MECHANISMS OF DRUG RESISTANCE IN GLIAL CELLS

by

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ABSTRACT

The most common human primary intracranial tumors are the gliomas. Despite intensive research, attempts to develop effective chemotherapy regimens for gliomas have met with limited success. In this thesis, two main approaches were taken to test the hypothesis that biochemical properties intrinsic to specific subtypes of glial tumors play a role in the resistance of these tumors to chemotherapeutic agents. The first approach was to examine biochemical properties inherent to the normal glial cell of origin. To accomplish this, the possibility of differential expression of drug resistance genes in cultures of purified rat glial cells was explored. Although the response of normal rat glial cells to BCNU appeared to be dependent on more than one mechanism of drug resistance, evidence of roles for O^6-methylguanine-DNA methyltransferase (MGMT) and the glutathione-glutathione-*S*-transferase detoxification pathway in response to BCNU were demonstrated. The second approach of this thesis was to examine the impact of a genetic alteration, found commonly in astrocytic gliomas, on the response of otherwise normal astrocytes to BCNU. Mutations of the *p53* gene, and subsequent loss of p53 function, are a common occurrence in astrocytic tumors. Therefore, the impact of the loss of p53 function on response of mouse astrocytes to BCNU was examined. Wild type p53 appeared to protect mouse astrocytes from the cytotoxic effects of BCNU in a gene-dose-dependent fashion. This resistance displayed by wild type astrocytes could not be explained by either p53-dependent apoptosis or differential cell cycle arrest. In addition, although results supported a role for p53 in the regulation of MGMT, the p53-dependent BCNU resistance displayed by mouse astrocytes appeared to be mediated by a non-MGMT mechanism. Studies presented here demonstrated that knockout *p53* astrocytes could undergo apoptosis both in the absence of drug and at increased levels in response to BCNU treatment. It is hypothesized that DNA damage in these knockout astrocytes may be detected by an alternative damage-sensing mechanism and subsequently, the cells are triggered to undergo p53-independent apoptosis. Together, these experiments provide support for the hypothesis that biochemical properties intrinsic to specific subtypes of glial tumors play a role in resistance to chemotherapeutic agents.

Keywords: cancer, tumor, brain, chemotherapy, drug resistance, alkylating agent, nitrosourea, BCNU, astrocytoma, oligodendroglioma, glial cell, astrocyte, oligodendrocyte progenitor, oligodendrocyte, O^6-methylguanine-DNA methyltransferase, glutathione, glutathione-*S*-transferase, p53, cell cycle arrest, apoptosis

This thesis is dedicated
in loving memory of

David John Briggs

...an unspoken promise made many years ago...

This one is for you, Grandpa.

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ABBREVIATIONS

+/+	wild type
+/−	heterozygous
−/−	knockout
ABC	ATP-binding cassette
ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)]
AGT	O^6-alkylguanine-DNA alkyltransferase
AMCA	7-amino-4-methylcoumarin-3-acetic acid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
%BAD	percent background aggregates and debris
bax	bcl-associated x
bcl-2	B-cell leukemia/lymphoma-2
bcl-x	B-cell leukemia/lymphoma-x
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
bFGF	basic fibroblast growth factor
BG	O^6-benzylguanine
bp	base pair(s)
BSA	bovine serum albumin
BSO	DL-buthionine-[S,R]-sulfoximine
BTSG	Brain Tumor Study Group
°C	degrees Celsius
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CD44	cluster of differentiation 44
CDC	cell-division-cycle
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
cGy	centigray
CIP1	cyclin-dependent kinase-interacting protein-1

cm	centimetre(s)
CNS	central nervous system
^{60}Co	cobalt-60
cpm	counts per minute
DAB	diaminobenzidine
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotides
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
ECOG	Eastern Cooperative Oncology Group
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ENU	*N*-ethyl-*N*-nitrosourea
ERCC	excision repair cross-complementing
FBS	fetal bovine serum
FGF	fibroblast growth factor
γ	gamma
g	gram(s)
GADD	growth arrest and DNA damage inducible
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GC	galactocerebroside
GFAP	glial fibrillary acidic protein
GST	glutathione-*S*-transferase
HEPES	N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
hr	hour(s)
Ig	immunoglobulin
IGF	insulin-like growth factor
JNK1	c-Jun kinase 1
kb	kilobase(s)
kD	kilodalton(s)
L	litre(s)
M	molar
mdm-2	murine double minute-2
MDR	multidrug resistance
MGAP	mouse glyceraldehyde-3-phosphate dehydrogenase
MGMT	O^6-methylguanine-DNA methyltransferase
μCi	microcurie(s)
μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
min	minute(s)
MMAC1	mutated in multiple advanced cancers 1
MNNG	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine
MNU	*N*-methyl-*N*-nitrosourea
MOPS	3-(N-morpholino)propanesulphonic acid

mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
MT	metallothionein
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	molecular weight
N	normal
NER	nucleotide-excision repair
ng	nanogram(s)
nm	nanometre(s)
O2A	oligodendrocyte-type 2 astrocyte
OTZ	(-)-2-oxo-4-thiazolidine-carboxylic acid
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RAN-2	rat neural antigen-2
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RTOG	Radiation Therapy Oncology Group
SDS	sodium dodecylsulphate
sec	second(s)
SSC	salt sodium citrate
Taq	*Thermus aquaticus*
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
VEGF	vascular endothelial growth factor

WAF1	wild type p53-activated fragmant
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 THESIS OVERVIEW

Despite intensive research, attempts to develop effective chemotherapy regimens for gliomas have met with limited success. Significant progress was made when Cairncross and Macdonald (1988) reported that anaplastic oligodendrogliomas responded to a chemotherapy regimen of procarbazine, CCNU, and vincristine (PCV). It has been confirmed since that oligodendrogliomas and mixed oligoastrocytomas respond predictably to nitrosourea-based chemotherapy, especially PCV, whereas astrocytomas are relatively chemoresistant. Presumably oligodendroglioma cells are inherently susceptible to the cytotoxic effects of PCV, but why? In addition, a subset of astrocytomas have been observed to benefit from chemotherapy. What intrinsic properties of this subset of astrocytomas set them apart from the majority of astrocytic tumors? The answers to these questions are undoubtedly important as they may lead to better therapies for oligodendrogliomas and to new treatment strategies for anaplastic astrocytomas and glioblastomas.

In this thesis, two main approaches were taken to test the hypothesis that biochemical properties intrinsic to specific subtypes of glial tumors play a role in the resistance of these tumors to chemotherapeutic agents. The first approach was to examine biochemical properties inherent to the normal glial cell of origin. To accomplish this objective, the possibility of differential expression of drug resistance mechanisms in astrocytes and oligodendrocytes was investigated. In Chapter 3, astrocytes and mixed cultures of oligodendrocyte lineage cells were isolated from neonatal rat. The role of a DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) in the response of these cells to the clinically relevant alkylating agent, BCNU was examined. In Chapter 4, purified cultures of astrocytes, oligodendrocyte progenitors, and oligodendrocytes were obtained from neonatal rat. A panel of six drug resistance genes (*MGMT*, *GST-*μ, *GST-*π,

p53, *MDR*, and *MT*) were probed for differential expression in these cells and the role of two mechanisms, MGMT and the glutathione-glutathione-*S*-transferase detoxification pathway, were examined in greater detail.

The second approach was to examine the impact of a specific genetic alteration, found commonly in astrocytic gliomas, on the response of otherwise normal astrocytes to BCNU. Mutations of the *p53* gene, and subsequent loss of p53 function, are a common occurrence in astrocytic tumors. To determine the impact of the loss of p53 function on response of astrocytes to BCNU, astrocytes were isolated from TSG-*p53* mice; this experimental system allowed me to examine the consequences of *p53* gene dosage on drug resistance on an otherwise normal cellular background. In Chapter 5, the effect of p53 status on the viability of mouse astrocytes exposed to BCNU was examined and furthermore, whether this effect was drug-specific or cell-specific was investigated. In addition, the effect of BCNU treatment on the expression of three p53-regulated genes (*mdm-2*, *WAF1*, and *GADD45*) that may play a role in the cell cycle response to DNA damage was determined and the cell cycle response of astrocytes to DNA damaging treatments (ie. BCNU and radiation) was explored. In Chapter 6, the effect of p53 status on the ability of mouse astrocytes to undergo apoptosis when exposed to BCNU was examined. Furthermore, a semi-quantitative analysis of apoptosis in mouse cells in response to BCNU was performed and correlated with p53 status. The effect of BCNU treatment on the expression of two p53-regulated genes (*bax* and *bcl-2*) and one additional cell death gene (*bcl-x*) that may play a role in the apoptotic response to DNA damage also was investigated. Finally, in Chapter 7, the effect of p53 status on MGMT protein and mRNA levels was determined and the consequences of altered MGMT activity on the response of astrocytes to BCNU explored.

The remainder of Chapter 1 provides an overview of the present state of knowledge in this area of research; Chapter 2 describes the materials and methods employed throughout this thesis; and Chapter 8 summarizes the overall conclusions of this research.

1.2 GLIAL NEOPLASMS

1.2.1 Introduction

The most common human primary intracranial tumors are the gliomas. This family of brain tumors contains three main subtypes, each classified according to their presumed cell of origin: astrocytomas from astrocytic cells, oligodendrogliomas from oligodendrocytic cells, and ependymomas from ependymal or subependymal cells. Gliomas comprised of more than one cell type are termed mixed tumors and are named for both cell types; for example, oligoastrocytomas are marked by the presence of both oligodendrocytic and astrocytic cells. These cellular origins of gliomas are debated still since immunohistochemical staining for markers of more than one glial cell lineage has been observed simultaneously in histologically pure gliomas (Sarkar *et al.*, 1988; Bishop and de la Monte, 1989; de la Monte, 1989; Kros *et al.*, 1990; Cruz Sanchez *et al.*, 1991; Cutarelli *et al.*, 1991; Piepmeier *et al.*, 1993). Most studies of the cellular origin of gliomas have focused on astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas (Sarkar *et al.*, 1988; Bishop and de la Monte, 1989; de la Monte, 1989; Cutarelli *et al.*, 1991; Piepmeier *et al.*, 1993). Using immunochemical markers for normal glial cells, Bishop and de la Monte (1989) suggested that the majority of astrocytomas (approximately 75%) arise from the type 1 astrocyte lineage and the remainder from type 2 astrocytes of the O2A cell lineage (refer to section 1.4.1 for details of glial cell types). de la Monte (1989) utilized the same techniques to study oligodendrogliomas and mixed oligoastrocytomas and suggested that they arise from cells of the oligodendrocyte progenitor (O2A) lineage.

1.2.2 Pathology

Astrocytic gliomas can be divided broadly into two groups: the more common diffuse astrocytomas and a second group of relatively well circumscribed tumors comprised of the rarer subtypes such as the pilocytic juvenile astrocytoma, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma (Lopes *et al.*, 1993). Within the classification of diffuse astrocytomas, the World Health Organization (WHO)

designations recognize three histological subtypes: low grade astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme (Lopes *et al.*, 1993; Kleihues *et al.*, 1993; Kleihues *et al.*, 1995; Berger *et al.*, 1996; Louis and Cavenee, 1997). Low grade astrocytomas display a tendency to diffusely infiltrate surrounding brain parenchyma, often resulting in the entrapment of pre-existing cells. Mitotic activity is absent but nuclear atypia may be present. Phenotypically, neoplastic cells may vary with respect to size, prominence, number of cell processes, and abundance of cytoplasmic glial filaments. Depending on the prevailing cell phenotype, astrocytomas can be further classified into two major variants, the more common fibrillary astrocytoma and the rarer gemistocytic astrocytoma. Anaplastic astrocytomas are distinguished from low grade tumors by increased cellularity, marked nuclear atypia and cellular pleomorphism, increased mitotic activity, and a moderate increase in vascular endothelial cell proliferation. The most malignant designation, glioblastoma multiforme, is used to denote tumors that are poorly differentiated and display an exaggeration of the features of anaplastic astrocytoma. However, tumor necrosis is the histological feature that distinguishes glioblastoma from an anaplastic astrocytoma.

Oligodendrogliomas and mixed oligoastrocytomas are diffuse tumors that are clinically and biologically the most closely related to the diffuse, fibrillary astrocytoma (Louis and Cavenee, 1997). Many of the histologic features used to grade astrocytomas also are used to grade oligodendrogliomas and mixed gliomas; these include cellularity, pleomorphism, mitotic activity, vascular changes, and necrosis (Berger *et al.*, 1996). Oligodendrogliomas have variable histologic appearances, the most common arrangement being a pseudolobular pattern of tumor cells surrounded by a vascular network (Lopes *et al.*, 1993; Berger *et al.*, 1996). In general, oligodendroglioma cells have round, regular, monotonous nuclei, distinct cellular borders with clearing of the cytoplasm and lack fibrillary cytoplasmic processes (Berger *et al.*, 1996). With increasing malignancy, oligodendrogliomas can become highly cellular and pleomorphic, approaching an appearance of glioblastoma multiforme with the presence of necrosis. Although some may classify necrotic oligodendrogliomas as glioblastoma multiforme, the preferred

classification is anaplastic oligodendroglioma (Berger *et al.*, 1996). Many, if not most, oligodendrogliomas contain a regional or intimate cellular mixture of astrocytoma cells. For the classification of mixed oligoastrocytoma, the proportion of the minor component is usually at least 10 to 25 percent.

Ependymomas are slowly growing tumors, usually well circumscribed. They have an almost epithelial appearance and are commonly calcified. Other histologic features may include perivascular pseudorosettes and ependymal rosettes (Berger *et al.*, 1996). The more aggressive anaplastic ependymoma is characterized by a marked increase in mitotic activity (Kleihues *et al.*, 1995).

1.2.3 Epidemiology

In 1997, an estimated 60 700 deaths from cancer will occur in Canada (National Cancer Institute of Canada, 1997). Of these, 1 380 are estimated to result from brain cancers, making brain tumor the ninth leading cause of cancer death among adult Canadians (National Cancer Institute of Canada, 1997). Cancers of the brain are the second most common cause of cancer death in children under 15 years of age (National Cancer Institute of Canada, 1996). The age-standardized incidence rate for Canadian males is estimated to be 8 per 100 000 people in 1997, with a slightly lower incidence rate of 6 per 100 000 in Canadian females (National Cancer Institute of Canada, 1997). It has been suggested that brain tumor incidence is on the rise in North America, especially among older patients (Greig *et al.*, 1990; Mao *et al.*, 1991). The possibility that improved diagnostic methods alone can account for these increases has been debated (Desmeules *et al.*, 1992; Helseth, 1995). In comparison, the average annual percent change in age-standardized incidence in Canada (1985-1992) has been 0.7 for males and -0.1 for females, neither figure showing a statistically significant change (National Cancer Institute of Canada, 1997). In 1997, the estimated ratio of deaths to newly diagnosed cases of brain tumor in Canada is 0.64, outranked only by cancers of the esophagus, pancreas, and lung, and multiple myeloma (National Cancer Institute of Canada, 1997).

Gliomas represent the majority of primary intracranial brain tumors, with astrocytic tumors being the most common (Louis and Cavenee, 1997). Relative proportions of astrocytomas are age-dependent with indolent astrocytoma variants and lower grade diffuse fibrillary astrocytomas being more prevalent in children and adults under the age of 40 and glioblastomas predominating in patients over 45 years of age. The incidence of most gliomas, with the exception of glioblastoma multiforme, actually decreases with increasing age. Oligodendrogliomas, mixed oligoastrocytomas, and ependymomas comprise approximately 10-15% of all gliomas. Patients with high grade gliomas have an extremely poor prognosis; only 32% of patients with glioblastoma multiforme are alive one year after diagnosis and their five year survival rate is 7% or less (Holowaty *et al.*, 1996). Patients with low grade gliomas, on the other hand, often can survive five to ten years or longer (Leighton *et al.*, 1997).

1.2.4 Risk Factors

The epidemiology of glioma has provided some suggestions of possible environmental or occupational risk factors but few definitive observations have been made. A higher than expected incidence of brain tumors has been observed as a result of exposure to pesticides, herbicides, and fertilizers (Musicco *et al.*, 1982), and various substances prevalent in the petrochemical industry (Moss, 1985) and health professions (McLaughlin *et al.*, 1987). The credibility of these observations is difficult to determine; aside from an association between vinylchloride and gliomas, there are no common chemical or environmental factors among these observations (Moss, 1985). Electromagnetic fields also have been implicated as a possible risk factor for gliomas (Savitz and Loomis, 1995) but many studies do not support these findings (Wrensch *et al.*, 1993).

A possible inherent risk factor for glioma has been suggested recently by Bondy *et al.* (1996). In a case-control study, they demonstrated that lymphocytes of glioma patients were more sensitive to the induction of DNA strand breaks by γ-irradiation. Perhaps an increased sensitivity to this and other mutagens could be a predisposing risk

factor for glioma.

Clues to a hereditary basis for glioma have come from the study of genetic and hereditary syndromes associated with an increased incidence of glial tumors (Bondy *et al.*, 1996). These syndromes include Li-Fraumeni syndrome, tuberous sclerosis, neurofibromatosis types 1 and 2, and Turcot's syndrome. In addition, multiple studies have described familial aggregations of brain tumors in the absence of a known predisposing syndrome (Ikizler *et al.*, 1992; Bondy *et al.*, 1996). These studies, although inconclusive, suggest that a role may exist for inherited genetic susceptibility in the genesis of brain tumors.

1.2.5 Molecular Tumor Biology

In 1976, Nowell proposed a model whereby the *in vivo* evolution of tumor cell populations occurs through the stepwise accumulation of growth-advantageous genetic changes in cells that were initially of normal genotype and phenotype. In general, the more growth-advantageous mutations that accumulated, the more aggressive or malignant would be the tumor. It is now known that the genetic changes that occur during the evolution of tumor cells can be grouped into two categories: those involving the activation through overexpression and/or mutation of gene products conferring growth advantage (ie. oncogenes) and those involving the functional loss or inactivation of gene products having a negative growth-regulatory function (ie. tumor suppressor genes) (James and Collins, 1993). Gliomas are no exception to this model and much work into the molecular biology of brain tumors has occurred in the last few years.

Of all glial tumors, the molecular biology of astrocytomas has received the most attention. One of the earliest genetic alterations to occur in astrocytomas is mutation of the *p53* gene or loss of chromosome 17p (*p53* is localized to 17p13). This alteration is found in approximately 25% of astrocytic gliomas of all malignancy stages (James *et al.*, 1988; el Azouzi *et al.*, 1989; James *et al.*, 1989; Fults *et al.*, 1989; Nigro *et al.*, 1989; Chung *et al.*, 1991; Hayashi *et al.*, 1991; Mashiyama *et al.*, 1991; Frankel *et al.*, 1992; Fults *et al.*, 1992; Saxena *et al.*, 1992; Sidransky *et al.*, 1992; von Deimling *et al.*,

1992a; Aka *et al.*, 1993; del Arco *et al.*, 1993; Hunter *et al.*, 1993; Louis *et al.*, 1993; Newcomb *et al.*, 1993; Wu *et al.*, 1993; Bello *et al.*, 1994; Koga *et al.*, 1994; Kraus *et al.*, 1994; Louis, 1994; Rasheed *et al.*, 1994). Overexpression of growth factors and their receptors are also a common feature of astrocytomas. Changes in expression of platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), insulin-like growth factors I and II (IGF-I and IGF-II), and vascular endothelial growth factor (VEGF) have been well documented (Antoniades *et al.*, 1992; Brem *et al.*, 1992; Gillaspy *et al.*, 1992; Hermanson *et al.*, 1992; Plate *et al.*, 1992; Trojan *et al.*, 1993; Plate and Risau, 1995; Westermark *et al.*, 1995). Of particular interest is PDGF; PDGF ligands and receptors are expressed at approximately equal levels in all grades of astrocytoma, suggesting that such overexpression is important in the initial stages of tumor formation (Louis and Cavenee, 1997).

One of the hallmarks of astrocytomas is their tendency to infiltrate the surrounding brain. These invasive abilities often are apparent in low grade as well as high grade astrocytomas, suggesting that the invasive phenotype is another feature acquired early in glial tumorigenesis. A variety of cell surface molecules are differentially expressed in astrocytomas, including CD44 glycoproteins, gangliosides, and integrins (Louis and Cavenee, 1997). In addition, many of the growth factors expressed in astrocytic tumors, such as FGF, EGF, and VEGF, have been shown to stimulate tumor cell migration (Pilkington, 1994).

A less common genetic change in astrocytomas involves chromosome 22. Allelic loss of chromosome 22q has been observed in about one quarter of astrocytomas, suggesting the presence of a glioma tumor suppressor gene within that region (James *et al.*, 1988).

The transition of astrocytoma to anaplastic astrocytoma is accompanied by a marked increase in malignant behavior and a number of molecular abnormalities have been associated with this tumor of higher grade. The most prevalent changes involve a critical cell cycle regulatory complex that includes the p16, CDK4, cyclin D1, and retinoblastoma (Rb) proteins (Venter *et al.*, 1991; Henson *et al.*, 1994; Jen *et al.*, 1994;

Schmidt *et al.*, 1994; Arap *et al.*, 1995; Collins, 1995; Nishikawa *et al.*, 1995; Sonoda *et al.*, 1995; James and Olson, 1996; Ueki *et al.*, 1996; Zhang *et al.*, 1996; Dirks *et al.*, 1997; Louis and Cavenee, 1997). Individual components of this pathway can be altered in up to half of anaplastic astrocytomas and in the majority of glioblastomas. A second common abnormality is allelic loss on 19q; loss of heterozygosity at this locus is observed in up to 40% of anaplastic astrocytomas and glioblastomas (von Deimling *et al.*, 1992c). This region has been suggested to contain a tumor suppressor gene important for progression of astrocytomas (Seizinger *et al.*, 1991). This gene may be unique to gliomas and is involved in all three of the major cerebral glial tumors (ie. astrocytoma, oligodendroglioma, and oligoastrocytoma) (Seizinger *et al.*, 1991).

Glioblastomas are the most malignant form of astrocytomas and display their own complement of genetic abberations. As mentioned above, the majority, if not all, glioblastomas have alterations in the p16-CDK4-cyclin D1-Rb pathway of cell cycle control. In addition, chromosome 10 is lost frequently in glioblastomas, occurring in 60 to 95% of glioblastomas but only rarely in anaplastic astrocytomas (James *et al.*, 1988; Fults *et al.*, 1992; Rasheed *et al.*, 1992; von Deimling *et al.*, 1992b; Bello *et al.*, 1994; von Deimling *et al.*, 1995). Attempts to identify possible tumor suppressor genes for glioma on chromosome 10 have been hampered by the fact that, in most cases, the entire chromosome is lost but evidence suggests that loss of heterozygosity involves at least three distinct regions (Rasheed *et al.*, 1992; Karlbom *et al.*, 1993). Recently, Steck *et al.* (1997) have identified one candidate tumor suppressor gene, *MMAC1*, at chromosome 10q23.3.

Since one of the hallmarks of glioblastoma is marked vascular endothelial cell proliferation, the upregulation of a number of growth factors and their receptors has been investigated (Louis and Cavenee, 1997). A paracrine mechanism has been suggested whereby growth factors such as VEGF and PDGF are frequently overexpressed in tumor cells while their receptors, flk-1 and flt-1 for VEGF and the PDGF-β receptor for PDGF, are upregulated on endothelial cells (Hermanson *et al.*, 1992; Plate *et al.*, 1992). VEGF and its receptors, in particular, appear to play a key role in the angiogenesis of

glioblastoma (Plate *et al.*, 1992; Plate *et al.*, 1994; Plate and Risau, 1995).

The most frequently amplified oncogene in highly malignant astrocytic tumors is the epidermal growth factor receptor (*EGFR*) gene, being amplified in approximately 40% of glioblastomas but in few anaplastic astrocytomas (Ekstrand *et al.*, 1991; von Deimling *et al.*, 1992b; Wong *et al.*, 1992; Kordek *et al.*, 1995; Schwechheimer *et al.*, 1995; James and Olson, 1996). EGFR overexpression is almost always accompanied by a loss of chromosome 10 in glioblastomas, suggesting that these alterations may be integral to malignant progression in some astrocytic tumors (Ekstrand *et al.*, 1991; von Deimling *et al.*, 1992b; Nishikawa *et al.*, 1994; von Deimling *et al.*, 1995). Genes less commonly amplified in glioblastomas include N-*myc*, *gli*, PDGF-α receptor, c-*myc*, *myb*, K-*ras*, c-*fos*, *CDK4*, and *mdm-2* (Orian *et al.*, 1992; Collins, 1995; Louis and Cavenee, 1997).

Subsets of glioblastoma multiforme can be classified on the basis of molecular genetic analysis (von Deimling *et al.*, 1993; van Meyel *et al.*, 1994; Kordek *et al.*, 1995; von Deimling *et al.*, 1995; Louis and Cavenee, 1997). For instance, loss of chromosome 17p and *p53* mutations occur in tumors with overexpression of the PDGF-α receptor (Hermanson *et al.*, 1996) and loss of chromosome 10 often is found in association with EGFR overexpression (von Deimling *et al.*, 1992b). However, loss of chromosome 17p almost never occurs in glioblastomas with EGFR overexpression: approximately one-third of glioblastomas display *p53*/chromosome 17p alterations, one-third have EGFR amplification, and one-third do not exhibit either change (von Deimling *et al.*, 1993). Of significant interest are the findings that glioblastomas with loss of chromosome 17p occur in younger patients and are hypothesized to arise via stepwise progression while those characterized by EGFR overexpression occur in older patients and are typically *de novo* tumors (von Deimling *et al.*, 1993; van Meyel *et al.*, 1994; Rasheed *et al.*, 1994; von Deimling *et al.*, 1995).

The most common molecular change found in oligodendrogliomas and oligoastrocytomas is the allelic loss of chromosomes 1p and 19q, affecting 40 to 80% of these tumors (Seizinger *et al.*, 1991; von Deimling *et al.*, 1992c; Reifenberger *et al.*,

1994; Kraus *et al.*, 1995). These regions are lost in low grade as well as anaplastic oligodendrogliomas and oligoastrocytomas at almost equal frequency, suggesting that these loci are important early in oligodendroglial tumorigenesis. Chromosome 1p and 19q losses are closely associated, indicating that tumor suppressor genes located in these regions might be involved in biologically distinct pathways, each of which must be altered for tumorigenesis (Reifenberger *et al.*, 1994; Kraus *et al.*, 1995). In contrast to astrocytomas, oligodendrogliomas rarely harbor *p53* mutations (Reifenberger *et al.*, 1994) but often accumulate wild type p53 protein (Pavelic *et al.*, 1994).

Ependymomas are a clinically diverse group of gliomas. Progression to tumors characteristic of glioblastoma are rare and have not been studied by molecular biology techniques (Louis and Cavenee, 1997). The most common genetic alteration in ependymomas is chromosome 22q loss (James *et al.*, 1990; Ransom *et al.*, 1992). In contrast to astrocytomas, mutations in the *p53* gene do not occur (Ohgaki *et al.*, 1991).

1.2.6 Treatment

1.2.6.1 Surgery

No other modality of cancer treatment can reduce tumor bulk as quickly as surgery. Well circumscribed tumors such as ependymomas and juvenile pilocytic astrocytomas often are amenable to gross total resection. Unfortunately, the infiltrative nature of most other gliomas precludes complete resection of the tumor in most instances and thus, a high rate of recurrence exists. When surgical cure is not possible, the main goals of surgery become tumor size reduction and consequent decompression of the brain (Levin *et al.*, 1997). Numerous retrospective studies have supported the concept that an aggressive resection has a positive impact on survival as well as quality of life (Berger *et al.*, 1996). In addition to the treatment goals of surgery, surgical biopsies are crucial for pathologic evaluation and treatment recommendations for all gliomas.

1.2.6.2 Radiation Therapy

Postsurgery external beam radiation for malignant gliomas has proven effective

in prolonging patient survival in several prospective randomized controlled trials and has become the standard therapy for glial tumors (Berger *et al.*, 1996). The treatment of low grade astrocytomas and oligodendrogliomas with radiation has been more controversial. There have been no randomized studies comparing the outcome of patients treated by surgery alone with those who received surgery plus radiation therapy and thus, treatment decisions are based on information obtained from retrospective reports (Berger *et al.*, 1996). It is agreed generally that patients with neurologic impairment, tumor progression, or malignant transformation should receive radiation therapy. The techniques of interstitial brachytherapy (Loeffler *et al.*, 1990) and stereotactic radiosurgery (Loeffler *et al.*, 1992) have been used to deliver intense focal radiation to the tumor. Initially, these methods appeared promising but have since been questioned due to the finding that most of the increase in patient survival could be traced to patient selection bias (Curran, Jr. *et al.*, 1992; Florell *et al.*, 1992).

1.2.6.3 Chemotherapy

Despite intensive research, attempts to develop effective chemotherapy regimens for gliomas have met with limited success. Early studies of the Brain Tumor Study Group (BTSG) found that a greater proportion of patients with malignant glioma survived 18 months or longer when chemotherapy was added to surgery and radiotherapy (Walker *et al.*, 1980; Green *et al.*, 1983). Although median survival did not change, 15% of patients receiving adjuvant chemotherapy were long-term survivors compared to 5% following radiation alone. Stenning *et al.* (1987) obtained complementary results from a meta-analysis of eight randomized trials reported between 1976 and 1985. They also described a significant, although limited, benefit to the addition of chemotherapy to radiation therapy; the 1-year and 2-year survival rates were increased 9 and 3.5 percent respectively. These results were interpreted to mean that a small subset of patients benefit from adjuvant chemotherapy but the question of which patients benefit remained.

More recently, some groups have investigated possible factors responsible for improved patient prognosis in response to adjuvant chemotherapy. Nelson *et al.* (1988),

in a re-evaluation of a randomized trial by the Radiation Therapy Oncology Group (RTOG) and Eastern Cooperative Oncology Group (ECOG), reported that adjuvant BCNU enhanced overall and proportionate survival at two years in patients with anaplastic glioma and glioblastoma. Patients aged 40-60 survived significantly longer with BCNU treatment; those aged less than 40 appeared to benefit; and those older than 60 showed no improvement in survival. A possible role for patient age in prognosis of glioma has been supported by others (Chang *et al.*, 1983; Grant *et al.*, 1995). In a meta-analysis of 16 randomized trials, Fine *et al.* (1993) concluded that adjuvant chemotherapy enhanced proportionate survival at one and two years independent of tumor grade. Finally, DeAngelis *et al.* (1996) re-examined two randomized trials by the BTSG and noted that adjuvant BCNU improved proportionate survival at 18 months across all major prognostic factors, including age less than 65, intermediate or high tumor grade, symptoms, and performance status levels. In conclusion, these studies suggested that prognostic factors for survival, with the possible exception of younger age, do not predict chemotherapeutic benefit.

Studies had largely ignored the possibility that specific histological subtypes of glioma might differ in their response to chemotherapy. Significant progress was made when Cairncross and Macdonald (1988) reported that anaplastic oligodendrogliomas responded to a chemotherapy regimen of procarbazine, CCNU, and vincristine (PCV). Subsequently, Glass *et al.* (1992) and Kim *et al.* (1996) observed that mixed oligoastrocytomas responded frequently to PCV. In addition, Mason *et al.* (1996) noted that symptomatic low-grade oligodendrogliomas often responded to PCV. At the current time, it is not known whether adjuvant PCV chemotherapy will result in prolonged patient survival but a randomized controlled trial for anaplastic pure and mixed oligodendrogliomas is being carried out by the RTOG in an attempt to answer this question.

1.2.7 Summary

Gliomas are the most common brain tumors of adults. The three main subtypes

of primary glial tumors are astrocytomas, oligodendrogliomas, and ependymomas. These tumors are a significant cause of cancer death; in 1997, the estimated ratio of deaths to newly diagnosed cases of brain tumors in Canada is 0.64, outranked only by cancers of the esophagus, pancreas, lung, and multiple myeloma (National Cancer Institute of Canada, 1997). Although there is little evidence for well established environmental or behavioral risk factors for glioma, there does appear to be a small population that may be predisposed to glioma due to inherited susceptibility. More is known about the molecular biology of brain tumors. Genetic alterations have been identified that can be linked to a specific pathologic subtype or malignancy stage of glioma. Finally, due to the poor prognosis of glial tumors, much emphasis has been placed on treatment improvement. Surgery can reduce tumor size and improve quality of life through the resultant decompression of the brain but the invasiveness of gliomas generally prevents total surgical resection. Conventional radiation and chemotherapy can offer temporary benefit but are seldom curative. Present and future studies designed to investigate why some gliomas benefit from treatment while others are refractory will lead to improved treatment regimens and patient prognosis.

1.3 EXPERIMENTALLY INDUCED BRAIN TUMORS

Since 1966, alkylating agents, principally the N-nitroso compounds, have been used to induce central nervous system (CNS) tumors in rodents (Druckrey *et al.*, 1966). The features of this experimental system as reviewed by Lantos (1986) are as follows: 1) the incidence, distribution, latency, and histology of these tumors are influenced by the species, age, and sex of the animals and by the dose and mode of application of the carcinogen; 2) the neoplasms induced by nitrosoureas are not distributed at random in the nervous system, but develop at certain favoured sites; most notably, the periventricular areas and the subcortical white matter in the cerebral hemispheres; 3) tumor development is often multicentric and more than one lesion can occur in one animal; 4) histologically, the cerebral tumors are most often gliomas; 5) the latency period is long, with cerebral gliomas developing in 245 $\pm$ 80 days. It was found that, although MNU had to be administered to adult animals in small repeated doses in order

to produce a high tumor yield, ENU could induce an almost 100% incidence of neural neoplasms in the offspring of mothers injected during the second half of gestation and in neonatal rats using a single dose (Druckrey *et al.*, 1966). Consequently, most studies to date have utilized the transplacental administration of ENU in order to chemically induce gliomas in rat.

Although the pathology of experimental neurogenic tumors induced by transplacental administration of ENU has demonstrated a wide range of morphological variability (Wechsler *et al.*, 1969), it is interesting to note that the cerebral glial tumors were more often mixed and pure oligodendrogliomas than astrocytomas (Wechsler *et al.*, 1969; Grossi-Paoletti *et al.*, 1970; Koestner *et al.*, 1971; Lantos, 1972; Druckrey, 1973; Lantos and Cox, 1976; Schiffer *et al.*, 1978; Usuki *et al.*, 1992). These findings led to the hypothesis that the two most susceptible targets in the brain to carcinogenic action were the primitive cells of the subependymal plate and oligodendroglial cells (Lantos, 1972). To investigate this hypothesis further, Burger *et al.* (1988) removed the forebrains from transplacentally exposed fetal rats one day after treatment and transplanted them into the brains of adult animals. Unexpectedly, the histological spectrum of neoplasms was reduced to a single tumor type, oligodendrogliomas. They reasoned the selective induction of oligodendrogliomas indicated that neoplastic transformation in the nervous system could occur in a differentiated glial cell or in a precursor cell committed to oligodendrocytic differentiation, and that transformation of a pluripotential stem cell was not necessary.

1.4 GLIAL CELL LINEAGES IN THE MAMMALIAN BRAIN

1.4.1 Glial Cells *In Vitro*

In 1980, McCarthy and de Vellis developed a method for separating rat astrocytes from O2A progenitors and their derivatives based on differential adhesion to tissue culture plates. In the years since, much study has gone into the development, differentiation, and cellular lineage of these glial cells in culture (Abney *et al.*, 1983; Raff *et al.*, 1983b; Raff *et al.*, 1983a; Eccleston and Silberberg, 1984; Raff *et al.*,

1984b; Raff *et al.*, 1984a; Miller *et al.*, 1985; Raff *et al.*, 1985; Williams *et al.*, 1985; ffrench Constant and Raff, 1986; Kennedy and Fok Seang, 1986; Lillien *et al.*, 1988; Bogler *et al.*, 1990; Lillien and Raff, 1990; Agresti *et al.*, 1991; Dutly and Schwab, 1991; Knapp, 1991; Madarasz *et al.*, 1991; Mozell and McMorris, 1991; Wolswijk *et al.*, 1991; Louis *et al.*, 1992; Wolswijk and Noble, 1992; McKinnon *et al.*, 1993) and the subject has been reviewed extensively (Miller *et al.*, 1989; Raff, 1989; Cameron and Rakic, 1991; Goldman and Vaysse, 1991; Noble, 1991; Noble *et al.*, 1991; Skoff and Knapp, 1991; Linskey and Gilbert, 1995). Two studies in particular advanced the ability to grow pure cultures of individual glial subtypes in culture: 1) Raff *et al.* (1983b) demonstrated that serum deprivation promoted oligodendrocytic differentiation in cultures of O2A progenitor cells and 2) Bogler *et al.* (1990) found that O2A progenitors proliferated in the presence of PDGF and basic fibroblast growth factor (bFGF) and differentiated into pure oligodendrocytes when bFGF was removed from the medium. With this knowledge, glial cell culture techniques were further refined by Barres *et al.* (1992) and it is now possible to culture large numbers of pure populations of astrocytes, oligodendrocyte progenitors, and oligodendrocytes from rat brain.

Two major branches of the mammalian glial cell lineage can be distinguished by the presence or absence of a surface marker detected by an antibody called A2B5 (Raff, 1989). A2B5 negative glial progenitors give rise to astrocytes (called type 1 astrocytes by some). A2B5 positive precursor cells, called oligodendrocyte or O2A progenitors, give rise to oligodendrocytes. In tissue culture, O2A progenitors also give rise to a second astrocytic cell type called type 2 astrocytes. The existence of type 2 astrocytes *in vivo* has been debated extensively and results remain inconclusive (Miller and Raff, 1984; Miller *et al.*, 1985; Miller *et al.*, 1989; Cameron and Rakic, 1991; Goldman and Vaysse, 1991; Noble, 1991; Skoff and Knapp, 1991; Espinosa de los Monteros *et al.*, 1993); thus, these cells are not discussed further. Astrocytes, oligodendrocyte progenitors, and oligodendrocytes have distinct morphologies and can be detected in tissue sections and cell culture using immunochemical techniques. Astrocytes are A2B5 negative, glial fibrillary acidic protein (GFAP) positive, and galactocerebroside (GC) negative.

Oligodendrocyte progenitors are A2B5 positive, GFAP negative, and GC negative. Oligodendrocytes are A2B5 negative, GFAP negative, and GC positive [Note: immature oligodendrocytes express A2B5 but this marker is lost as oligodendrocytes differentiate fully.]

1.4.2 Glial Cells *In Vivo*

Neuroglia in the brain function as supportive cells and comprise approximately one half of the mammalian brain by mass (Marieb and Mallatt, 1997). Each glial cell type has its own specialized role. The most abundant cells are the astrocytes, stellate cells of diverse morphology and function. The main role of these cells is to stabilize the neuronal microenvironment: they remove neurotransmitters (Marieb and Mallatt, 1997) and potassium ions (Newman, 1985; Ransom and Sontheimer, 1992) from the extracellular space following neuronal activity; remove neurotoxic molecules (Eddleston and Mucke, 1993); participate in carbohydrate and lipid metabolism (Bignami and Dahl, 1994); and synthesize neurotrophic factors and cytokines (Stockli *et al.*, 1991; Yoshida and Gage, 1992; Lin *et al.*, 1993). In addition, Janzer and Raff (1987) have provided evidence that astrocytes are responsible for inducing capillary and venule endothelial cells in the CNS to form the blood-brain barrier. Oligodendrocytes are the myelin producing cells of the CNS and each oligodendrocyte can form myelin sheaths around multiple axons (Marieb and Mallatt, 1997). These insulating layers of myelin facilitate rapid action potentials and the conservation of metabolic energy in neurons (Morell and Norton, 1980). Ependymal cells form a single layer of ciliated epithelium that lines the ventricles of the brain and the central canal of the spinal cord; specialized ependymal cells in the choroid plexus regulate the production of cerebrospinal fluid (Del Bigio, 1995; Marieb and Mallatt, 1997). These cells constitute a fairly permeable interface between the cerebrospinal fluid and the tissue fluid that bathes the cells of the CNS and function as a protective and regulatory barrier (Del Bigio, 1995; Marieb and Mallatt, 1997). Finally, microglia are phagocytic cells that migrate through the CNS and remove foreign and degenerated material (Marieb and Mallatt, 1997). As microglia are not fixed glial cells of the brain and do not contribute to the formation of the gliomas discussed

previously (refer to section 1.2), they are not discussed further.

1.5 MECHANISMS OF RESISTANCE TO BCNU

1.5.1 Introduction

The nitrosoureas (BCNU, CCNU, and methyl-CCNU being the most common) are lipid-soluble drugs capable of penetrating into the CNS for treatment of intracranial tumors. Of these, BCNU has been the most frequently studied chemotherapy agent in the treatment of glial tumors (Chang *et al.*, 1983; Walker *et al.*, 1978; Walker *et al.*, 1980). Other chemotherapeutic agents studied either alone or in combination displayed similar or less effective activity and often were more toxic than BCNU alone (Chang *et al.*, 1983; Green *et al.*, 1983; Leibel *et al.*, 1991; Nelson *et al.*, 1988; Shapiro *et al.*, 1989). For these reasons, the combination of conventional radiotherapy and BCNU became a standard treatment regimen for gliomas (Deutsch *et al.*, 1989). One exception to this finding has been the combination of procarbazine, CCNU, and vincristine (PCV) which in one study resulted in an improvement of outcome for patients with anaplastic gliomas (Levin *et al.*, 1990).

BCNU is highly unstable in aqueous medium, especially in the presence of plasma proteins, and its decomposition results in a large number of products (reviewed in Wiencke and Wiemels, 1995). The first stage of decomposition results in two products (Montgomery *et al.*, 1967). One of these is chloroethyldiazohydride, which gives rise to the reactive chloroethyl carbonium ion that is thought to be responsible for the alkylation of DNA. In addition, further degradation of chloroethyldiazohydride can lead to significant amounts of several volatile products, including acetaldehyde and chloroethanol. The second product from the first stage of decomposition is chloroethyl isocyanate. It is believed that protein modifications are the result of the carbamoylating activity of this compound. Metabolic deactivation of the chloroethylnitrosoureas is through either the cytochrome P-450 enzymes or by the more prevalent glutathione-*S*-transferases.

The main anti-tumor activity of BCNU is considered by most to be the formation

of DNA interstrand crosslinks (Colvin *et al.*, 1976; Erickson *et al.*, 1978; Erickson *et al.*, 1980; Bodell *et al.*, 1984; Beith *et al.*, 1997) and the initial, rapidly formed DNA modification O^6-chloroethyl guanine is thought to be necessary for the formation of these crosslinks (Erickson *et al.*, 1980). After the initial chloroethylation at the O^6 position of guanine, an intramolecular rearrangement occurs leading to an ethane bridge between the guanine and its partnered cytosine (Ludlum, 1990). The resultant crosslink has been identified as 1-[N^3-deoxycytidyl],2-[N^1-deoxyguanosinyl]-ethane (Tong *et al.*, 1982).

1.5.2 O^6-Methylguanine-DNA Methyltransferase

One of the best-studied mechanisms of nitrosourea resistance is the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT), also called O^6-alkylguanine-DNA alkyltransferase (AGT) (reviewed extensively by Yarosh, 1985; Lindahl *et al.*, 1988; Pegg, 1990a; Pegg, 1990b). This protein removes adducts from the O^6-position of guanine, one type of structural alteration formed when alkylating agents react with double stranded DNA. Of particular interest to BCNU resistance, MGMT repairs the O^6-chloroethyl adducts produced by the chloroethylnitrosoureas, preventing the ultimate cytotoxic lesions, guanine-cytosine interstrand DNA crosslinks (refer to section 1.5.1). MGMT acts by covalent transfer of the alkyl group from the modified base to a cysteine residue within the active site of the protein, restoring the integrity of the DNA and irreversibly inactivating the protein. Due to the stoichiometry of the reaction, the repair capacity of the cell depends on the number of active MGMT molecules present at the time of cytotoxic insult.

The cytotoxic and carcinogenic properties of the nitrosoureas appear related to MGMT activity: cells rendered MGMT positive by DNA transfection or transgenic expression become resistant to the nitrosoureas (Ding *et al.*, 1985; Kaina *et al.*, 1991; Dumenco *et al.*, 1993; Fritz *et al.*, 1993); resistant cells depleted of MGMT by pretreatment with O^6-methylguanine or O^6-benzylguanine are sensitized to the nitrosoureas (Dolan *et al.*, 1985; Yarosh *et al.*, 1986; Dolan *et al.*, 1990a; Dolan *et al.*, 1990b; Dolan *et al.*, 1991; Mitchell *et al.*, 1992; Baer *et al.*, 1993; Felker *et al.*, 1993;

Sarkar *et al.*, 1993; Marathi *et al.*, 1994; Wedge and Newlands, 1996); and cells susceptible to neoplastic transformation by nitrosoureas express low levels of MGMT (Swenberg *et al.*, 1982; Wani *et al.*, 1992).

MGMT is the mechanism of drug resistance in human glial tumors and cells that has received the most attention to date (Wiestler *et al.*, 1984; Bodell *et al.*, 1986; Aida and Bodell, 1987; Schold, Jr. *et al.*, 1989; Frosina *et al.*, 1990; Citron *et al.*, 1991; Ostrowski *et al.*, 1991; Friedman *et al.*, 1992b; Hotta *et al.*, 1993; Mineura *et al.*, 1993; Silber *et al.*, 1993; Hotta *et al.*, 1994; Bobola *et al.*, 1995; Citron *et al.*, 1995; Belanich *et al.*, 1996; Mineura *et al.*, 1996a; Mineura *et al.*, 1996b; Beith *et al.*, 1997). Most studies agree that one of the mechanisms responsible for resistance of gliomas to the chloroethylnitrosoureas is increased repair of O^6-alkylguanine products in DNA by MGMT (Bodell *et al.*, 1986; Aida and Bodell, 1987; Friedman *et al.*, 1992b; Mineura *et al.*, 1993; Hotta *et al.*, 1994; Mineura *et al.*, 1996a; Beith *et al.*, 1997), although some studies provide evidence that BCNU resistance is multifactorial (Hotta *et al.*, 1993; Bobola *et al.*, 1995) and that MGMT makes only a modest contribution to resistance (Bobola *et al.*, 1995). Additional studies have demonstrated a shorter time to treatment failure and death in patients with high MGMT activity, supporting the concept that MGMT correlates with resistance to the nitrosoureas (Belanich *et al.*, 1996). A wide range of MGMT activity has been found in brain tumor samples (Frosina *et al.*, 1990; Hotta *et al.*, 1994; Citron *et al.*, 1995); one study suggested that activity is higher in low grade gliomas than in high grade tumors (Mineura *et al.*, 1996b), one suggested the reverse (Frosina *et al.*, 1990), while still others have found no significant difference (Mineura *et al.*, 1993; Hotta *et al.*, 1994). In addition, Wiestler *et al.* (1984) and Frosina *et al.* (1990) found lower levels of MGMT activity in oligodendrogliomas than astrocytomas but this also has not been a universal finding (Mineura *et al.*, 1993; Mineura *et al.*, 1996a; Silber *et al.*, 1993). Low MGMT activity has been detected in normal human brain tissue (Grafstrom *et al.*, 1984; Wiestler *et al.*, 1984; Silber *et al.*, 1993; Silber *et al.*, 1996) but MGMT activity has not been studied in purified populations of glial cells.

1.5.3 Glutathione and Glutathione-*S*-Transferases

Tumor cell resistance to alkylating agents has been associated with changes in cellular metabolic systems that facilitate detoxification of cytotoxic drugs (Beck and Dalton, 1997), such as cytochrome P-450 enzymes (Hill *et al.*, 1975; Grant and Ironside, 1995) and the glutathione-glutathione-*S*-transferase pathway. Of these systems, the majority of investigations of nitrosourea resistance have concentrated on the effect of alterations in levels of glutathione and its associated glutathione-*S*-transferases (GSTs). The tripeptide glutathione plays a role in detoxification by non-enzymatic conjugation of alkylating agents and other electrophilic molecules with its thiolate moiety (Russo *et al.*, 1986a). This conjugation of drug with glutathione results in a more water soluble and less toxic product (Beck and Dalton, 1997). GSTs are a family of enzymes that catalyze the conjugation of these drugs, thereby enhancing the rate of detoxification. GST isoenzymes are broadly grouped by their isoelectric points into three classes: GST-π, acidic; GST-μ, neutral; and GST-α, basic (Mannervik *et al.*, 1992). Each isoenzyme class exhibits different but potentially overlapping substrate specificities.

Levels of glutathione and GSTs have been implicated in resistance to a variety of alkylating agents including cyclophosphamide (McGown and Fox, 1986; Friedman *et al.*, 1992a), nitrogen mustards (Ahmad *et al.*, 1987; Evans *et al.*, 1987), cisplatin (Teicher *et al.*, 1987; Moore *et al.*, 1989), melphalan (Friedman *et al.*, 1989) and the chloroethylnitrosoureas (Ali-Osman *et al.*, 1989; Ali-Osman, 1989; Smith *et al.*, 1989; Ali Osman *et al.*, 1990; Hara *et al.*, 1993). Ali-Osman *et al.* (1989) have reported increased resistance to nitrosoureas *in vitro* following the addition of glutathione and Russo *et al.* (1986b) were able to show an increased resistance to melphalan after increasing glutathione levels with oxothiazolidine-4-carboxylate (OTZ), an intracellular cysteine delivery system. Others have demonstrated decreased resistance to alkylating agents upon reduction of glutathione levels with buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase (Green *et al.*, 1984; Somfai-Relle *et al.*, 1984a; Somfai-Relle *et al.*, 1984b; Friedman *et al.*, 1989). In addition, inhibition of the GST enzymes with ethacrynic acid or piriprost has been found to reverse resistance to

alkylating agents (Tew *et al.*, 1988).

One interesting property of the chloroethylnitrosoureas in regard to glutathione is the ability to inactivate the enzyme glutathione reductase through the specific carbamoylation of the enzyme by the decomposition products, 2-chloroethyl isocyanate and cyclohexyl isocyanate (Babson and Reed, 1978). Babson *et al.* (1981) also were able to demonstrate that this effect resulted in a 70% decrease in glutathione levels. In contrast, Deneke *et al.* (1992) found that although BCNU treatment was effective at reducing glutathione reductase activity, a concentration-dependent increase in glutathione levels was observed after 24 hr of exposure and that this increase correlated with increases in cysteine uptake. It was suggested that in addition to the ability of BCNU to inactivate glutathione reductase, BCNU also may stimulate cysteine uptake and subsequent glutathione synthesis and that these two effects are perhaps totally independent of each other (Deneke *et al.*, 1992).

Glutathione and GST levels have been studied in human glioma tissues and cell lines and support for a role in resistance to alkylating agents has been found (Ali-Osman *et al.*, 1989; Friedman *et al.*, 1989; Ali Osman *et al.*, 1990). Studies by Strange *et al.* (1992) and Grant *et al.* (1995) demonstrated that the expression of GST-π was the predominant subclass expressed in astrocytic tumor cells, although GST-α and GST-μ also were expressed in some tumors. The localization of the placental form of GST-π, expressed normally during development, also has been demonstrated in glioma cell lines (Hara *et al.*, 1993). These findings are of particular interest since Ali-Osman *et al.* (1990) showed that only the expression of the GST-π subclass correlated significantly with BCNU resistance in human astrocytoma cell lines. Expression of GSTs also have been studied in normal human brain (Carder *et al.*, 1990; Strange *et al.*, 1992) and parallel that found in tumors; GST-π was the predominant subclass with GST-α and GST-μ subclasses being detected less frequently. Carder *et al.* (1990) also demonstrated expression of GST-π in normal human astrocytes.

1.5.4 Nucleotide-Excision Repair Proteins

A system that has received less attention as a mechanism of resistance to the chloroethylnitrosoureas involves nucleotide-excision repair (NER). It is known that interstrand crosslinks are removed from human DNA and based on evidence from bacterial systems, it is thought that these crosslinks are eliminated by the combined actions of the excision repair and recombination systems (Chaney and Sancar, 1996). NER removes bulky lesions from DNA in the form of 27- to 29-nucleotide-long oligomers by: incising the damaged strand on both sides of the lesion; excising the segment of damaged DNA with an excinuclease; filling the gap with DNA replication proteins; and ligating the repair patch (Sancar, 1994; Sancar, 1995; Chaney and Sancar, 1996). In addition, NER plays a back-up role for the repair of non-bulky lesions, including O^6-methylguanine residues (Chaney and Sancar, 1996). It is not surprising, therefore, that evidence suggests the NER pathway is involved in repair of at least some of the cytotoxic lesions formed by the chloroethylnitrosoureas (Bronstein *et al.*, 1992; Wu *et al.*, 1992; Cappelli *et al.*, 1995); a direct effect on resistance to the chloroethylnitrosoureas is less well defined, however.

The investigation into a possible role for NER in glial tumors has been limited to studies of the excision repair cross-complementing (ERCC) proteins. ERCC1, a member of the incision complex, and ERCC2, a member of the helicase complex, are both mapped to chromosome 19q, a genomic region of common abnormality in glioma (refer to section 1.2.5). Abnormalities in copy number of ERCC1 and ERCC2 have been demonstrated in glial tumors (Liang *et al.*, 1995). In addition, although mRNA levels of ERCC1 and ERCC2 are tightly coordinated in non-malignant brain tissue, it has been suggested that this coordination is lost in malignant brain tissues (Dabholkar *et al.*, 1995) and that progressive loss of coordination correlated with increased malignancy (Dabholkar *et al.*, 1996). The role that these changes may play in resistance of glial tumors to the chloroethylnitrosoureas has not been examined.

Although the base mismatch repair system has been shown to remove some of the bases modified by the chloroethylnitrosoureas, these lesions are generally not considered

cytotoxic and therefore, it is not likely that this system contributes substantially to resistance to these agents (Cappelli *et al.*, 1995). In addition to being able to repair DNA damage, acquired resistance to monofunctional alkylating agents such as MNU and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) has been linked to their ability to tolerate the potentially cytotoxic methylated base O^6-methylguanine in DNA (Karran and Bignami, 1992). This tolerance is imparted to cells defective for the DNA mismatch repair pathway (Branch *et al.*, 1993; Kat *et al.*, 1993; Hawn *et al.*, 1995). Since the O^6-chloroethyl adducts produced by the chloroethylnitrosoureas go on to form guanine-cytosine interstrand DNA crosslinks (refer to section 1.5.1), tolerance of the original adduct is not a viable method of resistance. Therefore, this method of resistance will not be discussed further.

1.6 ADDITIONAL MECHANISMS OF DRUG RESISTANCE RELEVANT TO GLIAL TUMORS

1.6.1 Introduction

In addition to the classical chloronitrosoureas, a number of other chemotherapeutic agents have been used in the treatment of glial tumors. These agents often are used in various combination therapies and include procarbazine (Cairncross and Macdonald, 1988; Macdonald *et al.*, 1990; Cairncross and Macdonald, 1991; Cairncross *et al.*, 1992; Glass *et al.*, 1992; Kim *et al.*, 1996; Mason *et al.*, 1996), cisplatin (Boiardi *et al.*, 1991; Garfield *et al.*, 1991; Dropcho *et al.*, 1992; Mortimer *et al.*, 1992; Spence *et al.*, 1992; Yung *et al.*, 1992; Nakagawa *et al.*, 1994; Hiesiger *et al.*, 1995), melphalan (Brown *et al.*, 1990), thiotepa (Saarinen *et al.*, 1990), diaziquone (Cairncross and Macdonald, 1988; Cairncross and Macdonald, 1991), dacarbazine (Chang *et al.*, 1983; Nelson *et al.*, 1988), temozolomide (Newlands *et al.*, 1992; O'Reilly *et al.*, 1993; Newlands *et al.*, 1996), vincristine (Cairncross and Macdonald, 1988; Macdonald *et al.*, 1990; Cairncross and Macdonald, 1991; Cairncross *et al.*, 1992; Glass *et al.*, 1992; Kim *et al.*, 1996; Mason *et al.*, 1996), and etoposide (Boiardi *et al.*, 1991; Nakagawa *et al.*, 1994).

Although many of these are alkylating agents and their efficacy would be influenced by many of the same mechanisms of resistance already discussed (refer to sections 1.5.2 through 1.5.5), some belong to other families of drugs. For this reason, it is useful to consider alternate mechanisms that may play a role in the resistance of glial tumors to these agents.

1.6.2 Multidrug Resistance (P-glycoprotein)

Simultaneous resistance of cancers to multiple unrelated chemotherapeutic agents is a common occurrence and has been attributed to a phenomenon known as multidrug resistance (MDR). The best characterized MDR mechanism is P-glycoprotein, a protein encoded by the *MDR1* gene. P-glycoprotein is a member of the ATP-binding cassette (ABC) superfamily (Higgins, 1992) and acts as a transmembrane, energy-dependent drug-efflux pump (Endicott and Ling, 1989; Gottesman and Pastan, 1993). The drugs most often involved in MDR are vinca alkaloids (eg. vincristine), anthracyclines (eg. doxorubicin), and epipodophyllotoxins (eg. etoposide); elevated levels of P-glycoprotein have been associated with resistance to these agents in many systems (Endicott and Ling, 1989; Gottesman and Pastan, 1993). P-glycoprotein has been detected mainly on capillary endothelial cells within human brain tumors (Nabors *et al.*, 1991; Tanaka *et al.*, 1994) and implicated in the resistance, at least in part, of glial tumors to vincristine, etoposide, and doxorubicin (Feun *et al.*, 1994; Kiwit *et al.*, 1994; Abe *et al.*, 1995; Walther *et al.*, 1995).

1.6.3 Multidrug Resistance-Associated Protein

A second, and more recently characterized, mechanism of MDR is the multidrug resistance-associated protein (MRP) (Cole *et al.*, 1992). MRP is also a member of the ABC superfamily and exhibits approximately 15% amino acid identity with P-glycoprotein (refer to section 1.6.2) (Cole *et al.*, 1992; Grant *et al.*, 1994). MRP, like P-glycoprotein, is an energy-dependent drug pump that extrudes drugs from the cell and is capable of causing resistance to a similar, although not identical, spectrum of drugs

(Grant *et al.*, 1994; Zaman *et al.*, 1994; Koike *et al.*, 1996; Utz *et al.*, 1996). The main functional property of MRP that distinguishes it from P-glycoprotein is the ability of MRP to export lipophilic compounds that have been conjugated to glutathione, suggesting a role for MRP as a glutathione *S*-conjugate carrier (Leier *et al.*, 1994; Muller *et al.*, 1994; Versantvoort *et al.*, 1995; Zaman *et al.*, 1995; Hollo *et al.*, 1996; Loe *et al.*, 1996). Very few studies have been performed to investigate a possible role for MRP in human glial tumors but preliminary evidence suggests that low level MRP expression is more common in glioma than MDR gene expression (Feun *et al.*, 1994). In addition, MRP has been implicated in the resistance, at least in part, of glial tumors to vincristine, etoposide, and doxorubicin (Abe *et al.*, 1994; Abe *et al.*, 1995).

1.6.4 Metallothionein

Although the physiologic role of metallothionein (MT) in resistance to the cytotoxic effects of anticancer drugs remains unclear and controversial, increased levels of MT have been associated with resistance to cisplatin and some alkylating agents (Kelley *et al.*, 1988; Kaina *et al.*, 1990; Lohrer *et al.*, 1990; Doz *et al.*, 1993). MTs are a family of low molecular weight, intracellular proteins characterized by an abundance of thiol groups (30% cysteine) and are, in fact, the principal source of thiol groups in eukaryotic cells (Kagi and Schaffer, 1988; Doz *et al.*, 1993). Originally, it was thought that MT and cytotoxic agents, including cisplatin and alkylating agents, interacted through simple covalent binding by the thiol groups (Zelazowski *et al.*, 1984); this mechanism would prevent the active molecules from reaching their DNA target in the cell. More recently, however, it has been suggested that the effect of MT on these agents may be more complex and the physiologic role of MT requires more study (Doz *et al.*, 1993). As with other tumor types, increased MT levels have been implicated in resistance of glial tumors to cisplatin (Phillips, 1991; Feun *et al.*, 1994).

1.6.5 Topoisomerase II

The DNA topoisomerases relieve the torsional stress on DNA during replication,

transcription, and cell division (Liu, 1989). Type II topoisomerases are necessary for the replication of DNA; they permit the creation of a double-strand break in the DNA, rotation of the two strands, and resealing of the helix (Liu, 1989; Tannock, 1992). Anthracyclines (eg. doxorubicin), epipodophyllotoxins (eg. etoposide), and aminoacridines (eg. amsacrine) all block topoisomerase II strand religation and stabilize DNA-protein complexes, resulting in cytotoxic lesions (Morrow and Cowan, 1990; Beck and Dalton, 1997). Resistance to these topoisomerase inhibiting drugs generally occurs in one of two ways: 1) topoisomerase levels are downregulated and the amount of stabilized complex formed is thus insufficient to initiate cell death; or 2) mutations of the topoisomerase allow it to resist stabilization by the drugs (Asano *et al.*, 1996b). Decreased topoisomerase II levels have been implicated in the resistance of glial tumors to etoposide (Mousseau *et al.*, 1993; Feun *et al.*, 1994; Asano *et al.*, 1996a; Asano *et al.*, 1996b).

In contrast, inhibition of topoisomerase II has been suggested to play a role in sensitizing brain tumor cells to DNA crosslinking anticancer agents (Ali Osman *et al.*, 1993). It was postulated that the topographical changes in DNA accompanying the unwinding of DNA by topoisomerase II would make the DNA segments more open and thus, would affect not only the extent and site of DNA damage but also the accessibility of the damaged DNA to repair proteins. The possibility of enhanced repair in the presence of topoisomerase II suggested a potential mechanism of increased resistance to the DNA crosslinking drugs.

1.7 THE p53 TUMOR SUPPRESSOR

1.7.1 Introduction

The p53 protein was first discovered in 1979 as part of a stable oligomeric complex with the SV40 large T-antigen that could be immunoprecipitated from extracts of SV40-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Since p53 was expressed at higher levels in tumor cells than in normal cells (Benchimol *et al.*, 1982) and some cancer patients presented with circulating antibodies directed against the

protein (Crawford *et al.*, 1982), it was thought to be necessary for cellular transformation. Original *p53* cDNA clones were found to immortalize cultured cells when co-transfected with other oncogenes such as *ras* and *E1A* and thus, *p53* became regarded as an oncogene (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984; Wolf *et al.*, 1984). This concept changed dramatically in 1989 when it was found that the clones used in previous experiments were actually mutants of *p53* (Hinds *et al.*, 1989). Subsequently, it was determined that p53 inhibited the growth of tumor cells (Baker *et al.*, 1990; Chen *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990) and suppressed the transformed phenotype of malignant cells (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). The *p53* gene was then reclassified properly as an anti-oncogene or tumor suppressor gene.

Since these initial findings, the role of p53 in human tumors has been studied extensively. The *p53* gene is now considered the most frequently mutated gene in many common malignancies, including tumors of the colon, breast, lung, esophagus, liver, and brain (Nigro *et al.*, 1989; Hollstein *et al.*, 1991; Levine *et al.*, 1991; Vogelstein and Kinzler, 1992). Approximately one half of these adult cancers contain a *p53* mutation, of which more than 80% are missense mutations clustered within evolutionarily conserved regions (Hollstein *et al.*, 1991; Hollstein *et al.*, 1996). Although mutations and allelic losses are the most common mechanisms for the inactivation of p53, the loss of normal p53 function also can result from the formation of protein complexes with viral oncoproteins (Levine, 1990), dislocation of p53 between cell compartments (Moll *et al.*, 1992), and inactivation and degradation through the overexpression of mdm-2 (Momand *et al.*, 1992; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). In addition to the loss of p53 function, mutations also exist that result in a gain of oncogenic function (Dittmer *et al.*, 1993; Hsiao *et al.*, 1994). It is this potential to transform cells by two mechanisms that may provide an explanation for the high frequency of *p53* mutations in human cancers (Greenblatt *et al.*, 1994).

Mutation frequencies also have been studied extensively in human glial neoplasms. Chromosome 17p deletions and *p53* gene mutations are found frequently in astrocytic

gliomas of all malignancy stages (James *et al.*, 1988; el Azouzi *et al.*, 1989; James *et al.*, 1989; Fults *et al.*, 1989; Nigro *et al.*, 1989; Chung *et al.*, 1991; Hayashi *et al.*, 1991; Mashiyama *et al.*, 1991; Frankel *et al.*, 1992; Fults *et al.*, 1992; Saxena *et al.*, 1992; Sidransky *et al.*, 1992; von Deimling *et al.*, 1992a; Aka *et al.*, 1993; del Arco *et al.*, 1993; Hunter *et al.*, 1993; Louis *et al.*, 1993; Newcomb *et al.*, 1993; Wu *et al.*, 1993; Bello *et al.*, 1994; Koga *et al.*, 1994; Kraus *et al.*, 1994; Louis, 1994; Rasheed *et al.*, 1994). In contrast, oligodendrogliomas rarely harbor *p53* mutations (Reifenberger *et al.*, 1994) but often accumulate wild type p53 protein (Pavelic *et al.*, 1994). Elucidating the consequences of such differential genetic changes in gliomas will not only enhance understanding of brain tumor biology but also may have implications for diagnosis, prognosis, and treatment.

1.7.2 The *p53* gene

Interestingly, p53 is found in vertebrates only (Soussi *et al.*, 1987). The *p53* gene is expressed, at least at low levels, in all tissues (Rogel *et al.*, 1985) and appears to be highly conserved between species (Soussi *et al.*, 1987). The human *p53* gene is located on the short arm of chromosome 17 at position 17p13.1 and encompasses 16 to 20 kb of DNA (McBride *et al.*, 1986; Miller *et al.*, 1986). It is comprised of 11 exons, the first of which is located 8 to 10 kb away from exons 2 through 11 and is non-coding. Transcription of the human *p53* gene is controlled by two different promoters (Reisman *et al.*, 1988) and results in a transcript of approximately 2.8 kb in length (Matlashewski *et al.*, 1984).

The mouse *p53* gene displays 81% homology to that of the human, has a similar genomic structure but spans approximately 12.5 kb of genomic DNA (Bienz *et al.*, 1984). It is localized to chromosome 11 and transcription results in a transcript approximately 2.0 kb in length. In addition to the gene found on chromosome 11, a processed, non-functional *p53* pseudogene is located on chromosome 14 (Zakut-Houri *et al.*, 1983). Alternatively spliced mRNA also has been documented in mouse cells (Arai *et al.*, 1986; Han and Kulesz Martin, 1992; Kulesz Martin *et al.*, 1994; Wu *et al.*, 1994;

Will *et al.*, 1995). This variant form of *p53* results in a protein shorter by nine amino acids and different by 17 amino acids at the C-terminus. Although a potential homologous alternative splice site is present in the human *p53* gene, the presence of alternatively spliced human mRNA remains uncertain (Will *et al.*, 1995).

1.7.3 The p53 protein

Human p53 is a 393 amino acid nuclear phosphoprotein within which multiple functional domains can be found. The N-terminus contains an acidic transcriptional activation domain within amino acids 1-43 (Unger, 1992) and central residues 100-300 comprise a sequence specific DNA binding domain (Bargonetti *et al.*, 1993; Halazonetis *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). The C-terminus encompasses an oligomerization domain within amino acids 320-360 (Sturzbecher *et al.*, 1992; Pavletich *et al.*, 1993; Wang *et al.*, 1993; Wang *et al.*, 1994) and a region from residues 330-393 capable of non-specific nucleic acid binding, reannealing, and strand-transfer (Wang *et al.*, 1993; Bakalkin *et al.*, 1994; Jayaraman and Prives, 1995; Lee *et al.*, 1995; Reed *et al.*, 1995). In addition, three nuclear localization signal sequences have been documented in the C-terminus of p53 (Shaulsky *et al.*, 1991) and regions that may harbor negative autoregulatory domains also have been suggested (Hupp *et al.*, 1992; Mosner *et al.*, 1995).

Levels of wild type p53 are generally low due to a short protein half life. The turnover of p53 *in vivo* is mediated by the ubiquitin proteolysis system (Maki *et al.*, 1996) and enzymes capable of participating in the *in vitro* ubiquitination of p53 have been documented (Scheffner *et al.*, 1990; Scheffner *et al.*, 1993; Scheffner *et al.*, 1995). Levels of p53 increase dramatically following DNA damage and this increase is thought to be due to p53 protein stabilization (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Khanna and Lavin, 1993; Lu and Lane, 1993; Hess *et al.*, 1994; Sakhi *et al.*, 1994; Yamaizumi and Sugano, 1994; Midgley *et al.*, 1995; Sugano *et al.*, 1995). Maki and Howley (1997) suggested that one possible mechanism for the stabilization of p53 following DNA damage is the repression of the ubiquitin-mediated

degradation of p53. They showed that UV and gamma radiation had differential effects on ubiquitinated p53 and suggested that the UV-induced stabilization of p53 is due to a specific loss of p53 ubiquitination. Although there are several mechanisms by which p53 ubiquitination could be lost following UV treatment, one strong and very intriguing possibility is that changes in the phosphorylation of p53 following UV irradiation could modify p53 in such a way that it can no longer be recognized by the ubiquitin-conjugating enzymes and the ubiquitin protein ligase involved in its ubiquitination (Maki and Howley, 1997).

Phosphorylation of the p53 protein appears to play a key role in the post-translational modification and regulation of p53 (Meek, 1994). p53 is a substrate for a number of serine kinases including CDC2 (Bischoff *et al.*, 1990), CDK2 (Wang and Prives, 1995), casein kinase II (Meek *et al.*, 1990), DNA dependent protein kinase (Lees-Miller *et al.*, 1990), protein kinase C (Baudier *et al.*, 1992), mitogen-activated protein kinase (Milne *et al.*, 1994), and JNK1 (Milne *et al.*, 1995). The ability of p53 to be phosphorylated by these kinases suggests a mechanism by which p53 may be regulated through intracellular signalling. In fact, mutations of these phosphorylation sites can influence DNA binding, transactivation, tumor suppression, and cell cycle progression (Hupp *et al.*, 1992; Fiscella *et al.*, 1993; Mayr *et al.*, 1995; Wang and Prives, 1995).

1.7.4 The Role of p53 in Response to DNA-Damaging Agents

1.7.4.1 Introduction

When DNA damage occurs in a cell, the alterations must be detected and signals sent to cellular control mechanisms in order that the cell can take measures to prevent the damage from being genetically fixed and propagated (Kastan *et al.*, 1995; Leonard *et al.*, 1995). The two principal options for a cell in response to DNA damage are: 1) for the cell to temporarily arrest its cell cycle to allow for repair of the damage; or 2) initiate a programmed cell death pathway (apoptosis). The p53 protein has been identified as the most significant player in both of these pathways and upregulation of p53 protein

in response to DNA damage has been well documented (Maltzman and Czyzyk, 1984; Khanna and Lavin, 1993; Lu and Lane, 1993; Hess *et al.*, 1994; Sakhi *et al.*, 1994; Yamaizumi and Sugano, 1994; Midgley *et al.*, 1995). The factors influencing whether cells undergo p53-dependent cell cycle arrest or apoptosis in response to DNA damage are probably multifaceted and likely include cell type, nature or extent of DNA damage, and growth factor environment (Yonish Rouach *et al.*, 1991; Collins *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993a; Lowe *et al.*, 1993b; Di Leonardo *et al.*, 1994; Canman *et al.*, 1995; Girinsky *et al.*, 1995). Consequently, p53 has been referred to as the "guardian of the genome" (Lane, 1992).

In addition to the cell cycle arrest and apoptotic responses initiated by p53 in response to DNA damage, recent studies have suggested that p53 may have a role in the actual detection of DNA damage. Nelson *et al.* (1994) demonstrated that DNA strand breaks, but not other DNA lesions, were necessary for induction of p53 protein in response to DNA damaging agents. Others have shown that the C-terminal domain of p53 can bind single-stranded DNA ends and catalyzes DNA renaturation and strand transfer (Bakalkin *et al.*, 1994; Jayaraman and Prives, 1995; Reed *et al.*, 1995). Lee *et al.* (1995) also demonstrated that the C-terminal domain of p53 recognized single-stranded regions caused by insertion and deletion lesions. These p53-DNA complexes were highly stable and displayed a half life of greater than two hours, suggesting that this interaction was specific. It was hypothesized that p53 may act to recruit other proteins to the lesion site, providing a signal for DNA damage.

1.7.4.2 Cell Cycle Arrest

One of the ways p53 plays a role in protecting cells and organisms from the deleterious effects of DNA damage is by regulating cell cycle progression. The requirement of a wild type p53 for the induction of cell cycle arrest in response to various kinds of DNA damage has been observed in many cell types (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992; O'Connor *et al.*, 1993; Slichenmyer *et al.*, 1993; Cross *et al.*, 1995; Fan *et al.*, 1995; Girinsky *et al.*, 1995;

Herzinger *et al.*, 1995; Stewart *et al.*, 1995; Pellegata *et al.*, 1996). These alterations in cell cycle presumably permit the optimal repair of damage before the cell reinitiates DNA synthesis (G_1 arrest) and/or begins mitosis (G_2 arrest), thus preventing the propagation of mutagenic lesions (Kuerbitz *et al.*, 1992).

The mechanism by which p53 is thought to exert its effects on cell cycle regulation is by activating the transcription of genes that influence cell cycle progression, such as *WAF1* and *GADD45* (Kastan *et al.*, 1995; Leonard *et al.*, 1995). The *WAF1* gene encodes the potent CDK inhibitor, p21 (el Deiry *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Noda *et al.*, 1994). The p21 protein binds the ternary kinase complex composed of a CDK, a cyclin, and proliferating cell nuclear antigen (PCNA); p21 acts as an assembling factor of the CDK complex but when the ratio of p21 to complex is more than one, p21 inhibits kinase activity (Zhang *et al.*, 1994). Thus, upregulation of p21 results in the inhibition of kinase activity and subsequent cell cycle arrest (Xiong *et al.*, 1993; Michieli *et al.*, 1994).

The *GADD45* gene was originally identified as an mRNA species whose expression was elevated in cells that had been growth arrested or had suffered DNA damage (Fornace, Jr. *et al.*, 1988; Fornace, Jr. *et al.*, 1989). *GADD45*, like p21, has been found to bind PCNA and inhibit DNA synthesis, although the mechanism by which this occurs is not well understood (Smith *et al.*, 1994; Chen *et al.*, 1995; Bates and Vousden, 1996). Interestingly, the binding of *GADD45* to PCNA also has been linked with the stimulation of DNA excision repair (Smith *et al.*, 1994). In addition, the binding of p21 to PCNA prevented the association of PCNA with DNA polymerase δ but not DNA polymerase ϵ, thereby inhibiting DNA replication while allowing DNA repair to proceed (Li *et al.*, 1994; Shivji *et al.*, 1994; Waga *et al.*, 1994). Thus, by inducing the expression of p21 and *GADD45*, p53 can potentially achieve the dual effect of arresting cell cycle progression and stimulating DNA repair.

1.7.4.3 Apoptosis

A second, and equally important, role for p53 in protecting cells and organisms

from the deleterious effects of DNA damage is through the induction of programmed cell death or apoptosis. Apoptosis is an active cell death pathway that is characterized morphologically by cell shrinkage, membrane blebbing, chromatin condensation, apoptotic body formation, and DNA degradation; dead cells are removed by macrophages without the induction of an inflammatory response (Sen and D'Incalci, 1992; Schwartzman and Cidlowski, 1993; Bortner *et al.*, 1995). In the event that a cell suffers substantial and irreparable DNA damage, p53 can trigger the apoptotic pathway to remove damaged cells and prevent the propagation of cells that have sustained mutation. In addition, some cells appear to be primed for apoptosis and will undergo programmed cell death in the presence of even minor DNA damage (Wang and Harris, 1996). The ability of p53 to induce apoptosis in response to various forms of DNA damage has been documented in many different cell types (Clarke *et al.*, 1993; Lowe *et al.*, 1993a; Lowe *et al.*, 1993b; Malcomson *et al.*, 1995; Norimura *et al.*, 1996).

The mechanism by which p53 induces apoptosis in response to DNA damaging agents is still unclear, although activities of p53 have been identified that may play a role in this process (Wang and Harris, 1996). The apoptotic pathway in humans that has received the most study to date includes multiple members of the bcl-2 family (Farrow and Brown, 1996) and is characterized by the interaction of the bcl-2 and bax proteins (Korsmeyer *et al.*, 1993). The relative amounts of bcl-2 and bax can dictate whether a cell will survive or undergo apoptosis in response to an environmental stimulus. Bcl-2 can participate in homo- and heterodimeric complexes with bax; bcl-2/bax heterodimers promote cell survival (Yin *et al.*, 1995) but the relative overexpression of bax results in the formation of bax/bax homodimers and subsequently, the induction of cell death (Oltvai *et al.*, 1993). p53 has been shown to induce the transcription of *bax* (Miyashita and Reed, 1995) and inhibit the expression of *bcl-2* (Miyashita *et al.*, 1994). It remains unclear, however, whether transcriptional activation of p53 target genes is required for p53-dependent apoptosis and thus, the ability of p53 to repress transcription of genes required for cell survival or participate directly as a component of the enzymatic machinery for apoptosis may provide alternative mechanisms for apoptotic induction

(Caelles *et al.*, 1994; Canman *et al.*, 1995).

1.7.4.4 p53-Regulated Drug Resistance Mechanisms

An alternative role for p53 in response to DNA-damaging agents may be to modulate response by influencing directly the regulation of drug resistance and DNA repair mechanisms. This has been suggested recently for a number of proteins. The first specific drug resistance mechanism implicated to be regulated by p53 was the *MDR1* gene (Chin *et al.*, 1992). Wild type p53 has been shown to repress the activity of the *MDR1* promoter *in vitro*, while mutant p53 was found to stimulate promoter activity (Chin *et al.*, 1992; Zastawny *et al.*, 1993; Nguyen *et al.*, 1994). These findings were supported by de Kant *et al.* when they found that *MDR1* expression correlated with mutant *p53* expression in colorectal cancer metastases (de Kant *et al.*, 1996). In contrast, wild type p53 stimulated transcription from a *MDR* promoter in a p53-negative cell line during co-transfection studies, whereas a mutant p53 could not stimulate transcription (Goldsmith *et al.*, 1995). Finally, Strauss and Haas (1995) demonstrated that *MDR1* transcription could only be stimulated by specific subtypes of p53 mutants.

More recently, p53 has been implicated to play a role in the regulation of the DNA repair protein MGMT (refer to section 1.5.2). Again, as with the *MDR* promoter, conflicting data has emerged. Harris *et al.* (1996) suggested that wild type p53 suppressed transcription of the MGMT gene whereas Rafferty *et al.* (1996) found that MGMT was induced in a p53 gene-dose-dependent manner in response to ionizing radiation.

In addition to being able to stimulate DNA excision repair through p21 and GADD45 as described in section 1.7.4.2, p53 also has been shown to function in the NER pathway (refer to section 1.5.4) by direct interaction with proteins of the repair complex. p53 can selectively bind proteins that are part of the basal transcription complex, including ERCC2, ERCC3, and p62 (Xiao *et al.*, 1994; Wang *et al.*, 1994; Wang *et al.*, 1995; Leveillard *et al.*, 1996; Wang and Harris, 1996), whereas some mutant p53 proteins lack this ability (Leveillard *et al.*, 1996). The p53 protein also has

been implicated in regulation of the human mismatch repair gene hMSH2 due to the ability of p53 to bind the hMSH2 promoter *in vitro* (Scherer *et al.*, 1996).

1.7.4.5 Summary

When DNA damage occurs in a cell, the alterations must be detected and signals sent to cellular control mechanisms in order that the cell can take measures to prevent the damage from being genetically fixed and propagated. The p53 protein seems to be a key player in the response of cells to DNA damage. The two main response pathways induced by p53 are cell cycle arrest and apoptosis. The factors influencing whether cells undergo p53-dependent cell cycle arrest or apoptosis in response to DNA damage are probably multifaceted and likely include cell type, nature or extent of DNA damage, and growth factor environment. In addition to cell cycle arrest and apoptosis, p53 also may play a role in the regulation of MDR expression and DNA repair; as much of this data has produced conflicting results, the significance of these findings remains to be determined. However, since *p53* is the most frequently mutated gene in many common human malignancies, these studies have important pathogenetic and therapeutic implications.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Culture Materials and Reagents

Surgical tools for animal dissections were purchased from Fine Science Tools Inc. (North Vancouver, British Columbia). All coverslips and plasticware used in these studies were precoated with 13.3 μg/ml poly-L-lysine obtained from Sigma Chemical Company (St. Louis, Missouri, USA). Circular coverslips (12 mm) were acquired from Fisher Scientific (Whitby, Ontario). Nunc cell culture plasticware, Dulbecco's Modified Eagles Medium (DMEM), L15 medium, Hank's Balanced Salt Solution, fetal bovine serum (FBS), antibiotics, and growth factors were purchased from Gibco BRL Life Technologies (Burlington, Ontario). All other cell culture reagents were obtained from Sigma Chemical Company. BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) was obtained from Bristol Laboratories of Canada (Montreal, Quebec) and MNU (*N*-methyl-*N*-nitrosourea) from Sigma Chemical Company. O^6-Benzylguanine (BG) was kindly provided by Dr. Robert C. Moschel of the National Cancer Institute - Frederick Cancer Research and Development Center (Frederick, Maryland, USA). Anti-galactocerebroside (GC) hybridoma cells (Ranscht *et al.*, 1982) were kindly provided by Dr. Mark Noble of the Huntsman Cancer Institute (Salt Lake City, Utah, USA). The anti-rat neural antigen-2 (anti-RAN-2) (Bartlett *et al.*, 1981) and A2B5 (Eisenbarth *et al.*, 1979) antibody hybridomas were purchased from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). Anti-GC antibody was obtained from Boehringer Mannheim (Laval, Quebec) and anti-glial fibrillary acidic protein (GFAP) antibody from Dimension Laboratories Inc. (Mississauga, Ontario). Rhodamine- and fluorescein-conjugated secondary antibodies, biotinylated anti-rabbit IgG and 7-amino-4-methylcoumarin-3-acetic acid (AMCA) streptavidin also were purchased from Dimension Laboratories Inc.

2.1.2 Molecular Biology Reagents

Restriction and modifying enzymes were obtained from Gibco BRL Life Technologies or Promega Corporation (Madison, Wisconsin, USA). Radiolabelled nucleotides ([α-^{32}P]dCTP and [γ-^{32}P]dATP) were purchased from Amersham Canada Limited (Oakville, Ontario). All other chemicals were of molecular biology grade and obtained from BDH Incorporated (Toronto, Ontario), Fisher Scientific, Gibco BRL Life Technologies and Sigma Chemical Company.

2.1.3 Primers, Probes and Plasmids

PCR primers and synthetic oligonucleotide probes for Northern hybridization were purchased from the Molecular Biology Core Facility of the Medical Research Council of Canada Group in Fetal and Neonatal Health and Development (London Regional Cancer Centre, London, Ontario). All oligonucleotides were prepared using a Gene Assembler Plus (Pharmacia LKB Biotechnology, Baie d'Urfé, Quebec). Sequences of the PCR primers are listed in Figure 2.1B. Sequences for the oligonucleotide probes are as follows:

MGMT	5' TTTCTTACCA GCAATTAGCA GCCCTGGCAG GCAACCCCAA AGC 3'
GST-μ	5' CGGCACGAAT CCGCTCCTCC TCTGTCTCTC CACACAGGTG 3'
GST-π	5' GGTAACCACC TCCTCCTTCC AGCTCTGGCC CTGGTCAGCC 3'
Bcl-2	5' CATTGGGTTG CTCTCAGGCT GGAAGGAGAA GATGCCAGG 3'
Bcl-x	5' GGCTCCATTC ACGGCCGGGC TATCCGCCAG GTGCCAGG 3'

cDNA templates for the synthesis of DNA probes were isolated from the appropriate plasmid by restriction endonuclease digestion, gel electrophoresis, and purification from low melting agarose (Gibco BRL Life Technologies). All plasmids were transformed into the *Escherichia coli* strain XL 1-Blue (Stratagene Cloning Systems, La Jolla, California, USA) unless otherwise noted. All cDNA inserts used for Northern hybridization were generously donated by other investigators. A 1.35 kb mouse *p53* partial cDNA in pUC-18 designated pMO53 was a gift from Dr. Sam Benchimol (Ontario Cancer Institute, Toronto, Ontario). A derivative of pMO53, pECM53, has been described by Johnson *et al.* (1991). The hamster *MDR* cDNA in pUC-9 designated

pEX/172 (Ng *et al.*, 1989) and the primers (A100 and B100) required to label the probe using a PCR protocol (refer to section 2.7.4) were kindly provided by Dr. Victor Ling (British Columbia Cancer Research Centre, Vancouver, British Columbia). The 1.05 kb mouse *GAPDH* fragment in pBR322 designated pM-GAP (Edwards *et al.*, 1985; Edwards and Denhardt, 1985) was donated by Dr. Ann Chambers (London Regional Cancer Centre, London, Ontario). A 1.3 kb mouse *mdm-2* cDNA in pBluescript SK− designated 11B (Fakharzadeh *et al.*, 1991) was provided by Dr. Donna George (University of Pennsylvania, Philadelphia, Pennsylvania, USA). The plasmid p21-9C (Huppi *et al.*, 1994), containing a 0.85 kb mouse *WAF1* cDNA in pGEM-4Z, was supplied by Dr. Konrad Huppi (National Cancer Institute, Bethesda, Maryland, USA). Dr. A. J. Fornace (National Cancer Institute, Bethesda, Maryland, USA) provided pXR45m (Papathanasiou *et al.*, 1991), a 1.2 kb *GADD45* cDNA isolated from Chinese hamster. The 0.94 kb mouse *bax* cDNA designated 70Z7 (Oltvai *et al.*, 1993) was donated by Dr. Stanley Korsmeyer (Washington University School of Medicine, St. Louis, Missouri, USA).

2.2 RAT PRIMARY CELL CULTURES

2.2.1 Isolation of Glial Cell Enriched Cultures

Two populations of glial cells, oligodendrocyte lineage enriched and astrocyte enriched, were isolated from newborn (1 to 3 day old) rat cerebrums using a method modified from McCarthy and de Vellis (1980). Cerebral hemispheres were isolated from unanesthetized Sprague-Dawley neonates and dissected free of meninges and blood vessels. The tissue was minced with a scalpel blade and treated with 0.025% trypsin for 30 min at 37°C. Trypsin inhibitor with 40 μg/ml DNase was added and incubated for 5 min to stop the digestion of the tissue. The tissue was centrifuged in a Beckman Model TJ-6 centrifuge (Beckman Instruments, Inc., Fullerton, California, USA) for approximately 1 min at 100g, the pellet resuspended in 40 μg/ml DNase and triturated in a 10 ml pipette to dissociate cells. The resultant single cell suspension was centrifuged at 100g for 5 min, resuspended in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml transferrin, 5 μg/ml insulin, 30 nM selenium, 30 nM tri-

iodothyronine and 50 U/ml penicillin/streptomycin, and seeded into polylysine-coated 80 cm^2 flasks ($2X10^7$ cells/flask). After five days, the FBS concentration was reduced to 1% to enhance oligodendrocyte differentiation (Raff *et al.*, 1983b). At day nine, oligodendrocyte lineage cells were separated from astrocytes by overnight rotary shaking (180 rpm) on a Gyrotory Shaker Model G2 (New Brunswick Scientific Company, Inc., Edison, New Jersey, USA) in DMEM containing 10% FBS, L-glutamine and 5 mM leucine methyl ester. Suspended cells (ie. oligodendrocyte lineage enriched) were decanted, filtered (100 μm pore nylon mesh; B&SH Thompson & Company Ltd., Scarborough, Ontario), plated for 30 min to allow contaminating astrocytes to readhere and decanted again; adherent cells (ie. astrocyte enriched) were harvested with 0.05% trypsin and 0.53 mM EDTA and recultured. Both were grown separately on coverslips for immunochemical analysis (refer to section 2.2.1.1).

2.2.1.1 Immunocytochemistry

A2B5 antibody (undiluted hybridoma supernatant), anti-GC antibody (diluted 1:40) and anti-GFAP antibody (1:300) were used to ascertain the percentage of oligodendrocyte lineage cells (A2B5-positive or GC-positive) and astrocytes (GFAP-positive, A2B5-negative) in the cultures. A2B5 antibody or anti-GC was added to unfixed cells and anti-GFAP after pre-treatment with 4% paraformaldehyde/0.05% Nonidet P40 detergent. To double label, these steps were done in sequence. Primary antibodies were detected as follows: A2B5 with rhodamine-conjugated goat anti-mouse IgM (diluted 1:250); anti-GC with fluorescein-conjugated goat anti-mouse IgG (1:400); and anti-GFAP with either rhodamine- or fluorescein-conjugated goat anti-rabbit IgG (1:400). Cells were visualized with fluorescence optics on a Leitz Diavert inverted microscope (Ernst Leitz Canada Ltd., Midland, Ontario).

2.2.2 Isolation of Purified Oligodendrocyte Progenitors and Oligodendrocytes

Populations of purified oligodendrocyte progenitors and oligodendrocytes were isolated from 7 day old rat corpus callosa using an immunopanning purification method modified from Barres *et al.* (1992). In the protocol described by Barres *et al.*, cells were

isolated from the optic nerves of postnatal Sprague-Dawley rats and three different antibody-coated plates were used sequentially for panning. The procedure yielded approximately 17 000 cells per animal and resulted in greater than 99% purity of oligodendrocyte progenitor cells. The experiments performed in this thesis required a much larger yield of purified progenitor cells and thus, cells were isolated from the corpus callosa of the animals and only the anti-RAN-2 panning plates were used in order to reduce the amount of cell death that occurred during the panning process. This modified protocol gave a greater than 100-fold increase in cell numbers while still maintaining a greater than 95% purity of oligodendrocyte progenitor cells.

Panning plates were prepared one day in advance by incubating 10 ml Tris buffer solution (50 mM, pH 9.5) with 50 μg of goat anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, California, USA) in a 10 cm Petri dish overnight at 4°C. The next day, each dish was washed 3 times with phosphate buffered saline (PBS) and incubated with anti-RAN-2 hybridoma supernatant (1:4) in DMEM containing 0.8 mg/ml bovine serum albumin (BSA) for 1 hr at 37°C. The addition of BSA was required to block the non-specific adherence of cells to the panning plates. The dishes were then washed 3 times with PBS and PBS was left on the dishes until required for panning. Twelve anti-RAN-2 plates were prepared for each prep of 12-18 rats (one litter).

Corpus callosa were isolated from 6-9 unanesthetized Sprague-Dawley neonates (half of a litter), minced finely and incubated in 750 μl of a 667 units/ml solution of collagenase in L15 medium for 45 min at 37°C. The tissue was then centrifuged at 1000 rpm in a Beckman Model TJ-6 centrifuge for 5 min and resuspended in 3 ml of a 30 unit/ml papain solution in DMEM containing L-cysteine and 0.013% DNase. This suspension was incubated for 1 hr at 37°C, centrifuged, and resuspended in 1 ml of papain inhibitor solution consisting of 2 mg/ml ovomucoid and 1 mg/ml BSA in L15 medium. The tissue was then triturated sequentially through 23 and 27 gauge needles to yield a single cell suspension. Two of these preps were performed in parallel for each litter of 12-18 rats.

The corpus callosa cell suspension was resuspended in Bottenstein-Sato (B-S) medium (Bottenstein and Sato, 1979) as modified by Lillien *et al.* (1988). This defined

medium consisted of 5.6 mg/ml glucose, 5 μg/ml insulin, 100 μg/ml transferrin, 100 μg/ml BSA, 0.06 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml selenium, 40 ng/ml thyroxine, and 30 ng/ml tri-iodothyronine in DMEM. In order to deplete astrocytes and meningeal cells, the suspension was divided evenly into six samples and incubated on anti-RAN-2 panning plates for 30 min at 37°C. This incubation also depletes microglia and macrophages which stick via their Fc receptors to the original IgG coating on the panning plate. The plates were agitated gently at 15 min to ensure access of the panning surface to all cells. The non-adherent cells were transferred to a second set of six anti-RAN-2 plates for an additional 30 min incubation. An anti-GC panning plate to deplete oligodendrocytes was not used in these experiments since differentiated oligodendrocytes tended to die during the isolation process and thus did not present a major contamination problem. All remaining cells were seeded into polylysine-coated 80 cm^2 flasks in B-S medium containing 0.5% FBS and 10 ng/ml each of PDGF-AA and bFGF.

Oligodendrocyte progenitors were grown in the presence of 10 ng/ml each of PDGF-AA and bFGF in order to promote cell proliferation and prevent differentiation. To obtain pure populations of oligodendrocytes, oligodendrocyte progenitors were first grown to the required cell number. Oligodendrocyte progenitors were then allowed to undergo differentiation into oligodendrocytes by removing bFGF from the medium. All medium was removed from the flasks and the progenitors were washed once with citrate saline buffer (15 mM sodium citrate, 130 mM KCl). Fresh B-S medium containing 0.5% FBS and 10 ng/ml PDGF-AA was added to the flasks and the cells were allowed to differentiate for approximately 4 days. The state of differentiation was monitored morphologically; oligodendrocytes displayed highly branched processes in contrast to the bipolar morphology displayed by oligodendrocyte progenitors. Since the oligodendrocytes began to undergo cell death shortly after reaching terminal differentiation, all experiments were completed within 1 week of the original change in morphology. In all cases, the requisite growth factors were replenished every 24 hr. Both cell types were grown on coverslips for various time intervals under the above mentioned conditions before being used for immunochemical analysis (refer to section 2.2.2.1). With this modified protocol, greater than 95% oligodendrocyte progenitor purity and greater than 90%

oligodendrocyte purity could be obtained.

2.2.2.1 Immunocytochemistry

A2B5 antibody (mouse IgM), anti-GC antibody (mouse IgG_3) and anti-GFAP antibody (rabbit IgG) were used to ascertain the percentage of oligodendrocyte precursors (A2B5-positive, GC- and GFAP-negative), oligodendrocytes (GC-positive, A2B5- and GFAP-negative) and astrocytes (GFAP-positive, A2B5- and GC-negative) in the pure cultures using a triple staining protocol. All dilutions and washes were carried out using Hank's staining solution (Hank's balanced salt solution without sodium bicarbonate, 5% calf serum, 4.76 g/L N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid (HEPES; MW 238.3), 5.2 g/L HEPES (MW 260.3), 0.05% sodium azide). Briefly, coverslips containing cells were washed once by dipping the coverslip in Hank's staining solution and cells were fixed with 4% paraformaldehyde for 15-20 min. After all subsequent incubations, the coverslips were washed by dipping in four changes of Hank's staining solution. A mixture of 1/2 GC-1/2 A2B5 hybridoma supernatants was added for 20 min, and then ice-cold methanol for 15 min at -20°C in order to permeabilize the cell membranes. A mixture of anti-GFAP (1:300), anti-mouse IgG_3-fluorescein (1:100) and anti-mouse IgM-rhodamine (1:100) was incubated for 20 min in the dark. All subsequent incubations were performed in the dark. Goat anti-rabbit biotin (1:100) was added for 20 min followed by AMCA streptavidin (1:50) for 40 min. After the final wash, coverslips were rinsed in distilled water, mounted onto slides with a drop of anti-fade (2.5% 1,4-diazabicyclo-[2.2.2] octane in glycerol) and sealed with nail polish. Cells were visualized with fluorescence optics on a Leitz Diavert inverted microscope.

2.3 MOUSE PRIMARY CELL CULTURES

2.3.1 Mouse Husbandry

Mice were housed, fed, marked and sacrificed according to guidelines established by the Canadian Council on Animal Care. All experimental protocols have been approved by the Animal Care Committee of the University of Western Ontario and copies of all approvals are included in Appendix I. The colony was housed in standard cages under

conditions of a 12 hr light/dark cycle and controlled temperature (22 ± 2°C). Mice were fed Purina rat chow and tap water *ad libitum*. The colony was tested periodically for circulating antibodies to *Mycoplasma pulmonis*, coronaviruses and Sendai virus (Murine Immunocomb, Orgenics Limited, Yavne, Israel); at no time was a positive test result obtained for this colony.

The colony of p53 deficient mice was established from a breeding pair of TSG-*p53* knockout mice purchased from GenPharm International (Taconic, Germantown, New York, USA). Homozygous wild type (+/+) *p53* mice were bred to obtain wild type offspring. Homozygous knockout (−/−) and heterozygous (+/−) mice were generated by breeding male −/− animals with +/− females. Mice were differentiated by genotyping tail DNA using a PCR-based method provided by GenPharm International (Timme and Thompson, 1994).

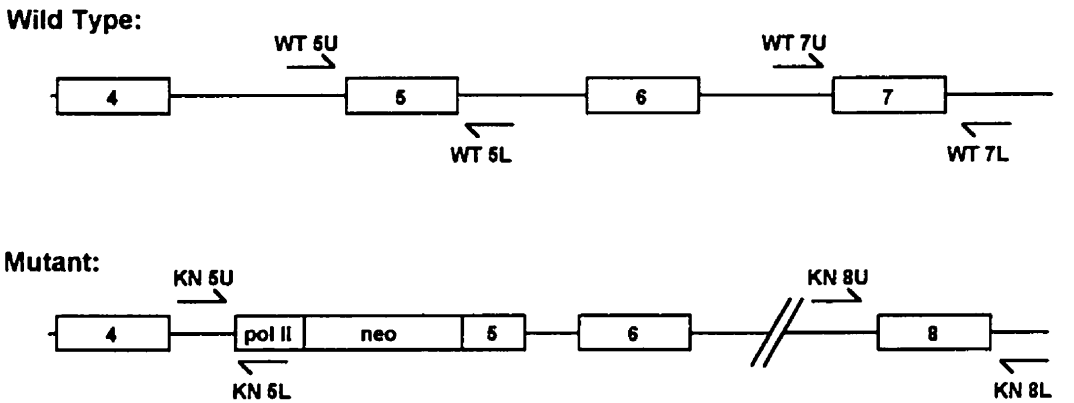
2.3.2 Mouse *p53* Genotyping

Mouse DNA was isolated by incubating a 0.5 cm piece of tail tip in 750 μl tail DNA extraction buffer (50 mM Tris-Cl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml Proteinase K) for 16 hr at 55°C. Following vigorous mixing, 250 μl 6 M NaCl were added, mixing was repeated and the sample was centrifuged 10 min at full speed in a Sorvall MC 12V microcentrifuge (Dupont Canada, Mississauga, Ontario). Avoiding the surface layer, 750 μl of the supernatant was removed, added to 500 μl isopropanol and the sample was mixed and centrifuged. The pellet of crude DNA was washed in 70% ethanol and resuspended in 200 μl T.1E (10 mM Tris-Cl, 0.1 mM EDTA, pH 7.5) for use in PCR.

To discriminate +/+, +/−, and −/− mice, target *p53* sequences were amplified from 2 μl of DNA (Figure 2.1). Two PCRs were required for each tail DNA sample. The first reaction targeted specifically the wild type allele by amplifying a 320 bp intron 4/exon 5 fragment deleted in the knockout allele (WT5 primer pair) as well as a 280 bp exon 7 fragment to control for amplification success (WT7 primer pair). The second reaction targeted specifically the knockout allele by detecting a 150 bp fragment

Figure 2.1 **Mouse *p53* Genotyping by PCR.** A schematic of the PCR approach demonstrating the positions of the PCR primers on the wild type and mutant *p53* alleles (A), sequences of the primers (B), and the expected pattern of bands obtained by agarose gel electrophoresis (C). Exons are represented by boxes (A); pol II, RNA polymerase II; neo, neomycin; WT, wild type; HET, heterozygote; KN, knockout; bp, base pairs.

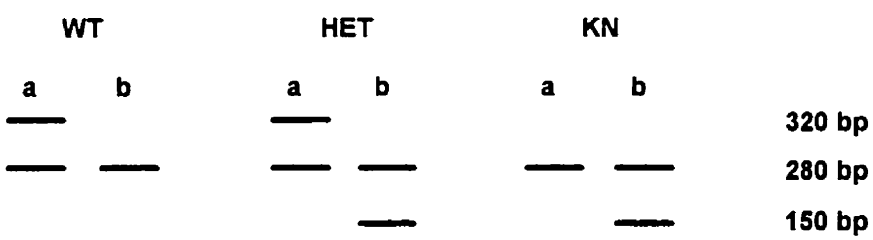
A



B

WT 5U:	5' - GTGTTTCATTAGTTCCCCACCTTGAC - 3'
WT 5L:	5' - ATGGGAGGCTGCCAGTCCTAACCC - 3'
WT 7U:	5' - CCCTACTCTACAACTAAAACTGAA - 3'
WT 7L:	5' - AAGCTGGGGAAGAAACAGGCTAAC - 3'
KN 5U:	5' - GTGGGAGGGACAAAAGTTCGAGGCC - 3'
KN 5L:	5' - TTTACGGAGCCCTGGCGCTCGATGT - 3'
KN 8U:	5' - CTAGTTTACACACAGTCAGGATGG - 3'
KN 8L:	5' - AAGAGGTGACTTTGGGGTGAAGCTC - 3'

C



consisting of sequences from both the endogenous *p53* and the construct used to disrupt *p53* (KN5 primer pair) as well as a 280 bp control fragment (KN8 primer pair). Each 25 μl reaction contained four oligonucleotide primers (1 μM each), 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.1% Triton X-100, 1 mM $MgCl_2$, four dNTPs (0.2 mM each) and 1 unit *Taq* DNA polymerase. An initial 7 min denaturation at 87°C was followed by 30 cycles of denaturation (92°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 6 min) with a final 10 min extension at 72°C in a Perkin Elmer DNA Thermal Cycler (Perkin-Elmer Corporation, Norwalk, Connecticut, USA). PCR products were electrophoresed through a 4% agarose horizontal slab gel (Horizon 11.14, Gibco BRL Life Technologies) comprised of NuSieve GTG:Seakem GTG (3:1) (FMC Bioproducts, Rockland, Maine, USA) and visualized with ethidium bromide.

2.3.3 Isolation of Astrocytes and Fibroblasts

All primary +/+, +/− and −/− cell cultures were isolated from the TSG-*p53* colony of mice. Enriched cultures of astrocytes were prepared from 4 to 6 day old mouse cerebrums using a method adapted from McCarthy and de Vellis (1980). Cerebral hemispheres were isolated from unanesthetized neonates, dissected free of meninges and blood vessels, and a single cell suspension was prepared by a combination of enzymatic (250 μg/ml trypsin, 40 μg/ml DNase) and mechanical dissociation. Fibroblast cultures were established from the cellular outgrowth of minced livers. All brains and livers were isolated separately and the resulting primary cultures were seeded into individual cell culture plates until *p53* genotype could be resolved by PCR (section 2.3.2); once determined, cells of identical genotype were pooled from littermates (2-6 mice) for each independent experiment. All cultures were grown in DMEM containing 10% heat-inactivated FBS and 50 U/ml penicillin/streptomycin.

2.4 CELL PROLIFERATION ASSAY

The proliferation rates of all rat and mouse primary glial cells were determined by performing cell counts over a 1 week period. Briefly, cells were seeded onto poly-

lysine coated 24-well plates at various densities. Each day for 7 days, cells from three individual wells were harvested with 0.05% trypsin and 0.53 mM EDTA and counted with a haemocytometer. The average cell count was plotted and the exponential growth phase determined. Approximate cell doubling times during exponential growth were calculated by averaging times obtained from at least three independent cell isolations.

2.5 CYTOTOXICITY ASSAY

BCNU was dissolved in absolute alcohol and diluted to 3.3 mg/ml with sterile water. MNU was prepared at a concentration of 6.6 mg/ml in sterile water. Both drugs were stored at -80°C and subsequent dilutions were made with culture medium at the time of cell treatment. BG stock solution was made in filter-sterilized dimethylsulfoxide (DMSO) at a concentration of 100 mM and stored at -80°C. At time of use, the BG was diluted to 1 mM in PBS and subsequent dilutions were made with culture medium. Twenty-five μM DL-buthionine-[S,R]-sulfoximine (BSO) and 5 mM (-)-2-oxo-4-thiazolidine-carboxylic acid (OTZ) were prepared in medium at the time of treatment. Irradiation was performed at a distance of 31 cm from an Eldorado 6 ^{60}Co source (Theratronics, Kanata, Ontario) with an average dose rate of 240 cGy/min.

To test for cytotoxicity, 96 well plates were seeded with cells and incubated at 37°C. Due to differences in cell proliferation rates (refer to section 2.4), all rat cells were seeded at 10^4 cells/well, wild type and heterozygous *p53* mouse cells at $7.5X10^3$ cells/well, and knockout *p53* mouse cells at $5X10^3$ cells/well. Forty-eight hr later, cells were treated and incubated for an additional 96 hr, at which time cytotoxicity was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. BCNU and MNU treatments were performed for 2 hr. Pretreatment with modulators consisted of a 2 hr incubation for BG, a 24 hr incubation for BSO and 4 hr for OTZ. Irradiation was performed for the length of time required to achieve the desired dose (range of approximately 12.5 sec to 8.3 min).

The toxic effects of the above treatments were compared using the MTT assay as modified for chemosensitivity testing as described by Cole (1986). Ninety-six hr after treatment, 100 μl of medium was removed, 25 μl MTT solution (2 mg/ml in PBS) added

and the plates were incubated for 2 hr at 37°C. To solubilize formazan crystals, 0.04 M hydrochloric acid in isopropanol was added to each well and thoroughly mixed. Plates were kept at 37°C for 1 hr and cell viability quantified by measuring light absorbance (570 nm) in a Bio-Rad Model 3550 automated microplate reader (Bio-Rad Laboratories, Richmond, California, USA). To control for differential growth rates, dose response curves were normalized by expressing absorbance values relative to non-treated control values. Cytotoxicity assay results were analyzed using the two-tailed *t*-test for independent means.

2.6 MGMT ASSAYS

Extracts were prepared from harvested rat cell pellets and rat tissues for the measurement of MGMT activity. Samples were washed in PBS, suspended in a buffer containing 70 mM HEPES, 1 mM dithiothreitol (DTT), 1 mM EDTA and 5% glycerol, and sonicated on ice for 30 sec (setting 30) using a 3mm tapered microtip attached to a Vibra-cell VC375 Cell Disrupter (Sonics & Materials Incorporated, Danbury, Connecticut, USA). Samples were frozen in liquid nitrogen and stored at -80°C until they were shipped on dry ice to Dr. Daniel Yarosh (Applied Genetics Incorporated, Freeport, New York, USA). MGMT activity was measured in the laboratory of Dr. Yarosh using a DNA adduct removal assay described by Myrnes *et al.* (1984). Extracts were incubated with calf thymus DNA containing O^6-[^{3}H]methylguanine for 30 min at 37°C. After the addition of 5% TCA and heating to 80°C for 30 min, tritiated methyl proteins were recovered by filtration through glass fibre filters (Whatman GF/C, Baxter Corporation, Mississauga, Ontario). Filters were washed with 5% TCA/ethanol and radioactivity counted in a xylene based cocktail containing 0.5% diphenyl oxazole and 0.02% 1-4-bis-2(5-phenyl oxazolyl) benzene. Activity was expressed as molecules of MGMT per cell.

Mouse primary cultures were harvested, washed once with citrate saline, and the cell pellet frozen in liquid nitrogen. Samples were stored at -80°C until they were shipped on dry ice to the laboratory of Dr. Anthony Pegg (The Milton S. Hershey Medical Center, Hershey, Pennsylvania, USA) for the measurement of MGMT activity. MGMT activity was determined by incubation of cell extracts with [^{3}H]methylated calf

thymus DNA for 30 minutes at 37°C followed by acid hydrolysis of the substrate and separation of free bases on HPLC as described by Dolan *et al.* (1986; 1990a). The activity was expressed as fmol O^6-methylguanine removed per mg protein. [^{3}H]methylated DNA substrate was obtained by reaction of calf thymus DNA with 5.9 mCi/mmol *N*-[*methyl*-^{3}H]*N*-nitrosourea (Amersham, Inc.). All MGMT results were analyzed using the two-tailed *t*-test for independent means.

2.7 NORTHERN BLOT ANALYSIS

2.7.1 RNA Isolation and Selection of mRNA

All glassware and solutions used in the isolation of RNA were autoclaved for 1 hr and solutions were treated with a 0.1% diethylpyrocarbonate (DEPC) solution where appropriate prior to use. Total RNA was isolated from cultured cells using TRIzol Reagent (Gibco BRL Life Technologies) according to manufacturer's instructions. Briefly, T80 flasks containing cells growing in monolayer were washed once in a citrate saline buffer. Four ml of TRIzol Reagent was added directly to the flask and cells were lysed by passing the suspension through a 10 ml pipette. After an incubation of at least 5 min at room temperature, the suspension was transferred to a glass Corex tube (Corning Costar Corporation, Richmond Hill, Ontario), 0.2 ml of chloroform was added per 1 ml of TRIzol Reagent, and the sample was vortexed for 15 sec. Following a 2 min incubation at room temperature, the sample was centrifuged at 12 000 rpm (Beckman Model J2-21 centrifuge) for 15 min at 4°C and the aqueous phase was transferred to a new Corex tube. RNA was precipitated from the aqueous phase by the addition of 0.5 ml of isopropanol per 1 ml of TRIzol Reagent used in the initial lysis. After a 10 min incubation at room temperature, the sample was centrifuged at 12 000 rpm for 10 min. The RNA pellet was then washed with 75% ethanol, dried and resuspended in DEPC-treated water.

Selection of rat mRNA was performed using Oligo(dT) Cellulose Columns (Gibco BRL Life Technologies). Columns were first equilibrated with 4 ml binding buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.3 M NaCl, 0.1% SDS) in 1 ml aliquots. RNA pellets were resuspended in 3 ml binding buffer, heated at 70°C for 5 min and chilled

on ice for 5 min. Samples were loaded onto the equilibrated columns under gravity flow and washed with 4 ml of binding buffer to elute non-messenger RNA. mRNA was eluted with 1.5 ml elution buffer (10 mM tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS) and the entire selection procedure was repeated a second time. The resultant mRNA was precipitated, resuspended in DEPC-treated water and quantitated by spectrophotometry (Beckman DU-65 Spectrophotometer, Beckman Instruments, Inc.). Samples were stored at -80°C for future use.

Selection of mouse mRNA was performed using the PolyATract mRNA Isolation System III (Promega Corporation). This system utilizes a biotinylated oligo(dT) primer and streptavidin coated paramagnetic particles to capture mRNA species at high efficiency. Briefly, 500 μl of total RNA was heated to 65°C for 10 min and then annealed with 3 μl biotinylated-oligo(dT) probe and 13 μl 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). The sample was mixed gently and incubated at room temperature until completely cooled. The streptavidin-paramagnetic particles were resuspended and washed with 0.5X SSC according to manufacturer's instructions. The entire annealing reaction was added to the washed particles and incubated at room temperature for 10 min. The paramagnetic particles were captured using the supplied magnetic separation stand. The particles were washed with 0.1X SSC to remove contaminating non-messenger RNA and the mRNA was eluted with RNase-free water. Samples of mRNA were quantitated by spectrophotometry and stored at -80°C.

2.7.2 Gel Electrophoresis of mRNA

RNA was analyzed as described by Sambrook *et al.* (1989). The desired quantity of RNA was aliquotted, dried (Speed-Vac Model RH40-11, Savant Instruments, Hicksville, New York, USA), and rehydrated in 2.25 μl of DEPC-treated water. The RNA was then resuspended in a formamide/formaldehyde mix comprised of 65% deionized formamide and 22% deionized formaldehyde in MOPS solution (0.25 M 3 [N-morpholino] propane-sulphonic acid (pH 8.0), 0.62 mM EDTA, 6.2 mM sodium acetate). The samples were heated to 55°C for 15 min, removed to ice and glyoxal loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene

cyanol in MOPS solution) was added. Samples and molecular weight RNA markers (0.24-9.5 kb, Gibco BRL Life Technologies) were loaded immediately onto a 1.1% agarose horizontal slab gel containing formaldehyde and electrophoresed at 50 mA for 3 to 4 hr.

2.7.3 Northern Transfer

Formaldehyde gels used to resolve mRNA for transfer were rinsed briefly in distilled water. The lane containing the RNA ladder was removed and stained in 1 μg/ml ethidium bromide in order to visualize the RNA standards. Transfer of the mRNA was carried out by capillary action onto Gene Screen Plus membranes (Dupont Canada) in 10X SSC buffer using a method modified from Alwine *et al.* (1977). Transfer proceeded overnight, at which time the blot was removed, rinsed briefly in 2X SSC and baked for 2 hr at 80°C in a Gravity Convection Oven (Economy Model 16EG, Canlab, Baxter Corporation, Mississauga, Ontario). The membrane was stored in sealed plastic until hybridization was performed.

2.7.4 Radioactive Labelling of Hybridization Probes

Labelling the 5' ends of oligonucleotide probes was carried out in a reaction mixture containing 5 pmol probe, kinase buffer (0.1 M Tris-HCl (pH 9.5), 10 mM $MgCl_2$), 10 mM DTT, 40 μCi [γ-^{32}P]dATP and 5-10 units T4 polynucleotide kinase (Gibco BRL Life Technologies). The reaction was incubated at 37°C for 30 min and was stopped by the addition EDTA to 80 mM final concentration.

A PCR-based method was used to label the pEX/172 (*MDR*) cDNA probe (Ng *et al.*, 1989). Each 50 μl reaction contained 100 pg pEX/172, primers A-100 (5' GTCCAGGGCTTCCTGGAC 3') and B-100 (5' CCATGGAGAAATAGATGC 3') at a concentration of 1 μM, dATP, dGTP, dTTP (each at 0.25 mM), 0.5 μM non-radioactive dCTP, 70 μCi [α-^{32}P]dCTP, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin and 2.5 units of *Taq* DNA polymerase. The mixture was subjected to 25 cycles of denaturation (94°C, 30 sec), annealing (48°C, 30 sec) and extension (72°C, 1 min).

Labelling of all other cDNA probes was performed using the Gibco Random

Primers DNA Labelling System (Gibco BRL Life Technologies) according to manufacturer's instructions. The reaction mixture containing 25 ng probe, dATP, dGTP, dTTP (0.02 mM each), random primers buffer (0.2 M HEPES, 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.4 mg/ml BSA, 5.4 OD_{260} units/ml oligodeoxyribonucleotide primers, pH 6.8), 50 μCi [α-^{32}P]dCTP and 3 units Klenow Fragment was incubated at 25°C for 1 hr. The reaction was stopped by the addition of stop buffer (Na_2EDTA to 20 mM final concentration).

Following all labelling reactions, unincorporated isotope was removed by spin chromatography on a column of 3 ml of Sephadex G-50 (Pharmacia LKB Biotechnology). The G-50 gel filtration matrix retains the unincorporated isotope and allows the labelled probe to pass through. As the percentage of incorporation of radionucleotide remaining after chromatography has been found to be greater then 90%, the degree to which the probes were labelled was determined by measuring directly an aliquot in a scintillation counter (LKB Wallac 1211 Rackbeta, Pharmacia LKB Biotechnology).

2.7.5 Hybridization of Blots

Prior to using a blot for the first time, Gene Screen Plus membranes were dampened with RNase-free water, wrapped in plastic wrap, and exposed to ultraviolet light for 5 min in an ultraviolet viewing cabinet (Spectroline Model CC-80, Spectronics Corporation, Westbury, New York, USA) in order to cross-link the bound RNA to the membrane. The membranes were then prehybridized to block non-specific binding at 42°C in 5-10 ml prehybridization buffer (50% formamide, 10% dextran sulphate, 1% SDS, 1 M NaCl, 100 μg/ml herring sperm DNA) in a rotating bottle hybridization oven (VWR Scientific Model 2710, Toronto, Ontario). After 1-4 hr, radiolabelled probe (10^6 cpm/ml) was added to the prehybridization solution and incubated with constant rotation for 16-20 hr at 42°C. Following hybridization, blots were washed according to the protocol appropriate for the particular probe. For 5'-end labelled probes, the blots were washed briefly 4 times in 2X SSC/0.1% SDS at room temperature, once for 30 min in 2X SSC/0.1% SDS at 65°C, once for 5 min in 2X SSC/0.1% SDS at room temperature, and once briefly in 2X SSC at room temperature. For random primed probes, a more

stringent protocol was used. These blots were washed twice for 5 min in 2X SSC at room temperature, twice for 30 min in 2X SSC/1.0% SDS at 60°C, and twice for 30 min in 0.1X SSC/0.1% SDS at room temperature. Following the washes, membranes were blotted dry on Whatman paper (Baxter Corporation), wrapped in plastic wrap, and placed on a Storage Phosphor Screen (Molecular Dynamics, Inc., Sunnyvale, California, USA).

For further probing of the same membrane, each probe was removed from the blot by vigorous washing in a boiling solution of 0.01X SSC/0.01% SDS for 2-3 min. This was repeated twice with fresh solution and the blot was exposed to a Storage Phosphor Screen to ensure the sufficient removal of the probe. Membranes were then placed in prehybridization solution and hybridized with the next probe as described.

2.7.6 PhosphorImage and Densitometry Analysis

Northern hybridization images were scanned off of the Storage Phosphor Screens using a Personal Densitometer SI Model 375A (Molecular Dynamics, Inc.). Images were quantitated using ImageQuant software (Molecular Dynamics, Inc.). All images used for quantitation were below saturation and corrected appropriately for background. Quantified signal intensities for each probe were normalized to a poly-dT probe for rat samples and a mouse glyceraldehyde-3-phosphate dehydrogenase (GADPH) probe for mouse samples. Images were assembled into composites using CorelDraw software (Corel Corporation, Ottawa, Ontario) and signal intensity data was analyzed using Microsoft Excel software (Microsoft Canada, Inc., Mississauga, Ontario).

2.8 FLOW CYTOMETRY

Nuclear DNA was stained with propidium iodide using the CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, San Jose, California, USA) following manufacturer's instructions. Briefly, cells were harvested at the appropriate times, washed once in citrate saline, and resuspended in Solution A (250 μl per sample). Solution A contained trypsin in a detergent buffer and was responsible for the enzymatic disaggregation of the cells and digestion of the cell membranes and cytoskeleton. This suspension was mixed gently and incubated at room temperature for 10 min. Solution B

(200 μl) was added, mixed gently, and incubated for an additional 10 min. Solution B was comprised of trypsin inhibitor and ribonuclease A in a stabilizing buffer and resulted in the inhibition of the trypsin and digestion of cellular RNA. Lastly, ice-cold Solution C (200 μl) was added, mixed gently and incubated on ice for 10 min in the dark. Solution C contained the propidium iodide in a stabilizing buffer and stoichiometrically bound the DNA at a final concentration of at least 125 μg/ml. Samples were filtered through a 74 μm mesh (B&SH Thompson & Company Ltd.) directly into a 12X75 mm tube and the tubes were stored on ice in the dark until flow analysis was performed (not greater than 30 min). Fifty thousand events per sample were analyzed with a Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, Florida, USA). Cell distribution was determined after correction for background aggregates and debris using Multicycle software (Phoenix Flow Systems, San Diego, California, USA). Flow cytometry results were analyzed using the two-tailed *t*-test for independent means.

2.9 DETECTION OF APOPTOSIS

2.9.1 ApopTag™ *In Situ* Apoptosis Detection Kit

To test for the presence of apoptotic cells, +/+, +/−, and −/− *p53* astrocytes were seeded onto coverslips in 24-well plates at a cell density of approximately 10^4 cells/coverslip. At the desired time after BCNU treatment, cells were assayed with the ApopTag™ *In Situ* Apoptosis Detection Kit (Oncor, Gaithersburg, Maryland, USA) according to manufacturer's instructions. Briefly, cells were washed once in PBS, fixed for 10 min in 4% neutral buffered formalin, and quenched in 2% hydrogen peroxide in PBS for 5 min at room temperature. Cells were then exposed to the supplied equilibration buffer for 10-15 sec at room temperature. Without washing, coverslips were immediately incubated with reaction buffer containing terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37°C for 1 hr. Negative controls consisted of identical cells assayed in the absence of the TdT enzyme (incubated with reaction buffer alone). The enzyme reaction was stopped by incubating the cells in the stop/wash buffer at 37°C for 30 min. Coverslips were washed 3 times in PBS and treated with anti-

digoxigenin-peroxidase at room temperature for 30 min. Cells were then washed again and exposed to 0.05% DAB substrate in PBS to which 0.02% fresh hydrogen peroxide had been added. Color development was monitored with a microscope and once the desired level of color was obtained, the coverslips were placed in distilled water and washed for at least 5 min. Cells were counterstained in 0.5% methyl green in 0.1M sodium acetate (pH 4.0) at room temperature for 10 min and washed in distilled water. Finally, coverslips were washed in 100% butanol, dehydrated in 3 changes of xylene and mounted on slides with Permount mounting medium (Fisher Scientific).

2.9.2 Cell Death Detection ELISA

In an attempt to achieve a more quantitative determination of the amount of apoptosis occurring in response to BCNU treatment than that provided by the ApopTag™ *In Situ* Apoptosis Detection Kit, the commercially available Cell Death Detection ELISA kit from Boehringer Mannheim was used. This photometric enzyme immunoassay allows for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (nucleosomes) after induced cell death.

Cells were seeded onto 35 mm plates at a cell density of $2.5X10^5$ and, 24 hr later, treated as desired. At the time of sample harvest, the medium was removed from the plate and centrifuged in a Beckman Model TJ-6 centrifuge (1500 rpm for 5 min) to collect any cellular fraction. Five hundred μl cold isolation buffer was added to the plate and cells were scraped into the buffer and collected in an Eppendorf tube. This solution was added to the cell pellet collected from the medium and placed at 4°C for 30 min with occasional inversion of the Eppendorf tube to mix gently. The sample was centrifuged at 4°C for 10 min in a Sorvall MC 12V microcentrifuge at full speed. Four hundred μl of the supernatant was removed to a new tube and the sample was frozen at -20°C until the ELISA could be performed.

The ELISA was performed according to manufacturer's instructions. Wells were coated with 100 μl of an anti-histone antibody solution for 1 hr at room temperature followed by 200 μl incubation buffer for 30 min. The wells were rinsed 3 times with washing solution and then incubated with 100 μl of sample solution (10X dilution of

original sample in incubation buffer) for 90 min at room temperature. Wells were washed again and 100 μl of an anti-DNA-peroxidase conjugate solution was added for 90 min. After a final wash, 100 μl of an ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)]) substrate solution was added with shaking (250 rpm) on a Gyrotory Shaker Model G2 until colour development was sufficient for analysis. The contents of the wells were homogenized by tapping carefully at the edges of the plate and measured at 405 nm in a Bio-Rad Model 3550 automated microplate reader. Incubation buffer was used as a blank and non-treated cells as negative controls. All samples were measured in triplicate. Results were expressed as enrichment factors which were calculated by expressing the average absorbance of the sample as a fraction of the average absorbance of the controls.

CHAPTER 3

THE ROLE OF O^6-METHYLGUANINE-DNA METHYLTRANSFERASE IN THE RESPONSE OF GLIAL LINEAGE CELLS TO BCNU

3.1 INTRODUCTION

Oligodendrogliomas (Cairncross and Macdonald, 1988; Macdonald *et al.*, 1990; Cairncross *et al.*, 1994; Cairncross, 1994; Mason *et al.*, 1996) and mixed oligoastrocytomas (Glass *et al.*, 1992; Kim *et al.*, 1996) respond predictably to nitrosourea-based chemotherapy, especially PCV (procarbazine, CCNU (lomustine) and vincristine). In contrast, astrocytomas are relatively chemoresistant (refer to section 1.2.6.3 for details). Moreover, experimental studies have demonstrated that tumors of oligodendroglial lineage are preferentially induced in rats following transplacental exposure to ethylnitrosourea (Druckrey, 1973; Burger *et al.*, 1988) (refer to section 1.3 for details). The results of these clinical and experimental studies suggest not only that oligodendroglial tumors are chemosensitive but that the normal cell giving rise to oligodendrogliomas (ie. cell of origin) may be sensitive to the transforming effects of the nitrosoureas. The biochemical basis for this apparent vulnerability to the nitrosoureas is not known.

The nitrosoureas alkylate DNA at O^6-guanine and repair at this site is mediated by MGMT (refer to section 1.5.2 for details). The cytotoxic and carcinogenic properties of the nitrosoureas appear related to MGMT activity; cells rendered MGMT positive by DNA transfection or transgenic expression become resistant to the nitrosoureas, resistant cells depleted of MGMT by pretreatment with O^6-methylguanine or O^6-benzylguanine are sensitized to the nitrosoureas and cells susceptible to neoplastic transformation by nitrosoureas express low levels of MGMT (refer to section 1.5.2 for details). In addition, procarbazine, the other major component of the PCV regimen, also produces cytotoxicity by alkyl substitution at O^6-guanine (Schold, Jr. *et al.*, 1989).

The purposes of this study were 1) to explore whether the differential response to BCNU displayed by gliomas was reproducible in normal rat glial lineage cells and 2) to ascertain whether this difference correlated with MGMT activity in these cells.

3.2 RESULTS

3.2.1 Immunochemical characterization of rat glial cell enriched cultures

Isolation of glial cell enriched cultures from neonatal rats was performed as described in section 2.2.1. The cellular composition of the cultures was determined by immunochemistry as shown in Figure 3.1 and described in section 2.2.1.1. Oligodendrocyte lineage cultures in these studies were approximately 80% pure. They contained 40% GC-positive, A2B5-negative, GFAP-negative oligodendrocytes, 10% A2B5-positive, GFAP-negative, GC-negative oligodendrocyte progenitors, and 30% A2B5-positive, GFAP-positive, GC-negative type 2 astrocytes. They also contained 5-10% GFAP-positive, A2B5-negative, GC-negative type 1 astrocytes and 10-15% unidentified cells. Cultures enriched for astrocytes (type 1; GFAP-positive, A2B5-negative, GC-negative) were at least 90% pure. Contaminants included small numbers of oligodendrocyte lineage and unidentified cells.

3.2.2 Effect of BCNU concentration on rat glial cell viability

To determine the effect of BCNU on glial cells, cultures of oligodendrocyte lineage cells and astrocytes were exposed to increasing concentrations of drug and cell viability was measured using the MTT assay (refer to section 2.5 for details). Oligodendrocyte lineage cells were found to be significantly more sensitive to BCNU than astrocytes ($p=0.002$ at 80 μg/ml; Figure 3.2). The 50% inhibitory concentration (IC_{50}) was 40 μg/ml for oligodendrocyte lineage cells and 102 μg/ml for astrocytes, a 2.5-fold difference.

3.2.3 MGMT activity in rat glial cell cultures

MGMT activity was measured in oligodendrocyte lineage cells and astrocyte cultures in order to investigate possible differences in MGMT levels between the two

Figure 3.1 **Immunochemical characterization of rat glial cell enriched cultures.** This figure demonstrates the double labelling of a typical culture before the oligodendrocyte lineage cells have been separated from the astrocytes (ie. both cell types grown on the same coverslip). Oligodendrocyte lineage cells are stained with A2B5 and rhodamine-conjugated goat anti-mouse IgM (A). Astrocytes are stained with anti-GFAP and fluorescein-conjugated goat anti-rabbit IgG (B). Bar, 50 μm.

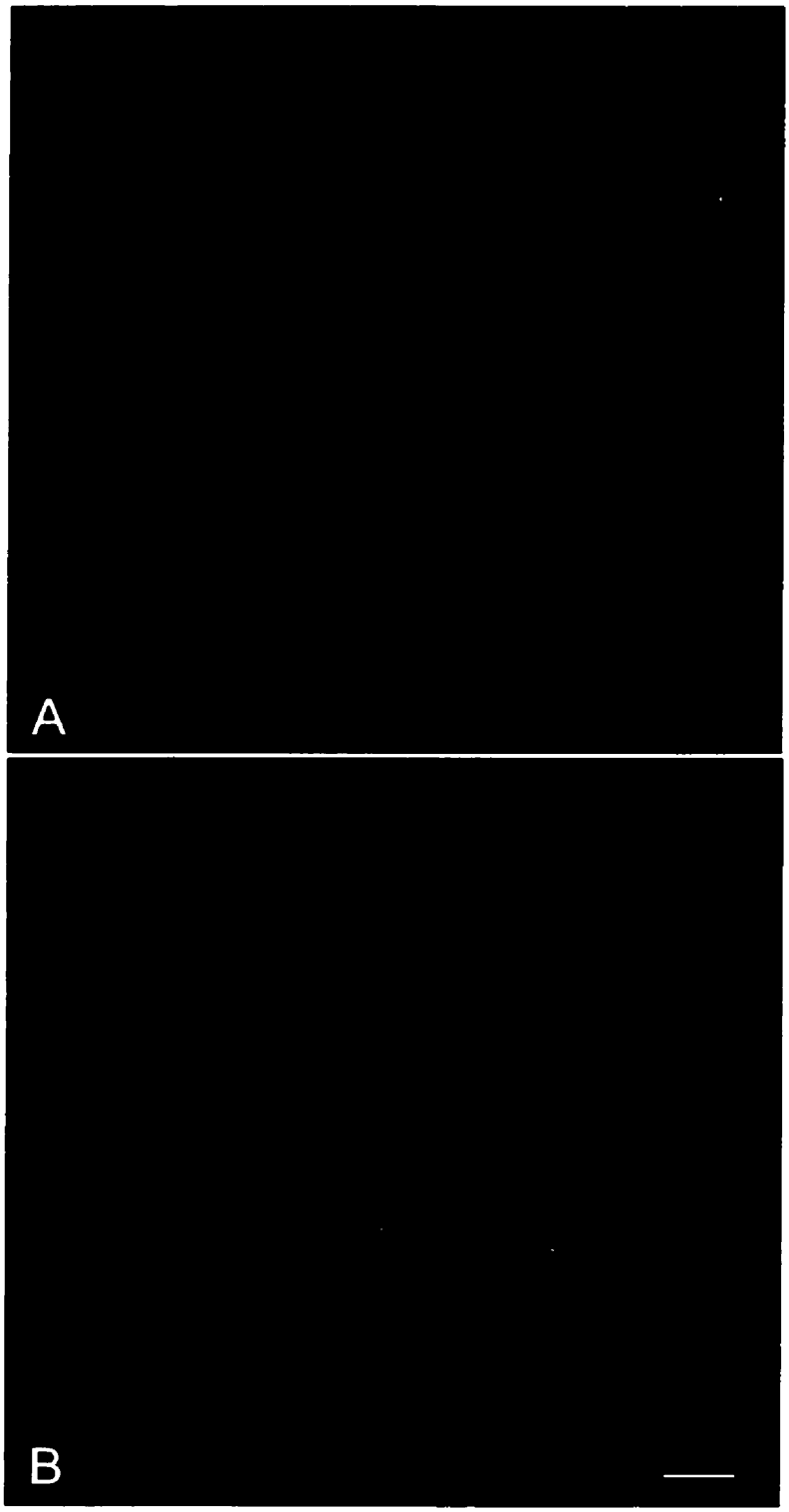
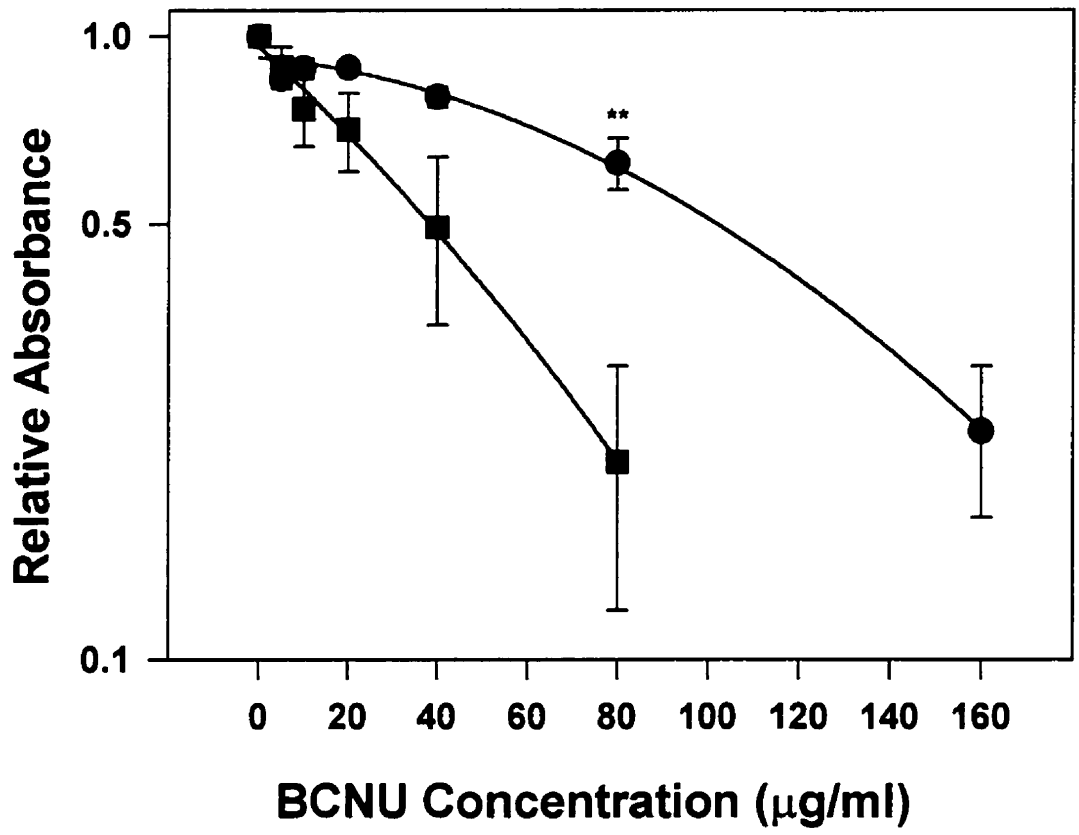

A
B

Figure 3.2 **Effect of BCNU concentration on rat glial cell viability.** Oligodendrocyte lineage cells were found to be significantly more sensitive to BCNU than astrocytes. **Circles represent average values for astrocyte enriched cultures (n=18) and squares depict oligodendrocyte lineage enriched cultures (n=6).** The absorbance of untreated cells was normalized to a value of 1.0. Bars depict the standard error of the mean and the double asterisks show the level of statistical significance ($p=0.002$).


1.0
0.5
0.1
Relative Absorbance
**
0
20
40
60
80
100
120
140
160
BCNU Concentration (μg/ml)

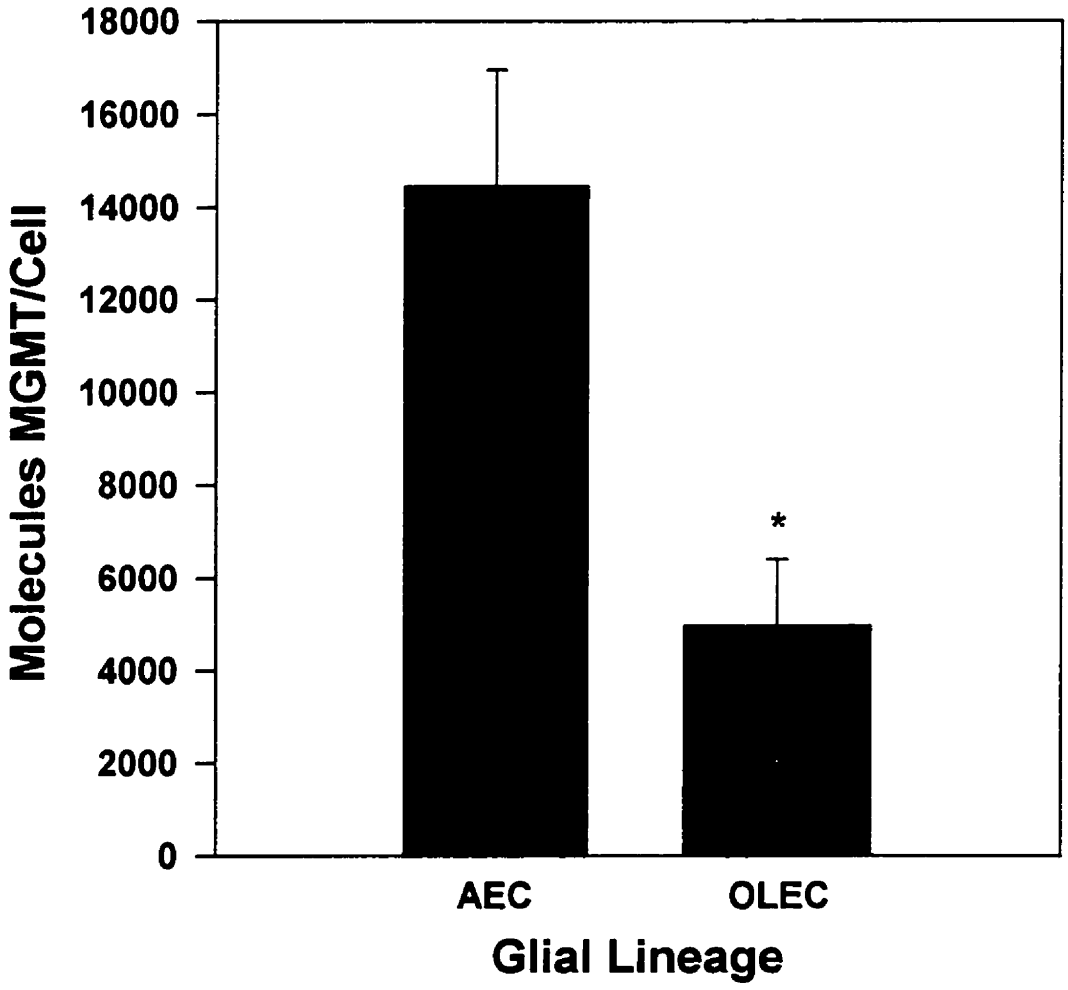
glial cell lineages (refer to section 2.6 for details). Oligodendrocyte lineage cells had low MGMT activity, approximately $5X10^3$ molecules per cell (Figure 3.3). MGMT levels in cells of the oligodendrocyte lineage were low relative to other rat cells (Grafstrom *et al.*, 1984), and approximately one-third of the $1.45X10^4$ value found for astrocytes ($p < 0.02$).

3.3 DISCUSSION

One intriguing development in neuro-oncology in recent years has been the observation that aggressive oligodendrogliomas (Cairncross and Macdonald, 1988; Macdonald *et al.*, 1990) and anaplastic mixed gliomas with an unequivocal oligodendroglial element (Glass *et al.*, 1992; Kim *et al.*, 1996) respond predictably to a combination chemotherapy regimen containing procarbazine, lomustine (CCNU) and vincristine, called PCV. Since oligodendrogliomas appear less vascular and less permeable than other gliomas (Paleologos *et al.*, 1992), superior drug delivery would seem an unlikely explanation for their chemosensitive nature. Presumably oligodendroglioma cells are inherently susceptible to the cytotoxic effects of PCV, but why? The answer to this question is undoubtedly important as it may lead to better therapies for oligodendrogliomas and to new treatment strategies for anaplastic astrocytomas and glioblastomas.

The observation that oligodendrogliomas are preferentially induced in rats following transplacental exposure to ethylnitrosourea suggested that developing oligodendrocytes have difficulty repairing nitrosourea-induced DNA alkylation and that oligodendrogliomas might be chemosensitive if this biochemical vulnerability were retained in tumors of the oligodendrocyte lineage. Since MGMT repair of DNA alkylation confers resistance to nitrosoureas it could be reasoned further that oligodendrocyte lineage cells and oligodendrogliomas might have low levels of MGMT activity. MGMT activity in human oligodendrocyte lineage cells has not been studied, but the results presented in section 3.2.3 demonstrated that low levels of MGMT activity are exhibited by rat oligodendrocyte lineage cells. In addition, several groups have measured low levels in human oligodendrogliomas (Wiestler *et al.*, 1984; Frosina *et al.*,

Figure 3.3 **MGMT activity in rat glial cell cultures expressed as molecules/cell.** MGMT activity in cells of the oligodendrocyte lineage was approximately one-third of that found for astrocytes. **The average values are shown for astrocyte enriched cultures (AEC; n=6) and oligodendrocyte lineage enriched cultures (OLEC; n=5).** Error bars depict the standard error of the mean and the asterisk the level of statistical significance ($p < 0.02$).


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Molecules MGMT/Cell
*
AEC
OLEC
Glial Lineage

1990; Mineura *et al.*, 1993; Silber *et al.*, 1993; Citron *et al.*, 1995; Mineura *et al.*, 1996a) and we have found low MGMT activity in PCV-responsive human oligodendrogliomas (Nutt *et al.*, 1995). Allowing for the fact that rat tissues and cells have a several-fold lower level of MGMT than human tissues and cells (Grafstrom *et al.*, 1984), both rat oligodendrocyte lineage cells and human oligodendrogliomas displayed low levels of MGMT activity relative to other rat and human tissues, respectively. The significance of this finding in relation to the clinical observation that oligodendrogliomas in humans appear more sensitive to PCV than anaplastic astrocytomas and glioblastomas, although intriguing, requires further study. Wiestler *et al.* (1984) and Frosina *et al.* (1990) found lower levels of MGMT activity in oligodendrogliomas than astrocytomas but this has not been a universal finding (Mineura *et al.*, 1993; Mineura *et al.*, 1996a; Silber *et al.*, 1993).

It is unlikely that the response of human cancers to chemotherapy is determined by a single biochemical process, such as MGMT. In addition to PCV and BCNU (Cairncross and Macdonald, 1988), oligodendrogliomas have been found to respond to a number of additional DNA damaging agents including diaziquone (Cairncross and Macdonald, 1988; Cairncross and Macdonald, 1991), melphalan (Brown *et al.*, 1990), thiotepa (Saarinen *et al.*, 1990), dacarbazine (New, 1995), and regimens containing cisplatin (Yung *et al.*, 1992; Peterson *et al.*, 1996). These findings raise the possibility that additional DNA repair mechanisms may be inefficient in oligodendroglial tumors. Studies of MGMT and other mechanisms of drug resistance in glial neoplasms, especially uncommon ones like oligodendrogliomas, are seriously limited by tissue availability. Moreover, contamination of samples by necrotic debris and non-tumor elements potentially complicate the interpretation of results. The observation that human oligodendrogliomas and rat oligodendrocyte lineage cells have similarly low MGMT activity may be noteworthy in this regard. If mechanisms of drug resistance in glial tumors reflect the biochemical properties of their cells of origin, then the results presented in this study suggest that normal glial cells may serve as a laboratory substitute for human gliomas.

CHAPTER 4

DIFFERENTIAL EXPRESSION OF DRUG RESISTANCE GENES IN CULTURES OF PURIFIED GLIAL CELLS

4.1 INTRODUCTION

Human oligodendrogliomas and mixed oligoastrocytomas respond predictably to nitrosourea-based chemotherapy, whereas astrocytomas are relatively chemoresistant (refer to sections 1.2.6.3 and 3.1 for details). One possible explanation for this difference in response is that oligodendroglioma cells are inherently susceptible to the cytotoxic effects of these alkylating agents. If this were true, one might hypothesize that mechanisms of drug resistance in glial tumors would reflect the biochemical properties of their cell of origin. In Chapter 3, it was shown that rat oligodendrocyte lineage cells were more sensitive to the cytotoxic effects of BCNU than astrocytes. In addition, levels of the DNA repair protein MGMT were reduced in oligodendrocyte lineage cells in comparison to astrocytes (Chapter 3; Nutt *et al.*, 1995). These results, although performed in rat and precursory in nature, provided support for this hypothesis.

It is unlikely that the response of human gliomas to chemotherapy is determined by a single biochemical process, such as MGMT. Additional mechanisms of resistance have been implicated for BCNU such as glutathione, GSTs, and nucleotide excision repair (refer to section 1.5). In addition to the classical chloronitrosoureas, a number of other chemotherapeutic agents have been used in the treatment of glial tumors. These agents often are used in various combination therapies and include procarbazine, cisplatin, melphalan, thiotepa, diaziquone, temozolomide, vincristine, and etoposide (refer to section 1.6.1). Although many of these are alkylating agents and their efficacy would be influenced by many of the same mechanisms of resistance as BCNU, some belong to other drug families. It is useful, therefore, to consider alternate mechanisms that may play a role in the resistance of glial tumors to chemotherapeutic agents.

The purposes of this study were 1) to explore the possibility of the differential expression of drug resistance genes in cultures of purified rat glial cells; 2) to investigate the response of purified rat astrocytes, oligodendrocyte progenitors, and oligodendrocytes to treatment with the bifunctional alkylating agent, BCNU, and the monofunctional alkylating agent, MNU; 3) to examine the effect of MGMT modulation on resistance to BCNU and MNU in these cells; and 4) to study the effect of glutathione modulation on resistance to BCNU in purified astrocytes.

4.2 RESULTS

4.2.1 Immunochemical characterization of cultures of purified rat glial cells.

Isolation of cultures of purified rat glial cells from neonatal rats was performed as described in sections 2.2.1 (astrocytes) and 2.2.2 (oligodendrocyte progenitors and oligodendrocytes). The cellular composition of the cultures was determined by immunochemistry as described in section 2.2.2.1 and representative cultures are shown in Figure 4.1. Astrocyte cultures (GFAP-positive, A2B5-negative, GC-negative) were at least 90% pure. Oligodendrocyte progenitors (O2A cells; A2B5-positive, GFAP-negative, GC-negative) were cultured to a purity of greater than 95% and oligodendrocytes (GC-positive, A2B5-negative, GFAP-negative) to a purity greater than 90%.

4.2.2 Differential expression of drug resistance genes in purified rat glial cells.

In order to explore the possibility of the differential expression of drug resistance genes in cultures of purified rat glial cells, RNA was isolated from rat tissues (liver and brain) and purified glial cells (astrocytes, oligodendrocyte progenitors and oligodendrocytes), poly-A selected, and subjected to Northern analysis (refer to section 2.7). The Northern blots were probed sequentially for transcripts encoding MGMT, GST-μ, GST-π, p53, MDR, and MT (Figure 4.2). Hybridization signals were normalized to a poly-dT probe and comparative results are shown in Figures 4.3 and 4.4. A differential pattern of mRNA expression was observed for each of the six probes. Levels of *MGMT*, *GST-π*, and *p53* mRNA were highest in oligodendrocyte progenitors: progenitors displayed an approximate 2-fold increase in *MGMT* expression over astrocytes and a 5-

Figure 4.1 **Immunochemical characterization of purified rat glial cell cultures.** This figure demonstrates astrocytes, oligodendrocyte progenitors, and oligodendrocytes purified from rat using the procedures described in sections 2.2.1 (astrocytes) and 2.2.2 (progenitors and oligodendrocytes). Astrocytes are stained with anti-GFAP and fluorescein-conjugated goat anti-rabbit IgG (A). Oligodendrocyte progenitors (O2A cells) are stained with A2B5 and rhodamine-conjugated goat anti-mouse IgM (B). Oligodendrocytes are stained with anti-GC and fluorescein-conjugated goat anti-mouse IgG_3 (C). Bar, 25 μm.

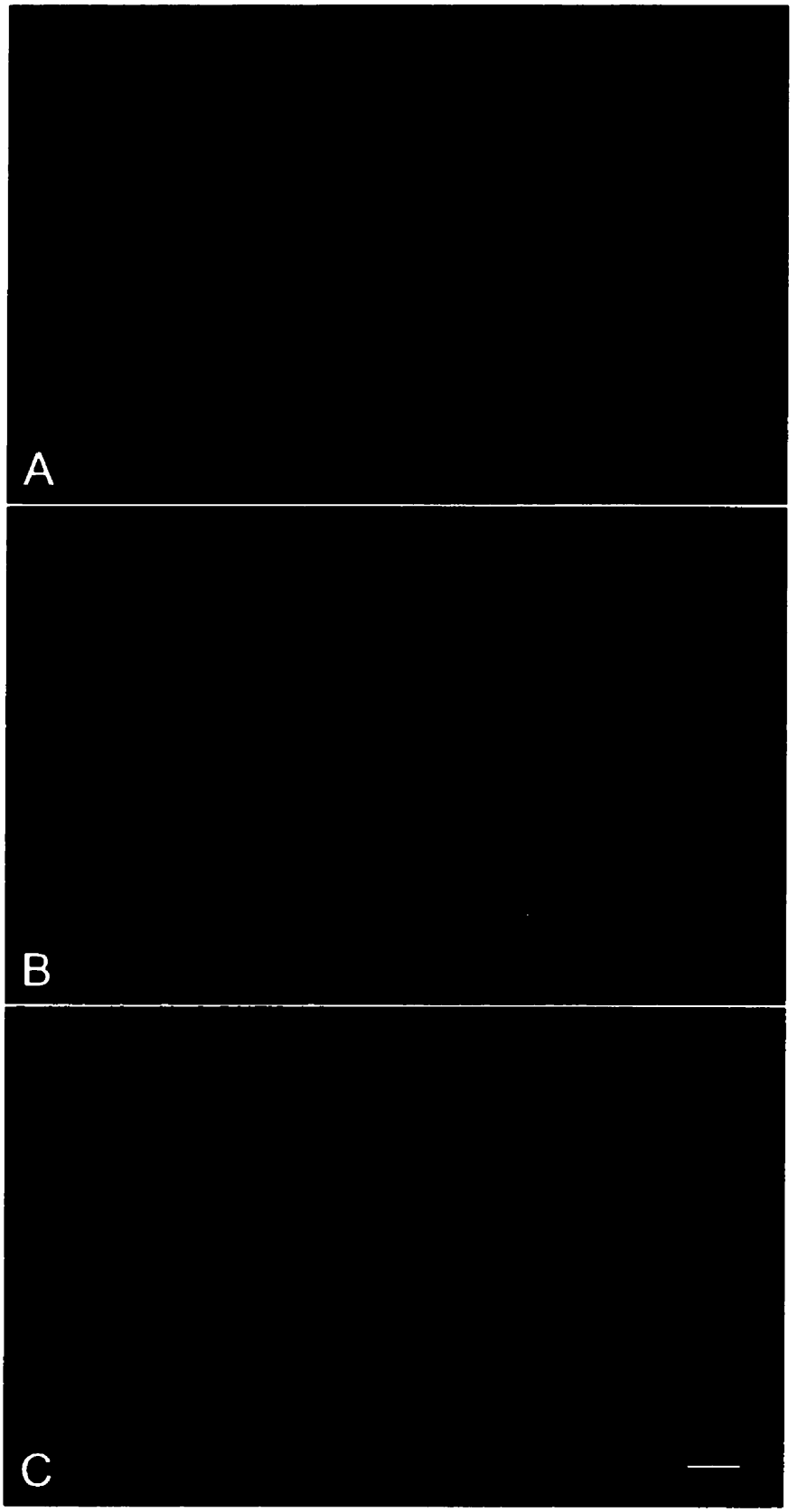

A
B
C

Figure 4.2 **Differential expression of drug resistance genes in cultures of purified glial cells.** RNA was isolated from rat tissues (liver and brain) and purified cells (astrocytes, progenitors, and oligodendrocytes), poly-A selected, and subjected to Northern analysis. Quantitated signal intensities for each probe were normalized to poly-dT as described in section 2.7.6 and comparative results are shown in Figures 4.3 and 4.4. Oligo, oligodendrocyte.

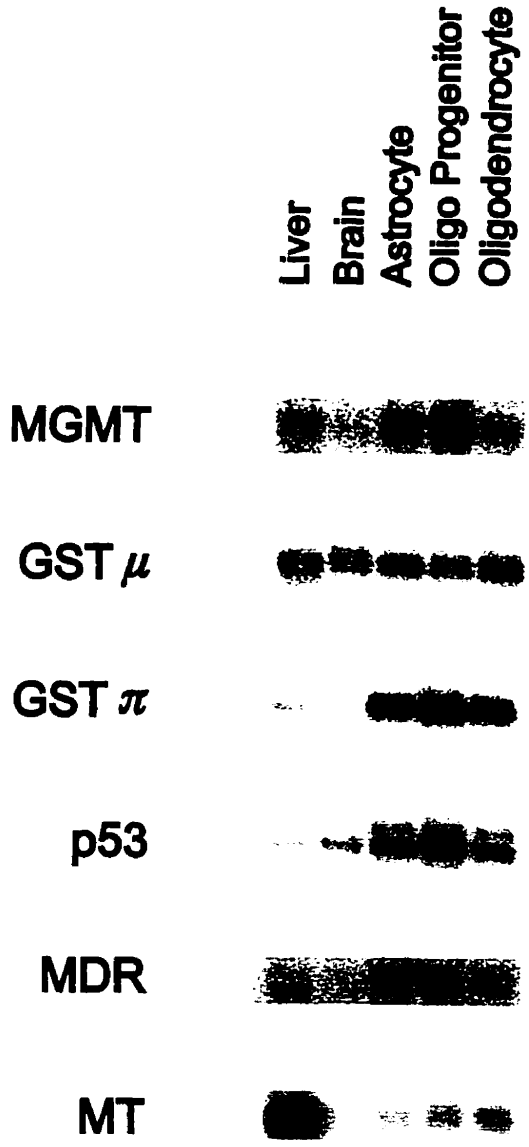

Liver
Brain
Astrocyte
Oligo Progenitor
Oligodendrocyte
MGMT
GST μ
GST π
p53
MDR
MT

Figure 4.3 **Densitometry analysis of *MGMT* and *GST* mRNA expression in purified rat glial cells.** A differential pattern of mRNA expression was observed for each of the three probes. Quantified signal intensities from Figure 4.2 were normalized to poly-dT as described in section 2.7.6 and values for astrocytes were arbitrarily set to 1.0. L, liver; B, brain; A, astrocytes; OP, oligodendrocyte progenitors; O, oligodendrocytes.

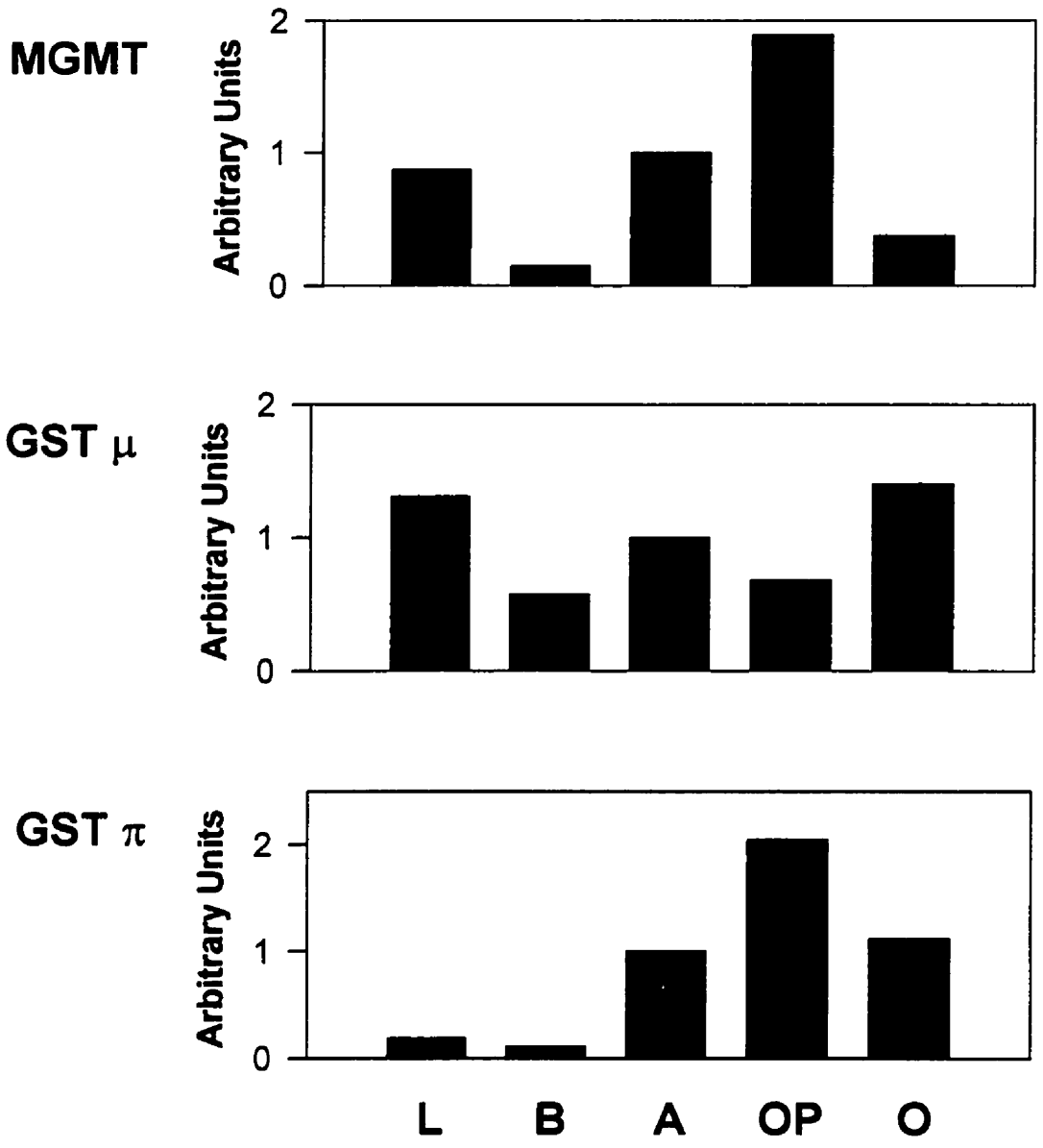
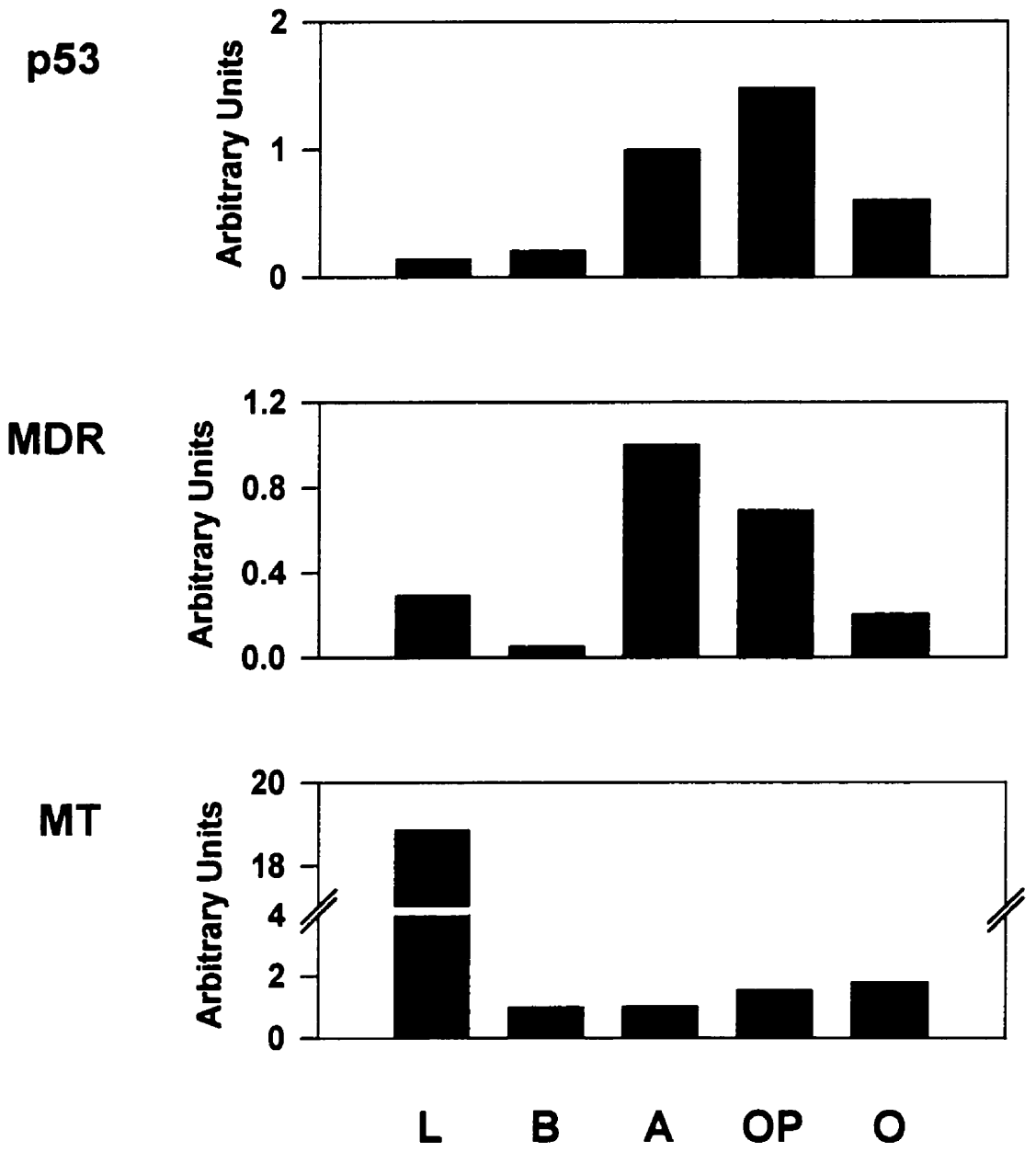

MGMT
Arbitrary Units
2
1
0
GST μ
Arbitrary Units
2
1
0
GST π
Arbitrary Units
2
1
0
L
B
A
OP
O

Figure 4.4 **Densitometry analysis of *p53*, *MDR* and *MT* mRNA expression in purified rat glial cells.** A differential pattern of mRNA expression was observed for each of the three probes. Quantified signal intensities from Figure 4.2 were normalized to poly-dT as described in section 2.7.6 and values for astrocytes were arbitrarily set to 1.0. L, liver; B, brain; A, astrocytes; OP, oligodendrocyte progenitors; O, oligodendrocytes.


p53
Arbitrary Units
2
1
0
MDR
Arbitrary Units
1.2
0.8
0.4
0.0
MT
Arbitrary Units
20
18
4
2
0
L
B
A
OP
O

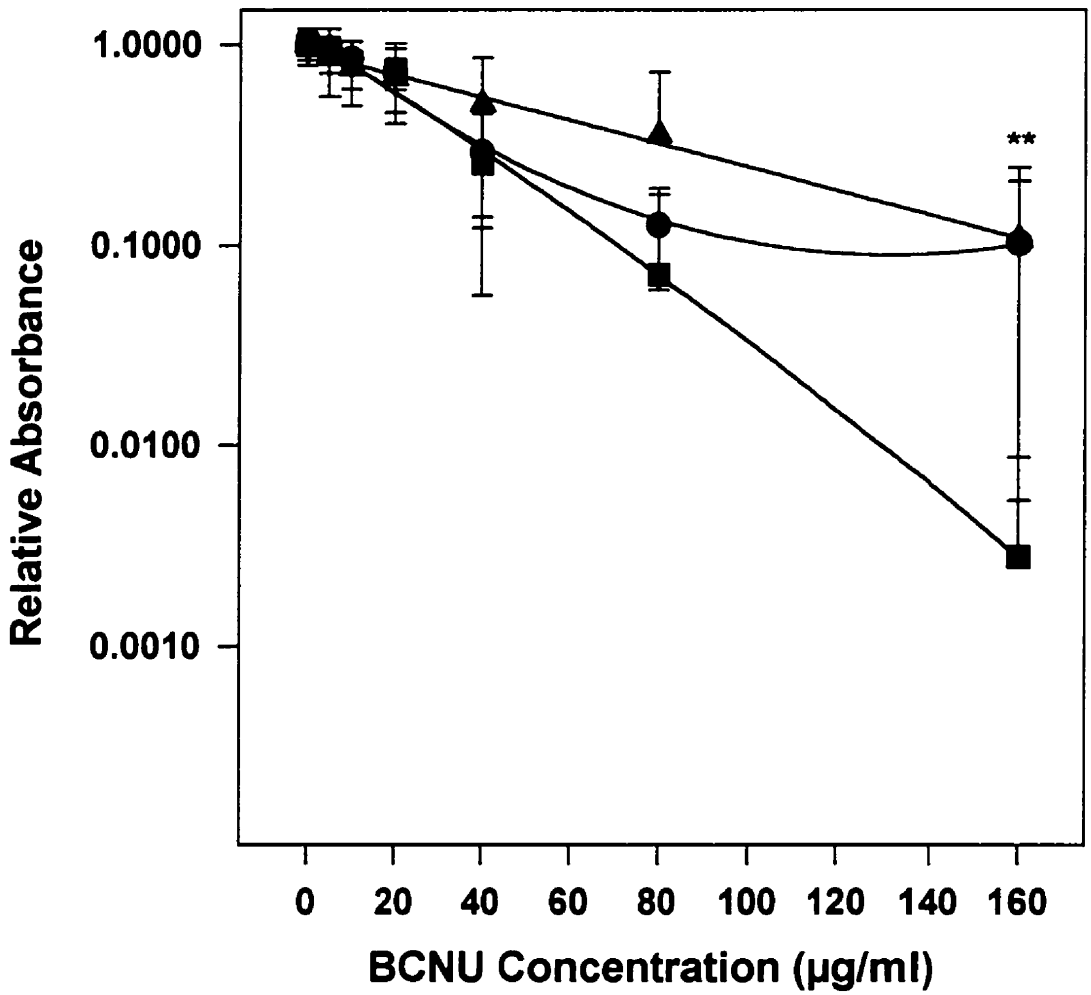
fold increase over oligodendrocytes; *GST-π* expression was approximately half that of progenitors in both astrocytes and oligodendrocytes; and a slight increase in *p53* expression was seen in progenitors over astrocytes while an approximate 2-fold increase was displayed over oligodendrocytes. Oligodendrocytes displayed the highest expression of *GST-μ* and *MT* mRNAs: *GST-μ* levels were only slightly lower in astrocytes, whereas oligodendrocyte progenitors expressed approximately half of that observed in oligodendrocytes; and *MT* mRNA levels were slightly lower in both astrocytes and oligodendrocyte progenitors. Only *MDR* expression was highest in astrocytes, with oligodendrocyte progenitors displaying a slightly lower level of expression and oligodendrocytes exhibiting approximately one-fifth of the astrocyte levels. In general, expression of the drug resistance genes tended to be lower in tissue samples than in samples from the cultured cells, with the notable exception of *MT*; *MT* expression was at least 10-fold higher in liver tissue than the levels displayed by the cultured glial cells.

Two mRNA transcripts were detected for each of *p53* and *MDR*. Although two transcripts have not been demonstrated previously in rat tissue, alternatively spliced forms of *p53* have been documented in mouse tissues (Han and Kulesz Martin, 1992; Will *et al.*, 1995). Two transcripts have been detected previously for *MDR* in rat and were shown to correspond to the *MDR1a* and *MDR1b* genes (larger and smaller transcripts respectively; Gant *et al.*, 1992).

4.2.3 Effect of BCNU and MNU treatment on the viability of purified rat glial cells.

Since a differential expression of drug resistance genes was found in cultures of purified rat glial cells, the response of purified rat astrocytes, oligodendrocyte progenitors, and oligodendrocytes to treatment with BCNU and MNU was investigated. Cells were isolated from rats, treated with increasing concentrations of either BCNU or MNU, and cell viability tested using the MTT assay (as described in sections 2.2.1, 2.2.2, and 2.5). Oligodendrocyte progenitors exhibited a trend toward increased resistance to BCNU when compared to astrocytes but this difference did not reach statistical significance (Figure 4.5). Both astrocytes and oligodendrocyte progenitors

Figure 4.5 **Effect of BCNU treatment on purified rat astrocytes, oligodendrocyte progenitors, and oligodendrocytes.** Oligodendrocyte progenitors exhibited a trend toward increased resistance to BCNU when compared to astrocytes but this difference did not reach statistical significance. Both astrocytes and oligodendrocyte progenitors displayed greater resistance to BCNU than did oligodendrocytes. The absorbance of untreated cells was normalized to a value of 1.0. Symbols represent the mean of at least three independent experiments, each performed in triplicate. **Circles represent astrocytes; triangles, oligodendrocyte progenitors; squares, oligodendrocytes.** Bars depict the standard error of the mean and asterisks the level of statistical significance (**, $P < 0.005$).


1.0000
0.1000
0.0100
0.0010
Relative Absorbance
**
0
20
40
60
80
100
120
140
160
BCNU Concentration (µg/ml)

displayed greater resistance to BCNU than did oligodendrocytes. All three cell types were extremely resistant to MNU although astrocytes and oligodendrocyte progenitors displayed a significantly greater resistance than oligodendrocytes at a concentration of 320 μg/ml (Figure 4.6).

In order to culture pure populations of glial cells successfully, each of the three glial cell types had to be grown in a defined, but distinct, cell culture medium (refer to sections 2.2.1 and 2.2.2). Due to these differences, the effect of medium on the viability of rat astrocytes in response to BCNU was determined. Astrocytes displayed a slight increase in resistance to BCNU when grown in the alternate B-S medium + 0.5% FBS supplemented with either PDGF and FGF or PDGF alone in comparison to the usual medium of DMEM + 10% FBS (Figure 4.7).

4.2.4 Effect of BG pretreatment on the response of purified rat glial cells to BCNU and MNU.

Since the expression of *MGMT* mRNA levels correlated with resistance to BCNU and MNU in the purified glial cells, the effect of BG pretreatment on the response of these cells to BCNU and MNU was determined. Cells were pretreated with 25 μM BG for two hours, exposed to increasing concentrations of either BCNU or MNU, and cell viability was determined using the MTT assay (refer to section 2.5 for details). BG appeared to increase the sensitivity to BCNU in all three cell types (Figure 4.8). In contrast, BG did not increase sensitivity to MNU in any of the cells (Figure 4.9).

4.2.5 Effect of BSO and OTZ pretreatment on the response of astrocytes to BCNU.

Although the levels of *GST* mRNAs did not appear to correlate directly with resistance to BCNU, modulation of glutathione has been demonstrated to alter resistance to a number of alkylating agents, including BCNU (refer to section 1.5.3). For this reason, the effect of BSO and OTZ pretreatment on the response of astrocytes was examined. Astrocytes were pretreated with either 25 μM BSO for 24 hours or 5 mM OTZ for four hours, exposed to increasing concentrations of BCNU, and cell viability was determined using the MTT assay (refer to section 2.5 for details). BSO pretreatment

Figure 4.6 **Effect of MNU treatment on purified rat astrocytes, oligodendrocyte progenitors, and oligodendrocytes.** All three cells types were extremely resistant to MNU although astrocytes and oligodendrocyte progenitors displayed a significantly greater resistance than oligodendrocytes at 320 μg/ml. The absorbance of untreated cells was normalized to a value of 1.0. Symbols represent the mean of at least three independent experiments, each performed in triplicate. **Circles represent astrocytes; triangles, oligodendrocyte progenitors; squares, oligodendrocytes.** Bars depict the standard error of the mean and asterisks the level of statistical significance (**, $P < 0.005$).

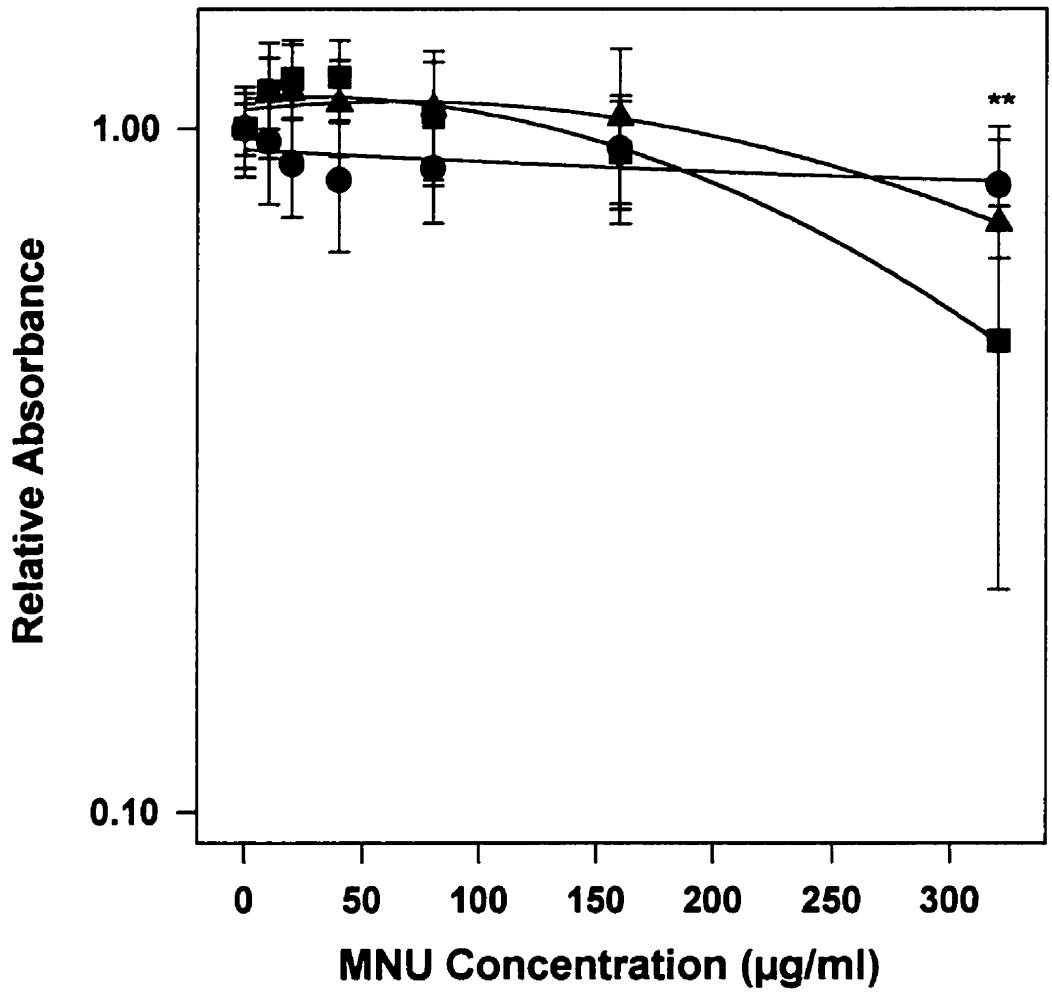

1.00
0.10
Relative Absorbance
**
0
50
100
150
200
250
300
MNU Concentration (µg/ml)

Figure 4.7 **The effect of cell culture medium on the viability of rat astrocytes in response to BCNU.** Astrocytes displayed a slight increase in resistance to BCNU when grown in the alternate cell culture media. The absorbance of untreated cells was normalized to a value of 1.0. Symbols represent the mean of at least two independent experiments, each performed in triplicate. **Circles represent astrocytes grown in DMEM + 10% FBS; squares, B-S medium + 0.5% FBS, PDGF, and bFGF; triangles, B-S medium + 0.5% FBS and PDGF.** Bars depict the standard error of the mean and asterisks the level of statistical significance (*, $P < 0.05$).

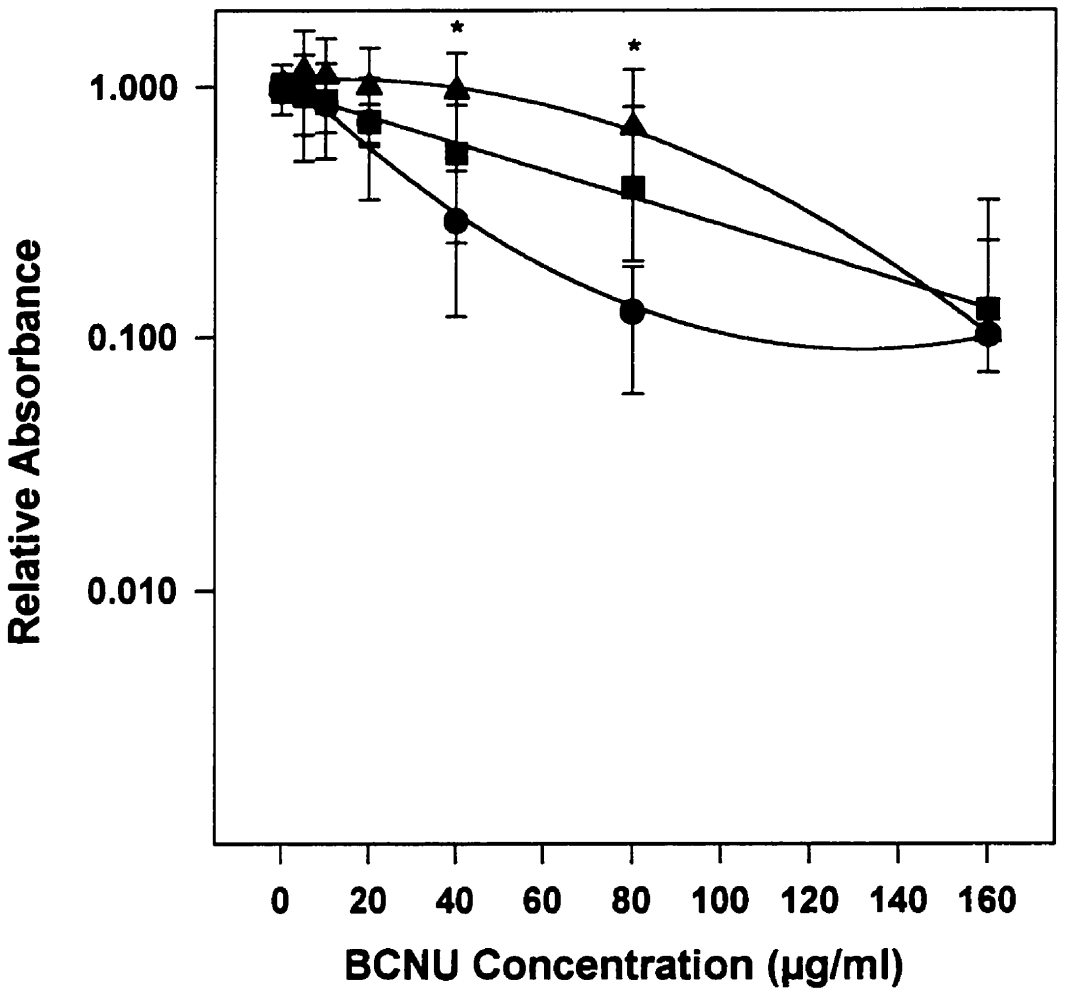
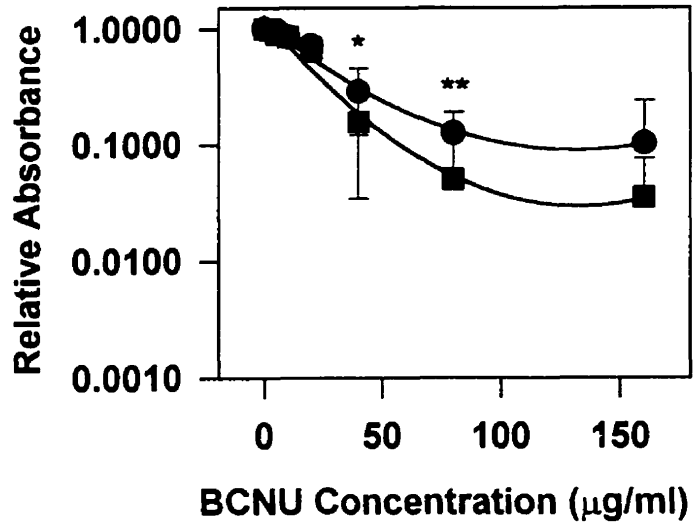
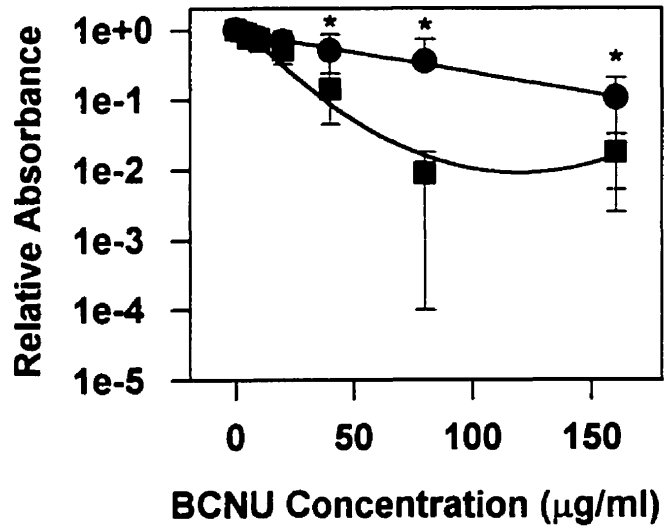

1.000
0.100
0.010
Relative Absorbance
*
*
0
20
40
60
80
100
120
140
160
BCNU Concentration (µg/ml)

Figure 4.8 **Effect of BG pretreatment on the response of purified rat glial cells to BCNU.** Astrocytes (A), oligodendrocyte progenitors (B), and oligodendrocytes (C) were pretreated with 25 μM BG for two hours and then exposed to varying concentrations of BCNU. BG appeared to increase slightly the sensitivity to BCNU in all three cell types. Symbols represent the mean of at least two independent experiments, each performed in triplicate. **Circles represent cells treated with BCNU alone; squares, 25 μM BG pretreatment.** Bars depict the standard error of the mean and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$).

A


1.0000
0.1000
0.0100
0.0010
Relative Absorbance
*
**
0
50
100
150
BCNU Concentration (μg/ml)

B


1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Relative Absorbance
*
*
*
0
50
100
150
BCNU Concentration (μg/ml)

C

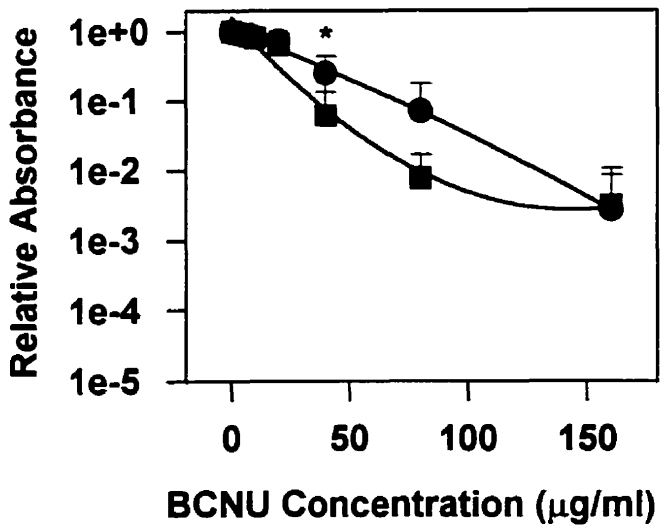
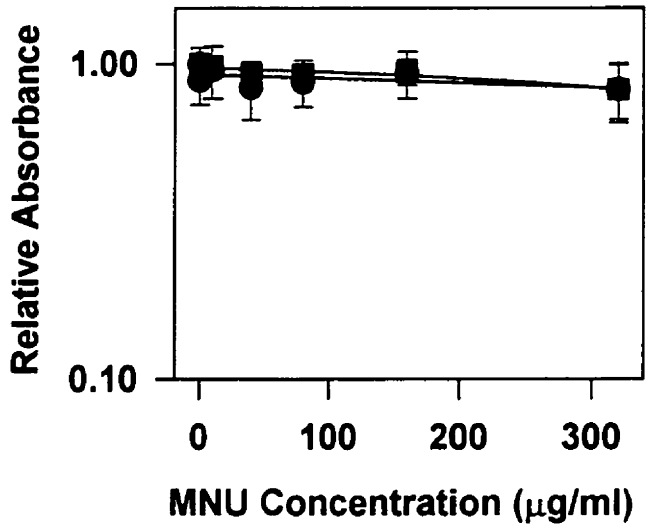
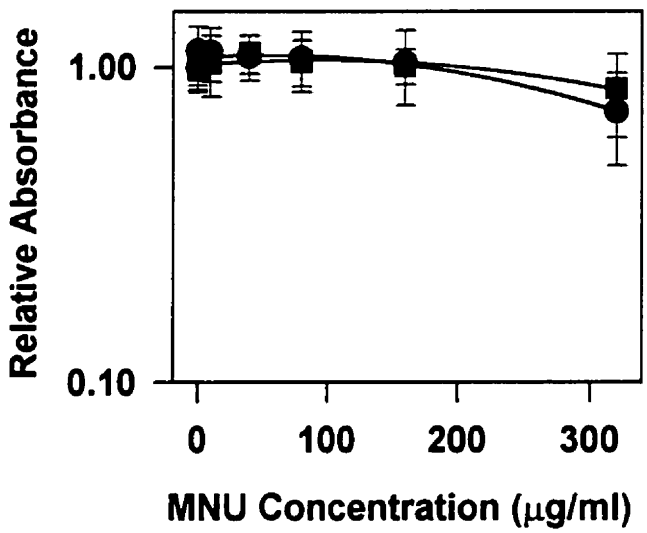

1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Relative Absorbance
*
0
50
100
150
BCNU Concentration (μg/ml)

Figure 4.9 **Effect of BG pretreatment on the response of purified rat glial cells to MNU.** Astrocytes (A), oligodendrocyte progenitors (B), and oligodendrocytes (C) were pretreated with 25 μM BG for two hours and then exposed to varying concentrations of MNU. BG did not significantly increase sensitivity to MNU in any of the three cells types. Symbols represent the mean of at least two independent experiments, each performed in triplicate. **Circles represent cells treated with MNU alone; squares, 25 μM BG pretreatment.** Bars depict the standard error of the mean.

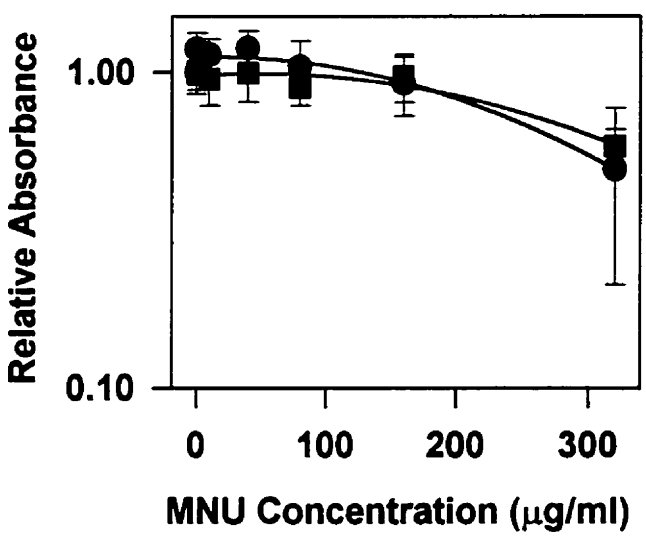
A


Relative Absorbance
1.00
0.10
0
100
200
300
MNU Concentration (μg/ml)

B


Relative Absorbance
1.00
0.10
0
100
200
300
MNU Concentration (μg/ml)

C


Relative Absorbance
1.00
0.10
0
100
200
300
MNU Concentration (μg/ml)

increased the sensitivity of astrocytes to BCNU but OTZ pretreatment displayed no effect (Figure 4.10).

4.3 DISCUSSION

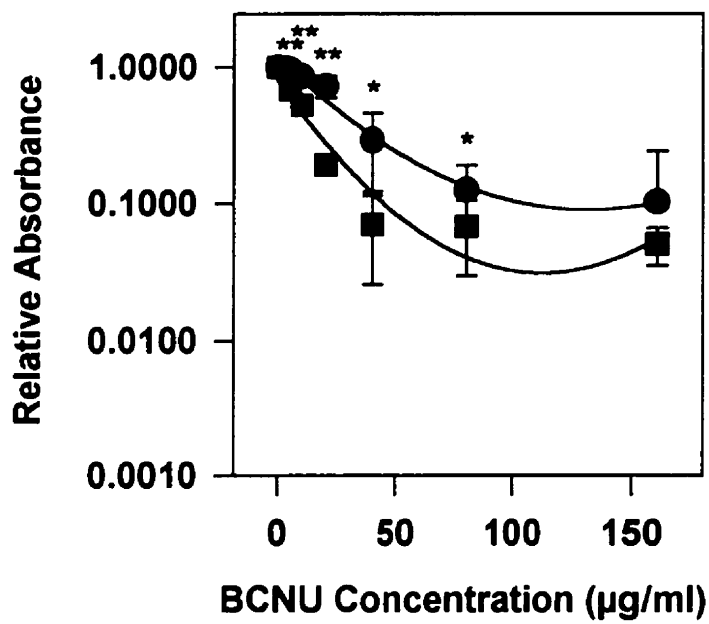
One of the disadvantages with the cell culture methodology utilized in Chapter 3 was that the oligodendrocyte lineage cell population was not pure. Since multiple cell types were present in the cultures, characteristics displayed by the oligodendrocyte lineage cells could not be attributed to a particular glial cell type. Due to the refinement of glial cell culture techniques by Barres *et al.* (1992), it became possible to culture large numbers of pure populations of astrocytes, oligodendrocyte progenitors, and oligodendrocytes from rat brain. All experiments presented in this chapter were performed on these pure populations of glial cells.

As discussed previously, it is unlikely that the response of human gliomas to chemotherapy is determined by a single biochemical process. Furthermore, if mechanisms of drug resistance in some subtypes of glial tumors reflect the biochemical properties of their cell of origin, then it would follow that a differential expression of drug resistance genes would be seen in subtypes of normal glial cells. This hypothesis was supported by experiments performed on pure cultures of rat glial cells. RNA was isolated from purified rat astrocytes, oligodendrocyte progenitors and oligodendrocytes, poly-A selected, and subjected to Northern analysis. Northern blots were probed sequentially for transcripts encoding MGMT, GST-μ, GST-π, p53, MDR, and MT. A differential pattern of mRNA expression was observed for each of the six probes. Rat oligodendrocytes expressed lower levels of *MGMT*, *p53*, and *MDR* than astrocytes. These findings demonstrated that more than one mechanism of drug resistance was deficient in oligodendrocytes and the possibility that some of these same mechanisms may be inefficient in oligodendroglial tumors is raised.

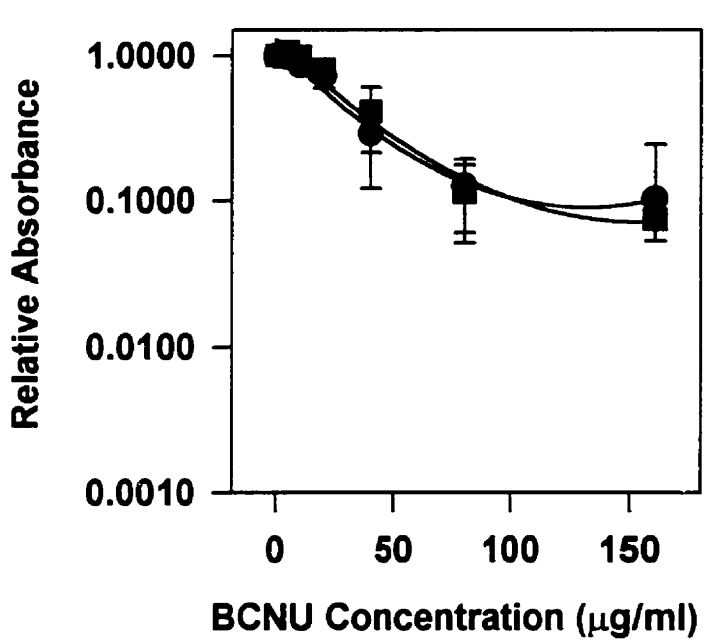
Since a differential expression of drug resistance genes was found in cultures of purified rat glial cells, the response of purified rat astrocytes, oligodendrocyte progenitors, and oligodendrocytes to treatment with BCNU and MNU was investigated. Both astrocytes and oligodendrocyte progenitors displayed greater resistance to BCNU

Figure 4.10 Effect of BSO and OTZ pretreatment on the response of rat astrocytes to BCNU. Astrocytes were pretreated with either 25 μM BSO for 24 hours (A) or 5 mM OTZ for four hours (B) and then exposed to varying concentrations of BCNU. BSO pretreatment increased significantly the sensitivity of astrocytes to BCNU but OTZ pretreatment displayed no effect. Symbols represent the mean of at least two independent experiments, each performed in triplicate. **Circles represent astrocytes treated with BCNU alone; squares, BSO or OTZ pretreatment.** Bars depict the standard error of the mean and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$).

A


Relative Absorbance
1.0000
0.1000
0.0100
0.0010
0
50
100
150
BCNU Concentration (µg/ml)

B


Relative Absorbance
1.0000
0.1000
0.0100
0.0010
0
50
100
150
BCNU Concentration (µg/ml)

than did oligodendrocytes. All three cell types were extremely resistant to MNU although astrocytes and oligodendrocyte progenitors displayed a significantly greater resistance than oligodendrocytes at a concentration of 320 μg/ml. Thus, it was demonstrated that subtypes of pure populations of rat glial cells displayed a differential response to both the bifunctional alkylating agent BCNU and the monofunctional alkylating agent MNU. As each of the three glial cell types were grown in a defined, but distinct, cell culture medium, the possibility that these differences in response to cytotoxic drug was due to dissimilar medium could not be excluded. Since astrocytes were the only cell type that could survive in each of the three media, the effect of cell culture medium on the resistance of astrocytes to BCNU was determined. Astrocytes displayed a slight increase in resistance to BCNU when grown in the alternate B-S medium + 0.5% FBS supplemented with either PDGF and bFGF (oligodendrocyte progenitor medium) or PDGF alone (oligodendrocyte medium) in comparison to the usual medium of DMEM + 10% FBS. There was still no significant difference, however, between the response of astrocytes and that of oligodendrocyte progenitors to BCNU and astrocytes remained significantly more resistant than oligodendrocytes in each of the three media. Therefore, it was concluded that the differential response of astrocytes, oligodendrocyte progenitors, and oligodendrocytes to cytotoxic drugs was not a result of differences in cell culture conditions and was, in fact, due to properties of the individual cell types.

The two mechanisms of drug resistance that have received the most study in relation to BCNU are MGMT and the GSTs (refer to sections 1.5.2 and 1.5.3 respectively). Since MGMT protein activity has been found to correlate with resistance to BCNU in cultures of mixed glial cells (refer to Chapter 3; Nutt *et al.*, 1995) and expression of *MGMT* mRNA levels correlated with resistance to BCNU and MNU in purified glial cells, the effect of pretreatment with the modulator, BG, on the response of these cells to BCNU and MNU was determined. BG has been shown to cause the depletion of MGMT activity (refer to section 1.5.2) and appeared to increase the sensitivity to BCNU, in all three cell types. In contrast, BG did not increase sensitivity to MNU in any of the cells. Thus, it was concluded that MGMT contributed, at least in part, to the resistance of rat glial cells to BCNU. Although the results obtained for MNU

seemed contradictory to those for BCNU, the extreme level of resistance displayed by glial cells to MNU may have precluded any modulation at the drug concentrations used; it is possible that even in the absence of MGMT, glial cells remain resistant to the cytotoxic effects of MNU at these concentrations.

Although the levels of *GST* mRNAs did not appear to correlate directly with resistance to BCNU, modulation of glutathione has been demonstrated to alter resistance to a number of alkylating agents, including BCNU (refer to section 1.5.3). For this reason, the effect of BSO and OTZ pretreatment on the response of astrocytes was examined. BSO has been shown to cause depletion of cellular glutathione levels and OTZ has been shown to increase these levels (refer to section 1.5.3). Interestingly, BSO pretreatment increased the sensitivity of astrocytes to BCNU but OTZ pretreatment displayed no effect. Thus, it appeared that the glutathione-GST detoxification system also played a role in the resistance of astrocytes to BCNU. The finding that depletion of glutathione increased sensitivity to BCNU but the induction of glutathione did not enhance resistance was of particular interest; since GSTs are a family of enzymes that catalyze the conjugation of cytotoxic drugs to glutathione, thereby enhancing the rate of detoxification, these results indicated that the levels of GSTs, not glutathione, may actually be the rate limiting step for detoxification in glial cells.

In summary, it was determined that rat glial cell subtypes exhibit differential expression of a number of drug resistance genes. In addition, a differential response to the alkylating agent BCNU was demonstrated. The increased resistance of astrocytes in comparison to oligodendrocytes was modulated, at least in part, by both BG and BSO, suggesting a role for both MGMT and the GSTs in the resistance of astrocytes to BCNU. Thus, the response of normal rat glial cells to BCNU appeared to be dependent on more than one mechanism of drug resistance. If mechanisms of drug resistance in some subtypes of glial tumors reflect the biochemical properties of their cell of origin, then these experiments provide additional support for the hypothesis that oligodendroglioma cells may be inherently susceptible to the cytotoxic effects of alkylating agents.

CHAPTER 5

THE EFFECT OF p53 STATUS ON THE VIABILITY AND CELL CYCLE RESPONSE OF MOUSE ASTROCYTES EXPOSED TO BCNU

5.1 INTRODUCTION

The p53 protein plays a role in protecting cells and organisms from the deleterious effects of DNA damage by regulating cell cycle progression and by triggering programmed cell death (refer to sections 1.7.4.2 and 1.7.4.3 for details). A p53-dependent G_1 arrest in some cell types in response to certain kinds of DNA damage is protective (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992); it prevents the propagation of mutations by allowing additional time in the cell cycle for DNA repair. In other cell types in response to DNA damage, p53-dependent apoptosis is protective to the organism (Clarke *et al.*, 1993; Lowe *et al.*, 1993a); it prevents the propagation of abnormal cells by facilitating their self-destruction. These normal functions for p53 may influence the response of human cancers to DNA-damaging therapies in opposite ways; p53 could confer resistance if G_1 arrest permits therapeutic DNA damage to be repaired, but also sensitivity if damaged cancer cells apoptose. The situation is further complicated because p53 often is abnormal in cancer cells; dysfunctional p53 could confer sensitivity if G_1 arrest cannot occur and treatment-induced DNA damage cannot be repaired, but also resistance if damaged cancer cells are unable to apoptose. Failure to apoptose seems to predominate in cancers with p53 abnormalities; abrogation of a functional p53 often is associated with treatment resistance (Lowe *et al.*, 1993a; O'Connor *et al.*, 1993; McIlwrath *et al.*, 1994; Nabeya *et al.*, 1995) and shorter survival (Chang *et al.*, 1995). This may not be true for all tumor types, however. Wild type p53 may confer resistance to treatment in some cancer cells (Biard *et al.*, 1994; Petty *et al.*, 1994; Fan *et al.*, 1995; Vikhanskaya *et al.*, 1995) and in evolving astrocytic gliomas, wild type p53 has been associated with shorter patient survival (van Meyel *et al.*, 1994). The effect of p53 status

on the response of astrocytomas to chemotherapy has not been studied.

The p53 protein has been shown to activate transcription of the *WAF1* and *GADD45* genes in response to some forms of DNA damage (refer to section 1.7.4.2), resulting in the induction of a cell cycle arrest. p53 also has been demonstrated to activate transcription of the *mdm-2* gene (Barak *et al.*, 1993). Mdm-2 is a cellular inhibitor of p53 in that it binds the transactivation domain of p53 and downregulates its ability to activate transcription (Momand *et al.*, 1992; Meltzer, 1994; Kussie *et al.*, 1996). In addition, binding by mdm-2 can promote the rapid degradation of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Thus, the potential exists for a tightly regulated cell cycle response to DNA damage; the activation of p53 can upregulate genes known to affect a cell cycle response as well as *mdm-2*, resulting in its own inactivation and degradation through an autoregulatory loop.

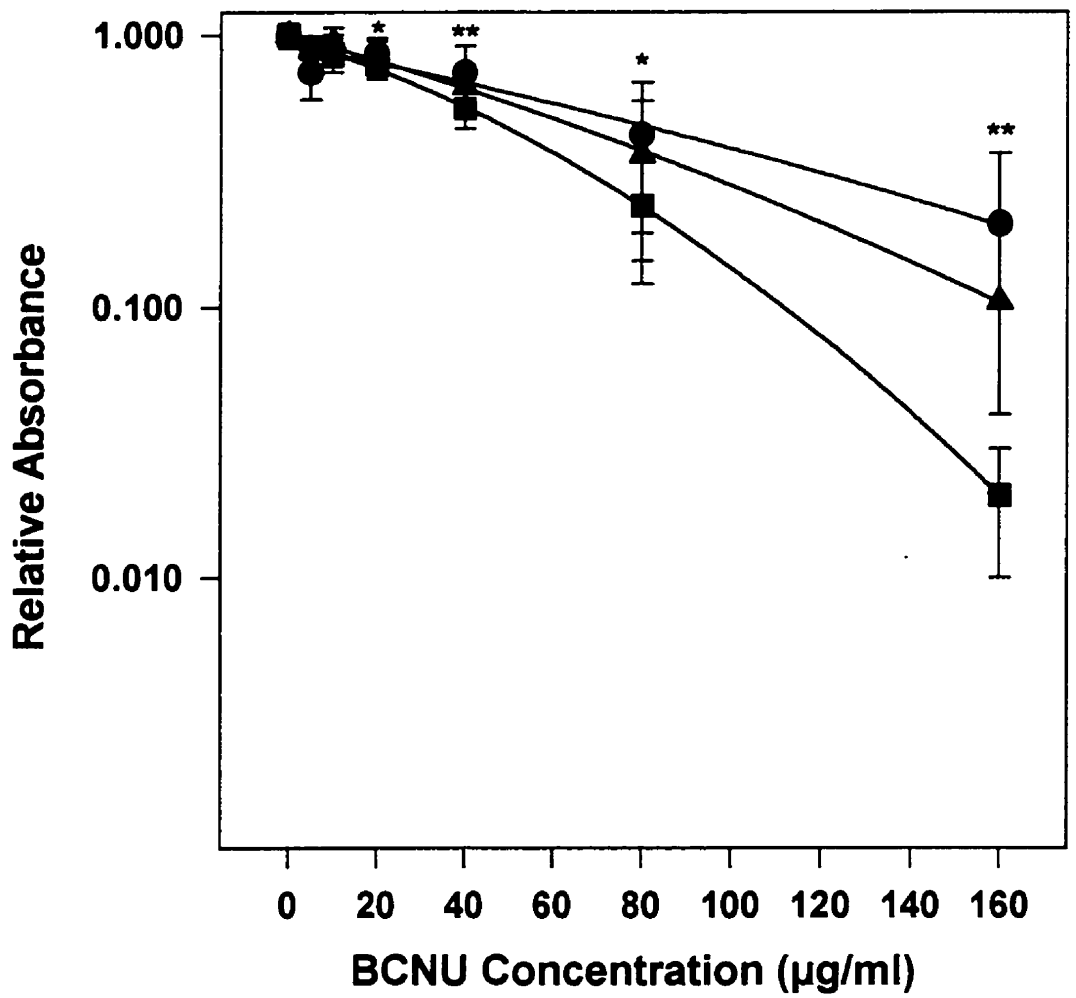
The purposes of this study were 1) to examine the effect of p53 status on the viability of mouse astrocytes exposed to BCNU, the standard cytotoxic drug for astrocytic gliomas; 2) to determine if this effect was drug-specific or cell-specific; 3) to explore the effect of BCNU treatment on the expression of three p53-regulated genes (*mdm-2*, *WAF1*, and *GADD45*) that may play a role in the cell cycle response to DNA damage; and 4) to examine the effect of p53 status on the cell cycle of astrocytes in response to DNA damaging treatments (ie. BCNU and radiation).

5.2 RESULTS

5.2.1 Effect of p53 on the viability of mouse astrocytes exposed to BCNU.

First, the effect of p53 on the viability of BCNU-treated astrocytes was determined. Astrocytes were isolated from mice with distinct *p53* genotypes (+/+, +/−, −/−), treated with increasing concentrations of BCNU and cell viability tested using the MTT assay (as described in sections 2.3.3 and 2.5). Homozygous wild type (+/+) *p53* astrocytes displayed significantly greater resistance to BCNU than knockout (−/−) astrocytes (Figure 5.1). Heterozygous (+/−) astrocytes demonstrated an intermediate level of resistance and were significantly more resistant to BCNU than −/−

Figure 5.1 **Effect of p53 status on the viability of mouse astrocytes treated with BCNU.** Homozygous wild type (+/+) *p53* astrocytes displayed significantly greater resistance to BCNU than homozygous knockout (−/−) astrocytes. Heterozygous (+/−) astrocytes displayed an intermediate level of resistance. The absorbance of untreated cells was normalized to a value of 1.0. Symbols represent the mean of at least five independent experiments, each performed in triplicate. **Circles represent +/+ cells; triangles, +/− cells; squares, −/− cells.** Bars depict the standard error of the mean and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$).


1.000
0.100
0.010
Relative Absorbance
*
**
*
**
0
20
40
60
80
100
120
140
160
BCNU Concentration (µg/ml)

astrocytes (Figure 5.1). Hence, wild type p53 appeared to protect mouse astrocytes from the cytotoxic effects of BCNU in a gene-dose-dependent fashion.

5.2.2 Effect of p53 on the viability of mouse astrocytes and fibroblasts in response to cytotoxic treatment.

To test whether the *p53* genotype/BCNU response pattern was BCNU-specific or astrocyte-specific, the effect of p53 on the viability of astrocytes treated with MNU and radiation, and that of fibroblasts treated with BCNU and radiation was determined. The *p53* genotype had no discernible effect on the response of astrocytes to MNU over the range of concentrations tested (Figure 5.2A). However, in all other situations $-/-$ cells were more resistant than $+/+$ cells: $-/-$ astrocytes exhibited greater resistance to radiation than $+/+$ astrocytes (Figure 5.2B); $-/-$ fibroblasts exhibited greater resistance to BCNU than $+/+$ fibroblasts (Figure 5.3A) and $-/-$ fibroblasts were more resistant than $+/+$ fibroblasts to radiation at lower doses, although not at higher doses (Figure 5.3B). Heterozygous astrocytes and fibroblasts displayed an intermediate level of resistance to all toxic agents (data not shown). In summary, mouse astrocytes appeared to have a unique response to BCNU; wild type p53 rendered astrocytes resistant to this drug while in no other situation tested were $+/+$ cells more resistant than $-/-$ cells.

5.2.3 Effect of BCNU treatment on p53-regulated gene expression.

In order to investigate whether BCNU treatment could influence the expression of p53-regulated genes, $+/+$ and $-/-$ astrocytes and fibroblasts were exposed to 40 μg/ml BCNU for 2 hr and samples were taken at defined times for Northern analysis (refer to section 2.7). The Northern blots were probed sequentially for transcripts encoding mdm-2, p21 (WAF1) and GADD45 (Figures 5.4 - 5.7). Hybridization signals were normalized to the *MGAP* signal from the same lane. Expression of *GADD45* remained relatively constant over the 24 hr time period in response to BCNU in all four cell types; densitometry was performed (refer to section 2.7.6) and any differences in signal intensities were not greater than 50% and were within the range of variation displayed by the control samples.. There was evidence, however, of a slight induction

Figure 5.2 **Effect of p53 status on the viability of mouse astrocytes in response to cytotoxic treatment.** There was no significant difference in the level of resistance exhibited by wild type (+/+) *p53* nor knockout (−/−) astrocytes to MNU over the range of concentrations used (A). Knockout astrocytes displayed greater resistance to radiation than +/+ astrocytes (B). The absorbance of untreated cells was normalized to a value of 1.0. Symbols represent the mean of at least five independent experiments, each performed in triplicate. **Circles represent +/+ cells; squares, −/− cells.** Bars depict the standard error of the mean and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$). Gy, grays.

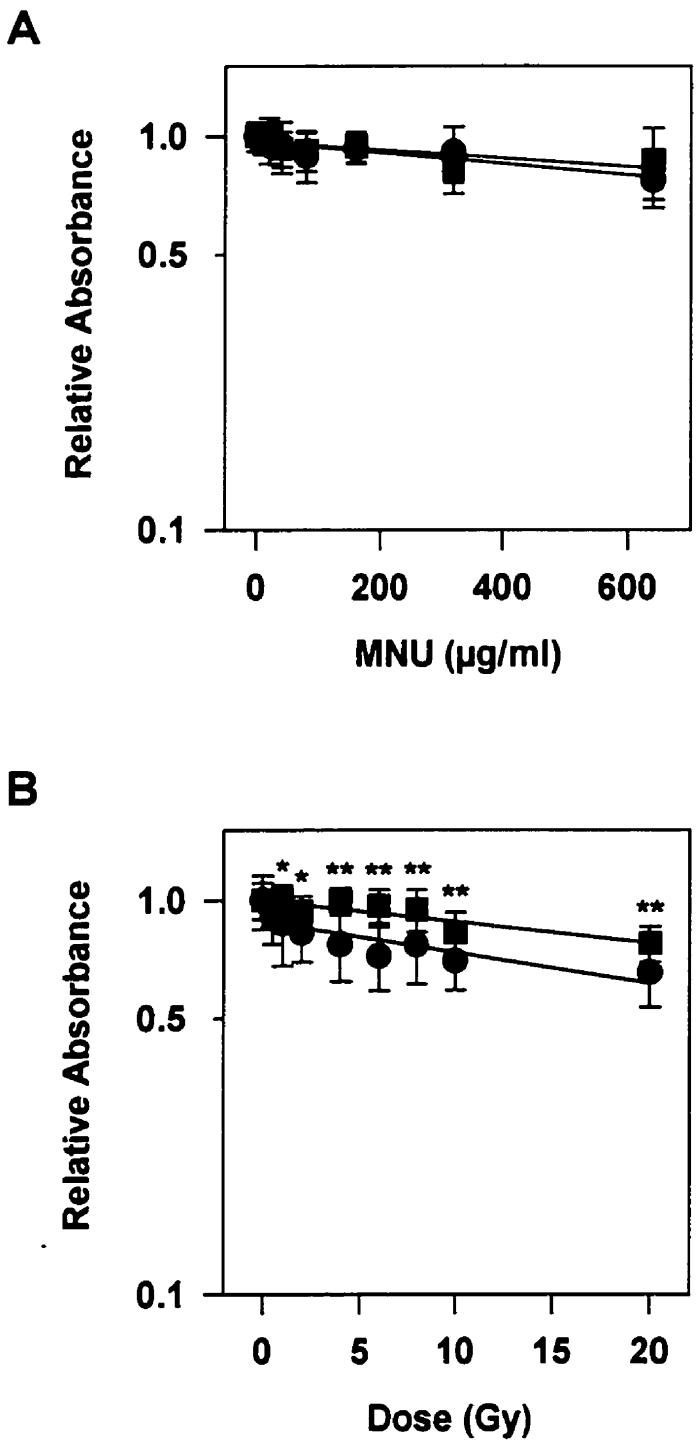
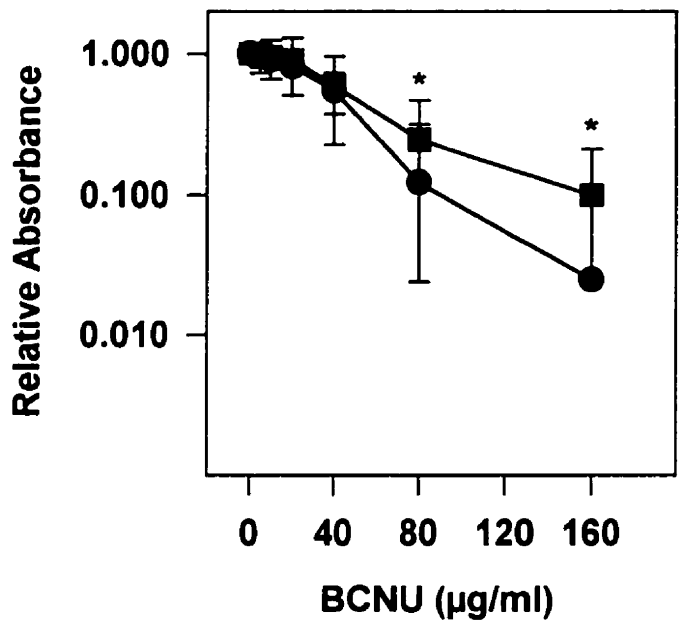

A
Relative Absorbance
1.0
0.5
0.1
0
200
400
600
MNU (µg/ml)
B
Relative Absorbance
1.0
0.5
0.1
0
5
10
15
20
Dose (Gy)

Figure 5.3 **Effect of p53 status on the viability of mouse fibroblasts in response to cytotoxic treatment.** Knockout fibroblasts (−/−) demonstrated greater resistance to BCNU than wild type (+/+) *p53* fibroblasts (A). In response to radiation, −/− fibroblasts displayed greater resistance than +/+ fibroblasts at lower doses, but no significant difference at higher doses (B). The absorbance of untreated cells was normalized to a value of 1.0. Symbols represent the mean of at least five independent experiments, each performed in triplicate. **Circles represent +/+ cells; squares, −/− cells.** Bars depict the standard error of the mean and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$). Gy, grays.

A


1.000
0.100
0.010
Relative Absorbance
*
*
0
40
80
120
160
BCNU (µg/ml)

B

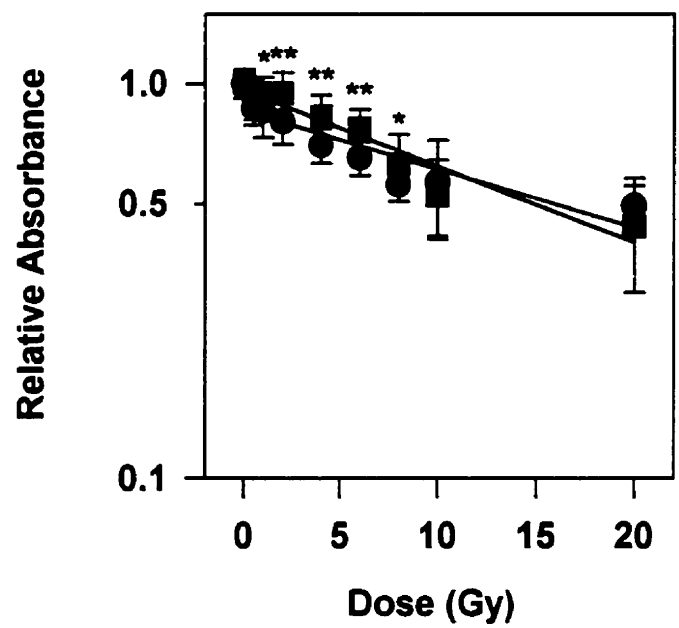

1.0
0.5
0.1
Relative Absorbance
***
**
**
*
0
5
10
15
20
Dose (Gy)

Figure 5.4 **Effect of BCNU treatment on the mRNA expression of *p53* and p53-regulated genes in wild type *p53* mouse astrocytes.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated mRNA sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Quantified signal intensities for each probe were normalized to *MGAP* and comparative results for *mdm-2* and *WAF1* are shown in Figures 5.8 and 5.9 respectively. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.

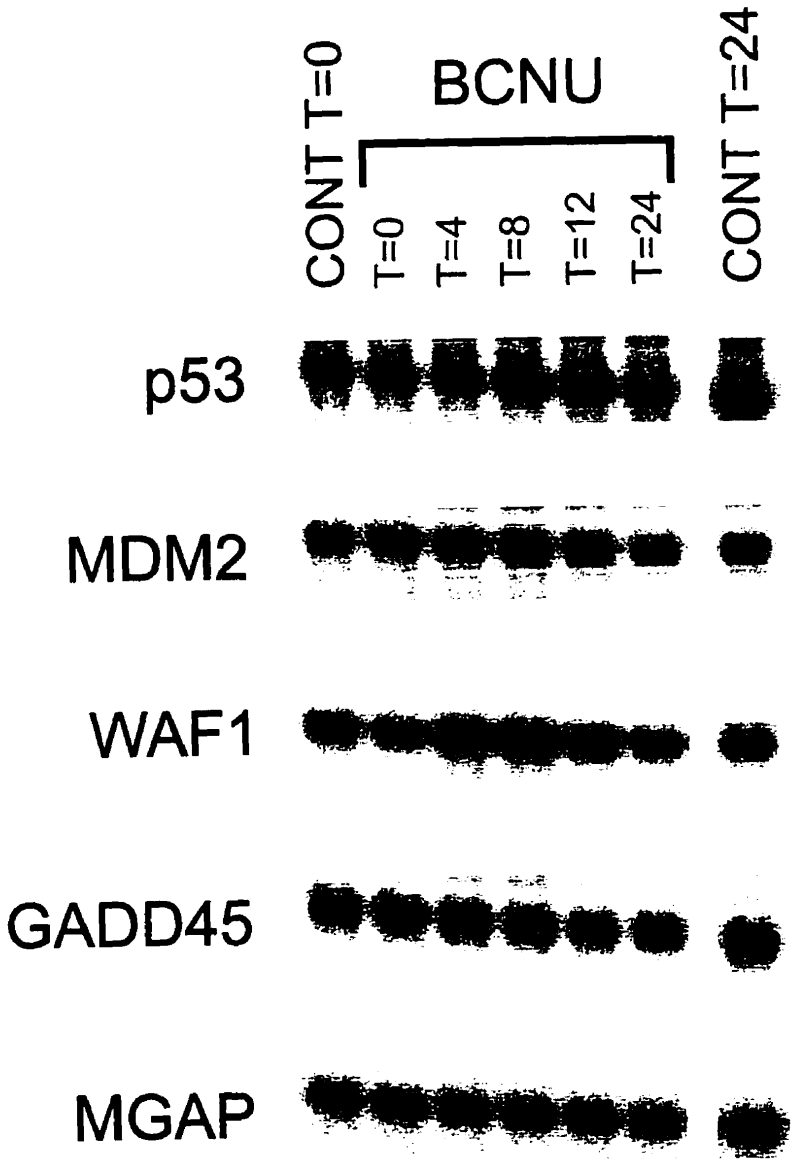

CONT T=0
BCNU
T=0
T=4
T=8
T=12
T=24
CONT T=24
p53
MDM2
WAF1
GADD45
MGAP

Figure 5.5 **Effect of BCNU treatment on the mRNA expression of p53-regulated genes in knockout *p53* mouse astrocytes.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Quantified signal intensities for each probe were normalized to *MGAP* and comparative results for *mdm-2* and *WAF1* are shown in Figures 5.8 and 5.9 respectively. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.

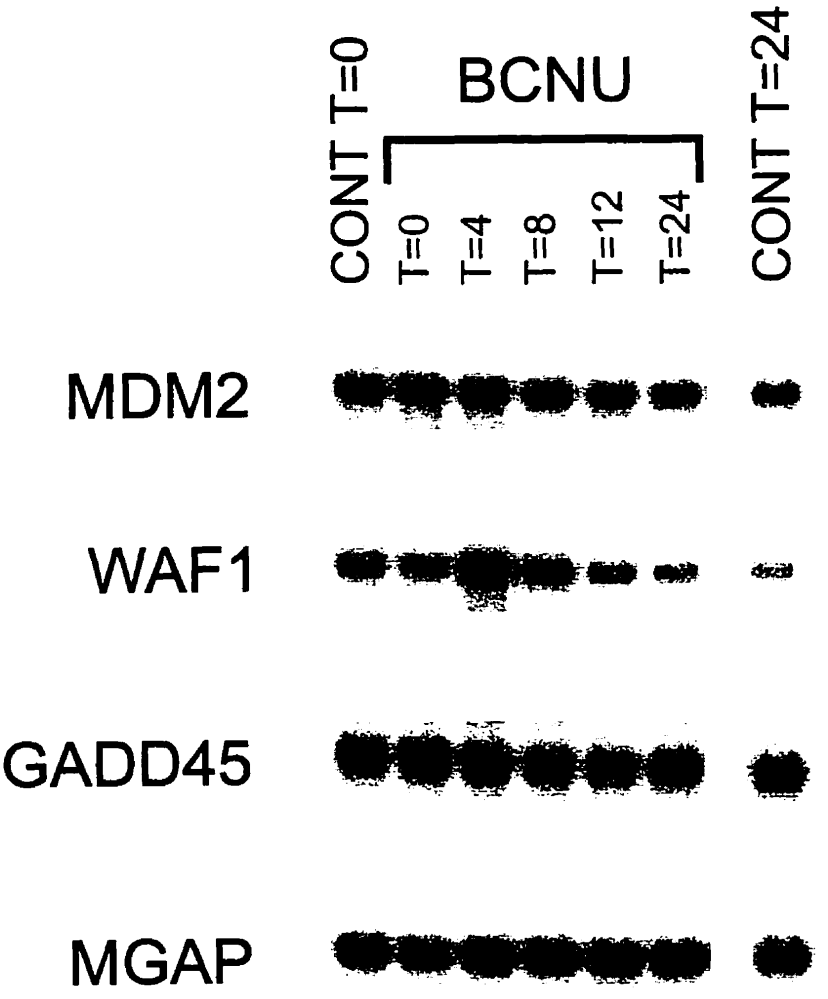
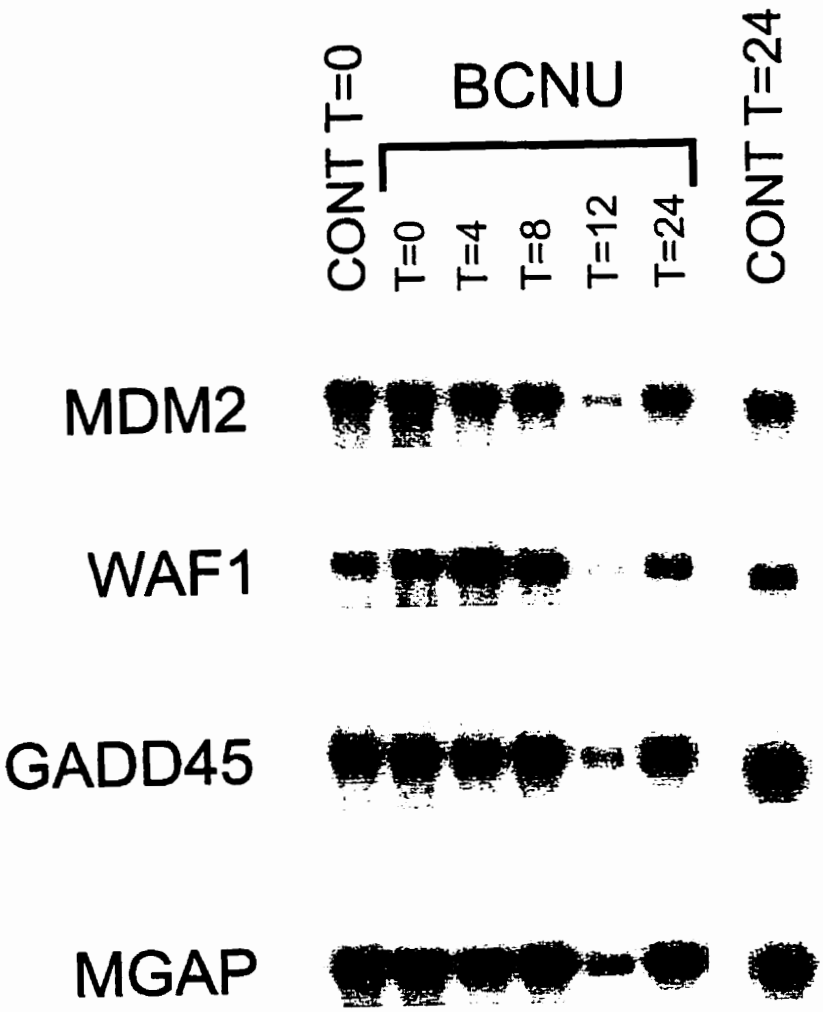

CONT T=0
BCNU
T=0
T=4
T=8
T=12
T=24
CONT T=24
MDM2
WAF1
GADD45
MGAP

Figure 5.6 **Effect of BCNU treatment on the mRNA expression of *p53* and p53-regulated genes in wild type *p53* mouse fibroblasts.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Quantified signal intensities for each probe were normalized to *MGAP* and comparative results for *mdm-2* and WAF1 are shown in Figures 5.8 and 5.9 respectively. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.


CONT T=0
BCNU
T=0
T=4
T=8
T=12
T=24
CONT T=24
p53
MDM2
WAF1
GADD45
MGAP

Figure 5.7 **Effect of BCNU treatment on the mRNA expression of p53-regulated genes in knockout *p53* mouse fibroblasts.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Quantified signal intensities for each probe were normalized to *MGAP* and comparative results for *mdm-2* and *WAF1* are shown in Figures 5.8 and 5.9 respectively. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.


CONT T=0
BCNU
T=0
T=4
T=8
T=12
T=24
CONT T=24
MDM2
WAF1
GADD45
MGAP

in *mdm-2* and a 2- to 5-fold induction in *WAF1* expression in response to BCNU (summarized in Figures 5.8 and 5.9 respectively).

The effect of BCNU treatment on the expression of *p53* mRNA also was determined. Wild type astrocytes and fibroblasts were exposed to 40 μg/ml BCNU for 2 hr and samples were taken at defined times for Northern analysis (refer to section 2.7). There was no evidence of *p53* mRNA induction; expression remained constant over a 24 hr time period in response to BCNU in both cell types (Figures 5.4 and 5.6).

5.2.4 Cell cycle distribution of mouse astrocytes treated with BCNU or radiation.

Since changes in the expression of the cell cycle *WAF1* gene were found in mouse astrocytes in response to BCNU (refer to section 5.2.3), the effect of p53 on cell cycle distribution in response to cytotoxic treatment was investigated. Astrocytes were isolated from mice with distinct *p53* genotypes (+/+, +/−, −/−), treated with either 20 μg/ml BCNU or 2 Gy radiation, and the effect on cell cycle distribution was determined by flow cytometry (refer to sections 2.3.3 and 2.8 for details). A *p53* gene-dose-dependent cell cycle pattern was observed in non-treated, control astrocytes; heterozygous (+/−) astrocytes consistently displayed a response intermediate to wild type (+/+) and knockout (−/−) astrocytes. This pattern remained relatively constant following treatment for both BCNU-treated and irradiated astrocytes (Figure 5.10). This apparent *p53* gene-dose-dependent effect likely reflects differences in growth rates of the cells; +/+, +/− and −/− astrocytes have doubling times of 60-65, 50 and 40 hours respectively (refer to section 2.4). The cell cycle response to treatment with BCNU was not, however, the same as that observed in response to radiation (Figures 5.11 and 5.12).

Whereas the induction of p21 often is consistent with a cell cycle arrest in G_1 (Di Leonardo *et al.*, 1994; Chen *et al.*, 1995), a G_1 arrest was not observed in +/+ astrocytes in response to BCNU (Figure 5.11A); instead, the percentage of cells in G_1 decreased and a G_2 arrest was evident 48 hours post BCNU treatment. Knockout astrocytes exhibited a similar G_2 arrest pattern (Figure 5.11B). These findings suggest that the cell cycle modification that occurred in astrocytes following treatment with

Figure 5.8 **Densitometry analysis of changes in *mdm-2* mRNA expression in response to BCNU treatment.** A slight induction in *mdm-2* mRNA expression was seen in response to BCNU in all four cell types. Quantified signal intensities from Figures 5.4 to 5.7 were normalized to *MGAP* as described in section 2.7.6 and the lowest control value for each individual cell type (panels A through D) was arbitrarily set to a value of 1.0. **Comparative results are given for wild type (A) and knockout (B) astrocytes and wild type (C) and knockout (D) fibroblasts.** Lanes are identified as: 1, control T=0; 2, BCNU T=0; 3, BCNU T=4; 4, BCNU T=8; 5, BCNU T=12; 6, BCNU T=24; 7, control T=24; T represents time in hours.

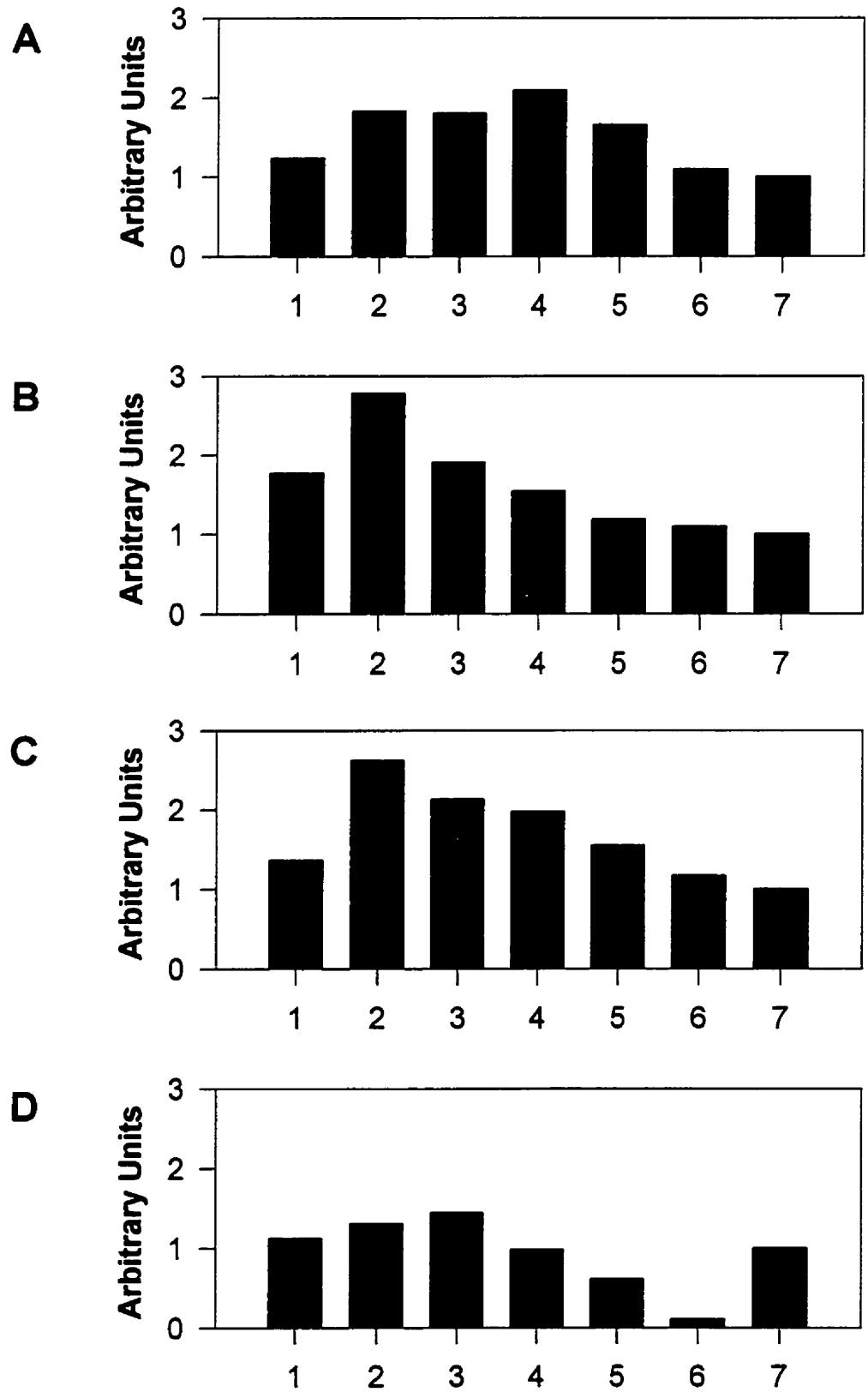

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Figure 5.9 **Densitometry analysis of changes in *WAF1* mRNA expression in response to BCNU treatment.** A slight induction in *WAF1* mRNA expression was seen in response to BCNU in all four cell types. Quantified signal intensities from Figures 5.5 to 5.8 were normalized to *MGAP* as described in section 2.7.6 and the lowest control value for each individual cell type (panels A through D) was arbitrarily set to a value of 1.0. **Comparative results are given for wild type (A) and knockout (B) astrocytes and wild type (C) and knockout (D) fibroblasts.** Lanes are identified as: 1, control T=0; 2, BCNU T=0; 3, BCNU T=4; 4, BCNU T=8; 5, BCNU T=12; 6, BCNU T=24; 7, control T=24; T represents time in hours.

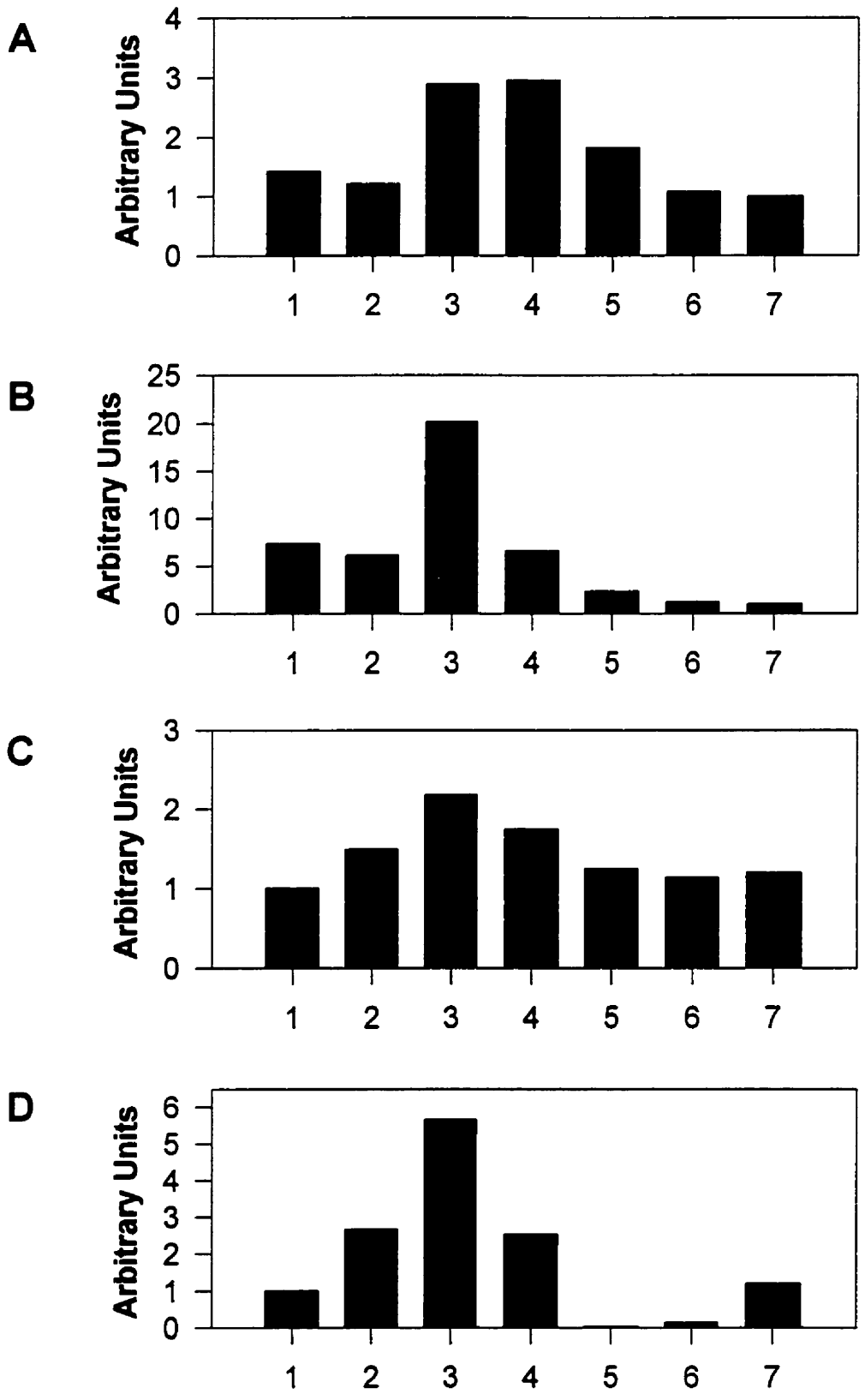
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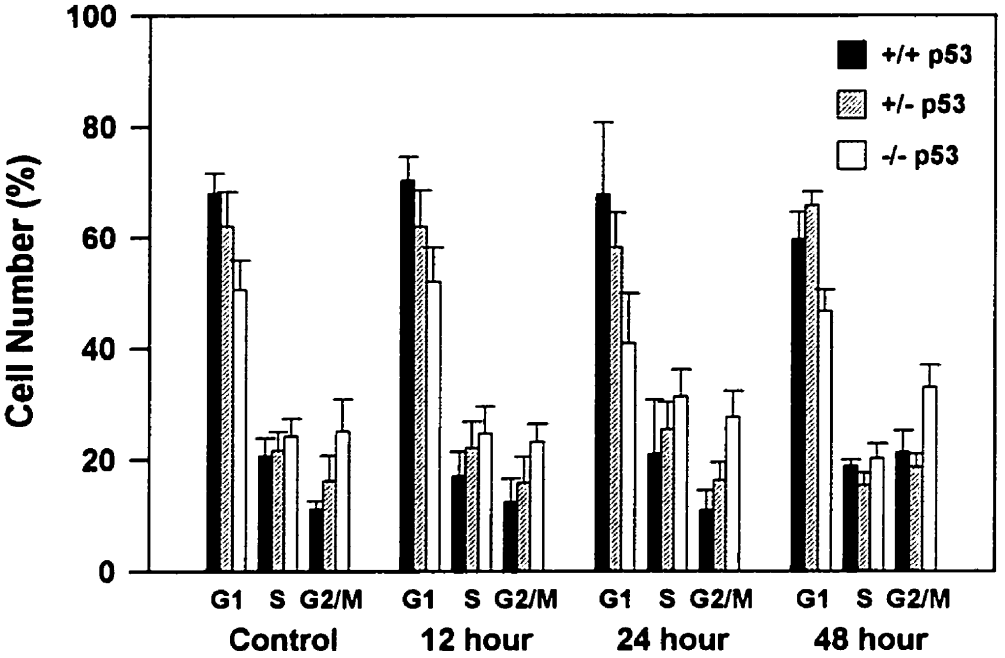
Figure 5.10 Summary of cell cycle distribution of mouse astrocytes treated with 20 μg/ml BCNU for 2 hrs (A) or 2 Gy radiation (B). A *p53* gene-dose-dependent cell cycle pattern was observed in non-treated, control astrocytes; heterozygous (+/−) astrocytes consistently displayed a response intermediate to wild type (+/+) and knockout (−/−) astrocytes. This pattern remained relatively constant following treatment for both BCNU-treated and irradiated astrocytes. Bars depict the mean of at least four independent experiments (± SEM). Detailed statistical examination of the cell cycle responses of +/+ and −/− astrocytes are depicted in Figures 6.2 and 6.3.

A


100
80
60
40
20
0
Cell Number (%)
+/+ p53
+/- p53
-/- p53
G1 S G2/M G1 S G2/M G1 S G2/M G1 S G2/M
Control
12 hour
24 hour
48 hour

B

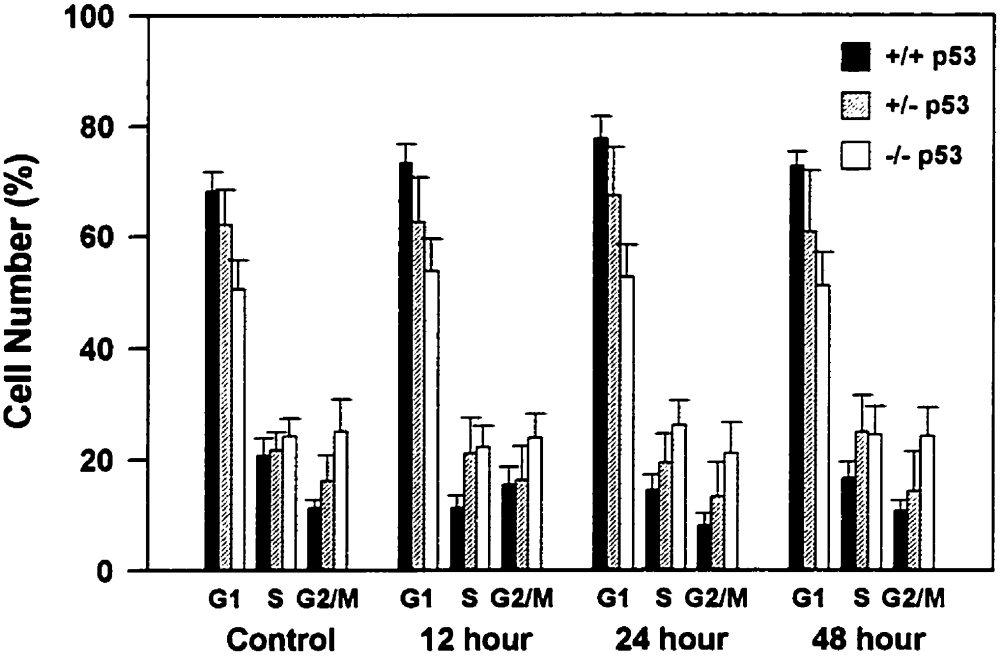
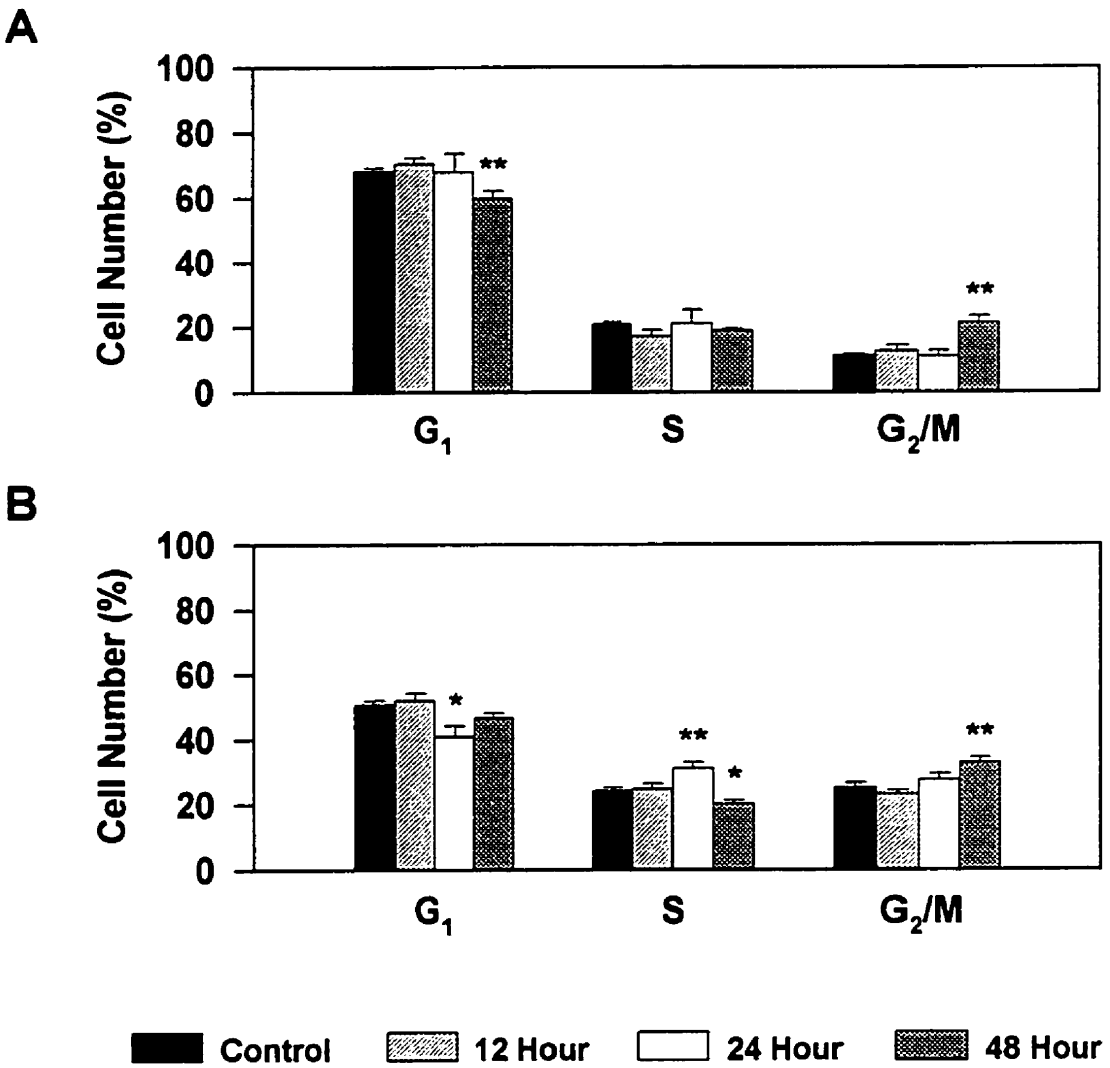

100
80
60
40
20
0
Cell Number (%)
+/+ p53
+/- p53
-/- p53
G1 S G2/M G1 S G2/M G1 S G2/M G1 S G2/M
Control
12 hour
24 hour
48 hour

Figure 5.11 Cell cycle distribution of mouse astrocytes treated with 20 μg/ml BCNU for 2 hrs. Homozygous wild type *p53* astrocytes showed a decrease in G_1 and an increase in G_2 at 48 hours (A); a similar trend was observed for homozygous knockout astrocytes (B). Bars depict the mean of at least four independent experiments ($\pm$ SEM) and asterisks significant change from non-treated astrocytes (*, $P < 0.05$; **, $P < 0.005$).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Cell Number (%)
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G1
S
G2/M
B
Cell Number (%)
100
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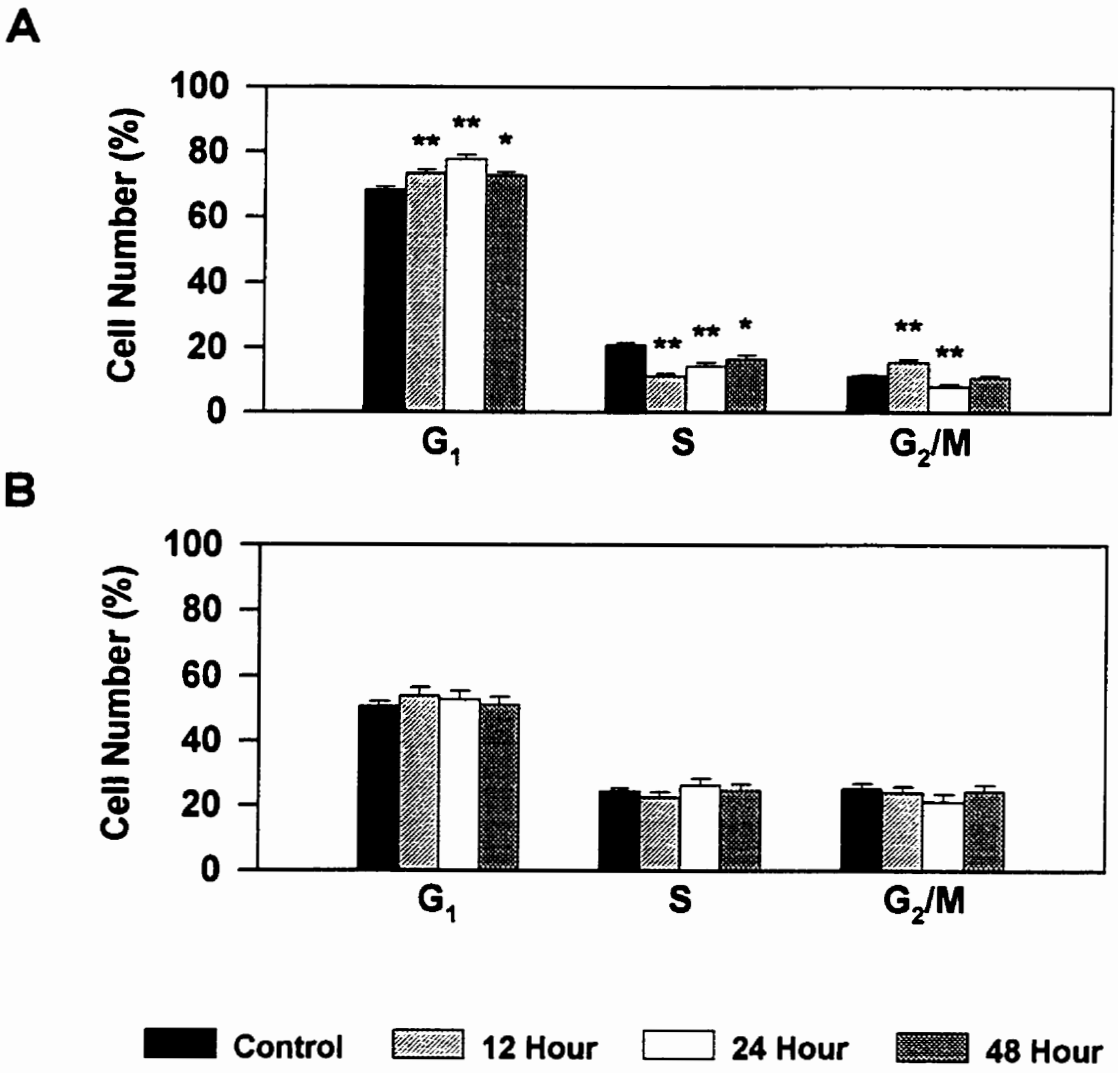
40
20
0
*
**
*
**
G1
S
G2/M
Control
12 Hour
24 Hour
48 Hour

Figure 5.12 Cell cycle distribution of mouse astrocytes treated with 2 Gy radiation. Irradiated wild type *p53* astrocytes accumulated in G_1; maximum G_1 accumulation occurred at 24 hours (A). The percentage of cells in S phase was significantly lower than non-treated astrocytes at all three time points, with the lowest value at 12 hours. There were no statistically significant changes in the cell cycle distribution of knockout astrocytes in response to radiation (B). Bars depict the mean of at least four independent experiments (± SEM) and asterisks significant change from non-treated astrocytes (*, $P < 0.05$; **, $P < 0.005$).


A
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Cell Number (%)
100
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*
**
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*
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**
G1
S
G2/M
Control
12 Hour
24 Hour
48 Hour

BCNU was a p53-independent response. The G_1 decrease appeared at 48 hours in +/+ astrocytes compared to 24 hours in −/− cells and a ripple effect was observed in the percentage of −/− astrocytes in S phase. The earlier G_1 decrease and the detection of S phase changes in −/− cells might be explained by the faster rate of growth of −/− as compared to +/+ astrocytes.

To test whether this *p53* genotype/cell cycle pattern seen in astrocytes was specific to BCNU, I studied the effect of p53 on cell cycle changes in irradiated astrocytes. In contrast to BCNU, +/+ astrocytes exhibited a G_1 arrest in response to radiation which peaked 24 hours post-treatment; the percentage of cells in G_1 was returning to control levels 48 hours post-radiation (Figure 5.12A). At 12 hours, G_1 arrest was accompanied by a decrease in the percentage of astrocytes in S phase and a transient G_2 arrest. There were no cell cycle changes seen in −/− astrocytes in response to radiation (Figure 5.12B), implying that those observed in +/+ astrocytes were dependent on a functional p53. In summary, BCNU and radiation had dissimilar effects on the cell cycle of astrocytes; while the response to BCNU was comparable in both +/+ and −/− cells and therefore p53-independent, the response to radiation was clearly p53-dependent.

5.3 DISCUSSION

The p53 protein plays a key role in protecting cells and organisms from the deleterious effects of DNA damage. The functional significance of this role is especially important in the resistance of human cancers to DNA-damaging treatments since the *p53* gene is now considered the most frequently mutated gene in many common malignancies, including glial tumors. The effect of p53 status on the response of astrocytomas to chemotherapy has not been studied. Malignant glial tumors and cell lines often harbor multiple genetic defects (refer to section 1.2.5), thus impeding the examination of the effects of an isolated gene or protein. In order to examine the specific effects of p53 on drug resistance, I chose to utilize cells isolated from TSG-*p53* mice (refer to section 2.3.1); this experimental system allowed me to examine the consequences of *p53* gene dosage on drug resistance on an otherwise normal cellular background.

First, the response of normal and p53 deficient mouse astrocytes to the alkylating

agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a clinically useful DNA-damaging drug to which some human astrocytomas are resistant and some are relatively sensitive, was determined. Astrocyte cultures were isolated from the cerebrums of +/+, +/−, and −/− *p53* neonatal mice and treated with increasing concentrations of BCNU. Wild type *p53* astrocytes were significantly more resistant to BCNU than −/− *p53* astrocytes, with +/− astrocytes exhibiting an intermediate level of resistance. Hence, wild type p53 appeared to protect mouse astrocytes from the cytotoxic effects of BCNU in a gene-dose-dependent fashion. In addition, these results suggested that +/+ astrocytes were not undergoing p53-dependent apoptosis; one would expect +/+ astrocytes to be more sensitive to BCNU than −/− cells if they were preferentially undergoing programmed cell death.

The effect of p53 on the response of cells to toxic stimuli may be cell-specific or insult-specific (Lowe *et al.*, 1993a; O'Connor *et al.*, 1993; Biard *et al.*, 1994; McIlwrath *et al.*, 1994; Petty *et al.*, 1994; Fan *et al.*, 1995; Nabeya *et al.*, 1995; Vikhanskaya *et al.*, 1995) and sometimes there is no significant relationship between p53 status and treatment resistance (Brachman *et al.*, 1993; Slichenmyer *et al.*, 1993; Petty *et al.*, 1994; Fan *et al.*, 1995; Vikhanskaya *et al.*, 1995). Therefore, to test whether the *p53* genotype/BCNU response pattern was BCNU-specific or astrocyte-specific, the effect of p53 on the viability of astrocytes treated with MNU and radiation, and that of fibroblasts treated with BCNU and radiation was determined. Mouse astrocytes appeared to have a unique response to BCNU; wild type p53 rendered astrocytes resistant to this drug while in no other situation tested were +/+ cells more resistant than −/− cells.

Interestingly, astrocytes and fibroblasts displayed greater than anticipated resistance to radiation and small differential responses between +/+ and −/− cells. These results may reflect the inability of the MTT assay to distinguish between healthy, dividing cells and viable, non-dividing cells; the utilization of primary cell cultures necessitated use of the non-clonogenic MTT assay.

In light of its effects on the chemosensitivity of mouse astrocytes, might the status of the *p53* gene in human astrocytomas influence tumor response to BCNU, the standard

drug therapy for this tumor type? Mutations of p53 are an early molecular event in the genesis of many astrocytomas (Chung *et al.*, 1991; Frankel *et al.*, 1992; Louis *et al.*, 1993; Van Meir *et al.*, 1994) but are less commonly observed in the tumors of older patients than in the tumors of younger patients (Chung *et al.*, 1991; Louis *et al.*, 1993). Older patients with high grade astrocytomas, often with normal p53, are reported to be less likely to benefit from treatment with BCNU than younger patients with histologically similar tumors (Chang *et al.*, 1983; Nelson *et al.*, 1988; Grant *et al.*, 1995), often harboring p53 mutations. In addition, Russell *et al* (1995) studied glioma cell lines and xenografts and found that those containing normal p53 tended to be more resistant to procarbazine, another clinically useful alkylating agent, than glioma cell lines and xenografts with mutated p53. Their results in astrocytic glioma cell lines, coupled with my findings in primary cultures of mouse astrocytes, suggest that p53 status may influence the response of human cancers to chemotherapy in a cell- and drug-specific manner.

In order to investigate whether BCNU treatment could influence the expression of p53-regulated genes that may play a role in the cell cycle response to DNA damage, the mRNA expression of *mdm-2*, *WAF1*, and *GADD45* following treatment with BCNU was examined. There was evidence of a slight induction in *mdm-2* and a 2- to 5-fold induction in *WAF1* expression in response to BCNU but expression of *GADD45* remained constant over the 24 hr time period. Increased *mdm-2* expression could be detected in the first treated samples that were harvested immediately following the two hour BCNU incubation. Since mdm-2 acts in an autoregulatory loop with p53, these results provide evidence for a very tightly regulated p53 response. The induction of *WAF1* also was rapid in fibroblasts; increased levels could be found in the first samples following BCNU incubation. In comparison, astrocytes demonstrated a slightly delayed induction of *WAF1*; increased expression was observed by four hours following treatment and had returned to normal levels by 24 hours. There was no change in the expression of *p53* over the course of treatment as expected; any evidence of p53 induction in response to DNA damage has been shown to occur at the level of protein stabilization (refer to sections

1.7.3 and 1.7.4.1).

The relative delayed induction of *WAF1* in astrocytes, combined with the almost immediate induction of *mdm-2* suggested that a role for a G_1 arrest in response to BCNU in astrocytes may not be crucial. For this reason, the effect of p53 on the cell cycle of mouse astrocytes in response to DNA damaging treatments (ie. BCNU and radiation) was examined. Although astrocytes demonstrated the characteristic G_1 arrest in response to radiation, a G_2 arrest was observed in response to BCNU. G_2 arrest has been noted previously in various cell types in response to the nitrosoureas (Tobey, 1975; Tobey and Crissman, 1975), including BCNU (Barranco and Humphrey, 1971; Tobey, 1975; Tobey and Crissman, 1975; Nomura *et al.*, 1978; Hoshino *et al.*, 1981), and in response to some other alkylating agents (Tobey, 1975). Nomura *et al* (1978) demonstrated that BCNU-treated 9L rat glioma cells arrested in G_2 by 48 hours; G_2 arrest persisted for 24 hours and then returned to control levels. Transient delays in the G_1 and S phases lasting less than four hours in total also have been observed (Barranco and Humphrey, 1971; Tobey and Crissman, 1975). The design of my experiments would not have detected minor delays of this type. Lastly, leftward "skewing" of the G_2/M peak has been noted previously (Tobey and Crissman, 1975) and also was seen in my flow cytometry studies (data not shown); this finding has been interpreted by Tobey and Crissman (1975) as a late S/G_2 accumulation, not exclusively a G_2 arrest. In summary, the cell cycle changes found in BCNU-treated astrocytes appeared to be characteristic of the nitrosoureas (Tobey, 1975).

BCNU and radiation had dissimilar effects on the cell cycle of astrocytes; while the response to BCNU was comparable in both +/+ and −/− *p53* cells, the response to radiation was p53-dependent. Mouse astrocytes were clearly able to undergo cell cycle arrest in response to BCNU but this response was not p53-dependent. Therefore, the differential resistance to BCNU of +/+, +/−, and −/− astrocytes could not be explained by a p53-dependent cell cycle arrest, G_1 or otherwise.

Wild type p53 rendered mouse astrocytes resistant to BCNU in a gene-dose-dependent fashion yet resistance could not be explained by either p53-dependent apoptosis

or differential cell cycle arrest. How then does p53 render +/+ astrocytes resistant to BCNU? Perhaps the slower rate of proliferation of +/+ as compared to −/− astrocytes contributes to drug resistance; slower cell cycling in the presence of p53 would allow more time for repair of DNA damage and as a consequence enhance genomic stability in +/+ astrocytes. If this were true, one might hypothesize that −/− astrocytes would become genetically unstable in response to BCNU. This hypothesis is supported by the findings of Tobey and Crissman (1975) that demonstrated DNA polyploidy in cells treated with BCNU. Since p53-independent apoptotic pathways have been documented (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Selvakumaran *et al.*, 1994), one might reason further that damage in these unstable cells can be detected by an alternative DNA damage-sensing mechanism and these cells may be triggered subsequently to undergo p53-independent programmed cell death.

CHAPTER 6

THE EFFECT OF p53 STATUS ON THE APOPTOTIC RESPONSE OF MOUSE ASTROCYTES EXPOSED TO BCNU

6.1 INTRODUCTION

The p53 protein plays a role in protecting cells and organisms from the deleterious effects of DNA damage by regulating cell cycle progression and by triggering programmed cell death (refer to sections 1.7.4.2, 1.7.4.3, and Chapter 5). In Chapter 5, wild type *p53* was demonstrated to render mouse astrocytes resistant to BCNU in a gene-dose-dependent fashion. These results suggested that +/+ astrocytes were not displaying a significant amount of p53-dependent apoptosis; one would expect +/+ astrocytes to be more sensitive to BCNU than −/− cells if they were preferentially undergoing programmed cell death. In addition, using flow cytometry experiments to examine cell cycle response, it was found that both +/+ and −/− mouse astrocytes were clearly able to affect cell cycle arrest in response to BCNU and therefore, this response was not p53-dependent. Thus, it seemed that the resistance displayed by +/+ astrocytes in response to BCNU could not be explained by either p53-dependent apoptosis or differential cell cycle arrest. As a result, I hypothesized that perhaps the slower rate of proliferation of +/+ as compared to −/− astrocytes contributed to drug resistance; slower cell cycling in the presence of p53 would allow more time for repair of DNA damage and as a consequence enhance genomic stability in +/+ astrocytes. If this were true, I reasoned further that damage in these unstable −/− cells may be detected by an alternative DNA damage-sensing mechanism and these cells may be triggered subsequently to undergo a p53-independent programmed cell death. It was my intent, in the studies summarized in this chapter, to investigate the possibility of increased programmed cell death in −/− astrocytes in response to BCNU.

The p53 protein has been shown to influence the expression of two members of the bcl-2 family involved in the regulation of apoptosis (refer to section 1.7.4.3); the

transcription of *bax* has been shown to be induced by p53 (Miyashita and Reed, 1995) and inhibition of *bcl-2* expression has been documented (Miyashita *et al.*, 1994). Mechanisms of p53-independent apoptosis do, however, exist (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Selvakumaran *et al.*, 1994) as well as a number of additional members of the bcl-2 apoptotic family (Craig, 1995). One member of this family that has received much attention is bcl-x. Two alternatively spliced forms of *bcl-x* exist; the long form, bcl-x_L, enhances viability whereas the short form, bcl-x_S, acts in a dominant negative fashion to inhibit the effects of bcl-x_L (Boise *et al.*, 1993; Craig, 1995).

The purposes of this study were 1) to examine the effect of p53 status on the ability of mouse astrocytes to undergo apoptosis when exposed to BCNU; 2) to perform a semi-quantitative analysis of apoptosis in mouse cells in response to BCNU and correlate with p53 status; and 3) to explore the effect of BCNU treatment on the expression of two p53-regulated genes (*bax* and *bcl-2*) and one additional cell death gene (*bcl-x*) that may play a role in the apoptotic response to DNA damage.

6.2 RESULTS

6.2.1 Percent BAD values in mouse astrocytes after BCNU treatment or radiation.

In order to investigate the effect of p53 status on the ability of mouse astrocytes to undergo apoptosis when exposed to BCNU, I began by examining in more detail the % BAD values taken from the original flow cytometry experiments performed in Chapter 5 (refer to section 5.2.4). Percent BAD is a flow cytometry measure of *b*ackground *a*ggregates and *d*ebris (BAD) calculated by the Multicycle software program (refer to section 2.8); the % BAD calculation also takes into consideration the presence of aneuploid and apoptotic cells. In this analysis, % BAD values were significantly higher in −/− control, non-treated astrocytes than +/+ control astrocytes; −/− control astrocytes displayed a slightly greater than 4-fold increase over +/+ cells (Table 6.1). In response to treatment with BCNU or radiation, % BAD values increased significantly over control values for BCNU treated −/− astrocytes only; BCNU treated −/− astrocytes displayed almost twice the % BAD value of that for non-treated −/− cells

Table 6.1 Percent BAD[a] in Astrocyte Flow Cytometry Analysis

	p53 Genotype		
Treatment	(+/+)	(+/−)	(−/−)
Control	4.52 ± 0.86	8.92 ± 1.11[b]	19.3 ± 2.50[b]
BCNU	9.10 ± 4.62	7.13 ± 1.71	34.6 ± 3.48[c]
Radiation	7.56 ± 2.21	9.45 ± 0.83	15.1 ± 3.24

Percent BAD values are reported for cells 48 hours post-treatment. Values were calculated using the Multicycle software. Shown is the mean ± SEM of at least four independent experiments. Statistically significant differences from non-treated control values are noted.

[a] Percent *b*ackground *a*ggregates and *d*ebris

[b] $P < 0.005$ in comparison with +/+ control

[c] $P < 0.005$ in comparison with −/− control

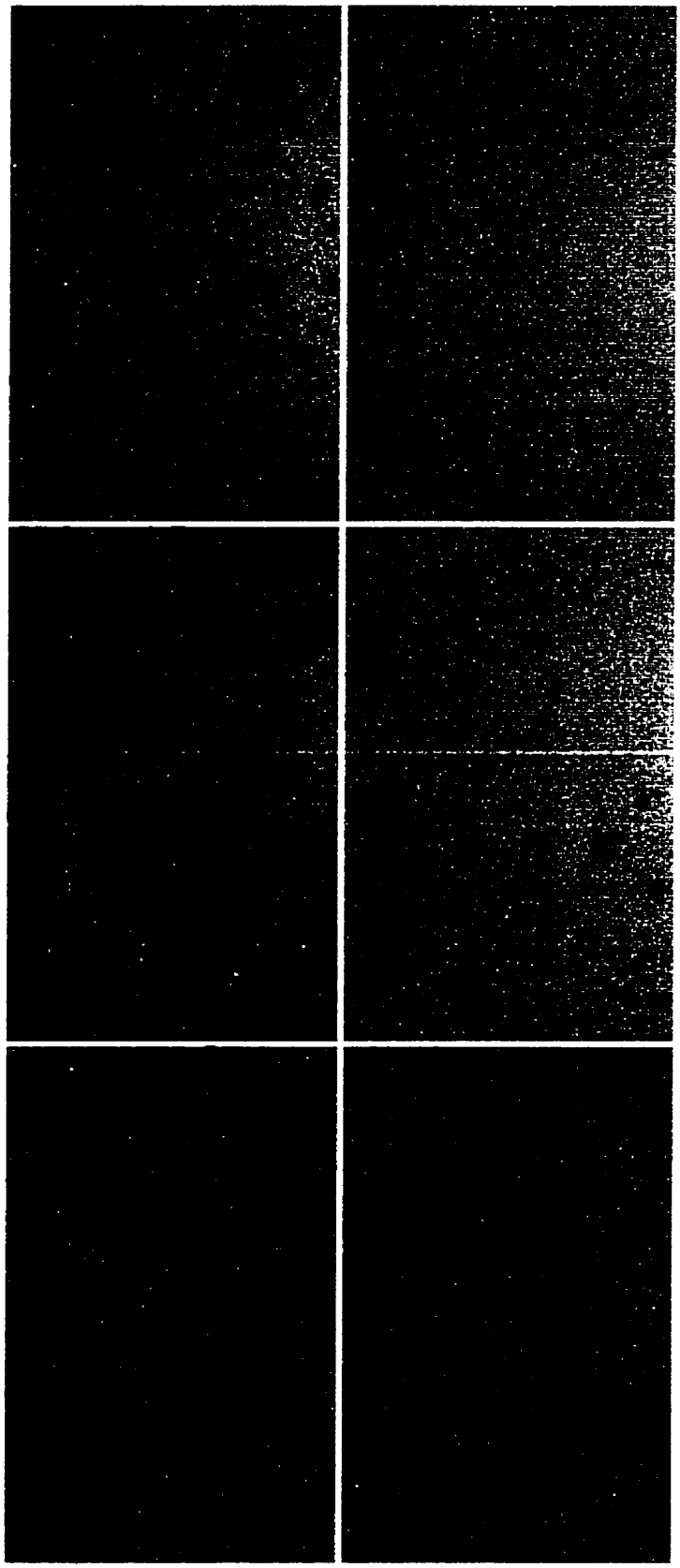
(Table 6.1).

6.2.3 Detection of apoptosis in mouse astrocytes after BCNU treatment.

Since there appeared to be evidence for programmed cell death in −/− astrocytes, this finding was investigated further by detecting immunohistochemically the presence of apoptotic astrocytes following treatment with BCNU. Astrocytes were seeded onto coverslips, treated with BCNU, and assayed at various times after treatment for the presence of apoptotic cells with the ApopTag™ *In situ* Apoptosis Detection Kit (refer to section 2.9.1 for details). Positive staining for apoptotic cells was found for all three *p53* genotypes (Figure 6.1). In addition, it appeared that −/− astrocytes may be more susceptible to apoptosis than +/+ astrocytes; −/− cells tended to display more positive staining than +/+ cells at equal BCNU doses and positive staining was evident in −/− astrocytes at lower BCNU concentrations than +/+ cells (data not shown).

As evidenced in Figure 6.1, most of the cells that had undergone apoptosis seemed to lift off their substrate in culture and therefore, were not represented in the final immunohistochemical staining. Although it appeared that −/− astrocytes may be more susceptible to apoptosis in response to BCNU than +/+ cells, the ApopTag™ Kit was unsuitable for true quantitation of apoptotic cells; it was possible that damaged, but not apoptotic, cells were being preferentially washed off the coverslips during staining. In an attempt to achieve a more quantitative determination of the amount of apoptosis occurring in response to cytotoxic treatment, the commercially available Cell Death Detection ELISA kit was used (refer to section 2.9.2); this colorimetric assay measured apoptosis within a cell culture well and therefore, did not require cells to remain adherent to their substrate after treatment. Wild type and −/− astrocytes were exposed to either 20 μg/ml BCNU or 2 Gy radiation and fibroblasts (+/+ and −/−) to 20 μg/ml BCNU; a semi-quantitative analysis was then performed using the Cell Death Detection ELISA kit in order to determine the amount of apoptosis induced by each treatment (refer to section 2.9.2). Astrocytes exposed to BCNU showed a marked increase in the presence of apoptotic cells in comparison to non-treated controls; enrichment factors of approximately 2 to 7 were demonstrated over the three time periods sampled for the two

Figure 6.1 **Detection of apoptotic mouse astrocytes after treatment with BCNU.** The presence of apoptotic cells (brown DAB staining) in response to BCNU was detected using the ApopTag™ *In situ* Apoptosis Detection Kit as described in section 2.9.1. Non-treated cells counterstained with 0.5% methyl green in 0.1M sodium acetate (pH 4.0) are shown in the left panels and cells treated with 160 μg/ml BCNU are shown in the right panels after a four day recovery. Apoptosis was detected in cells of all three *p53* genotypes: **wild type (A); heterozygous (B); and knockout (C).** Bar, 50 μm.



cell genotypes (Figure 6.2). Little, if any, enrichment of apoptosis was evident in astrocytes exposed to radiation and fibroblasts exposed to BCNU. Of particular interest, $-/-$ astrocytes exhibited greater amounts of apoptosis in response to BCNU than did $+/+$ astrocytes. These findings of increased programmed cell death in $-/-$ astrocytes in response to BCNU confirmed what had been suggested visually by the ApopTag™ data.

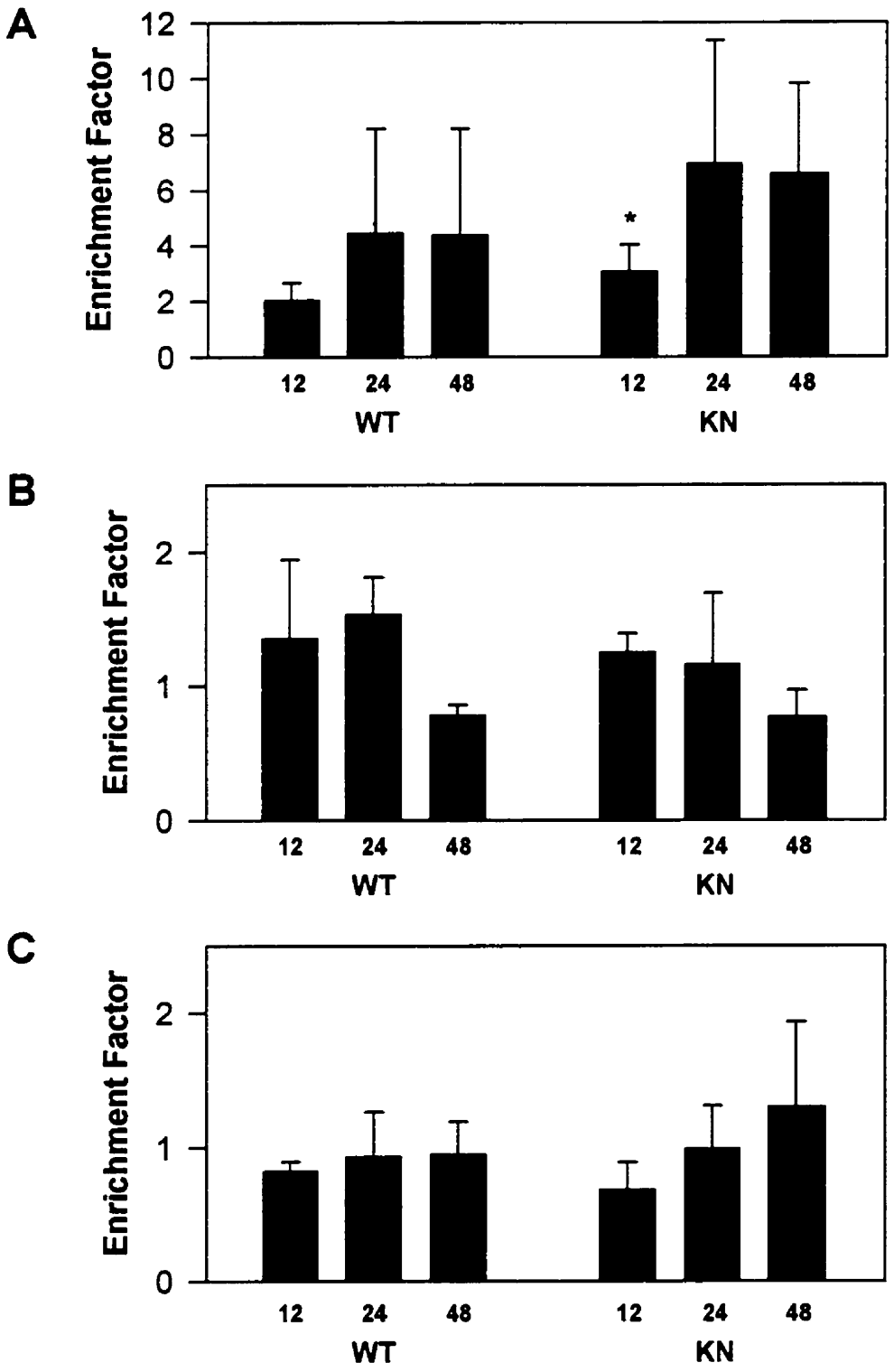
6.2.4 Effect of BCNU treatment on the expression of *bax*, *bcl-2*, and *bcl-x*.

In order to investigate whether BCNU treatment could influence the expression of two p53-regulated genes (*bax* and *bcl-2*) and one additional cell death gene (*bcl-x*), $+/+$ and $-/-$ astrocytes and fibroblasts were exposed to 40 μg/ml BCNU for 2 hr and samples were taken at defined times for Northern analysis (refer to section 2.7). One Northern blot was probed sequentially for transcripts encoding bax and bcl-x and a second blot was probed for transcripts encoding bcl-2 (Figures 6.3 - 6.6). Two transcripts were detected for each of the three genes, as previously described (Negrini *et al.*, 1987; Oltvai *et al.*, 1993; Frankowski *et al.*, 1995), although the larger *bcl-2* transcript was barely detectable in fibroblasts. Hybridization signals were normalized to the *MGAP* signal from the same lane. Expression of all three genes remained relatively constant over the 24 hr time period in response to BCNU in all four cell types; densitometry was performed (refer to section 2.7.6) and any differences in signal intensities were not greater than 50% and were within the range of variation displayed by the control samples.

6.3 DISCUSSION

Percent BAD is a flow cytometry measure of *b*ackground *a*ggregates and *d*ebris (BAD) calculated by the Multicycle software program (refer to section 2.8); the % BAD calculation also takes into consideration the presence of aneuploid and apoptotic cells. Although generally speaking a quality control measure, % BAD values may be an important indicator of genomic instability or apoptotic cell death. In order to investigate

Figure 6.2 **Semi-quantitative analysis of apoptosis in mouse cells.** An attempt to quantitate the extent of apoptosis occurring in response to cytotoxic treatment was made using the Cell Death Detection ELISA as described in section 2.9.2. Enrichment factors are expressed as multiples of 1.0, where 1.0 represents the amount of apoptosis occurring in non-treated, control cells. Astrocytes displayed an enrichment of apoptotic cells in response to 20 μg/ml BCNU (A). Knockout astrocytes exhibited greater amounts of apoptosis in response to BCNU than did wild type astrocytes (statistically significant at 12 hours; *, $P < 0.05$). Astrocytes treated with 2 Gy radiation (B) and fibroblasts treated with 20 μg/ml BCNU (C) did not display any substantial enrichment over non-treated, control cells. Bars depict the mean of 2 or 3 independent experiments each performed in triplicate ($\pm$SEM). WT, wild type; KN, knockout; 12, 24, 48, time in hours after cytotoxic treatment.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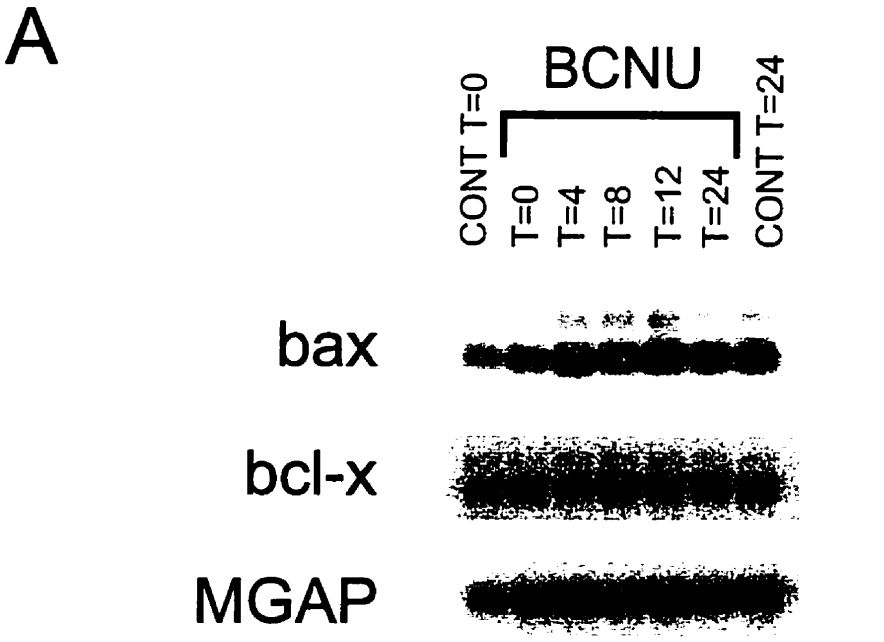
48
WT
KN

Figure 6.3 **Effect of BCNU treatment on the mRNA expression of *bax*, *bcl-x*, and *bcl-2* in wild type *p53* mouse astrocytes.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated mRNA sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Panels A and B represent results obtained from two different Northern blots. Expression of all three genes remained relatively constant over the 24 hr time period in response to BCNU. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.


A
BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bax
bcl-x
MGAP

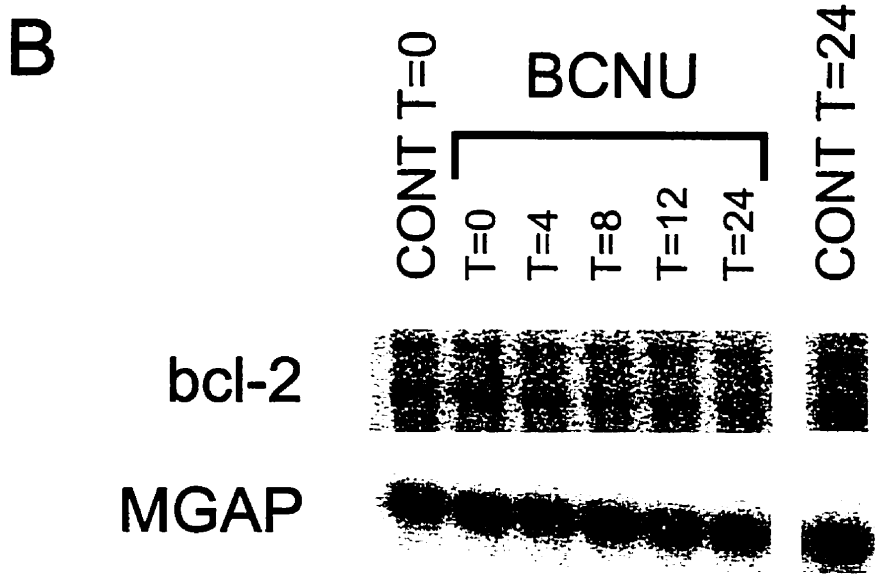
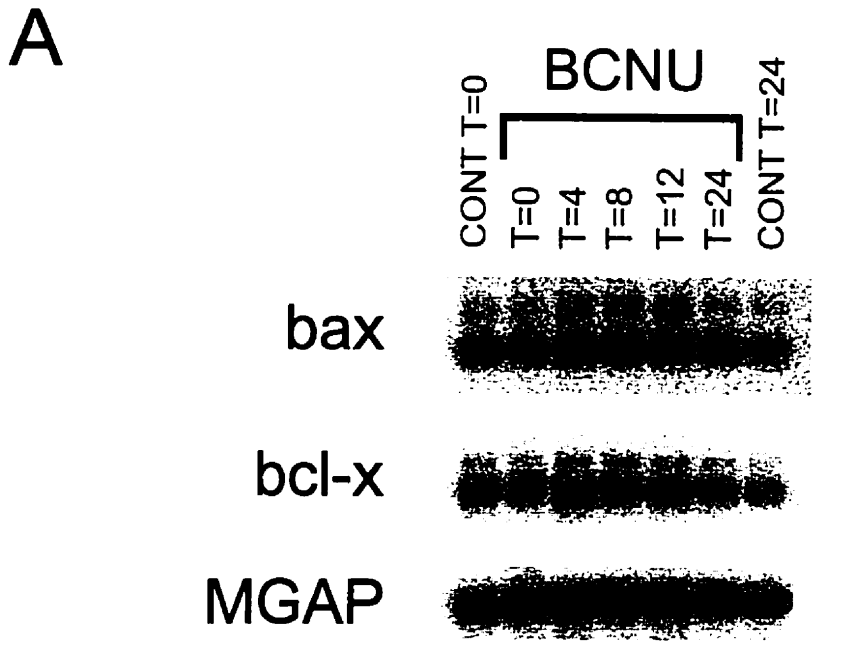

B
BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bcl-2
MGAP

Figure 6.4 **Effect of BCNU treatment on the mRNA expression of *bax*, *bcl-x*, and *bcl-2* in knockout *p53* mouse astrocytes.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated mRNA sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Panels A and B represent results obtained from two different Northern blots. Expression of all three genes remained relatively constant over the 24 hr time period in response to BCNU. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.


A
BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bax
bcl-x
MGAP

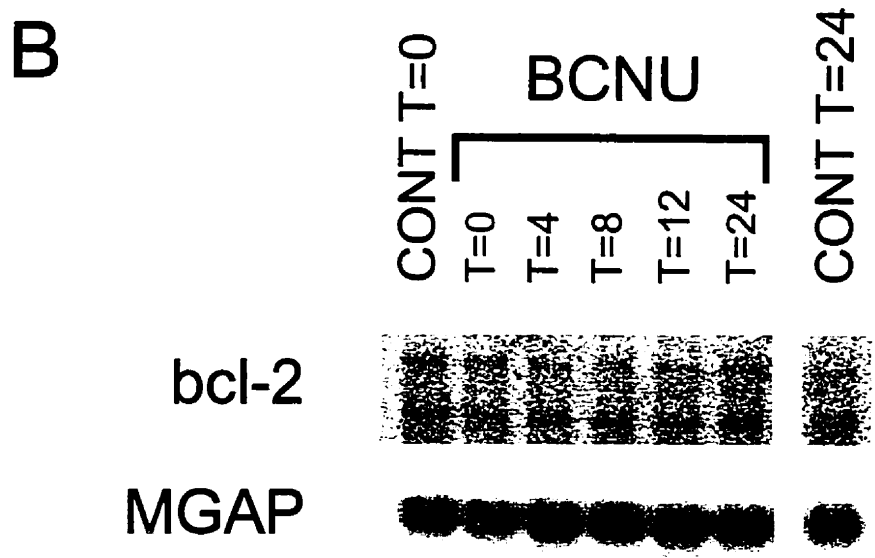
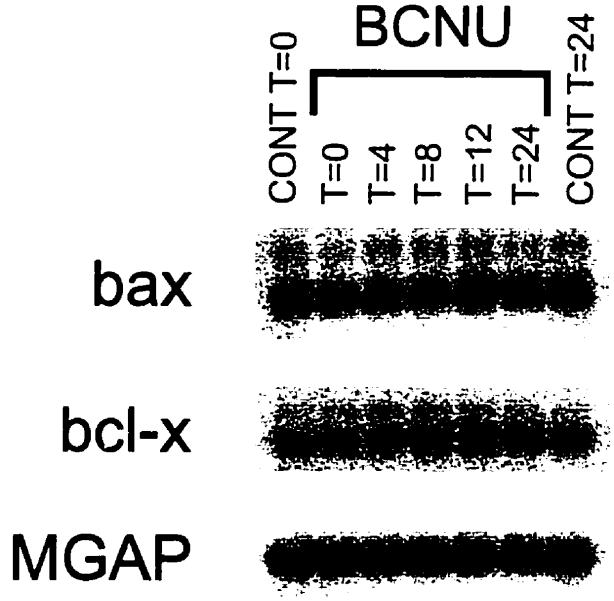

B
BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bcl-2
MGAP

Figure 6.5 **Effect of BCNU treatment on the mRNA expression of *bax*, *bcl-x*, and *bcl-2* in wild type *p53* mouse fibroblasts.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated mRNA sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Panels A and B represent results obtained from two different Northern blots. Expression of all three genes remained relatively constant over the 24 hr time period in response to BCNU. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.

A


BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bax
bcl-x
MGAP

B

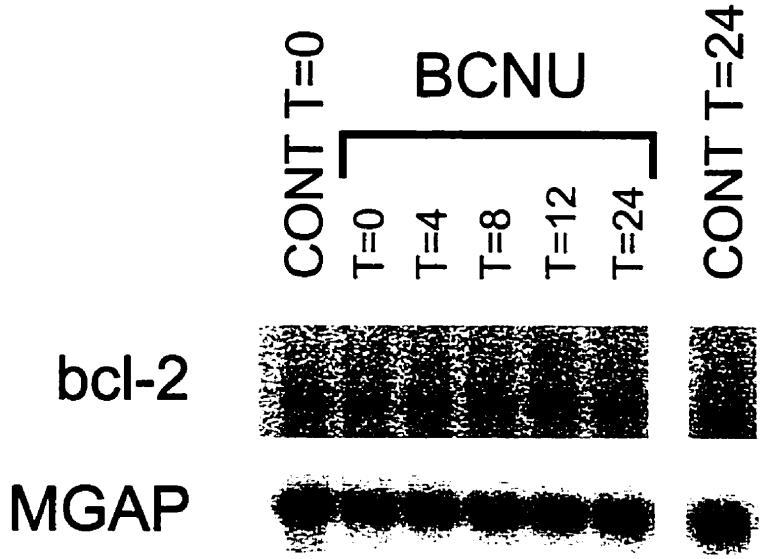
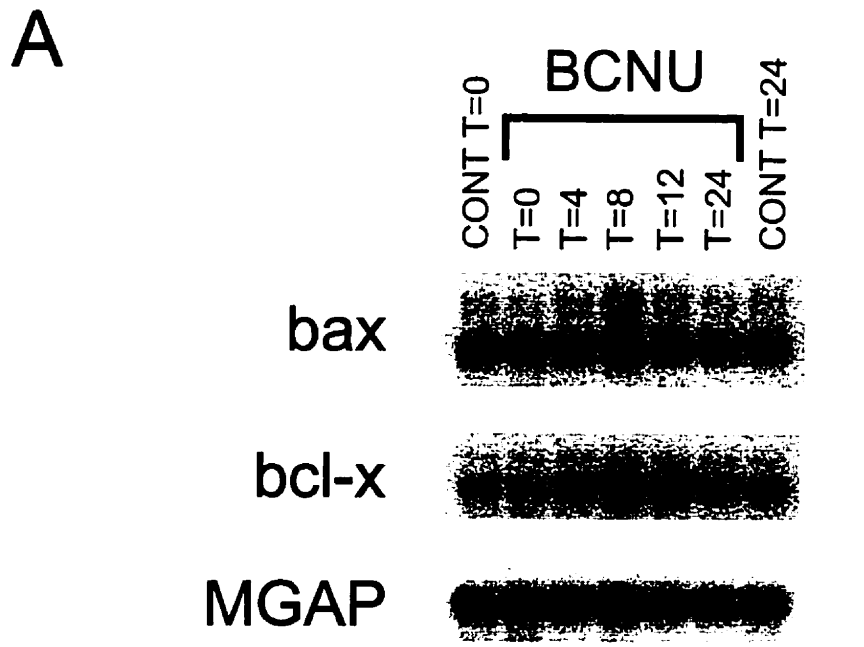
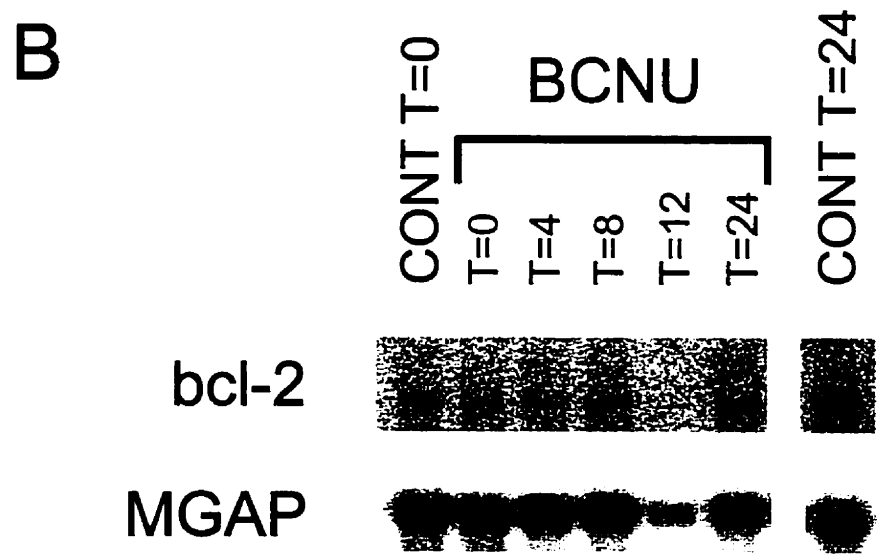

BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bcl-2
MGAP

Figure 6.6 **Effect of BCNU treatment on the mRNA expression of *bax*, *bcl-x*, and *bcl-2* in knockout *p53* mouse fibroblasts.** Cells were treated with 40 μg/ml BCNU for two hours. The first treated mRNA sample (t=0) was harvested at the end of the drug incubation and successive samples were taken at the specified times (in hours). Panels A and B represent results obtained from two different Northern blots. Expression of all three genes remained relatively constant over the 24 hr time period in response to BCNU. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.


A
BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bax
bcl-x
MGAP


B
BCNU
CONT T=0
T=0
T=4
T=8
T=12
T=24
CONT T=24
bcl-2
MGAP

the effect of p53 status on the apoptotic response of mouse astrocytes exposed to BCNU, I examined in more detail the % BAD values taken from the flow cytometry experiments performed in Chapter 5. In this analysis, % BAD values were significantly higher in −/− control astrocytes than +/+ control astrocytes. While this difference in % BAD could be due to enhanced aggregation of −/− cells, spontaneous apoptosis or emergence of aneuploid −/− astrocytes seem more likely explanations since the loss of functional p53 is associated with genomic instability (reviewed in Hartwell, 1992). Of greater interest in these flow cytometry experiments were the % BAD values after BCNU treatment; the % BAD increased significantly for −/− astrocytes only. Polyploidy or cell death induced by BCNU would lead to high % BAD values in astrocytes; Tobey and Crissman (Tobey and Crissman, 1975) observed polyploidy after treatment with BCNU and presented data suggesting that polyploid cells and cells arrested in G_2 were among the first to die after exposure to BCNU.

The possibility of increased programmed cell death in −/− astrocytes in response to BCNU was supported qualitatively by immunohistochemical staining with the ApopTag™ *In Situ* Apoptosis Detection Kit and confirmed quantitatively using the Cell Death Detection ELISA. These results demonstrated that p53 was not required for apoptosis in mouse astrocytes and suggested the possibility of p53-independent apoptosis as a preferred cell death pathway for these cells in response to BCNU. To investigate the role of potential effectors in this pathway, the effect of BCNU treatment on the expression of three genes known to be involved in the apoptotic response to DNA damage was examined. The expression of all three genes, *bax*, *bcl-2*, and *bcl-x*, remained constant over the 24 hr period after treatment with BCNU. Therefore, differential induction of these cell death genes in response to BCNU did not explain the increased survival of +/+ astrocytes. It is interesting to note, however, in one experiment that examined basal levels of *bcl-2* mRNA expression (data not shown), levels of both transcripts were approximately 5-fold higher in +/+ than −/− astrocytes; this difference in basal levels was not seen for *bax* and *bcl-x* (data not shown). This finding is of particular interest in light of the fact that, although +/+ fibroblasts also displayed

a slightly higher level of expression of the smaller *bcl-2* transcript (approximately 3.5-fold), the larger transcript was barely detectable in both +/+ and −/− cells. Perhaps this increased expression of *bcl-2* in non-treated astrocytes is enough to protect +/+ cells from undergoing programmed cell death in response to BCNU and therefore induction of additional bcl-2 is not required; this finding also could explain the presence of greater amounts of apoptosis in non-treated −/− astrocytes. In addition, the bcl-2 family includes many proteins and is continually expanding as new members are isolated (Farrow and Brown, 1996). Whether any of these other proteins play a role in the protection of +/+ astrocytes in response to BCNU remains to be determined.

In summary, wild type p53 was found to render mouse astrocytes resistant to BCNU in a gene-dose-dependent fashion yet this resistance could not be explained by either differential cell cycle arrest or p53-dependent apoptosis. I hypothesized that perhaps the slower rate of proliferation of +/+ as compared to −/− astrocytes contributed to drug resistance; slower cell cycling in the presence of p53 would allow more time for repair of DNA damage and as a consequence enhance genomic stability in +/+ astrocytes. If this were true, I reasoned further that damage in these unstable −/− cells may be detected by an alternative DNA damage-sensing mechanism and these cells may be triggered subsequently to undergo a p53-independent programmed cell death. It has been demonstrated by others that the presence of wild type p53 does, in fact, enhance genomic stability (reviewed in Hartwell, 1992) and the studies presented here confirmed that −/− astrocytes could undergo apoptosis both in the absence of drug and at increased levels in response to BCNU treatment. The actual mechanism for increased apoptosis in −/− mouse astrocytes in response to BCNU remains unclear and awaits further study; increased basal *bcl-2* expression in +/+ astrocytes provides one possible explanation for the differential response to BCNU, as does the potential for additional members of the bcl-2 family to alter the balance of cell survival/death.

CHAPTER 7

THE EFFECT OF p53 STATUS ON O^6-METHYLGUANINE-DNA METHYLTRANSFERASE ACTIVITY IN MOUSE ASTROCYTES

7.1 INTRODUCTION

The p53 protein plays numerous roles in protecting cells and organisms from the deleterious effects of drug-induced DNA damage. As discussed in chapters 5 and 6, p53 may function to inhibit cell cycle progression, presumably allowing time for DNA repair, or induce apoptosis in response to DNA damage. In addition, p53 may regulate directly alternate drug resistance mechanisms such as MDR or DNA repair (Chin *et al.*, 1992; Zastawny *et al.*, 1993; Nguyen *et al.*, 1994; Xiao *et al.*, 1994; Wang *et al.*, 1994; Goldsmith *et al.*, 1995; Strauss and Haas, 1995; Wang *et al.*, 1995; Leveillard *et al.*, 1996; Scherer *et al.*, 1996; Wang and Harris, 1996) (refer to section 1.7.4.4). These many functions of p53 may influence the response of human cancers to DNA-damaging therapies in a very intricate manner.

It is known that a subset of astrocytomas respond to alkylating agents but why some astrocytomas are more sensitive than others is not well understood (Nelson *et al.*, 1988; Fine *et al.*, 1993; DeAngelis *et al.*, 1996). Since chromosome 17p deletions and mutations of the p53 tumor suppressor gene are fairly common in astrocytomas (James *et al.*, 1988; el Azouzi *et al.*, 1989; James *et al.*, 1989; Fults *et al.*, 1989; Nigro *et al.*, 1989; Chung *et al.*, 1991; Hayashi *et al.*, 1991; Mashiyama *et al.*, 1991; Frankel *et al.*, 1992; Fults *et al.*, 1992; Saxena *et al.*, 1992; Sidransky *et al.*, 1992; von Deimling *et al.*, 1992a; Aka *et al.*, 1993; del Arco *et al.*, 1993; Hunter *et al.*, 1993; Louis *et al.*, 1993; Newcomb *et al.*, 1993; Wu *et al.*, 1993; Bello *et al.*, 1994; Koga *et al.*, 1994; Kraus *et al.*, 1994; Louis, 1994; Rasheed *et al.*, 1994), the effect of p53 status on the viability of mouse astrocytes exposed to the clinically useful, bi-functional alkylating agent BCNU was examined. Wild type p53 rendered mouse astrocytes resistant to BCNU

in a gene-dose-dependent fashion, yet resistance could not be explained by either differential cell cycle arrest or p53-dependent apoptosis (refer to chapters 5 and 6; Nutt *et al.*, 1996).

Repair of alkyl adducts at the O^6-guanine position of DNA is mediated by O^6-methylguanine-DNA methyltransferase (MGMT). Nitrosoureas alkylate DNA at this position and the cytotoxic and carcinogenic properties of these drugs appear related to MGMT activity (Swenberg *et al.*, 1982; Ding *et al.*, 1985; Dolan *et al.*, 1985; Dolan *et al.*, 1990b; Kaina *et al.*, 1991; Wani *et al.*, 1992) (refer to section 1.5.2). Russell *et al.* (1995) found that MGMT levels correlated negatively with response to procarbazine, another clinically useful alkylating agent, in primary human brain tumors and glioma cell lines when grown as xenografts. In addition, they found a strong trend toward an increased p53 mutation incidence in tumors with increased growth delay in procarbazine-treated xenografts and no MGMT activity. More recently, there have been studies to suggest that p53 may play a role in the regulation of MGMT activity (Harris *et al.*, 1996; Rafferty *et al.*, 1996) (refer to section 1.7.4.4). Since MGMT-mediated repair has been shown to play a role in resistance to alkylating agents, the effect of p53 status on MGMT activity in mouse astrocytes was examined.

The purposes of this study were 1) to determine the effect of p53 status on MGMT protein and mRNA levels; 2) to investigate whether depletion of MGMT protein with BG could sensitize mouse astrocytes to BCNU; and 3) to explore the possibility of p53-dependent changes in MGMT levels in response to BCNU treatment.

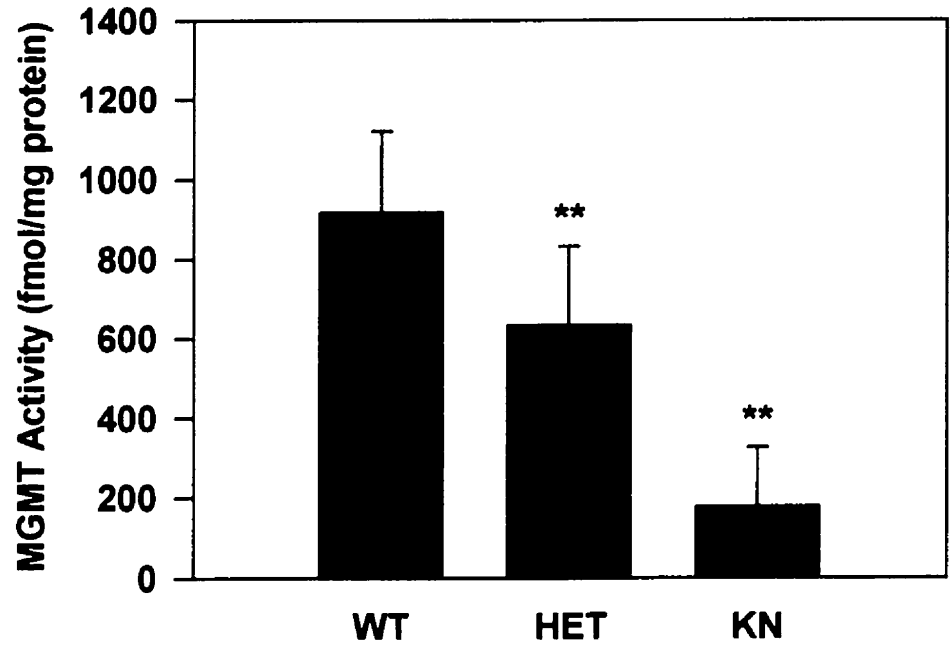
7.2 RESULTS

7.2.1 Effect of p53 status on MGMT protein and mRNA in mouse astrocytes and fibroblasts.

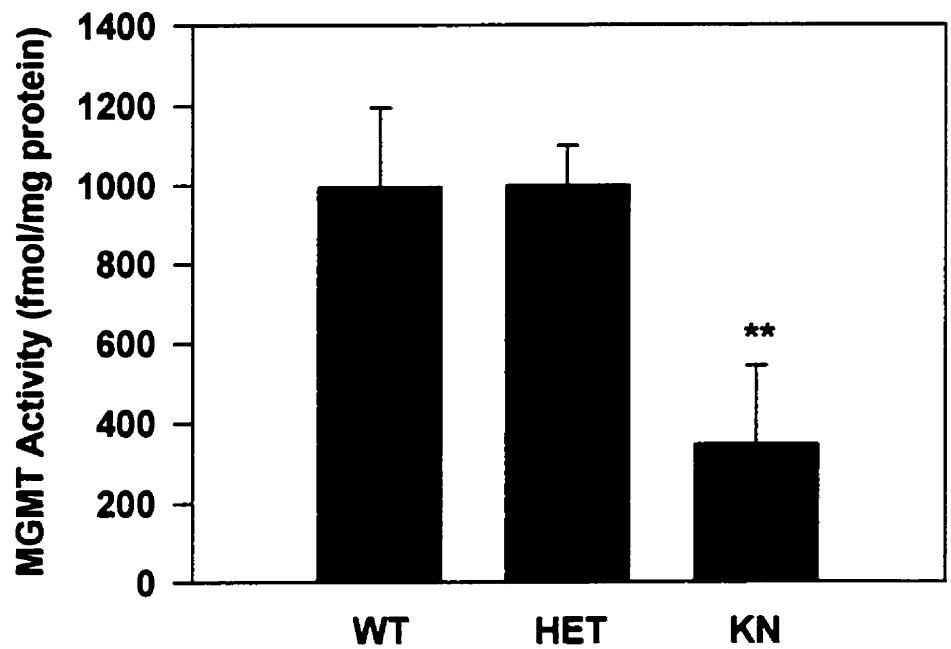
To determine the effect of p53 on MGMT activity, astrocytes and fibroblasts were isolated from mice with distinct *p53* genotypes (+/+, +/−, −/−) and MGMT levels were measured in each cell type (as described in sections 2.3.3 and 2.6 respectively). Homozygous wild type *p53* astrocytes displayed significantly greater MGMT activity than

Figure 7.1 **Effect of p53 on MGMT activity in mouse astrocytes (A) and fibroblasts (B).** Homozygous wild type (+/+) *p53* astrocytes displayed significantly greater MGMT activity than homozygous knockout (−/−) astrocytes while heterozygous (+/−) cells exhibited intermediate activity. There was no significant difference in MGMT activity between +/+ and +/− fibroblasts but both displayed significantly higher activity than −/− fibroblasts. Bars depict the mean of at least eight replicates from a minimum of two independent experiments (±SEM) and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$). WT, wild type; HET, heterozygous; KN, knockout.

A


MGMT Activity (fmol/mg protein)
1400
1200
1000
800
600
400
200
0
**
**
WT
HET
KN

B


MGMT Activity (fmol/mg protein)
1400
1200
1000
800
600
400
200
0
**
WT
HET
KN

−/− astrocytes while +/− cells exhibited intermediate activity (Figure 7.1A). Hence, p53 appeared to enhance MGMT activity in a gene-dose-dependent manner in astrocytes. In comparison, a gene-dose-dependent relationship was not evident in fibroblasts; there was no significant difference in MGMT activity between +/+ and +/− cells (Figure 7.1B). However, +/+ and +/− fibroblasts both displayed significantly greater activity than −/− fibroblasts (Figure 7.1B) suggesting that MGMT activity was enhanced in the presence of p53. Although +/+ levels were comparable in astrocytes and fibroblasts, −/− astrocytes exhibited half the MGMT activity of −/− fibroblasts.

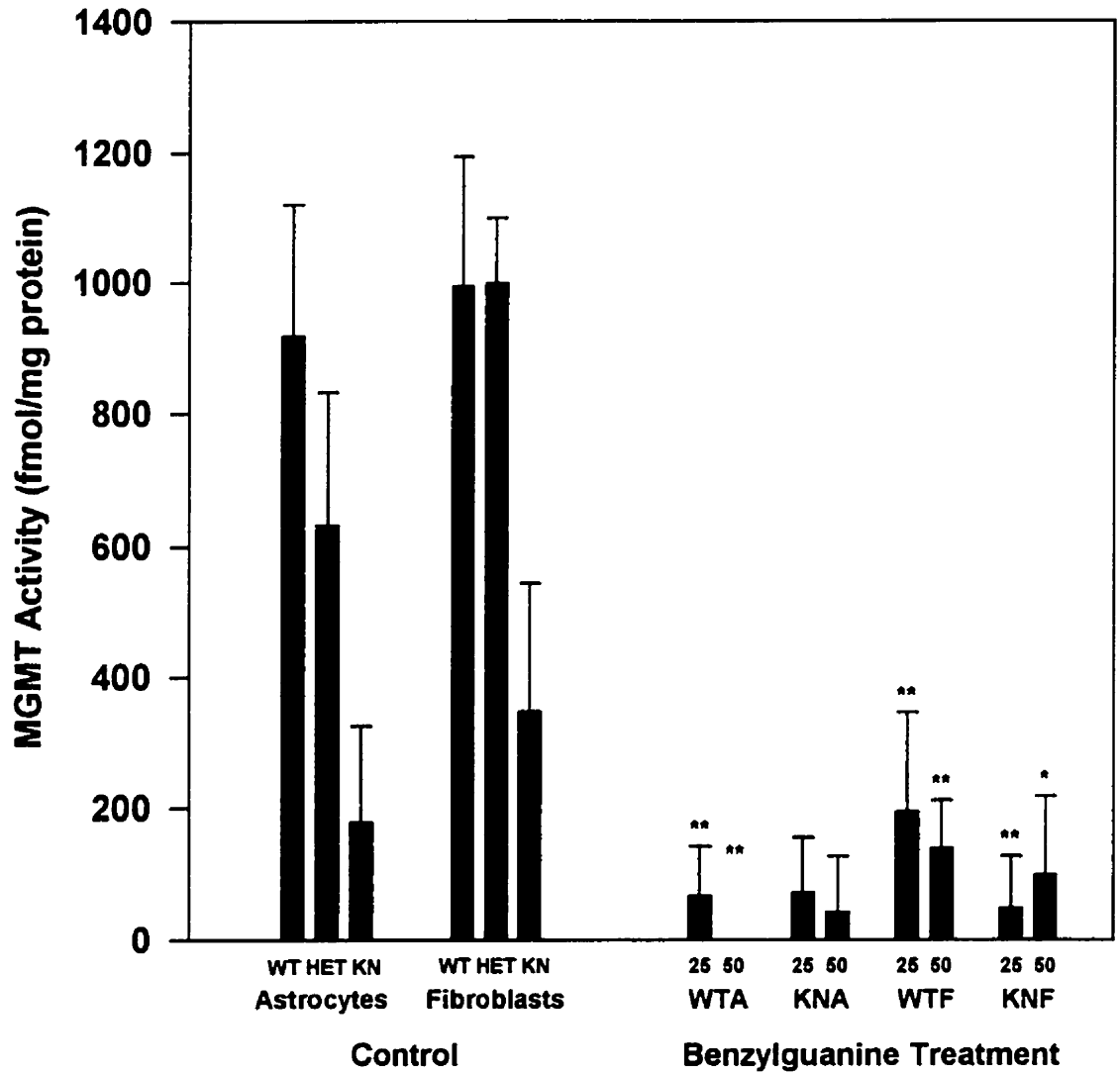
The effect of p53 on MGMT mRNA expression also was examined. Astrocytes and fibroblasts were isolated and mRNA was analyzed as described in section 2.7. Northern blot procedures were performed twice and, consistent with the MGMT activity results, +/+ cells displayed higher levels of MGMT mRNA than −/− cells (data not shown). Knockout fibroblasts also displayed consistently higher MGMT mRNA levels than did −/− astrocytes (data not shown).

7.2.2 Effect of BG pretreatment on the response of mouse astrocytes and fibroblasts to BCNU.

Since MGMT activity correlated with response to BCNU in mouse astrocytes (as described in section 5.2.1), cells were pretreated with BG to determine if depletion of MGMT would increase sensitivity to BCNU. First, cells were exposed to BG and MGMT activity was measured to ensure that BG would deplete MGMT. Astrocytes and fibroblasts were treated with either 25 μM or 50 μM BG for two hours, cells were harvested and MGMT activity was measured (refer to section 2.6 for details). BG treatment significantly depleted MGMT activity in +/+ *p53* astrocytes and fibroblasts and −/− *p53* fibroblasts (Figure 7.2). There was no significant depletion of the already low MGMT activity displayed by −/− astrocytes; the basal MGMT level found in −/− astrocytes was not significantly different from those levels found in the more MGMT-positive cells following depletion of greater than 90% of MGMT activity.

To determine if depletion of MGMT would sensitize cells to BCNU, astrocytes and fibroblasts were pretreated with 25 μM or 50 μM BG for 2 hr, exposed to increasing

Figure 7.2 **Effect of BG treatment on MGMT activity in mouse astrocytes and fibroblasts.** Cells were treated with either 25 or 50 μM BG for two hours prior to the measurement of MGMT activity. BG treatment significantly depleted MGMT activity in wild type *p53* astrocytes and fibroblasts and knockout *p53* fibroblasts. There was no significant depletion of the already low MGMT activity displayed by knockout astrocytes. Control MGMT levels from Figure 7.1 are shown for comparison. Bars depict the mean of at least two replicates from a minimum of two independent experiments ($\pm$SEM) and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$). WT, wild type; HET, heterozygous; KN, knockout; A, astrocytes; F. fibroblasts.


MGMT Activity (fmol/mg protein)
1400
1200
1000
800
600
400
200
0
WT HET KN
Astrocytes
WT HET KN
Fibroblasts
25 50
WTA
25 50
KNA
25 50
WTF
25 50
KNF
Control
Benzylguanine Treatment

concentrations of BCNU and cell viability tested using the MTT assay (refer to section 2.5 for details). BG did not significantly increase sensitivity to BCNU in any of the combinations tested. In addition to the +/+ astrocytes and fibroblasts and −/− astrocytes and fibroblasts (Figures 7.3 and 7.4 respectively), +/− cells also were tested and an increased sensitivity to BCNU was not evident (data not shown).

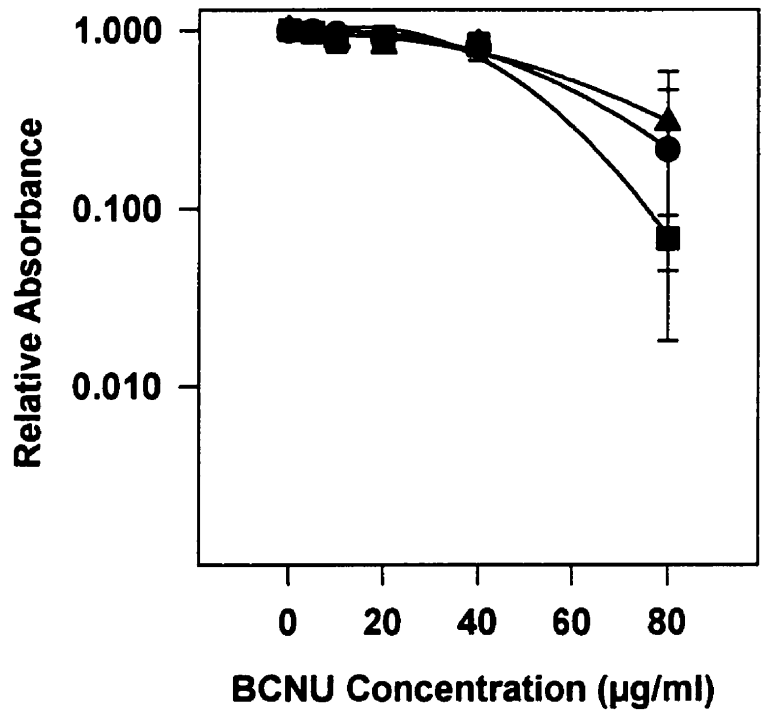
7.2.3 Effect of BCNU and BG treatment on MGMT levels.

Since there have been reports of MGMT induction in response to treatment with DNA-damaging agents (Margison *et al.*, 1985; Dexter *et al.*, 1989; Stammberger *et al.*, 1990; Fritz *et al.*, 1991; Stammberger and Tempel, 1991; Chan *et al.*, 1992; Wilson *et al.*, 1993; Grombacher *et al.*, 1996), cells were treated with BCNU to determine if there were any changes in MGMT levels. First, cells were examined for changes in *MGMT* mRNA expression. Wild type and −/− astrocytes and fibroblasts were exposed to either 40 μg/ml BCNU or 25 μM BG for 2 hr and samples were taken at defined times for Northern analysis (refer to section 2.7). There was no evidence of *MGMT* mRNA induction; expression remained constant over a 24 hr time period in response to both treatments in all four cell types (Figure 7.5).

In addition to exploring mRNA levels, cells also were examined for changes in MGMT activity following treatment with BCNU. There was no significant change in MGMT activity in +/+ and −/− astrocytes in response to BCNU (Figure 7.6). There was an indication of a slight, but non-significant, decrease in MGMT activity in +/+ astrocytes which may be evidence of the inactivation of some MGMT molecules following repair of BCNU-induced DNA damage. This conclusion is supported by the finding that a reduction in activity was not seen in response to treatment with 5 Gy radiation (Figure 7.6); radiation does not alkylate DNA at the O^6 position of guanine. Furthermore, there was no evidence of a substantial induction of MGMT activity in fibroblasts (Figure 7.7). The same pattern of a slight decrease in MGMT activity in response to BCNU was seen for +/+ fibroblasts and although an increasing trend is seen in −/− fibroblasts, the levels of MGMT activity do not increase to the levels displayed

Figure 7.3 **Effect of BG pretreatment on the response of wild type *p53* (A) and knockout (B) mouse astrocytes to BCNU.** Cells were pretreated with either 25 or 50 μM BG for two hours and then exposed to varying concentrations of BCNU. BG did not significantly increase sensitivity to BCNU in any of the combinations tested. Symbols represent the mean of at least two independent experiments, each performed in triplicate. **Circles represent cells treated with BCNU alone; triangles, 25 μM BG pretreatment; squares, 50 μM BG pretreatment.** Bars depict the standard error of the mean.

A


1.000
0.100
0.010
Relative Absorbance
0
20
40
60
80
BCNU Concentration (µg/ml)

B

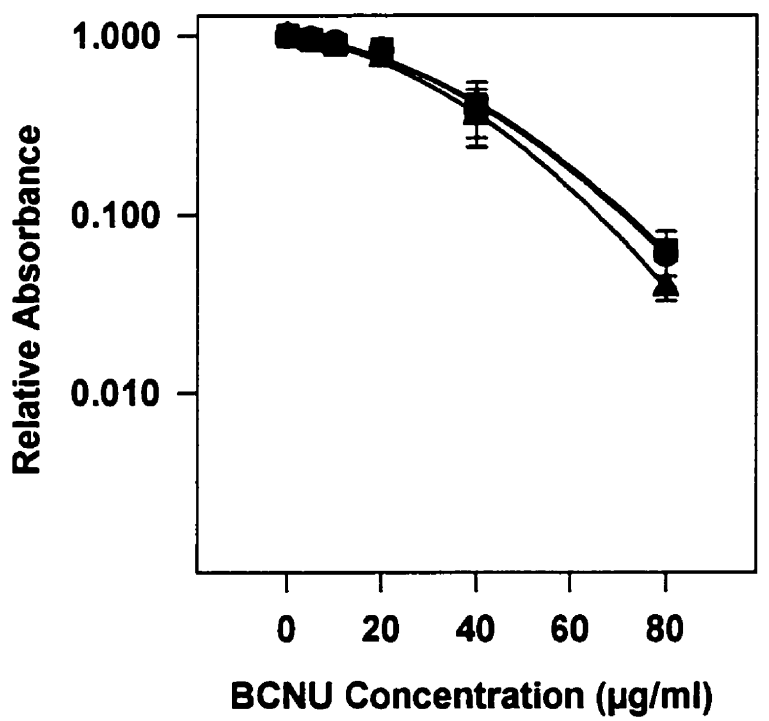
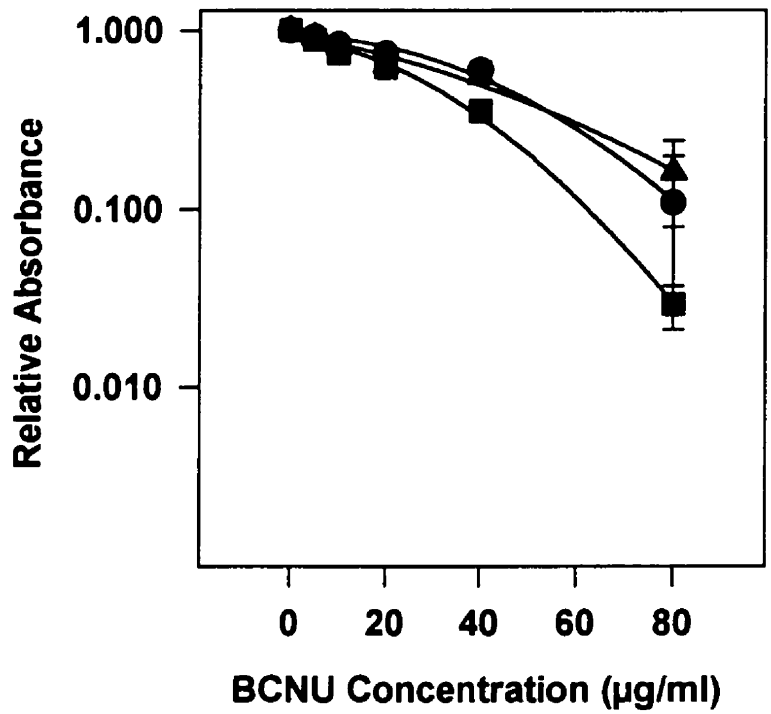

1.000
0.100
0.010
Relative Absorbance
0
20
40
60
80
BCNU Concentration (µg/ml)

Figure 7.4 **Effect of BG pretreatment on the response of wild type *p53* (A) and knockout (B) mouse fibroblasts to BCNU.** Cells were pretreated with either 25 or 50 μM BG for two hours and then exposed to varying concentrations of BCNU. BG did not significantly increase sensitivity to BCNU in any of the combinations tested. Symbols represent the mean of at least two independent experiments, each performed in triplicate. **Circles represent cells treated with BCNU alone; triangles, 25 μM BG pretreatment; squares, 50 μM BG pretreatment.** Bars depict the standard error of the mean.

A


1.000
0.100
0.010
Relative Absorbance
0
20
40
60
80
BCNU Concentration (µg/ml)

B

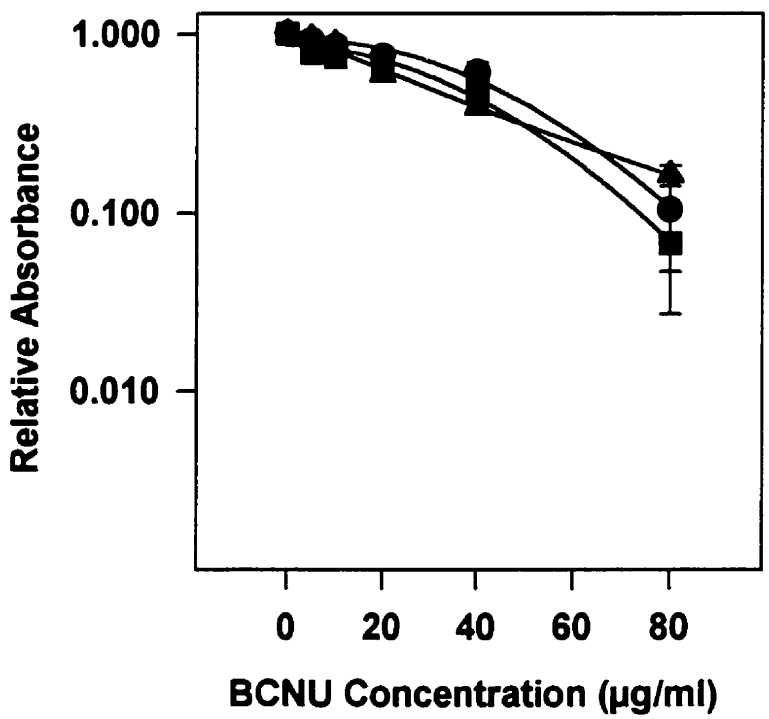

1.000
0.100
0.010
Relative Absorbance
0
20
40
60
80
BCNU Concentration (µg/ml)

Figure 7.5 **Effect of BCNU and BG treatment on *MGMT* mRNA expression.** Cells were exposed to either 40 μg/ml BCNU or 25 μM BG for two hours. The first treated mRNA sample (t=0) was harvested at the end of the incubation and succeeding samples were taken at the specified times (in hours). Quantified signal intensities for *MGMT* were normalized to *MGAP* in **wild type *p53* mouse astrocytes (A), knockout astrocytes (B), wild type fibroblasts (C), and knockout fibroblasts (D)**. *MGMT* mRNA expression remained constant throughout both treatments in all four cell types. Cont, control; MGAP, mouse glyceraldehyde-3-phosphate dehydrogenase.

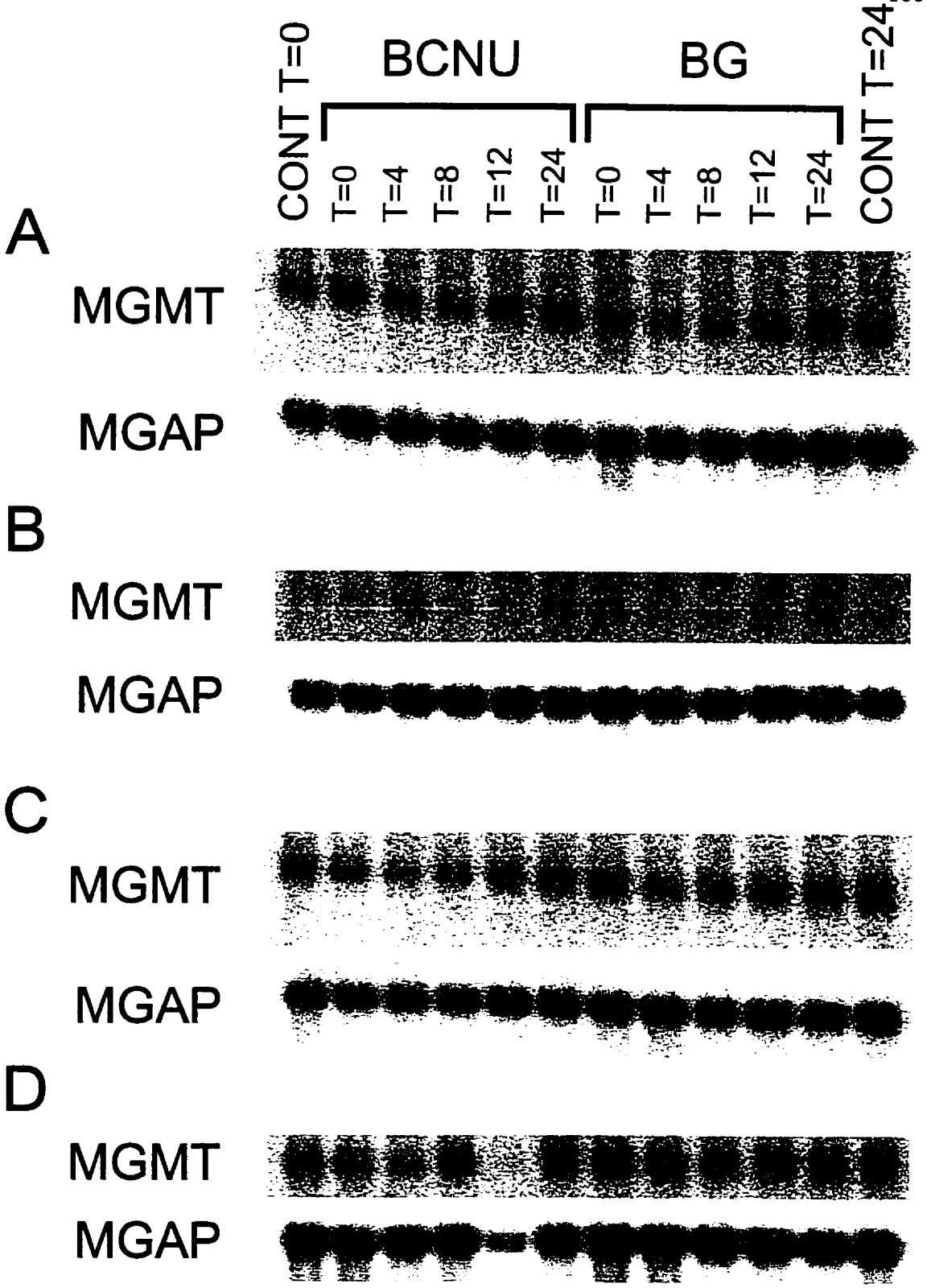
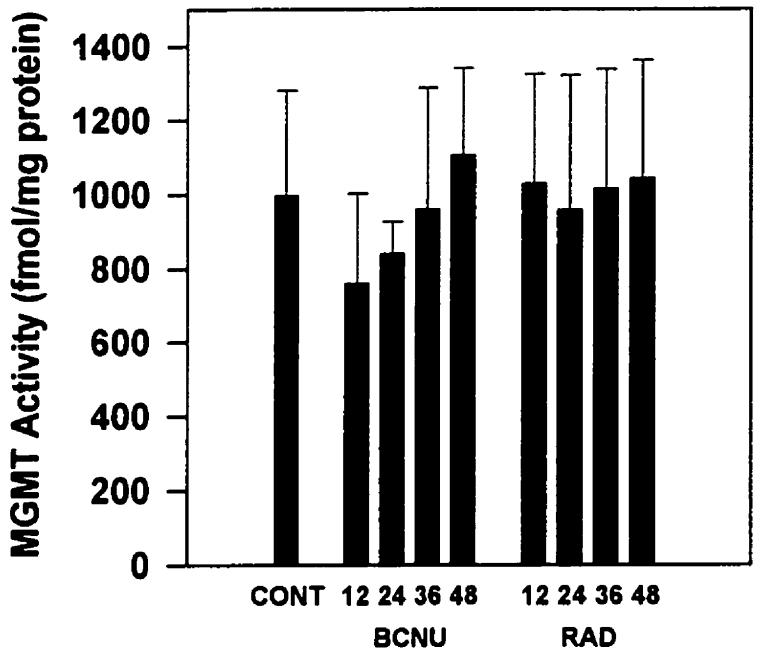

CONT T=0
BCNU
BG
CONT T=24
T=0
T=4
T=8
T=12
T=24
T=0
T=4
T=8
T=12
T=24
A
MGMT
MGAP
B
MGMT
MGAP
C
MGMT
MGAP
D
MGMT
MGAP

Figure 7.6 **Effect of BCNU treatment and radiation on MGMT activity in wild type *p53* (A) and knockout (B) mouse astrocytes.** Cells were treated with either 40 μg/ml BCNU for two hours or 5 Gy radiation. MGMT activity was measured at successive time points as indicated (in hours). There was no significant change in MGMT activity in response to treatment. Bars depict the mean of at least four replicates from a minimum of two independent experiments ($\pm$SEM). Gy, grays; cont, control; rad, radiation.

A


MGMT Activity (fmol/mg protein)
1400
1200
1000
800
600
400
200
0
CONT 12 24 36 48 12 24 36 48
BCNU
RAD

B

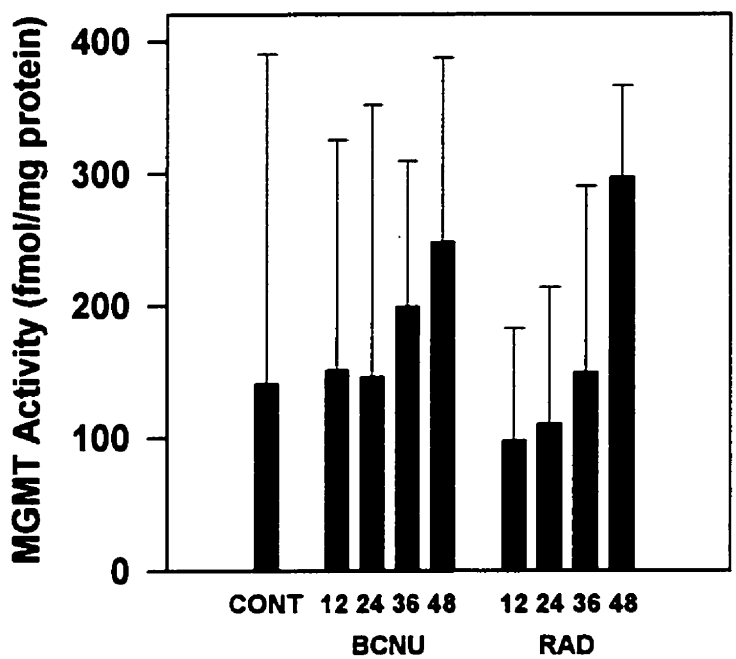
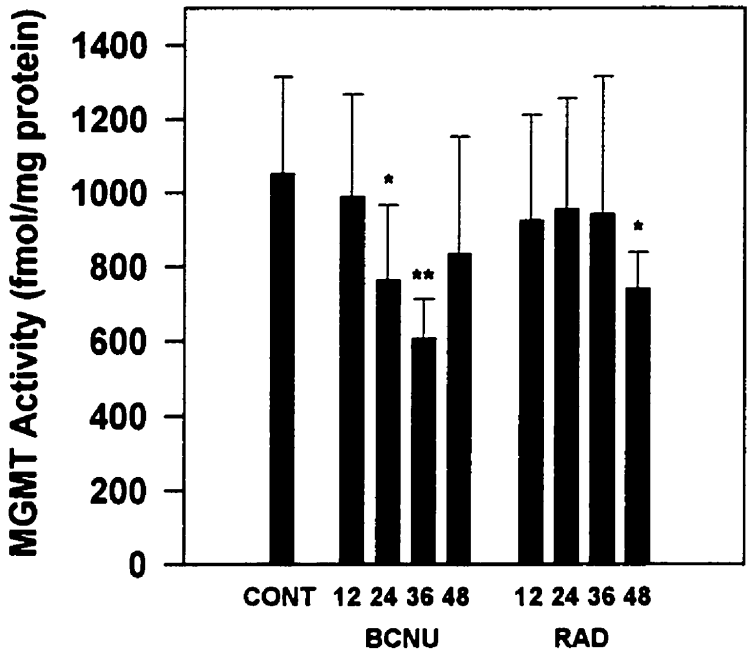
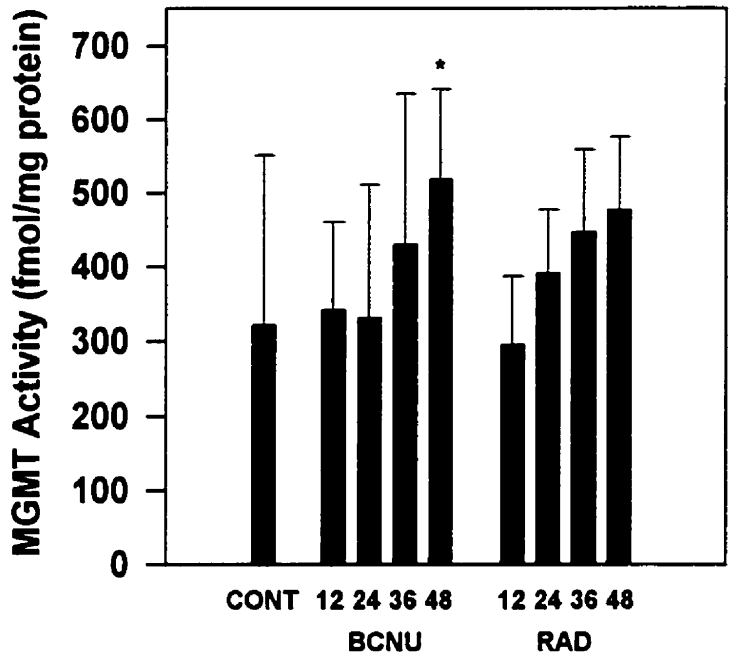

MGMT Activity (fmol/mg protein)
400
300
200
100
0
CONT 12 24 36 48 12 24 36 48
BCNU
RAD

Figure 7.7 **Effect of BCNU treatment and radiation on MGMT activity in wild type *p53* (A) and knockout (B) mouse fibroblasts.** Cells were treated with either 40 μg/ml BCNU for two hours or 5 Gy radiation. MGMT activity was measured at successive time points as indicated (in hours). Although there was some statistically significant changes in MGMT activity in response to treatment, the magnitude of the changes were quite small and there was no evidence of a substantial induction in MGMT activity. Bars depict the mean of at least four replicates from a minimum of two independent experiments ($\pm$SEM) and asterisks the level of statistical significance (*, $P < 0.05$; **, $P < 0.005$). Gy, grays; cont, control; rad, radiation.

A


MGMT Activity (fmol/mg protein)
1400
1200
1000
800
600
400
200
0
*
**
*
CONT 12 24 36 48 12 24 36 48
BCNU
RAD

B


MGMT Activity (fmol/mg protein)
700
600
500
400
300
200
100
0
*
CONT 12 24 36 48 12 24 36 48
BCNU
RAD

by +/+ cells. Heterozygous cells also were tested at the 24 and 48 time points and there was no change in MGMT activity (data not shown).

7.3 DISCUSSION

The p53 protein plays numerous roles in protecting cells and organisms from the deleterious effects of drug-induced DNA damage, one of which may be to regulate the expression of alternate drug resistance mechanisms. Since MGMT-mediated repair has been shown to play a role in resistance to alkylating agents and recently, there have been studies to suggest that p53 may be involved in the regulation of MGMT activity, the effect of p53 status on MGMT activity in mouse astrocytes was examined. Homozygous wild type *p53* astrocytes displayed significantly greater MGMT activity than −/− astrocytes while +/− cells exhibited intermediate activity. Hence, p53 appeared to enhance MGMT activity in a gene-dose-dependent manner in astrocytes. These results correlated well with the *p53* gene-dose-dependent resistance to BCNU displayed by mouse astrocytes. A gene-dose-dependent relationship for MGMT activity was not evident in fibroblasts; although +/+ and +/− fibroblasts both displayed significantly greater activity than −/− fibroblasts, there was no significant difference in MGMT activity between +/+ and +/− cells. Of particular interest, +/+ levels were comparable in astrocytes and fibroblasts but −/− astrocytes exhibited half the MGMT activity of −/− fibroblasts; basal levels of MGMT activity in −/− astrocytes could be considered MGMT-negative as they were not significantly different from those levels found in the MGMT-positive cells following depletion of greater than 90% of MGMT activity. Perhaps MGMT was contributing to the resistance of +/+ mouse cells to BCNU and only −/− astrocytes were sensitive due to their apparent lack of MGMT activity. This would explain the differential response exhibited by −/− astrocytes and fibroblasts in response to BCNU; −/− fibroblasts did not appear to display the same sensitivity to BCNU as did −/− astrocytes (refer to section 5.2.2).

Since MGMT activity correlated with response to BCNU in mouse astrocytes, cells were pretreated with BG to determine if depletion of MGMT would increase sensitivity to BCNU. Although BG pretreatment was shown to deplete MGMT activity

in +/+ astrocytes and fibroblasts and −/− fibroblasts, BG did not significantly increase sensitivity to BCNU in any of the combinations tested. These results suggested that although p53 status could affect basal levels of MGMT in astrocytes and fibroblasts, MGMT was not playing a role in the resistance of these cells to BCNU.

MGMT has been shown to be induced in response to some DNA damaging treatments (Margison *et al.*, 1985; Dexter *et al.*, 1989; Stammberger *et al.*, 1990; Fritz *et al.*, 1991; Stammberger and Tempel, 1991; Chan *et al.*, 1992; Wilson *et al.*, 1993; Grombacher *et al.*, 1996); it was possible that MGMT activity was being induced in response to either BG or BCNU and therefore, any attempts to deplete MGMT were, in turn, being overcome by the cells. This possibility was excluded as induction of neither MGMT mRNA nor protein levels was found after BG and BCNU treatment.

In summary, *p53* appeared to enhance MGMT activity in a gene-dose-dependent manner in astrocytes. Although these results correlated well with the *p53* gene-dose-dependent resistance to BCNU displayed by mouse astrocytes, depletion of MGMT by BG pretreatment did not significantly increase sensitivity to BCNU. This inability to increase sensitivity was not due to the subsequent induction of additional MGMT protein. Thus, although these results support a role for p53 in the regulation of MGMT, the p53-dependent BCNU resistance displayed by mouse astrocytes appears to be mediated by a non-MGMT mechanism. The significance of these results are not lost however; Preuss *et al.* (1996) recently suggested that the protective effect of MGMT varied depending on the alkylating agent in question, with BCNU being less affected by MGMT modulation than other alkylating agents of this class. It would be of interest, therefore, to investigate whether modulation of the p53-dependent differential expression of MGMT demonstrated in this study could confer sensitivity to additional alkylating agents.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Despite intensive research, attempts to develop effective chemotherapy regimens for gliomas have met with limited success. Significant progress was made when Cairncross and Macdonald (1988) reported that anaplastic oligodendrogliomas responded to a chemotherapy regimen of procarbazine, CCNU, and vincristine (PCV). It has been confirmed since that oligodendrogliomas and mixed oligoastrocytomas respond predictably to nitrosourea-based chemotherapy, especially PCV, whereas astrocytomas are relatively chemoresistant. Presumably oligodendroglioma cells are inherently susceptible to the cytotoxic effects of PCV, but why? In addition, a subset of astrocytomas have been observed to benefit from chemotherapy. What intrinsic properties of this subset of astrocytomas set them apart from the majority of astrocytic tumors? The answers to these questions are undoubtedly important as they may lead to better therapies for oligodendrogliomas and to new treatment strategies for anaplastic astrocytomas and glioblastomas.

In this thesis, two main approaches were taken to test the hypothesis that biochemical properties intrinsic to specific subtypes of glial tumors play a role in the resistance of these tumors to chemotherapeutic agents. The first approach was to examine biochemical properties inherent to the normal glial cell of origin. In cultures of semi-pure glial cells isolated from rat, oligodendrocyte lineage cells were found to be significantly more sensitive to BCNU than astrocytes. In addition, oligodendrocyte lineage cells had low MGMT activity, approximately one-third of the levels found for astrocytes. In cultures of purified rat glial cells, purified oligodendrocytes also were demonstrated to be more sensitive to BCNU than astrocytes, while oligodendrocyte progenitors and astrocytes displayed no significant difference in response to BCNU. When a panel of six drug resistance genes (*MGMT*, *GST-μ*, *GST-π*, *p53*, *MDR*, and *MT*) was probed for expression in these cells, a differential pattern of mRNA expression was observed for each of the six probes. The roles of MGMT and the glutathione-glutathione-*S*-transferase

detoxification pathway in response to BCNU were examined further. Depletion of both MGMT and glutathione were demonstrated to increase sensitivity to BCNU in astrocytes, suggesting a role for these two mechanisms in the resistance of rat glial cells to alkylating agents. The evidence provided from the differential expression of drug resistance genes suggested that more than one mechanism of resistance may be deficient in oligodendrocytes. In addition, numerous other mechanisms exist that may play a role in drug resistance in these cells but were not explored in this study (refer to sections 1.5 and 1.6). Thus, the response of normal rat glial cells to BCNU appeared to be dependent on more than one mechanism of drug resistance. If mechanisms of drug resistance in some subtypes of glial tumors reflect the biochemical properties of their cell of origin, then these experiments provide additional support for the hypothesis that oligodendroglioma cells may be inherently susceptible to the cytotoxic effects of alkylating agents.

The second approach of this thesis was to examine the impact of a specific genetic alteration, found commonly in astrocytic gliomas, on the response of otherwise normal astrocytes to BCNU. Mutations of the *p53* gene, and subsequent loss of p53 function, are a common occurrence in astrocytic tumors. Therefore, the impact of the loss of p53 function on response of astrocytes to BCNU was examined using cells isolated from TSG-*p53* mice (refer to section 2.3.1). Wild type p53 appeared to protect mouse astrocytes from the cytotoxic effects of BCNU in a gene-dose-dependent fashion. These results suggested that +/+ astrocytes were not undergoing p53-dependent apoptosis; one would expect +/+ astrocytes to be more sensitive to BCNU than −/− cells if they were preferentially undergoing programmed cell death. In addition, when the effect of p53 status on cell cycle response to BCNU treatment was examined, mouse astrocytes were shown to undergo cell cycle arrest in response to BCNU but this response was not p53-dependent. Thus, it seemed that the resistance displayed by +/+ astrocytes in response to BCNU could not be explained by either p53-dependent apoptosis or differential cell cycle arrest.

How then would p53 render +/+ astrocytes resistant to BCNU? It was hypothesized that perhaps the slower rate of proliferation of +/+ as compared to −/−

astrocytes contributed to drug resistance; slower cell cycling in the presence of p53 would allow more time for repair of DNA damage and as a consequence enhance genomic stability in +/+ astrocytes. If this were true, it was reasoned further that damage in these unstable −/− cells may be detected by an alternative DNA damage-sensing mechanism and these cells may be triggered subsequently to undergo a p53-independent programmed cell death. The studies presented here confirmed that −/− astrocytes could undergo apoptosis both in the absence of drug and at increased levels in response to BCNU treatment. The actual mechanism for increased apoptosis in −/− mouse astrocytes in response to BCNU remains unclear and awaits further study; increased basal *bcl-2* expression in +/+ astrocytes provides one possible explanation for the differential response to BCNU, as does the potential for additional members of the bcl-2 family to alter the balance of cell survival/death.

Finally, it has been suggested that p53 may regulate directly alternate drug resistance mechanisms. Since MGMT-mediated repair has been shown to play a role in resistance to alkylating agents and recently, there have been studies to suggest that p53 may be involved in the regulation of MGMT activity, the effect of p53 status on MGMT activity in mouse astrocytes was examined. The presence of p53 appeared to enhance MGMT activity in a gene-dose-dependent manner in astrocytes. Although these results correlated well with the *p53* gene-dose-dependent resistance to BCNU displayed by mouse astrocytes, depletion of MGMT with BG did not sensitize these cells to BCNU and thus, the p53-dependent BCNU resistance displayed by mouse astrocytes appeared to be mediated by a non-MGMT mechanism. The finding that mouse astrocytes were not sensitized to BCNU following BG pretreatment was of particular interest since, in Chapter 4, rat astrocytes were found to be sensitized, at least to some degree, following the same treatment regimen. These results suggested that some inter-species variation may exist in regard to preferred mechanisms of choice for drug resistance. This variability has been documented previously for MGMT; Gerson *et al.* (1986) showed that, compared with human and rat tissues, all mouse tissues contained low levels of MGMT activity.

Why might some gliomas benefit from chemotherapy treatment whereas others

do not? Taken together, the results presented in this thesis suggest that properties inherent to the normal glial cell of origin and specific genetic alterations occurring during tumorigenesis both may play a role in the development of a resistant tumor. In conclusion, these results provide support for the hypothesis that biochemical properties intrinsic to specific subtypes of glial tumors play a role in the resistance of these tumors to chemotherapeutic agents.

APPENDIX I

CERTIFICATION OF APPROVAL OF ANIMAL RESEARCH

The UNIVERSITY *of* WESTERN ONTARIO

Council on Animal Care • Animal Care and Veterinary Services
Director - Michele M. Bailey, D.V.M.
Clinical Veterinarian - Susan H. Fussell, D.V.M.

January 15, 1992

Dear Dr. Cairncross:

Your "Application To Use Animals for Research or Teaching" entitled:

"DNA Methyltransferase and Glutathione Transferase Activity in Rat Glial Cell Cultures"

has been approved by the University Council on Animal Care. This approval expires in one year on the last day of the month. The number for this project is # 92013-1

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears on this approval please contact this office when grant approval is received. If the application for funding is not successful and if you wish to proceed with the project, request that an internal scientific peer review be performed by your animal care committee.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED

Rats – S.D. 60

REQUIREMENTS/COMMENTS

M Bailey

c.c. A. Parbtani
P. Coakwell

Health Sciences Centre • London, Ontario • Canada • N6A 5C1 • Telephone: (519) 661-3034 • Fax: (519) 661-3797

The UNIVERSITY of WESTERN ONTARIO

Council on Animal Care • Animal Care and Veterinary Services
Director - Michele M. Bailey, D.V.M.
Clinical Veterinarian - Susan H. Fussell, D.V.M.

September 11, 1992

Dear Dr. Cairncross:

A MODIFICATION to your "Application to Use Animals for Research or Teaching" entitled:

"DNA Methyltransferase and Blutathione Transferase Activity in Rat Glial Cell Cultures"

has been approved. The protocol #92013-1 and expiry date of January 1993 remains unchanged.

APPROVED CHANGES INCLUDE:

---------- 1. Name change to " "

X
---------- 2. Animal use change(s)

Within Year Modification to ADD 7 - 14 day old rats.
40 litters

---------- 3. Procedural

---------- 4. Other

X
---------- 5. Comments

Rats greater than 7 days old will be euthanized with CO_2.

c.c. A. Parbtani
P. Coakwell

Health Sciences Centre • London, Ontario • Canada • N6A 5C1 • Telephone: (519) 661-3034 • Fax: (519) 661-3797

The UNIVERSITY *of* WESTERN ONTARIO

Council on Animal Care • Animal Care and Veterinary Services
Director - Michele M. Bailey, D.V.M.
Clinical Veterinarian - Susan H. Fussell, D.V.M.

March 3, 1993

Dear Dr. Cairncross:

Your "Application To Use Animals for Research or Teaching" entitled:

"DNA Methyltransferase and Glutathione Transferase Activity in Glial Cell Cultures"

has been approved by the University Council on Animal Care. This approval expires in one year on the last day of the month. The number for this project is # 93050-3

1. **This number must be indicated when ordering animals for this project.**
2. **Animals for other projects may not be ordered under this number.**
3. **If no number appears on this approval please contact this office when grant approval is received. If the application for funding is not successful and if you wish to proceed with the project, request that an internal scientific peer review be performed by your animal care committee.**
4. **Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.**

ANIMALS APPROVED

Rats – S.D. – 150 litters

REQUIREMENTS/COMMENTS

c.c. A. Parbtani
P. Coakwell

Health Sciences Centre • London, Ontario • Canada • N6A 5C1 • Telephone: (519) 661-3034 • Fax: (519) 661-2028

The UNIVERSITY *of* WESTERN ONTARIO

Council on Animal Care • Animal Care and Veterinary Services
Director - Michele M. Bailey, D.V.M.
Clinical Veterinarian - Susan H. Fussell, D.V.M.

March 18, 1994

Dear Dr. Cairncross:

Your "Application to Use Animals for Research or Teaching" entitled:

"DNA Methyltransferase and Glutathione Transferase Activity in Glial Cell Cultures"

has been approved by the University Council on Animal Care. This approval expires in one year on the last day of the month. The number for this project is # 94075-3

1. **This number must be indicated when ordering animals for this project.**
2. **Animals for other projects may not be ordered under this number.**
3. **If no number appears on this approval please contact this office when grant approval is received. If the application for funding is not successful and if you wish to proceed with the project, request that an internal scientific peer review be performed by your animal care committee.**
4. **Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.**

ANIMALS APPROVED

Rats – S.D. – 150 litters

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

c.c. A. Parbtani
P. Coakwell

Health Sciences Centre • London, Ontario • Canada • N6A 5C1 • Telephone: (519) 661-3034 • Fax: (519) 661-2028

The UNIVERSITY *of* WESTERN ONTARIO

Council on Animal Care • Animal Care and Veterinary Services
Director - Michele M. Bailey, D.V.M.
Clinical Veterinarian - Susan H. Fussell, D.V.M.

May 1, 1995

Dear Dr. Cairncross:

Your "Application to Use Animals for Research or Teaching" entitled:

"DNA Methltransferase and Glutathione Transferase Activity in Glial Cell Cultures
Funding Agency: LRCC Endowment Fund - A572

has been approved by the University Council on Animal Care. This approval expires in one year on the last day of the month. The number for this project is # 95084-5

1. **This number must be indicated when ordering animals for this project.**
2. **Animals for other projects may not be ordered under this number.**
3. **If no number appears on this approval please contact this office when grant approval is received. If the application for funding is not successful and if you wish to proceed with the project, request that an internal scientific peer review be performed by your animal care committee.**
4. **Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.**

ANIMALS APPROVED

Rats - S.D. - 125 litters Mice - TSG-p53 (C57Bl) - 150 litters

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

1. **SOP#320-01 "Euthanasia" applies to this protocol. If you do not already have a copy they are available from Mr. Paul Coakwell.**
2. **Inaccurate protocol numbers in the body of your protocol have been corrected.**
3. **Pups less than 7 days old must be decapitated with scissors.**

J. Martens

c.c. A. Parbtani
P. Coakwell
Office of Research Services

COPY

April 15, 1996

Dear Dr. Cairncross:

Your "Application to Use Animals for Research or Teaching" entitled:

"Mechanisms of Drug Resistance in Glial Cells"
Funding Agency: LRCC Endowment Fund

has been approved by the University Council on Animal Care. This approval expires in one year on the last day of the month. The number for this project is # 96080-5. This replaces #95084-5.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears on this approval please contact this office when grant approval is received. If the application for funding is not successful and if you wish to proceed with the project, request that an internal scientific peer review be performed by your animal care committee.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED

Rats - Sprague Dawley, 7 days old, M/F - 150 litters
Mice - TSG-p53 (C57BI), 4-6 days old, M/F - 150 litters

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Renewal - J. Cairncross, P. Coakwell
Approval Letter - P. Coakwell

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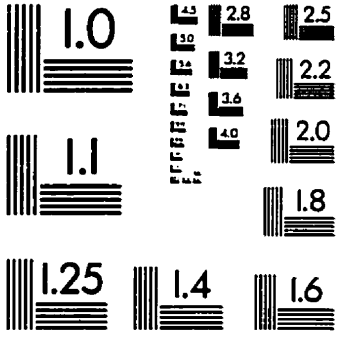

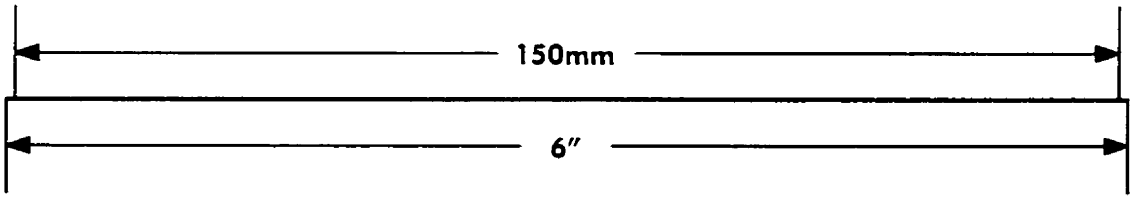

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