weak staining of macrophages</td>
<td></td>
</tr>
<tr>
<td>Fresh/frozen human brain (11 specimens)</td>
<td>C219</td>
<td>7 of 11, positive in blood vessels, including hippocampus, cerebellum, amygdala and cortex.</td>
<td>Nabors (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 of 11, parenchymal cells negative.</td>
<td></td>
</tr>
<tr>
<td>Cultured mouse and pig cerebral capillary endothelial cells</td>
<td>C219</td>
<td>All constitutively express moderate levels of PGP. Staining was heterogeneous.</td>
<td>Hegmann (1992)</td>
</tr>
<tr>
<td>Isolated rat brain capillaries</td>
<td>C219</td>
<td>Overexpression of PGP on luminal membranes only.</td>
<td>Beaulieu (1997)</td>
</tr>
</tbody>
</table>
Brain tumours

Astrocytic tumours

Astrocytic tumours have been shown to express the MDR1 gene, although the reported incidence of PGP expression varies widely between studies (Matsumoto et al 1991, Henson et al 1992). The results from 8 studies investigating the expression of the MDR1 gene astrocytoma are shown in Table 1.15. Discrepancies between Northern blotting and immunocytochemistry (Becker et al 1991 and Nabors et al 1991) may be accounted for by the different sensitivities of the techniques as previously discussed. Interestingly the highest number of PGP positive cells, 27.6%, was seen in a GBM in a patient who had received previous radiotherapy and chemotherapy including VCR (Matsumoto et al 1991) which might indicate an example of acquired resistance.

The expression of PGP in endothelial cells within these tumours was recorded, for example in 17 of 22 GBMs, 13 of 14 grade III astrocytomas and 3 of 3 grade II astrocytomas (Henson et al 1992), it was suggested that the presence of an intact BBB was essential to the expression of PGP in endothelial cells so where there was more breakdown of the BBB, as in the very malignant GBMs, there may be less PGP in the endothelial cells. However, in another study 5 of 6 GBMs expressed PGP in endothelial cells (Nabors et al 1991), and no difference in PGP expression was found between endothelial cells of 19 low grade and 34 high grade astrocytomas (von Bossanyi et al 1997). In a larger study PGP expression has been demonstrated in the majority of newly formed capillaries of malignant brain tumours, 25 of 29 (86%) grade III and IV astrocytomas and also in 3 of 6 tumours metastatic to the brain, in comparison none of 137 non-brain tumours expressed PGP in new capillaries (Toth et al 1996).

Other brain tumours

In one study of 33 ependymal tumours 29 expressed PGP including all five recurrent tumours. One recurrent tumour showed strong expression of PGP though its corresponding primary tumour had been negative (Geddes et al 1994). PGP expression has been seen in a variety of other brain and spinal tumours including, menigioma, metastatic adenocarcinoma, oligodendrogliaoma and ependymoma, and again all endothelial cells expressed this protein (Table 1.16).
**Table 1.15 Expression of the MDR1 gene in astrocytomas.**

<table>
<thead>
<tr>
<th>Tissue/Cells</th>
<th>Techniques</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM cell lines GB-1 and U373MG</td>
<td>Northern blotting</td>
<td>GB-1 had 26 fold more MDR1 mRNA than U373MG</td>
<td>Matsumoto et al (1990)</td>
</tr>
<tr>
<td></td>
<td>Southern blotting</td>
<td>Each had 1 copy of the MDR1 gene</td>
<td></td>
</tr>
<tr>
<td>6 Fresh frozen GBMs 2 Fresh frozen Gr. II Ast</td>
<td>Northern blotting Immunocytochemistry using MAb C219 on GBMs only</td>
<td>No expression of MDR1 mRNA detected in any tumour 1 of 6 GBMs PGP positive in tumour cells 5 of 6 GBMs PGP positive in endothelial cells</td>
<td>Nabors et al (1991)</td>
</tr>
<tr>
<td>5 astrocytoma cell lines</td>
<td>Western blotting using MAb C219 Immunocytochemistry using MAb MRK16</td>
<td>1 MDR cell line, high PGP expression 4 sensitive cell lines with no PGP All expressed PGP (range 6.7-27.6% of cells positive)</td>
<td>Matsumoto et al (1991)</td>
</tr>
<tr>
<td>Fresh frozen specimens 12 GBMs, 2 Gr. III Ast</td>
<td>Northern blotting Immunocytochemistry using MAb C219</td>
<td>3 of 5 GBMs and 1 of 2 Gr.III Ast expressed MDR1 mRNA All expressed PGP (range 0.3-15.4% of cells positive)</td>
<td>Becker et al (1991)</td>
</tr>
<tr>
<td>Fresh frozen specimens 22 GBMs 14 Gr. III Ast 3 Gr. II Ast</td>
<td>Immunocytochemistry using MAb HYB-241</td>
<td>2 of 22 GBMs and no Gr.II or III Ast expressed PGP in tumour cells. 17 of 22 GBMs and 13 of 14 Gr.III Ast PGP expressed in all capillaries.</td>
<td>Henson et al (1992)</td>
</tr>
<tr>
<td>Paraffin embedded GBMs</td>
<td>Immunocytochemistry using MAb JSB-1</td>
<td>14 of 15 GBMs expressed some level of PGP</td>
<td>Kiwit et al (1994)</td>
</tr>
<tr>
<td>Paraffin embedded 4 Gr.II Ast 1 Gr.III Ast 3 GBMs</td>
<td>Immunocytochemistry using MAb C219 and MRK16 mRNA by RT-PCR</td>
<td>No PGP expressed in tumour cells but all samples expressed PGP in capillaries</td>
<td>Tanaka et al (1994)</td>
</tr>
<tr>
<td>Fresh frozen specimens 22 GBMs 9 Gr. III Ast 2 Gr. II Ast</td>
<td>mRNA by RT-PCR</td>
<td>All samples expressed MDR1 mRNA. Different areas from the same tumour showed varying results.</td>
<td>Kirches et al (1997)</td>
</tr>
<tr>
<td>Paraffin embedded 7 pilocytic Ast, 9 Gr. II, 9 Gr. III ast, 25 GBMs</td>
<td>Immunocytochemistry using MAb JSB-1</td>
<td>Mean % of PGP positive neoplastic cells for each grade: Pilocytic 1%, Gr.II 0.4%, Gr.III 14.6%, GBM 25.7% PGP expressed in all capillaries.</td>
<td>von Bossanyi et al (1997)</td>
</tr>
</tbody>
</table>

Ast=astrocytoma, RT=Reverse transcription.
Table 1.16 Expression of the MDR1 gene in other brain and spinal tumours.

<table>
<thead>
<tr>
<th>Tissue/Cells</th>
<th>Techniques</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh frozen specimens 2 oligodendrogliomas</td>
<td>Immunocytochemistry using MAb MRK16</td>
<td>7% of cells expressed PGP</td>
<td>Matsumoto et al (1991)</td>
</tr>
<tr>
<td>Fresh frozen specimens 2 oligodendrogliomas 6 meningiomas 3 shwannomas 3 met. adeno. 1 craniopharyngioma</td>
<td>Northern blotting (14 specimens) Immunocytochemistry using MAb C219 (9 specimens)</td>
<td>MDR1 mRNA detected in 1 of 6 meningiomas and 1 of 3 meta. adeno. PGP expressed in vessels and tumour cells in 1 of 1 oligodendroglioma, 3 of 6 meningiomas, and in vessels only in 1 of 6 meningiomas.</td>
<td>Nabors et al (1991)</td>
</tr>
<tr>
<td>Fresh frozen specimens 16 PNETs</td>
<td>Western blotting using JSB-1 MDRI mRNA by RT-PCR</td>
<td>PGP expressed in 2 of 16 specimens 6 of 12 specimens expressed MDRI mRNA. One expressed MDRI mRNA in recurrent but not in primary.</td>
<td>Tishler et al (1992)</td>
</tr>
<tr>
<td>Paraffin embedded 33 ependymomas</td>
<td>Immunocytochemistry using MAbC219 and JSB-1</td>
<td>PGP expressed in 29 of 33 tumours in some ependymal rosettes and epithelial-lined tubules and clefts and all endothelial cells.</td>
<td>Geddes et al (1994)</td>
</tr>
<tr>
<td>Fresh frozen specimens 8 ependymomas 18 PNETs</td>
<td>Immunocytochemistry using MAbMRK16</td>
<td>No PGP expression in tumour cells. &gt;50% of vessels expressed PGP in 3 of 8 ependymomas and 1 of 18 PNETs</td>
<td>Billson et al (1994)</td>
</tr>
</tbody>
</table>

PNET = primitive neuroectodermal tumour, met. adeno. = metastatic adenocarcinoma

Functional studies.

In an early study into ADR accumulation in human glioma, three glioma cell lines were investigated, one was relatively sensitive to ADR, (G-MCF) and two (G-CCM and G-UVW) were resistant to ADR and other MDR drugs, although the PGP status of the cells was not known. No relationship between sensitivity and ADR accumulation was found, but the addition of verapamil which reverses PGP function significantly increased ADR sensitivity in the MDR cell lines (Merry et al 1986). A sensitive human GBM cell line transfected with an expression-vector plasmid containing the human MDR1 gene became multidrug resistant. The level of PGP was increased with no amplification of the gene, and again resistance was reversed with verapamil (Reddy et al 1991).
Response to ACNU, Cisplatin and VCR with and without verapamil or nimodipine, was compared to the expression of PGP in 15 short term GBM cell cultures. Six of the cultures expressed PGP and could be chemosensitized, seven lines had no PGP expression and could not be chemosensitized, and 2 of 15 did not respond to the calcium channel antagonists even though they expressed PGP (Kiwit et al 1994). Sensitization to all the drugs was seen even though ACNU and Cisplatin are not transported by PGP, implying that some other resistance mechanisms are affected by these calcium channel blockers. However, none of the cell cultures had a positive response to these chemosensitizers in the absence of PGP expression.

**Non-PGP-mediated MDR**

**Patterns of cross resistance**

Many cancer cells are cross-resistant to a number of different drugs but this may not involve the *MDR1* gene or PGP. They may show a more limited range of cross resistance, for example resistance to drugs which share a common mechanism of action, and non-PGP-mediated MDR appears to be a more common event in human rather than animal cells (Harrison et al 1995), and in non-selected rather than selected cell lines (Izquierdo et al 1996).

**Atypical MDR**

The term atypical MDR is used to describe a specific form of MDR which does not involve PGP or drug efflux from the cell. Atypical MDR cells will have an altered or decreased topoisomerase II, which is a nuclear enzyme involved in transcription, DNA repair, and cell proliferation. Topoisomerase II relaxes DNA by nicking both strands of the double helix then religating to allow DNA replication and repair of damaged DNA. Inhibitors of topoisomerase II prevent the DNA strands from being religated. Binding of topoisomerase II to DNA also decreases the availability of DNA sequences for alkylation by some drugs. Topoisomerase II is the target for many of the drugs also implicated in classical MDR, for example, etoposide, DOX and mitoxantrone, but not the Vinca alkaloids (Twentyman 1993). All these drugs inhibit topoisomerase II by different mechanisms, but all act after strand breakage has occurred, so DNA replication cannot continue (Harrison 1995).
Multidrug Resistance Associated Protein

Another protein linked with MDR is multidrug resistance associated protein (MRP) (Cole et al 1992). MRP is a 190 kD integral membrane protein encoded for by a gene localised to chromosome 16 and over-expression appears to be the result of gene amplification as both DMs and HSRs were seen in highly resistant cell lines (Barrand et al 1994). In a cells transfected with MRP, the amount of MRP was closely associated with the degree of drug resistance (Grant et al 1994). MRP was discovered when the small cell lung cancer (SCLC) cell line H69AR, which was selected in DOX, was found to be resistant to DOX and VCR but did not express PGP, nor did chemosensitising agents like Cyclosporin A reverse DOX resistance. The mRNA that was consistently over-expressed in these resistant cells was sequenced and the resulting protein was named MRP (Cole et al 1992). Like PGP, MRP is a member of the ABC superfamily of transport proteins and despite their lack of primary structure similarity, MRP confers a similar resistance phenotype, including resistance to vinca alkaloids, anthracyclines and epipodophyllotoxins, but not taxol and mitoxantrone (Deeley and Cole 1997). MRP is predominantly present in the endoplasmic reticulum of resistant cells as well as in the plasma membrane (Krishnamachary and Center 1993) and may be involved in drug sequestration into vesicles. MRP has no apparent binding regions for free drugs and co-transport of drugs may occur along with reduced glutathione (GSH) (Zaman et al 1996).

MRP is expressed at low levels in all normal human tissues, and at different levels in human cancer biopsies, e.g. low levels in melanoma, and cancers of the breast, kidney, bladder, testis, ovary and colon, intermediate levels in non-small cell lung cancer and acute myelocytic leukaemia, and high levels in chronic lymphocytic leukaemia (Nooter et al 1995). MRP was also expressed in 49 of 56 (88%) human cell lines compared to 14 of 55 (25%) expressing PGP (Izquierdo et al 1996). MRP may also be involved in astrocytoma cell resistance (Abe et al 1994). Increased levels of MRP mRNA were shown in 7 of 7 glioma cell lines resistant to etoposide, VCR and ADR, and levels of MRP mRNA corresponded to the degree of resistance. Interestingly, the increased expression of the MRP protein was not a result of gene amplification. No MDR1 gene expression was seen in any of the cell lines. Drug accumulation studies implied that MRP may be involved in drug transport from the nuclei to the cytoplasm. Preliminary studies showed low levels of MRP expression in 12 of 15 brain tumour samples, although the diagnoses of these were unspecified, suggesting that MRP expression is more common than PGP expression in gliomas (Feun et al 1994).
**Lung Resistance Associated Protein**

Recently another protein, Lung Resistance Associated Protein (LRP) has been found in non-PGP MDR cells which express MRP (Slovak et al 1995). Both MRP and LRP map to the short arm of chromosome 16 but do not appear to be co-amplified. LRP shows homology with a rat vault protein involved in nucleo-cytoplasmic transport suggesting that it may also be involved in drug transport. The LRP associated drug resistance phenotype is broad, including VCR and DOX and drugs not associated with PGP or MRP resistance e.g. carboplatin, cisplatin and melphalan, and in a study of 61 human cancer cell lines including seven derived from CNS tumours, LRP expression was a superior predictor of *in vitro* resistance compared to PGP or MRP (Izquierdo et al 1996). The role of this protein in mediating drug resistance in brain tumours is unknown.

**Tumour suppressor genes**

When a tumourigenic cancer cell was fused with a normal cell it was noted that the hybrid grew well but was no longer tumourigenic (Harris et al 1969). Occasionally however, a hybrid would form tumours in animals. These hybrids had lost one or more chromosomes derived from the normal parent and this suggested that the normal chromosome contained a gene to suppress formation of tumours, a so called tumour suppressor gene. A two hit hypothesis for inactivation of both tumour suppressor gene alleles has been proposed as a mechanism for the development of some familial cancers like retinoblastoma (Knudson 1971). The first mutation is either germline (inherited) or somatic, and the second rate limiting event is always somatic. The first mutation is recessive and tumour suppressor activity is only lost after the second event, which may include mutation of the remaining allele, loss of the whole chromosome or loss of function of the gene product. The high incidence of cancer in Li-Fraumeni Syndrome (LFS) families is thought to be caused by germline p53 gene mutations. This syndrome confers a predisposition to cancer including gliomas, primitive neuroectodermal tumours, adrenocortical cancer and breast cancer (Birch 1992). The properties of tumour suppressor genes and their comparison with proto-oncogenes can be seen in Table 1.17. Examples of some common tumour suppressor genes are listed in Table 1.18.
Table 1.17 Properties of tumour suppressor genes and proto-oncogenes.

<table>
<thead>
<tr>
<th>Property</th>
<th>Tumour suppressor genes</th>
<th>Proto-oncogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mutational events needed to contribute to the cancer</td>
<td>two</td>
<td>one</td>
</tr>
<tr>
<td>Function of the mutant allele</td>
<td>loss of function, acts recessively</td>
<td>gain of function, acts dominantly</td>
</tr>
<tr>
<td>Mutation can be inherited</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Somatic mutation contributes to cancer</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Tissue specificity?</td>
<td>Inherited form has tissue preference</td>
<td>Acts in many tissues</td>
</tr>
</tbody>
</table>

(Adapted from Levine 1993)

Table 1.18 Tumour suppressor genes.

<table>
<thead>
<tr>
<th>Tumour suppressor gene</th>
<th>Location</th>
<th>Inherited tumours</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb</td>
<td>13q14</td>
<td>retinoblastoma, osteosarcoma</td>
<td>regulates transcription factors</td>
</tr>
<tr>
<td>p53</td>
<td>17p13</td>
<td>brain, osteosarcoma, adrenocortical, breast</td>
<td>transcription factor</td>
</tr>
<tr>
<td>WT-1</td>
<td>11p13</td>
<td>Wilm’s tumour, nephroblastoma</td>
<td>transcription factor</td>
</tr>
<tr>
<td>NF-1</td>
<td>17q</td>
<td>neurofibromatosis</td>
<td>GTPase activating protein (GAP) with ras</td>
</tr>
<tr>
<td>APC</td>
<td>5q21</td>
<td>adenomatous polyposis</td>
<td>unknown</td>
</tr>
<tr>
<td>DCC</td>
<td>18q21</td>
<td>unknown, somatic mutations in colon</td>
<td>adhesion like membrane protein</td>
</tr>
</tbody>
</table>

p53
The nuclear phosphoprotein p53 was first discovered in extracts of transformed cells. It reacted with antiserum from animals inoculated with purified simian virus 40 (SV40) T protein (Lane and Crawford 1979). p53 was bound to the SV40 oncogene product, the large T antigen, forming an oligomeric complex. Genomic clones encoding p53 have been isolated from mice that can immortalise rat cells in culture (Jenkins et al 1984). The transfected cells showed no signs of senescence and doubled every 18-20 hours (non-transfected cells had a doubling time of >60 hours), but were non-tumourigenic when injected into syngeneic or nude rats. However, if p53 transfected cells were then transfected with cloned activated c-Ha-ras they became tumourigenic. From this data it appeared that p53 was a dominantly acting
oncogene, but then it was discovered that the transforming clones were all of mutant p53 (Hinds et al 1989).

**Function**

Wild type (normal) p53 protein is a negative regulator of the cell cycle. The protein binds to DNA in a sequence specific manner (Kern et al 1991), whilst point mutated p53 proteins do not (El-Deiry et al 1992). p53 is not essential to early development or normal cell division, as mice without p53 develop normally but they are susceptible to cancer (Donehower et al 1992). The amount of normal p53 protein rises in cells after DNA damage, and arrests the cell cycle at G1, this allows repair of DNA before the synthesis phase of the cycle (Kastan et al 1991, Lane 1992). If the DNA is not repaired correctly the cell may undergo apoptosis (programmed cell death), if the p53 is inactivated by mutation or by binding to virus proteins the cycle cannot be arrested. These cells will then accumulate mutations and chromosomal rearrangements, leading to the rapid selection of malignant clones (Figure 1.7.) (Yonish-Rouach et al 1991).

Another property of normal p53 is that of a transcriptional activator. The p53 protein binds specifically to a region upstream of the transcription start site for the human ribosomal gene cluster and in a concentration dependent manner stimulates transcription. Mutant p53 proteins did not stimulate transcription, nor did normal p53 when complexed with T antigen (Farmer et al 1992).

As indicated above some functions of p53 are now known although the exact mechanism of cell cycle arrest remains unclear. Other cellular regulators may be involved including MDM2. The MDM2 gene was first identified in spontaneously transformed mouse cells where the protein encoded by this gene was shown to bind to p53. MDM2 was localised to chromosome 12q13-14 and this region was found to be amplified in several human sarcomas (Oliner et al 1992). The MDM2 protein did not immunoprecipitate with antibodies against p53 but when MDM2 and p53 were mixed they precipitated together. MDM2 appears to inactivate the transcriptional activity of p53 (Momand et al 1992). Figure 1.8 shows some of the factors known to be involved in p53 regulatory pathways that lead to apoptosis.
Figure 1.7. Simple model of p53 function

1. **Normal cell division**

2. **Normal cell response to DNA damage**
   - DNA damage
   - p53 level rises
   - G1 arrest
   - Repair before division
   - Apoptosis

3. **Cells with inactivated p53**
   - DNA damage
   - No p53
   - No G1 arrest
   - Division with damage
   - Mitotic failure and cell death
Figure 1.8 Factors involved in p53 regulatory pathways

Cell Cycle blocks cell cycle progression via inhibition of cyclin dependent kinases (cdk) and PCNA.

Apoptotic pathway

Activates WAF1 (p21/cip1)

MDM2

MDM2

inactive p53

bax

bax

bcl2

bcl2

bcl2

bax

cell death
Mutant p53 proteins inhibit the activity of normal p53 in a dominant negative fashion, by forming inactive hetero-oligomers of normal and mutant proteins. Mutant p53 protein has a longer half life than the wild type, several hours compared to about 20 minutes (Hinds et al 1990), and therefore large amounts of the mutant form can accumulate in transformed cells and tumours (Levine 1993).

Genetics
The human p53 gene has been localised to chromosome 17p13.1, the resulting 53kD protein is 393 amino acids long. The gene contains 11 exons, of these exons 5-8 are the most evolutionarily conserved regions and also contain most of the mutations, (98% of mutations studied), with 5 hot spots for mutations at amino acid residues, 175, 248, 249, 273 and 282 (Hollstein et al 1991). A number of different types of mutations in the gene have been found and depend on the tissue of origin of the cancer. Carcinomas usually lose one allele and have a missence mutation in the remaining allele which produces a mutant protein, and sarcomas usually have deletions, insertions and gene rearrangements, with point mutations being rare (Levine 1993).

Methods for detecting p53 protein
Many antibodies to detect wild-type and mutant p53 are now commercially available and the techniques for the detection of the protein are similar to those used for PGP. Mutant p53 or stabilised wild type p53 are more easily detected by immunocytochemistry because of their longer half lives. A diagram showing some of the antibodies to p53 and their binding regions can be found in Figure 1.9. Table 1.19 indicates the binding characteristics of these antibodies to p53.

Loss of heterozygosity (LOH) of the p53 gene
Loss of tumour suppressor activity commonly occurs by mutation in one p53 allele and the loss of the remaining allele (von Deimling et al 1992). This allelic loss can be assessed using a form of Southern blotting and although the status of the remaining allele is unknown, loss of one allele clearly indicates a potential for loss of p53 function. LOH is based on the fact that often two alleles have specific polymorphisms and will run out into 2 different bands on a gel so are heterozygous. Normal DNA encoding p53 is extracted from blood and is then electrophoresed along with tumour DNA. If one allele is missing in the tumour then heterozygosity is lost and only one band will appear, see Figure 1.10.
Figure 1.9 Antibodies to human p53 and their approximate binding regions.

![Antibodies to human p53 and their approximate binding regions.](image)

Figure 1.10 LOH technique to study loss of p53 allele.

![LOH technique to study loss of p53 allele.](image)

Light shading = p53 allele. Allelic loss of one p53 allele can be seen on the Southern blot.
Table 1.19 Binding characteristics of antibodies to human p53

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Binding characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0-1 (monoclonal)</td>
<td>human only wild type and mutant denatured or native conformation</td>
<td>Vojtesek et al 1992</td>
</tr>
<tr>
<td>PAbl801 (monoclonal)</td>
<td>human only wild type and mutant denatured or native conformation</td>
<td>Banks et al 1986</td>
</tr>
<tr>
<td>PAb240 (monoclonal)</td>
<td>human and mouse mutant only under non-denaturing conditions mutant and wild type when denatured</td>
<td>Gannon et al 1990</td>
</tr>
<tr>
<td>PAb421 (monoclonal)</td>
<td>human and mouse mutant and wild type denatured or native conformation</td>
<td>Harlow et al 1981</td>
</tr>
<tr>
<td>CM-1 (polyclonal)</td>
<td>human only mutant and wild type denatured or native conformation</td>
<td>Midgley et al 1992</td>
</tr>
</tbody>
</table>

Mutations in the p53 gene in cancer
Missense mutations caused by base substitutions in exons 5-8 of the p53 gene have been studied in a variety of sporadic cancers. These mutations alter the amino acid sequence at different points along the conserved regions suggesting that no single domain is responsible for maintaining the tumour suppressor function. Mutational trends were seen in the different tumour types which may hold clues to understand tumour aetiology including carcinogen exposure and tissue specific clonal selection (Hollstein et al 1991, Lasky and Silbergeld 1996), see Table 1.20.

Table 1.20 p53 mutational trends in different cancers.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>31/39 mutations (79%) = G:C to A:T transitions</td>
</tr>
<tr>
<td>Lymphomas/Leukaemias</td>
<td>30/53 mutations (57%) = G:C to A:T transitions</td>
</tr>
<tr>
<td>Lung</td>
<td>SCLC 6/13 mutations (46%) = G:C to A:T transitions</td>
</tr>
<tr>
<td></td>
<td>non-SCLC 17/30 mutations (57%) = G:C to T:A transversions</td>
</tr>
<tr>
<td>Oesophageal</td>
<td>16/37 mutations (43%) = G:C to A:T transitions</td>
</tr>
<tr>
<td>Liver</td>
<td>14/19 mutations (74%) = G:C to T:A transversions</td>
</tr>
</tbody>
</table>

(SCLC = Small Cell Lung Cancer, transitions = purine for purine or pyrimidine for pyrimidine, transversion = purine for pyrimidine or pyrimidine for purine)

(Adapted from Hollstein et al 1991)
Accumulation of p53 protein has been studied using immunocytochemistry, where no mutational analysis has been undertaken it is often assumed that the more stable p53, the mutant form, is present. Another study has shown that wild-type p53 is almost exclusively nuclear whereas mutant p53 exists in both nucleus and cytoplasm (Zerrahn et al 1992). Table 1.21 shows expression of p53 using antibody CM-1 in 212 fixed, paraffin embedded tumours. None of 49 cases of adjacent normal tissue had any p53 accumulation (Bartek et al 1991).

Table 1.21 Expression of p53 using antibody CM-1.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Number of cases</th>
<th>p53 expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Negative</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>45</td>
<td>33</td>
</tr>
<tr>
<td>Stomach carcinoma</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>Melanoma</td>
<td>38</td>
<td>8</td>
</tr>
<tr>
<td>Testicular carcinoma</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>8</td>
<td>12.5</td>
</tr>
<tr>
<td>Uterine carcinoma</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Soft-tissue sarcoma</td>
<td>11</td>
<td>64</td>
</tr>
</tbody>
</table>

negative = no positive cells, variable = 1-90% of cells positive but variable intensity, homogeneous = >90% of cells strongly positive
(Adapted from Bartek et al 1991)

Mutations in the p53 gene in brain tumours
Loss of heterozygosity on chromosome 17p and p53 mutation appear to be early events in astrocytoma tumour progression because frequencies of loss and mutation are similar in all astrocytoma grades. Mutations in the p53 gene are found in one third of all diffuse fibrillary astrocytomas. Of 68 p53 mutations in 65 astrocytomas, 54 were missense, 12 were nonsense or frameshift and 2 were intronic (Louis 1994). Table 1.22 shows the frequency of p53 mutations and LOH in human brain tumours. There are no brain specific p53 mutations, although there are three mutational hotspots at codons 175, 248 and 173, similar to colon cancer, but in brain tumours mutations at codon 273 are most common, compared to codon 175 in colon cancer (Bögler et al 1995). Although there is a correlation between LOH and p53 mutation in gliomas, in one study 27 of 38 (71%) grade III and IV astrocytomas retained both 17p alleles and 9 of these 27 (33%) had a mutation in one of the alleles. Of the 11 tumours with 17pLOH, 7 (64%) had a mutation (Frankel et al 1992). Where LOH is found with no p53 mutation suggests the possibility of a second tumour suppressor
gene on Cm17p, and where there is no LOH but mutation in one p53 allele suggests that mutant p53 protein is acting in a dominant negative manner. p53 protein has been detected, using MAb CM-1, in 15-40% of grade II astrocytomas, 35-60% of grade III astrocytomas and 45-70% of GBMs. Although there appears to be a trend that the more malignant tumours are more often positive the staining can be heterogeneous and the number of positive cells varies within the grades (Louis 1994).

Table 1.22 Frequency of p53 mutations and LOH in human brain tumours.

<table>
<thead>
<tr>
<th>Tumour type and grade</th>
<th>p53 mutations</th>
<th>LOH on Cm17p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Astrocytoma I</td>
<td>6/25</td>
<td>24</td>
</tr>
<tr>
<td>Astrocytoma II</td>
<td>51/172</td>
<td>30</td>
</tr>
<tr>
<td>Astrocytoma III</td>
<td>51/165</td>
<td>31</td>
</tr>
<tr>
<td>GBM</td>
<td>83/250</td>
<td>33</td>
</tr>
<tr>
<td>Oligodendroglioma I</td>
<td>0/16</td>
<td>0</td>
</tr>
<tr>
<td>Oligodendroglioma II</td>
<td>4/35</td>
<td>11</td>
</tr>
<tr>
<td>Oligodendroglioma III</td>
<td>2/16</td>
<td>13</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>3/27</td>
<td>11</td>
</tr>
<tr>
<td>Ependymoma</td>
<td>0/15</td>
<td>0</td>
</tr>
</tbody>
</table>


**p53 and drug resistance**

The fact that p53 is involved in the response to DNA damage and that this damage can be caused by many cytotoxic drugs suggests there could be a relationship between p53 and chemosensitivity. In lymphoma cells, p53 mutations were associated with decreased sensitivity to DNA damaging agents like cyclophosphamide and cisplatin (Fan et al 1994). In ovarian cancer cells transfected with mutant p53, radiation induced G1 arrest was lost (McIllwrath et al 1994). However, p53 has not only been implicated in response to DNA damaging agents, normal human fibroblasts with non-functional p53, bound to SV40 T antigen, were seven-fold more sensitive to paclitaxel than controls (Wahl et al 1996). These studies show that loss of functional p53 paradoxically results in either increased or decreased resistance to chemotherapeutic drugs and this may reflect p53's selective regulation of certain genes (Thottassery et al 1997).
Such a specific relationship has been found between p53 and the *MDR1* gene. Transfection studies in human fibroblasts showed that mutant p53 specifically stimulated the *MDR1* promoter, and wild type p53 had a specific repressive effect. The c-Ha-ras-1 oncogene also stimulated the *MDR1* gene promoter but was not specific for this promoter alone (Chin et al 1992). It is not known how this activation occurs but it does not appear to be dependent upon a consensus p53 binding sequence (Zastawny et al 1993). Further studies using human lung, colon and ovary carcinoma cells indicated that p53 bound to an initiator sequence of the *MDR1* gene (Goldsmith et al 1995). A functional study of the relationship between p53 and PGP was carried out in rat hepatoma cells with wild-type p53 and PGP expression (Thottassery et al 1997). A mutant p53 gene was introduced into these cells causing marked increases in levels of PGP and *MDR1* mRNA and increased resistance to MDR implicated drugs, sensitivity was restored by a PGP inhibitor. Observed differences in p53-mediated cell death by drugs that are PGP substrates may therefore depend upon whether a cell expresses PGP. Other studies have found no link between p53 status and *MDR1* expression in mammary, endometrial nor cervical tumours (Schneider et al 1994) nor B-cell chronic lymphocytic leukaemia (El Rouby et al 1993).

The cells expressing mutant p53 in the study by Thottassery et al (1997) were also found to have increased sensitivity to methotrexate which is not a PGP substrate, thus indicating that mutant p53 not only regulates PGP expression but may have other effects upon the cell. There is now further evidence that p53 status specifically affects other mechanisms of resistance. Increased levels of wild-type p53 made human melanoma cells resistant to genistein, a soybean derivative which inhibits protein tyrosine kinase and topoisomerase II (Rauth et al 1997). Wild-type p53, but not mutant, can suppress O⁶-methyl guanine methyl transferase (MGMT) gene promoter activity in three human cell lines, therefore increasing sensitivity to nitrosoureas (Harris et al 1996).

In astrocytic tumours mutations in the p53 gene correlated with increased resistance to DNA damaging drugs but increased sensitivity to anti-microtubule agents, indicating that cell death caused by the latter agents is not mediated by functional p53 (Iwadate et al 1988). Further evidence for this is given by the ability of paclitaxel to activate an apoptosis inducing cytokine independent of p53 (Lanni et al 1997). In a conflicting study using mouse astrocyte cultures, those expressing wild-type p53 were significantly more resistant to BCNU than mutant p53 expressing or p53 knockout cells (Nutt et al 1996).
**Relationship between tumour recurrence and drug resistance.**

High grade astrocytomas invariably recur. Recurrence usually occurs within a 2cm margin of the original tumour (Liang et al 1991) although in 5-10% of patients recurrence occurs at sites remote from the original tumour, presumably as a result of diffuse invasion (Salcman et al 1995). Low grade fibrillary astrocytomas have a marked propensity for progression to high grade tumours although this may occur 10-15 years after the original diagnosis. The process that leads to tumour progression and recurrence seems to be related to a specific pattern of genetic changes (Figure 1.11).

Kleihues has suggested that there are two separate pathways involved, one for de novo GBMs (1° GBMs) and one for GBMs that are a result of progression (2° GBMs). 1° GBMs overexpress EGFR and have no p53 mutations whereas 2° GBMs have no increased EGFR but do have p53 mutations (Kleihues et al 1993, Reifenberger et al 1996). Confirmation of there being two separate pathways to GBM was given by von Deimling et al (1995) where further molecular studies implicated several new genetic changes distinguishing 1° from 2° GBMs (Figure 1.12).

In the present study grade III and IV astrocytomas which recur with the same grade as the primary diagnosis are of most interest. Recurrent tumours are more densely cellular and are histologically more homogeneous than at the time of diagnosis (Burger and Scheithauer 1994) and the cells that repopulate these recurrent tumours differ in their chromosomal number from those present at diagnosis. How this clonal expansion occurs is unknown but investigations into this apparent cell selection may have prognostic significance. Data produced from metaphase studies of short-term cultures derived from primary and recurrent tumours in the same patients, indicate that the cells which repopulate the recurrence often have additional copies of chromosomes 7 and 22, overproduce platelet derived growth factor (PDGF) and are resistant to nitrosoureas (Scheck et al 1993). It is unknown whether these changes are seen in the solid tumour. Genes coding for growth factors and growth factor receptors are located on chromosomes 7 and 22 implying that these cells may have a growth advantage, for example epidermal growth factor receptor (EGFR) and PDGFA are coded for by genes at 7p12 and 7p22 respectively (Maxwell et al 1990), PDGFB is coded for by a gene at 22q13 (Mapstone et al 1991). The MDR1 gene is also located on chromosome 7 at 7q21 (Callen et al 1987).
Figure 1.11 Glial tumour progression.

Adapted from Collins and James (1993) (- = loss of genetic material from that chromosome, MDM2 = mouse double minute 2, EGFR = epidermal growth factor receptor, PDGFR = platelet derived growth factor receptor)

Figure 1.12 Model of dual pathway to GBM.

Adapted from Von Deimling et al 1995.

(LOH=loss of heterozygosity, RB=retinoblastoma gene, EGFR=epidermal growth factor receptor, CDK4=cyclin dependant kinase-4, mdm2=mouse double minute-2)
Aims

High grade astrocytomas are intrinsically resistant to chemotherapeutic drugs, although the basis of this resistance remain unclear. This study aims to examine a number of factors which have been implicated in this resistance.

1. The patterns of resistance found in vitro using a chemosensitivity assay against a panel of short term cultures derived from adult high grade astrocytomas will be determined. Three drugs will be assessed, VCR, DOX and CCNU. Two of these, namely VCR and CCNU, are presently included in chemotherapy regimens for high grade astrocytoma. The third drug, DOX, will be used in this study because resistance to this drug is seen in cells which display the Multidrug Resistance (MDR) phenotype.

2. The expression of P-glycoprotein will be assessed using immunocytochemistry. A panel of three antibodies, JSB-1, C219 and MRK-16, which recognise different epitopes of PGP will be used. Non-PGP MDR will be examined using immunocytochemistry to assess the expression of multidrug resistance associated protein (MRP).

3. Mutations in p53 are common in high grade astrocytoma and it is known that mutant p53 protein can initiate transcription of the MDR-1 gene promoter leading to PGP overexpression. This relationship will be investigated in high grade astrocytomas by comparing the patterns of expression of these two proteins. Expression of p53 protein will be assessed using a panel of three antibodies, DO-1 and PAb421, which recognise both wild type and mutant p53, and PAb240 which recognises mutant forms only.

4. Mutation of the p53 gene has been shown to be involved in resistance to DNA damaging drugs. In the absence of normal p53 there is no cell cycle arrest in response to DNA damage, DNA repair is not initiated nor is entry into the apoptotic pathway, therefore cell division may continue with the DNA damage unrepaired. This study will therefore examine the relationship between p53 protein expression and chemosensitivity.

5. Specific genetic changes are known to be involved in the progression of low grade to high grade astrocytomas. Little is known about the genetic events which lead to the recurrence of high grade astrocytomas. In metaphase studies of paired cell
cultures derived from high grade astrocytomas, at the time of diagnosis and again at recurrence in the same patients, additional copies of chromosomes 7 and 22 have been found. These cells also overproduce platelet derived growth factor and are resistant to nitrosoureas. This study will examine the copy numbers of these two chromosomes, in formalin fixed, paraffin embedded tumour sections using fluorescence \textit{in situ} hybridisation (FISH) with DNA centromere probes. Paired samples from the primary tumour and the tumour at recurrence will be examined to determine if the copy number changes seen \textit{in vitro} occur \textit{in situ} and therefore may be implicated in the development of drug resistance.
MATERIALS AND METHODS
Cell culture
Tumour specimens
Adult human glial tumour resection specimens or stereotactic biopsies were collected at the time of operation and transferred into antibiotic rich media in sterile 30ml universal containers. This transport media was Ham’s F10 nutrient mixture containing kanamycin (50μg/ml), penicillin/streptomycin (100 IU/ml/100μg/ml) and amphotericin B (2.5μg/ml). The specimens were processed immediately on arrival at the laboratory but remained viable for up to 5 days in the collection media. This allowed for specimens to be sent from different hospitals around the country.

All further cell work was carried out under sterile conditions in a Class II laminar flow hood (Gelaire, ICN Flow). All pipetting was done using sterile, single use, plastic pipettes with electronic pipette aids.

Setting up short term cell cultures
Short term cell cultures were originated from the tumour specimens. Three methods for initiating growth were used depending upon the size of the specimen, firstly the coverslip method for specimens less than 5mm diameter, (often used for stereotactic biopsy specimens), secondly, the collagenase method for larger resections, and thirdly the direct plating method for specimens in suspension.

1. Coverslip method
Two dabs of sterile silicon grease were placed on to the base inside a 25cm² flask. The tumour specimen was washed with Ham’s F10 nutrient mixture and placed between the dabs of grease. A sterile 9x22mm glass coverslip was placed onto the specimen and secured by pressing into the grease as shown below;

5mls of Ham’s F10 nutrient mixture plus 10% foetal calf serum were added to the flask and incubated at 37°C.
2. **Collagenase method.**  
The specimen was placed in a sterile Petri dish and sliced using crossed scalpel blades until fine enough to be pipetted. The finely sliced specimen was placed into a 30ml universal container with 3mls Ham’s F10 with 10% FCS and 2000 units of collagenase type 1A (one unit liberates peptides from collagen equivalent to 1.0μM of leucine in 5 hours at pH 7.4 at 37°C), and incubated at 37°C for 1-2hrs. After incubation 7mls Ham’s F10 with 10% FCS was added to stop any further enzyme reaction, and centrifuged at 1000rpm in a Wifug 500E centrifuge, equivalent to 400G, for 5mins. The supernatant was removed and the pellet resuspended in 10mls Ham’s F10 with 10% FCS. The cell suspension was transferred to a 25cm² culture flask and incubated at 37°C.

3. **Direct plating method.**  
The suspended specimen was centrifuged in the original collection universal container at 400G for 5mins. The supernatant was removed and the pellet resuspended in 5mls Ham’s F10 with 10% FCS. The cell suspension was transferred to a 25cm² culture flask and incubated at 37°C.

**Cell counting**  
Cells were counted using a Coulter Counter ZM, calibrated using the cell line U251MG as a typical example of glioma cells. The machine was set up to count all the cells in a 0.5ml sample taken from a suspension of single cells in an isotonic solution (Isoton II). For general use 0.4ml of a 10ml single cell suspension was added to 19.6ml of Isoton II in a counting pot. The counter gave a reading of the number of cells in 500μl. The following calculation gives the total number of cells in the original 10mls:

\[
\text{Reading from counter} = X \\
X = \text{no. of cells in 0.5ml taken from 20mls (0.4 + 19.6) in the counting pot.} \\
\therefore \text{multiply } X \text{ by 20/0.5} = 40 \\
\text{but only 0.4mls were taken from the original 10ml cell suspension} \\
\therefore \text{multiply } X \text{ by 10/0.4} = 25 \\
\Rightarrow \text{number of cells in the original 10mls} = 40 \times 25 \times X = 1000X
\]

For example a reading of 3987 would indicate \(3.987 \times 10^6\) cells in the 10mls.
Maintenance of cultures.
Newly set up flasks were continuously monitored for cells adhering to the flask and for cells growing out from clumps of tissue, then the remaining media was aspirated and fresh growth media (Ham's F10 with 10% FCS) was added. This removed any debris, dead tissue and red blood cells. Subsequently cultures were fed with fresh growth media approximately once a week depending on the rate of growth.

Once the cultures were confluent they were passaged, prior to subculturing or freezing. Remaining media was aspirated and the cells were washed twice with Hank's balanced salt solution (HBSS), rinsed with trypsin 0.25%, then incubated with 3mls trypsin at 37°C for 15-30mins or until the cells had rounded up and were non-adherent. Seven mls of media were added to the flask and all the fluid was transferred to a 30ml universal and centrifuged at 1000rpm for 5mins. The supernatant was removed and the pellet resuspended in 10mls media, 0.4mls were removed for counting the cells using a Coulter counter ZM. To subculture, the cells were then divided into new 75cm² flasks seeding approximately 0.5-1million cells per flask equivalent to 7000-13000cells/cm². To freeze the cells, the cell suspension was recentrifuged and the pellet resuspended in FCS with 10% dimethyl sulphoxide (DMSO) to a final concentration of 1 million cells per ml, this was aliquoted into cryotubes, 1ml per tube, which were packed in polystyrene and frozen slowly down to -70°C, after 24 hrs the cryotubes were transferred into liquid nitrogen tanks where they were stored. Each time trypsin was used the passage (P) number of the culture was increased by one.

Growing cells from frozen.
To grow cells from frozen stock, the cryotube containing the cells of interest was removed from the liquid nitrogen storage tank and placed directly into a beaker of warm water and allowed to thaw. The cell suspension from the cryotube was pipetted into a 30ml universal container and 9mls of growth media were added. This was centrifuged as before and the supernatant containing much of the DMSO was removed. The resultant pellet was resuspended in 10mls of fresh growth media and transferred to a 75cm² flask. The following day the media was aspirated to remove more DMSO and any dead cells and 10mls of fresh growth medium was added.

Mycoplasma testing
Each culture was routinely tested for mycoplasma, usually before being frozen down. The fluorescent dye Hoechst No. 33258 was used, this binds specifically to
DNA. Mycoplasma contain DNA so if the culture is infected the mycoplasma on the surface of cells or between cells will appear as dotted or filamentous blue fluorescence, distinct from the much larger fluorescing cell nuclei. A slide that is negative for mycoplasma will have bright blue fluorescent nuclei on a black background. Cells from the resuspended pellet were grown on sterile 13mm diameter coverslips in 24 well plates for 3-4 days, in 1ml of growth media at 37°C, until approximately 60% confluent. The coverslips were rinsed in HBSS without phenol red and the cells fixed in ice-cold methanol at -20°C for 10mins. HBSS without phenol red was used because phenol red is fluorescent and this may affect the result. The coverslips were again rinsed in HBSS without phenol red and incubated at room temperature with Hoechst dye 250ng/ml for 20-30mins. The coverslips were rinsed 3 times in HBSS without phenol red and twice in deionised water and were mounted, cells downwards, in 50% glycerol in phosphate buffered saline (PBS) mountant onto glass slides. Excess mountant was blotted with a tissue. The coverslips were sealed onto the slides with nail varnish and stored in the dark at 4°C overnight, or viewed unsealed immediately. The slides were visualised using a UV fluorescence microscope with DAPI filter and X10 eyepiece with X40 objective lens and under oil immersion with a X100 objective lens. All cells used were found to be negative for mycoplasma contamination.

**Chemosensitivitv testing using the MTT Assay.**

This assay is based on the ability of living cells to reduce the yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), to a blue formazan product which is proportional to the number of living cells present. The formazan can be quantified spectrophotometrically (Denizot and Lang 1986). The protocol is adapted from Nikkah et al (1992a).

**Preparation of drugs for chemosensitivity assay**

**Vincristine (VCR)**

Vincristine sulphate solution (1mg/ml) for injection was stored at 4°C. For use in the assay this was further diluted with growth media. A stock solution of 100 or 200μg/ml was made and stored at -70°C for up to 1 month. Dilutions of this stock solution were made up fresh on day 1 of the assay and stored at -20°C for the duration of the assay.

**Doxorubicin (DOX)**

Doxorubicin hydrochloride solution for injection (2mg/ml) was stored at 4°C. For use in the assay this was further diluted with growth media. A stock solution of
100μg/ml was made and stored at -70°C for up to 1 month. Dilutions of this stock solution were made up fresh on day 1 of the assay and stored at -20°C for the duration of the assay.

CCNU
Capsules of CCNU were stored at 4°C. The contents of one 10mg capsule were emptied into a universal container. 10mls ethanol were added and mixed gently. This was centrifuged at 3000rpm equivalent to 3000G for 10mins. The supernatant was then pipetted into a new universal container and this stock solution (1mg/ml) was stored at -20°C for up to one month. For use in the assay the stock solution was further diluted using growth media. Dilutions were made up fresh on day 1 of the assay and stored at -20°C for the duration of the assay.

Storage and stability of drug solutions
All solutions were prepared aseptically and filter sterilisation was not used as some filters have been shown to adsorb drugs (Bosanquet 1985). During the 3 day drug exposure in the assay the drug solutions were kept in the dark and were frozen and thawed only twice then discarded. Under these storage conditions, the drugs used in the study remain stable (Hunter et al 1994).

Serial dilutions and ranges of drug concentrations used.
Different concentration ranges were used to try to ensure maximum cell kill at the highest concentration and minimum cell kill at the lowest concentration. For VCR, 11 different ranges were used (Table 2.1). For DOX, 12 different ranges were used (Table 2.2). For CCNU, 5 different ranges were used (Table 2.3). These concentration ranges are shown in diagrammatic form in Figure 2.1.

MTT assay protocol
Cells were plated into 96 well plates using 1500 cells per well in a volume of 100μl, equivalent to 4688 cells/cm². Plates were sealed with adhesive mylar sheets. For each individual culture 2 plates were set up for each drug, plus a control plate to monitor growth. The growth curve protocol is described later. These were then incubated for 48 hours to allow the cells to adhere and reach exponential phase of growth. A block of 6 by 8 wells was then chosen for the assay. Three day drug exposure followed by a four day recovery in drug free media was used.
Day 1. Media was aspirated from test plates. Six serial dilutions of drug were added, 100μl per well, lowest concentration in row F, highest concentration in row A. Media was aspirated from rows G and H (control wells), and fresh media was added. Days 2 and 3. As Day 1.

Day 4. Media was aspirated from all test plates. Wells were washed twice with HBSS and fresh media was added.

Day 5. All wells were aspirated and fresh media was added.

Day 8. Media was aspirated from all test plates. 100μl of 1mg/ml MTT in Ham's F10, was added to each well and incubated at 37°C for 4 hours. Each well was carefully aspirated and the remaining formazan crystals were dissolved in 100μl DMSO per well. Each plate was placed on a plate shaker for a few seconds. The plate reader was blanked against fresh MTT solution (1mg/ml) in an empty well. The absorbance was read at 570nm using an MR600 plate reader (Dynatech). A summary of the protocol is shown in Figure 2.2.

Table 2.1  VCR concentration ranges used in the chemosensitivity assay.

<table>
<thead>
<tr>
<th>VCR range</th>
<th>Concentrations (ng/ml)</th>
<th>Dilution factor</th>
<th>Cultures tested at each range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100000→10000→1000→100→10→1</td>
<td>10</td>
<td>IN1951</td>
</tr>
<tr>
<td>B</td>
<td>100000→5000→250→12.5→0.625→0.03125</td>
<td>20</td>
<td>IN1951</td>
</tr>
<tr>
<td>C</td>
<td>100000→2000→40→0.8→*0.008</td>
<td>50 (*100)</td>
<td>IN2134, IN2281, IN1461, IN1760, IN1728, IN1951</td>
</tr>
<tr>
<td>D</td>
<td>50000→500→5→0.05→*0.00005</td>
<td>100 (*1000)</td>
<td>IN2281, IN1728</td>
</tr>
<tr>
<td>E</td>
<td>2500→125→6.25→0.3125→0.00625→0.000125</td>
<td>20</td>
<td>IN1265, IN2094</td>
</tr>
<tr>
<td>F</td>
<td>1000→20→0.4→0.008→0.00016→0.000016</td>
<td>50</td>
<td>IN1265, IN1472</td>
</tr>
<tr>
<td>G</td>
<td>400→*40→10→2.5→0.625→0.156</td>
<td>4 (*10)</td>
<td>IN1528, IN2045, IN1612, IN1752, IN2103, IN2127, IN2103, IN2112</td>
</tr>
<tr>
<td>H</td>
<td>200→40→8→1.6→0.32→0.16</td>
<td>5</td>
<td>IN1682</td>
</tr>
<tr>
<td>I</td>
<td>200→4→0.08→0.0016→0.000032</td>
<td>50</td>
<td>IN1472</td>
</tr>
<tr>
<td>J</td>
<td>100→20→4→0.8→0.16→0.016→0.0032</td>
<td>5</td>
<td>IN1979, IN859, IN2094, IN2045, IN2093, IN1612, IN1682, IN1902</td>
</tr>
<tr>
<td>K</td>
<td>10→2→0.4→0.08→0.016→0.0032</td>
<td>5</td>
<td>IN1979, IN1752, IN1902, U251MG</td>
</tr>
</tbody>
</table>

Column 3 shows the dilution factors and column 4 shows the cultures tested at each range.
Table 2.2 DOX concentration ranges used in the chemosensitivity assay.

<table>
<thead>
<tr>
<th>DOX range</th>
<th>Concentrations (ng/ml)</th>
<th>Dilution factor</th>
<th>Cultures tested at each range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40000→ 4000→ 400→ 40→ 4→ 0.4</td>
<td>10</td>
<td>IN1951, IN1461</td>
</tr>
<tr>
<td>B</td>
<td>40000→ 800→ 16→ 0.32→ 0.0064</td>
<td>50</td>
<td>IN1951</td>
</tr>
<tr>
<td>C</td>
<td>20000→ 400→ 8→ 0.16→ 0.0032</td>
<td>50</td>
<td>IN2132, IN2134</td>
</tr>
<tr>
<td>D</td>
<td>4000→ 571.4→ 81.63→ 11.66→ 1.666→ 0.238</td>
<td>7</td>
<td>IN1760, IN1728, IN2094, IN1752, IN2281, IN2026, IN2058</td>
</tr>
<tr>
<td>E</td>
<td>2000→ 2000→ 20→ 2→ *0.5</td>
<td>10 (*4)</td>
<td>IN1461</td>
</tr>
<tr>
<td>F</td>
<td>1000→ 250→ 62.5→ 15.63→ 3.906→ 0.9766</td>
<td>4</td>
<td>IN1951</td>
</tr>
<tr>
<td>G</td>
<td>1000→ 20→ 0.4→ 0.008→ 0.00016</td>
<td>50</td>
<td>IN1265, IN1472</td>
</tr>
<tr>
<td>H</td>
<td>500→ 50→ 5→ 0.5→ 0.05</td>
<td>10</td>
<td>IN1728, IN2094, IN2132, IN2037</td>
</tr>
<tr>
<td>I</td>
<td>400→ 100→ 25→ 6.25→ 1.563→ 0.3906</td>
<td>4</td>
<td>IN1528, IN859, IN2127, IN1902, IN1682, IN1612, IN2093, IN2045, IN1979</td>
</tr>
<tr>
<td>J</td>
<td>200→ 40→ 8→ 1.6→ 0.32</td>
<td>5</td>
<td>IN1528</td>
</tr>
<tr>
<td>K</td>
<td>100→ 10→ 1→ 0.1→ 0.01</td>
<td>10</td>
<td>IN2112, IN2127, U251MG</td>
</tr>
<tr>
<td>L</td>
<td>10→ 2→ 0.4→ 0.08→ 0.016→ 0.0032</td>
<td>5</td>
<td>IN1979, IN2045, IN1902, IN2112, U251MG</td>
</tr>
</tbody>
</table>

Column 3 shows the dilution factors and column 4 shows the cultures tested at each range.
Table 2.3  CCNU concentration ranges used in the chemosensitivity assay.

<table>
<thead>
<tr>
<th>CCNU range</th>
<th>Concentrations (µg/ml)</th>
<th>Dilution factor</th>
<th>Cultures tested at each range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50→ 5→ 0.5→ 0.05→ 0.005</td>
<td>10</td>
<td>IN2134, IN2281</td>
</tr>
<tr>
<td>B</td>
<td>40→ 10→ 2.5→ 0.625→ 0.1563→ 0.01953</td>
<td>4 (*8)</td>
<td>IN2281, IN2132, IN1472, IN1265, IN2037, IN2112, IN1728, IN2094</td>
</tr>
<tr>
<td>C</td>
<td>25→ 8.33→ 2.778→ 0.9259→ 0.3086→ 0.1029</td>
<td>3</td>
<td>IN2026, IN2058, IN1461, IN2127, U251MG, IN859, IN1760, IN1528, IN1979, IN2045, IN2093, IN1612, IN1682, IN1752, IN1752, IN1902, IN1951</td>
</tr>
<tr>
<td>D</td>
<td>20→ 6.667→ 2.222→ 0.7407→ 0.2469→ 0.0823</td>
<td>3</td>
<td>IN1940, IN2112, IN1979, IN1902, IN1951, U251MG</td>
</tr>
<tr>
<td>E</td>
<td>15→ 3.75→ 0.9375→ 0.2344→ 0.0586→ 0.01465</td>
<td>4</td>
<td>IN2037, IN2103, IN2127, IN1528, IN1612, U251MG</td>
</tr>
</tbody>
</table>

Column 3 shows the dilution factors and column 4 shows the cultures tested at each range.
Figure 2.1  Ranges of drug concentrations used for the chemosensitivity assays.

Each x represents a single drug concentration used.
Figure 2.2 Summary of MTT chemosensitivity assay protocol

Plate cells into wells and incubate for 48 hours

3 day drug exposure

4 day recovery

Incubate with MTT for 4 hours

Add DMSO and measure absorbance at 570nm

Calculations to find \( \text{ID}_{50} \) values

The plate reader gave an absorbance reading for all the test and control wells, a total of 48. Table 2.4 shows an example of the absorbance values for a single plate and how the calculations were made.

**Table 2.4** An example of the MTT absorbance values for a single plate and how the calculations were made.

<table>
<thead>
<tr>
<th>Row</th>
<th>0.036</th>
<th>0.029</th>
<th>0.036</th>
<th>0.030</th>
<th>0.016</th>
<th>0.024</th>
<th>mean</th>
<th>Cell survival %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.025</td>
<td>0.026</td>
<td>0.020</td>
<td>0.015</td>
<td>0.010</td>
<td>0.000</td>
<td>0.017</td>
<td>5.0</td>
</tr>
<tr>
<td>B</td>
<td>0.031</td>
<td>0.025</td>
<td>0.028</td>
<td>0.017</td>
<td>0.007</td>
<td>0.009</td>
<td>0.019</td>
<td>5.9</td>
</tr>
<tr>
<td>C</td>
<td>0.124</td>
<td>0.098</td>
<td>0.074</td>
<td>0.048</td>
<td>0.050</td>
<td>0.060</td>
<td>0.076</td>
<td>23.0</td>
</tr>
<tr>
<td>D</td>
<td>0.291</td>
<td>0.294</td>
<td>0.221</td>
<td>0.301</td>
<td>0.282</td>
<td>0.260</td>
<td>0.275</td>
<td>83.6</td>
</tr>
<tr>
<td>E</td>
<td>0.325</td>
<td>0.356</td>
<td>0.355</td>
<td>0.315</td>
<td>0.333</td>
<td>0.357</td>
<td>0.340</td>
<td>103.5</td>
</tr>
<tr>
<td>F</td>
<td>0.403</td>
<td>0.359</td>
<td>0.322</td>
<td>0.317</td>
<td>0.289</td>
<td>0.287</td>
<td>0.331</td>
<td>**</td>
</tr>
<tr>
<td>G</td>
<td>0.321</td>
<td>0.376</td>
<td>0.293</td>
<td>0.307</td>
<td>0.339</td>
<td>0.321</td>
<td>0.326</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.329</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>100%</strong></td>
<td></td>
</tr>
</tbody>
</table>

*average of control wells' absorbance values representing **100% cell survival.
For each plate the average absorbance value for the control wells in rows G and H was calculated (Table 2.4*), this was assumed to represent 100% cell survival. The averages of the test rows were then also expressed as a percentage of the control rows. These cell survival values shown in the final column were then plotted against the corresponding drug concentration to give the MTT assay curve. The ID$_{50}$ value was determined by taking the two actual readings from either side of the 50% survival point, (in this case 23.0 and 83.6) and fitting a curve between these points using the graph programme Cricket Graph, the point of 50% cell survival could be calculated from the curve fit equation.

**Growth curves**

For the MTT assay to be valid it is important that the cells in all wells on a microtitre plate remain in the exponential phase of growth, because if the control wells become confluent and the culture enters plateau phase then it will appear to be more resistant than it actually is by shifting the dose response curve to the right.

Cells were seeded into the wells of a 96 well plate as for the MTT assay. Cell counts were performed on each day of the assay. Media was aspirated from 6 wells of the growth plate. These wells were washed twice with HBSS which was then discarded. 100μl of trypsin were added to each well and incubated at 37°C until cells had rounded up and detached. The trypsin/cell suspension from all 6 trypsinised wells (0.6mls) was added to 19.6mls isoton II and counted using the Coulter Counter ZM.

To find the number of cells per well the calculations are as follows:

**Reading from counter = X**

\[ X = \text{no. of cells in 0.5ml taken from 20.2mls (0.6 + 19.6) in the counting pot.} \]

\[ \therefore \text{multiply } X \text{ by } 20.2/0.5 = 40.4 \]

but this is the number of cells in 6 wells

\[ \therefore \text{divide } X \text{ by 6} \]

\[ \Rightarrow \text{number of cells per well} = 40.4/6 \times X \]

\[ = 6.7333X \]

Each well is 0.32cm$^2$ \[ \therefore \text{the number of cells per cm}^2 = 6.7333X + 0.32 \]

\[ = 21.04X \]
Figure 2.3 Example of a growth curve showing phases of growth and doubling time calculation.
An example of a growth curve is shown in Figure 2.3. The curve has been plotted on a log scale and the exponential phase of growth appears as a straight line. Cells in the lag phase are adapting to the culture conditions and attaching to the wells, during exponential phase optimal growth and cell division occurs. At plateau phase the wells become confluent and the number of daughter cells from cell division is approximately the same as the number of dying cells, producing an equilibrium with no apparent increase in cell number per unit area. To calculate the doubling time a line is fitted by eye through the points of the exponential phase, this is shown in red. On this line two points are chosen to show that the number of cells has doubled, in this case 1000 and 2000 cells per cm². These are extrapolated down to the horizontal axis to find the doubling time. In this case it is approximately 48 hours.

**Immunocytochemistry**

Cells were seeded into eight well glass chamber slides at 5000 cells per chamber in approximately 1ml of media. The cells were allowed to settle and grow for 3-4 days until approximately 60% confluent. The cells were fixed in cold acetone/methanol (1:1) for 2 mins, followed by a short wash in PBS (Lane D, personal communication).

**p53**

Three monoclonal antibodies were used, D0-1 and PAb421 which recognise both wild type and mutant forms of p53, and PAb240 which solely recognises the mutant form. All antibodies were a gift from David Lane (University of Dundee, UK). Antibodies were in the form of tissue culture supernatant and were used undiluted.

Slides were incubated with monoclonal antibodies (100μl per chamber) for 2 hours at room temperature (RT). Bound antibody was visualised with 100μl biotinylated sheep anti-mouse Ig, at 1:50 dilution in 5% FCS in PBS for 2 hours at RT. Finally the slides were incubated with streptavidin fluorescein, 1:50 dilution in PBS for 40mins at RT. After each incubation the slides were washed once in PBS with 0.1% Tween 20 and twice in PBS alone. Slides were air dried and mounted in Citifluor.

Scoring of strong nuclear staining was as follows; no staining = 0, 1-5% of nuclei positive = 1, 6-50% = 2, 51-100% = 3. For statistical calculations scores of 0 and 1 were deemed negative for overexpression while scores of 2 and 3 were deemed positive.

**P-Glycoprotein**

Three monoclonal antibodies were employed, JSB-1 in the form of ascites diluted 1 in 10 and C219 10μg/ml which recognise internal epitopes of PGP and MRK-16,
5μg/ml (a gift from Takashi Tsuruo, University of Tokyo, Japan), which recognises an external epitope. An irrelevant antibody clone CT6 (anti-Guinea pig lymphocyte), diluted 1 in 10, which recognises a guinea pig lymphocyte subset was used as a negative control. All antibodies were diluted in PBS containing 5% FCS.

Slides were incubated with monoclonal antibodies for 2 hours at RT. Bound antibody was visualised with 100μl biotinylated sheep anti-mouse Ig, 1:50 dilution in 5% FCS in PBS for 30mins at RT, followed by streptavidin fluorescein, 1:50 dilution in PBS for 40mins at RT. After each incubation the slides were washed once in PBS with 0.1% Tween 20, and twice in PBS alone. The slides were counterstained with propidium iodide, 40μg/ml in water for 1-2 seconds, then washed thoroughly in PBS. Slides were air dried and mounted in Citifluor.

**MRP**

Anti-human MRP monoclonal antibody, clone MRPm6, which reacts with an internal epitope at the carboxy terminal end of MRP was used at a 1:20 dilution. The immunocytochemistry protocol was exactly the same as for PGP. A summary of the immunocytochemistry protocol is shown in Figure 2.4.

**Figure 2.4** Summary of the immunocytochemistry protocol.
Fluorescence In Situ Hybridisation
Paraffin embedded material

Thirteen formalin fixed, paraffin embedded high grade glioma pairs (primary tumour and corresponding recurrent), were kindly provided by T Revesz (Institute of Neurology, London, UK) and P Wilkins (Atkinson Morley’s Hospital, London, UK). 5µm sections were mounted onto APES (3-aminopropyltriethoxysilane) coated slides. Centromere-specific DNA probes for chromosomes 7 and 22 were kindly donated by A Baldini (Baylor College, Houston, Texas, USA) and were labelled with biotin-11 dATP by nick translation using the Life Technologies BioNick kit.

The probes contained 20ng/µl DNA dissolved in Tris/EDTA (TE) buffer and were stored at -20°C. For each hybridisation 80ng of chromosome 22 DNA or 60ng of chromosome 7 DNA were used. The probe DNA was mixed in an Eppendorf tube with 3µl Cot1 DNA to compete for repetitive sequences so preventing non-specific binding. Then 2 volumes of ethanol were added to precipitate the DNA. The eppendorf was then placed in a spin vac and centrifuged until dry. The dried probe and Cot1 DNA was then dissolved in 11µl of hybridisation buffer (10% dextran sulphate, 2x Standard Saline Citrate (SSC), 50% formamide, 1% Tween-20) and kept on ice.

The slide with the paraffin section on was baked for one hour at 65°C in an oven, then dewaxed in xylene for 2x10mins in a coplin jar on a gently shaking platform. The slide was then similarly dehydrated in ethanol for 2x5mins prior to digestion with pepsin/HCl (4-25mg/ml pepsin in 0.2M HCl). 100µl of pepsin/HCl was pipetted directly onto the section, covered with a 22x50mm coverslip and incubated at 37°C for between 10 and 120mins. The concentration of pepsin and the incubation time differed for each section and was arrived at empirically. The slides were rinsed 5x in deionised water and 5x in PBS followed by dehydration through an alcohol gradient, 70%, 95% and 100% in coplin jars.

Both probe DNA, in the Eppendorf tube, and target DNA, on the slide, were heat denatured prior to hybridisation, the slides on a hot plate for 30 minutes at 80°C and the probe for 10 minutes at 80°C in a waterbath. The probe was immediately added to the slide, covered with a 22x22mm coverslip and sealed with cowgum. The slides were returned to the hotplate for 10 minutes before hybridisation in a humid chamber at 37°C overnight.
Post-hybridisation washes took place in coplin jars, and those at room temperature on a shaking platform. First wash was 2xSSC at room temperature for 5mins, then 0.1xSSC at 55°C for 2x 10mins. The first wash was then repeated. Finally the slides were rinsed in PBS. The slides were not allowed to dry out.

**Immunochemical detection**

Immunochemical detection involved 3 layers to amplify the signal. First the slides were preincubated with 100μl SSCTM under a 22x50 coverslip for 10mins at 37°C, followed by a short wash in SSCT. This was to block any non-specific binding sites. All antibodies were diluted in SSCTM and each incubation used 100μl under a 22x50 coverslip for 30mins at 37°C. The first layer was avidin-FITC (2μg/ml), the second was biotinylated anti-avidin (5μg/ml) and finally avidin-FITC (2μg/ml) again. After the first two incubations the slides were washed for 3 x 5mins in SSCT. After the final antibody incubation the slides were washed for 5 mins in SSCT then 2 x 10mins in PBS. The slides were dehydrated through an ethanol series and were mounted in Citifluor containing 0.5μg/ml propidium iodide. Averages of 124 and 125 nuclei were counted per section in several fields of view for the Cm7 and Cm22 probe respectively. The hybridisation method is summarised in Figure 2.5.

**Interpretation of hybridisation signals**

It was assumed that the number of signals per nucleus was identical to the exact copy number of that chromosome. Nuclei with no signals indicate failure of hybridisation due either to truncation of the nuclei or inaccessibility of the DNA. Nuclei with one signal represent monosomy if present in more than 20% of nuclei, (Arnoldus et al, 1991).

**Photography**

For microscopic examination and photography of immunocytochemistry and FISH slides, a Zeiss Axioskop microscope fitted with a MC80 35mm camera was used. Filter set 15 was used to visualise red fluorescence of the counterstain propidium iodide only, filter set 09 was used to visualise red fluorescence plus green fluorescence of FITC. The projective had a magnification of x2.5. Objectives of x10, x20, x40 and x100 oil immersion were used for photography. The film used was Fujicolor 400 iso, and automatic exposure was used for the immunocytochemistry slides and manual exposure of 15 or 30 seconds was used for FISH slides.

**Statistics**

The software package Minitab was used for all statistical calculations.
Figure 2.5 Summary of the Fluorescence In Situ Hybridisation Protocol.

- 5μm Paraffin section
- Dewax in xylene and alcohol
- Proteolytic digestion
- Accessible double stranded DNA target
- Denature target and probe DNA
- Single stranded DNA target
- Hybridise with probe
- Immunochemical detection using fluorochromes
- Photography
RESULTS

Growth of cultures

Twenty-six short term cultures derived from 21 GBM, 4 grade III astrocytomas and one grade III oligoastrocytoma were used. Table 3.1 shows patient information relating to these cultures. One established cell line U251MG derived from a GBM was also used (Bigner et al. 1981a). Growth curves for all 27 cell cultures are shown in Figure 3.1, the day one cell count values represent the number of adherent cells present 48 hours after plating approximately 4688 cells/cm² (1500 cells per well). The day one values differ indicating that the cultures may have different seeding efficiencies (Freshney 1994), the cell line U251MG appears to exhibit the highest seeding efficiency, with IN1752 and IN1728 having comparatively low seeding efficiencies. However, all subsequent experiments were carried out with exponentially growing cells eliminating any effect seeding efficiency may have. There is no apparent correlation between doubling time and seeding efficiency.

Table 3.1 Information on short term cultures.

<table>
<thead>
<tr>
<th>Culture Number</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age at diagnosis</th>
<th>Location</th>
<th>Passage levels used</th>
<th>DT (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN859</td>
<td>GBM</td>
<td>F</td>
<td>72</td>
<td>R frontal</td>
<td>8, 9</td>
<td>18</td>
</tr>
<tr>
<td>IN1265</td>
<td>GBM</td>
<td>F</td>
<td>70</td>
<td>R occipital</td>
<td>6, 7, 8, 12, 13</td>
<td>54</td>
</tr>
<tr>
<td>IN1461</td>
<td>GBM</td>
<td>F</td>
<td>44</td>
<td>L parietal</td>
<td>5, 6, 9, 10, 11</td>
<td>66</td>
</tr>
<tr>
<td>IN1472</td>
<td>GBM</td>
<td>F</td>
<td>46</td>
<td>unknown</td>
<td>6, 7, 8, 13</td>
<td>48</td>
</tr>
<tr>
<td>IN1528</td>
<td>GBM</td>
<td>M</td>
<td>61</td>
<td>R temporo-parietal</td>
<td>7, 8, 10</td>
<td>42</td>
</tr>
<tr>
<td>IN1612</td>
<td>GBM</td>
<td>M</td>
<td>53</td>
<td>R post-temporal</td>
<td>7, 9, 10, 11, 12</td>
<td>30</td>
</tr>
<tr>
<td>IN1682</td>
<td>GBM</td>
<td>F</td>
<td>48</td>
<td>R parietal</td>
<td>5, 6, 9, 10, 11</td>
<td>48</td>
</tr>
<tr>
<td>IN1728</td>
<td>GBM</td>
<td>M</td>
<td>49</td>
<td>R cerebral hemisphere</td>
<td>5, 6, 7, 8</td>
<td>66</td>
</tr>
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<td>8, 9, 10, 11, 12</td>
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<tr>
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<td>GBM</td>
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<td>58</td>
<td>L frontal</td>
<td>5, 6, 7</td>
<td>66</td>
</tr>
<tr>
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<td>42</td>
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<td>90</td>
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<tr>
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<td>Astro GrII</td>
<td>M</td>
<td>30</td>
<td>R parietal</td>
<td>6, 7, 8, 9, 11</td>
<td>60</td>
</tr>
<tr>
<td>IN1979</td>
<td>GBM</td>
<td>M</td>
<td>46</td>
<td>L temporo-parietal</td>
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<td>50</td>
</tr>
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<td>M</td>
<td>64</td>
<td>R temporo-parietal</td>
<td>3, 9</td>
<td>126</td>
</tr>
<tr>
<td>IN2037</td>
<td>GBM</td>
<td>F</td>
<td>58</td>
<td>R frontal</td>
<td>3, 5</td>
<td>62</td>
</tr>
<tr>
<td>IN2045</td>
<td>GBM</td>
<td>M</td>
<td>25</td>
<td>L frontal</td>
<td>4, 6, 8, 9, 11</td>
<td>54</td>
</tr>
<tr>
<td>IN2058</td>
<td>Oligoastro GrII</td>
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<td>49</td>
<td>R temporal</td>
<td>6, 7</td>
<td>66</td>
</tr>
<tr>
<td>IN2093</td>
<td>GBM</td>
<td>M</td>
<td>55</td>
<td>R frontal</td>
<td>5, 6, 9</td>
<td>48</td>
</tr>
<tr>
<td>IN2094</td>
<td>Astro GrII</td>
<td>F</td>
<td>41</td>
<td>L temporo-parietal and fronto midline</td>
<td>3, 4, 5, 6</td>
<td>60</td>
</tr>
<tr>
<td>IN2103</td>
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<td>M</td>
<td>56</td>
<td>R parietal</td>
<td>4, 5</td>
<td>84</td>
</tr>
<tr>
<td>IN2112</td>
<td>GBM</td>
<td>F</td>
<td>61</td>
<td>R fronto-parietal</td>
<td>3, 5, 6, 7</td>
<td>48</td>
</tr>
<tr>
<td>IN2127</td>
<td>Astro GrII</td>
<td>F</td>
<td>46</td>
<td>L parietal</td>
<td>4, 5, 7</td>
<td>60</td>
</tr>
<tr>
<td>IN2132</td>
<td>GBM</td>
<td>M</td>
<td>55</td>
<td>L parietal-occipital</td>
<td>4, 5, 8</td>
<td>84</td>
</tr>
<tr>
<td>IN2134</td>
<td>Astro GrII</td>
<td>M</td>
<td>59</td>
<td>R medial frontal</td>
<td>3, 4</td>
<td>54</td>
</tr>
<tr>
<td>IN2281</td>
<td>GBM</td>
<td>F</td>
<td>69</td>
<td>R temporal</td>
<td>3, 4, 5, 6</td>
<td>60</td>
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</tbody>
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Figure 3.1 Growth curves for all cultures
Figure 3.2 Doubling times for all cultures

Doubling Time (hrs)

Culture Number

P= passage level of cultures used.
The doubling time for each cell culture was calculated and the values are shown in Figure 3.2. The doubling times ranged from 18 hours (IN859) to 126 hours (IN2026), with a mean of 58 hours and median of 60 hours. Interestingly, the cell line U251MG with a doubling time of 24 hours was not the fastest growing culture even though it is an established line with a passage level over 600. The doubling times of the short term cultures were measured between P4 and P9 and within this short range there was no relationship between increasing passage levels and shorter doubling times (Figure 3.2).

**Chemosensitivity**

Twenty-two cultures were assessed for sensitivity to VCR, 25 cultures to DOX and 27 cultures to CCNU. No results were obtained for 5 cultures with VCR and for 2 cultures with DOX this was due to poor growth of the cultures during the assay. Figures 3.3, 3.4 and 3.5 represent all the MTT assay curves using VCR, DOX and CCNU respectively. All but one culture (IN1472) was derived from samples taken before therapy was started. The patient from which IN1472 was derived had received radiotherapy 3.5 years previously. For each drug the individual graphs are shown in ascending order of mean ID$_{50}$ value. Each line represents a single experiment, and duplicate colours indicate duplicate experiments carried out on two different 96 well plates at the same time. It is important to remember when comparing the graphs that different drug concentration ranges were used to achieve both minimum and maximum cell kill in one experiment. For example Figure 3.3. Nos. 5 and 6, IN1265 and IN859 respectively, have similar ID$_{50}$ values but the graphs differ in shape because a much wider drug concentration range was used for IN1265. Occasionally different drug ranges were used for different experiments on the same culture, for example Figure 3.4 Nos. 9 and 20, again to try to improve the assay curves. The graphs in Figures 3.3, 3.4 and 3.5 show there was good reproducibility between experiments on individual cultures, with the exception of one culture IN2112 (Figure 3.5 No. 20) representing only 4 out of a total of 356 individual assays. As there is no biological explanation for the differences in the graphs for this culture, it must be due to experimental error, using the mean CCNU ID$_{50}$ for this culture in the results and discussion did not affect the overall conclusions.

Figure 3.3 shows all the MTT assay curves using VCR. The classic dose response curve, where both minimum and maximum cell kill are achieved within a relatively narrow concentration range, can be seen with IN859 (Figure 3.3 No. 6), there is a very steep gradient around the 50% cell kill level and the ID$_{50}$ value can easily be interpolated from the graph. This part of the curve usually shows the least
deviation between experiments. A flatter slope usually indicates that a wider drug concentration range has been used, compare, for example, the different ranges used with IN2094 (Figure 3.3 No. 12). IN1461 (Figure 3.3 No. 18) is an example of a resistant culture, with a relatively high ID₅₀ value, very high concentrations of VCR are needed to produce cell kill compared to the more sensitive culture IN859 (Figure 3.3 No. 6). Even at the highest concentration of VCR (100µg/ml) about 25% of the cells survived. Similarly, IN1265 (Figure 3.3 No. 5) is an example of a culture relatively sensitive to VCR with a low ID₅₀ value, but again at high drug concentrations more than 25% of cells survived. Explanations for those cases where cell survival did not approach 0% even at high drug concentrations and maximum cell survival did not approach 100% can be found in the following chapter.

Figure 3.4 shows all the MTT assay curves using DOX. Again the classic dose response curve can be seen with IN859 (Figure 3.4 No.12) and also IN1612 (Figure 3.4 No. 8) and IN2045 (Figure 3.4 No. 4). IN1265 (Figure 3.4 No. 1) and IN1472 (Figure 3.4 No. 2) are very sensitive to DOX when the ID₅₀s are compared to the other cultures. IN1752 (Figure 3.4 No. 22) is one of the more resistant cultures, the graph shows how at even the highest concentration of DOX (>1µg/ml) 25% of the cells can apparently survive. The MTT assay curves for all cell lines using CCNU can be seen in Figure 3.5. The majority of the cultures produced classic dose response MTT assay curves.

The ID₅₀ ranges for each drug are shown in Table 3.2. There are vast differences between the most sensitive and the most resistant cultures with all three drugs, with VCR there is a 5x10⁵ fold difference, with DOX a 6x10⁴ fold difference and with CCNU a 14 fold difference. However, by comparing the mean and median values, the data for VCR and DOX do not appear to be normally distributed and the significance of this will be addressed later. The ID₅₀ values for each drug are shown in ascending order in Figure 3.6. One culture (IN1472) is obviously much more sensitive than the others to VCR. There are 15 cultures, IN1612, IN1528, IN1979, IN1265, IN859, U251MG, IN2045, IN1752, IN2112, IN1902, IN2094, IN2127, IN2093, IN2103, and IN1682, in the intermediate range and 6 cultures, IN2134, IN1461, IN1760, IN1728, IN2281 and IN1951 that are very resistant to VCR. With DOX there are two very sensitive cultures, IN1265 and IN1472 with ID₅₀ values of 0.0064ng/ml and 0.0088ng/ml respectively. The remaining cultures are comparatively resistant with an ID₅₀ range of 2.8-361ng/ml, there are no obvious divisions in this group. The final graph shows the ID₅₀s for CCNU and here again there are no obvious divisions between resistant and sensitive cultures.
Table 3.2 ID$_{50}$ ranges for all cell cultures.

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID$_{50}$ range</th>
<th>Mean</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine</td>
<td>4x10^{-2} to 2.152x10^{4} ng/ml</td>
<td>3.294x10^{3} ng/ml</td>
<td>4.57 ng/ml</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>6.4x10^{-3} to 3.61x10^{2} ng/ml</td>
<td>50.28 ng/ml</td>
<td>16.4 ng/ml</td>
</tr>
<tr>
<td>CCNU</td>
<td>1.65 to 23.06 μg/ml</td>
<td>7.8 μg/ml</td>
<td>5.62 μg/ml</td>
</tr>
</tbody>
</table>
Figure 3.3  MTT assay curves for all cell cultures using VCR.

1. IN1472

2. IN1612

3. IN1528

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.3  MTT assay curves for all cell cultures using VCR.

4. IN1979

5. IN1265

6. IN859

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.3 MTT assay curves for all cell cultures using VCR.

7. U251MG

8. IN2045

9. IN1752

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.3 MTT assay curves for all cell cultures using VCR.

10. IN2112

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.3  MTT assay curves for all cell cultures using VCR.

13. IN2127

14. IN2093

15. IN2103

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.3 MTT assay curves for all cell cultures using VCR.

16. IN1682

17. IN2134

18. IN1461

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.3  MTT assay curves for all cell cultures using VCR.

19. IN1760

20. IN1728

21. IN2281

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.3 MTT assay curves for all cell cultures using VCR.

22. IN1951

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.4  MTT assay curves for all cell cultures using DOX.

1. IN1265

2. IN1472

3. IN1902

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
4. IN2045

5. IN2112

6. U251MG

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.4 MTT assay curves for all cell cultures using DOX.

7. IN2093

8. IN1612

9. IN2127

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.4 MTT assay curves for all cell cultures using DOX.

10. IN1979

11. IN2132

12. IN859

(Each line represents one experiment. Duplicate colours = duplicate experiments. 
P = passage levels of cultures used).
Figure 3.4  MTT assay curves for all cell cultures using DOX.

13. IN2058

14. IN2134

15. IN2037

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.4 MTT assay curves for all cell cultures using DOX.

16. IN1528

17. IN2094

18. IN1682

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.4 MTT assay curves for all cell cultures using DOX.

19. IN2281

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.4  MTT assay curves for all cell cultures using DOX.

22. IN1752

23. IN1760

24. IN1728

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.4 MTT assay curves for all cell cultures using DOX.

25. IN1461

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.5  MTT assay curves for all cell cultures using CCNU.

1. IN859

2. IN1472

3. IN1612

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.5 MTT assay curves for all cell cultures using CCNU.

4. IN1940

5. IN1528

6. IN1752

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.5 MTT assay curves for all cell cultures using CCNU.

7. IN1902

8. IN2103

9. IN2094

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.5 MTT assay curves for all cell cultures using CCNU.

10. IN1951

11. IN1979

12. IN2134

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.5  MTT assay curves for all cell cultures using CCNU.

13. U251 MG

14. IN2045

15. IN2093

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.5  MTT assay curves for all cell cultures using CCNU.

16. IN2127

17. IN2058

18. IN2281

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used).
Figure 3.5 MTT assay curves for all cell cultures using CCNU.

19. IN1682

20. IN2112

21. IN2037

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.5 MTT assay curves for all cell cultures using CCNU.

22. IN2132

23. IN1461

24. IN2026

(Each line represents one experiment. Duplicate colours = duplicate experiments. 
P = passage levels of cultures used.)
Figure 3.5 MTT assay curves for all cell cultures using CCNU.

25. IN1760

26. IN1265

27. IN1728

(Each line represents one experiment. Duplicate colours = duplicate experiments. P = passage levels of cultures used.)
Figure 3.6 ID$_{50}$ values ±SE for all cell cultures with VCR, DOX and CCNU.

Values are shown in ascending order and a log scale is used for VCR and DOX.
The ranges of ID\textsubscript{50} values for all cultures and drugs are shown in Figure 3.7. The mean and median values as well as the clinically achievable dose for VCR and CCNU are shown. The VCR ID\textsubscript{50} values appear to fall into two groups, one resistant group (ID\textsubscript{50} > 100ng/ml) and the other sensitive (ID\textsubscript{50} < 10ng/ml), and this apparent dual population may explain the variance between the mean and median. There is one culture which is extremely sensitive to VCR and two cultures which are extremely sensitive to DOX. No divisions are apparent in the cultures when tested with CCNU. Some points on this graph are overlapping or very close together and therefore difficult to resolve, so Figure 3.8 shows the same values as cumulative proportions, where each ID\textsubscript{50} value can clearly be seen. With this cumulative graph the proportion of cultures exhibiting a given ID\textsubscript{50} range can be found, for example approximately 75% of cultures had ID\textsubscript{50} values below about 10ng/ml.

**Statistical evidence for the distribution of ID\textsubscript{50} and doubling time data.**

In order to apply any statistical tests to the doubling time and chemosensitivity data, assumptions made about the distribution of the data must be explained. Most statistical tests assume that the data is normally distributed. Tests for normality on samples where n<30 are not usually conducted as it is almost impossible to show that an individual measurement is not from a normal distribution. However, there is good reason to believe that the VCR ID\textsubscript{50} data does not come from a single normal population, it is likely that two populations are present within this data, one population is relatively resistant to VCR and includes the six highest ID\textsubscript{50} values and one is relatively sensitive to VCR and includes the remaining 16 cultures. To show if there is evidence for different populations within one set of data Parzen’s windows can be applied (Duda and Hart 1973). This method estimates the probability distribution of the data and shows the data as a continuous histogram, i.e. the area under the curve = 1. Figure 3.9 shows VCR ID\textsubscript{50} data using Parzen’s windows, the sensitive cultures in the large peak to the left and the resistant cultures in the smaller peak to the right. Both populations appear to be normally distributed, good evidence for a bimodal distribution. DOX ID\textsubscript{50} data may also be bimodal, although the two most sensitive cultures may simply be outliers (Figure 3.7) and this appears to be the case as no evidence of separate peaks can be seen using Parzen’s windows (Figure 3.10).

There is no reason to believe that the CCNU ID\textsubscript{50} data is not normally distributed (Figure 3.7) and this is shown in the simple barchart in Figure 3.11. Similarly the doubling times come from an apparently normal distribution (Figure 3.12).
Figure 3.7  Ranges of the ID$_{50}$ values for all cultures with all three drugs.

Legend:
- \( \text{DOXVCR} \) = mean
- \( \text{CCNU} \) = median
- Arrow = clinically achievable plasma levels (Stewart et al 1983, Hori et al 1987)
Figure 3.8 Cumulative proportions of ID$_{50}$ values at or below a particular drug dose for VCR, DOX and CCNU.
Figure 3.9 An estimate of the probability distribution of the VCR ID$_{50}$ values using a non-parametric method, Parzen's windows.

*probability = probability of ID$_{50}$ value falling within a given range

Figure 3.10 An estimate of the probability distribution of DOX ID$_{50}$ values using a non-parametric method, Parzen's windows.

*probability = probability of ID$_{50}$ value falling within a given range
Figure 3.11  Bargraph showing the normal distribution of the CCNU ID\textsubscript{50} data.

Intervals are 1 µg/ml from the number in the centre of the bar. For example 1 represents the interval 1 to 2 µg/ml, 2 represents >2 to 3 µg/ml.

Figure 3.12  Bargraph showing the normal distribution of the Doubling Time data.

Intervals are 10 hours from the number in the centre of the bar. For example 10 represents the interval 10 to 20 hours, 20 represents >20 to 30 hours.
To ensure that the MTT assay was not affected by the doubling times of the cultures, the ID$_{50}$ values for all drugs and cultures along with doubling times were compared in Figure 3.13. The two populations of ID$_{50}$ values, sensitive to VCR and resistant to VCR are shown on separate graphs. For a cell cycle specific drug like VCR it is possible that the fastest growing cultures may be more sensitive than the slower growing ones. To show any real correlation Pearson's correlation coefficient, $r$, and measurement of variance, $r^2$, were calculated. To test whether the correlation is significant, $p$ values were calculated from an F-test and are shown in Table 3.3.

Table 3.3 Correlation between ID$_{50}$ values and doubling time.

<table>
<thead>
<tr>
<th>VCR ID$_{50}$ sensitive</th>
<th>VCR ID$_{50}$ resistant</th>
<th>DOX ID$_{50}$</th>
<th>CCNU ID$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling Time</td>
<td>p = 0.038</td>
<td>p = 0.56</td>
<td>p = 0.27</td>
</tr>
<tr>
<td>S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

S = significant, NS = not significant

Thus, only for the VCR sensitive cultures was there statistically significant evidence of any positive correlation between ID$_{50}$ values and doubling times. However this may be due to a single outlier with the highest doubling time (Figure 3.13).

Patterns of resistance
To determine if cultures derived from malignant astrocytomas display the classic MDR cross resistant pattern including resistance to both vinca alkaloids and anthracyclines, the ID$_{50}$ values for VCR and DOX were compared (Figure 3.14). The ID$_{50}$ values for VCR are arranged in ascending order along with the corresponding DOX ID$_{50}$ values. Of the six cultures that are most resistant to VCR, IN2134, IN1461, IN1760, IN1728, IN2281 and IN1951, three are also most resistant to DOX, namely IN1461, IN1760 and IN1728. Figure 3.15 shows this relationship more clearly. The data naturally falls into 4 groups or resistance patterns, these are circled and labelled A-D. Group A contains the 6 cultures most resistant to VCR, and group B contains cultures with intermediate resistance to VCR, however, both Groups A and B have similar levels of resistance to DOX. This suggests that different mechanisms of resistance may operate in these two groups of cultures. Both groups A and B could be described as showing classical MDR, however an additional resistance mechanism resulting in marked resistance to VCR may be implicated in Group A cultures. For the remaining two cultures, the relationships between VCR and DOX ID$_{50}$ values are sufficiently dissimilar as to warrant inclusion in two separate groups. Group C contains a culture markedly sensitive DOX but with intermediate resistance to VCR, as in Group B. Group D contains the only culture markedly sensitive to both drugs.
Figure 3.13 Relationship between ID50 values and doubling times for all drugs and cultures.

- VCR ID$_{50}$ (ng/ml)
  - Sensitive: $r^2 = 0.27$
  - Resistant: $r^2 = 0.09$

- ADR ID$_{50}$ (ng/ml)
  - $r^2 = 0.05$

- CCNU ID$_{50}$ (μg/ml)
  - $r^2 = 0.12$

$r^2 = \text{correlation coefficient}$
Figure 3.14 Comparison of VCR and DOX ID$_{50}$ values. Shown in ascending order of VCR ID$_{50}$ value.
Figure 3.15 Relationship between VCR and DOX ID\textsubscript{50} values.

Four groups A-D with different resistance patterns are shown.
As CCNU is not implicated in classical MDR and no single mechanism could explain resistance to both CCNU and VCR, or to CCNU and DOX, there is no reason to expect a relationship between ID$_{50}$ values for CCNU and VCR (Figure 3.16), and for CCNU and DOX (Figure 3.17). In Figure 3.16 the data are scattered and although some cultures that are highly resistant to VCR tend to be fairly resistant to CCNU a mechanistic link between these two parameters is unlikely. It is more likely to be a reflection of the multiple drug resistant mechanisms which operate in malignant glioma cultures. Figure 3.17 appears to confirm this, as there is no general relationship between DOX and CCNU sensitivity. The solid arrow points to culture IN1265 which is very sensitive to DOX but relatively resistant to CCNU. The open arrow indicates culture IN1472 which is sensitive to both drugs.

The ID$_{50}$ values are useful for comparing the relative resistances of the cultures, but the shape of the MTT curves can also be informative. Figures 3.18, 3.19 and 3.20 show the meaned MTT assay dose response curves for each culture using VCR, DOX and CCNU respectively. Some of the cultures were assayed using different drug concentration ranges so the curves are not as smooth as in the individual experiments in Figures 3.3, 3.4 and 3.5. For each culture the mean values for all data points were calculated. In Figure 3.18, the six most VCR resistant cultures, as defined by their ID$_{50}$ values, have a distinctive shape compared to the others. The curves are much flatter and barely touch either 80% or 20% cell survival. This may be significant in describing the type of culture. These six cultures also appear to be less likely to have all the cells killed by the highest drug concentration used, 100μg/ml, or for most of the cells to survive at the lowest concentrations.

In Figure 3.19, the three most DOX resistant cultures, IN1461, IN1760 and IN1728, as defined by their ID$_{50}$ values, have very steep gradients and fairly classic dose response curves, in contrast to those cultures most resistant to VCR. The mean MTT assay curves with CCNU (Figure 3.20.) are all of similar shape, there are no cultures which obviously deviate from the norm.
Figure 3.16 Relationship between VCR and CCNU ID$_{50}$ values.
The black arrow points to IN1265 and the white arrow points to IN1472, these are the two cultures markedly sensitive to DOX.
Figure 3.18 Mean MTT assay curves for each culture with VCR
Figure 3.19 Mean MTT assay curves for all cultures with DOX.
Figure 3.20 Mean MTT assay curves for all cultures with CCNU
P-glycoprotein expression

PGP expression was assessed in all cultures with the exception of IN1940, this was due to poor growth of this culture. The staining patterns with all three antibodies are shown in Table 3.4. Different staining patterns were seen with all three antibodies with little agreement between them. Positive staining appeared to suggest the presence of PGP in the cytoplasm of several of the cultures, however, no cell membrane staining, which would be expected from a membrane bound protein, was seen with any of the antibodies.

Table 3.4 Staining patterns with all three anti-P-glycoprotein antibodies.

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>-ve control (no 1° Ab.)</th>
<th>JSB-1</th>
<th>C219</th>
<th>MRK16</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN859</td>
<td>-ve to c+</td>
<td>c+ pp+++</td>
<td>c+</td>
<td>nd</td>
</tr>
<tr>
<td>IN1265</td>
<td>-ve</td>
<td>c+ occ. to pp+++</td>
<td>c+</td>
<td>nd</td>
</tr>
<tr>
<td>IN1461</td>
<td>c+ to pp+</td>
<td>c+ pp+++</td>
<td>pc+ to c++</td>
<td>c++ to pc+++</td>
</tr>
<tr>
<td>IN1472</td>
<td>-ve</td>
<td>c+ occ. pp+++</td>
<td>c+</td>
<td>c+</td>
</tr>
<tr>
<td>IN1528</td>
<td>c+</td>
<td>c+</td>
<td>c+</td>
<td>nd</td>
</tr>
<tr>
<td>IN1612</td>
<td>-ve</td>
<td>c+</td>
<td>c+ occ. pp+++</td>
<td>nd</td>
</tr>
<tr>
<td>IN1682</td>
<td>c+</td>
<td>c+ occ. to all pp+++</td>
<td>c+ pc+ pp+++</td>
<td>c+ occ. pp++</td>
</tr>
<tr>
<td>IN1728</td>
<td>c+</td>
<td>c+ pp+++</td>
<td>nd</td>
<td>c+</td>
</tr>
<tr>
<td>IN1752</td>
<td>-ve</td>
<td>c+ pc++ pp+++</td>
<td>c+</td>
<td>c+++ occ. pc+++</td>
</tr>
<tr>
<td>IN1760</td>
<td>c+</td>
<td>c+ pp++ pc++</td>
<td>c+</td>
<td>nd</td>
</tr>
<tr>
<td>IN1902</td>
<td>-ve</td>
<td>c+ 50% pp+++</td>
<td>c+</td>
<td>c+</td>
</tr>
<tr>
<td>IN1940</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>IN1951*</td>
<td>c+</td>
<td>c+ pc+ occ. to pp++</td>
<td>-ve to c+</td>
<td>c+ to pp+++</td>
</tr>
<tr>
<td>IN1979</td>
<td>c+ occ. pc+</td>
<td>c+ occ. pc+</td>
<td>c+ occ. pc+</td>
<td>c+++ occ. pp+++</td>
</tr>
<tr>
<td>IN2026</td>
<td>c+</td>
<td>c+ pp+++</td>
<td>c++</td>
<td>c+++</td>
</tr>
<tr>
<td>IN2037</td>
<td>c+</td>
<td>c+ pp+++</td>
<td>pc++</td>
<td>c+++</td>
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<tr>
<td>IN2045</td>
<td>occ. pc+</td>
<td>c+ occ. pc+</td>
<td>c+ occ. pc+</td>
<td>c+ occ. pc+</td>
</tr>
<tr>
<td>IN2058+</td>
<td>c+</td>
<td>c+ pp+++</td>
<td>c+ to pc++</td>
<td>c+</td>
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<tr>
<td>IN2093</td>
<td>c+</td>
<td>pc+ occ. pp+++</td>
<td>-ve to pc++</td>
<td>pc+</td>
</tr>
<tr>
<td>IN2094*</td>
<td>-ve to c+</td>
<td>c+ occ. to pp+++</td>
<td>c+</td>
<td>c+</td>
</tr>
<tr>
<td>IN2103</td>
<td>-ve to c+</td>
<td>c+ occ. pp+++</td>
<td>c+ to pc++</td>
<td>c+</td>
</tr>
<tr>
<td>IN2112</td>
<td>-ve to c+</td>
<td>c+ pp+++</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>IN2114*</td>
<td>c+</td>
<td>c+ occ. pc+++</td>
<td>pc+</td>
<td>c++</td>
</tr>
<tr>
<td>IN2132</td>
<td>c+</td>
<td>c+ occ pp+++</td>
<td>c+</td>
<td>nd</td>
</tr>
<tr>
<td>IN2134*</td>
<td>occ. pc+</td>
<td>c+</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>IN2281</td>
<td>c+</td>
<td>c+ pp++</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>U251MG</td>
<td>occ. pc+</td>
<td>nd</td>
<td>c+ p++</td>
<td>c+ occ. pc+++</td>
</tr>
<tr>
<td>SuSa</td>
<td>-ve</td>
<td>c+ occ pc++pp++</td>
<td>-ve to c+ to pc+</td>
<td>c+++ to pc++</td>
</tr>
</tbody>
</table>

+= weak positive, ++ = intermediate positive, +++ = strong positive, c = homogeneous cytoplasmic staining, pc = punctate cytoplasmic staining, pp = punctate perinuclear staining, occ. = occasional cells. Where no indication of the proportion of cells is given, assume most cells exhibit that staining pattern. All cultures derived from Grade IV Astrocytoma except * = Grade III, † = oligoastrocytoma Grade III, SuSa = PGP negative teratoma cell line. nd = no data. Examples of staining patterns: c+ pc++ pp+++ Plate 3.1, c+ occ. to pp+++ Plate 3.2, -ve to pc+ Plate 3.3, c+ Plate 3.4, c++ occ. pp+++ Plate 3.5, c++ to pc+++ Plate 3.6.
All PGP and MRP immunocytochemistry was carried out on sub-confluent cultures during exponential growth. There was no apparent difference in the patterns of staining between Grade III and Grade IV astrocytomas nor with the oligoastrocytoma.

JSB-1 generally gave strong punctate staining in the cytoplasm and especially in the perinuclear region. This pattern was seen in 20 of the 25 astrocytoma cultures tested with JSB-1. Plates 3.1 and 3.2 show this characteristic staining. Plate 3.1 shows IN1752 with bright punctate staining in the majority of cells, no membrane staining was visible. Most positive cells tended to be the larger cells with the most cytoplasm. Plate 3.2 shows IN1265 with a minority of cells with bright cytoplasmic staining. Neither of these cell lines was especially resistant to VCR, IN1265 was also exceptionally sensitive to DOX therefore this cell culture would not be expected to exhibit classical MDR with concomitant expression of PGP. However, IN1725 was resistant to DOX, but if PGP were present the culture should also be comparatively resistant to VCR, and this was not the case.

With the C219 antibody the staining pattern was different, bright punctate staining was less common and the majority of cultures were negative or showed only faint homogeneous cytoplasmic staining or faint punctate staining. This pattern was seen in 14 of 22 astrocytoma cultures tested. Plates 3.3 and 3.4 show that there was very little staining compared to JSB-1. Plate 3.3 shows IN2093 was clearly negative with C219, likewise Plate 3.4 shows the majority of IN1752 cells have only faint background staining. Two of the cells appear to have brighter staining, but again there was no characteristic membrane localisation.

Finally, MRK-16 was used, this antibody gave the most bright homogeneous staining between both individual cells and between cultures, with only occasional punctate staining. This pattern was seen in 6 of 17 astrocytoma cultures tested, faint homogeneous cytoplasmic staining alone was seen in 5 of 17 cultures. Plate 3.5 shows the culture IN1979, sensitive to both VCR and DOX, homogeneous staining in the cytoplasm of all cells can be seen. The resistant culture IN1461 is shown in Plate 3.6 again homogeneous bright staining was seen throughout the cytoplasm. The conclusion is that this must represent non-specific binding as clearly both resistant and sensitive cultures have stained, and neither have characteristic membrane staining. Although automatic photographic exposure was used, artefactual brightness suggesting positive staining was not significant as backgrounds to all photos appear black.
To confirm that PGP was not being expressed in the cultures, three negative controls were employed, firstly omission of the primary antibody, secondly inclusion of a teratoma cell line (SuSa) in the panel of cultures which was known not to express PGP (Hosking et al 1994), and finally an irrelevant mouse IgG1 antibody recognising Guinea pig lymphocytes (clone CT6). Omission of the primary antibody generally gave negative or only faint homogeneous staining, but in 5 of 27 cases punctate staining was found, in 4 out of 5 of these cases punctate staining was also found with one or more of the anti-PGP antibodies. The teratoma cell line stained with all three antibodies with similar staining patterns to the astrocytoma cell cultures, this indicates that the antibodies were either non-specific or recognised an epitope distinct from PGP. The negative control CT6 clone was only available for use on 5 astrocytoma cultures, IN1951, IN1728, IN2134, IN2281 and IN1760, the first four of these showed faint homogeneous and/or intermediate punctate cytoplasmic staining, IN1760 also showed intermediate punctate peri-nuclear staining. Plates 3.7 and 3.8 show the culture IN1760 with JSB-1 and CT6. Both show similar staining patterns implying that the staining must have been non-specific and the conclusion was that PGP was not expressed in any of the cultures. To compare the negative control IN1760 with no 1° antibody see Plate 3.10.

MRP expression

MRP expression was assessed in the 6 cultures which were most resistant to VCR, IN2134, IN1461, IN1760, IN1728, IN2281 and IN1951. None were found to express this protein. Plate 3.9 shows IN1760 with MRP6 with faint background staining and this is comparable to the negative control with no primary antibody in Plate 3.10.
Plate 3.1 IN1752 P7 with JSB-1 (original magnification x50)

Plate 3.2 IN1265 P6 with JSB-1 (original magnification x50)
Plate 3.3 IN2093 P6 with C219 (original magnification x100)

Plate 3.4 IN1752 P7 with C219 (original magnification x50)
Plate 3.5 IN1979 P8 with MRK-16 (original magnification x50)

Plate 3.6 IN1461 P9 with MRK-16 (original magnification x50)
(PI filtered out)
Plate 3.7 IN1760 P8 with JSB-1 (original magnification x50)

Plate 3.8 IN1760 P8 with CT6 negative control (original magnification x50)
Plate 3.9  IN1760 P8 with MRP6 (original magnification x50)

Plate 3.10  IN1760 P8 with no 1° antibody. (original magnification x50)
**p53 expression**

Immunocytochemistry was carried out on twenty-one cultures using at least one anti-p53 antibody. All p53 immunocytochemistry was carried out on sub-confluent cultures during exponential growth. To quantify the staining only nuclear staining was scored because the nucleus is the normal site of action of p53. Table 3.5 shows the scores for nuclear staining with all three antibodies.

**Table 3.5** p53 nuclear staining with all three antibodies.

<table>
<thead>
<tr>
<th>Culture number</th>
<th>DO-1 wild type and mutant</th>
<th>PAb 421 wild type and mutant</th>
<th>PAb 240 mutant only</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN 859</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>nd</td>
</tr>
<tr>
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</tr>
<tr>
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<td>3</td>
<td>3</td>
</tr>
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<td>2</td>
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<td>IN 2093</td>
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<td>1</td>
</tr>
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<td>IN 2127*</td>
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<td>1</td>
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<td>1</td>
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<td>1</td>
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<tr>
<td>U251MG</td>
<td>3</td>
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</tr>
</tbody>
</table>

0 = no positive nuclei, 1 = single nuclei-5% of nuclei, 2 = 6-50% nuclei, 3 = 51-100% nuclei, nd = no data. All cultures derived from Grade IV Astrocytoma except * = Grade III, † = oligoastrocytoma Grade III. Examples of staining scores: 3=Plate 3.11, 2=Plate 3.15, 1=Plate 3.19.

Each antibody showed the full range of scoring possibilities from single positive nuclei up to 100% of all nuclei. All three antibodies detected p53 protein in nucleus and cytoplasm. Of the 16 culture which were tested with all three antibodies, only 5, IN1760, IN1902, IN2093, IN2127 and U251MG, had full agreement between the scores. Scores of 2 or 3 (>50% of nuclei positive) were deemed to indicate overexpression of p53 protein, and from this it can be shown that 6 of 21 cultures tested with DO-1, 8 of 18 cultures tested with PAb421 and 10 of 17 cultures tested with PAb240 overexpressed p53 protein.
There was no clear difference between grade III and grade IV astrocytomas nor with the oligoastrocytoma in terms of staining patterns, although all the cultures derived from grade III astrocytomas showed staining in less than 5% of the cells (Figure 3.2), and the single culture derived from the oligoastrocytoma had nuclear staining in less than 5% of the cells with 2 of the 3 antibodies and between 6 and 50% of nuclei with the third.

Different staining patterns with the three antibodies can be seen in Plates 3.11 to 3.22. Plates 3.11, 3.12 and 3.13 show U251MG using PAb240, PAb421 and DO-1 respectively. The nuclear staining is strong and homogeneous in all three cases, with a corresponding p53 score =3. Although the nuclear staining was similar the cytoplasmic staining differed, with PAb240 there is little or no cytoplasmic staining (Plate 3.11), with PAb421 faint punctate, almost fibrous cytoplasmic staining can be seen (Plate 3.12), and with DO-1 bright homogeneous cytoplasmic staining (Plate 3.13). These patterns were commonly seen but were not absolute for each antibody. Plates 3.14, 3.15 and 3.16 show culture IN1612 with the three antibodies. Plate 3.14 shows the characteristic punctate nuclear staining often found when using PAb240. This may indicate lower levels of p53 than in Plate 3.11 for example, where the nuclear staining is very bright and homogeneous, however, most nuclei are obviously positive and a score of 3 was assigned. With PAb421 (Plate 3.15) nuclei were occasionally positive along with punctate or fibrous perinuclear and cytoplasmic staining. DO-1 (Plate 3.16) gave a similar staining pattern to PAb421. Both Plate 3.15 and Plate 3.16 scored 2 for nuclear staining.

Plates 3.17, 3.18 and 3.19 show the different staining patterns achieved with a single antibody, PAb421. In Plate 3.17, IN2045 scored 3 and nuclear staining is clearly seen, with only occasional cytoplasmic staining visible. In contrast, Plate 3.18 (IN1951) scored 1 with only occasional nuclear staining, although bright fibrous cytoplasmic staining is apparent. Plate 3.19 (IN1760) also scored 1 for nuclear staining, but here the cytoplasmic staining is less bright although clearly punctate and fibrous.

Plates 3.20 show IN1760 with DO-1 scoring only 1 for nuclear staining although strong cytoplasmic staining can be seen. Plate 3.21 shows IN1528 with PAb240, this culture scored 3 although the nuclear staining is heterogeneous with some nuclei having punctate staining and some with more homogeneous fluorescence. Finally, IN2094, Plate 3.22, shows the strong cytoplasmic staining often found with DO-1 with only occasional nuclear positivity. This culture scored 1 for nuclear staining.
Plate 3.11  U251MG with anti-p53 PAb240  
(original magnification x50)

Plate 3.12  U251MG with anti-p53 PAb421  
(original magnification x50)
Plate 3.13. U251MG with anti-p53 DO-1
(original magnification x50)

Plate 3.14. IN1612 P7 with anti-p53 PAb240
(original magnification x50)
Plate 3.15 IN1612 P7 with anti-p53 PAb421
(original magnification x50)

Plate 3.16 IN1612 P7 with anti-p53 DO-1
(original magnification x50)
Plate 3.17 IN2045 P11 with anti-p53 PAb421
(original magnification x50)

Plate 3.18 IN1951 P9 with anti-p53 PAb421
(original magnification x50)
Plate 3.19 IN1760 P11 with anti-p53 PAb421
(original magnification x50)

Plate 3.20 IN1760 P11 with anti-p53 DO-1
(original magnification x50)
Plate 3.21  IN1528 P8 with anti-p53 PAb240  
(original magnification x50)

Plate 3.22  IN2094 P5 with anti-p53 DO-1  
(original magnification x50)
Relationship between p53 expression and chemosensitivity

Overexpression of p53 has been shown in these astrocytoma cultures but does this have any relationship to their chemosensitivities? The p53 immunocytochemistry scores and the corresponding ID$_{50}$ values for VCR, DOX and CCNU are compared in Tables 3.6, 3.7 and 3.8 respectively.

Sensitivity to VCR appears to correlate with overexpression of p53 (Table 3.6). From Table 3.7 there appears to be less relationship between DOX sensitivity and p53 overexpression, and likewise with CCNU in Table 3.8. It is easier to visualise these results in graph form (Figures 3.21, 3.22 and 3.23). In Figure 3.21 overexpression of p53 tends to be found in VCR sensitive cultures, while less than 6% of the nuclei of resistant cultures are p53 positive. The patterns in Figures 3.22 and 3.23 are more difficult to interpret although PAb240 does not appear to indicate any relationship between chemosensitivity and p53 overexpression.

The Mann-Whitney test was used to find if the ID$_{50}$ values of p53 positive and negative cultures were significantly different. This test is a non-parametric, two sample rank test for the difference between the two population medians. This test was used because the data may not be normally distributed and the values for p53 staining are only ranked scores and are not truly numerical, therefore a simple parametric test between two means would not be applicable (Siegel 1956). The two samples, ID$_{50}$ values for p53 positive cultures and ID$_{50}$ values for p53 negative cultures, were ranked together, the smallest value ranking 1. Then if the sum of the ranks of the p53 positive sample was less than the sum of the ranks of the p53 negative sample this indicated that the ID$_{50}$ values of p53 positive cultures were statistically less than the ID$_{50}$ values of p53 negative cultures, i.e. overexpression of p53 is found in drug sensitive cultures. The results from the Mann-Whitney test are shown in Table 3.9. The VCR ID$_{50}$ values for DO-1 positive and negative cultures were significantly different as were those for DOX and PAb421, and DOX and PAb421. When the aberrant CCNU ID$_{50}$ of culture IN1265 was omitted from the calculations the results for CCNU and DO-1, and CCNU and PAb421 were also significant. No statistically significant differences were found when PAb240 was used.
<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>DO1 score (wt/m)</th>
<th>421 score (wt/m)</th>
<th>240 score (m)</th>
<th>VCR ID50 ng/ml</th>
</tr>
</thead>
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<td>2</td>
<td>1</td>
<td>0.04</td>
</tr>
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</tr>
<tr>
<td>IN 1528</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0.998</td>
</tr>
<tr>
<td>IN 1979</td>
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<td>3</td>
<td>1.84</td>
</tr>
<tr>
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<td>2.57</td>
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<td>2.66</td>
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<td>3.91</td>
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<td>1</td>
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<td>4.28</td>
</tr>
<tr>
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<td>1</td>
<td>nd</td>
<td>4.85</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>4.9</td>
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<td>8.15</td>
</tr>
<tr>
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<td>1</td>
<td>2</td>
<td>8.83</td>
</tr>
<tr>
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<tr>
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<td>nd</td>
<td>15293.0</td>
</tr>
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<td>nd</td>
<td>21520.0</td>
</tr>
<tr>
<td>IN 2058</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>nd</td>
</tr>
<tr>
<td>IN 2132</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>nd</td>
</tr>
</tbody>
</table>

Table 3.6 VCR ID50s ranked in order of sensitivity with corresponding p53 scores. (wt= wild type, m= mutant)
Figure 3.21 p53 status and VCR sensitivity
Figure 3.22 p53 status and DOX sensitivity
Figure 3.23 p53 status and CCNU sensitivity
Table 3.9 Mann-Whitney test for significant differences between the ID₅₀ values of p53 positive and p53 negative cultures for all drug and antibody combinations.

<table>
<thead>
<tr>
<th></th>
<th>DO-1</th>
<th>PAb421</th>
<th>PAb240</th>
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<tr>
<td>CCNU</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(*p=0.0068)</td>
<td>(*p=0.0281)</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>NS</td>
<td>SS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.0368</td>
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</tr>
<tr>
<td>VCR</td>
<td>SS</td>
<td>SS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>p=0.0097</td>
<td>p=0.0009</td>
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</tr>
</tbody>
</table>

(NS = Not Significant, SS = Statistically Significant, *=p values when culture number IN1265 is omitted from calculations)

Cytogenetic studies on recurrent GBMs
Thirteen pairs of paraffin embedded, primary and corresponding recurrent GBMs were used. Information relating to these samples is shown in Table 3.10.

Table 3.10 Information on paraffin embedded samples.

<table>
<thead>
<tr>
<th>Paraffin block number</th>
<th>sex</th>
<th>age</th>
<th>diagnosis</th>
<th>time from 1st operation to 2nd operation (months)</th>
<th>1st operation to death (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°525/88 Rec. 169/89</td>
<td>F</td>
<td>44</td>
<td>GBM GBM</td>
<td>12m</td>
<td>18m</td>
</tr>
<tr>
<td>1°259/82 Rec. 34/84</td>
<td>M</td>
<td>41</td>
<td>GBM GBM</td>
<td>12m</td>
<td>19m</td>
</tr>
<tr>
<td>1°534/88 Rec. 87/90</td>
<td>F</td>
<td>39</td>
<td>GBM GBM</td>
<td>20m</td>
<td>41m</td>
</tr>
<tr>
<td>1°160/91 Rec. 176/93</td>
<td>M</td>
<td>31</td>
<td>GBM GBM</td>
<td>24m</td>
<td>31m</td>
</tr>
<tr>
<td>1°33/90 Rec. 539/90</td>
<td>M</td>
<td>51</td>
<td>GBM GBM</td>
<td>7m</td>
<td>11m</td>
</tr>
<tr>
<td>1°794/89 Rec. 28/91</td>
<td>M</td>
<td>48</td>
<td>GBM GBM</td>
<td>12m</td>
<td>18m</td>
</tr>
<tr>
<td>1°291/87 Rec. 306/88</td>
<td>F</td>
<td>54</td>
<td>GBM GBM</td>
<td>13m</td>
<td>19m</td>
</tr>
<tr>
<td>1°89/11175 Rec. 90/45</td>
<td>M</td>
<td>56</td>
<td>GBM GBM</td>
<td>4m</td>
<td>8m</td>
</tr>
<tr>
<td>1°91/427 Rec. 91/500</td>
<td>F</td>
<td>51</td>
<td>GBM GBM</td>
<td>1m</td>
<td>4m</td>
</tr>
<tr>
<td>1°154/83 Rec. 144/84</td>
<td>M</td>
<td>64</td>
<td>GBM GBM</td>
<td>13m</td>
<td>14m</td>
</tr>
<tr>
<td>1°159/88 Rec. 25/90</td>
<td>M</td>
<td>37</td>
<td>GBM GBM</td>
<td>20m</td>
<td>23m</td>
</tr>
<tr>
<td>1°93/646 Rec. 94/40</td>
<td>M</td>
<td>60</td>
<td>GBM GBM</td>
<td>2m</td>
<td>8m</td>
</tr>
<tr>
<td>1°93/261 Rec. 93/485</td>
<td>F</td>
<td>56</td>
<td>GBM GBM</td>
<td>4m</td>
<td>11m</td>
</tr>
</tbody>
</table>
Fluorescent signals from the centromeres of the hybridised chromosomes can be seen as yellow/green spots on red propidium iodide counterstained nuclei. Signals were counted by focusing up and down through the whole thickness of the sections, and hence multiple signals are often difficult to visualise in a single plane of a photograph. Plate 3.23 shows tumour 91/427 hybridised with the Cm7 probe, and in the single plane of this photograph most nuclei have 2 or 3 copies and occasionally 4 or more copies. Plate 3.24 shows 91/500 hybridised with Cm7 probe, again most nuclei have 2 or 3 copies. Plate 3.25 shows tumour 306/88 hybridised with Cm7 probe, the nuclei in the centre are trisomic. Plate 3.26 shows tumour 144/84 hybridised with Cm22 probe, these nuclei are all monosomic. Plate 3.27 shows 159/88 hybridised with Cm22 probe two signals can be seen in most nuclei. Plate 3.28 shows 160/91 hybridised with Cm22 probe 3 nuclei in the centre are trisomic.

Figure 3.24 shows the number of copies of chromosome 7 in thirteen paired sections. Nuclei with trisomy were seen in both primary and recurrent tumours, this is a common feature of high grade gliomas (Bigner et al 1988a). Sub-populations of cells with multiple copies of chromosome 7 (>3 copies) were also found, for example in Figure 3.24f the primary tumour is predominantly diploid and the recurrent is tetraploid, and in Figure 3.24i the primary is triploid and the recurrent is tetraploid. A maximum of seven copies of chromosome 7 were seen in tumours 794/89 (Figure 3.24f), 90/45 (Figure 3.24h), 91/500 (Figure 3.24j), 25/90 (Figure 3.24l), 93/261 and 93/485 (Figure 3.24m). The tumours can be divided into two groups based on the shapes of the graphs, Group A, those which have a sharp cut off point at 3 signals per nucleus, with only rare nuclei in the primary and/or the recurrent having 4 or more signals (Figure 3.25 a, c, d, g, j and k), and Group B, those with a sizeable proportion of nuclei with 4 or more signals in the primary and/or the recurrent (Figure 3.25 b, e, f, h, i, l and m). However, there appears to be no difference between these two groups in terms of survival, age or treatment, and although there is a predominance of males in group B the number of cases is rather small (Table 3.11).

Table 3.11 Clinical similarities of group A and group B patients

<table>
<thead>
<tr>
<th></th>
<th>Group A (1° and/or rec tumours rarely have &gt; 3 copies of Cm7)</th>
<th>Group B (1° and/or rec tumours commonly have &gt; 3 copies of Cm7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall survival (months)</td>
<td>17.8</td>
<td>16.9</td>
</tr>
<tr>
<td>Time from 1st to 2nd op. (months)</td>
<td>11</td>
<td>11.28</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>50.5</td>
<td>47</td>
</tr>
<tr>
<td>Sex</td>
<td>F4 M2</td>
<td>F1 M6</td>
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<tr>
<td>Radiotherapy after 1st op.</td>
<td>6 of 6</td>
<td>4 of 7</td>
</tr>
<tr>
<td>Chemotherapy after 1st op.</td>
<td>2 of 6</td>
<td>2 of 7</td>
</tr>
</tbody>
</table>

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Since both primary and recurrent tumours were often triploid for Chromosome 7, it was of interest to see if the recurrent tumours had more nuclei with more than 3 copies of chromosome 7. The percentages of nuclei with 3 or more and 4 or more copies of chromosome 7 are shown in Table 3.12. In 7 of the 13 paired samples there was a higher proportion of cells with 3 or more copies of chromosome 7 in the recurrent tumour than in the matching primary sample taken at diagnosis, but of more interest is the finding that in 10 of the 13 pairs the recurrrents had more nuclei with 4 or more copies than the corresponding primaries. Although there appears to be a difference between primary and recurrent gliomas based on the number of copies of chromosome 7 in the nuclei, using a two sample T-test there was no statistical difference between either 3 or more copies (p=0.33), or 4 or more copies (p=0.4).

**Table 3.12** Percentage of nuclei with 3 or more and 4 or more copies of chromosome 7.

<table>
<thead>
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<th>Tumour sample</th>
<th>3 or more copies</th>
<th>4 or more copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° 525/88</td>
<td>40.3</td>
<td>0</td>
</tr>
<tr>
<td>rec. 169/89</td>
<td>53.7</td>
<td>4.6</td>
</tr>
<tr>
<td>1° 259/82</td>
<td>50</td>
<td>23.9</td>
</tr>
<tr>
<td>rec. 34/84</td>
<td>25</td>
<td>3.5</td>
</tr>
<tr>
<td>1° 534/88</td>
<td>58.1</td>
<td>4.2</td>
</tr>
<tr>
<td>rec. 87/90</td>
<td>54.3</td>
<td>11.9</td>
</tr>
<tr>
<td>1° 160/91</td>
<td>50</td>
<td>23.7</td>
</tr>
<tr>
<td>rec. 176/93</td>
<td>40</td>
<td>25</td>
</tr>
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<td>2.2</td>
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<tr>
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<td>2.4</td>
</tr>
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<td>68.5</td>
<td>41.3</td>
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Plate 3.23 91/427 hybridised with Cm7 probe
(original magnification x250)

Plate 3.24 91/500 hybridised with Cm7 probe
(original magnification x250)
Plate 3.25 306/88 hybridised with Cm7 probe
(original magnification x250)

Plate 3.26 144/84 hybridised with Cm22 probe
(original magnification x250)
Plate 3.27 159/88 hybridised with Cm22 probe
(original magnification x250)

Plate 3.28 160/91 hybridised with Cm22 probe
(original magnification x250)
Figure 3.24 Thirteen paired tumour sections showing number of copies of chromosome 7 per nucleus as a percentage of all cells counted.

a

\[
\begin{align*}
\text{No. signals per nucleus} & \\
\% \text{ of cells} & \\
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7
\end{align*}
\]

\begin{align*}
\square \text{ Cm7 525/88} \\
\blacksquare \text{ Cm7 169/89}
\end{align*}

b

\[
\begin{align*}
\text{No. signals per nucleus} & \\
\% \text{ of cells} & \\
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7
\end{align*}
\]

\begin{align*}
\square \text{ Cm7 259/82} \\
\blacksquare \text{ Cm7 34/84}
\end{align*}

c

\[
\begin{align*}
\text{No. signals per nucleus} & \\
\% \text{ of cells} & \\
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7
\end{align*}
\]

\begin{align*}
\square \text{ Cm7 33/90} \\
\blacksquare \text{ Cm7 539/90}
\end{align*}

d

\[
\begin{align*}
\text{No. signals per nucleus} & \\
\% \text{ of cells} & \\
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7
\end{align*}
\]

\begin{align*}
\square \text{ Cm7 534/88} \\
\blacksquare \text{ Cm7 87/90}
\end{align*}

- Primary tumour
- Recurrent tumour
Figure 3.24 Thirteen paired tumour sections showing number of copies of chromosome 7 per nucleus as a percentage of all cells counted.
Figure 3.24. Thirteen paired tumour sections showing number of copies of chromosome 7 per nucleus as a percentage of all cells counted.

- **i**
  - % of cells
  - No. signals per nucleus
  - Cm7 93/646
  - Cm7 94/40

- **j**
  - % of cells
  - No. signals per nucleus
  - Cm7 91/427
  - Cm7 91/500

- **k**
  - % of cells
  - No. signals per nucleus
  - Cm7 154/83
  - Cm7 144/84

- **l**
  - % of cells
  - No. signals per nucleus
  - Cm7 159/88
  - Cm7 25/90

Primary tumour
- Recurrent tumour
Figure 3.24  Thirteen paired tumour sections showing number of copies of chromosome 7 per nucleus as a percentage of all cells counted.
Visualisation of the chromosome 22 probe on the paraffins proved technically more difficult, and although the Cm22 probe is a smaller probe than the Cm7 probe and therefore may be more difficult to discern, this alone would not account for the lack of hybridisation. Increasing the amount of Cm22 probe from 40ng to 80ng did increase the signal to some extent. Serial sections were used for both Cm22 and Cm7 probes and optimum pepsin digestion was arrived at empirically and yet clear Cm7 signals were often visible where Cm22 had failed to hybridise under identical conditions. Pepsin concentrations ranged from 4-25mg/ml with a range of incubation times of 10-120 minutes. Under-digestion meant that no hybridisation occurred and over-digestion meant that nuclear structure was lost and nuclei did not counterstain homogeneously. The different digestion conditions were needed as the samples differed in their cellularity, and the thickness of the sections even from a single block was not always a uniform 5μm. Examples of the different digestion conditions used to give good Cm7 hybridisation are as follows: 525/88 used 10mins at 4-5mg/ml, 34/84 used 30-90 mins at 10mg/ml and 154/83 used 30-45mins at 20mg/ml.

Of the 10 tumours that yielded results with the Cm22 probe there was only one pair of primary and corresponding recurrent tumour, 291/87 and 306/88 respectively. The remaining 8 tumours include one recurrent, 144/84, the remainder all being primary tumours. Figure 3.25 shows the number of copies of Cm22 per nucleus in these 10 tumours. The primary 291/87 is predominantly monosomic whereas the corresponding recurrent 306/88 has more disomic nuclei (Figure 3.25a). However, the only other recurrent tumour 144/84 (Figure 3.25i) is monosomic. In 159/88 (Figure 3.25c) the number of nuclei with no signals is high indicating possible failure of hybridisation. Of the remaining 6 primaries, two are predominantly monosomic for Cm22 259/82, 794/89 (Figure 3.25e and h). Four are predominantly disomic for Cm22, 525/88, 160/91, 33/90 and 534/88 (Figure 3.25b, d, f and g respectively). Occasional nuclei with 3 or 4 copies of Cm22 were found in 6 of the tumours this differs greatly with the majority of tumours which had 3 or more copies of Cm7.

There is no clear relationship between number of copies of Cm22 and Cm7 in the same tumour. A tumour with rarely more than 3 copies of Cm7 (group A) may be monosomic (144/84) or disomic (534/88) for Cm22. Likewise a tumour with greater than 3 copies of Cm7 (group B) may also be monosomic (259/82) or disomic (160/91) for Cm22.
Figure 3.25. Ten tumour sections showing number of copies of chromosome 22 per nucleus as a percentage of all cells counted.
Figure 3.25. Ten tumour sections showing number of copies of chromosome 22 per nucleus as a percentage of all cells counted.

- **g**: Primary tumour (534/88)
- **h**: Recurrent tumour (794/89)
- **i**: Recurrent tumour (144/84)

- % of cells
- No. signals per nucleus

- □: Primary tumour
- ■: Recurrent tumour
**Patient follow up and clinical correlations**

From a total of 38 patients, cell cultures were derived from 26 and paraffin blocks of the primary and recurrent tumour from 13. This included one patient where both cell culture, IN1461, and paraffin block, 525/88, were obtained. Follow up summaries are shown in Figures 3.26 (cell cultures) and 3.27 (paraffins), including details of re-operation for recurrence, radiotherapy treatment and PCV chemotherapy. A question mark indicates that it was unclear from the records that the patients had received chemotherapy. This accounted for 5 of the cell cultures, IN1682, IN1728, IN2112, IN1752 and IN2132. In addition two patients, IN2058 and IN2094, received an alternative drug temozolamide. One patient, IN1940, was completely lost to follow up. Survival times from first operation for all patients ranged from 1 day to 41 months, with a mean and median of 11 and 7 months respectively. The clinical data from the two sets of patients, those from which cell cultures were derived and those from which paraffin blocks were obtained, are compared in Table 3.13. The only difference between these two sets of patients appears to be the survival times which are more than twice as long in the patients from which paraffin blocks were obtained. This can be explained because these are a highly selected group of patients whose recurrences were operated upon and paraffin blocks made available. This in effect eliminated all those cases where the patient died from the primary tumour or other complications, or where the recurrence was not biopsied or debulked.

**Table 3.13** Summary of clinical data from the two sets of patients.

<table>
<thead>
<tr>
<th></th>
<th>Cultures</th>
<th>Paraffins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>25-72</td>
<td>31-64</td>
</tr>
<tr>
<td>mean (years)</td>
<td>52.2</td>
<td>48.6</td>
</tr>
<tr>
<td>median (years)</td>
<td>54</td>
<td>51</td>
</tr>
<tr>
<td>Survival from first operation (months)</td>
<td>8.3 (<strong>11.6</strong>)</td>
<td>17.3</td>
</tr>
<tr>
<td>Sex</td>
<td>14F 12M</td>
<td>5F 8M</td>
</tr>
<tr>
<td>PCV chemo after first operation.</td>
<td>5/26</td>
<td>4/13</td>
</tr>
<tr>
<td>Radiotherapy after first operation.</td>
<td>17/26</td>
<td>10/13</td>
</tr>
<tr>
<td>Radiotherapy and PCV after first operation.</td>
<td>4/26</td>
<td>4/13</td>
</tr>
</tbody>
</table>

(* when survival of 2 months or less were excluded)

The survival times in Figure 3.27 compared to Figure 3.26 appear to be much improved with chemotherapy, however, this may be due to the overall longer survival and the selection of these patients. In Figure 3.26 where there was less selection the survival times are much shorter, only 6 of 25 surviving over 10 months compared to 10 of 13 in Figure 3.27. In addition, of the cell culture patients
only two of 25 known cases were re-operated on. The median survival times for the cell culture group of patients is comparable to other clinical studies (Walker et al 1978 and Chang et al 1983). In the cell culture group, patients with grade III astrocytomas survived longer (mean 13 months) than the GBMs (mean 6.5 months). Relapse Free Intervals (RFI) were not known for most patients, however in those cases which were re-operated upon it can be assumed that RFI ended just prior to this second operation.

It appears that the three shortest survival times may be due to the fact that these patients received no radiotherapy, but conversely, if patients survived less than 1-2 months this may mean that they did not recover from surgery, or they may be too unwell for further treatment. Therefore for the clinical comparisons those patients who survived for 2 months or less after their primary operation have been excluded, this affects IN859, IN1760, IN2112, IN2026, IN2127, IN2134, IN2281 and IN2037. As a group, these patients are slightly older than the others from the cell culture group, with a mean age of 60.6 years and median of 60 years. Two of these patients did not recover consciousness after surgery (IN2037 and IN2127).

Relationships between survival time, age and chemotherapy for all patients are shown in Figure 3.28. It is clear that the younger patients survived longer irrespective of treatment with chemotherapy, mean and median survival for ≤ 45 years = 21.3 and 19 months respectively, mean and median survival for > 45 years = 10.6 and 8 months respectively, Mann-Whitney test of medians (p=0.02). However, it also appears that younger patients are more likely to have received chemotherapy. The patients with grade III astrocytomas and grade III oligoastrocytoma had above average survival and the patients with grade III astrocytomas were amongst the youngest. The majority of patients received radiotherapy, those who did not are marked with an asterisk. The median age and survival for the two groups of patients are shown on the graph, again emphasising the similarity in age and the difference in survival time.

There appears to be an inverse relationship between overexpression of p53 and survival. Figure 3.29 shows data where both p53 nuclear score and survival data were known for each antibody, excluding survival of 2 months or less. With DO-1 (wild type and mutant) the 4 cases with overexpression of p53 (scores of 2 and 3) have amongst the shortest survival times, but although there appears to be a strong trend for over expression of p53 to be linked with shorter survival, this is not statistically significant (Mann-Whitney p=0.22). With 240 (mutant only) there is also
no statistical significance (Mann-Whitney p=1.0). However, with 421 (wild type and mutant) the difference between the medians is significant (Mann-Whitney p=0.04). Unfortunately RFIs were not known for all these cases so could not be included.

Expression of p53 is independent of the passage level of the cultures tested (Figure 3.30). There is no difference between the passage levels of those cultures overexpressing p53 and those with no overexpression, Mann Whitney for DO-1 (p=0.69), for 421 (p=0.62) and for 240 (p=0.33). Similarly the age of the patient does not affect p53 expression (Figure 3.31). Mann-Whitney for DO1 (p=0.38), for 421 (p=0.46) and for 240 (p=1.0).

Figure 3.32 compares in vitro drug responses with survival times and RFIs in those patients who received PCV chemotherapy. It appears that in this small sample in vitro chemosensitivity does not correlate with patient outcome. All patients in this figure were aged between 41 and 55 and included four grade IV astrocytomas and one grade III astrocytoma. There are marked time differences between RFI and survival, indicating the importance of choice of endpoint in clinically correlative studies. These five patients did not receive identical treatment which makes correlation more difficult, this clinical information is shown in Table 3.14.

**Table 3.14.** Clinical information on the five patients with astrocytomas for whom both in vitro drug response and chemotherapy protocols were known.

<table>
<thead>
<tr>
<th>Culture number</th>
<th>diagnosis</th>
<th>Age at 1st op.</th>
<th>Radio-therapy</th>
<th>Chemotherapy</th>
<th>survival (months)</th>
<th>RFI (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN1461 GBM</td>
<td>44</td>
<td>yes</td>
<td>4 courses PCV</td>
<td></td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>IN1472 GBM</td>
<td>46</td>
<td>no</td>
<td>VCR and CCNU 1 month after diag. Procarbazine 3 months later.</td>
<td></td>
<td>5</td>
<td>unknown</td>
</tr>
<tr>
<td>IN1979 GBM</td>
<td>46</td>
<td>yes</td>
<td>2-3 courses PCV</td>
<td></td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>IN2093 GBM</td>
<td>55</td>
<td>yes</td>
<td>PCV unknown how many courses.</td>
<td></td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>IN2094 gr III</td>
<td>41</td>
<td>yes</td>
<td>1 course PCV. Temozolomide 8 months after diag. Etoposide 11 months after diag.</td>
<td></td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

Some of these five cases (Figure 3.32) have clinical complications that should be taken into account. Each will be discussed individually: IN1472, apparently has a very short survival (5 months) even though it is relatively sensitive to CCNU and VCR in vitro. This is the only case of the five that had no radiotherapy for the high grade glioma, and this could be the single most important factor in the survival. However, clinically this case is very complicated, because 3.5 years before the GBM
was operated upon and diagnosed, (from which IN1472 was derived), another tumour was biopsied and classified as a gliosarcoma. This was reclassified as a ganglioglioma 2.5 years later and the patient was then given radiotherapy. The diagnostic anomaly is under review. Therefore the GBM, IN1472, may be better classed as a recurrent in which case the survival time measured from the first operation for "gliosarcoma" would be 46 months and not 5 months as shown in Figure 3.32. No chemotherapy was given prior to the diagnosis of GBM and no cell culture from either of the previous operations was available.

For IN1979 and IN2093 there are no extra clinical factors to be taken into account and the patients' estimated RFIs were only 1 month and 2 months less than survival time respectively. The patient from which IN1979 was derived only received 2 or 3 courses of PCV before relapse. The RFI for the patient from which IN2094 was derived, was approximately 3 months, however, this was a grade III astrocytoma and would probably be expected to do better than the grade IV tumours. PCV was given 2 months after the first operation, and after relapse an alternative chemotherapy of Temozolamide and then Etoposide were also given 9 months and 12 months respectively after the operation, it is unknown how well the patient was during this latter chemotherapy.

Finally, IN1461 recurred and was operated upon 12 months after the first operation, giving paraffin 525/88 used in the present study. This patient only received 4 courses of PCV before recurrence of the tumour. The RFI for this patient was 11 months compared to survival of 18 months.

The lack of a relationship found in this comparison (Figure 3.32) may simply reflect the small number of cases available and the different treatments each patient received. Comparing Figures 3.29 and 3.32 suggests that with this small group of cases the p53 nuclear score is a more telling prognostic factor than in vitro drug testing, especially when using antibodies DO-1 and 421, with overexpression of p53 being a bad prognostic factor. The comparison of in vitro drug responses with survival times in patients who did not receive PCV chemotherapy is shown in Figure 3.33. and there appears to be no relationship as would be expected.

A rapidly growing tumour is likely to mean a poor prognosis, therefore the relationship between DT of cell cultures and survival may be of importance. Figure 3.34 shows that there does not appear to be a relationship between survival and DT. The longest survival at 36 months (IN1951) is more likely due to this being a grade
III astrocytoma. To show if there is any real correlation Pearson's correlation coefficient, r, and measurement of variance, r^2, were calculated. There was no significant correlation between survival >2 months and DT (r^2=0.16, p=0.11) nor all survivals and DT (r^2=0.02, p=0.47).

p53 protein is involved in regulation of the cell cycle in response to DNA damage, so the relationship between p53 nuclear expression and DT of the cell cultures was investigated. Figure 3.35 shows this relationship with all three antibodies. Overexpression of the p53 protein does appear to be found in the faster growing cells. This trend is significant with the two antibodies recognising both mutant and wild type p53 protein, Mann-Whitney for DO-1 (p=0.01) for 421 (p=0.03), but not with 240 the antibody recognising mutant only (p=0.39). Overexpression of p53 does not occur during normal cell division therefore this apparent relationship may not be causal.
Figure 3.26 Patient follow up summary for cell cultures.
Figure 3.27  Patient follow up summary for paraffins.
Figure 3.28 Relationship between survival and age, with and without chemotherapy

* = no radiotherapy, grIII = astrocytoma grade III, OA = oligoastrocytoma grade III, ——— = median survival and age for cultures included on graph, _____ = median survival and age for paraffins included on graph, patients surviving 2 months or less are excluded. Chemotherapy was given after the first operation and before recurrence.
Figure 3.29 Relationship between p53 nuclear expression and survival with each antibody.

(Patients surviving 2 months or less are excluded, score 2/3 = overexpression, score 0/1 = no overexpression, continuous lines = mean survival for scores of 0/1 and 2/3, dotted lines = median survival for scores of 0/1 and 2/3)
Figure 3.30  Passage level versus p53 expression for all three antibodies.

(2 or 3 = indicates number of cultures represented by single cross)
Figure 3.31. Relationship between p53 nuclear expression and age at first operation with each antibody.

(Score 2/3 = overexpression, score 0/1 = no overexpression, continuous lines = mean age for scores of 0/1 and 2/3, dotted lines = median age for scores of 0/1 and 2/3)
Figure 3.32. Comparison of in vitro drug response with survival times and RFIs in those patients who received chemotherapy.
Figure 3.33 Comparison of in vitro drug responses with survival times in those patients who did not receive PCV chemotherapy.

(Patients surviving 2 months or less are excluded).
Figure 3.34 Comparison between doubling times of the cell cultures and survival.

(Patients surviving for 2 months or less are excluded).
Figure 3.35. Relationship between p53 nuclear expression and doubling time of cultures with each antibody.

Score 2/3 = overexpression, score 0/1 = no overexpression, continuous lines = mean survival for scores of 0/1 and 2/3, dotted lines = median survival for scores of 0/1 and 2/3)
DISCUSSION

Cell cultures and their relation to astrocytomas in situ.

Growth of cell cultures

It has been possible to produce short-term cultures from operative samples of 26 malignant astrocytomas, but how representative are these cells of the tumours from which they were derived? It has been known for thirty years that more than 90% of grade III and IV astrocytomas can be grown as short term cultures and approximately half will give rise to established cell lines (Manuelides 1965, Ponten and Macintyre, 1968, Bigner et al 1981). It has also become clear that short-term cultures derived from malignant astrocytoma display biological features which are consistent with their neoplastic nature. They are capable of growth on confluent monolayers of normal glia (MacDonald et al 1985), they produce factors capable of inducing angiogenesis on chick chorioallantoic membrane (Frame et al 1984), they produce plasminogen activator (Frame et al 1984), are less sensitive to growth inhibition at high cell density (Westermark 1973) and some, at least, are capable of producing tumours when implanted subcutaneously or intracranially in nude mice (Bigner et al 1981). Short-term cultures derived from malignant glioma have been shown to be aneuploid, often with distinct characteristic karyotypic abnormalities (see below).

Whilst their neoplastic nature seems not to be in doubt, do cells in these cultures express features consistent with astroglial differentiation? Normal astrocytes express glial fibrillary acidic protein (GFAP) (Eng et al 1971), a component of cytoskeletal intermediate filaments. Studies have shown that cells derived from malignant glioma usually do not express this antigen after prolonged culture in vitro. Westphal et al (1990) have demonstrated that whilst GFAP positive cells were present in significant numbers in about 50% of primary cultures derived from either grade III or IV astrocytomas, by the time these cultures had reached passage level 8, GFAP positive cells constituted less than 1% of cells in about 10% of the cultures. The cell type which had come to predominate in these cultures were GFAP negative but expressed cell surface fibronectin. Frame et al (1984) also found that GFAP was rarely expressed in cultures derived from high-grade glioma, but that these cell lines exhibited both high-affinity GABA uptake which was inducible by steroids and glutamine synthetase activity, characteristics of neuro-ectodermal cell populations. Similarly, in a study which screened established glioma cell lines, only 2 of 15 of these lines expressed GFAP (Bigner et al 1981). It appears therefore that both short-term and established cell lines from malignant glioma are most usually composed of GFAP negative cells which express cell surface fibronectin. There is little doubt
that these are tumour cells and not contaminating adventitious cells like fibroblasts as they are aneuploid (Guner et al 1977, Kennedy et al 1987) and retain chromosome 10 abnormalities in culture which are present in the tumour at the time of biopsy (Noble et al 1995). This had led authors to postulate that these cultures are derived from either glial precursor cells or that the switch from in situ to in vivo conditions produces an antigenic shift in response to either changes in the extracellular matrix or diffusible factors like growth factors.

Although no systematic attempt has been made in the present study to repeat the type of characterization studies described above, previous studies in our laboratories using short-term cultures derived from high grade gliomas in exactly the same way have confirmed the neoplastic and astroglial characteristics described above (Kennedy et al 1987). An examination of the morphological appearance of these short-term cultures confirmed the presence of morphologically different types of cells within an individual culture, confirming their heterogeneous nature. Cell culture doubling times ranged between 18 and 126 hours (see Figure 3.2) and were consistent with other studies (Yung et al 1982, Frame et al 1984).

**Why use cell cultures?**

Although the expression of drug resistance genes like P-glycoprotein, glutathione-S-transferase and DNA repair enzymes can be detected using immunocytochemistry or molecular probes in archival material, it is often difficult to assess what the relationship is between these results and drug resistance. The number of cells positive for a particular antigen is likely to vary from section to section and there is no clear indication of how many positive cells per unit area are required to designate a sample as drug resistant or sensitive. Similarly, it is often not clear if the number of positive cells is important or the intensity of staining. What intensity of staining is required to categorize a particular cell as drug resistant or sensitive? There are also technical artefacts which might produce either false positive or false negative results and care must be taken to perform adequate control studies to ensure that the staining is not non-specific or that epitopes are either not lost or not unmasked sufficiently during preparation (Cordon-Cardo et al 1990). More importantly, it has become clear that drug resistance is multifactorial and tests focussed on a particular resistance marker might have limited applicability in cells which have developed resistance by multiple mechanisms. It is also not clear if the consequences of over-expression of a single drug resistance marker is the same in different tissues. These difficulties can be overcome largely by the use of assays
which measure chemosensitivity in a cell culture system where cells are treated with drugs and the residual viability determined.

**Short term cultures versus established cell lines**

Inevitably, cell culture systems represent a selected population of tumour cells with the capacity to grow in artificial conditions where the cells are removed from their microenvironment and deprived of their intercellular cues. Established glioma cell lines are likely to be composed of highly selected cells often grown for many years in culture and although they express many of the characteristics of neoplastic astrocytic-like cells, their relationship with tumour cells *in situ* is likely to be tenuous. It would be preferable to use cells *in vitro* for drug sensitivity studies that are closest to tumour cells *in situ*. The ideal cultures to use might be primary cultures, although these, of course, still only represent a selected sub-population of cells from the tumour. However, it is usually not practical to use primary cultures if only because there are only a limited number of cells available from each tumour biopsy which make it impossible to carry out the requisite number of replicates for a large panel of drugs tested at several different concentrations. Secondly, the kinetics of cells in primary culture are complex and this is reflected in unstable drug sensitivities *in vitro* as compared to using cells passaged once or twice before assay (Morgan et al 1983). The third reason why primary cultures are unsuitable is that they are contaminated with blood or non-viable cell debris. Short term cultures with low passage levels (between 3 and 13) were used in the present study. These cultures provide sufficiently large numbers of cells for the appropriate number of experimental replications and for cell banking purposes, but remain sufficiently close in cellular makeup to the original tumour. It is interesting that a number of studies have shown that low passage glioma cell lines are relatively stable *in vitro*. For example, culture doubling times have been shown not to vary between passage level 4 and 12 (Yung et al 1982) and characteristic karyotypic changes have been shown to be maintained over a wide range of passage levels (Shapiro and Shapiro 1985, Westphal et al 1994). The present study confirms this. There is no evidence that either culture doubling time, or more importantly, sensitivity to either CCNU, DOX or VCR (Figures 3.3, 3.4 and 3.5) changes significantly with passage level. Indeed, the variation in chemosensitivity between replicates of virtually all the short-term lines tested was no greater than that seen between replicates of the established cell line, U251MG over the passage range 631–635. This provides compelling evidence that short-term glioma cells lines, at least at the passage levels tested, provide a stable source of cells for chemosensitivity and other biological studies of human malignant glioma.
Chemosensitivity assay in vitro

Choice of endpoint
The clonogenic cell survival is often described as the "best" assay to measure chemosensitivity in vitro (Roper and Drewinko 1976). The reasoning behind this was that following the proposal of a stem-cell model for cancer (Steel 1977) that it was most important to know about the sensitivity of stem cells, that is, those cells with unlimited capacity to divide and the proliferation of which would ultimately precipitate tumour recurrence. However, it is now recognized that it is far from clear that clonogenic assays are measuring the sensitivity of stem cells (Selby et al 1983). This coupled with the poor plating efficiencies of human solid tumours and the technical difficulties in preparing single cell suspensions from most human tumours in sufficient numbers to carry out meaningful chemosensitivity assays (Selby et al 1983) has led to a reassessment of their usefulness as routine assays for chemosensitivity. There are several reasons why short term glioma cultures are not suited to clonogenic assays, principally their low plating efficiencies (typically in the range 0.5-0.006%, Rosenblum et al 1980) and relatively long population doubling times which would mean that with a mean doubling time of 59 hours for the 26 short term cultures in the present study, the minimum time needed for a single cell to form a colony of at least 50 cells would be approximately 15 days (six cell doublings). A clonogenic assay would therefore take at least twice as long as other chemosensitivity assays based on bulk growth of cells. Another drawback of clonogenic assay is the necessity to trypsinize the cells following drug exposure to obtain a single cell suspension which inevitably compromises cell viability. This has led to the development of a number of non-clonogenic chemosensitivity assays more suited to monolayer cell cultures and which can be semi-automated. One example of these is the MTT assay which appears to give comparable results to other assays where the measurement of endpoint is determined by measuring cellular protein or counting viable cells (Alley et al 1988). Indeed by adopting an approach of using long term (> 1 cell doubling) exposure to drugs followed by a similar period of recovery, it has been shown that drug sensitivity determined by assays using endpoints as diverse as isotope uptake with $^3$H-leucine measured by scintillation spectrometry or $^{35}$S-methionine measured by scintillation autofluorography (Morgan et al 1983), protein staining with sulphorhodamine B (Haselsberger et al 1996), cell counting (Darling, personal communication) or MTT reduction (Nikkah et al 1992b) produced results which closely agreed with monolayer cloning. Perhaps this is not surprising as in essence all chemosensitivity assays simply measure the number of viable cells remaining at the end of the assay and it is probably unimportant which endpoint is used if the assay is designed correctly.
Although it would be ideal to have both drug exposure and recovery taking place over a fixed number of cell doublings, in practice this would be impossible to design into a routine assay used to screen cultures with a wide range of doubling times. In the present study, a 3 day drug exposure period and 4 day recovery period were chosen to allow the vast majority of cultures, (23 of 27, see Figure 3.2) to complete at least one cell cycle with and without drug.

In the present study, there was no correlation between the sensitivity of these cultures to either CCNU or DOX and although there was no correlation between doubling time and sensitivity in those cultures which were resistant to VCR, in those cultures which fell into the more VCR-sensitive group there was a relationship between doubling and chemosensitivity. This is probably artefactual, the result if there being few cultures with intermediate sensitivities to VCR. Using $^3$H-leucine or $^{35}$S-methionine incorporation to examine chemosensitivity of five glioma cell lines against six drugs including vinblastine, a three day drug exposure, corresponding to at least one cell doubling in most of the cultures and a 3-5 day recovery period was used (Morgan et al 1983). Although a culture with the long doubling time of 132 hours was the most resistant to 5-fluorouracil, in most cases neither a steep slope on the drug response graph, nor a low ID$_{50}$ value after a 3 day drug exposure correlated with short doubling times (Morgan et al 1983).

It is clear that for any chemosensitivity study which attempts to ask biological questions relevant to clinical treatment, that the range of drug concentrations which is tested are similar to those likely to be achieved in patients. The concentration ranges chosen for CCNU and VCR in the present study included the known peak plasma levels of clinically recommended doses. DOX is not used in the treatment of malignant astrocytoma as it penetrates brain very poorly and was included because of its association with the MDR phenotype. For many drugs, the peak plasma levels are not necessarily a reflection of the levels that reach the tumour, but with the lipid-soluble nitrosoureas these values might be expected to be similar. However, there does not seem to have been a systematic examination of the levels of the lipid-soluble nitrosoureas like BCNU and CCNU attainable in human brain tumours, although levels in the region 5-10 $\mu$g/ml have been suggested as clinically achievable in malignant glioma (Kimmel et al 1987). Table 4.1 shows the maximum achievable plasma and tumour drug levels of two water soluble nitrosoureas and vinblastine administered at clinically recommended doses.
Table 4.1 Peak plasma and brain tumour levels of ACNU, TCNU and VBL.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Plasma concentration</th>
<th>Tumour concentration</th>
<th>Tumour type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACNU</td>
<td>1.7-8.3µg/g</td>
<td>1.6-19*µg/g</td>
<td>GBM</td>
<td>Hori et al (1987)</td>
</tr>
<tr>
<td>TCNU</td>
<td>0.1-3µg/ml</td>
<td>0.5µg/ml</td>
<td>high grade astrocytoma</td>
<td>Whittle et al (1990)</td>
</tr>
<tr>
<td>VBL</td>
<td>5ng/ml</td>
<td>68ng/g</td>
<td>epitheloid sarcoma</td>
<td>Stewart et al (1983)</td>
</tr>
</tbody>
</table>

TCNU = tauromustine, *achieved with mannitol to open BBB,

The results of the present study imply that if these levels of drugs did reach the tumour cells then high grade glioma chemotherapy would be very effective. For example, if concentrations of 19µg/ml of CCNU or 68 ng/ml of VCR could be achieved clinically more than 50% of cells would be killed in 26 of 27 cultures and 16 of 22 cultures respectively (see Figure 3.6.).

It is interesting to see that the ranges of ID\textsubscript{50} values in the present study do correspond well with other published studies. Table 4.2 compares the present results (column two) with five other studies. The ranges for CCNU are very similar. However, the ranges for VCR do appear to differ, although there is an overlap between the studies, the present study has the highest ID\textsubscript{50}s (relatively resistant) and Morgan's study the lowest ID\textsubscript{50}s (relatively sensitive). Comparing the medians it appears that the present study has highly resistant outliers, and Morgan's study has highly sensitive outliers, both situations tending to increase the range of ID\textsubscript{50} values.
<table>
<thead>
<tr>
<th>VCR ID&lt;sub&gt;50&lt;/sub&gt;</th>
<th>0.04 - 22000 (4.57) ng/ml</th>
<th>nd</th>
<th>0.00004-4.1 (0.41) ng/ml</th>
<th>nd</th>
<th>nd</th>
<th>1 - 16 (10.0) ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNU ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.65 - 23.06 (5.6) µg/ml</td>
<td>3.4-23.8 µg/ml</td>
<td>2 - &gt;23 µg/ml</td>
<td>&lt;0.2 - &gt;50 (0.9) µg/ml</td>
<td>84% of cultures had ID&lt;sub&gt;50&lt;/sub&gt; values &gt;5µg/ml*</td>
<td>4 - 14 µg/ml</td>
</tr>
<tr>
<td>Assay method</td>
<td>MTT</td>
<td>Direct cell count</td>
<td>3&lt;sup&gt;5&lt;/sup&gt;S-methionine incorporation into cell protein</td>
<td>14C-leucine or 3H-uridine incorporation into cell protein and RNA respectively</td>
<td>MTT</td>
<td>Sulforhodamine binding to cell protein</td>
</tr>
<tr>
<td></td>
<td>3 day drug</td>
<td>1 hour drug</td>
<td>3 day drug</td>
<td>3 day drug</td>
<td>1 hour drug</td>
<td>3 day drug</td>
</tr>
<tr>
<td></td>
<td>4 day recovery</td>
<td>1 day recovery</td>
<td>1-3 day recovery</td>
<td>9 day recovery</td>
<td>7 day recovery</td>
<td>3 day recovery</td>
</tr>
<tr>
<td>Number of cultures tested</td>
<td>22 (VCR)</td>
<td>58</td>
<td>20</td>
<td>12</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4.2 Comparison of ID<sub>50</sub> ranges from chemosensitivity studies on high grade astrocytoma cell cultures. nd=not determined, numbers in brackets are median values. *

ACNU/BCNU
These differences may be explained in part by the different recovery times used, because although both studies used 3 day drug exposure, Morgan et al (1983) used the recovery times of 1, 2 or 3 days. The shorter recovery times of 1 or 2 days may overestimate chemosensitivity, compared to the present study, by limiting the time for any division of surviving cells. In agreement with the present study, a chemosensitivity trial at MRC Cambridge 1991 (unpublished) also found IN1951 to have the highest VCR ID$_{50}$ (>10µg/ml) by a factor of 1x10$^5$ out of 5 high grade astrocytoma cultures tested.

The cultures exhibited inherently wide ranges of sensitivities to chemotherapeutic drugs (Figure 3.6). With DOX and VCR the differences between the most sensitive and the most resistant cultures are 6x10$^4$ fold and 5x10$^5$ fold respectively. It was necessary to compare 'resistant' and 'sensitive' cultures in this study, which entailed using arbitrary cut off points, cultures with ID$_{50}$ values ≤ median ID$_{50}$ for each drug were classed as sensitive, cultures with ID$_{50}$ values > median ID$_{50}$ for each drug were classed as resistant. Using this classification, all the resistant cultures were contained within the following ranges; DOX ID$_{50}$ range 17.24-361ng/ml, VCR ID$_{50}$ range 4.85-2.15x10$^4$ ng/ml and CCNU ID$_{50}$ range 6.18-23µg/ml.

The ID$_{50}$ value (drug dose which inhibits MTT conversion to formazan product by 50%) was used as the index of chemosensitivity. This point of the curve is the most stable and is not influenced by changes in drug concentration to the same extent as the ID$_{25}$ or ID$_{75}$ which are on rapidly changing parts of the dose-response curve.

Ideally a drug response curve would include concentrations of drug which kill all the cells (0% survival), and a concentration that kills no cells (100% survival) (Figure 3.3 No.7). Whilst it is clear that at low drug concentrations, viability approaches 100% and rarely exceeds this when compared to control values quite often at the highest drug concentrations used viability does not fall to zero. In some case, increasing the concentration range would decrease viability (see Figure 3.3, 15, IN 2103) however, in other cases increasing drug concentration does not produce increased cell kill (Figure 3.3, 2, IN 1612). This seems to be a feature more commonly seen in assays using VCR or DOX but not CCNU. Although this may be the result of blanking errors, untreated wells were used for blanking, it is more likely to be the result of other factors like pH or residual serum in the test wells which produce artefactual reduction of MTT to formazan production in the absence of cells (Twentyman and Luscombe 1987). However, all results are relative to control wells on the same plate, and each plate was blanked in the same way, so this cannot affect

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the ID\textsubscript{50} values. Position on the 96 well plate may also affect results, in particular test wells at the edges of the plates can give spurious readings, as a result of the difficulty of sealing these wells adequately with Mylar film. In order to minimise this problem, in the present study a block of 8 by 6 wells in the middle of the plates were used as test wells, with two additional rows of control wells for each drug. The drug concentration ranges were also chosen so that the ID\textsubscript{50} value would fall in the central wells of this block. The non-linearity of cellular MTT reduction means that it cannot be used to detect very small numbers (< 150 cells/ well) of cells (Haselsberger et al 1996). This is unlikely to be a significant factor in the present study as this would tend to underestimate viability for any given concentration of drug. It is also unlikely to be due to the inability of short-term cultures from malignant glioma to reduce MTT to the formazan product, a problem with some other cells (Scudiero et al 1988) as all control wells gave sufficiently high optical densities to produce good quality dose response curves. The relationship between cell number and absorbance in the MTT assay is also not linear at high cell densities, with a tendency for absorbance to plateau out with increasing cell number. This would tend to overestimate resistance, but does not occur at the relatively low cell densities reached by these cultures (see growth curves, Figure 3.1).

There is a remarkable degree of consistency between replicate dose-response curves carried out on separate occasions over a period of time and on different passage levels of cells. Certainly the passage level does not seem to influence sensitivity to any of the three drugs tested.

There was also little evidence that any of the four grade III astrocytomas and the single case of a mixed grade III oligo-astrocytoma were particularly sensitive to either CCNU or DOX as their ID\textsubscript{50} values fell in the middle of the range of sensitivities seen for grade IV tumours. However, all four cultures derived from grade III astrocytomas tended to be relatively resistant to VCR, with one of these cultures, IN1951 the most resistant of the whole panel to VCR. This was not related to their culture doubling times as these ranged from 54 to 66 hours, again in the middle of the range of doubling times observed for cultures derived from grade IV tumours. This suggests that for the nitrosoureas, there is little evidence of chemosensitivity at the cellular level for lower grade gliomas. This is in agreement with a number of reports that have also failed to demonstrate cellular sensitivity in lower grade astrocytomas (Kornblith et al 1981, Rosenblum et al 1983) although more recently Shapiro et al (1993) have reported that 7/10 cultures derived from
anaplastic astrocytomas were resistant to BCNU in vitro although this panel of cultures may include some derived from grade II astrocytomas.

Patterns of cross resistance in cultures derived from malignant glioma
Although a considerable amount is known about the mechanisms by which high grade gliomas become resistant to alkylating agents both in vitro and in situ, little is known about how these tumours become resistant to the Vinca alkaloids and agents which inhibit topoisomerase. This is surprising bearing in mind the encouraging clinical results produced by agents like etoposide, a topoisomerase II inhibitor (Finn et al 1986) and more recently in vivo data using irinotecan (CPT-11), a topoisomerase I inhibitor, when used in combination with alkylating agents like BCNU (Coggins et al 1998).

A major aim of this project was to determine the role of membrane glycoproteins in modulating the resistance of malignant glioma to natural product cytotoxic drugs. An important initial question, therefore, is do high grade astrocytoma cultures exhibit MDR? Below is a summary of the two most commonly studied MDR phenotypes:

**Classical MDR (PGP dependent)**
1. Resistance to a range of functionally and structurally unrelated drugs, including Vinca alkaloids, epipodophyllotoxins, anthracyclines and antibiotics.
2. Decreased cellular drug accumulation.
3. Increased efflux of drug.
4. Over-expression of P-glycoprotein.

**Atypical MDR (PGP independent)**
1. Resistance to a range of functionally and structurally unrelated drugs, including epipodophyllotoxins, anthracyclines, but not Vinca alkaloids.
2. No change in cellular drug accumulation.
3. No change in efflux of drug.
4. Altered topoisomerase II

In the present study, when the culture ID\(_{50}\) values for VCR and DOX were compared it was clear that the cultures could be divided up into four groups on the basis of their patterns of cross resistance. The composition of these groups are shown in Table 4.3 using data taken from Table 3.1 and Figure 3.6.
Table 4.3 Resistance patterns in cell cultures

<table>
<thead>
<tr>
<th>Resistance pattern</th>
<th>Group</th>
<th>Cell cultures exhibiting pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX+ VCR+</td>
<td>A</td>
<td>IN1461, IN1760, IN1728, IN1951, IN2134, IN2281</td>
</tr>
<tr>
<td>DOX+ VCR(\text{int})</td>
<td>B</td>
<td>IN859, IN1528, IN1612, IN1682, IN1752, IN1902, IN1979, IN2045, IN2093, IN2094, IN2112, IN2127, U251 MG</td>
</tr>
<tr>
<td>DOX(^{-}) VCR(\text{int})</td>
<td>C</td>
<td>IN1265</td>
</tr>
<tr>
<td>DOX(^{-}) VCR(^{-})</td>
<td>D</td>
<td>IN1472</td>
</tr>
</tbody>
</table>

\(+ = \text{marked resistance}, \ \text{int} = \text{intermediate resistance}, \ - = \text{sensitive}\)

Taking these two MDR phenotypes into account when interpreting the chemosensitivity data in Figure 3.15, one group of cultures (A) appears to be markedly resistant to both DOX and VCR. This suggested the possibility of classical (or typical) MDR and although only two drugs implicated in classical MDR were tested it is circumstantial evidence that PGP would be found in the cultures.

A second group of cultures (B) are certainly markedly resistant to DOX but perhaps no more resistant than group A cultures, however they do appear to be significantly more sensitive to VCR. Group B cells display a drug resistance phenotype more akin to atypical MDR. The anthracyclines like DOX intercalate adjacent DNA base pairs which produces an inhibition of the activity of topoisomerase II by stabilization of the topoisomerase II/DNA cleavable complex leading to the formation of DNA strand breaks (Booser and Hortobagyi 1994, Nielsen et al 1996, Richardson and Johnson 1997). However, resistance to anthracyclines appears to be multifactorial, at least \textit{in vitro}. Whilst in the clinical setting, classical MDR mediated by PGP appears to be the only mechanism of resistance, alterations in either the expression of, or structural alterations in, topoisomerase II and increased ability to repair DNA have been implicated in anthracycline resistance \textit{in vitro}, but as yet not clinically. The two isoforms of topoisomerase II can be differentially expressed, the \(\alpha\) isoform is more sensitive to DOX, so a shift in expression from II\(\alpha\) to II\(\beta\) can confer resistance and mutations of topoisomerase have also been found (Harrison 1995). Very recently, loss of mismatch repair (MMR) has been shown to be associated with low-level resistance to DOX (Drummond et al 1996) although why this should be so is unclear, perhaps the MMR proteins are able to detect the drug-induced cleavable complex or because the initial intercalation produces a distortion in the DNA which is capable of detection by MMR proteins (Fink et al 1998).
It is interesting to note that the differences in sensitivity between groups A and B is not based on differences in sensitivity to DOX, but rather to differences in their sensitivities to VCR. Group A cultures are markedly more resistant to VCR than Group B cultures.

The Vinca alkaloids act as spindle poisons by binding with high affinity to tubulin. This prevents the polymerisation of tubulin to form microtubules and depolymerises those microtubules which are already formed which leads to the arrest of cells in mitosis (Owelen et al 1976). VCR and VBL share a common pair of binding sites on each tubulin dimer which is distinct to the binding sites of either COL and epipodophyllotoxin. However, whilst PGP over-expression is an important mechanism of resistance to VCR, topoisomerase II alterations are not thought to play a role in the development of resistance to this drug. Other mechanisms which may be important include changes in the structure of beta tubulin or cellular composition of various subtypes of tubulin have been associated with the development of VCR resistance in cells without a drug retention defect. In rhabdomycosarcoma cells made resistant to VCR in vivo, the resistant cells lacked the less acidic beta-tubulins found in the parental cells but had gained three additional acidic isoforms (Houghton et al 1985). In Chinese hamster cells selected for resistance to VCR, the tubulin in the cytosolic protein derived from the resistant cells failed to bind tritiated VCR, whilst the tubulin derived from the parental cells did. Parental cell cytosols contained a single isoform of beta-tubulin whilst the resistant cells contained in addition to the parental isoform also contained three additional basic isoforms (Pain et al 1988). More recently, alterations in both the alpha tubulin isoforms (Ohta et al 1994) and beta tubulin isoforms have been observed in cell lines made resistant to taxol, an agent which stabilises polymerised tubulin into microtubule bundles (Ranganathan et al 1998).

It is clear from the data presented in this thesis that there is no evidence of cross-resistance between either VCR or DOX and CCNU. This might be expected as the major modulator of resistance to CCNU is the DNA repair enzyme O6-alkylguanine transferase (AT) (Feun et al 1994) which is not implicated in resistance to DOX or VCR. This enzyme prevents the formation of DNA interstrand cross-links by removing chloroethyl adducts from the O6 position of guanine produced by CCNU. However, AT may not be the only mechanism of resistance to CCNU. Glutathione-S-transferases (GST) are involved in many oxidation-reduction reactions within the cell and may deactivate some drugs producing drug resistance. BCNU is inactivated
by glutathione (GSH) dependant denitrosation (Smith et al 1989). Of the three classes of this enzyme, GST\(_\pi\), GST\(\alpha\), and GST\(\mu\), only GST\(\pi\) expression correlates with resistance to BCNU in high grade astrocytoma cell lines (Ali-Osman et al 1989). High expression of GST\(\pi\) has also been found using immunocytochemistry in virtually all high-grade gliomas in situ, but not in low grade astrocytomas (Grant and Ironside 1995). Increased levels of glutathiones may confer resistance to a wide range of chemotherapeutic agents by protecting the cells from free radicals produced by cytotoxic drugs. Although in colon carcinoma cells selected for DOX resistance, there were no difference in GSH levels, but there was a highly significant increase in GST\(\pi\) and GST\(\alpha\) (Peters and Roelofs 1992). Elevated levels of DNA polymerase-\(\beta\) (\(\beta\)-pol) have also been implicated in the development of resistance to nitrosoureas, presumably because this increases DNA repair after excision of damaged nucleotides (Gomi et al 1996). Increased levels of \(\beta\)-pol RNA transcripts have also been shown following exposure to cisplatin (Gomi et al 1996).

Occasionally, there are reports of chemically induced rodent glioma models with unusual patterns of cross resistance. For example, in a study using C6 cells selected for resistance to ACNU in addition to the expected cross resistance to MeCCNU, but not to other nitrosoureas, the cells also became moderately cross resistant to DOX, VBL and VP-16, but not resistant to other alkylating agents like cisplatin (Saito et al 1991). The uptake and efflux of ACNU was similar in the parental and the resistant cell lines and the lack of cross resistance to other nitrosoureas suggests that neither membrane efflux nor AT expression were involved in the development of resistance in these cells. Although topoisomerase II has not conventionally been thought to be involved in \textit{Vinca} alkaloid resistance, Taki and colleagues (1998) have recently described a sub-line of the C6 rat glioma which was produced by \textit{in vivo} exposure to increasing concentrations of etoposide, which shows cross resistance to VCR but not to DOX. These cells did not express PGP and although the total amount of the enzyme was the same in both the resistant cells and the parental cell line, there was a 16-fold reduction in topoisomerase II catalytic activity in the resistant cells. The significance of data like this is difficult to assess as such unusual patterns of cross-resistance are rarely seen in human cell lines and only then when these have been selected for resistance by culture in the presence of high doses of genotoxic drugs. The potential for the production of unusual types of DNA damage in larger amounts than that seen during clinical treatment under these circumstances suggests that these type of data are unlikely to provide much information which is useful in determining the mechanisms by which human tumours develop cytotoxic drug resistance at comparatively low levels.
Membrane glycoprotein staining

Typical membrane staining of PGP was not seen in the present study with any of the three antibodies, although some of these cultures display patterns of cross resistance which are consistent with classical MDR. From Table 3.4, it is clear that the semi-quantitative methods used to score the cultures for PGP reveal very little difference between groups A, B, C and D in terms of the quality, quantity or distribution of reaction product. Similarly tumour grade did not seem to influence staining pattern.

All three antibodies, however, did produce evidence of cytoplasmic staining. It has been suggested that in cells which exhibit only low levels of resistance, only amplification of mdr-1 RNA by reverse transcription PCR is sensitive enough to detect expression (Alvarez et al 1995). However in the present study some cultures were highly resistant to both DOX and VCR, up to $6 \times 10^4$ and $5 \times 10^5$ fold more resistant respectively than the most sensitive cultures in the study. Uniform positive cytoplasmic staining with JSB-1 and MRK-16 in drug selected cells that were 4-6 fold resistant to DNR and VCR, and plasma membrane staining in cells which are more than 10 fold resistant to these drugs has been observed (Broxterman et al 1989) suggesting that if PGP was present in the highly resistant astrocytoma cultures in the present study, it would be detectable by immunocytochemistry. The lack of membrane staining is unlikely to be the result of fixation artefact as the fixation of the cells and the immunocytochemistry protocols used in the present study were comparable to other studies using a variety of human cancer cell lines. Fixation of cells with acetone (Grogan et al 1990), acetone and ethanol (Thiebaut et al 1987), acetone or methanol (Scheper et al 1988), were successful with all three antibodies, JSB-1, C219 and MRK-16. Although with MRK-16, formalin or air drying alone may be preferred (Schinkel et al 1993). The lack of PGP expression in cell line U251MG in the present study is in agreement with Wu et al (1992) using the same three antibodies.

Why was it not possible to demonstrate the presence of PGP in cultures derived from human malignant glioma? The patterns of cross resistance suggest that Group A cultures might reasonably be expected to express PGP. Similar patterns of cross resistance have been shown in cultures derived from malignant glioma by others (Merry et al 1984) which are consistent with the MDR phenotype. Similarly, it has been possible to sensitisie these cultures to DOX using verapamil (Merry et al 1986) although in this study the two lines in which verapamil had this effect, sensitisation appeared to be mediated by two separate mechanisms one consistent with PGP acting as an energy dependent drug pump and one not.
PGP has been found to be expressed in frozen sections or paraffin wax embedded material of astrocytic tumours and astrocytoma cell lines. However, using C219 and JSB-1, Becker et al (1991) found both plasma membrane and cytoplasmic staining in between 0.3 and 15.4% (mean 4.7%) whilst using MRK 16, Matsumoto et al (1991) found that only between 6.5 and 21.3 % (mean 11.6%) stained positive for PGP. Others have found that occasional tumours stained almost completely (Henson et al 1992) and others have found that virtually all cells in a whole panel of tumours stained positive (Kiwit et al 1994). Whilst it is possible to dismiss series where there was homogeneous staining of cells across a panel of tumours as a technical artefact, this raises important questions in regard to quantifying immunocytochemical appearance in relation to drug sensitivity. For example, what level of PGP expression per cell is necessary to confer MDR and what percentage of cells must over-express PGP for the tumour or culture to be drug resistant? In a review by van der Heyden (1995) the minimum definition of PGP positivity, independent of chemosensitivity data, ranged between studies from a single cell to 30% of cells. Where drug selected cells are used there does appear to be a correlation between PGP staining and resistance (Grogan et al 1990), but little is known about the relationship in non-selected cells. Levels of cellular drug resistance may correlate to density of PGP molecules in the membrane rather than the absolute number of PGP molecules per cell (Noonan et al 1990). Using mRNA PCR techniques they showed that parental sensitive KB cells expressed one molecule of mdr-1 mRNA per cell, but 20 fold resistant KB cells selected with COL had 74 molecules per cell. However, the density of actual PGP molecules was not described and little research has been carried out in this area. Secondly, it is not known what percentage of cells, in a tumour or culture, must express PGP for resistance to be effected, but if small fractions of cells can render the tumour resistant then this may be related to the ability of the PGP expressing cells to repopulate the tumour after drug treatment.

It has been found recently that the major obstacle to the detection of PGP is the unexpectedly poor specificities of most of the antibodies available to detect PGP. These antibodies, which have been widely used in studies aimed at comparing PGP expression with drug response or with clinical outcome following chemotherapy, have been shown to have significant cross-reactivities with epitopes unrelated to drug resistance. As early as 1989, concern had been expressed about the use of C219 which had been shown to stain type I (slow twitch) class skeletal muscle fibres, tissue which is known not to contain PGP (Thiebaut et al 1989). Additional, apparent cross reactivity has also been detected in pancreatic acini, seminal vesicle and testis. Using
immunoblotting, it was possible to show that C219 reacted with an approximately 200 KD band in skeletal and cardiac muscle which migrated in the same position as heavy chain myosin. More recently, this situation has been further complicated by a report which demonstrates that C219 also cross reacts with the 185 KD c-erbB2 product in MDA-MB-435 human breast cancer cells transfected with the c-erbB2 cDNA (Liu et al 1997). Peptide sequence analysis has shown that C219 recognises a shared epitope between these two molecules (Liu et al 1997).

JSB-1, which shows marked cytoplasmic staining in the panel of cell lines investigated here, also appears to have a major cross reactivity to a protein not involved in drug resistance. The antibody appears to cross react with a 130 KD protein which is present in rat liver mitochondrial inner membrane/matrix fractions. This appears to be pyruvate carboxylase, an enzyme present in large amounts in mitochondria (Rao et al 1995). Another PGP “specific” antibody, C494, also appears to cross-react with pyruvate carboxylase, although JSB-1 and C494 appear to recognise distinct but closely proximate epitopes (Rao et al., 1994). It is therefore of significance that brain endothelial cells which often stain with JSB-1 in tissue section have 3-5 fold more mitochondria than systemic endothelial cells, necessary to support their numerous energy dependent transport mechanisms (Thapar et al 1995).

Although these antibodies cross react with proteins of different molecular weight to PGP and so are unlikely to be confused with PGP in western blotting studies, clearly their use for staining cultured cells, frozen sections or archival material has greatly been compromised. In situations where strong staining is confined to the cell membrane this is likely to reflect specific staining for PGP. However, the significance of weak homogeneous cytoplasmic or granular cytoplasmic staining is more difficult to interpret. It may well be that this reflects staining of pyruvate carboxylase. This highly conserved enzyme is found in a wide variety of prokaryotes and virtually all eukaryotic tissues (Attwood 1995, Wallace et al 1998) including astrocytes (Wiesinger et al 1997). It plays an important role in gluconeogenesis by catalysing the formation of oxaloacetate from pyruvate and HCO3-. It is interesting that despite the high levels which have been found in astrocytes, that staining with JSB-1 in the glioma cell cultures proved to be extremely heterogeneous. Some cultures had very faint, homogeneous, cytoplasmic staining whilst other cultures had very strong punctate staining either in the cytoplasm or in the perinuclear region. There was often marked heterogeneity in the degree of staining within a single culture. It may be that this is a reflection that the phenotypic and genotypic heterogeneity of malignant
glioma described earlier extends to expression of pyruvate carboxylase or it may be that some, low level expression of PGP is present in these cultures. If this does occur, it is quite plain that it is restricted to the cytoplasm of glioma cells, perhaps in the Golgi apparatus or as discrete cytoplasmic vesicles as described below.

There may be additional biological factors which could account for the differences between PGP expression in situ and in vitro. Perhaps cells which express PGP differ in some biological characteristics which make their survival in vitro more difficult. For example, the disruption of the three dimensional structure brought about by processing cells for culture might in some way down-regulate the expression of PGP. This may be the result of changes in cell to cell or cell to extracellular matrix contact. Certainly, changes in the expression of integrins has been reported in MCF-7 human breast cancer cells made resistant to DOX (Narita et al 1998) although the effect of this on PGP expression was not determined. In another study, Kaaijk et al (1996) produced so-called organotypic multicellular spheroids from fragments of fresh glioma tissue obtained during surgery on seven patients. Although these cells had never grown as monolayers, about 5% of cells within the spheroids expressed PGP and none of the spheroids expressed MRP. It may be because in vitro culture conditions remove some of the intercellular signals like growth factors which in situ might maintain PGP expression. For example, insulin-like growth factor I up-regulates PGP expression leading to the inhibition of apoptosis in MCLM colon cancer cells (Guo et al 1998) and retinoic acid and C6 conditioned medium have been shown to increase the levels of PGP in an immortalised rat brain endothelial cell line (El Hafny et al 1997). It is also possible, although there is as yet no direct experimental evidence, that PGP expressing cells, although present in the tumour, do not adapt to culture readily and are progressively lost during the initial stages of cell culture. This may be because they grow more slowly in vitro, have lower plating efficiencies or that media supplemented with foetal calf serum and consequently rich in platelet-derived growth factors are not optimal in maintaining the long term viability of cells which express PGP.

In a proportion of glioma cultures examined in the present series, there is some evidence of positive staining in the cytoplasm and this appears to be organised into discrete structures often around the nucleus. Could this be PGP expressed cytoplasmically? Initial studies suggested that JSB1 produced a cytoplasmic staining pattern which was thought to be associated with early development of low level MDR (Broxterman et al 1989, Grogan et al 1990) although these cells with cytoplasmic staining were later found to have no mdr-1 mRNA by PCR which
suggests that the antibody was recognising an antigen other than PGP (Baas et al 1990). However, more recently, additional evidence has been produced to suggest that the site of action of PGP may not solely be at the cell surface. Molinari et al (1994) have shown that PGP can be found in the Golgi apparatus of MDR cells but not sensitive cells. In the resistant cells, DOX accumulates cytoplasmically in the Golgi apparatus and this accumulation can be reversed by ATP-depletion or treatment with verapamil indicating that it is PGP which sequesters the drug. This can occur in cells which do not express PGP on their cell surfaces (Molinari et al 1998). In human myeloma cells selected in the presence of DOX and verapamil, DOX appears to accumulate in the cytoplasm in contrast to the parental, sensitive cells where it accumulates almost exclusively in the nucleus. This process correlates with a two and a half-fold increase in PGP in the cytoplasm (Abbaszadegan et al 1996). The intracytoplasmic structures which are responsible for this are probably PGP containing cytoplasmic vesicles, distinct from endocytic vesicles, in which the PGP molecules are orientated in such a way that drugs are transported and accumulate within the interior of the vesicles (Shapiro et al 1998). The net result of this sequestration of cytotoxic drug within the cytoplasm is a reduction of the net amount of free drug within the cytoplasm which could potentially reach the nucleus, effectively producing drug resistance.

Do PGP lined vesicles exist in the cytoplasm of high-grade glioma cultures and, if so, what relevance are they in modulating resistance to natural product cytotoxic drugs? In spite of the clear lack of external cell membrane staining in these cells, it is impossible to exclude the possibility of some PGP being localised in the cytoplasm of these cells, principally because of the poor specificity of at least two of the commercially available antibodies. It is interesting that MRK 16, an antibody directed against an external epitope of PGP, seems to produce some cytoplasmic staining in permeablised cells. As yet, there have been no reports of confounding cross reactivities with this antibody.

A further interesting possibility might be that contaminating PGP positive endothelial cells may be present in these tumour cultures. There do appear to be a small, but stable, sub-population of endothelial cells present in short-term cultures derived from malignant glioma (Guner et al 1977). These cells have endothelial specific Weibel-Palade bodies.

These questions will not be resolved until more extensive studies have been carried out using approaches like western blotting where the antibodies can be used in
combination with electrophoretic separation to confirm that the molecular weight of the bound antigen is 170 KD. However, care must also be exercised with this approach as pyruvate carboxylase with a molecular weight of 130 KD could be confused with the 130 KD core-glycosylated PGP, the precursor of PGP which is converted into its mature form by N-linked glycosylation (Richert et al 1988).

MRP may be involved in resistance to VCR and DOX, and although none was detected by immunocytochemistry in the present study, it is unknown how sensitive this technique is and what levels of resistance can be detected by expression of MRP. Interestingly MRP is involved in transport of drugs conjugated with glutathione (Zaman et al 1996), and this may include cationic molecules like VCR (Loe et al 1996). Resistance to DOX has also been associated with increased levels of GST isoenzymes (Tew et al 1994).

There is only a single antibody which is widely available for immunocytochemical studies of MRP. This antibody is much less well characterised that those supposedly specific for PGP and it remains to be seen if this antibody is any more specific than those directed against PGP. Interestingly, it appears that MRP appears to be present in all of a panel of unselected melanoma cell lines examined by Molinari et al (1998) and that whilst verapamil was able to alter the distribution of DOX in these cells which also express PGP cytoplasmically, two modulators of MRP function, probenecid and genistein did not.

**p53 expression**

The patterns of p53 expression in short term glioma cell cultures have been examined using three different antibodies (Table 3.5). However, the staining patterns of the three antibodies were not always consistent. It might be expected that DO-1 and PAb421 results would agree because they both recognise mutant and wild type p53. This, however was not always the case and indeed this apparent disagreement between antibodies has been demonstrated by others in high-grade gliomas using frozen sections with antibodies PAb240, PAb1801 and PAb421 (Ellison et al 1992). PAb1801 recognises an epitope at the N-terminal of p53. A comparison of the present study and Ellison's study are shown in Table 4.4.
Table 4.4 Staining patterns found with three anti-p53 antibodies.

<table>
<thead>
<tr>
<th>PAb240</th>
<th>DO-1 or *PAb1801</th>
<th>PAb421</th>
<th>No. of cases</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>—</td>
<td>—</td>
<td>2 (12.5)</td>
<td>13 (27)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>—</td>
<td>0 (0)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4 (25)</td>
<td>12 (25)</td>
</tr>
<tr>
<td>—</td>
<td>+</td>
<td>+</td>
<td>1 (6.3)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>+</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>+</td>
<td>—</td>
<td>+</td>
<td>3 (18.8)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5 (31.3)</td>
<td>16 (33)</td>
</tr>
<tr>
<td>—</td>
<td>+</td>
<td>—</td>
<td>1 (6.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>total 16 (100)</td>
<td>48 (100)</td>
</tr>
</tbody>
</table>

Numbers in brackets are percentages, *Ellison et al 1992 used PAb1801.

Similar proportions were negative for all three antibodies and positive for all three antibodies in both studies.

In the present study, of the 16 cases with results for all three antibodies, four gave different results for PAb421 and DO-1. Three of these were positive with PAb240 and PAb421 but negative with DO-1, and one was positive with DO-1 but negative with PAb240 and PAb421. The disagreement between the antibodies can be partially explained because DO-1 and PAb421 recognise epitopes at the two extremes of the p53 protein, DO-1 at the N-terminal and 421 at the C-terminal (Figure 1.9). A truncated protein could be formed as the result of a frame shift mutation causing premature stop codons and termination of transcription (Louis et al 1993). This type of mutation would explain DO-1 positive staining with PAb421 and/or PAb240 negative staining, but it cannot explain the three cases which were negative for DO-1 and positive for PAb240 and PAb421. Other explanations could be that the two antibodies only recognise specific mutations, or if the p53 protein had hidden epitopes caused by structural mutations.

In theory, using these specific antibodies to look for mutant and wild type p53 in tumour specimens should be a simple way of qualifying the presence of each form of the protein. If DO-1 and PAb421 recognised all mutant forms and wild type p53, and PAb240 recognised solely non-denatured mutant forms, the only expected staining patterns would be as shown in Table 4.5.
Table 4.5 Expected p53 staining patterns.

<table>
<thead>
<tr>
<th>DO-1</th>
<th>PAb421</th>
<th>PAb240</th>
<th>p53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>mutant only or</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>both forms</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>wild type only</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>wild type or none</td>
</tr>
</tbody>
</table>

Applying this to the 16 glioma cell cultures which were tested with all three antibodies, 4/16 (25%) expressed mutant or both forms, 1/16 (6%) had only wild type, 5/16 (31%) wild type or none, and 6/16 (38%) gave ambiguous results in that the antibody staining did not follow any of these patterns. This does not clarify whether the p53 was mutant or wild type, although the cell line U251MG which showed the strongest nuclear staining, does contain a mutation in exon 8 at codon 273, resulting in replacement of arginine by histidine (Asai et al 1994). When DO-1 and PAb421 disagree it is reasonable to assume that the p53 must definitely be mutant as there must be only one form of the wild type protein in which case the antibodies would agree. However, wild type and mutant forms do exist together, and both forms may undergo changes in tertiary protein structure which may alter antibody affinity and access to specific epitopes (Ellison et al 1992). In one study PAbl801 did not detect one particular type of nonsense mutation in astrocytomas, so underestimating the presence of the mutant form (Louis et al 1993). Perhaps as many as 18% of p53 mutations in astrocytomas may be of this type (Louis 1994).

None of the three cultures derived from grade III astrocytomas appeared to accumulate high levels of p53 protein although the culture derived from the mixed grade III oligo-astrocytoma did have significant numbers of cells which stained positive with one antibody, PAb 240. The observations are surprising as p53 mutations appear to be as common in grade III gliomas as in grade IV tumours (Bögler et al 1995).

In addition to the heterogeneity of expression which occurs between cultures, there can be a range of staining within a single tumour section or monolayer of cells, with individual cells with no p53 expression, punctate staining or dense, even staining. Disagreements between molecular analysis and immunocytochemistry are also not uncommon and usually fall into two categories: 1. False negatives, where mutations have been found through DNA sequencing but immunocytochemistry is negative, and 2. False positives where immunocytochemistry is positive but no mutations are
found. Of 34 grade II, III and IV astrocytomas results of immunocytochemistry and mutation analysis agreed in only 56% of cases (Louis et al 1993). Explanations for false negatives include truncated p53 protein (described above), or a mutation which changes the antigenicity of the protein (Louis et al 1993). Mutations may cause unstable protein due to an inability to form oligomeric complexes (Milner et al 1991). Polymorphisms detected as abnormal migration on SSCP gels may be classed as a mutation but in fact may not code for a mutant protein (Louis et al 1993), but DNA sequencing would be required to determine this.

False positives may occur because mutations outside the hotspot regions of exons 5-8 are not found when only these exons are studied. However, this would not account for many cases of false positives because less than 5% of mutations are found outside of this region (Hollstein et al 1991). False positives also arise if DNA from normal tissue around the tumour or tissue with heterogeneous expression of p53 is sequenced, normal sequences will mask any mutations (Dix et al 1994). However, with well separated, optimized SSCP analyses as little as 1.5% mutant DNA when mixed with normal DNA can be detected (Dix et al 1994). When heterogeneously staining tissue was studied good correlation between immunocytochemistry and mutational analysis was found if >5% of cells were positive for p53 with immunocytochemistry (Kyritsis et al 1996).

Without confirmation by DNA sequencing of the presence of mutations in the p53 gene, it is unwise to assume the presence of mutant p53 only on the basis of immunocytochemistry with a single "specific" antibody. However, this does not completely invalidate the technique because important information about the site and the 'amount' of p53 and the heterogeneity of accumulation can only be assessed using immunocytochemistry, whereas the presence of a mutation in a gene found using molecular techniques does not reveal whether the gene is expressed at all.

Although in the present study p53 accumulation was seen in both the cytoplasm and nuclei, only nuclear staining was scored. This was deemed necessary to enable quantification of the staining and because the nucleus is the site of action for p53 (Shaulsky et al 1990a). Similar patterns of p53 expression to those in the present study have been found in a variety of malignant tumours, including homogeneous, heterogeneous and focal staining (Porter et al 1992). In their study tumour cells were considered positive if they had nuclear positivity, although some cytoplasmic staining was noted. Similarly to the present study, discrepancies between some of
the antibodies, in this case PAb1801 and PAb421, could not be explained. They also found that compared to the other antibodies, PAb240 gave more intense staining.

The present study has shown heterogeneous staining between cells within individual short term cultures (Table 3.2), and this may be due to subsets of cells with differential expression of the p53 gene. Elevated levels of wild type p53 in growth stimulated Balb/c 3T3 cells accumulated in the nucleus during S phase (Shaulsky et al 1990b), and similar heterogeneity in nuclear staining has been shown in three different glioblastomas all with the same, known mutation, indicating that both mutant and wild type p53 expression can be cell cycle specific (Louis et al 1993 and Ali et al 1994). Heterogeneous staining in the present study may be due to the fact that the cultures were not synchronized and therefore does not necessarily indicate the presence of mutant p53.

The fact that mutant p53 has an increased half life means it is more likely to accumulate in the cells and give positive results with immunocytochemistry. In contrast, normal levels of wild type p53 protein may be undetectable using this technique (Iggo et al 1990, Porter et al 1992 and Ali et al 1994). Studies comparing immunocytochemistry and molecular analysis of the p53 gene in gliomas have shown differing results, Louis et al (1993) and Rubio et al (1993) found that although 30% of astrocytomas were positive with PAb1801, these were negative with the mutant specific PAb240 and did not have mutations in the p53 gene indicating that this was wild type p53 accumulation. Gene mutations have also been identified in 6 of 17 (35%) glioma cell lines with increased p53 protein levels (Anker et al 1993). From these studies it appears that similar proportions of gliomas express either wild type or mutant p53, and the present study showed a similar amount of positive staining with DO-1, 6 of 21 cultures, 29% (see Table 3.2), suggesting that both forms are being expressed (Martinez et al 1991).

If some of the accumulated p53 in the present study is in fact wild type then it must be non-functional although by what mechanism this has occurred is not clear. The elevated levels of p53 observed may not be the result of over-expression but of stabilisation. The half life of p53 in cells expressing the viral protein E1A has been shown to exceed 2 hours, and be detectable by immunocytochemistry although p53 gene transcription was not increased (Lowe et al 1993). Other proteins which are able to bind and stabilise p53 have been found, including those associated with viruses like SV40LT, human papilloma virus (HPV) E6 and adenovirus E1B (Reich and Levine 1984, Debbas and White 1993), and cellular proteins including MDM2 and
heat shock protein 70 (Oliner et al 1992, Finlay et al 1989, Martinez et al 1991). Over-expression of MDM2 was found in cell lines derived from GBMs with no p53 mutations (Reifenberger et al 1993). However, these cell lines were negative for p53 protein implying that the half life of p53 was not increased by complexing with MDM2 (He et al 1994). Stabilisation may also take place between mutant and wild type p53, rendering the wild type non-functional (Martinez et al 1991). Another protein which has recently been found to interact physically with p53 is p33, the product of a tumour suppressor gene ING1. p33 co-precipitates with p53 on immunoblots but unlike MDM2, p33 cooperates with p53 in transcriptional activation (Garkavtsev et al 1998). Chromatin may also stabilise p53 in the nucleus of transformed cells (Rotter et al 1983). However, the possibility of p53 stabilisation by virus-derived proteins in gliomas is unlikely as transforming viruses are not thought to be implicated in the pathogenesis of these tumours in humans (Anker et al 1993, Louis et al 1993).

Of course, tumours where p53 protein cannot be detected may have normal levels of unstabilised wild type protein, a mutant form not detected by antibody or may have homozygous deletion of the p53 gene (Porter et al 1992). However, in the present study any accumulation of p53 protein is plainly non-functional and for all intents and purposes functions as a mutant form.

Subcellular localisation of p53
Both nuclear and cytoplasmic accumulation of p53 has been observed in paraffin embedded human glioblastomas with known p53 DNA mutations (Ali et al 1994). The localisation was dependant on whether mutant or wild type p53 was present and also on the type of mutation. Indeed, different tumours with the mutation in the same codon of p53 had different intracellular distributions, some localising in the nucleus and some in the cytoplasm, suggesting that intracellular micro-environment may also be important in p53 localisation (Ali et al 1994). In those cells with one normal and one mutated p53 allele, the cytoplasmic staining may be due to the wild type p53 being sequestered by the mutant form. Cytoplasmic staining was also noted in 3 of 12 GBMs each with 1 wild type and 1 mutant p53 gene, but they contained disproportionately more wild type than mutant, so in this case it would be unlikely that the mutant form was acting in a dominant negative manner (Saxena et al 1992). MDM2 may be involved in the cytoplasmic sequestering of wild type and mutant p53 (Oliner et al 1992) preventing p53 reaching its site of action (Moll et al 1992). The cytoplasmic staining seen in the present study could be due to
this sequestering, but as cytoplasmic staining was always found together with strong nuclear staining this explanation is unlikely.

Have these cell lines acquired mutations as a result of in vitro culture?

It is unlikely that the cell lines used in the present study acquired p53 mutations as a result of prolonged culture as it has been shown that the p53 gene locus usually remains genetically stable during long term cell culture. In one study, five of six glioma cell lines contained a mutation identical to that in their respective primary tumours despite having reached passage levels as high as 221 (Anker et al 1993). Additionally, two of these cell lines derived from tumours which were heterozygous for p53 mutation had maintained the normal allele over 6 years in culture. This preservation of p53 gene status indicates that the cultures in the present study are likely to express the same p53 as the tumour in situ. Similar results have been obtained with a number of other cell systems including murine hepatomas which did not develop p53 mutations on prolonged culture in vitro (Kress et al 1992) and CML cell lines which maintained identical p53 mutations to those found in fresh cells taken from patients (Sen et al 1995). In the case of astrocytomas, which are heterogeneous in nature, the presence of p53 mutations in cell cultures derived from tumours with apparently only wild-type p53, may not indicate introduction of new mutations but a selective expansion of those clones containing a p53 mutation which at low levels are difficult to detect using conventional means. Using a yeast p53 transcription assay it has been possible to show that in a single case of a primary astrocytoma without an apparent p53 mutation, the tumour recurred with 76% of cells containing mutant p53 (Tada et al 1996).

Primary (de novo) or secondary GBMs?

Twenty-one of the 26 short term cultures in the present study were derived from Grade IV astrocytomas (GBMs). Only one case had evidence of a pre-existing tumour which was either a ganglioglioma or a gliosarcoma rather than a low grade astrocytoma. Therefore 20 of 21 GBMs, having no clinical history of previous astrocytic tumours, have probably arisen de novo. These may comply with the dual pathway theory of GBM development (Kleihues et al 1993). The theory suggests that 1° GBMs have no p53 mutation, of the 20 'de novo' GBMs, 15 were assessed with mAb DO-1 and only 4 of these over-expressed p53 protein, therefore 11 of 15 probably expressed normal p53 which is usually undetectable with immunocytochemistry, and could be classed as 1° GBMs. Despite the unknown EGFR status, it appears that the majority of the GBMs in the present study fit with
this dual pathway theory. Unfortunately, not all GBMs will fall into one or the other category, approximately one third have p53 mutations, one third have EGFR amplification and one third have neither of these genetic changes (Louis and Gusella 1995). If the 4 of the 15 'de novo' GBMs over-expressing p53, do not prove to have p53 mutations in future studies and the over-expression implies that wild type protein is not acting normally, then this would indicate that other mechanisms may be controlling tumourigenesis in these cells.

**Relationship between p53 expression and chemosensitivity *in vitro*.**

In the present study there is a positive correlation between increased levels of p53 in the nuclei and increased sensitivity to CCNU and VCR, but not DOX (see Table 3.6). It has been proposed that if normal p53 is inactivated by mutation, stabilisation or deletion, then the cells become more resistant to a wide range of cytotoxic drugs and radiation. Fan et al (1994) showed that in human lymphoma cells, gamma rays, nitrogen mustard, cis-platinum and etoposide caused strong G1 arrest in cells with normal p53, and if there was sufficient DNA damage which could be repaired during the G1 arrest, p53 would initiate apoptosis. On the other hand, cells with mutant p53 did not undergo G1 arrest or apoptosis. Using a larger panel of 28 anticancer agents the sensitivity of 34 high grade astrocytomas was assessed using FACS analysis to determine the proportion of apoptotic cells it was found that the mean number of effective agents against cells with mutant p53 was only 0.58, as compared to 5 effective agents effective against cells with wild type p53 (Iwadate et al 1996). These results fit in well with the simplified model for normal p53 activity within the cell described in the Introduction (Figure 1.7).

However, the findings presented in this thesis seem to contradict this. Accumulation probably as a result of mutation appears to correlate with sensitivity to two drugs, CCNU and VCR but not DOX. An interesting theory to explain this would suggest that it is possible that cells with mutant p53 which are treated with DNA damaging drugs may continue through the cell cycle without arrest and because no DNA repair can take place these cells, if there is enough DNA damage, rapidly undergo apoptosis. If there is only wild type, functional p53 present, any damage may be repaired before progression through the cell cycle and so these cells would appear resistant. This can clearly explain the sensitivity to CCNU, but it is more difficult to explain why this produces sensitivity to drugs like VCR which do not target DNA directly The exact mechanism by which the amount of DNA damage is detected and how this leads to increased expression of p53 to either
activate G1 arrest and repair or induce apoptosis is unknown. It is therefore possible that it is not the amount of DNA damage which is the triggering factor but the down-stream consequences of this damage perhaps reflected in disruption of the mitotic apparatus or even changes relating to cell proliferation which are detected.

There is now a body of information which suggests that p53 inactivation may be, at least in certain tissues, a predictor of chemosensitivity. Three studies by Petty et al (1994) are in agreement with the present study. Firstly, the mouse prostate R2 cell line, with a p53 mutation and activated ras, when compared to an R2 cell line without the p53 mutation, was more sensitive to cisplatin, 4-hydroperoxycyclophosphamide (4-HC), DOX, 5-fluorouracil (5-FU), methotrexate and VCR. Secondly, similar results were found using homozygous p53 disrupted fibroblasts. Thirdly, breast tumour extracts with p53 mutations were also more sensitive than those with normal p53. It was suggested that in the R2 mutant p53 cell line which had a very short doubling time, and had been multiply passaged, p53 disrupted fibroblasts may have increased karyotypic abnormalities which may explain the increased sensitivity. The increased sensitivity to VCR was explained by the possibility of increased mitotic failure in the presence of spindle active agents along with the increased karyotypic abnormalities. Petty et al (1994) also explained the apparent sensitivity to alkylating agents of breast tumour extracts with p53 mutations, by the fact that clinically, sensitivity is only short term and may not persist as the p53 mutation leads to genomic instability and enhanced genomic mechanisms of resistance. However, genomic instability is just as likely to produce mutations that cause drug sensitivity. The present study has shown that the over-expression of p53, and the corresponding chemosensitivity are not short term phenomena in high grade astrocytomas, because drug sensitivity was maintained over several cell passage levels (Figures 3.3 No8, 3.4 No4, and 3.5 No19) thus indicating that genomic instability, if present, does not affect chemosensitivity in this system.

The sensitivity of human foreskin fibroblasts and mouse embryo fibroblasts to taxol, carboplatin, cisplatinum and melphalan has been shown to be enhanced in cells where the wild type p53 had been inactivated (Wahl et al 1996, Hawkins et al 1996) although this does not occur in EBV-immortalised lymphoblastoid cells derived from patients with Li-Fraumeni syndrome which carry heterozygous germ line mutation for p53 (Delia et al 1996). This has lead to the suggestion that p53 inactivation has different effects on chemosensitivity in different types of cells (Wu and El-Deiry 1996). It may also mean that the outcome with regard to drug
sensitivity might be different in tumour cells than normal cells which have artificially disrupted p53 genes. It is known that high grade gliomas have a complex pattern of molecular genetic changes which have been outlined earlier in this thesis. The intracellular effect of this is superimposed over the effect of inactivated p53 gene and this may be the reason that sensitivity to certain drugs, but not others, correlates with inactivation of the p53 gene in malignant glioma cultures. This is likely to be further complicated by the recent observation that the position of the mutation within the p53 gene influences whether a cell will become more resistant or more sensitive to a particular drug (Wang et al 1998)

There is also the question of how p53 inactivation brings about cell death in cells which have developed resistance to cytotoxic drugs. Some mutant p53 human lymphoma cell lines which were resistant to nitrogen mustard are relatively sensitive to etoposide whilst for other cell lines the reverse was true (Fan et al 1994). This inverse relationship was not seen in the wild type cells with normal p53, and could be partly explained if the nitrogen mustard resistant cells had increased topoisomerase II levels. This would enhance DNA cross link repair, but there would be increased cell kill with etoposide because of increased levels of etoposide-stabilised topoisomerase II-DNA cleavable complexes. Disruption of p53 by transfection with HPVE6, or mutant p53 gene, sensitised breast cancer cells to cisplatin but not to other DNA damaging agents. The non-transfected cells had normal p53 function but did not undergo p53 dependent apoptosis. DNA damage caused by cisplatin is repaired by nucleotide excision therefore in these cells, enhanced sensitivity was explained by lack of G1 checkpoint control or defective DNA repair, or both (Fan et al 1995). Lack of G1 checkpoint may be overcome by activation of G2 checkpoint delaying entry into mitosis. In some breast cancer lines apoptosis does not readily occur despite normal p53 expression, and this may also be the case with malignant astrocytomas.

Another suggestion is that the effect of p53 inactivation may be different for different types of cytotoxic drug. For example, the sensitivity of drugs which damage DNA directly are likely to be enhanced by p53 mutation while mutation in the p53 gene would decrease the sensitivity of cells to drugs like antimetabolites which do not damage DNA and require p53-dependent induction of apoptosis (Mueller and Eppenberger 1996).

Human testicular teratomas have been shown to express very high levels of nuclear p53 protein although they never have p53 mutations (Schenkman et al 1995). These
tumours are extremely sensitive *in situ* to platinum-based chemotherapy and over 90% of patients with these tumours are cured. Studies on murine teratocarcinoma cell lines which also accumulate p53 protein in the absence of any mutation in the gene indicates that this protein is functionally active and following treatment with DNA-damaging agents the inactivation of p53 transcriptional activity is reversed producing apoptosis (Lutzker and Levine 1996). This would certainly explain the marked sensitivity of teratomas to cytotoxic chemotherapy. It is possible that some of the glioma cultures in the present study accumulate p53 protein in the same manner. However, in contrast to teratomas, p53 mutations are a common feature of malignant astrocytomases and others have shown that there is a strong correlation between large numbers of cells with positive nuclear staining and p53 mutations in high grade gliomas *in situ* (Baxendine-Jones et al 1997). This suggests that the protein detected in the present study is mutant and that if accumulation of wild-type p53 protein occurs it is only likely to be in a small sub-population of gliomas.

Many factors are involved in the cell death regulatory pathway (Figure 1.8) and the role of some of these in malignant glioma is now becoming clear. An early step in the cell death pathway is transcriptional activation of WAF1 by wild type p53 as a response to DNA damage. WAF1 blocks the cell cycle at G1 by inhibiting cdk/cyclin complexes. Cells lacking normal p53 activity cannot induce WAF1 in response to DNA damage, however, WAF1 can be induced independently of p53 via growth factor stimulation including PDGF and fibroblast growth factor (FGF). This may be a feedback system as over-expression of WAF1 causes growth arrest (Bögler et al 1995). WAF1 is over-expressed in the nuclei of high grade astrocytomases, it is not found in mitotic cells, in GBMs over-expression occurs with both mutant or wild type p53, in astrocytomase grade III WAF1 is only over-expressed if p53 is wild type. This implies that in GBMs p21 (the gene coding for WAF1) is activated independently of normal p53. High grade astrocytomases often over-express growth factors, which may in turn induce WAF1 which blocks proliferation. Therefore therapies targeting proliferating cells in these tumours are likely to fail (Jung et al 1995). Cells over-expressing WAF1 may also appear drug-resistant because WAF1 may protect cells from drug induced p53 regulated apoptosis (Gomez-Manzano et al 1997). Studies to find the crucial differences in elements of the cell death pathway between normal and cancer cells are complicated by dual amplification of some genes involved. For example in GBMs, MDM-2 is co-amplified with SAS and CDK4, all found in close proximity on chromosome 12. The protein encoded by SAS may be important in signal transduction and growth control (Reifenberger et al 1994).
Bcl2, which inhibits the apoptotic pathway, has been found in 7 of 7 human gliomas expressing wild type p53 but in only 1 of 7 which expressed mutant p53 (Alderson et al 1995). Implying that high grade astrocytomas with wild type p53 do not necessarily have functional cell death pathways. Bcl2 dimers or Bcl2/bax dimers are both anti-apoptotic, but bax dimers are pro-apoptotic therefore the ratio of expression of these two factors is crucial. The phosphorylation of bcl2 also leads to loss of function, this increases apoptosis. The drug taxol which inhibits microtubule depolymerisation also induces bcl2 phosphorylation. VBL and VCR also induce bcl2 phosphorylation and therefore cell death (Haldar et al 1997). Other drugs that damage DNA do not induce bcl2 phosphorylation but induce apoptosis through a different mechanism involving p53. Phosphorylation of bcl2 may explain VCR sensitivity found in p53 over-expressing cultures in the present study. The cell death pathway may be induced by factors other than p53, and the phosphorylated bcl2 could not then block this pathway. The presence of bax would also increase the likelihood of the pathway progressing to cell death.

Some of these pathways leading to apoptosis are shown in Figure 4.1. How these other factors and pathways may affect chemosensitivity is only beginning to be assessed, although recent studies suggest that factors which block apoptosis may be candidates for therapeutic intervention in the future. Fas is a cell surface cytokine receptor protein of the nerve growth factor/tumour necrosis factor (TNF) superfamily. Fas-ligand (FasL) a cytotoxic cytokine similar to TNF, can trigger apoptosis via Fas. A cytotoxic Fas antibody was used in one study to show that a critical level of cell surface expression of Fas is necessary to induce apoptosis in malignant glioma cells in vitro. Low levels of Fas expression were found in 3 of 7 cell lines tested, these three were resistant to the cytotoxic antibody and expressed a non-functional Fas lacking a trans-membrane domain (Weller et al 1995).
Figure 4.1 Factors involved in the apoptotic pathway.

Programmed Cell Death Pathway

Red arrows indicate induction of apoptosis, Blue arrows indicate blocking of apoptosis. Caspase cascade includes 10 members. Two are shown, Caspase 1 = ICE, Caspase 2 = ICH
Additionally a synergistic effect has been shown between Fas-ligand and individual chemotherapeutic drugs like DOX, VCR, etoposide and BCNU, in cell lines with no wild-type p53 activity and/or increased bcl-2 expression, both often associated with chemoresistance (Roth et al 1997). This synergistic effect could potentially be exploited in vivo, inducing growth inhibition or even cell death. Another synergistic effect between the drug cisplatin and apoptosis factor ICE (Interleukin-1 beta converting enzyme) has been shown in malignant glioma cells (Kondo et al 1995). Cisplatin was found to induce the expression of ICE, which is a member of the pro-apoptotic caspase cascade.

One original aim of the present study had been to determine if mutation in the p53 gene resulted in stimulation in the MDR1 promoter, producing elevated levels of PGP. In the present study there is no evidence that those cultures which accumulate p53 protein within their nuclei, almost certainly a sign of p53 mutation, differed in their pattern of expression of "PGP" as evidence by staining with any of the three anti-PGP antibodies. There was no evidence of either increased numbers of cells with reaction product, an increase in the amount of reaction product per cell or any evidence of a redistribution of reaction product, for example from the cytoplasm to the cell membrane. Despite the shortcoming discussed above of the reagents which are used to detect PGP in cell culture, there is little or no evidence for alterations in the p53 gene influencing the expression of PGP in human malignant glioma in vitro. Since the original description of an up-regulation of PGP expression in NIH 3T3 cells in response to alterations in the p53 gene (Chin et al 1992), others have confirmed that this can occur in human chronic lymphocytic leukaemia (Wallner et al 1994), some colorectal cancers (de Kant et al 1996, Oka et al 1997) and primary breast cancer (Linn et al 1996), although others have failed to confirm this (De Angelis et al 1995) However, it is clear that in other types of human cancer there is no association between these factors, for example in B-cell chronic lymphocytic leukaemia (el Rouby et al 1993), myelodysplastic syndromes (Preudhomme et al 1993), endometrial and cervical tumours (Schneider et al 1994), hepatocellular carcinoma (Soini et al 1996), epithelial ovarian carcinoma (Schneider et al 1997). Perhaps the effect of p53 mutation on MDR-1 over-expression is cell type specific.

Chin et al (1992) also reported that mutated c-Ha-Ras-1 was also capable of upregulating MDR1 gene expression although unlike p53 this was not specific for MDR-1. This is unlikely to be particularly important in human malignant gliomas where mutations in c-Ha-Ras-1 are uncommon (Fults et al 1992), although activated Ras GTP is often elevated in malignant glioma cell lines and surgical specimens and
may be an important factor influencing the growth of malignant gliomas (Guha et al 1997).

Clinical correlations
Is there a relationship between in vitro studies using cell cultures derived from malignant glioma and clinical outcome? For example, is in vitro chemosensitivity testing of predictive value to individuals and can it be used to aid future treatment programmes? Although this was not a primary aim of this study, a number of other studies have shown that in vitro tests are predictive of treatment response in patients with malignant glioma. Kornblith et al (1981) were able to show in a small group of patients treated with BCNU or CCNU that using a cell counting assay there was a strong correlation between in vitro sensitivity and clinical outcome. Of the 9 patients sensitive in vitro (≥25% cell kill at 6.6μg/ml BCNU), 6 of them (67%) responded clinically, whereas all 5 patients whose tumours were resistant in vitro (<25% cell kill at 6.6μg/ml) failed to respond clinically. Similar results were presented by Rosenblum et al (1983) using a clonogenic assay. Of the 12 cultures sensitive in vitro, 5 of 12 (42%) patients responded clinically, whereas again all 10 (100%) of non-responsive patients showed resistance in vitro. When five patients who had received chemotherapy before biopsy were excluded 5 of the 7 (71%) remaining patients responded as predicted by sensitivity in vitro, and 6 of 6 (100%) non-responders were predicted by resistance in vitro.

In these studies, response to chemotherapy was determined on the basis of neuro-radiological changes, although there is now evidence that there is little or no relationship between transient responses seen immediately after chemotherapy and patient survival (Grant et al 1997). However, more extensive studies of the relationship between chemosensitivity testing and outcome in patients with malignant glioma have shown that an in vitro test based on 35S-methionine uptake has demonstrated that there was a relationship between sensitivity to either CCNU or procarbazine and length of relapse free interval (RFI). In that study of 40 patients treated with procarbazine, CCNU and VCR, patients whose tumour cells were sensitive to PCB and/or CCNU in vitro, had significantly longer RFIs than those who did not respond in vitro. No similar correlation was found with VCR (Thomas et al 1985). Chemosensitivity appeared to be an independent prognostic variable as this correlation was as the result of imbalances in known prognostic factors such as patient age or exact histological grade.
In the present study there was no correlation between *in vitro* sensitivity to any of the three drugs and survival or relapse free interval (RFI) in five patients on whom there was chemosensitivity data at the time of diagnosis and whom subsequently underwent chemotherapy as part of their routine therapy. It was not surprising that it was not possible to detect a relationship in such a small group of patients. These patients were also treated at several different centres with differences in their total doses of radiotherapy, number of courses of chemotherapy and differences in their clinical features. One patient had a grade IV astrocytoma which apparently evolved from a ganglioglioma/gliosarcoma mentioned previously.

There was however a marked relationship between p53 staining and clinical outcome at least for two of the antibodies used, DO-1 and 421. This does not seem to be because p53 status is a consequence of patient age, a known prognostic sign in patients with malignant cerebral glioma.

p53 staining has been examined as a possible independent prognostic variable in a large number of series of patients using both frozen sections and archival material. Most of these studies have relied on using a variety of antibodies to stain sections in order to determine the proportion of positive nuclei. In most cases, it is not clear what criteria were used to designate a nucleus as positive and individual authors have been inconsistent as regards the number of stained nuclei that are required to designate a particular sample as positive. The overall results of these studies indicate that p53 staining is not an independent prognostic variable (Chozick et al 1994, al Sarraj and Bridges 1995, Danks et al 1995, Ellison et al 1995, Cunningham et al 1997). A small number of studies do find that p53 staining was predictive of survival, although many of these studies used cut off points as low as 1% positive cells to designate a tumour as p53 positive (Soini et al 1994, Korkolopolou et al 1997).

It seems paradoxical that p53 staining predicts sensitivity to CCNU and VCR *in vitro*, but also is indicative of poor survival in these patients. This to a great extent is likely to be selection problems with the patients used in this study. Of the 26 patients studied in the chemosensitivity experiments only 5 received post-radiation chemotherapy and this in a somewhat inconsistent manner at different centres (see above). One of the cases who had a short survival, and over expressed p53 was the culture derived from the ganglioglioma/gliosarcoma. It does seem that excluding this case, only one patient, IN1979 who had high levels of p53 protein actually received chemotherapy. It seems likely that whilst p53 accumulation produces chemosensitivity *in vitro*, this does not predict survival unless the patient receives
adequate chemotherapy. A biological reason for this might be that increased p53 accumulation produces enhanced tumour growth which enhances the effects of chemotherapy if it is given but if not, produces early tumour recurrence. There is some evidence that p53 accumulation and Ki-67 labeling index are linked in situ (Jaros et al 1992), although in the present study there is no evidence that p53 accumulation is linked with population doubling time in vitro.

**Relationship between tumour recurrence and drug resistance**

A major aim of this project was to determine if the near diploid cells with over represented numbers of chromosomes 7 and 22 which have been identified in vitro can be found in biopsy material from malignant glioma and also to determine if these cells were present in greater numbers in tumours at the time of recurrence. As these cells in vitro were often resistant to BCNU and malignant gliomas at the time of recurrence are also refractory to further treatment with nitrosoureas, the identification of this minority sub-population of cells present in the tumour at the time of diagnosis is likely to be of considerable importance. In vitro these cells can be identified in cultures from about a third of all malignant gliomas, although the proportion of these cells within a tumour can be variable constituting between 2 and 36% of cells in the primary cultures (Shapiro et al 1993). However, treatment of these cultures with repeated and increasing doses of BCNU increases the proportion of these cells in vitro (Pu et al 1983). In vitro studies have shown that these cells with additional copies of chromosomes 7 and 22 produce a polypeptide growth factor similar to platelet-derived growth factor (Scheck et al 1993). As the genes for the A and the B chain of PDGF are located on chromosomes 7 and 22 respectively this leads to an attractive hypothesis to explain why high grade gliomas recur. They recur because a minority cell population of near diploid cells, with additional copies of chromosomes 7 and 22 which are resistant to BCNU and in an autocrine manner are able to respond to PDGF, are present in the tumour in small numbers at the time of diagnosis. Following treatment with BCNU or simply the passage of time, these cells grow up to repopulate the tumour.

It remains an important question to determine if this process is a cell culture artefact. The process of cell culture is often accused of significantly changing the cells compared to their in vivo state, for example by selection of those cells most suited to the in vitro culture environment. It has been suggested that cells lose gross structural chromosomal abnormalities, because of the inability of cells containing these abnormalities to survive in vitro. Presumably because cells with very
abnormal karyotypes are likely to deplete DNA repair mechanisms and bring about apoptosis. In a sequential study of cultures prepared from eight untreated GBMs, 2-3 day direct cultures were compared karyotypically with 4-15 day cultures and whilst in all eight cases, the 2-3 day cultures contained cells with structural abnormalities these were present in only four of the eight 4-15 day cultures (Li et al 1997). There is more evidence, however, that suggests that the converse is true, for example, stability of p53 mutations have been shown to be maintained over very long periods of culture (Anker et al 1993). Similarly, Westphal et al (1994) have shown that the number of random aberrations per metaphase were similar in low and high passage cultures, ranging from 7-23 and 6-36 respectively. In order to test the in vitro observations that chromosomes 7 and 22 were over-represented in human malignant glioma at the time of recurrence a panel of 13 paired samples from the same patients with malignant glioma were examined for changes in the numbers of these chromosomes using alpha satellite probes which can be applied to interphase nuclei without the need for an intervening period of culture. This study represents the first systematic examination of ploidy levels for these chromosomes in paired samples of human malignant glioma.

Chromosome 7
The numbers of copies of chromosome 7 which were gained by recurrent samples is consistent with the increased number seen by Shapiro et al (1993) in cultures which have been treated with BCNU. Trisomy of 7 is common in samples both at the time of diagnosis and at recurrence, there appeared to be two patterns of chromosome 7 gains. Firstly, there are some tumours which appeared to have approximately no more than 3 copies of chromosome 7 at the time of diagnosis and at recurrence, although the proportion of those cells with trisomy 7 was higher at the time of recurrence. In these cases there are few if any cells with more than three copies of chromosome 7 at either diagnosis or recurrence. The other group of tumours often have multiple copies of chromosome 7 with perhaps half the nuclei having more than 3 copies of this chromosome. In these samples, there was usually also a tendency to have larger numbers of chromosome 7 present at the time of recurrence.

Although it is not possible to make comments about the ploidy of these tumours with respect to any chromosomes other than 7 and 22, it is extremely likely that chromosome 7 was over-represented in these samples, because monosomy Cm22 and trisomy Cm7 occurred in the same specimens, (144/84, 259/82, 794/89 and 291/87). This pattern of Cm22 monosomy and Cm7 trisomy cannot be the result of a
simple polyploidisation and is most likely because these cells are near diploid, with the loss of one copy of Cm22 and the gain of one copy of Cm7.

Over-representation of complete copies of Cm7 is often found in malignant astrocytomas, yet the significance of this is not understood. In three different solid samples of GBM, using FISH with a centromere probe to Cm7, 3 signals were seen in 23-38% of tumour nuclei, 4 signals in 4-17% and 5 signals in 4-6%. In the endothelial cells in the samples 5-8% of nuclei also exhibited trisomy Cm7 and 2-6% had 4 copies (Pylkkänen et al 1995), similar results for normal tissue have been shown (Sauter et al 1996). In a similar study of 28 samples of Grade II, III and IV astrocytomas gains of Cm7 were found in all grades, as were occasional nuclei with 5 copies (Perry et al 1997). More than four copies of Cm7 were also found in the present study. Over-representation of Cm7 is not however specific to astrocytomas. Cm7 polysomy is found in other cancers and may simply be a marker of neoplastic transformation (Perry et al 1997), as less than 5% of normal tissue has Cm7 polysomy (Sauter et al 1996). There also appears to be a link between gain of Cm7 and loss of Cm10, monosomy 10 is uncommon without trisomy 7, although this is not true vice versa (Hecht et al 1995). Monosomy is only seen in grade III and IV astrocytomas (Perry et al 1997), suggesting that loss of Cm10 occurs after gain of Cm7 and a co-operative mechanism may be involved. Clinical significance of these genetic changes is unclear, in one study gain of Cm7 did not predict survival yet loss of Cm10 did (Perry et al 1997), in another neither change was clinically significant (Bigner et al 1988).

Although other chromosomes are over-represented in high grade astrocytomas these are at much lower levels than Cm7, for example of 71 malignant astrocytomas with numeric changes, 33 (45%) had 3 or 4 copies of Cm7 compared to only 6 (8.5%) with extra copies of other chromosomes usually chromosomes 19, 20 and 21 (Scheck et al 1993). The persistence of this Cm7 over-representation suggests that it must convey some advantage to the cell. Presumably it is simply the additional number of chromosomes which produces the growth advantage through a gene dosage effect with three or four additional copies of chromosome 7 producing three or four times the basal levels of growth factors and thereby enhancing the growth of those cells.

The presence of the gene which codes for the PDGFA chain located at 7p22 suggests one potential route by which these cells develop a growth advantage. Platelet-derived growth factor appears to be an important mitogen in malignant astrocytomas. PDGF is a 30 kDa protein which consists of disulphide bonded dimers
of A and B chains. PDGF binds to two receptors, the alpha and beta receptor coded for by genes on Cm4q and Cm5q respectively. PDGF A-chains bind to the alpha receptors whilst the B chains bind to both the alpha and beta receptors (Raivich and Kreutzberg 1994). Although both chains are produced by glioma cells, PDGFA is over-expressed to a greater extent than the B chain in glioma cell lines (Mapstone et al 1991, Nister et al 1991, Hermanson et al 1992). Over-expression of PDGFRα RNA has been found in all grades of astrocytoma, but highest in GBM. DNA amplification was not the mechanism responsible for over-expression. Tumour malignancy may correlate with the expression of the ligand not the receptor, because PDGFRα is expressed in all grades of astrocytoma but PDGFA and/or B chains are increasingly expressed as malignancy increases (Maxwell et al 1990, Mapstone et al 1991).

The presence of the c-erb B gene which codes for EGFR located at 7p12 suggests an alternative or potentially complementary mechanism by which these cells with additional copies of chromosome 7 develop a growth advantage. However, over-expression of EGFR in GBMs is not necessarily related to trisomy 7. Sauter et al (1996) found that there was no association between Cm7 copy number and either the proportion of EGFR amplified cells, the average number of EGFR signals per cell, or the average EGFR copy number per Cm7 centromere. Polysomy of Cm7 has also been found in cells where EGFR is not amplified (Sauter et al 1996). Similarly, EGFR amplification has not been shown to correlate to shorter relapse free interval in patients with GBM (Weber et al 1996). The role of EGFR amplification in modulating cell proliferation in GBMs in situ and in vitro (Sauter et al 1996) remains unclear. There also seems to be a mutually exclusive relationship between PDGF related mitogenesis and EGFR expression. In most astrocytomas there is over-expression of one of the two growth factor receptors, but only one case of 83 was found with both PDGFRα expression and EGFR amplification (Hermanson et al 1996). PDGFRα expression correlated with LOH on 17p and mutation in remaining p53 gene, suggesting further evidence for genetic subsets of astrocytomas, one group with EGFR amplification plus LOH on Cm 10, and another group with LOH on 17p and/or PDGFRα over-expression (Hermanson et al 1996).

A third growth factor gene on chromosome 7 which may have relevance to the pathogenesis of malignant glioma is hepatocyte growth factor (HGF) or scatter factor and its receptor the product of the c-Met oncogene both of which map to chromosome 7. HGF gene maps to chromosome 7q21.1 (Fukuyama et al 1991) and the gene for its receptor, is located a 7q21-q31. C-Met appears to be expressed in neoplastic glial cells (Nabeshima et al 1997) and the gene has been shown to be
amplified in biopsy samples (Muleris et al 1994, Collins 1995). Others have reported that it is most abundant in cells in both the vascular rich and peripheral regions of the tumour but not in cells with distinctly malignant features (Hirose et al 1998). It may also be involved in promoting tumour cell migration (Koochekpour et al 1997, Lamszus et al 1998). It has also been shown that HGF and c-Met are both expressed in human glioma in situ and in vitro (Koochekpour et al 1997). This suggests that polypoidy of Cm7 might result in increased expression of HGF and its receptor but whether this has a direct effect on glioma cell growth or motility or indirectly on the tumour vasculature remains to be seen.

Chromosome 7 contains a number of non-growth factor genes which might be of relevance to glioma recurrence. On the short arm of chromosome 7, there are two genes TTIM1, a gene involved in invasion and metastasis (Habets et al 1992) and oncomodulin a calcium binding protein (Ritzler et al 1992), although neither are implicated, as yet, in glial oncogenesis.

**Chromosome 22**

In contrast to the results obtained with chromosome 7, cells from malignant glioma were essentially diploid or monosomic for chromosome 22 (Figure 3.25). In 5 of 8 samples taken at the time of diagnosis there was evidence of a very small population of cells with three nuclei per cell and 3 of 8 samples there were very small numbers of cells with four copies of chromosome 22. This is in accordance with low levels of chromosome 22 polyploidy reported by Jenkins et al (1989) and Li et al (1997). Two samples from recurrent gliomas were analysed, one which had a paired sample from the time of diagnosis which did appear to have a higher proportion of cells with 2, 3 and 4 copies of chromosome 22 at the time of recurrence (Figure 3.25a) whilst the other recurrent sample was monosomic for 22 (Figure 3.25i). It is clear that there is little evidence for a high proportion of cells at the time of diagnosis having multiple copies of chromosome 22. It is impossible to be sure that a feature of recurrence of high grade gliomas does not involve an expansion of a clone or clones of cells with additional copies of chromosome 22 present in very small numbers at the time of diagnosis although the limited data from recurrent tumours presented here is not strong evidence for this.

How is it possible, therefore to account for apparent loss of chromosome 22 material in circumstances where it might be reasonable to expect increase in the number of this chromosome? Although it has been possible to demonstrate multiple copies of chromosome 7 at the same time as disomy or monosomy of chromosome 22 the
centromere probe for Cm 22 has proved rather more technically difficult to visualise than chromosome 7 despite optimisation of the hybridisation protocol. However, it is unlikely that simple technical artefact could account for the loss of chromosome 22 material. Similarly, interphase pairing of signals has not been reported to occur with chromosome 22 although it has for chromosome 17 in human brain and brain tumour material (Dalrymple et al 1994). The use of isolated nuclei could prevent any problem of nuclear truncation common when paraffin sections are used as targets for FISH, as may be the case in the present study. The constitutive heterochromatin of the human centromeric region has been recognized as a highly heteromorphic structure. This appears to have no clinical significance, but produces considerable variation in centromere size which will occasionally result in a centromere which is too small to detect (Verma and Luke 1992). Although this may result in an occasional missed centromere, it cannot account for the widespread loss of chromosome 22 material described in this thesis.

The loss of one copy of chromosome 22, coupled with the possibility of a mutation in the second allele is highly suggestive of the presence of a tumour suppressor gene. Loss of Cm22 reported here is in accordance with data produced from a number of cytogenetic studies which have shown monosomy 22 (Rey et al 1993). LOH studies have also demonstrated that there are terminal and interstitial deletions in adult grade III and IV astrocytomas, with 22q11.2-13.1 a region of consistent loss (Muhammad et al 1997). A region at 22q13.1 has been found to be lost in low and high grade astrocytomas and ependymomas in children (Warr et al 1997). Both these regions are distinct from that implicated in the development of Type 2 neurofibromatosis (TSG22A at 22q11.2-q13) or that associated with development of sporadic meningiomas (TSG22B at 22q11) (Rey et al 1993).

It has been reported (Shapiro JR, personal communication) that although chromosome 22 material is over represented in samples derived from recurrent malignant glioma, much of this material is inserted into other chromosomes. This has been visualised by using a chromosome 22 paint on metaphase spreads from glioma which show that sequences from chromosome 22 do appear to be distributed across the genome. Whether it is integrated into specific sites or distributed randomly is not yet clear. The same pattern of chromosome 22 disruption has been seen in cultures selected for BCNU resistance although it is not clear in this case whether this disruption occurs as a consequence of the cytocholastic action of the drugs used to select the cells and whether it is restricted to chromosome 22. If this can be confirmed, it certainly explains the failure of this study to detect additional
centromeres in samples of recurrent gliomas. At the same time it suggests a possible
mechanism for the activation of potential genes which might be involved in
malignant progression. Translocation of chromosomal material across the genome
has been recognised as an important mechanism for the activation of oncogenes or
interference with the function of tumour suppressor genes (Rabbitts 1994).
Chromosome 22p11–p13.1 is a rich area for putative genes which might be involved
in tumour recurrence. In addition to being the site of the PDGFB gene (22q13.1), two
other growth factors, galectin I, a beta galactoside-binding lectin (Barondes et al 1994)
and platelet-derived endothelial cell growth factor I (Stenman et al 1992) map in this
area as do loci important in Ewing’s sarcoma (Delattre et al 1994) and RRP22, a
member of the RAS family which is expressed only in the CNS and GAR 22 a
mouse growth-arrest specific (Gas2) gene highly expressed in growth arrested
fibroblasts (Zucman-Rossi et al 1996). However, with the exception of PDGFB, none
of these genes have been implicated in the pathogenesis of malignant brain
tumours.

Effect of treatment on the numbers of chromosomes 7 and 22
There did not appear to be a link between clinical treatment with PCV and increased
Cm7 polysomy in the paraffin sections. However, only a small number, 4 of 13
patients (525/88 and 169/89, 259/82 and 34/84, 534/88 and 87/90, and 159/88 and
25/90, see Table 3.7) were treated with PCV between operations. In paired samples
taken from patients who did not receive therapy between the diagnostic and
recurrence, all had multiple copies of copies of chromosome 7 (between 25 and 50%
of cells with greater than 4 copies of chromosome 7) in both samples. However,
irrespective of treatment there were more copies of Cm7 in recurrent than in
primary tumours, indicating that repopulation of the tumour could be from
preferential clonal expansion of the cells with Cm7 polysomy. Recurrent tumours
have also been found to have more breaks at Cm7q than primary tumours, this may
be caused by alkylating agents because radiation is thought unlikely to cause this
specific rearrangement. Radiation causes more balanced structural changes
including translocations, inversions, insertions and deletions (Li et al 1997). In
untreated high grade astrocytomas, there is often over representation of Cm7 and
under representation of Cm22 with a minor sub population of cells with over
representation of both Cm7 and Cm22. After selection with increasing
concentrations of BCNU (range 1-70µg/ml) in vitro, the cells with over-expression
of both chromosomes became the dominant population, with both PDGFA (on
Cm7) and PDGFB (on Cm22) genes expressed in the cultures (Scheck et al 1993) no
other genes were assessed for expression. However, in that study of only 3 GBM
derived cell lines, there were several different clones per cell line and other
chromosomal changes occurred after selection with BCNU including in one near diploid clone, gains of Cm5, Cm11 and Cm17. Although polysomy Cm7 and Cm22 dominated the cell population after drug selection in vitro, this may bear little clinical relevance because in contrast to the in vitro treatment of the cells, in vivo drug treatment is not continuous and increasing dose is not used.

The under representation of Cm22 in untreated cells was confirmed in paraffin embedded tumours in the present study. However, only two recurrent tumours, 144/84 and 306/88, were assessed for Cm22 copy number and neither had previous chemotherapy, therefore no conclusion can be drawn about recurrence or the effect of treatment on Cm22 representation in the present study. In another karyotypic study where radiotherapy and chemotherapy had been administered in vivo, no involvement of Cm22 was found in 6 of 6 recurrent tumours (Li et al 1997), and a study using CGH again showed no gains of Cm22 in recurrent tumours, but these patients may not have received chemotherapy (Kim et al 1995).

It is of concern that there is a marked difference in the survival time between those patients whose samples were derived pre and post-treatment and those patients whose unselected samples were used for the cell culture and chemosensitivity experiments. It does suggest that those patients who had second operation represent a selected group of patients, not because of their age, but perhaps because of favourable position of the tumour or better performance status. Although there certainly is no neuropathological difference in their appearance from other high-grade it is possible that these tumour are a subset of malignant glioma which are biologically less aggressive.

Interestingly although both chromosomes 7 and 22 have a number of genes which are known to be involved in glioma pathogenesis and a larger number of genes which although only poorly characterised at least have the oncogenic potential with the exception of mdr1, the gene which encodes PGP which is located at 7p21, none have genes which might be implicated directly with cytotoxic drug resistance. It might be argued that an additional copy of mdr1 might be of potential advantage to a cell, results presented earlier in this thesis suggest that PGP plays at best a very minor role in the modulating drug resistance in malignant glioma. Although there are no genes known to be of importance in the development of drug resistance, it may be that PDGF may protect the glioma cells from apoptotic cell death. It is known that a variety of growth factors are known to protect a variety of cells from cell death, at least in experimental systems. The increase in the number of chromosome
7 at least suggests a potential mechanism by which proliferation and drug resistance might be linked. PDGF induces FOS which appears to be involved in the activation of DNA repair enzymes involved in the development of cis-platinum-resistance in ovarian cancer cells in vitro (Scanlon et al 1991).
Summary of achievements
This study was undertaken to investigate the role of membrane glycoproteins and p53 status in the resistance to cytotoxic drugs of cultures derived from human adult malignant glioma. A panel of 26 cultures produced from biopsy specimens taken at the time of diagnosis, before any radiotherapy or chemotherapy, were examined for sensitivity to three cytotoxic agents, CCNU, DOX and VCR. There was a wide range of in vitro sensitivities to all three drugs. Some cultures displayed patterns of cross resistance consistent with classical MDR, although this did not seem to be associated with elevated levels of either P-glycoprotein or MRP at the cell surface. Overexpression of p53 protein was found to be associated with sensitivity to CCNU and VCR, but not DOX suggesting that there is an association between elevated levels of non-functional p53 and the triggering of apoptosis. Changes in copy number of chromosomes 7 and 22 in in vitro experiments have been implicated in precipitating recurrence in high grade glioma and these studies have been extended to paired samples of paraffin wax-embedded archival samples taken from the same patient at diagnosis and again at the time of recurrence. Using specific centromere DNA probes to chromosomes 7 and 22, it was possible to show that increases in the numbers of chromosome 7 occur during the process of recurrence, there is little evidence for increases in ploidy for chromosome 22.
Suggestions for future work

1. Membrane glycoproteins in malignant glioma
From the present studies it is apparent that PGP and MRP are not expressed on the cell membranes of short-term cultures derived from high grade gliomas. However, it is possible that PGP might be expressed at low levels in the cytoplasm or organised into cytoplasmic vesicles, which might be capable of sequestering drugs like DOX within the cytoplasm of tumour cells. Western blotting or RT-PCR might answer some of these questions. Functional studies using fluorescent drugs could show sequestration or efflux of drugs this would help to clarify the role of membrane glycoproteins in cultured malignant glioma. A wider examination of MRP and other drug resistance-related membrane glycoproteins like LRP expression is required in malignant glioma as this might be of important in the transport of glutathione complexes. The expression of these proteins in other types of brain tumour, particularly paediatric brain tumours needs to be undertaken.

2. Atypical MDR and other mechanisms of resistance in malignant glioma
Assessment of the presence of altered or reduced levels of topoisomerase II or increased AT levels could be carried out on cultured cells to find out which resistance mechanisms might be involved in failure of chemotherapy in malignant glioma. Similarly it seems likely that altered tubulin might account for the marked resistance of some of the cultures in the present study to VCR. A systematic investigation of this has not been carried out in glioma, but might yield therapeutically useful information.

3. The role of apoptosis in malignant glioma
The role of apoptotic cell death following treatment with cytotoxic drugs like VCR, DOX or CCNU has not been assessed in human malignant glioma. Techniques for assessing whether cell death occurs via apoptosis, based on the apoptotic phenomenon of DNA fragmentation, should be investigated in these cell cultures. Apoptotic factors, other than p53, may prove to be of importance in malignant glioma drug resistance. Commercially available antibodies could be used to screen the cultures for these factors including bcl2, bax and WAF1. Interfering with these factors and pathways may be of future therapeutic use.

4. P53 and chemosensitivity
Although it is likely that most p53 protein accumulation is the result of a mutation in the p53 gene, this needs to be confirmed by SSCP analysis and DNA sequencing. It is possible that a sub-set of glioma cultures accumulate p53 wild-type protein, if so
what genetic mechanisms produce this accumulation? MDM2, which is known to bind to and stabilise p53 protein is a candidate for further examination in this subset of tumours. Is there a relationship between other common molecular genetic changes that occur in malignant glioma, for example, loss of Cm10 sequences, alteration in the Rb gene, loss of p16 and chemosensitivity? What effect does p53 inactivation have on chemosensitivity in cells with one or other of these genetic changes? Can the levels of p53 protein be modulated in these cells? Might p53 be a target for gene therapy for example, by replacement of a mutant gene with intact wild-type p53? Clearly there needs to be a study involving larger numbers of patients where the relationship between survival, chemosensitivity and p53 staining in vitro are examined.

5. Cytogenetic studies of recurrence in malignant glioma

What other differences are there between these paired samples in terms of proliferation markers, p53 expression or the expression of other oncogenes or tumour suppressors? More samples from patients who recurred and were operated on more quickly could be assessed to see if there is a cytogenetic difference between more aggressive and less aggressive recurrent GBM.

If Cm22 is distributed throughout the genome, is this specific for Cm22? What about other chromosomes? Are fragments of Cm22 inserted at specific points which might interfere with the operation of tumour suppressor genes or dominantly acting oncogenes. Is insertion random or is it different in different patients?

Isolation of Cm7+ Cm22+ cells from biopsies could be attempted by flow cytometry. Other characteristics of these cells could then be assessed for example expression of growth factors and receptors. What are the characteristics of these different populations of cells in terms of phenotype and genotype with particular regard to sensitivity to drugs and resistance mechanism. Are there specific characteristics of these cells which should be used to detect them in tumours at the time of diagnosis and make a prediction as to how quickly a tumour would recur.

Is PDGF or HGF acting as protective factors producing drug resistance? Although this question is not easy to address in situ, in vitro studies aimed at investigating this using administration of endogenous growth factors or temperature sensitive mutants of PDGF expression could be used to show whether permissive/non permissive temperatures change chemosensitivity.
An investigation of the numbers of Cm7 and Cm22 in isolation may be somewhat naive. The genetic aberrations seen in malignant glioma are complex and it is clear that these tumours can be sub-divided into different groups with different histories or prognoses. The observations of abnormalities in Cm7 and Cm22 despite being specific aberrations associated with tumour recurrence and the development of drug resistance should not be looked at in isolation as the consequences of polyploidy of 7 or loss of Cm22 might have different consequences in cells with different backgrounds of genetic change. Clearly this is an area which needs investigation. For example, the p53 gene status of these tumours may be important.

Can we use CGH to profile the differences between tumours at the time of diagnosis and recurrence using paired samples from this study. Recently it has been suggested that Cm22 monosomy, demonstrated by either karyotyping or ISH with centromere probes, may be misleading. Preliminary studies using a Cm22 paint probe, which binds to the full length of the chromosome, has shown parts of Cm22 inserted into other chromosomes (Shapiro et al 1997). The use of both CGH and ISH in further investigations will reduce the possibility of misinterpreting ISH results when centromere probes are used alone. CGH will indicate whether the tumour is monosomic or disomic for Cm22. With CGH new areas of amplification and deletion have been found in astrocytomas, which with further investigation may prove to be significant, these include amplification at 11q13 and 11q22-q23, and specific deletion on Cm10 at 10q25-q26 in GBMs (Weber et al 1996). It is hoped that the established techniques of karyotyping and FISH used in conjunction with the newer CGH analysis will find other patterns of genetic anomalies in high grade astrocytomas which could be exploited diagnostically and therapeutically.
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Acknowledgements

I would like to thank my supervisor Dr. John Darling and all the members of the University Department of Neurosurgery, Institute of Neurology, for their support.

This work was funded by the Brain Research Trust.
Appendix I

SOURCES

Sterile plastic ware

Plastic pipettes, 1ml, Cat. No. F7521
10ml, Cat. No. F7551
25ml, Cat. No. F7525
Culture flasks, 25cm², Cat. No. F3013E
75cm², Cat. No. F3082
175cm², Cat. No. F3084
24 well plates, Cat. No. F3847
60 mm Petri dishes, Cat. No. F3802

_Falcon, Becton Dickinson Ltd._

30ml Universal containers, Cat. No. UN030
60ml containers, Cat. No. CT60

_Ross Labs. Ltd._

96 well plates, Cat. No. 1-68055
8 well chamber slides, Cat. No. 7-048085
Cryotubes, Cat. No. 3-66656

_Nunc Ltd., Life Technologies_

7ml Bijou containers, Cat. No. 129A

_Sterilin_

Sterile media and reagents

Foetal Calf Serum (Australian), Cat. No. 10099-075
Nutrient Mixture Ham's F-10 with 25mM HEPES Cat. No. 22390-025
Hanks' Balanced Salt Solution, Cat. No. 24020-091
Hanks' Balanced Salt Solution without phenol red Cat. No. 14025-050
Trypsin (0.25%) 2.5g/litre, Cat. No. 25050-022
Penicillin 5000 Units/ml and Streptomycin 5000µg/ml Cat. No. 15070-022

_Life Technologies._

Kanamycin (5000µg/ml) Cat. No. 16-720-48
Amphotericin B (250µg/ml) Cat. No. 16-723-48

ICN Biologicals

Drugs

Vincristine Sulphate (Oncovin) 2mls of 1mg/ml solution.
_Eli Lilly._

Doxorubicin Hydrochloride 5mls of 2mg/ml solution.
_Farmitalia._

CCNU, 10mg capsules.
_Lundbeck_

Immunocytochemistry

JSB-1 Cat. No. RPN 1232
JSB-1 clone CT6
MRP clone m6
_(Monosan) TCS Biologicals_

P-glycocheck C219
_Centocor diagnostics_

Streptavidin-Fluoroscein
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Appendix II
Reagent preparation

Collagenase Stock (2000 units/ml)
The specific activity of collagenase was stated on the data sheet, this differed from batch to batch. The collagenase was dissolved in sufficient HBSS to give the required concentration. For example 1g collagenase with a specific activity of 380 units/mg solid would be dissolved in 190mls HBSS.

\[
\text{Total activity in 1g} = 1000 \times 380 \text{ U} \\
= 380000 \text{ U} \\
2000 \text{ U/ml required} \therefore \text{dissolve in } (380000 + 2000) = 190\text{mls HBSS.}
\]

When the collagenase was completely dissolved the solution was aliquoted into 30ml universal containers and centrifuged at 3000rpm in a Wifug 500E centrifuge, equivalent to 3000G, for 15 mins. The supernatants were then filtered once through 0.45μm then through 0.2μm filters and stored in 1ml aliquots in bijou bottles at -20°C.

Growth media (Ham's F-10 with 10% FCS.)
Ham's F-10 with HEPES was used because the presence of this buffer allows a closed culture system to be used. This means that CO\textsubscript{2} does not have to be used in the incubators, cutting down the risk of contamination. To make up the growth media, a 500ml bottle of FCS was allowed to thaw at room temperature. This was sufficient to make up 9x 500ml bottles of growth media. 9 x 500ml bottles of Ham's F-10 were labelled A-I and dated. 55mls FCS were added to each bottle of Ham’s F-10 to give a final concentration of 10% FCS. For example:

\[
\frac{55}{500 + 55} \times 100 = 9.9 = 10\%
\]

5mls were removed from each bottle, put into 30ml universals and incubated at 37°C for a few days to check for sterility. If contamination was seen the corresponding bottle was discarded. Bottles of growth medium were stored at +4°C.

Hoechst Dye
Stock solution 5mg Hoechst Dye (Bisbenzimide Hoechst No. 33258) was dissolved in 100mls HBSS without phenol red. Mixed for 30mins at room temperature and stored at 4°C in the dark.
Staining solution 0.5mls of stock solution were added to 100mls HBSS without phenol red and mixed for 30mins at room temperature. The solution was filtered through 0.45μm filter and stored at 4°C in the dark.

PBS (phosphate-buffered saline)
To make a 10XPBS stock solution, 80g sodium chloride, 2g potassium chloride, 14.4g disodium hydrogen phosphate and 2.4g potassium dihydrogen phosphate were made up to 1 litre with deionised water. pH adjusted to 7.4 using HCl. For a 1X working solution this stock was diluted 1 in 10 with deionised water. Both solutions were stored at room temp.

TE Buffer (10mM tris/ 1mM EDTA)
1.2114g tris and 380.2mg EDTA were made up to 1 litre with deionised water. pH adjusted to 8.0 with HCl. Stored at room temp.

3M sodium acetate (NaAc)
408.24g sodium acetate (hydrated) made up to 1 litre in deionised water. Stored at room temp.

20xSSC Buffer (3M sodium chloride/ 0.3M sodium citrate)
175.32g sodium chloride and 88.23g sodium citrate made up to 1 litre in deionised water. pH adjusted to 7.0 with HCl. Stored at room temp. This stock was diluted as necessary with deionised water for working concentrations.

0.2M HCl
3.15mls Concentrated HCl (Density 1.16g/ml) added to 496.85mls deionised water. Stored at room temp.
Hybridisation mix
(10% dextran sulphate, 2X SSC, 50% formamide, 1% Tween 20)
1g dextran sulphate, 1ml 20X SSC, 5ml formamide, 100μl Tween 20 made up to 10ml with deionised water. Mixed well and pH adjusted to 7.0. This was stored in 200μl aliquots in Eppendorf tubes at -20°C.

SSCT (4x SSC, 0.05% Tween 20).
100mls 20x SSC plus 250μl Tween 20 made up to 500mls with deionised water.

SSCTM (SSCT, 5% Marvel (non fat dried milk))
0.5g Marvel in 10mls SSCT, shaken well and warmed to 40°C.