APOPTOSIS IN MEDULLOBLASTOMA:

IN VITRO ANALYSIS

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Graduate Studies and Research
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By
Wei Wang
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The Head
Department of Pathology
University of Saskatchewan
Saskatoon, SK, Canada
S7N 5E5
ABSTRACT

The poor prognosis associated with medulloblastoma (MB), a primitive neuroectodermal neoplasm, has stimulated the evaluation of improved treatment strategies. The potential of lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, as a possible anticancer drug against MB cell lines (Daoy, UW228, D283 Med and D341 Med) and two primary MB cultures was evaluated in vitro. Analysis of cell morphologic changes, cell viability, DNA fragmentation and flow cytometry demonstrated growth inhibition and induction of apoptosis in MB cells as well as a population of lovastatin 'resistant' MB cells after lovastatin treatment. Cell cycle arrest in G1 was concomitant with apoptosis. Mevalonate prevented lovastatin-induced changes in MB, thus confirming that blockage of the mevalonate pathway is a critical step in the mechanism of lovastatin-induced MB apoptosis, and that HMG-CoA reductase activity is necessary for MB survival. Within the mevalonate pathway, blocking protein farnesylation with manumycin A and blocking G-protein function by the depletion of GTP using mycophenolic acid (MPA) were followed by inhibition of proliferation and induction of apoptosis. Cell death induced by manumycin A was uniformly more rapid and efficient than that induced by lovastatin. The cell cycle was also arrested in G1 by MPA. Lovastatin, manumycin A and MPA synergistically induced MB apoptosis. These results indicate that blocking farnesylation of proteins, especially G-proteins, at least partially accounts for MB apoptosis that is induced by blocking the mevalonate pathway. Studies of the molecular mechanisms involved in apoptosis induced by these compounds demonstrated the following: 1) regulation of HMG-CoA reductase gene expression was perturbed; 2) the caspase-3 pathway was activated; 3) apoptosis was p53-independent; 4) Bcl-2 and Bax did not regulate apoptosis; 5) up-regulation of the CKIs p21WAF1 and P27KIP1 accompanied cell cycle arrest and growth inhibition; 6) c-myc overexpression did not influence likelihood of apoptosis; and 7) the regulation of ras gene expression did not account for MB apoptosis induced by these compounds. The efficient induction of apoptosis by lovastatin favors this drug as a potential new therapeutic intervention for MB.
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LIST OF ABBREVIATIONS

ANOVA.............................. analysis of variance
AP........................................ alkaline phosphatase
ATCC........................................ American Type Culture Collection
A.U........................................ arbitrary units
BCIP................................. 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BDNF........................................ brain-derived neurotrophic factor
bp........................................ base pair(s)
BSA......................................... bovine serum albumin
CAK......................................... cyclin dependent kinase-activating kinase
CKI........................................ cyclin dependent kinase inhibitor
CDK......................................... cyclin dependent kinase
CNS........................................ central nervous system
DAB........................................ 3,3'-diaminobenzidine
D-MEM............................... Dulbecco's modified Eagle's medium
dNTP........................................ deoxynucleotide triphosphates
EDTA........................................ ethylenediaminetetraacetic acid
EGF........................................ epidermal growth factor
EGFR........................................ epidermal growth factor receptor
FBS......................................... fetal bovine serum
FTase......................................... farnesyltransferase
FPTase................................. protein farnesyltransferase
g........................................... gravitational force
G........................................... GTP-binding
GAPDH............................... glyceraldehyde-3-phosphate dehydrogenase
GGTase..................................... geranylgeranyltransferase
GGPTase................................. protein geranylgeranyltransferase
GFAP....................................... glial fibrillar acidic protein
HMG-CoA........................... 3-hydroxy-3-methylglutary coenzyme A
hr............................................... hour
ICE................................. interleukin-1β converting enzyme
IL........................................ interleukin
IMP........................................ inosine monophosphate
IMPDH................................. inosine monophosphate dehydrogenase
kb........................................ kilobases
kDa........................................ kilodaltons
LDL...................... low-density lipoprotein
LOH...................... loss of heterozygosity
Mab...................... monoclonal antibody
MAP...................... mitogen activated protein
MB...................... medulloblastoma
MDM2.................... murine double-minute-2
MHC...................... major histocompatibility complex
MOPS.................... 3-[[N-Morpholino]propanesulfonic acid
MPA...................... mycophenolic acid
MuLV..................... murine leukovirus
NBT...................... p-nitro blue tetrazolium chloride
NF...................... neurofilament
NGFR..................... nerve growth factor receptor
NSE...................... neuron-specific enolase
NT...................... neurotrophin
PARP..................... poly-(ADP ribose) polymerase
PBS...................... phosphate-buffered saline
PCR...................... polymerase chain reaction
PDGF..................... platelet-derived growth factor
PEG...................... polyethylene glycol
PI...................... propidium iodide
PI3K..................... phosphatidylinositol 3’-kinase
PKC..................... protein kinase C
PMSF.................... phenylmethylsulphonylfluoride
PRPP..................... 5-phosphoribosyl-1-pyrophosphate
RGD...................... arginine-glycine-aspartic acid
RIPA..................... radioimmunoprecipitation assay
r²...................... Pearson correlation coefficient squared
RT...................... reverse transcription
SD...................... standard deviation
SDS...................... sodium-dodecyl sulfate
SDS-PAGE................ sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
SEM...................... standard error of the mean
SRE...................... sterol regulatory element
TGF...................... transforming growth factor
TBS...................... Tris-buffered saline
TBST......................... TBS-Tween 20
TNF........................... tumor necrosis factor
TNFR.......................... tumor necrosis factor receptors
Tris........................... tris (hydroxymethyl) aminomethane
1.0 INTRODUCTION

1.1 Characteristics of medulloblastoma

Cancer is the second most frequent cause of death in children under 15 years of age (Boring et al., 1991), and neoplasms of the central nervous system are the most common type of cancer to affect children, following leukemia (Boring et al., 1991; Young and Miller, 1975). Among central nervous system neoplasms, medulloblastoma (MB), a primitive neuroectodermal tumor of the cerebellum with incompletely understood pathogenesis, is the most common embryonal tumor.

1.1.1 Epidemiology

The annual incidence of MB is 5 cases per 1 million children (Stevens et al., 1991). It accounts 15% to 25% of pediatric central nervous system neoplasms (Farwell et al., 1977; Friedman et al., 1991; Jay and Becker, 1990). The prognosis of MB is unpredictable for individual patients. Although survival rates as high as 80% have been noted in prospective single-arm studies on MB (Packer et al., 1994), the overall 5-year survival rate for children is 50-70% (Packer, 1990; Reddy and Packer, 1998) and long-term survival in patients with advanced disease is only about 30% (Friedman et al., 1991). The mortality of recurrent MB approaches 100% (Torres et al., 1994).

MB affects both children and adults, although it is not common in adults. According to a population-based study of MB (Roberts et al., 1991), the range of age at time of diagnosis in 532 cases was 0 to 86 years, however, the mean age at diagnosis of patients was 7.3 years of age with peaks at 3 and 7 years. Of all patients, 2/3 were under 19 years old. Similar findings have been reported in numerous studies.
Male predilection is observed in childhood malignant brain tumors as a whole, and males are particularly affected by childhood MB (Finlay, 1986), with a 2:1 male preponderance noted (Young and Miller, 1975).

MB occurring in siblings has been reported. No pattern of inheritance was identified, but disease presented at similar ages, and patient age at death was similar (Tijssen, 1986). Other forms of cancer occurring in close relatives of siblings afflicted by MB have also been reported (Hung et al., 1990), raising the possibility of a role for heredity in the etiology of MB.

MB has been associated with a number of heritable disorders, both autosomal dominant and autosomal recessive. For example, MB is associated with Gorlin syndrome (nevoid basal cell carcinoma syndrome), an autosomal dominant disorder of the skin and skeletal system (Louis and von Deimling, 1995), and with glioma-polyposis Turcot syndrome, an autosomal recessive disorder.

1.1.2 Biology

Although lateral cerebellar hemisphere lesions may be seen in older children and adolescents, MB arises most frequently in the midline of the cerebellum (Friedman et al., 1991). These neoplasms appear as friable, soft tumors, usually without central necrosis, cyst formation or calcification. In most patients, MB displays the characteristics of highly cellular neoplasms with small round cells, hyperchromatic nuclei and absence of endothelial proliferation. MB cells can express protein markers of neuronal differentiation, including synaptophysin and neurofilament (often in MB cell lines), as well as astrogial, ependymal, and rarely muscle differentiation (Coffin et al., 1990; Cruz et al., 1989; Gould et al., 1990; Molenaar et al., 1989). For example, in an immunohistochemical study of 53 MB biopsies, the neuronal protein synaptophysin was present in 94% of neoplasms, while 38% of the cases expressed vimentin, 21% GFAP, and 9% desmin; none expressed neurofilament protein or cytokeratin; 8% were immunoreactive for S-100 (Coffin et al.,
1990). The majority of primary MB samples usually do not show the expression of neurofilaments, the terminal neuronal differentiation marks (Pietsch et al., 1994). This is different from neoplasms arising from 'mature' neurons. These features suggest that developmental arrest of neuronal precursors in the developing cerebellum accompanies neoplastic transformation (Trojanowski et al., 1992).

1.1.2.1 Histogenesis and pathogenesis

MBs originate from the roof of the fourth ventricle in children. A previously accepted histogenetic derivation was from medulloblasts, i.e. undifferentiated, proliferating embryonal cells with the capacity to differentiate into spongioblasts and neuroblasts. Three hypotheses regarding the histogenesis of MB have been proposed (Graham and Lantos, 1997). They are 1) an origin from the external granular layer of the cerebellum; 2) an origin from subependymal matrix cells which reside throughout the embryonal CNS, including the fourth ventricle to give rise to neurons and glial cells; 3) an origin from the ventricular matrix/velum medullae.

The different phenotypes of MB have suggested that they are derived from different CNS progenitor cells, and that they may be the various abnormalities of cellular differentiation that are acquired during neoplastic transformation. Since MB emerges predominantly in early childhood, the induction of MB may result from genetic lesions that arise in developing CNS progenitor cells, thereby preventing these precursors from executing normal programs of lineage commitment and differentiation in the CNS (Trojanowski et al., 1994). MB consists of cells that are morphologically similar to the primitive neuroepithelial cells normally seen in early stages of neural embryogenesis, supporting the notion that they result from a disturbance in the process of normal neuronal or glial differentiation.

After the demonstration of nestin expression (an intermediate filament protein found in neuroepithelial stem cells) in MB (Fults et al., 1992; Lendahl et al., 1990; Valtz et
al., 1991), it has been suggested that MB is the malignant counterpart of multipotential neural progenitor cells. One established human MB cell line, PFSK, expressed nestin, but not antigens typically found in terminally differentiated neurons or glia (Fults et al., 1992), indicating that MB contains neuroepithelial stem cells prior to commitment to a neuronal or glial lineage. Valtz (1991) reported that the rat cerebellar cell line ST15A expresses nestin, and can differentiate, giving rise to different cell types (e.g. neurons, glia, and muscle cells) found in MB. These results support a neuroectodermal stem cell origin for MB.

Neurotrophins and their receptors are widespread in the developing and mature CNS. They play a role in regulating neuronal progenitor cell proliferation, migration, differentiation and survival. Studies using dissociated and organotypic cultures of rat cerebellum demonstrated that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) influence developing granule cells at distinct stages of differentiation (Segal et al., 1992). While early granule neurons in the external germinal layer responded to BDNF, more mature granule cells responded to NT-3. BDNF, but not NT-3, enhanced survival of granule cells. The sequential sensitivity to BDNF and NT-3 may be critical in the development of the cerebellar cortex. BDNF may promote the initial commitment to the neuronal lineage, while NT-3 may influence the subsequent differentiation of granule cells.

Recent evidence suggests that neurotrophins may also influence the biologic behavior of MB. In a study of 27 biopsy samples of MB, immunoreactive neurotrophin receptors TrkA (for NGF), TrkB (for BDNF), and TrkC (for NT-3) were observed in neoplastic cells in 27%, 62%, and 48% of these MBs, respectively. Immunoreactive BDNF, NT-3, and NT-4/5 were detected in 22%, 9%, and 19% of biopsies, respectively (Washiyama et al., 1996). These findings imply that signal transduction pathways mediated by neurotrophins and/or their receptors might influence the induction or progression of MB.

Another piece of evidence that MB is related to cerebellar development is the expression of the zic gene, which is highly homologous to the Drosophila pair-rule gene
Opa, and touted as a potential biomarker for the cerebellar granule cell lineage as well as for MB. Zic is a novel zinc finger protein that displays a highly restricted expression pattern. It is confined to nuclei of the cerebellar granule cell lineage, ranging from progenitor cells in the external germinal layer to the postmigratory cells in the internal granular layer. Zic protein was detected in MB (26/29 cases), whereas no other neoplasms examined (over 70 cases including primitive neuroectodermal neoplasms in other sites) expressed this protein (Yokota et al., 1996).

1.1.2.2 Experimental models

Unlike other related primitive neuronal neoplasms such as neuroblastoma where many cell lines and transplantable xenograft models have been successfully established, only a few MB cell lines have been generated so far. TE-671, the first “MB cell line” reported (McAllister et al., 1977), had been used extensively for cell biology and chemotherapeutic sensitivity studies, but subsequent studies revealed that TE-671 cell line was a derivative of RD, a classic rhabdomyosarcoma line (Stratton et al., 1989). Currently, several MB cell lines, including Daoy (Jacobsen et al., 1985), D283 Med (Friedman et al., 1985), and D341 Med (Friedman et al., 1988), are available from the ATCC, and widely used as research models. In addition, cell line UW228 has been established and also used as a model (Keles et al., 1993; Keles et al., 1995; Silber et al., 1992). Table 1.1 (adapted from Pietsch et al., 1994 and Friedman et al., 1991) summarizes the characteristics of these MB cell lines. Daoy represents a model of a 'glial' phenotype much more than a 'neuronal' phenotype, while UW228, D283 Med and D341 Med may represent a more advanced stage of neuronal differentiation.

These cell lines provide models for the analysis of MB biology in vitro. Insights into the histogenesis, cell biology, and drug resistance of MB have been gained through the study of cell lines. The use of cell lines eliminates the problem of 'contaminating' normal central nervous system (CNS) cells because immunohistochemical
markers do not distinguish between subsets of neuronal precursors (Bell et al., 1989; Kleinert, 1991; Kleinert, 1991). Significantly, the cell lines D283 Med and D341 Med possess the same karyotype as the original neoplasm specimen, confirming that they were derived from the resected tumor. Both intracranial and subcutaneous xenograft models in athymic rats and mice have been established for D283 Med and D341 Med. In addition, the reported nude rat model of leptomeningeal dissemination of human neoplasm xenografts (Fuchs et al., 1990) allows the intrathecal transplantation of neoplasm cells and subsequent administration of experimental treatments.

Table 1.1. Summary of the Characteristics of Medulloblastoma Cell Lines

<table>
<thead>
<tr>
<th>cell line</th>
<th>UW228</th>
<th>Daoy</th>
<th>D283 Med</th>
<th>D341 Med</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro Growth</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adherent</td>
<td>Adherent</td>
<td>Suspension</td>
<td>Suspension</td>
</tr>
<tr>
<td>Neural-associated markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSE</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurofilament L</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurofilament M</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurofilament H</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glial-associated markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>S-100</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Other intermediate filaments</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Surface markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF receptor</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>EGFR receptor</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MHC class I</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MHC class II</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Karyotype</td>
<td>Aneuploid</td>
<td>Tetraploid</td>
<td>Diploid</td>
<td>Diploid</td>
</tr>
</tbody>
</table>

However, cell lines may represent subclones of cells with oncogene amplification (e.g. c-myc in D283 Med and D341 Med, EGFR in Daoy) which are capable of survival in vitro, and which may not be representative of primary neoplasms. To
explore cancer biology, fresh neoplasm tissues cultured in vitro should also be used as experimental models where possible.

1.1.3 Genetics

The molecular pathogenesis of MB is not clear. Early reports describing MB karyotypes demonstrated that the majority of tumors have near-diploid stem lines, with only occasional examples of near-tetraploid stem lines. However, routine incorporation of chromosome banding has allowed more precise analysis of chromosomal abnormalities in MB.

Loss of heterozygosity (LOH) on chromosome arms 17p, 11p, 1q and 9q and cytogenetic abnormalities of chromosome 1 have been detected in MB (Albrecht et al., 1994; Cogen et al., 1992; Fults et al., 1992; Griffin et al., 1988; Kraus et al., 1996; McDonald et al., 1994; Schofield et al., 1995), but only LOH 17p is frequent (Albrecht et al., 1994). The susceptible locus, which localizes to 17p13.3 (distinct from the p53 locus at 17p13.1), was found to be deleted in approximately 50% of MB (Albrecht et al., 1994; McDonald et al., 1994). The data strongly support the hypothesis that a second tumor suppressor gene located on chromosome 17p is involved in the pathogenesis of MB. Less frequently, LOH on 11p can be detected in 27% of MB (Fults et al., 1992).

MB has been associated with Gorlin syndrome, the gene for which has been mapped to chromosome 9q (Gailani et al., 1992). A subset of sporadic desmoplastic MB and MB in patients with Gorlin syndrome have been shown to exhibit LOH on chromosome 9q (Schofield et al., 1995). Recently, the human homolog of the Drosophila-patched gene (PTC) was identified on 9q22 as the tumor suppressor gene responsible for Gorlin syndrome (Hahn et al., 1996; Johnson et al., 1996; Unden et al., 1996; Unden et al., 1997). Although site-directed mutation of PTC promotes the development of MB (Goodrich et al., 1997), LOH at the Gorlin syndrome locus does not seem to be important in the pathogenesis of sporadic MB without desmoplastic features.
(Albrecht et al., 1994), and analysis of PTC in these neoplasms is under way. LOH on 1q has been detected in MB by one research group (Kraus et al., 1996), but it is not known whether a locus on this chromosomal arm is indeed affected in a significant fraction of MBs.

Candidate genes that are important in the pathogenesis of other brain neoplasms have not been found to be altered in MB. Oncogene amplification is a relatively uncommon mechanism of oncogene activation in pediatric brain neoplasms (Wasson et al., 1990). Although about 25% of MB strongly express Ras (Macaulay et al., 1996), only about 10% have activating ras mutations (Ioascon et al., 1991). Less than 10% of MB show amplification of the c-myc and n-myc oncogenes or the epidermal growth factor receptor gene (Bigner and Vogelstein, 1990; Raffel et al., 1990; Wasson et al., 1990). p15 INK4b, p16 INK4, CDK4, cyclin D1, MDM2 (murine double-minute-2), p53 and RB1 tumor suppressor genes are not commonly affected in MB (Adesina et al., 1994; Batra et al., 1995; Cogen et al., 1992; Cogen et al., 1990; Jen et al., 1994; Ohgaki et al., 1991; Sato et al., 1996). p53 mutation has been observed in less than 10% of primary MB (Ohgaki et al., 1991; Raffel et al., 1993; Saylors et al., 1991). Bcl-2, a lymphoma-associated proto-oncogene which under normal circumstances appears to protect lymphoid precursors from programmed cell death, is expressed in some MB cells (Heck et al., 1994; Nakasu et al., 1994), suggesting that failure of apoptosis may be involved in MB tumorigenesis (Arends and Wyllie, 1991).

1.1.4 Treatment

After surgical resection of MB, common therapeutic strategies involve radiation and a variety of single chemotherapeutic agents. However, the use of radiation therapy is limited on young patients, due to the deleterious effects on intellectual development and unacceptable long-term sequelae (Nishiyama et al., 1994). Adjuvant chemotherapy, therefore, has been tried on early childhood MB. However, for several reasons,
therapeutic progress of MB has lagged behind that achieved with other childhood tumors (Tomlinson et al., 1992). One reason is that a portion of MB is protected by the blood-brain barrier. Another obstacle to progress is the fact that, at presentation, many children require acute decompression and surgery, a factor which limits or precludes preoperative chemotherapy. Although a beneficial effect of chemotherapy has recently been documented, whether this will be accompanied by improvements in the long-term prognosis for intellectual development remains to be seen (Packer et al., 1994). In addition, chemotherapy may be associated with dose-limiting toxicity (Cohen et al., 1990), and may induce expression of drug resistance genes (Tishler and Raffel, 1992; Tishler et al., 1992a).

Research efforts are seeking an alternate strategy of therapy for MB. One new therapeutic approach uses monoclonal antibodies conjugated to drugs or radionuclides as a delivery system to enter the target. For obvious reasons, directing such antibodies against normal brain tissues is undesirable, so an MB specific antigen is being sought to act as a suitable immunogen. Recently, one MB specific antibody has been developed (Kishima et al., 1999; Moriuchi et al., 1993; Shimizu et al., 1998), but it remains to be seen whether adequate dosages of linked chemotherapeutic agents or radionuclides can be delivered to neoplasm. Low uptake in MB because of the complex nature of transcapillary transport in brain neoplasms, and catabolism of radioisotopes from monoclonal antibodies in vivo, may also limit wide clinical application of this approach (Jay and Becker, 1990; Moseley et al., 1990).

Recently, an alternative strategy of therapy for MB has been proposed following preliminary investigations. 3-hydroxy-3-methylglutary coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in the cholesterol biosynthesis pathway, is highly expressed in MBs (Dimitroulakos and Yeger, 1996; Macaulay et al., 1994). The proposed strategy employs HMG-CoA reductase to block mevalonate biosynthesis, derivatives of which are involved in cellular growth control (Goldstein and Brown, 1990; Keyomarsi et
al., 1991; O'Donnell et al., 1993).

1.2 Regulation of the mevalonate pathway

Mevalonate derivatives (shown in Figure 1.1) include isoprenoids that are vital for diverse cellular functions, ranging from cholesterol synthesis to growth control. To ensure a constant production of the multiple isoprenoid compounds (such as isopentenyladenine, geranylgeranylated proteins, farnesylated proteins) at all stages of growth, cells must precisely regulate mevalonate synthesis while avoiding over-accumulation of potentially toxic products such as cholesterol (Brown and Goldstein, 1980). The molecular mechanisms for mevalonate regulation are being unraveled, because the crucial genes that control mevalonate synthesis have been cloned (Gil et al., 1986; Reynolds et al., 1984). Several mechanisms for feedback regulation have been suggested to ensure the production of sufficient mevalonate for several end-products (Goldstein and Brown, 1990). Manipulation of this regulatory system could be useful in treating certain forms of cancer (Goldstein and Brown, 1990).

1.2.1 Balance of external and internal cholesterol

Animal cells face a complex problem in regulating mevalonate synthesis because cholesterol could be derived from synthesis within the cell, as well as from plasma low-density lipoprotein (LDL) which enters the cell by receptor-mediated endocytosis (Figure 1.1). Each cell must balance these external and internal sources so as to sustain mevalonate synthesis while avoiding sterol over-accumulation. This balance is achieved via feedback regulation of HMG-CoA synthase, HMG-CoA reductase, and also LDL receptors (Figure 1.1). In the absence of LDL, animal cells maintain high activities of HMG-CoA synthase and reductase, thereby synthesizing mevalonate for production of cholesterol as well as non-sterol products. When LDL is present, these two enzyme activities decline by more
2 Acetyl-CoA

\[ \xrightarrow{\text{Acetoacetyl-CoA Synthase}} \]

HMG-CoA

\[ \xrightarrow{\text{HMG-CoA Reductase}} \] HMG-CoA Reductase Inhibitors:

- Lovastatin
- Pravastatin
- Simvastatin

Isopentenyl-diphosphate

\[ \xrightarrow{\text{Dimethylallyl diphosphate}} \]

Isopentenyl Adenine (tRNA) (for protein translation)

Geranyl diphosphate \(\xrightarrow{\text{Farnesyl-diphosphate}}\) Geranylgeranyl-diphosphate

- Geranylgeranylated proteins
  (for cell cycle regulation and signal transduction etc.)

\[ \xrightarrow{\text{Heme-a}} \] Heme-a (transport electrons in the mitochondrial respiratory chain)

Farnesyl diphosphate \(\xrightarrow{\text{Dolichyl-diphosphate}}\)

- Dolichyl-diphosphate (carry oligosaccharides in the process of N-linked protein glycosylation)

\[ \xrightarrow{\text{Decaprenyl}} \]

- Decaprenyl \(\xrightarrow{\text{Ubiquinones}}\)
  Ubiquinones (transport electrons in the mitochondrial respiratory chain)

\[ \xrightarrow{\text{FPTase}} \]

- Farnesylated proteins (for signal transduction etc.)

\[ \xrightarrow{\text{Lanosterol}} \]

Manumycine A

\[ \xrightarrow{\text{Cholesterol}} \]

LDL Receptor

Plasma LDL

Figure 1.1. Overview of the Mevalonate Pathway in Animal Cells
(adapted from Maltese, 1990 and Goldstein & Brown, 1990)
than 90%, and cells produce only the small amounts of mevalonate needed for non-sterol end-products (Brown and Goldstein, 1980). When cellular sterols rise or when cell growth ceases and cholesterol demand declines, the LDL receptor gene is repressed, averting cholesterol over-accumulation (Goldstein and Brown, 1984).

In addition to regulating mevalonate synthesis, cells regulate mevalonate disposition. The enzymes of the non-sterol pathways generally have higher affinities than those of the sterol pathway for mevalonate-derived substrates (Brown and Goldstein, 1980). When mevalonate is limited, it is preferentially shunted into the high-affinity non-sterol pathways. After prolonged incubation of cell with sterols, squalene synthase further limits the incorporation of mevalonate into sterols (Brown and Goldstein, 1980).

1.2.2 Sterol-mediated regulation of transcription

The mechanisms of sterol regulation of the LDL receptor, HMG-CoA synthase and HMG-CoA reductase (Gil et al., 1986; Reynolds et al., 1984; Sudhof et al., 1985) gene products are being investigated. One mechanism is transcriptional regulation. It has been found that there is a short segment containing a closely related sequence at the 5' flanking regions in these three genes, designated sterol regulatory element-1 (SRE-1) (Goldstein and Brown, 1984). Like enhancers, SRE-1 sequences can function in either orientation (Goldstein and Brown, 1984). In the promoters of LDL receptor gene and HMG-CoA synthase gene, SRE-1 enhances transcription in the absence of sterols but not when they are present (Smith et al., 1988). The role of SRE-1 in the promoter of HMG-CoA reductase gene is not well established, but there is a suggestion that it actively represses transcription in the presence of sterols (Osborne et al., 1988).

1.2.3 Post-transcriptional control of HMG-CoA reductase

HMG-CoA reductase is a highly regulated enzyme in nature. The use of HMG-CoA reductase inhibitors from fungi has revealed the complexity of this regulation. In
cultured cells, these inhibitors block the synthesis of mevalonate and trigger adaptive reactions that yield a 200-fold increase in reductase protein within a few hours. This induced protein is inactive in cells because it is blocked by inhibitors (Brown et al., 1978; Nakanishi et al., 1988). The 200-fold increase in reductase protein is due to the synergistic effect of smaller changes occurring at three levels: induction of transcription produces high levels of mRNA; each mRNA is translated at higher rate; and the enzyme molecules are degraded more slowly (Nakanishi et al., 1988).

HMG-CoA reductase activity is controlled by several feedback-regulation mechanisms. Physiologically, full suppression of enzyme activity requires both a sterol derived from LDL and a non-sterol metabolite synthesized from mevalonate (Goldstein and Brown, 1990; Nakanishi et al., 1988). Recent evidence indicates that sterol and non-sterol metabolites act at different levels. Sterols repress transcription through effects on SRE-1 (Goldstein and Brown, 1990); however, sterols can not totally repress transcription. The rate of translation of HMG-CoA reductase mRNA is controlled by the cell's demand for non-sterol isoprenoids. When mevalonate production is blocked by a reductase inhibitor, the reductase mRNA is efficiently translated even in the presence of sterols, but when the non-sterol requirements are satisfied by administration of exogenous mevalonate or together with sterols, translation of reductase mRNA is reduced (Nakanishi et al., 1988). Both sterol and non-sterol metabolites are required for the accelerated degradation of reductase. Sterols accelerate reductase degradation in part by diverting the mevalonate into a non-sterol regulatory product, but sterol alone does not accelerate degradation of the reductase (Nakanishi et al., 1988). Acceleration requires the addition of exogenous mevalonate, presumably for incorporation into a non-sterol product (Goldstein and Brown, 1990). The discovery of farnesylated proteins raises new mechanistic possibilities in this regard (see below).

1.2.4 Mevalonate requirement in cell growth
Mevalonate is an important compound for cell growth and cycling (Fairbanks et al., 1984; Habenicht et al., 1980; Maltese et al., 1985; Quesney et al., 1979). Blocking or absence of the activity of HMG-CoA synthase or reductase will 1) arrest cell cycling in vitro even if growth factors are provided; 2) prevent cell growth in the absence of lipoproteins unless adequate amounts of mevalonate are supplied; and 3) suppress tumor growth in vivo (Maltese et al., 1985). Mevalonate requirements are partially reduced by cholesterol supplied in LDL. Only when mevalonate is available can cells eliminate their HMG-CoA reductase activity.

As shown in Figure 1.1, mevalonate as the precursor of isoprenoid groups (such as isopentenyl-diphosphate, dimethylallyl-diphosphate, geranyl-diphosphate and farnesyl-diphosphate) plays a central role in isoprenoid biosynthesis. The mevalonate pathway allows the synthesis of several classes of end-products in addition to cholesterol. They include 1) isopentenyladenine which is found in some types of tRNA. 2) dolichyl phosphates which supply oligosaccharides in the process of N-linked protein glycosylation, 3) geranylgeranylated and farnesylated proteins such as G-proteins and lamin B which are involved in signal transduction pathways for cell proliferation and survival, and 4) polyisoprenoid side chains of ubiquinone and heme-a which transport electrons in the mitochondrial respiratory chain (Goldstein and Brown, 1990; Maltese, 1990).

Although the potential roles of these diverse isoprenoid products in cell cycling remain to be clarified, bulk products such as ubiquinone or dolichol are unlikely to play an important role in cell cycling, because cells should have a sufficient storage pool of these metabolites to allow at least one round of cell division (Goldstein and Brown, 1990). Furthermore, in the absence of mevalonate, the protective effect on cell growth and morphology was not achieved by supplying the cells with cholesterol, isopentenyl-adenine, ubiquinone or dolichol (Maltese, 1990). Rather, some regulatory molecules, which must be synthesized at a precise phase of the cell cycle to allow DNA synthesis are more likely to
play a role. It has been suggested as a possible mechanism of cell cycle inhibition that blocking mevalonate synthesis prevents isoprenylation of proteins such as G-proteins and nuclear envelope proteins, after discovering that these proteins are covalently attached to isoprenyl residues, which anchor them to cell membranes (Goldstein and Brown, 1990; Maltese, 1990).

1.2.5 Isoprenylated proteins

Several isoprenylated proteins with different molecular masses have been reported. These proteins include Ras proteins, nuclear lamins, Ras-related low molecular mass GTP-binding proteins (G-proteins), heterotrimeric G proteins and several others (Avraham and Weinberg, 1989; Beck et al., 1988; Chardin et al., 1988; Chardin and Tavitian, 1989; Didsbury et al., 1989; Hancock et al., 1989; James et al., 1994; Kawata et al., 1988; Maltese and Robishaw, 1990; Mumby et al., 1990; Pizon et al., 1988; Vorburger et al., 1989; Wolda and Glomset, 1988; Yamane et al., 1990; Zahraoui et al., 1989).

Protein isoprenylation can be subclassified into geranylgeranylation or farnesylation (see Figure 1.1). Protein isoprenylation is mediated by a series of post-translational modifications (Zhang and Casey, 1996). All isoprenylated proteins contain a CaaX (an acronym that refers to Cys (C), a usually aliphatic amino acid (a), and another amino acid (X)) carboxyl-terminal amino acid sequence motif, or CC or CxC residues, which serve as a signal sequence for protein isoprenylation (Maltese, 1990). Proteins containing the CaaX motif are varying groups of proteins whereas CC or CxC-containing proteins are almost all members of the Rab family of small G-proteins that participate in intracellular membrane trafficking (Zhang and Casey, 1996).

Three known enzymes, protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase-I) and protein geranylgeranyltransferase type II (GGTase-II), catalyze the reaction of protein isoprenylation. For farnesylation, the process includes: 1) immediately after translation, when X is serine or methionine or glutamine, the
addition of the 15-carbon isoprenyl compound farnesyl (derived from farnesyl diphosphate) to the sulphydryl group of cysteine in the carboxyl-terminal CaaX motif. This reaction is catalyzed by the enzyme FTase; 2) subsequent to farnesylation, proteolytic removal of the aaX amino acids and methylation of the carboxyl group of cysteine. This type of modification is common to a number of proteins, such as Ras proteins, nuclear lamins A and B, RhoB and Rap2, phosphorylase kinase, rhodopsin kinase, cGMP phosphodiesterase, and the γ subunit of transducin (Kohl et al., 1995). By a similar process, when X is a leucine, a 20-carbon geranylgeranyl group is used by the enzyme GGTase-I for geranylgeranylation of many additional proteins, including some G proteins such as Rac, Rho, and Rap involved in signaling pathways (Rilling et al., 1990). In the case of CC or CxC-containing proteins, GGTase-II transfers a geranylgeranyl group from GGPP to both cysteine residues of CC or CxC in a process mechanistically distinct from that of the CaaX proteins. Additionally, proteins containing the CxC motif are methylated at the C-terminal prenylcysteine while CC-containing proteins are not (Zhang and Casey, 1996). The prenyl group is required for protein modification and cellular localization to perform appropriate biological functions.

1.2.5.1 Ras proteins

The Ras proteins, including H-Ras, K-Ras4A, K-Ras4B and N-Ras, are small (molecular weight of 21 kDa) guanine nucleotide-binding proteins (G-proteins) involved in the transduction of growth-proliferative signals from membrane to nucleus (Barbacid, 1987). K-Ras4A and K-Ras4B are the products of a single K-ras gene through a single alternatively spliced transcript by which exon 4a or 4b is spliced onto exon 3 during mRNA processing (Barbacid, 1987). Ras proteins cycle between active (GTP-bound) and inactive (GDP-bound) states via the protein's intrinsic GTPase activity and a number of accessory proteins. Ras proteins are stably localized to the inner surface of the cell membrane where they transduce growth signals from tyrosine kinase receptors to mitogen activated protein
(MAP) kinases and other downstream molecules en route to the nucleus (Marshall, 1994; Khosravi et al., 1998) (see Figure 1.2).

**Figure 1.2.** Overview of Ras signal transduction pathway (adapted from Khosravi et al., 1998)

Isoprenylation is the first step in a series of post-translational processing events required for the stable association of Ras with the cell membrane (Hancock et al., 1989). Blocking mevalonate synthesis results in cytosolic accumulation of nonisoprenylated Ras precursor (23-24 kDa) (Casey et al., 1989; Hancock et al., 1989; Leonard et al., 1990; Schafer et al., 1989; Ura et al., 1994). This results in loss of transforming activity of
oncogenic Ras protein (Casey et al., 1989; Schafer et al., 1989).

1.2.5.2 Nuclear lamins

The association of isoprenylated proteins with the nuclear matrix (Maltese and Sheridan, 1987) raised the possibility that these proteins were components of nuclear lamins. It has been identified that lamin A and lamin B are posttranslationally modified through isoprenylation (Beck et al., 1988; Wolda and Glomset, 1988). Isoprenylation of lamins plays an important role in determining their association with the nuclear envelope and nuclear envelope function.

In the case of lamin B, the isoprenyl group is retained after processing, and may therefore contribute to the relatively stable association of this protein with the nuclear membrane (Maltese, 1990). For lamin A, isoprenylation initially directs the translation product (prelamin A) to the nuclear membrane, but the entire C-terminal region containing the isoprenoid is subsequently removed. Continued association of mature lamin A with the nuclear envelope depends on protein-protein interactions, for example the interactions of the α-helical domain (Holtz et al., 1989).

1.2.5.3 Ras-related low molecular mass G-proteins

Mammalian cells contain many low molecular weight G-proteins, such as Rho, Rap, Rab, Ral and Rac, which are different from p21Ras (H, K and N) structurally. However, regions contributing to the guanine nucleotide-binding site exhibit homologies to Ras. All these proteins have C-terminal CaaX motifs and could undergo farnesylation and geranylgeranylation (Avraham and Weinberg, 1989; Chardin et al., 1988; Chardin and Tavitian, 1989; Didsbury et al., 1989; Kawata et al., 1988; Maltese and Sheridan, 1990; Maltese et al., 1990; Mumby et al., 1990; Pizon et al., 1988; Yamane et al., 1990; Zahraoui et al., 1989).
1.2.5.4 Heterotrimeric G proteins

The trimeric guanine nucleotide-binding proteins (G proteins) consist of \( \alpha, \beta, \) and \( \gamma \) subunits, which play essential regulatory roles in receptor-mediated signal transduction pathways in mammalian cells. Among these, the \( \alpha \) subunit and two \( \gamma \) subunits contain C-terminal CaaX motifs which could undergo isoprenylation (Maltese and Robishaw, 1990; Mumby et al., 1990; Yamane et al., 1990). It has been suggested that isoprenylation of \( \gamma \) subunits confers membrane affinity on the \( \beta\gamma \) complex. The \( \alpha \) subunit of mammalian G proteins does not appear to be isoprenylated, reflecting the fact that it undergoes a different type of modification (Maltese, 1990).

1.2.6 Functional consequences of protein isoprenylation

1.2.6.1 Membrane targeting and cellular localization

Most isoprenylated proteins are localized at cell membranes, at least for a portion of their lives, and the isoprenoid modification is essential for this membrane association. This property was first demonstrated with Ras proteins, and has been confirmed with many other isoprenylated proteins (Clarke, 1992; Glomset and Farnsworth, 1994). All steps of protein isoprenylation (prenylation, proteolysis and methylation) are important for stable membrane association (Zhang and Casey, 1996). However, additional factors must be important in directing these proteins to specific cell membranes. For example, farnesylated Ras proteins are localized to the plasma membrane; geranylgeranylated trimeric G-proteins are associated with the plasma membrane; and geranylgeranylated Rap proteins are localized on Golgi (Zhang and Casey, 1996). Analysis of chimeras of Rab proteins has revealed that the highly variable C-terminal domain of these proteins determines their specific membrane localization (Chavrier et al., 1991). In addition, for the case of lamin B, a nuclear localization signal in the protein is required for its appropriate subcellular localization (Holtz et al., 1989).
However, localization of some isoprenylated proteins can be modulated. Rab proteins need to cycle between membrane and cytosol as part of their mechanism of action (Lino-Gonzalez and Scheller, 1999). SOS (the product of gene son-of-sevenless) and GDP dissociation inhibitor (GDI) proteins are required to bind to Rab and induce its dissociation from the membrane (Lino-Gonzalez and Scheller, 1999; Zhang and Casey, 1996).

1.2.6.2 Protein-protein interactions

Most isoprenylated proteins play important roles in signal transduction. The following observations support the importance of a prenyl group in protein-protein interactions for signal transduction.

SOS forms a complex with farnesylated, but not with unprocessed, K-Ras, and SOS catalyzes the nucleotide exchange much more efficiently on farnesylated K-Ras than on the unfarnesylated form (Porfiri et al., 1994). As noted above, cellular protein GDI can form a complex with isoprenylated Rabs but not un-isoprenylated ones. GDI retains GDP-bound isoprenylated Rabs within the cytosol by preventing them from binding to membranes, and can induce dissociation of the GDP-bound form of isoprenylated Rabs from membranes (Lino-Gonzalez and Scheller, 1999; Wiedemann and Cockcroft, 1998; Zhang and Casey, 1996). Processing of prenylated proteins can also influence their assembly into multi-subunit complexes. Although both isoprenylated and un-isoprenylated γ subunit can form dimers with the β subunit of trimeric G-proteins, only isoprenylated γ in the βγ complex can interact with the α subunit to form trimeric G-proteins (Casey, 1994).

1.2.7 Conclusion

In summary, the mevalonate pathway plays an important role in cell growth and proliferation. The blocking of this pathway thus presents an attractive target for potential novel cancer treatments.
1.3 HMG-CoA reductase inhibitor: Lovastatin

1.3.1 Metabolism of lovastatin

Lovastatin, a fungal metabolite derived from Aspergillus terreus, is one of several competitive inhibitors of HMG-CoA reductase (Tobert et al., 1982). When given orally, lovastatin is absorbed from the gastrointestinal tract and is hydrolyzed in the liver to its active β-hydroxyacid form. Lovastatin undergoes extensive first-pass metabolism in the liver, and less than 5% of the oral dose has been reported to reach the circulation (Reynolds, 1996). Peak plasma concentrations occur within 2 to 4 hours, and steady-state concentrations are achieved after 2 to 3 days with once daily administration. Although the half-life of lovastatin form is 3 hours (USPDI, 1994), its duration of action is 4 to 6 weeks after withdrawal of continuous therapy (USPDI, 1994). Both lovastatin and its β-hydroxyacid metabolite are extensively bound to plasma proteins (Reynolds, 1996). It is mainly excreted in the bile; about 83% of the administered dose has been recovered from the feces and about 10% from the urine (USPDI, 1994). Because lovastatin is lipophilic, it is capable of penetrating the blood-brain barrier in concentrations that may have pharmacologic effects (Botti et al., 1991), however, parenteral administration may be necessary to achieve effective CNS dosages (Desager and Horsmans, 1996; Sumi et al., 1992).

1.3.2 Potential of lovastatin as an anti-cancer drug

Because the mevalonate pathway involved in cellular growth control is blocked by inhibitors of HMG-CoA reductase, these inhibitors have been suggested to be used as potential antineoplastic agents in the therapy of human cancer (Goldstein and Brown, 1990; Hancock et al., 1989; Keyomarsi et al., 1991; O’Donnell et al., 1993; Schafer et al., 1989; Torres et al., 1994).
Lovastatin is currently used to treat patients with hypercholesterolemia. The dose is 10 to 20 mg initially and up to 80 mg daily. It is only rarely accompanied by significant side effects (blurred vision, impotence, and insomnia have been reported) (Reynolds, 1996). Lovastatin affects not only cholesterol biosynthesis, but also the production of non-steroidal mevalonate derivatives (Tobert et al., 1982). Therefore, it can affect major signaling pathways within the cell. As discussed in detail above, several signal transduction proteins (e.g. Ras-proteins, nuclear lamins and heterotrimeric G proteins) require the post-translational modification by isoprenylation in order to anchor to plasma or nuclear membranes (Khosravi et al., 1992). Mevalonate, the product of the HMG-CoA reductase catalyzed reaction, is the essential precursor for the synthesis of isoprenoids. Thus, inhibition of HMG-CoA reductase by lovastatin leads to reduced isoprenylation of these proteins, and leaves them unable to execute their mitogenic or oncogenic activity (Khosravi et al., 1992). As evidence to support this theory, lovastatin has been shown to block signal transduction through the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor, insulin receptor, and lipopolysaccharide pathways (Law et al., 1992; McGuire et al., 1993; Vincent et al., 1991), which presumably account for the described effects of this drug on the growth of tumor cells both in vivo and in vitro. Illustrative of its potential as an anti-neoplastic agent, lovastatin has been shown to block cell cycling in G1 and at G2/M for a variety of tumor cell lines in vitro (Maltese, 1984; Newman et al., 1994; Sumi et al., 1992). Most importantly, it has been reported that lovastatin caused growth arrest and neuronal differentiation in neuroblastoma cell line NUB-7 (Dimitroulakos et al., 1994), and suppressed the growth of murine neuroblastoma tumors in vivo (Maltese et al., 1985). This suggested a possible role in growth and differentiation for the downstream products of HMG-CoA reductase.

In addition to its inhibitory effects on tumor cell proliferation, lovastatin has been shown to induce apoptosis in human acute T-cell leukemia (Bansal et al., 1989), HL-
promyelocytic cells (Perez and Mollinedo, 1994) and human malignant glioma cells (Jones et al., 1994).

In a preliminary survey by Macaulay, Northern blotting showed that expression of HMG-CoA reductase was higher in the developing cerebellum than elsewhere in the nervous system, and higher than systemic tissue including liver. Since cell proliferation within the cerebellum is high during this period, these cells may be uniquely dependent on HMG-CoA reductase activity to maintain mitotic activity before terminal differentiation. It has been demonstrated that high levels of HMG-CoA reductase mRNA are present in human neuroblastoma cells (Dimitroulakos and Yeger, 1996) and MB-derived cell lines (Macaulay et al., 1994). Therefore, it was postulated that HMG-CoA reductase activity is critical to MB cell survival, and inhibition of HMG-CoA reductase would reduce MB cell proliferation and induce cell death. This important postulate is a component of the hypotheses tested in this thesis (section 1.9).

1.4 Farnesyl transferase inhibitor: Manumycin A

The mevalonate derivatives geranyl and farnesyl pyrophosphate are required for the isoprenylation of a variety of membrane-bound proteins, including several signal transduction proteins that influence cellular proliferation. A number of FPTase inhibitors have been characterized. Selective and potent FPTase inhibitors can be divided into categories: CaaX peptidomimetics or pseudopeptides (James et al., 1993; Kohl et al., 1993), and farnesylpyrophosphate analogs (Manne et al., 1995; Tamanoi, 1993). Several of these compounds modulate critical aspects of transformation-related functions of p21 Ras in cell cultures, including inhibition of anchorage-dependent and -independent growth, reversal of transformed morphology and restoration of the actin cytoskeleton (James et al., 1993; Kohl et al., 1993; Manne and others, 1995; Prendergast et al., 1994).

Recently, a potent and specific FPTase inhibitor, manumycin A, was isolated from a strain of Streptomyces and shown to be identical to the antibiotic compound
manumycin (Hara et al., 1993). Using a cell-free system, it was demonstrated that manumycin A acts as a competitive inhibitor of FPTase with respect to farnesyl-diphosphate, and as a non-competitive inhibitor with respect to p21 Ras. Although it has some activity against GGPTase, the IC50 of manumycin A for yeast FPTase is 5 μM, and for rat brain FPTase is 35 μM, whereas it is 180 μM for bovine brain GGPTase (Hara et al., 1993). In human cell cultures, 30 μM manumycin A has no effect on protein geranylgeranylation (Nagase et al., 1996), thus, manumycin A at this concentration selectively inhibits protein farnesylation.

1.5 GTP depletion with mycophenolic acid

As noted above, G-proteins control cell growth through several mechanisms. GTP is required for G-protein function (see Figure 1.2), thus depletion of GTP will abolish G-protein function.

**Figure 1.3.** Schematic representation of biosynthetic pathways for guanine nucleotides
Mycophenolic acid (MPA), an inhibitor of IMP dehydrogenase, blocks the conversion of IMP to GMP, which is the precursor of GDP and GTP (see Figure 1.3), and thus depletes cellular GTP. MPA is a new immunosuppressive agent widely used in clinical therapy (Allison and Eugui, 1994). This compound also has significant antitumor activity, for example the growth inhibition of human leukemia cells and the induction of neuroblastoma cell apoptosis (Catapano et al., 1995; Messina et al., 1998).

1.6 Regulation of the cell cycle

The cell cycle is typically divided into four phases. The periods associated with DNA synthesis (S Phase) and mitosis (M Phase) are separated by varying length G1 and G2 phases. The orderly progression of the cell cycle is critical for accurate transmission of genetic information, and consequently, a number of checkpoints have evolved to ensure that the initiation of a particular cell cycle event is dependent on the successful and accurate completion of the others.

1.6.1 Cyclins and CDKs in cell cycle control

The key players regulating passage through major checkpoints in the cell cycle are a family of CDKs (cyclin dependent kinases), and their obligate activating partners, the cyclins (Figure 1.4). A CDK must be associated with a cyclin partner to become an active holoenzyme. In mammalian cells, the cyclins oscillate dramatically in phase with the cell cycle, different cyclins accumulate periodically, and different cyclin/CDK complexes are assembled and activated at specific points of the cell cycle (Hunter and Pines, 1994; Sherr, 1994). The D-type cyclins with their catalytic partners CDK4 and CDK6 function as cells leave G0 and progress through G1. D cyclins are absent in quiescent cells and their expression is stimulated by growth factors. In contrast to other cyclins, their levels do not fall abruptly at any point of the cell cycle and only a modest peak of accumulation is observed in late G1. However, their expression is dependent on the growth factor supply.
and consequently, if growth factors are removed, D cyclin levels drop rapidly, regardless of the stage of the cell cycle. Cyclin E is another mammalian G1 cyclin, which is synthesized later than D-type cyclins, peaks in late G1. Cyclin E associates with CDK2. Cyclin E/CDK2 is activated in late G1. After cells have entered S phase, cyclin E is degraded and CDK2 then associates with cyclin A. Cyclin A synthesis is initiated during late G1 and its associated kinase activity is first detected in S phase. Cyclin A/CDK2 operates in S phase, and is required for DNA replication. Cyclin A also binds Cdc2 (also named CDK1) with peak activity in G2 and then it is rapidly degraded in G2. Cyclin B binds Cdc2 forming mitosis-promoting factor (MPF), which triggers entry into M phase. Finally, Cyclin B destruction is required to exit mitosis (Grana and Reddy, 1995; Hall and Peters, 1996; Hunter and Pines, 1994; Sherr, 1996). Many cyclin/CDK complexes are inactivated by degradation of the cyclin. Cyclins A and B are degraded by cell cycle-dependent proteolysis, while cyclins D and E are intrinsically unstable throughout the cycle.
(Hall and Peters, 1996).

The activity of cyclin/CDK complexes not only depends on the synthesis of cyclin, but also is regulated by phosphorylation/dephosphorylation of critical threonine and tyrosine residues on the CDK catalytic subunit (Solomon, 1993). This regulatory network in mammalian cells is not completely understood.

1.6.2 CKIs in cell cycle control

One further level of cell cycle control is the expression of specific CDK inhibitors (CKIs) (Figure 1.4). Two families of CKIs have been identified in mammalian cells (Grana and Reddy, 1995; Hall and Peters, 1996; Sherr, 1996). One group, comprising related proteins p21WAF1, p27KIP1 and p57KIP2, appears to function as broad specificity inhibitors of cyclin/CDK complexes.

p21WAF1 has at least two separate roles, acting as a CDK inhibitor and a PCNA inhibitor. Two separate domains of p21WAF1 are responsible for these two inhibitory activities. p21WAF1 induction may be either p53-dependent or -independent. p21WAF1 expression induces G1 arrest and terminal differentiation (Grana and Reddy, 1995).

p27KIP1 shares partial homology with p21WAF1, and acts as a cyclin E/CDK2 inhibitor that binds to this complex in TGF-β or contact-inhibited G1 arrest. In these G1 arrested cells, cyclin E and CDK2 are expressed, but there are no cyclin E/CDK2 active complexes. p27KIP1 is present in quiescent cells and its levels drop upon growth factor stimulation. p27KIP1 is probably sequestered by cyclin D1/CDK4 or CDK6 complexes, which need to overcome a p27KIP1 threshold to become active when cells progress through G1. cAMP-induced G1 arrest is also mediated by p27KIP1, which binds to cyclin D1/CDK4 complexes and prevents them from being activated by CDK-activating kinase (CAK) (Grana and Reddy, 1995). In G1 arrest induced by other compounds, for example lovastatin, the p27KIP1 family is also responsible for the inhibition of cyclin/CDK
complexes (Hengst and Reed, 1996).

p57KIP2 can interact with and inhibit several cyclin/CDK complexes. Its overexpression blocks cells in G1. p57KIP2 is expressed in terminally differentiated cells, and is probably involved in cell cycle exit of specific cell types (Grana and Reddy, 1995).

The second family of CKIs has four members, namely p15INK4b, p16INK4, p18 and p19. They bind directly to, and are specific inhibitors of, CDK4 and CDK6. p15INK4b expression is induced 30 fold after treatment of human keratinocytes with TGF-β, and this correlates with increased association with its targets CDK4 or CDK6 and with inhibition of CDK6 kinase activity. p18 and p19 mRNA expression and p19 protein levels peak at S phase during the macrophage cell cycle. The expression of p16INK4 and p18 has a dependent relationship with pRb expression (Grana and Reddy, 1995).

Thus, CKIs play an important role mediating negative growth signals that result in cell cycle arrest at different G1 points. Some of these points may allow cells to exit the cell cycle and undergo specific differentiation programs or apoptosis.

1.7 Biochemistry of cell death through apoptosis

Nearly all physiological cell deaths in animals proceed by the process of programmed cell death, named apoptosis (Kerr et al., 1972), during which the dying cells are silently cleared without any accompanying inflammatory response (Green, 1998). In recent years, the significance of apoptosis has become increasingly recognized. Apoptosis is particularly important in the developing nervous and musculoskeletal systems (Raff et al., 1993) and in both the development and the effective functioning of the immune system (Golstein et al., 1991; Williams, 1994). Failure of apoptosis is likely to contribute both to the initial development of cancer and to the appearance of cancer cells resistant to cytotoxic therapy (Williams, 1991). On the other hand, inappropriate induction of apoptosis may be involved in degenerative diseases. Apoptosis may also play an important role in the HIV-induced pathology of AIDS (Thompson, 1995). Understanding the fundamental molecular
mechanisms of apoptosis has therefore become an important challenge, and is likely to lead to as yet unforeseen benefits.

1.7.1 Cellular consequences of apoptosis

During apoptosis, intracellular reorganization occurs. Chromatin becomes fragmented and condensed, the organelles shrink, and the cell-surface blebs leading to budding off of membrane-bound ‘apoptotic bodies’. All these processes likely proceed in parallel (Hale et al., 1996) and may be independent. For example, DNA fragmentation is not required for the cytoplasmic changes of apoptosis (Jacobson et al., 1994).

1.7.1.1 Nuclear changes and DNA fragmentation

During apoptosis, chromatin condenses and the nuclear envelope breaks down. In this process, lamin disassembly occurs by proteolysis and is apparently irreversible (Kaufmann, 1989). The proteolysis is catalyzed by lamin protease (LamP). Lamin B binds to specific DNA sequence motifs called matrix attachment regions, which mediate the interaction of chromatin with the nuclear matrix (Hale et al., 1996). Therefore, it has been suggested that the degradation of lamin may promote the formation of large fragments of DNA by releasing matrix attachment regions to allow access to endonucleases (Neamati et al., 1995).

The biochemical marker of apoptosis in many cells is the formation of distinct DNA fragments. DNA is cleaved between the nucleosomes resulting in regularly-sized DNA fragments of multiples of approximately 180 bp. Large DNA fragments (30-50 and 200-300 kb), smaller fragments (100 bp) produced by a novel nuclease cleavage, and even single-strand-cleavage events are also observed during apoptosis (Solis et al., 1995). As both large and oligonucleosomal fragments can be independently produced under some conditions (Bortner et al., 1995), the large fragments may not be precursors of the oligonucleosomal fragments.
It is widely assumed that the DNA fragment formation in apoptosis is the result of endogenous neutral \( \text{Ca}^{2+}/\text{Mg}^{2+} \) dependent endonuclease activity which is capable of inducing double strand breaks at internucleosomal sites (Arends et al., 1990). The endonuclease produces \( 3'\)-OH DNA breaks, and can be inhibited by zinc (Earnshaw, 1995). Several candidate endonucleases have been reported, including DNase I, DNase II, Nuc 18 and Nuc 1 (Hale et al., 1996). The nuclease which is responsible for large fragment formation is not identified so far.

1.7.1.2 Cytoplasmic changes

One of the most noticeable morphological features of apoptosis is the fragmentation of cell into apoptotic bodies. A rearrangement of the microfilament network of cell must occur during this process and may be responsible for some of the observed changes (Hale et al., 1996). Microtubule disrupting agents induce apoptosis, suggesting that the disruption of the microtubule network is among the events which lead to apoptosis (Martin and Cotter, 1990).

Transglutaminases are a family of \( \text{Ca}^{2+} \) dependent glutamine and glutamyl transferases. They catalyze post-translational modifications, for example the formation of \( N^\epsilon-(\gamma\text{-glutamyl})\)lysine isodipeptide cross links in proteins between the \( \gamma \)-amido of a donor glutamine and the \( \epsilon\)-NH\(_2\) of an acceptor lysine, which form irreversible cross links conferring high resistance to mechanical breakage and chemical attack (Fesus et al., 1989). Tissue-type or type II transglutaminase, a cytosolic protein, selectively accumulates in cells undergoing apoptosis (Piacentini et al., 1994). At the onset of apoptosis there is a large increase in type II transglutaminase mRNA and an elevation in intracellular \( \text{Ca}^{2+} \) sufficient to activate the enzyme. The overexpression of transglutaminase induces the cytoplasmic changes characteristic of cells undergoing apoptosis (Gentile et al., 1992; Melino et al., 1994). The cross linking of intracellular proteins stabilizes the cytoplasm of dying cells, preventing the leakage of harmful intracellular elements into the extracellular environment.
which could lead to an inflammatory response. Actin, annexin II, vinculin, fibronectin and other uncharacterized proteins have been suggested as cross-linked intracellular elements (Knight et al., 1993).

1.7.1.3 Cell membrane alterations

There are extensive cell membrane alterations during apoptosis. Cells detach from neighboring cells, from the culture substrate in vitro or from the extracellular matrix in vivo. Membranes lose specialized structures such as microvilli. Apoptotic cells display cell-surface markers that are important in phagocytic recognition of phagocytic cells (for example macrohage, liver endothelial cells) (Savill et al., 1993).

Integrins are responsible for the recognition of phagocytosis because the phagocytosis of apoptotic bodies can be inhibited by peptides and proteins containing an arginine-glycine-aspartic acid (RGD) sequence (Savill et al., 1990).

A change in the lipid composition of apoptotic cell outer plasma membranes, and a reduced anionic charge, which implies the loss of terminal sialic residues in apoptotic cells, are also implicated in phagocytosis of apoptotic cells (Hale et al., 1996). During apoptosis phosphatidylserine is exposed on the outer membrane surface, which appears to be important in eliciting a phagocytic response (Fadok et al., 1992). The removal of sialic acid residues may unmask other sugar residues such as N-acetylglucosamine and N-acetylgalactosamine which could then interact with a phagocytic recognition lectin on the macrophage surface (Duvall et al., 1985). It has been suggested that lectins are important in the phagocytosis of apoptotic bodies (Dini et al., 1995).

1.7.2 Signal transduction in apoptosis

Cells undergo apoptosis when they receive (or fail to receive) appropriate information from the environment, such as the appearance or disappearance of hormones or cytokines, or a change in direct intercellular interactions.
Examples of death signaling are:

1) sustained release or influx of calcium, which activates endonucleases and induces apoptosis (Lynn et al., 1989). When mitochondria become overloaded with calcium, abnormal mitochondrial metabolism activates apoptosis (Berridge et al., 1998).

2) cAMP elevation. In thymocytes and myeloid leukemia cells, agents that stimulate adenylate cyclase or cause elevation of cAMP cause apoptosis in the rat myelocytic leukemic cell line IPC-81 and thymocytes (Lanotte et al., 1991; McConkey et al., 1990).

3) Activation of phospholipase (PL)-A2. Inhibitors of PL-A2 inhibit TNF-induced cytotoxicity in BALB/c 3T3 cells (Palombella and Vilcek, 1989).

4) Ceramide production. Ceramide is a second messenger released by the hydrolysis of membrane sphingolipids (especially sphingomyelin), and it activates a protein kinase cascade, and then inhibits growth, induces differentiation of leukemia cells, modulates protein phosphorylation and regulates gene transcription (Hale et al., 1996).

5) Fas-like ligand. The FAS/TNF receptor 1 (TNFR1) family of cell-surface receptors mediates cell death and survival (Nagata and Golstein, 1995). Both FAS and TNFR1 contain a 'death domain' in the cytoplasmic region, which is important in inducing apoptosis by activating downstream caspases (Chou et al., 1998; Golstein et al., 1995) (see Figure 1.5).

Examples of viability signals include:

1) Production of phorbol esters. Phorbol esters block calcium ionophore- or glucocorticoid-induced DNA fragmentation in thymocytes (McConkey et al., 1989; McConkey et al., 1992), and spontaneous apoptosis of chicken bursa cells (Asakawa et al., 1993).

2) Tyrosine kinase activity. Tyrosine kinase inhibitors mediate the loss of protective effects of IL-2 and IL-3 on apoptosis (for example in hematopoietic cells), probably by blocking the expression of bcl-2 (Otani et al., 1993).
3) Ras proteins. Ras proteins transfer signals to Raf kinase (Khosravi et al., 1994; Khosravi and Der, 1994). Raf-1 kinase promotes the proliferation of IL-3-dependent myeloid cells and suppressed apoptosis induced by IL-3 withdrawal (Cleveland et al., 1994). The activation of PI3K pathway by Ras contributes to neuronal survival (Dudek et al., 1997). Ras-related proteins inhibit apoptosis via the regulation of the Bcl-2 protein and the consequences of this regulation (Wang et al., 1995; Wang et al., 1994).

**Figure 1.5.** Overview of caspase activation and apoptosis (Bantel et al., 1999; Chou et al., 1999; Chou et al., 1998; Green, 1998; Stennicke and Salvesen, 1998).

1.7.3 Gene expression in apoptosis

After demonstrating that inhibitors of macromolecular synthesis retard the process of apoptosis (Cohen and Duke, 1984), it has been suggested that the process of
apoptosis is under some form of genetic control. Several genes involved in the regulation of apoptosis have been identified, such as pRB, ras, p53, myc, E2F, fos, jun, and the mammalian family of ced-9-related genes.

Retinoblastoma protein (pRB) not only controls cell cycle progression but also plays a critical role in the development and differentiation of certain cell types. Deficient pRB results in cell division and cell death via apoptosis in the haematopoietic and nervous systems (Clarke et al., 1992), suggesting that pRB inhibits proliferation and apoptosis.

p53 phosphoprotein is a transcription factor (Levine et al., 1991). It functions to promote differentiation and apoptosis in certain cellular contexts (Yonish et al., 1991) and as a cell cycle regulator that can induce cell cycle arrest in G1 phase following DNA damage (Kastan et al., 1991). If the damage cannot be repaired, then apoptosis is triggered.

The expression of myc, E2F, fos, and jun transcription factors is important for cell proliferation, but also drives the process of apoptosis in some experimental systems. For example, a) the continued presence of myc under conditions of growth arrest, as in murine myeloid cells or Rat-1 fibroblasts deprived of growth factor, will induce apoptosis (Askew et al., 1991; Evan et al., 1992; Hermeking and Eick, 1994); b) Over-expression of E2F drives quiescent cells into S phase, prevents cycling cells from exiting the cell cycle, transforms rat embryo fibroblasts, and ultimately leads to apoptosis (Kowalik et al., 1995); c) IL-2- and IL-6-dependent myeloma cell lines undergo apoptosis after growth factor deprivation, accompanied by rapid and transient induction of fos and jun. Inhibition of fos and jun gene expression protects the cells from apoptosis, suggesting an active role for fos and jun in the onset of apoptosis (Colotta et al., 1992).

The identified human ced-9-related genes include bcl-2, bcl-x, mcl-1, bax, bak and bak-2. bcl-2 is a lymphoma-associated proto-oncogene, which prevents apoptosis and prolongs the survival of non-cycling cells (Vaux et al., 1988). bcl-x produces two protein products, Bcl-xL and Bcl-xS. Like Bcl-2, Bcl-xL is also a negative regulator of apoptosis
(Boise et al., 1993). In contrast, Bcl-xS allows apoptosis to proceed in IL-3-deprived cells even in the presence of Bcl-2 (Boise et al., 1993). Mcl-1 is a negative regulator of apoptosis and can delay apoptosis when c-myc is overexpressed (Reynolds et al., 1994). Baxα, the principle product of the bax gene, binds to Bcl-2 (Oltvai et al., 1993) and acts in opposition to Bcl-2 (Oltvai et al., 1993). Overexpression of bax allows apoptosis to proceed after IL-3 withdrawal from IL-3-dependent cells even in the presence of dysregulated expression of bcl-2 (Oltvai et al., 1993). Interestingly, p53 is a direct transcriptional activator of human bax gene (Miyashita and Reed, 1995), and the expression of the bax gene serves as a marker of p53-dependent apoptosis (Grasso and Mercer, 1997; Liebermann et al., 1995; Yln et al., 1997). bak is expressed in a wide range of tissues (Farrow et al., 1995; Kiefer et al., 1995). Like Bax, Bak accelerates the apoptotic death of IL-3 deprived cells and partially blocks the inhibition of apoptosis by Bcl-2 (Chittenden et al., 1995). Bak also accelerates apoptosis in growth-factor-deprived neurons (Farrow et al., 1995).

1.7.4 Activation and regulation of proteases in apoptosis

The signals that cause apoptosis act through a variety of pathways, but all converge on a common end point which is the activation of proteases. Almost all mammalian cells express several cell death proteases, even when they are not undergoing apoptosis. The proteases can then be activated without having to be synthesized anew, and apoptosis can be induced without influencing transcription from protease genes (Jacobson et al., 1994; Vaux and Weissman, 1993). Intracellular proteases might play a critical role in the initiation of apoptosis. Proteins shown to be cleaved during apoptosis include poly-(ADP ribose) polymerase (PARP) (Lazebnik et al., 1994), lamin B (Neamati et al., 1995), a 70-kDa protein (Casciola et al., 1994), topoisomerasers I and II, histone H1 (Kaufmann, 1989), protein kinase cβ1, cPLa2 (Voelkel et al., 1995), fodrin (Martin et al., 1995) and a 58-kDa protein kinase PITSLRE (Lahti et al., 1995). The most widely studied group of
proteases in apoptosis is the cysteine proteases (Bruno et al., 1992; Sarin et al., 1993).

In mammals 13 different cysteine proteases, the homologues of Ced-3, have been identified (named caspase-1 to caspase-13). They have distinct roles in apoptosis and inflammation (Thornberry and Lazebnik, 1998). Human caspase-2, -8, -9 and -10 function as initiators of apoptosis, caspase-3, -6 and -7 function as effectors of apoptosis, and caspase-1, -4 and -5 function in cytokine activation (Stennicke and Salvesen, 1998). Human counterparts of caspase-11 and -12 have not known yet. Over-expression of caspase genes can cause apoptosis, and this death can be inhibited by interfering with protease function.

All of these cysteine proteases are initially translated as inactive precursor polypeptides. After cleavage at aspartate residues and assembly into heterotetramers, they become active and trigger apoptosis. The active cysteine is in the middle of a conserved QACRG motif common to all the proteases. All of these cysteine proteases cleave their substrates distal to aspartate residues (Munday et al., 1995; Nicholson et al., 1995; Sleath et al., 1990). The requirement for all known Ced-3-like proteases to be processed at aspartate residues, together with their ability to cleave at aspartate residues, suggests that some of them could activate themselves (e.g. caspase 8 (Yang et al., 1998)) or act on each other in a hierarchical cascade. Mature caspase-1 (also called ICE) can activate pro-caspase-1 as well as pro-caspase-3 (also called CPP32) (Cerretti et al., 1992; Faucheu et al., 1995; Miller et al., 1993; Tewari et al., 1995). Such interactions between cysteine proteases have been observed both in vitro and in cellular systems.

Caspase-2 or -8 or -9 or -10 is activated when they receive apoptotic signals, and then they activate a proteolytic cascade including the cleavage of caspase-3, caspase-6 and caspase-7 (Green, 1998) (see Figure 1.5).

Activated caspase-3 can cleave and inactivate PARP, an enzyme that is used for DNA repair (Lazebnik et al., 1993; Lazebnik et al., 1994; Nicholson et al., 1995). PARP cleavage products can be used as a marker of apoptosis (Kaufmann et al., 1993). The
cleavage of PARP results in the separation of its DNA binding motifs from the poly(ADP ribos)ylating catalytic domains. Poly(ADP ribos)ylation negatively regulates the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent endonuclease implicated in the internucleosomal DNA cleavage which occurs during apoptosis; therefore loss of PARP function could result in the activation of the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent endonuclease in dying cells. Intriguingly, although active caspase-3 alone can cleave PARP, on its own it does not cause apoptotic changes in isolated nuclei, suggesting that caspase-3 must act on a substrate within the cytoplasmic extract to precipitate apoptotic changes in nuclei (Nicholson et al., 1995), or that activation of caspase-3 alone is not sufficient for apoptosis (Hale et al., 1996).

Like caspase-3, caspase-7 can also cleave and inactivate PARP, resulting in the activation of endonuclease and DNA fragmentation (Stennicke and Salvesen, 1998). In addition, caspase-3 and caspase-7 have other effects such as activating kinases and inactivating mRNA splicing (Stennicke and Salvesen, 1998) (see Table 1.2).

Table 1.2. Summary of the Effects of Apoptosis Effector Caspases

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Substrate</th>
<th>Cleavage site</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>proIL-1β</td>
<td>YVHD/A</td>
<td>Activates IL-1β</td>
</tr>
<tr>
<td></td>
<td>proIGIF (IL-18)</td>
<td>LESD/N</td>
<td>Activates IGIF</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>DFF</td>
<td>DETD/S</td>
<td>Initiates DNA fragmentation</td>
</tr>
<tr>
<td>and-7</td>
<td>PARP</td>
<td>DEVD/G</td>
<td>Inactivates DNA repair</td>
</tr>
<tr>
<td></td>
<td>PKCG</td>
<td>DMQD/N</td>
<td>Activates kinase</td>
</tr>
<tr>
<td></td>
<td>MEKK</td>
<td>DTVD/G</td>
<td>Activates kinase</td>
</tr>
<tr>
<td></td>
<td>U1-70kDa</td>
<td>DGPD/G</td>
<td>Inactivates mRNA splicing</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Lamin A</td>
<td>VEID/N</td>
<td>Nuclear collapse</td>
</tr>
<tr>
<td></td>
<td>Keratin 18</td>
<td>VEID/A</td>
<td>Cytoskeletal collapse</td>
</tr>
</tbody>
</table>

The activation of caspase-6 cleaves lamin A and thus results in nuclear collapse; the activation of caspase-6 can also cleave keratin 18 and lead to cytoskeletal collapse (Stennicke and Salvesen, 1998) (see Table 1.2).

Mature caspase-1 cleaves proIL-1β to produce mature IL-1β. However, overexpression of caspase-1 induces apoptosis in rat fibroblast cells which do not express
proIL-1β (Miura et al., 1993). Thus, IL-1β does not appear to play a crucial role in induction of apoptosis (Black et al., 1989). Caspase-1 probably has an additional as yet undefined substrate which functions in the control of apoptosis in some circumstances, or there is some redundancy in the genes involved in the control of apoptosis by the caspase family (Hale et al., 1996; Vaux and Strasser, 1996).

The effects of caspase-1, -3, -6 and -7 are summarized in Table 1.2 (adapted from Stennicke and Salvesen, 1998).

1.8 Summary

A summary of the above points directly relating to this thesis follows:

1. MB is the most common malignant brain tumor in children, and has a guarded prognosis with standard radiation therapy and chemotherapy. Thus, the development of novel therapeutic strategies is required.

2. The mevalonate pathway plays an important role in cell proliferation and survival. Because of the high levels of HMG-CoA reductase mRNA in MB and the developing cerebellum, we proposed blocking this pathway in MB therapy.

3. Blocking the mevalonate pathway can be achieved by lovastatin inhibition of HMG-CoA reductase. Some specific functions dependent on mevalonate, such as protein isoprenylation and G-protein function, may be critical for cell growth and survival. Inhibition of these processes presents an attractive target for MB therapy.

4. Caspase activation is an important event in apoptosis, and DNA fragmentation (laddering) is a consequence of apoptosis; both can be used as biochemical markers of apoptosis.

5. Fresh primary MBs and MB cell lines representing a variety of phenotypes serve as experimental models for studies of MB biology.
1.9 Hypotheses

I hypothesize:

1) that HMG-CoA reductase activity is critical to MB cell survival, and that blocking mevalonate pathway by inhibition of HMG-CoA reductase will reduce MB cell proliferation and induce cell death via apoptosis.

2) that protein isoprenylation (for example farnesylation) and G-protein function are more critical for MB cell growth and survival.

3) that ras, bcl-2, bax, p53, c-myc, CDK inhibitor genes and HMG-CoA reductase gene expression mediate isoprenylation inhibition-induced apoptosis and inhibition of proliferation.

1.10 Research objectives

In order to examine lovastatin effects and the possibility of this potential therapeutic approach, four different MB cell lines, Daoy, UW228, D341 Med and D283 Med, and two primary MBs were used as models to test the above hypotheses.

The following objectives were addressed:

A. Initial studies

To evaluate whether lovastatin reduces MB cell proliferation and induces cells to undergo apoptosis.

B. Mechanism of lovastatin-induced apoptosis

1). To determine whether physiological regulation of HMG-CoA reductase gene expression occurs during isoprenylation inhibition-induced apoptosis. To achieve this goal, HMG-CoA reductase expression was evaluated at the transcription level after lovastatin and manumycin A treatment.

2). To determine whether lovastatin-induced changes were the result of specific inhibition of HMGCoA reductase. This was achieved by attempting to overcome the
effects of lovastatin by exogenous administration of mevalonate, the product of the biochemical reaction catalyzed by HMGCoA reductase.

3). To explore whether blocking protein isoprenylation accounts forlovastatin-induced cell death in MB; and if so, which isoprenylation reaction (farnesylation and/or geranylgeranylation) may be responsible for MB survival.

4). To assess whether blocking G-protein function accounts for MB apoptosis following lovastatin administration.

C. Molecular regulation in isoprenylation inhibition-induced apoptosis

1). To investigate whether downregulation of bcl-2 expression occurs in MB apoptosis.

2). To evaluate whether isoprenylation inhibition-induced apoptosis is p53-dependent, by determining the expression of p53 and bax.

3). To analyze whether cell cycle changes are concomitant with isoprenylation inhibition-induced MB apoptosis, and if so, whether CKIs genes such as p16, p21WAF1 and p27KIP1 contribute to cell cycle changes.

4). To assess whether the caspase-3 pathway is activated in isoprenylation inhibition-induced MB apoptosis.

5). To evaluate whether the difference of gene expression in test cell lines influences their sensitivities to lovastatin.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Unless stated otherwise, all chemicals used were analytical or reagent grade, purchased from either BDH Chemical Company (Toronto, ON), or Fisher Scientific Company (Edmonton, AB).

2.2 Cell lines and cell culture passage

2.2.1 Cell lines

2.2.1.1 Daoy (ATCC HTB-186)

The Daoy cell line was established from a biopsy of a solid tumor tissue in the posterior fossa of a 4-year-old boy with desmoplastic cerebellar MB (Jacobsen et al., 1985). Although the original tumor had characteristics of both neuronal and glial differentiation, these were not retained by the cell line. Daoy represents a glial phenotype of MB, rather than a neuronal phenotype. It is a hypertetraploid human cell line with a modal chromosome number between 93 and 99. The frequency of cells with higher ploidies is 2.0%. It grows as an adherent monolayer (American Type Culture Collection, 1997).

2.2.1.2 UW228

The UW228 cell line was kindly provided by Dr. J. R. Silber (Keles et al., 1995), Department of Neurological Surgery, University of Washington, Seattle, WA. This
cell line was derived from a biopsy of a solid mass of MB tissue in the posterior fossa of a 9-year-old female (Keles et al., 1995). UW228 represents a neuronal phenotype of MB. It has diploid DNA content, and grows as adherent monolayer.

2.2.1.3 D283 Med (ATCC HTB-185)

The D283 Med cell line was originally established (Friedman et al., 1985) from a peritoneal metastasis from a 6-year-old male with MB (Friedman et al., 1985). This cell line is positive for expression of neurofibrillary proteins (NF-L, -M, -H), glutamine synthase and neuron specific enolase but negative for glial fibrillary acidic protein and S-100 protein. It represents a neuronal phenotype of MB. D283 Med cells aggregate in suspension with some adherent epithelial like cells and spontaneous spheroids. Thus, D283 Med is partially attached cell line; when grown on glass cover slips, attachment is more conspicuous. This is a hypodiploid cell line with a frequency of higher ploidies of 5.4%. Its modal chromosome number is 45 (range from 41 to 46) (American Type Culture Collection, 1999).

2.2.1.4 D341 Med (ATCC HTB-187)

The D341 Med cell line was originally derived from tumor tissue obtained at craniectomy from a 3-year-old male with metastatic MB (Friedman et al., 1988). Like D283 Med, the D341 Med cell line grows mostly in suspension as spheroids with some adherent cells. Thus, D341 Med is also a partially attached cell line, and attachment is more conspicuous when grown on glass coverslips. This is a hyperdiploid human cell line with higher ploidies occurring with a frequency of 8.5%. The modal chromosome number is 49 (range from 44 to 50), occurring in 66% of metaphases examined (American Type Culture Collection, 1999). The cell line is positive for expression of neurofibrillary proteins (NF-L, -M, -H), glutamine synthase and neuron specific enolase but negative for glial fibrillary acidic proteins and S-100 protein. The D341 Med also represents a neuronal phenotype of
MB, but apparently at a less advanced stage of neuronal differentiation than D283 Med (Friedman et al., 1988).

2.2.1.5 MRC-5 (ATCC CCL-171)

MRC-5 was kindly provided by Dr. J. Xiang, Saskatoon Cancer Center, University of Saskatchewan, Saskatoon, SK. It is a normal human fibroblast cell line derived from normal human lung tissue. It is diploid, and the karyotype is that of a normal human male. This cell line grows as an adherent monolayer (American Type Culture Collection, 1998).

2.2.2 Cell culture and passage

All cell lines were propagated in Dulbecco's modified Eagle's medium (D-MEM)/12 nutrient mixture supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U of penicillin G per ml, and 50 µg of streptomycin per ml (all media and chemicals for cell culture were from Gibco BRL, Gaithersburg, MD, unless otherwise stated) and grown in either 25 ml or 75 ml tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ). Cells were incubated at 37°C in an incubator with an atmosphere of 5% CO₂ and maximum humidity. Adherent cells (MRC-5, Daoy and UW228) were passaged by trypsinization. When the monolayers were about 80-90% confluent, the old medium was aspirated, cells were rinsed once with Hank’s balanced salt solution and treated with 1 x trypsin-EDTA (0.5 g of trypsin and 0.2 g EDTA per liter) in Hank's balanced salt solution. After removing the trypsin-EDTA, appropriate numbers of cells were taken up with a pipette and deposited into fresh medium. Cells were generally split 1:10 at one week intervals; culture medium was otherwise renewed every 3 days. For partially attached cell lines D283 Med and D341 Med, floating cells were aspirated, spun and resuspended in fresh medium; adherent cells were dislodged by trypsinization, and cell pellets were resuspended; floating cells and adherent cells were recombined and then
returned to the flasks at 1:10 amount. These partially attached cells were also passaged every week, and medium renewed every 3 days.

2.3 Primary medulloblastomas and culture

2.3.1 Primary medulloblastomas

Two primary MBs (98-8 and 98-2627), were resected from a 7-year-old male and a 12-year-old female respectively at the Royal University Hospital, University of Saskatchewan, Saskatoon, SK. Histological examination revealed that 98-8 was composed of uniform cells with minimal cytoplasm and hyperchromatic pleomorphic angulated nuclei. Similarly, 98-2627 consisted of densely packed cells with small hyperchromatic round to slightly elongated nuclei and scant cytoplasm. Immunohistochemical analysis of fixed sections showed that occasional 98-8 cells exhibited glial differentiation (GFAP positive), while occasional 98-2627 cells showed neuronal differentiation (synaptophysin positive).

2.3.2 Primary medulloblastoma culture

Portions of unfixed neoplasms from 98-8 and 98-2627 were minced and incubated in flasks containing Dulbecco's modified Eagle's medium (D-MEM)/F12 nutrient mixture supplemented with 10% FBS, 2 mM L-glutamine, 50 U of penicillin G per ml, and 50 μg of streptomycin per ml. Cells were cultured to subconfluency at 37°C in an incubator with an atmosphere of 5% CO₂ and maximum humidity. Medium was replaced with fresh medium every 3 days; floating cells were collected and returned to their original flasks. 98-8 and 98-2627 were passaged only 0-2 times by trypsinization (see above) before pharmacological agents were administered.

2.4 Lovastatin treatment of medulloblastoma cells
2.4.1 Activation of lovastatin

Lovastatin is inactive in its pro-drug (lactone) form (kindly provided by W. L. Henckler, Merck Research Laboratories, Rahway, NJ). It was converted to its active (dihydroxy open acid) form using NaOH (Keyomarsi et al., 1991) as follows: 52 mg of lovastatin was dissolved in 1.04 ml of 95% ethanol; 813 μl of 1N NaOH was added into the lovastatin solution; after mixing, about 550 μl of 1N HCl was then added to neutralize to pH 7.2; finally, distilled water was added to yield 13 ml of 10 mM lovastatin. The resulting lovastatin solution was aliquotted and stored at -20°C.

2.4.2 Lovastatin administration

When they were grown to subconfluency (60-70% of confluence) in flasks, MB cells were treated with lovastatin for various intervals, up to 7 days. For attached cell lines, media were aspirated and replenished with fresh medium containing varying concentrations of lovastatin. For partially attached cell lines, floating cells were aspirated, spun and resuspended in medium, then returned to the flasks containing varying concentrations of lovastatin. For experiments extending beyond 48 hours, the medium was removed at this time, floating cells were pelleted by centrifugation, resuspended in fresh medium containing lovastatin at the appropriate concentration, and then replaced into flasks (Macaulay et al., 1999). Vehicle alone (see section 2.4.1) was used to treat cells to compare with lovastatin administration.

2.5 Mevalonate treatment of medulloblastoma cells

2.5.1 Preparation of mevalonate

Mevalonate was purchased from Sigma, St. Louis, MO. In vitro it is directly absorbed by cells and converted into its active form, mevalonic acid (Faust and Krieger, 1987). Thus, mevalonate was directly dissolved in 95% ethanol to form a 1 M solution,
then aliquotted and stored at -20°C.

2.5.2 Mevalonate administration

After subconfluent cells in flasks were pretreated with lovastatin alone for different time periods, they were then co-incubated with medium containing 20 μM of lovastatin and 2 mM of mevalonate together. The duration of lovastatin pretreatment was varied for each cell line to establish time windows, after which mevalonate was unable to overcome or reverse the effects of lovastatin. The medium containing lovastatin and mevalonate was replaced every 48 hours where necessary. When replacing medium, floating cells were aspirated, spun and returned to their original flasks (Wang and Macaulay, 1999a). Ethanol was supplied to cells as the control of mevalonate treatment.

2.6 Manumycin A treatment of medulloblastoma cells

2.6.1 Preparation of manumycin A

Manumycin A was purchased from Sigma, St. Louis, MO. Manumycin A was directly dissolved in methanol as described (Mitsuzawa and Tamanoi, 1995), to form a 2 mM solution, then aliquotted and stored at -20°C.

2.6.2 Manumycin A administration

Like lovastatin administration, when MB cells were grown to subconfluency (60-70% of confluence) in flasks, they were treated with manumycin A at various times up to 48 hours. For attached cell lines, media were aspirated and replenished with fresh medium containing varying concentrations of manumycin A. For partially attached cell lines, floating cells were aspirated, spun and resuspended in medium, then returned to flasks containing varying concentrations of manumycin A (Wang and Macaulay, 1999). Methanol alone was used as the control of manumycin A treatment.
2.7 Mycophenolic acid treatment of medulloblastoma cells

2.7.1 Preparation of mycophenolic acid

MPA was purchased from Sigma, St. Louis, MO, and directly dissolved in methanol according to the manufacturer’s instructions, to form a 40 mM solution, then aliquotted and stored at -20°C.

2.7.2 Mycophenolic acid administration

Like lovastatin and manumycin A administration, when MB cells were grown to subconfluency (about 30% of confluence) in flasks, they were treated with MPA at various times up to 120 hours. For the cells cultured on cover slips, they were treated with MPA at various times up to 1 week. For attached cell lines, media were aspirated and replenished with fresh medium containing varying concentrations of MPA. For partially attached cell lines, floating cells were aspirated, spun and resuspended in medium, then returned to the flasks containing varying concentrations of MPA. Methanol alone was used as the control of MPA treatment.

To determine whether MPA induced changes were due to its specific effect of GTP depletion, 500 μM of guanosine or adenosine or deoxyguanosine (Sigma, St. Louis, MO) were supplemented into MPA treatment.

For experiments extending beyond 48 hours, the medium was removed at this time and floating cells were centrifuged. Fresh medium containing MPA at the appropriate concentration was replaced into flasks, after floating cells were resuspended.

2.8 Synergistic treatment of medulloblastoma cells

When MB cells were grown to subconfluency (30-40% of confluence) in flasks, they were treated for 48 hours with low concentrations of either: lovastatin;
manumycin A; MPA; lovastatin plus manumycin A; lovastatin plus MPA; manumycin A plus mycophenolic acid; or lovastatin plus both manumycin A and MPA. For attached cell lines, media were aspirated and replenished with fresh medium containing the above compounds. For partially attached cell lines, floating cells were aspirated, spun and resuspended in medium, then returned to the flasks containing varying compounds. Ethanol and methanol plus the vehicle of lovastatin were supplied to cells as control treatments.

2.9 Antisense c-myc treatment of medulloblastoma cells

2.9.1 Antisense and sense c-myc oligonucleotides

Based on the cDNA sequence of c-myc, the oligonucleotide 5'-AAC GTT GAG GGG CAT-3' is complementary to the translation initiation site of c-myc mRNA, and was used as a c-myc-specific antisense inhibitor of translation. A sense oligonucleotide 5'-ATG CCC CTC AAC GTT-3' with a secondary structure identical to that of the antisense oligonucleotide served as control. The antisense oligonucleotide used in this experiment has been successfully used by numerous other groups for specific inhibition of c-myc expression (Heikkila et al., 1987; Holt et al., 1988; Kaptein et al., 1996; Koster et al., 1996; Wickstrom et al., 1988). To increase stability of oligonucleotides, both antisense and sense oligonucleotides were modified with phosphorothioate. Oligonucleotides purified by high-pressure liquid chromatography were purchased from GIBCO BRL, Gaithersburg, MD. Oligonucleotides were dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

2.9.2 Antisense c-myc treatment of medulloblastoma cells

When MB cells were grown to subconfluency (about 60% of confluence) in flasks, they were treated for 12 hours with 25 μM antisense oligonucleotide or sense
oligonucleotide. Following this treatment, medium was removed, floating cells were spun and resuspended in medium, then returned to the flasks containing 20 μM lovastatin plus 25 μM antisense oligonucleotide or sense oligonucleotide. After treatment at varying time points, cells were harvested (as described below) for DNA analysis. The vehicle treatment of lovastatin was used as the control.

2.10 Cell viability assays

Cell survival was quantified by trypan blue exclusion (Armstrong et al., 1992). Briefly, at the indicated time points, flasks containing control or treated cells were scraped with a rubber policeman, allowing aspiration of both adherent and non-adherent cells, which were isolated by centrifugation, rinsed with PBS, and resuspended in PBS. A 100 μl aliquot of this cell suspension was mixed with an equal volume of 0.08% trypan blue in Hank’s balanced salt solution. After staining for 10 minutes, viable cells which exclude trypan blue were counted with a hemocytometer (Macaulay et al., 1999).

2.11 Immunohistochemical assay

After MB cells cultured on glass cover slips were treated with MPA, control and treated cells were fixed in 100% ethanol for 2 x 10 minutes at room temperature, and rehydrated using PBS for 2 x 5 minutes. The slips were incubated with universal blocking solution supplied in the DAKO LSAB 2 kit (DAKO Diagnostics Canada Inc., Mississauga, ON) at room temperature for 5 minutes in a moist chamber to block the non-specific antibody binding sites. The slips were then incubated at room temperature for one hour with GFAP- or NFL-/NFH- specific monoclonal antibodies. The GFAP monoclonal antibody (DAKO Diagnostics Canada Inc., Mississauga, ON) was diluted 1:1000 in PBS containing 1% BSA while NFL/NFH monoclonal antibody (DAKO Diagnostics Canada Inc., Mississauga, ON) was diluted 1:200. After the slips were washed three times in PBS, link (biotinylated anti-mouse and anti-rabbit IgGs) supplied in the DAKO LSAB 2 kit
was applied. The slips were incubated for 30 minutes and then washed as before. Then, immunoperoxidase labeling was performed with streptavidin-peroxidase conjugated reagent supplied in the DAKO LSAB 2 kit for 30 minutes. The substrate for peroxidase was 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO), 0.03% in PBS plus 0.018% fresh H₂O₂. After about 5 minutes of DAB-peroxide treatment, slips were incubated with 2% CuSO₄ for 5 minutes, and then subsequently counterstained with Meyer's hematoxylin, dehydrated by treatment in a graded alcohol series (70%, 75%, 95% and 100%) and cleared by four changes of xylene. Coverslips were mounted with Entellan on microscope slides.

2.12 Purification and analysis of cellular DNA for 'laddering'

Cells in logarithmic growth phase were treated with lovastatin, or lovastatin plus mevalonate, or manumycin A, or mycophenolic acid. The control and treated cells were then scraped with a rubber policeman. The media were removed, and both adherent and non-adherent cells were pelleted by centrifugation, then rinsed with PBS. DNA extraction was performed as follows: cells were lysed with 1.0 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K), incubated at 50°C for 18 h, and extracted twice with 1 volume of phenol/chloroform (GIBCO BRL, Gaithersburg, MD) and once with chloroform. Following treatment with RNase A (40 μg/ml) (GIBCO BRL, Gaithersburg, MD) at 37°C for 1 h, the supernatant was then retreated with phenol and chloroform, and precipitated by adding 1/2 volume of 7.5 M ammonium acetate and 2.5 volume of 100% ethanol at -70°C for 0.5 h. The precipitate was then pelleted at top speed for 20 minutes using a microcentrifuge (Hettich EBA 12/12R series). The precipitate was rinsed with 75% ethanol, dried 10 minutes, and resuspend in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. About 10 μg DNA was electrophoresed on a 1.4% agarose (Sigma, St. Louis, MO) gel containing 0.2 μg/ml ethidium bromide in 1xTAE buffer. A DNA ladder (1 kb) (GIBCO
BRL, Gaithersburg, MD) was used as a marker. Gels were placed on an ultraviolet transilluminator (FOTODYNE FOTO/UV26), visualized by UV fluorescence, and photographed with a Polaroid camera system (model DS-34) using Polaroid type 667 film.

2.13 Flow cytometry: analysis of cell cycle and apoptosis

Flow cytometric analysis was performed according to the method of Buchkovich et al (Buchkovich et al., 1989). Controls with the same replating times were used for all experiments. Control and treated cells were collected by centrifugation and washed in ice-cold Hank's balanced salt solution with 0.3 mM EDTA and 0.1% NaN3. 5 x 10^6 cells were resuspended in 500 μl of ice-cold buffer and fixed by the gradual addition of ice-cold 100% ethanol (-20°C) to a final concentration 80% while vortexing. After 1-3 days at 4°C, the fixed cells were pelleted and washed once with the ice-cold PBS, and resuspended in 1 ml of this buffer containing 10 μg/ml RNase A (GIBCO BRL, Gaithersburg, MD) and 5 μg/ml propidium iodide (PI; Sigma, St. Louis, MO) from a 10x stock solution of 50 μg PI /ml of 40 mM sodium citrate pH 7.0. After digestion and staining at 37°C for 30 minutes, the samples were analyzed by flow cytometry [Coulter(R) Epics(R), Hialeah, FL]. Data were analyzed by the “overlapped peak” multicycle fitting option.

2.14 Western Blotting

2.14.1 Antibodies

The following mouse monoclonal antibodies (Mabs) were used for the detection of different proteins. Mab pan-ras (Ab-2) detects total p21 Ras with molecular weight 21 kDa (Oncogene Science Diagnostics, Cambridge, MA). Mab M7001 reacts with 53 kDa p53 (DAKO Diagnostics Canada Inc., Mississauga, ON). Mab M7203 is specific for human p27KIP1 with molecular weight 27 kDa (DAKO Diagnostics Canada Inc.,
Mississauga, ON). Mab M887 reacts with human Bcl-2 oncoprotein with molecular weight 25 kDa (DAKO Diagnostics Canada Inc., Mississauga, ON). Mab Bax (Ab-2) recognizes human Bax protein with molecular weight 21 kDa (Oncogene Science Diagnostics, Cambridge, MA). Mab c-myc (Ab-1) recognizes 67 kDa C-myc oncoprotein and its cleavage products (Oncogene Science Diagnostics, Cambridge, MA). Mab WAF1 (Ab-1) recognizes the 21 kDa protein product of the human p21WAF1 gene (Oncogene Science Diagnostics, Cambridge, MA). The polyclonal rabbit anti-human p16INK4 antibody (PHARMINGEN Canada, Mississauga, ON) was used to detect the 16 kDa protein product of human p16INK4 gene. The polyclonal goat anti-human caspase-3 p11 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the 32 kDa human pro-caspase-3 and its 11 kDa subunit p11.

2.14.2 Preparation of cell lysates

At the indicated post-treatment times, control and treated cells were scraped into medium and pelleted by centrifugation at 1,000 rpm for five minutes (international centrifuge, International Equipment Company), washed three times with ice-cold PBS, and resuspended in RIPA buffer supplemented with 1mM freshly made phenylmethylsulphonylfluoride (PMSF) according to a modification of a previously described method (Magee et al., 1987). Cells were solubilized for one hour on ice, and then centrifuged at 12,000 g (Hettich EBA 12/12R Series) for 15 minutes at 4°C. The supernatants were removed to clean microtubes and stored at -20°C.

2.14.3 Determination of protein concentrations

The protein concentrations of cell lysates were determined by using a Bio-Rad DC protein assay kit as follows. Cell lysates were diluted 1:5 in TBS. Serial dilutions of BSA from 0.2 mg/ml to 1.5 mg/ml were made in 0.2% NP-40 buffer (RIPA buffer diluted 1:5 in TBS) and used as protein standards. Protein samples and standards were pipetted
into microwell plates (Nunclon U96), 5 μL per well, followed by the addition of 25 μL of reagent A' and 200 μL of reagent B (supplied with the kit and prepared as the manufacturer's instruction) to each well. The reagents were mixed by gentle agitation of the plate. After incubation for 15 minutes at room temperature, absorbences at 600 nm were read by the EIA Reader (Bio-Rad Model 2550). A standard curve was constructed by plotting the OD600 values versus BSA concentrations. The protein concentrations of the unknown samples were interpolated using the standard curve.

2.14.4 SDS-PAGE (Minigel)

Each sample of cell lysates was diluted to a concentration of 1 mg/ml with RIPA buffer, mixed with an equal volume of 2 x SDS-gel loading buffer, and heated for 5 minutes at 100°C. Protein samples, including prestained SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA), were separated by SDS-PAGE using a discontinuous system with a 10% acrylamide separating gel and a 3% acrylamide stacking gel in a mini-V8.10 Vertical Gel Electrophoresis apparatus (Gibco BRL, Gaithersburg, MD). The gel was run at a constant voltage of 120 for about 1.5 hours.

2.14.5 Immobilization of proteins on nitrocellulose membranes

A piece of supported nitrocellulose-1 membrane (Gibco BRL, Gaithersburg, MD) that was cut to the same size as the gel was equilibrated in ice cold transfer buffer, together with 6 pieces of 3 MM filter paper and two fiber pads. The electroblotting apparatus was assembled according to the manufacturer's instructions (Gibco BRL Mini-V8.10 Blot Module). Proteins were electrophoretically transferred from the gel to the nitrocellulose membrane at 160 volts for 1.5 hours.

2.14.6 Immunoblotting

Following the transfer of proteins from gels to membranes, the membranes
were blocked in TBS containing 5% skim milk and 0.05% Tween-20 overnight at 4°C. The blocking solution was decanted, and monoclonal antibodies and polyclonal antibodies noted above (according to the manufacturer's instructions, diluted 1:100-1:1000 in TBST containing 0.5% skim milk) were added. After incubation for four hours at room temperature with gentle shaking, the primary antibodies were removed and the membranes were washed three times with TBST. The membranes were then incubated with biotinylated anti-mouse, anti-rabbit or anti-goat IgG H+L (Vector Laboratories, Burlingame, CA), based on the source of first antibody, at a dilution of 1:1000 in TBST containing 0.5% skim milk for one hour at room temperature on a rocking platform. After three washes in TBST, membranes were incubated with ABC-AP solution provided by Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA) for 30 minutes. Following three washes in TBST, membranes were then equilibrated in alkaline phosphatase buffer and the AP substrates p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) (Gibco BRL, Gaithersburg, MD) were added. The reaction was allowed to proceed at room temperature until dark purple bands were visible. Color development was stopped by washing membranes with several changes of deionized water.

2.15 Reverse transcription and polymerase chain reaction (RT-PCR)

2.15.1 Primers used in RT-PCR

Based on the cDNA sequences of human HMG-CoA reductase, K-Ras, H-Ras, N-Ras and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primers were designed and synthesized by Gibco BRL, Gaithersburg, MD. For the amplification of HMG-CoA reductase cDNA, 3' flanking sequence 5'-GGC CTC TCT GAA GAA ATA GCC TGC GGA GAT-3' was selected as its sense primer, and the sequence 5'-CCA TGC AGA CTC CTC AGA TCT GAA CAC AGT-3' was used as its antisense primer. The expected
product of HMG-CoA reductase cDNA was 525 bp. For the amplification of GAPDH cDNA, 5'-ACC ACC ATG GAG AAG GCT GG-3' was used as antisense primer in combination with the sense primer 5'-CTC AGT GTA GCC CAG GAT GC-3'. This primer pair spans 3 introns (#5, 6 and 7) of GAPDH gene. The expected product of GAPDH cDNA was 528 bp.

To detect total Ras RNA levels, a common sequence for K-Ras4A, K-Ras4B, H-Ras and N-Ras mRNA was used for primers. The antisense primer for total Ras RNA was 5'-AAT TTG CTC TCT GTA GTG GT-3' which corresponds to a consensus located in exons 2 and 3 in all Ras genes (primer RT1), and the sense primer was 5'-TGA CGG AAT ATA AAC TGG TG-3' which corresponds to a consensus located at the beginning of the first exon of all Ras genes (primer F1). The expected product was 299 bp. The antisense primer of total Ras RNA (RT1) was used in combination with sense primer 5'-GGA GAT AGG CAT GCT GAA A-3' (primer F2) for K-Ras RNA (K-Ras4A and K-Ras4B). The expected product was 319 bp. The primer RT1 combined with the sense primer 5'-GAT CTT GAG GTT ATT GCT G-3' (primer F3) were used for N-Ras RNA, obtaining a 326 bp product in RT-PCR. The combination of primer RT1 and sense primer 5'-TAG GTC AGG AGA ACC TGT A-3' (primer F4) was used for H-Ras RNA, producing a 354 bp expected product by RT-PCR. As the internal standard in Ras cDNA amplification, GAPDH was detected using the above primer pair.

### 2.15.2 Total cellular RNA extraction

Total cellular RNA was extracted from cultured MB cells using the acid guanidinium-phenol-chloroform method with the TriZol reagent (GIBCO BRL, Gaithersburg, MD). The control and treated cells were collected at indicated time points as described above. Both adherent and non-adherent cells (10⁶ to 10⁷) were washed three times with RNase-free PBS and lysed in 1 ml of TriZol reagent by passing the cell lysate several times through a pipette, and then incubated at room temperature for 5 minutes.
ml of chloroform was then added into cell lysates, vigorously mixed by hand for 15 seconds and incubated for 2 to 3 minutes at room temperature. The samples were centrifuged at no more than 12,000 g for 15 minutes at 4°C. The colorless upper aqueous phase was transferred to a fresh RNase-free tube, mixed with 0.5 ml of isopropyl alcohol, incubated for 10 minutes at room temperature, and then centrifuged at no more than 12,000 x g for 10 minutes at 4°C. The RNA pellet was washed once with 1 ml of RNase-free 75% ethanol after removing the supernatant, and then centrifuged at no more than 7,500 g for 5 minutes at 4°C. At the end of the procedure, RNA pellets were briefly dried for 5 to 10 minutes, and dissolved in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 minutes at 55°C. Following the measurement of RNA concentrations, RNA solutions were mixed with a 2.5 fold volume of 100% ethanol, and stored at -20°C.

2.15.3 Determination of RNA concentration

RNA concentrations were determined by measuring the absorbance of RNA at 260 nm and 280 nm using a UV/VIS spectrometer (UNICAM 8700 series). RNA samples were considered to be essentially free of contaminating proteins if the ratio of A260/ A280 was > 1.75. The TriZol reagent usually isolates RNA with an A260/ A280 ratio of 1.6 - 1.8. Since a RNA solution with an OD260 value of one contains about 40 µg mRNA per ml, the RNA concentrations were determined by the equation:

\[
[RNA] \text{(µg/ml)} = \text{dilution factor} \times 40 \times A_{260}
\]

2.15.4 RT-PCR methods

To amplify cDNA, reverse transcription was performed first for cDNA generation. Total cellular RNA (1 µg) was carried in 20 µl reaction containing 1 x PCR buffer with 5 mM MgCl₂, 1 mM each of deoxynucleotide triphosphates (dNTP), 20 units of RNase inhibitor, 50 units of MuLV reverse transcriptase and 50 pMol of random
hexamer oligodeoxynucleotides (all reagents for cDNA generation were from Perkin Elmer, Branchburg, NJ). After preincubation at 21°C for 10 minutes, the reverse transcriptase reaction was performed for 15 minutes at 42°C, and extinguished by incubating at 99°C for 5 minutes on a Perkin-Elmer GeneAmp PCR System 2400. The products were immediately chilled on ice, and stored at -20°C.

For HMG-CoA reductase and GAPDH cDNA amplification, PCR was performed in 50 µL reaction volumes using Perkin-Elmer PCR tubes on a Perkin-Elmer GeneAmp PCR System 2400. Microtubes contained 2 µl of RT reaction products as template DNA, 1 x PCR buffer, 180 µM of each of deoxynucleotide triphosphates (including the dNTP left over from the RT reaction), 20 pMol of each antisense and sense primer pair (of either HMG-CoA reductase or GAPDH), and 2.5 units of Taq DNA polymerase (all reagents in this step were from GIBCO BRL, Gaithersburg, MD). Cycle conditions for HMG-CoA reductase cDNA amplification were 94°C for 90 seconds, 60°C for 60 seconds, and 72°C for 120 seconds for 30 cycles. Cycle conditions for GAPDH cDNA amplification were 94°C for 90 seconds, 60°C for 45 seconds, and 72°C for 60 seconds for 32 cycles. After the last cycle, samples were incubated for 10 minutes at 72°C to extend incomplete products. Aliquots of PCR reaction products (1 µL) were used as template DNA for further PCR amplification. The PCR amplification products were electrophoresed through 1.4% agarose gels containing 0.2 µg/ml ethidium bromide in 1xTAE buffer, evaluated by UV illumination of the gel, and photographed using Polaroid film. A 100 bp DNA ladder (GIBCO BRL, Gaithersburg, MD) was used as a marker.

2.15.5 Semi-quantitative RT-PCR

To determine the relative levels of p21 Ras mRNA, a semi-quantitative RT-PCR method was used. The levels of GAPDH mRNA were measured in parallel as an internal control. Following the above cDNA generation, PCR for cDNA amplification was performed on the above PCR System in 50 µl of reaction containing 2 µl of cDNA products
as template DNA, 1 x PCR buffer with 2 mM of MgCl₂, additional 50 µM of each dNTP, 1.25 units of AmpliTaq Gold™ DNA polymerase (all agents in this PCR were from Perkin Elmer, Branchburg, NJ), 20 pMol of GAPDH primers and 50 pMol of Ras primers. Following preincubation at 95°C for 9 minutes, amplification proceeded for serial cycles (35 to 41), consisting of 94°C for 30 seconds, 49°C for 20 seconds, and 72°C for 30 seconds, to obtain data within the linear range of the assay. After the last cycle, samples were incubated for 7 minutes at 72°C to extend incomplete products. An aliquot of each PCR product was size-fractionated by electrophoresis in 1.4% agarose gel containing 0.1 µg/ml ethidium bromide, visualized by UV irradiation and photographed using Polaroid film. A 100 bp DNA ladder (GIBCO BRL, Gaithersburg, MD) was used as a marker.

2.16 Northern blotting

2.16.1 Formaldehyde-agarose gel electrophoresis of RNA

Following RNA extraction (see section 2.15.2), 30 µg of RNA in 75% ethanol at -20°C was precipitated by adding 0.1 volume of 3 M RNase-free sodium acetate (pH 5.2), collected by centrifugation at 12,000 g for 15 minutes at 4°C, and washed once with RNase-free 70% ice-cold ethanol. The air-dried RNA pellets were dissolved in RNase-free sample buffer (1 x MOPS {10 x stock solution: 0.2 M MOPS (Sigma, St. Louis, MO), 10 mM EDTA, 50 mM NaAc, pH 7.0}, 7% formaldehyde, 50% formamide, and 10% dye mix {stock solution: 50% glycerol, 1mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol}), and heated to 60°C for 15 minutes. Samples were snap-cooled on ice, and loaded onto a 1.2% agarose/1.8% formaldehyde gel containing ethidium bromide (0.1 µg/ml). Gels were electrophoresed at 5 V/cm for 2.5 to 3 hours in 1 x MOPS buffer. Following electrophoresis, gels were washed several times with RNase-free 1 x phosphate buffer (50 mM NaH₂PO₄-NaOH, pH 6.5, 5 mM EDTA), and the RNA was visualized by UV illumination of the gel to check for degradation of the RNA.
2.16.2 Northern transfer

After electrophoresis, the gel was put upside down on a supporter covered with 3 M papers, and then Hybond\textsuperscript{TM}-N+ membrane (Amersham, Buckinghamshire, UK) soaked with RNase-free 6 x SSC was put on the surface of gel to set up capillary blotting system. Capillary blotting was achieved in RNase-free 10 x SSC for at least 12 hours. After the northern transfer, the membrane was washed once with 6 x SSC, and then air-dried for 30 minutes. RNA was visualized by UV illumination of the membrane to ensure that complete transfer had occurred. Membranes were baked for 2 to 4 hours at 80\degree C, then stored in RNase-free bags at -70\degree C until they were used for hybridization.

2.16.3 Use of RT-PCR products as probes

2.16.3.1 Purification of RT-PCR products

Following PCR amplification of GAPDH and HMG-CoA reductase cDNA (see section 2.15.4), amplified cDNA fragments were purified using polyethylene glycol (PEG) precipitation (Lis, 1980; Paithankar and Prasad, 1991). 100 \mu l of PCR products were added to a tube containing 50 \mu l of 30 mM MgCl\textsubscript{2} and PEG 8000 (25%), mixed and incubated for 10 minutes at room temperature, and then centrifuged at top speed for 10 minutes in a microcentrifuge (Hettich EBA 12/12R series). Supernatants were completely removed, and 20 \mu l TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added. After flicking a few times, the liquid phase was transferred to a fresh tube, and then the effect of purification was determined by electrophoresis. DNA concentrations of samples were determined by measuring the absorbance of DNA at 260 nm and 280 nm using a UV/VIS spectrometer (UNICAM 8700 series). Since a DNA solution with an OD\textsubscript{260} value of one contains about 50 \mu g DNA per ml, the DNA concentrations were determined by the equation: [DNA] (\mu g/ml) = dilution factor \times 50 \times A\textsubscript{260}. The purified RT-PCR products
were stored at -20°C until used for probe labeling

2.16.3.2 Probe labeling

The RT-PCR amplified cDNA fragments of GAPDH and HMG-CoA reductase were used as probes for Northern blotting. The probes were labeled using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany). 25 ng of either GAPDH or HMG-CoA reductase cDNA fragments was denatured by heating, and then incubated mixtures of α32P-dCTP (Amersham, Buckinghamshire, UK), dNTP, reaction mixture and klenow enzyme supplied by the kits in 20 μl of volume at 37°C for 30 to 50 minutes. Following the stop of reaction with 1 μl of 0.5 M EDTA, the unincorporated nucleotides were removed by spun-column chromatography using MicroSpin™ S-300 HR Columns (Pharmacia Biotech, Baie d’Urfe, Quebec). Briefly, a S-300 HR column was pre-centrifuged for 1 minute at 735 g in a 1.5 ml decapped microtube. The reaction mixture with 40 μl of TE buffer was loaded on the column which was placed in a 1.5 ml new decapped microtube. The column was then centrifuged at 735 g for 2 minutes, and the purified samples as probes were collected at the bottom of the support tube.

2.16.4 Hybridization, washing and blot visualization

Hybond™-N+ membranes with fixed RNA were wetted with 6 x SSC and placed in a plastic bag containing Hybrisol solution (Oncor, Gaithersburg, MD) 1 ml/10 cm² membrane. Following prehybridization for 2 hours in a 42°C water bath with gentle shaking, 10⁶ cpm/ml of radiolabeled probes with 1 ml of Hybrisol solution were denatured by heating at 100°C for 10 minutes and added to pre-warmed fresh Hybrisol solution, and then put into hybridization bags. Membranes with probes were then incubated for a further 16-18 hours at 42°C, transferred to trays, washed twice with 2 x SSC/0.1% SDS at room temperature for 15 minutes, followed by 4 x 15 minute washes for HMG-CoA reductase probe in 0.1 x SSC/0.1% SDS at 52°C. Membranes were then air-dried and exposed to
Kodak X-ray film until desired exposures were obtained. They were then boiled in RNase-free water for 2 x 10 minutes to strip probes. Membranes were then reprobed with GAPDH probe as indicated above. Membrane wash conditions for GAPDH probe were: twice with 2 x SSC/0.1% SDS at room temperature for 15 minutes, followed by 2 x 30 minute washes with 0.1 x SSC/0.1% SDS at 65°C. Results were again visualized by exposing membrane to Kodak X-ray film.

2.17 Densitometric analysis

Densitometric evaluation of Western blot analysis, Northern blot analysis, and RT-PCR analysis was performed with gel documentation system (Gel Doc 2000, Bio-Rad Laboratories, Mississauga, ON). Data from two independent experiments were pooled for each densitometric study. For each blot, the bands on each membrane, film or gel were quantified twice after subtracting background, and then averaged. Relative quantity of density signals in arbitrary units (A.U.) were analyzed with computer software Quantity One (version 4.0) (Bio-Rad Laboratories, Mississauga, ON).

2.18 Statistical analysis

Independent experiments for cell growth and flow cytometric analysis (n=3), and densitometric analysis (n=2) were analyzed by analysis of variance (ANOVA). One-way and two-way ANOVA were performed with computer software SYSTAT (version 5.02) (SYSTAT Inc., Evanston, IL). If there was a significant difference (p<0.05), Scheffe multiple range test was then performed. For comparison of two independent samples, a Student's T-test was performed. Correlation between data sets was assessed using the Pearson correlation coefficient.
3.0 RESULTS

3.1 Lovastatin-induced changes of MB cell lines

3.1.1 Morphological changes

Growth of all cell lines was established, and morphologic characteristics were identical to published descriptions in each case (see section 2.2). Further characterization by western blot, immunohistochemistry and molecular analysis revealed no significant variation from published reports (see below and discussion). No appreciable variability in the characteristics and responses of the various cell lines was observed with increasing passages in culture.

At $\geq 10 \ \mu$M lovastatin treatment, significant morphological changes were observed (Figure 3.1). For attached cell lines (Daoy and UW228), cell bodies rounded, and cells became detached from flasks. For the partially attached cell lines (D283 Med and D341 Med), all attachment on flask was lost and cell bodies shrank. Different sensitivities to lovastatin were observed among the cell lines, although all showed dose-dependent effects. Morphological changes were induced in UW228 and Daoy 12-24 hr earlier than in D283 Med and D341 Med. At a concentration of 1 $\mu$M there was no observed effect on morphology of any cell line. Thus, despite the differences in morphology between the untreated cell lines, lovastatin-treated cells of each cell line showed similar features suggestive of apoptosis.

3.1.2 Effects of lovastatin on cell viability

Daoy and UW228 cell lines are adherent cell lines. Daoy doubling time is 34 hr
Figure 3.1. Morphologic Changes Induced by Lovastatin.
The top row shows untreated cells, while the bottom row shows cells following exposure to 20 μM of lovastatin, after
the stated time interval (phase contrast microscopy). Note extensive cell rounding of attached cell lines, and rounding
and fragmentation of cell lines which grow both lightly attached and in suspension. Daoy x 48 hr. UW228 x 36 hr.
D283 Med x 72 hr. D341 Med x 96 hr.
D283 and D341 are partially adherent cell lines, and their doubling times are 52 hr and 37 hr respectively (American Type Culture Collection, 1999). As ATCC and Keles et al. reported, Daoy (3 to 4 day culture) is GFAP negative, UW228, D283 Med and D341 Med are NF positive in our lab.

Treatment of each MB cell line with 10-40 μM of lovastatin resulted in significant declines in viable cell number (p<0.01, two-way ANOVA (time, [lovastatin])), as assessed by trypan blue exclusion. Control cells increased in numbers between 24-96 hr (Figure 3.2), similar to the growth properties described by ATCC and Keles et al.

1 μM of lovastatin had no apparent effect on cell numbers (p>0.1). 10-40 μM of lovastatin abrogated the expected increase in viable cell numbers (Figure 3.2) after 24 hr. and thereafter the numbers of viable cells diminished markedly (p<0.01). UW228 and Daoy responded after about 24 hr treatment, while the responses of D283 Med and D341 Med were delayed, with no statistically significant reduction until 48 to 72 hr. Eventually, all four cell lines showed reduction of viable cell numbers to 10% of passaged numbers after 20 to 40 μM lovastatin treatment. When compared to the numbers of viable cells in control flasks at maximum time intervals, the effect of 20 to 40 μM lovastatin for each cell line was to reduce the number of viable cells by more than 95%. These data expand on preliminary findings using a Live Cell/Dead Cell assay (Macaulay et al., 1994).

There was also some variability in sensitivity to increasing lovastatin concentration. UW228 cells required only 10 μM for maximum response (when compared to 20 μM, p>0.1, Student's T-test), while the other cell lines required at least 20 μM of lovastatin for maximal response (when compared to 10 μM, p<0.05, Student's T-test).

It is unlikely that the initial effect of lovastatin was on cell membrane integrity, since >90% of Daoy and >80% of UW228 at 24 hr, as well as >90% of D341 Med and >80% of D283 Med at 48 hr, excluded trypan blue, before morphologic changes appeared (Figure 3.2).
Figure 3.2. Cell Death Induced by Lovastatin. Viability of MB cells was determined by trypan blue exclusion, in untreated cells (0 μM) and after exposure to 1-40 μM of lovastatin for up to 96 hr. Daoy and UW228 were more sensitive, while D283 Med and D341 Med showed negligible effects until 72 hr of treatment. Data points: Means of three experiments. Bars: 2xSEM.
3.1.3 Apoptosis

To further characterize lovastatin-induced cell death in MB cell lines, cellular DNA fragmentation was analyzed by flow cytometry (Zamai et al., 1993) and DNA gel electrophoresis.

3.1.3.1 Flow cytometric analysis

The results of flow cytometry showed that, for each cell line, 20 μM lovastatin induced the appearance of a subdiploid peak, although at different times for each cell line (Figure 3.3). This peak, representing nuclear fragmentation due to apoptosis as opposed to necrosis (Zamai et al., 1993), was seen in UW228 and Daoy after 36-40 hr treatment with lovastatin, while D283 Med and D341 Med required about 72 hr treatment. The more rapid appearance of this 'apoptosis peak' in UW228 and Daoy further emphasized their increased sensitivity to lovastatin compared to D283 Med and D341 Med.

Table 3.1. Summary of Lovastatin-Induced Apoptosis in a Dose- and Time-Dependent Manner (flow cytometric analysis of three experiments, PI staining)

<table>
<thead>
<tr>
<th>A (dose)</th>
<th>Lovastatin Concentration</th>
<th>Daoy (40 h) Apoptosis Percentage</th>
<th>UW228 (40 h)</th>
<th>D283 Med (72 h)</th>
<th>D341 Med (72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 μM</td>
<td>0</td>
<td>3.2 ± 1.9</td>
<td>0</td>
<td>12.2 ± 3.0</td>
<td>0</td>
</tr>
<tr>
<td>10 μM</td>
<td>10.1 ± 3.5</td>
<td>56.7 ± 5.2</td>
<td>13.3 ± 2.8</td>
<td>12.2 ± 3.0</td>
<td>0</td>
</tr>
<tr>
<td>20 μM</td>
<td>22.6 ± 1.8</td>
<td>71.7 ± 7.1</td>
<td>64.4 ± 7.2</td>
<td>31.5 ± 3.7</td>
<td>0</td>
</tr>
<tr>
<td>40 μM</td>
<td>32.9 ± 4.7</td>
<td>90.2 ± 2.1</td>
<td>80.5 ± 3.0</td>
<td>43.1 ± 3.5</td>
<td>0</td>
</tr>
<tr>
<td>r²</td>
<td>0.96</td>
<td>0.83</td>
<td>0.91</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B (time)</th>
<th>20 μM lovastatin treatment (hr)</th>
<th>Daoy</th>
<th>Apoptosis Percentage</th>
<th>UW228</th>
<th>D283 Med</th>
<th>D341 Med</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0.6 ± 0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>2.2 ± 2</td>
<td>11.4 ± 1.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>12.5 ± 1.4</td>
<td>64.1 ± 7.7</td>
<td>5.5 ± 3.4</td>
<td>0.4 ± 0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>39.9 ± 2.0</td>
<td>15.0 ± 4.9</td>
<td>5.5 ± 2.3</td>
<td>31.5 ± 3.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>64.4 ± 7.2</td>
<td>51.1 ± 10.6</td>
<td>31.5 ± 3.7</td>
<td>31.5 ± 3.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>r²</td>
<td>0.74</td>
<td>0.74</td>
<td>0.76</td>
<td>0.66</td>
<td>0.66</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Note: r²: correlation coefficient factor. Data: Mean ± SD.
Figure 3.3. Flow Cytometric Changes Induced by Lovastatin. Percentages of cells in G0/G1, S and G2/M as well as apoptosis are shown. The left column of graphs represents control cells (same time point as lovastatin treatment). The right column of graphs represents cells treated with 20 μM of lovastatin for the following durations: Daoy, 40 hr; UW228, 36 hr; D283 Med, 72 hr; D341 Med, 72 hr. The subdiploid shaded peak which appears to the left of the tall control peak (G1) represents the apoptotic fraction. This figure represents one of three experiments, summarized in table 3.1.
In all experiments (n=3), the size of the apoptosis peak increased with increasing dosages between 10 and 40 μM after 40 hr treatment (Results are summarized in Table 3.1A.). Similarly, the size of the apoptosis peak resulting from treatment with 20 μM of lovastatin increased progressively over time (Table 3.1B).

3.1.3.2 Electrophoretic analysis of DNA

DNA degradation was evident in all cell lines after treatment with >10 μM of lovastatin (Figure 3.4). In all four cell lines, nucleosomal oligomers, indicating apoptotic cleavage of chromosomal DNA at internucleosomal loci (Kaufmann, 1989), appeared at variable times correlating with morphological and flow cytometric evidence of apoptosis (Figure 3.5). Thus, similar to morphologic, viability and flow cytometric changes, DNA 'laddering' was dose and time dependent. Control cells cultured equivalent lengths of time without lovastatin failed to demonstrate DNA laddering. DNA ladder formation was more obvious in sensitive cell lines (UW228 and Daoy) compared to less sensitive cell lines (D283 Med and D341 Med). This may relate to the interval between treatment and cell death, since DNA ladder formation in D283 Med was more obvious than in D341 Med, which took 96 hr to develop.

3.1.4 Cell cycle effects

The effects of lovastatin on cell cycle changes are summarized in Table 3.2 according to the analysis of flow cytometry. Table 3.2 shows that 20 μM of lovastatin blocked the S phase entry (p<0.01) and induced cell cycle arrest in G1 (p<0.01) in all four cell lines. When compared to the cells at time 0, the cell cycle distribution of surviving cells in cultures exposed to 20 μM of lovastatin was unchanged during the initial 6-12 hr of treatment. However, as the time of exposure to lovastatin increased, the proportion of cells in S phase decreased (p<0.01) concomitant with an increase in G1-phase cells (p<0.01). The percentage of cells in G2 plus M showed no significant changes in Daoy and D341

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Figure 3.4. DNA Fragmentation Induced by Lovastatin. Ethidium bromide-stained agarose gels of MB DNA after treatment with varying concentrations of lovastatin. All four cell lines show 'laddering' of DNA indicative of apoptosis: Daoy x 48 hr; UW228 x 40 hr; D283 Med x 72 hr; D341 Med x 72 hr. Lane concentrations: (1) untreated; (2) 1 μM; (3) 10 μM; (4) 20 μM; (5) 40 μM; (M) marker.
Figure 3.5. Time Course of DNA Laddering Induced by Lovastatin. Ethidium bromide-stained agarose gels of DNA; cells were treated with 20 μM of lovastatin after varying intervals. Time intervals for lanes in Daoy, UW228 and D283 Med: (1) 0 hr; (2) 12 hr; (3) 24 hr; (4) 36 hr; (5) 48 hr; (6) 72 hr; (7) Untreated x 72 hr. Time intervals for lanes in D341 Med: (1) 0 hr; (2) 24 hr; (3) 48 hr; (4) 72 hr; (5) 96 hr; (6) Untreated x 96 hr. (M) marker. Laddering is first apparent at earlier intervals for Daoy and UW228 (36 hr) compared to D283 Med (48 hr) and D341 Med (72 hr).
Med (p>0.1); a decrease in UW228 (p<0.01); and an increase in D283 Med (p<0.01).

Table 3.2. Summary of the Effects of Lovastatin (20 µM) on Cell Cycle (flow cytometric analysis of three experiments, PI staining)

<table>
<thead>
<tr>
<th>Distribution of Cells (%)</th>
<th>Daoy</th>
<th>Time (hr)</th>
<th>UW228</th>
<th>Time (hr)</th>
<th>D283 Med</th>
<th>Time (hr)</th>
<th>D341 Med</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>12 (con)</td>
<td>12 (lov)</td>
<td>0</td>
<td>6 (con)</td>
<td>6 (lov)</td>
<td>0</td>
<td>12 (con)</td>
</tr>
<tr>
<td>G1</td>
<td>70.2±1.0</td>
<td>57.8±1.8</td>
<td>69.6±1.8</td>
<td>71.6±2.3</td>
<td>78.8±1.1</td>
<td>62.2±2.9</td>
<td>83.4±3.4*</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>23.4±2.0</td>
<td>38.8±1.5</td>
<td>22.7±1.9</td>
<td>22.2±2.8</td>
<td>14.2±1.8</td>
<td>32.6±1.7</td>
<td>10.2±2.8*</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>6.4±1.3</td>
<td>3.5±1.4</td>
<td>7.6±1.2</td>
<td>6.2±1.1</td>
<td>7.0±1.4</td>
<td>5.3±1.3</td>
<td>6.5±0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>6 (con)</td>
<td>6 (lov)</td>
<td>0</td>
<td>24 (con)</td>
<td>24 (lov)</td>
<td>0</td>
<td>36 (con)</td>
</tr>
<tr>
<td>G1</td>
<td>68.4±3.0</td>
<td>56.5±2.8</td>
<td>70.2±1.9</td>
<td>59.6±0.6</td>
<td>83.9±1.7</td>
<td>73.2±3.0</td>
<td>86.6±2.5*</td>
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<tr>
<td>S</td>
<td>20.1±0.7</td>
<td>31.5±4.5</td>
<td>19.7±1.1</td>
<td>28.9±2.7</td>
<td>10.3±1.4</td>
<td>17.0±1.9</td>
<td>8.9±1.6*</td>
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</tr>
<tr>
<td>G2</td>
<td>11.5±2.3</td>
<td>13.0±3.8</td>
<td>10.1±2.8</td>
<td>11.5±2.8</td>
<td>5.8±2.7</td>
<td>9.8±1.3</td>
<td>4.5±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12 (con)</td>
<td>12 (lov)</td>
<td>0</td>
<td>36 (con)</td>
<td>36 (lov)</td>
<td>0</td>
<td>48 (con)</td>
</tr>
<tr>
<td>G1</td>
<td>49.8±2.9</td>
<td>44.6±1.3</td>
<td>49.3±2.4</td>
<td>53.4±3.4</td>
<td>58.9±1.2</td>
<td>48.6±3.0</td>
<td>60.8±3.5*</td>
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<tr>
<td>S</td>
<td>46.0±2.6</td>
<td>51.4±1.6</td>
<td>47.4±2.2</td>
<td>45.9±1.5</td>
<td>25.0±3.0</td>
<td>45.7±2.8</td>
<td>16.5±2.4*</td>
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</tr>
<tr>
<td>G2</td>
<td>3.9±1.0</td>
<td>4.1±2.3</td>
<td>3.3±1.2</td>
<td>2.9±0.6</td>
<td>16.1±1.8</td>
<td>5.7±3.1</td>
<td>22.7±4.0*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>12 (con)</td>
<td>12 (lov)</td>
<td>0</td>
<td>36 (con)</td>
<td>36 (lov)</td>
<td>0</td>
<td>48 (con)</td>
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<tr>
<td>G1</td>
<td>72.9±3.2</td>
<td>68.3±3.8</td>
<td>74.2±3.6</td>
<td>59.3±2.6</td>
<td>80.1±1.3*</td>
<td>56.7±3.0</td>
<td>81.8±1.1*</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>21.0±2.6</td>
<td>24.8±2.2</td>
<td>18.9±2.2</td>
<td>31.2±2.2</td>
<td>12.7±1.8*</td>
<td>34.7±2.7</td>
<td>11.0±2.1*</td>
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<tr>
<td>G2</td>
<td>6.1±1.3</td>
<td>7.0±1.8</td>
<td>6.8±1.5</td>
<td>9.5±1.1</td>
<td>7.3±1.3</td>
<td>8.6±2.1</td>
<td>7.5±1.6</td>
<td></td>
</tr>
</tbody>
</table>

Note: *: p<0.01, two-way ANOVA (time, lovastatin). Data: Mean ± SD. Con: control. Lov: lovastatin.

3.1.5 Lovastatin 'resistant' Daoy cells

To naturally select lovastatin "resistant" cells, the treated cells that were floating were centrifuged and cultured in fresh media. Fresh media failed to rescue Daoy and UW228 treated for 48 hr, D283 Med treated for 84 hr, and D341 Med treated for 96 hr, consistent with their inability to exclude trypan blue. Cultures of the few attached Daoy cells, after 48 hr of 20 µM of lovastatin treatment, were subjected to repeated cycles of
lovastatin followed by fresh medium. The selected "resistant" cells had similar characteristics as the parental cells. Significantly, 20 μM of lovastatin could still inhibit proliferation of these selected 'resistant' cells, followed by as much as a 90% decline in viable cell number when treatment was prolonged to 72 hr. Flow cytometric analysis showed that treatment with 20 μM of lovastatin arrested the cell cycle in G1, and was still able to induce apoptosis of surviving 'resistant' Daoy cells, but required longer times (72 hr) than naive cells (Figure 3.6A). DNA gel electrophoresis also showed that surviving 'resistant' Daoy cells exhibited reduced sensitivity to lovastatin, but still underwent apoptosis after 20-40 μM of lovastatin treatment for 72 hr (Figure 3.6B), compared to 36 hr for naive cells (Figure 3.5).

3.1.6 bcl-2 expression in MB apoptosis

Western blotting showed that Bcl-2 remained stable in Daoy, UW228 and D283 Med during the initial treatment with lovastatin. Bcl-2 was decreased in Daoy and UW228 (p<0.05) and there was no significant change in Bcl-2 in D283 Med (p>0.05) after apoptosis appeared (Figure 3.7). Bcl-2 in D341 Med was difficult to detect because larger amounts of cell lysates were required, and showed negligible differences (p>0.05) between lovastatin-treated and untreated cells. These results suggested that bcl-2 is highly expressed in some but not in all MBs, and that bcl-2 expression is not sufficient to block lovastatin-induced apoptosis in MBs expressing bcl-2.

3.1.7 D283 Med and D341 Med relative insensitivity and c-myc

As noted above, D283 Med and D341 Med were relatively resistant to lovastatin compared to UW228 and Daoy. This difference in lovastatin sensitivity may be attributed to the differences in oncogene expression, such as c-myc, between the different cell lines.

3.1.7.1 D283 Med and D341 Med insensitivity and c-myc
**Figure 3.6.** Lovastatin Induced Changes in ‘Resistant’ Daoy.  
A: Flow Cytometric Changes. Percentages of cells in Go/G1, S and G2/M are shown. The left graph represents untreated cells. The right graph represents cells treated with 20 μM of lovastatin for 72 hr.  

B: DNA Fragmentation. Ethidium bromide-stained agarose gel of DNA after treatment with varying concentrations of lovastatin. Lane 1: untreated x 48 hr; 2: 1 μM x 48 hr; 3: 10 μM x 48 hr; 4: 20 μM x 48 hr; 5: 40 μM x 48 hr; 6: 20 μM x 72 hr; 7: 40 μM x 72 hr; M: marker.
Figure 3.7. *bcl-2* Expression Changes Induced by Lovastatin.

Western blotting for *Bcl-2* proteins; cells were treated with 20 μM of lovastatin after varying intervals. 20 μg of cell lysates of each Daoy, UW228 and D283 Med were loaded while 50 μg of D341 Med lysates were used. After densitometric analysis of the blots, the relative density was normalized to lane 5 of each cell line on each membrane. Columns: means of two different experiments; bars: SEM. Time intervals for lanes in Daoy and UW228: (1) 0 hr; (2) 8 hr; (3) 12 hr; (4) 24 hr; (5) 36 hr; (6) Untreated x 36 hr. Time intervals for lanes in D283 Med: (1) 0 hr; (2) 24 hr; (3) 36 hr; (4) 48 hr; (5) 72 hr; (6) Untreated x 72 hr. Time intervals for lanes in D341 Med: (1) 0 hr; (2) 24 hr; (3) 48 hr; (4) 72 hr; (5) 96 hr; (6) Untreated x 96 hr.

*: p<0.05. Student’s T-test was performed for the comparison with untreated controls (lane 6).

A. U.: arbitrary units.
It was previously reported that the *c-myc* gene is overexpressed in D283 Med and D341 Med (Bigner et al., 1990; Friedman et al., 1988). Therefore, *c-myc* gene expression was compared among all tested cell lines. Western blotting revealed that *c-myc* is relatively overexpressed in D283 Med and D341 Med, compared to sensitive cell lines Daoy and UW228 (Figure 3.8), raising the possibility that *c-myc* gene overexpression may confer relative resistance to lovastatin.

### 3.1.7.2 Blocking *c-myc* expression does not increase sensitivity

Following the treatment with 25 µM of *c-myc* antisense oligonucleotides on D283 Med and D341 Med (Figure 3.9A lanes 3 and 4), *c-myc* gene expression was markedly reduced to one third of control levels (without antisense *c-myc* treatment, Figure 3.9A lanes 1 and 6, and *c-myc* sense oligonucleotides treatment, Figure 3.9A lanes 2 and 5). However, DNA gel electrophoresis showed that reducing *c-myc* expression did not change the sensitivity of D283 Med and D341 Med to lovastatin. Apoptotic DNA fragmentation occurred after lovastatin treatment 72 hr for D283 Med and 84 hr for D341 Med; similarly, DNA laddering was seen when 25 µM of *c-myc* antisense or sense oligonucleotides accompanied 20 µM lovastatin of D283 Med for 72 hr and D341 Med for 84 hr (Figure 3.9C lanes 4, 5 and 6). Blocking *c-myc* expression with *c-myc* antisense oligonucleotides did not shorten the time required for lovastatin-induced DNA fragmentation (Figure 3.9C lane 1), compared to lovastatin alone (Figure 3.9C lane 3). *c-myc* sense oligonucleotide had no effect (Figure 3.9C lane 2). Like untreated control cells (Figure 3.9C lane 9), no DNA laddering was evident after administration of *c-myc* antisense and sense oligonucleotides alone (Figure 3.9C lanes 7 and 8).

Western blotting using a caspase-3 antibody, which recognizes the pro-caspase-3 and its active subunit p11, showed that caspase-3 was not cleaved to its active form by 20 µM lovastatin treatment after 48 hr for D283 Med, or 60 hr for D341 Med (Figure 3.9B lane 6); these represent early time points of apoptosis in these two cell lines. Beyond these
Figure 3.8. c-myc Expression in medulloblastoma Cell Lines. Western blotting for C-myc proteins; 30 μg of cell lysates of each cell line were loaded. The relative density was normalized to lane 2 after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. Lane 1: Daoy; Lane 2: UW228; Lane 3: D283 Med; Lane 4: D341 Med; Lane M: molecular weight markers. A. U.: arbitrary units. *: p<0.01, by Student's T-test compared to lane 2. Some bands with molecular weight of 36.8-52 kDa are C-myc subunits.
Figure 3.9. \(c\)-myc Expression and Lovastatin-Induced Apoptosis.

Left column represents D283 Med, right column is for D341 Med. All cell lines were treated with 20 \(\mu\)M lovastatin, 25 \(\mu\)M \(c\)-myc antisense and sense oligonucleotides for the stated time interval. A: Western blotting for C-myc. The relative density was normalized to lane 1 on each membrane after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. The above and below bands of C-myc are C-myc other forms and its subunits (cleavage products). B: Western blotting for caspase-3. C: Agarose gels of DNA laddering. Lanes in A and B show 48 hr treatment of D283 and 60 hr treatment of D341 Med, which are (1) Untreated; (2) sense \(c\)-myc; (3) antisense \(c\)-myc; (4) lovastatin plus antisense \(c\)-myc; (5) lovastatin plus sense \(c\)-myc; (6) Lovastatin. Lanes in C: (1) lovastatin plus antisense \(c\)-myc, D283 Med x 48 hr, D341 Med x 60 hr; (2) lovastatin plus sense \(c\)-myc, D283 Med x 48 hr, D341 Med x 60 hr; (3) lovastatin, D283 Med x 48 hr, D341 Med x 60 hr; (4) lovastatin plus antisense \(c\)-myc, D283 Med x 72 hr, D341 Med x 84 hr; (5) lovastatin plus sense \(c\)-myc, D283 Med x 72 hr, D341 Med x 84 hr; (6) lovastatin, D283 Med x 72 hr, D341 Med x 84 hr; (7) antisense \(c\)-myc, D283 Med x 72 hr, D341 Med x 84 hr; (8) sense \(c\)-myc, D283 Med x 72 hr, D341 Med x 84 hr; (9) Untreated D283 Med x 72 hr, D341 Med x 84 hr.

A. U.: arbitrary units. *: \(p<0.01\). Student’s T-test was performed for the comparison with controls (lane 1).
points, caspase-3 was cleaved to its active form (see below manumycin A treatment). 25 μM of c-myc antisense oligonucleotides blocked c-myc expression, but did not shorten the time required for lovastatin-induced apoptosis in D283 Med and D341 Med. Thus, for shorter treatment, no caspase-3 cleavage was seen with c-myc antisense oligonucleotides plus 20 μM of lovastatin treatment of D283 Med and D341 Med (Figure 3.9B lane 4). Like untreated cells (Figure 3.9B lane 1), the controls (c-myc antisense oligonucleotide treatment and c-myc sense oligonucleotide treatment as well as c-myc sense oligonucleotide plus lovastatin treatment) did not induce caspase-3 cleavage (Figure 3.9B lanes 3, 2 and 5).

3.2 The mevalonate pathway in lovastatin-induced MB apoptosis

3.2.1 Mevalonate prevents lovastatin-induced morphological changes

Morphological changes of MB cells treated with lovastatin have been stated above. Each cell line showed a different sensitivity to lovastatin: shrinkage of Daoy and UW228 cells commenced after about 24 hr treatment with lovastatin, while D283 Med and D341 Med cell shrinkage and detachment from flasks required more than 48 hr. In all tested cell lines, these morphological changes were reversible by co-incubation of lovastatin with mevalonate, but only if the duration of lovastatin pre-treatment was limited to a critical time window (Figure 3.10). Mevalonate failed to reverse the morphological changes in those cells that were treated with lovastatin for longer times, and were showing apoptotic bodies as assessed by phase contrast microscopy (Figure 3.10). Cells that were not pre-treated with lovastatin did not show morphological changes when they were treated with lovastatin and mevalonate.

3.2.2 Mevalonate prevents lovastatin-induced apoptosis

Apoptosis was assessed by flow cytometric analysis and DNA gel
Figure 3.10. Morphological Changes to Lovastatin and Mevalonate.
1) Normal cultured cells. 2) After exposure to lovastatin (20 μM) for 24 hr (Daoy and UW228) or 48 hr (D283 Med and D341 Med), cells begin to detach and round up. 3) Mevalonate (2 mM) rescues cells treated for 24 hr (Daoy and UW228) or 48 hr (D283 Med and D341 Med) with lovastatin, restoring original morphology. 4) No change is seen if lovastatin and mevalonate are co-administered for 48 hr (Daoy and UW228) or 96 hr (D283 Med and D341 Med). 5) Lovastatin alone induces marked morphologic changes after 48 hr (Daoy and UW228) or 96 hr (D283 Med and D341 Med), indicative of apoptosis. 6) After 48 hr (Daoy and UW228) or 96 hr (D283 Med and D341 Med) of lovastatin treatment, mevalonate is unable to restore original morphology.
electrophoresis.

3.2.2.1 Flow cytometric analysis

Following pre-treatment with lovastatin for limited durations, MB cells were again exposed to lovastatin and mevalonate for durations equivalent to those which induced flow cytometric evidence of apoptosis when lovastatin was administered alone. All four MB cell lines failed to exhibit an ‘apoptosis peak’ under these conditions (Table 3.3). In contrast, for cells treated with lovastatin for longer time periods the ‘apoptosis peak’

| Table 3.3. Summary of the Effect of Mevalonate on Lovastatin-induced Apoptosis (flow cytometric analysis of three experiments, PI staining) |
|---|---|---|---|---|
| Daoy |
| Distribution of Cells (%) | A | B | C | D | E |
| Lov (24 hr) | Lov | Lov (24 hr) + Lov/Mev (48 hr) | Lov | Lov (48 hr) + Lov/Mev (48 hr) | Lov/Mev |
| Apoptosis | 0 | 0.1 ± 0.2 * | 47.4 ± 5.5 | 37.5 ± 6.4 ** | 0 |
| UW/228 |
| Distribution of Cells (%) | A | B | C | D | E |
| Lov (12 hr) | Lov (12 hr) + Lov/Mev (36 hr) | Lov | Lov (36 hr) + Lov/Mev (36 hr) | Lov/Mev |
| Apoptosis | 0 | 0.2 ± 0.3 * | 59.1 ± 2.0 | 44.0 ± 2.6 ** | 0 |
| D283 Med |
| Distribution of Cells (%) | A | B | C | D | E |
| Lov (36 hr) | Lov (36 hr) + Lov/Mev (72 hr) | Lov | Lov (72 hr) + Lov/Mev (48 hr) | Lov/Mev |
| Apoptosis | 0.5±0.3 | 0.5 ± 0.6 * | 61.1 ± 2.0 | 40.9 ± 6.2 ** | 0 |
| D341 Med |
| Distribution of Cells (%) | A | B | C | D | E |
| Lov (36 hr) | Lov (36 hr) + Lov/Mev (72 hr) | Lov | Lov (72 hr) + Lov/Mev (48 hr) | Lov/Mev |
| Apoptosis | 0 | 0.1 ± 0.2 * | 32.8 ± 3.8 | 18.0 ± 3.1 ** | 0 |

Note: *: p>0.1, p was from comparison of group B and E. **: p<0.01, p was from comparison of group D and E. Data: Mean ± SD. Lov: lovastatin. Mev: mevalonate.
remained despite co-incubation with lovastatin and mevalonate (Table 3.3). However, when comparing group C and D, the percentage of apoptotic cells was significantly decreased in UW228 (p<0.01), D283 Med (p<0.01) and D341 Med (p<0.05), likely because surviving cells began proliferating again when mevalonate was provided in media. Cell cycle changes induced by lovastatin were also abolished; thus, G1 arrest in all four cell lines was overcome by mevalonate, as was G2 arrest in D283 Med (Figure 3.11). Cell cycle changes and apoptosis peaks did not develop when cells were treated with lovastatin and mevalonate together without lovastatin pre-treatment.

3.2.2.2 Electrophoretic analysis of DNA

As noted above, DNA fragmentation characteristic of apoptosis follows lovastatin treatment of MB cell lines. Mevalonate prevented lovastatin induced apoptotic DNA fragmentation when it was administered to cells pre-treated with lovastatin for limited durations (Figure 3.12), but failed to reverse lovastatin-induced apoptotic DNA fragmentation after longer lovastatin pretreatment (Figure 3.12 lanes 5&6). Co-administration of lovastatin and mevalonate failed to induce DNA laddering (Figure 3.12 lanes 4&7), indicating that mevalonate can prevent lovastatin-induced apoptosis if administered before the cell death program has been executed. For each cell line, a critical time window was identified beyond which mevalonate can no longer salvage lovastatin-treated cells; for the attached cell lines Daoy and UW228 it was between 24 and 48 hr, whereas it was considerably longer for the partially attached cell lines D283 Med and D341 Med, between 48 and 96 hr.

3.2.3 Mevalonate alters HMG-CoA reductase mRNA levels

The RT-PCR amplified GAPDH and HMG-CoA reductase cDNA used to probe Northern blots is shown in figure 3.13. Levels of HMG-CoA reductase mRNA in Daoy,
Figure 3.11. Cell Cycle Changes after Lovastatin and Mevalonate.
Flow cytometric analysis reveals an increasing proportion of cells in G1 (open squares), a decreasing proportion of cells in S (open circles) in all tested cell lines, while the proportion of cells in G2 (open triangles) decreases in Daoy (24 and 48 hr) and UW228 (12 and 36 hr) or increases in D283 Med and D341 Med (36 and 72 hr) after treatment of lovastatin (20 μM). When lovastatin (20 μM)-treated cells, Daoy (24 hr), UW228 (12 hr) and D283 Med (36 hr) as well as D341 Med (36 hr), were replenished with a mixture of lovastatin (20 μM) and mevalonate (2 mM), cell cycle parameters approached control values by 48 hr (solid symbols).
*: p<0.01, Student's T-test for the comparison of the data of different cell cycle phases at lov+mev and T2. Data points: means of three experiments. Bars: SEM
Figure 3.12. DNA Laddering after Lovastatin and Mevalonate Treatment.
Extracted DNA is intact at: Time 0 (lane 1); after lovastatin (20 μM) x 24 hr (Daoy & UW228) or x 48 hr (D283 Med & D341 Med) (lane 2); after lovastatin x 24 hr (Daoy & UW228) or x 48 hr (D283 Med & D341 Med) followed by lovastatin and mevalonate (2 mM) x 48 hr (lane 3); and after lovastatin and mevalonate co-incubation x 24 hr (Daoy & UW228) or x 48 hr (D283 Med & D341 Med) (lane 4). Lovastatin x 48 hr (Daoy & UW228) or x 96 hr (D283 Med & D341 Med) induces DNA laddering (lane 5). Mevalonate cannot rescue cells pre-treated with lovastatin alone x 48 hr (Daoy & UW228) or x 96 hr (D283 Med & D341 Med) (lane 6). No laddering is seen following lovastatin and mevalonate co-incubation x 48 hr (Daoy & UW228) or x 96 hr (D283 Med & D341 Med) without lovastatin pre-treatment (lane 7); DNA from untreated cells incubated for 48 hr (Daoy & UW228) or 96 hr (D283 Med & D341 Med) is also intact (lane 8); M: molecular weight marker.
Figure 3.13. RT-PCR Production of cDNA Probe.
Ethidium bromide-stained agarose gels of RT-PCR cDNA. 1: HMG-CoA reductase cDNA, 525 bp. 2 & 4: Molecular weight marker (100 bp laddering). 3: GAPDH cDNA, 528 bp.
D283 Med and D341 Med were increased (p<0.05) after MB cells were treated with up to 40 μM lovastatin (Figure 3.14 lane 2). Curiously, HMG-CoA reductase mRNA levels in the adherent cell line UW228 were apparently unaffected (p>0.1) by lovastatin (Figure 3.14 lane 2). In addition, more pronounced increases (3.5 folds) were seen in the partially attached cell lines D283 Med and D341 Med, compared with the adherent cell line Daoy (2.5 folds). In all four cell lines, HMG-CoA reductase mRNA was reduced below baseline when cells pretreated with lovastatin for limited duration were then incubated with lovastatin and mevalonate (Figure 3.14 lane 3). Fresh cells co-incubated with both lovastatin and mevalonate showed similar levels of HMG-CoA reductase mRNA as untreated cells (Figure 3.14 lanes 1&4).

3.3 Protein farneslylation and lovastatin-induced MB apoptosis

3.3.1 Lovastatin blocks protein isoprenylation in MB apoptosis

In order to investigate the biochemical mechanisms of lovastatin-induced apoptosis, Western blotting was performed on some proteins involved in the mevalonate pathway in apoptotic MB cells. p21 Ras was identified as a reliable indicator of protein processing during isoprenylation. The molecular weight of p21 Ras was larger than normally processed p21 Ras in all MB cell lines after ≥10 μM of lovastatin treatment (Figure 3.15), indicating that most p21 Ras proteins failed to undergo post-translational isoprenylation (Gutierrez et al., 1989). The possibility that protein isoprenylation, dependent on the mevalonate pathway, may play an important role in MB survival, led to the investigation of the role of farnesylation in these cells.

3.3.2 Blocking protein farnesylation induces MB apoptosis

3.3.2.1 Manumycin A blocks protein farnesylation
Figure 3.14. HMG-CoA Reductase mRNA levels after 20 μM Lovastatin and 2 mM Mevalonate. Northern-blotting for HMG-CoA reductase and GAPDH mRNA. The relative expression of HMG-CoA reductase was normalized to GAPDH expression in each lane after densitometric analysis of the bands. Columns: means of two different experiments; bars: SEM. Note the upregulation of HMG-CoA reductase following lovastatin administration in Daoy, D283 Med and D341 Med, and the marked down-regulation accompanying mevalonate coadministration in all four cell lines.

Lane 1: untreated; lane 2: lovastatin (24 h for Daoy and UW228; 48 hr for D283 med and D341 Med); lane 3: lovastatin and mevalonate co-incubation x 36 hr, after lovastatin pre-treatments (see lane 2); and lane 4: lovastatin and mevalonate co-incubation (24 hr for Daoy and UW 228; 48 hr for D283 Med and D341 Med) without lovastatin pretreatment.

GAPDH expression reveals minimal variability in loading.

*: p<0.05, Student’s T-test for the comparison of lane 1 and lane 2. **: p<0.05, Student’s T-test for the comparison of lane 2 to both lane 3 and 4. A.U.: arbitrary units.
Figure 3.15. Lovastatin Blocks p21 Ras Isoprenylation.
Western blotting for p21 Ras proteins. A: Daoy, lovastatin x 36 hr. B: UW228, lovastatin x 36 hr. C: D283 Med, lovastatin x 60 hr. D: D341 Med, lovastatin x 72 hr. 1: untreated. 2: 1 μM lovastatin. 3: 10 μM lovastatin. 4: 20 μM lovastatin. 5: vehicle.
C: cytosol (unprocessed) isoform. M: membrane (processed) isoform.
To explore the importance of isoprenylation in MB cell apoptosis, and to distinguish the roles of protein farnesylation and geranylgeranylation, manumycin A, a selective inhibitor of protein farnesylation (Hara et al., 1993), was administered to cultured MB cells. Similar to the effects ofLovastatin, the molecular weight of most p21 Ras was larger than the normally processed form following ≥10 μM of manumycin A treatment, but was unchanged following 1 μM of manumycin A (Figure 3.16). The small amount of processed p21 Ras seen following manumycin treatment may reflect protein geranylgeranylation (see discussion).

3.3.2.2 Manumycin A-induced morphological changes

After treatment of MB cells with varying concentrations of manumycin A, significant morphological changes and inhibition of proliferation were observed at ≥10 μM manumycin A (Figure 3.17). For attached cells Daoy and UW228, cell bodies became rounded and shrunken, cells detached from flasks, and growth was inhibited (Figure 3.17A and B). For D283 Med and D341 Med, all attachment on flask was lost, cell bodies shrank, and cell proliferation was inhibited (Figure 3.17C and D). Morphological changes were induced by 12 hr of treatment in all cell lines, more rapidly than seen following Lovastatin treatment. All cells showed similar sensitivities to manumycin A in a dose-dependent manner. The treatment of manumycin A at a concentration of 1 μM had little effect on morphology in any cell line, and maximal changes were achieved at 20 μM of manumycin A treatment.

3.3.2.3 Effects of manumycin A on cell viability

Treatment of each MB cell line with 10-20 μM manumycin A resulted in significant declines (p<0.01) in viable cell quantity, as assessed by trypan blue exclusion. Control cells increased in quantity between 12-24 hr (Figure 3.18). 1 μM manumycin A had no apparent effect on cell quantity (p>0.1). 10 μM manumycin A abrogated the
Figure 3.16. Manumycin A Blocks p21 Ras Farnesylation.
Figure 3.17. Manumycin A Induces Morphologic Changes.
The top row shows control (vehicle-treated) cells. The bottom row shows cells following exposure to 20 μM manumycin A for 24 hr. Note extensive cell rounding of Daoy and UW228, and rounding and fragmentation of D283 Med and D341 Med.
Figure 3.18. Cell Death Induced by Manumycin A. Viability of MB cells was determined by trypan blue exclusion, in untreated cells (0 μM) and after exposure to 1-20 μM manumycin A for 24 hr. Data points: means of three experiments. Bars: 2xSEM.
expected increase in viable cell quantity (Figure 3.18) after 6 hr (p<0.05 was for the comparison of 10 μM treatment and control at 12 hr time point) while 20 μM manumycin A decreased viable cell quantity sooner and thereafter the quantity of viable cells diminished markedly after 24 hr. All tested cell lines showed similar responses. Following exposure to 20 μM manumycin A for 24 hr, Daoy, UW228, D341 Med and D283 Med showed reduction of viable cell quantity to 10%, 20%, 40% and 20% of passaged quantity, respectively. When compared to the quantity of viable cells in control flasks at maximum time intervals, the effect of 20 μM manumycin A for each cell line was to reduce the quantity of viable cells by 85-95%.

There was some variability in sensitivity to increasing dosages, with UW228 and D283 Med requiring only 10 μM for near-maximum response after 24 hr (p>0.05 for the comparison of 10 μM and 20 μM treatment at time 24 hr), while Daoy and D341 Med required 20 μM of manumycin A for maximal response (p<0.01 for the comparison of 10 μM and 20 μM treatment at time 24 hr) (Figure 3.18).

3.3.2.4 Manumycin A-induced apoptosis

To characterize manumycin A-induced changes in these cells, cellular DNA was analyzed using flow cytometry and DNA gel electrophoresis.

3.3.2.4.1 Flow cytometric analysis

For each cell line, ≥10 μM of manumycin A induced the appearance of a subdiploid 'apoptosis peak' at similar time points (about 12 hr) in all cell lines (Figure 3.19), suggesting that all cell lines have similar sensitivity to manumycin A. The size of the apoptosis peak increased progressively with increasing dosages between 10 and 20 μM, and the size of the apoptosis peak resulting from 20 μM of manumycin A treatment increased progressively between 12 and 36 hr (Table 3.4).
Figure 3.19. Flow Cytometry of Manumycin A-Induced DNA Fragmentation. The left column of graphs represents control cells (24 hr). The right column of graphs represents cells treated with 20 μM manumycin A (24 hr). The subdiploid shaded peak which appears to the left of the tall control peak (G1) represents the apoptotic fraction. This figure represents one of three experiments, summarized in table 3.4 and figure 3.21.
Table 3.4. Summary of Manumycin A-Induced Apoptosis in a Dose- and Time-Dependent Manner (flow cytometric analysis of three experiments, PI staining)

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<td>0 µM</td>
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<td>13.1 ± 4.7</td>
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<td>20 µM</td>
<td>78.0 ± 6.6</td>
<td>42.9 ± 4.0</td>
<td>37.9 ± 3.0</td>
<td>56.4 ± 6.7</td>
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<tr>
<td></td>
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</table>

B: Time (20 µM manumycin A)

<table>
<thead>
<tr>
<th>Manumycin A Treatment</th>
<th>Daoy Apoptosis Percentage</th>
<th>UW228</th>
<th>D283 Med</th>
<th>D341 Med</th>
</tr>
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<tr>
<td>36 hr</td>
<td>78.0 ± 6.6</td>
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<td>56.4 ± 6.7</td>
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<td>24 hr</td>
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<td>17.4 ± 3.3</td>
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<td>12 hr</td>
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<td>2.5 ± 2.7</td>
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<td>18.4 ± 5.4</td>
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<td>0</td>
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</table>

Note: r²: Pearson correlation coefficient squared. Data: Mean ± SD.

3.3.2.4.2 Electrophoretic analysis of DNA

DNA fragmentation ('laddering'), indicating apoptotic cleavage of chromosomal DNA, was observed in all 4 cell lines after 20 µM of manumycin A treatment (Figure 3.20A). Manumycin A-induced DNA laddering was also time-dependent (Figure 3.20B), correlating with morphological and flow cytometric evidence of apoptosis.

3.3.2.5 Effects of manumycin A on the cell cycle

Unlike lovastatin-induced cell cycle changes, the results of flow cytometry showed that, for each cell line accompanying apoptosis, 20 µM of manumycin A did not block the S phase entry and had no effect on G1 and G2 phase (Figure 3.19). The cell cycle distribution at varying time points of manumycin A treatment is summarized in Figure 3.21, according to the analysis of flow cytometry. The cell cycle distribution in cultures exposed to 20 µM of manumycin A was unchanged during treatment, compared to untreated control and vehicle treated control (p>0.1). Thus, no consistent effects on the cell cycle accompanied manumycin A-induced apoptosis, in contrast to changes induced by
Figure 3.20A. Manumycin A Induces Apoptotic DNA Fragmentation.
Ethidium bromide-staining of agarose gels shows laddering of DNA after incubation with manumycin A for 36 hr. 1: untreated. 2: vehicle. 3: 10 μM manumycin A. 4: 20 μM manumycin A. 5: marker.
Figure 3.20B. Time Course of Apoptotic DNA Fragmentation Induced by Manumycin A. Ethidium bromide-staining of agarose gels shows laddering of DNA after cells were incubated with 20 μM manumycin A for varying intervals. Time intervals for lanes: (1) 0 hr; (2) 6 hr; (3) 12 hr; (4) 24 hr; (5) 36 hr; (6) 48 hr; (7) Untreated x 48 hr; (8) Molecular weight marker. Laddering is first apparent at 12 to 24 hr interval.
Figure 3.21. Cell Cycle Analysis of Manumycin A Treated Cells. 
G1 (open squares), S (solid diamonds) and G2 (open circles) phase entry is not blocked by manumycin A, compared to controls (columns 2 and 3). 
Data points: Means of three experiments. Bars: SEM.
lovastatin.

3.3.2.6 Manumycin A effects on HMG-CoA reductase expression

[32P]-labeled human HMG-CoA reductase cDNA probe was hybridized with total cellular RNA from manumycin A-treated MB cells. HMG-CoA reductase mRNA levels in all four tested cell lines was decreased (p<0.05) after 10 and 20 μM of manumycin A treatment (Figure 3.22), with some variability among these cell lines. As the control, GAPDH mRNA levels were unchanged under all treatment conditions of manumycin A. Thus, manumycin A-induced physiological regulation of HMG-CoA reductase gene expression is distinct from that seen with lovastatin treatment. The likely mechanism is discussed below (see discussion).

3.3.2.7 Caspase-3 activation in MB apoptosis

Caspase-3 antibody was used to determine its activation status in manumycin A-treated MB cells. Figure 3.23A shows that caspase-3 was cleaved to its active p11 subunit in 20 μM of manumycin A treatment for 24 hr, indicating activation of the apoptotic cascade (Hale et al., 1996). The p20 subunit of caspase-3 is not recognized by the p11 antibody, and thus was not detected.

Caspase-3 was also cleaved to its active subunit by treatment with 20 μM lovastatin, although at different times for each cell line (Figure 3.23B). For attached cell lines Daoy and UW228, 36 hr treatment was required while 96 hr is needed for D283 Med and D341 Med.

Therefore, the caspase-3 pathway is activated in apoptosis induced by blocking protein farnesylation with either lovastatin or manumycin A. This is consistent with, but does not definitely prove, a common mechanism for entering the apoptotic cascade for both agents.
Manumycin A Induces HMG-CoA Reductase Downregulation.

Northern-blot analysis reveals the mRNA levels of HMG-CoA reductase and GAPDH after treatment with manumycin A for x 18 hr. The top row shows HMG-CoA reductase expression, while the bottom row displays GAPDH expression (control). The relative expression of HMG-CoA reductase was normalized to GAPDH expression after densitometric analysis of the bands. Columns represent the means of two different experiments, and bars indicate SEM.

Columns:
1: untreated. 2: vehicle. 3: 10 μM manumycin A. 4: 20 μM manumycin A.

*: p<0.01, Student's T-test for the comparison with untreated controls (lane 1).
A.U.: arbitrary units.

Figure 3.22: Manumycin A Induces HMG-CoA Reductase Downregulation.
Figure 3.23A. Manumycin A Induces Caspase-3 Activation.
Western blotting of CPP32 and its subunit p11 after manumycin A x 24 hr.
1: untreated. 2: 20 μM manumycin A. 3: vehicle.

Figure 3.23B. Lovastatin Induces Caspase-3 Activation.
Western blotting of CPP32 and its subunit p11 after lovastatin treatment.
Daoy, 36 hr. UW228, 36 hr. D283 Med, 96 hr. D341 Med, 96 hr.
1: 20 μM lovastatin. 2: vehicle. 3: untreated.
3.4 \textit{p21 ras} involvement in MB apoptosis

The alterations in the relative levels of \textit{ras} mRNA and its patterns were analyzed with RT-PCR in lovastatin and manumycin A treated cell lines, Daoy, UW228, D283 Med and D341 Med.

3.4.1 Total \textit{p21 ras} expression

With varying concentrations of lovastatin or manumycin A treatment, the total \textit{ras} mRNA levels failed to show any significant change ($p>0.1$, ANOVA), compared to control (Figure 3.24). Furthermore, with the treatment of 20 \textmu M of lovastatin or manumycin A, which is sufficient to induce apoptosis in all tested cell lines (Macaulay et al., 1999; Wang and Macaulay, 1999), the total \textit{ras} mRNA levels showed a constitutive state without any significant change ($p>0.1$, ANOVA) at varying time points, similar to the levels in control cells (Figure 3.25). This finding indicates that \textit{ras} gene transcription or total mRNA was stable despite the blockage of \textit{ras} protein isoprenylation. However, it does not exclude the possibility that particular \textit{ras} genes may have been differentially regulated by mevalonate derivatives.

3.4.2 Single \textit{p21 ras} expression

To address this question, the relative levels of \textit{N-ras}, \textit{K-ras} and \textit{H-ras} mRNA were assessed with RT-PCR in lovastatin- and manumycin A-treated cells. No significant changes ($p>0.1$, ANOVA) of these \textit{ras} mRNA were observed in both lovastatin and manumycin A treatment, compared to control (Figure 3.26). However, \textit{N-ras} gene expression was much higher than \textit{K-ras} and \textit{H-ras} gene expression in each cell line; \textit{N-ras} and \textit{H-ras} gene expression was similar when cell lines were compared to one another; and \textit{K-ras} gene expression was at low levels in each cell line, but were difficult to detect in UW228 and D283 Med.
Figure 3.24. Total ras mRNA in Medulloblastoma Cells.

Ethidium bromide-stained agarose gels of RT-PCR cDNA products after cells were treated with lovastatin or manumycin A for indicated time intervals. The top row represents lovastatin treatment (24 hr in Daoy and UW228, 48 hr in D283 Med and D341 Med), while the bottom row shows manumycin A treatment for 24 hr. The relative expression of ras at the different concentration lovastatin and manumycin A was normalized to GAPDH expression after densitometric analysis of the bands. Columns: Means of two different experiments; bars: SEM. Lanes in lovastatin treatment: (1) Untreated; (2) Vehicle; (3) 10 μM; (4) 20 μM; (5) 40 μM; (6) Marker. Lanes in manumycin A treatment: (1) Untreated; (2) Vehicle; (3) 1 μM; (4) 10 μM; (5) 20 μM; (6) marker. GAPDH: 528 bp; ras: 299 bp.

A.U.: arbitrary units.
Figure 3.25. Time Course of Total ras mRNA in Medulloblastoma Cells.
Ethidium bromide-stained agarose gels of RT-PCR cDNA products after cells were treated with 20 μM lovastatin or 20 μM manumycin A for indicated time intervals. The top row represents lovastatin treatment, while the bottom row shows manumycin A treatment. The relative expression of ras was normalized to GAPDH expression after densitometric analysis of the bands. Columns: means of two different experiments; Bars: SEM. Time intervals for lanes in lovastatin treatment: (1) time 0; (2) 6 hr; (3) 12 hr; (4) 24 hr; (5) 48 hr in D283 Med & D341 Med, or untreated x 24 hr in Daoy & UW228; (6) Untreated x 48 hr. Time intervals for lanes in manumycin A treatment: (1) time 0; (2) 6 hr; (3) 12 hr; (4) 24 hr; (5) Untreated x 24 hr. GAPDH: 528 bp; ras: 299 bp. M: Marker. A.U.: arbitrary units.
Figure 3.26. N-, K- and H-ras mRNA after 20 μM Lovastatin or 20 μM Manumycin A.
Ethidium bromide-stained agarose gels of RT-PCR cDNA products after cells were treated with test reagents for indicated time intervals. The relative expression of ras was normalized to GAPDH expression after densitometric analysis of the bands. Columns: means of two different experiments; bars: SEM. GAPDH: 528 bp; N-ras: 326 bp; K-ras: 319 bp; H-ras: 354 bp. Lanes in Daoy and UW228: (1) Lovastatin x 24 hr; (2) Lovastatin vehicle x 24 hr; (3) Untreated x 24 hr; (4) Manumycin A vehicle x 24 hr; (5) Manumycin A x 24 hr; (M) Marker (100 bp laddering). Lanes in D283 Med and D341 Med: (1) Lovastatin x 48 hr; (2) Lovastatin vehicle x 48 hr; (3) Untreated x 48 hr; (4) Manumycin A vehicle x 24 hr; (5) Manumycin A x 24 hr; (M) Marker (100 bp laddering). A.U.: arbitrary units.
3.5 Lovastatin- and manumycin A-induced MB apoptosis and p53

Apoptosis can be classified as either p53-dependent or p53-independent. The ideal approach to classifying a given apoptotic phenomenon involves direct modulation of p53 function; this is beyond the scope of the current work. However, p53 is a transcription factor, and specifically regulates expression of the death gene \textit{bax} in p53 dependent apoptosis. Thus, an alternative approach to modulating p53 function is to detect \textit{bax} gene expression.

3.5.1 \textit{p53} expression

Following lovastatin and manumycin A treatment, Western blotting showed that significantly increased \textit{p53} expression was induced after administration of 20μM lovastatin in D341 Med (48 hr), and after administration of 20 μM manumycin A (12 hr) in D341 Med (p<0.05). In contrast, no significant change (p>0.05) was induced in Daoy, UW228 and D283 Med in either lovastatin or manumycin A incubation (Figure 3.27), a slightly increased \textit{p53} expression was observed in UW228 treated with lovastatin (24 hr) and manumycin A (12 hr). However, \textit{p53} levels were reduced to normal, even to below baseline, after longer treatments with lovastatin (36 hr for Daoy and UW228, 72 hr for D283 Med and D341 Med) and manumycin A (24 hr). \textit{p53} levels were stable in untreated controls and vehicle treated controls. Although these results were partly contradictory, and the timing of changes precluded a consistent effect among cell lines, further evidence of \textit{p53} involvement was sought.

3.5.2 \textit{bax} expression

Although \textit{p53} expression is up-regulated in lovastatin- and manumycin A-treated UW228, and D341 Med, Western blotting failed to show any significant change in \textit{bax} gene expression in both lovastatin and manumycin A treatment in all cell lines (p>0.1, ANOVA) (Figure 3.28). These results suggested that lovastatin- and manumycin
Figure 3.27. Lovastatin (20 μM) and Manumycin A (20 μM) Induced Changes of p53 Expression.
Western blotting for p53 proteins. The relative p53 level was normalized to lane 8 on each membrane after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. 12 hr treatment of manumycin A resulted in p53 up-regulation in UW228 and D341 Med (lane 4), but no change in Daoy and D283 Med (lane 4). Following exposure time increase to 24 hr (lane 3), p53 levels in all cell lines were reduced, even below baseline in Daoy, UW228 and D283 Med. Lanes 1 and 2 represent untreated cells (24 hr) and 24 hr treatment of manumycin A vehicle, respectively. p53 expression was increased in 24 hr treatment of lovastatin in UW228 and 48 hr in D341 Med (lane 6), while no change in 24 hr treatment in Daoy and 48 hr in D283 Med (lane 6). Following longer lovastatin treatment (36 hr for Daoy and UW228, 72 hr for D283 Med and D341 Med) (lane 7), p53 levels were reduced to normal in Daoy, UW228 and D341 Med, or below baseline in D283 Med. Lanes 5 and 8 represent untreated cells and lovastatin vehicle treatment (Daoy and UW228 x 36 hr, D283 Med and D341 Med x 72 hr), individually. A.U.: arbitrary units. *: p<0.05, Student’s T-test for the comparison with untreated controls (lanes 1 and 5).
Figure 3.28. Lovastatin and Manumycin A Induced Changes of bax Expression.
Western blotting for Bax proteins. The relative Bax band density was normalized to lane 8 on each membrane after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. No change of bax expression was seen in both 20 μM lovastatin and Manumycin A treatment for varying intervals. Time intervals for lanes: (1) Untreated x 24 hr; (2) Vehicle of manumycin A x 24 hr; (3) Manumycin A x 24 hr; (4) Manumycin A x 12 hr; (5) Untreated, x 36 hr for Daoy and UW228, x 72 hr for D283 Med and D341 Med; (6) Lovastatin, x 24 for Daoy and UW228, x 48 hr for D283 Med and D341 Med; (7) Lovastatin, x 36 hr for Daoy and UW228, x 72 hr for D283 Med and D341 Med; (8) Vehicle of lovastatin, x 36 hr for Daoy and UW228, x 72 hr for D283 Med and D341 Med. A.U.: arbitrary units.
A-induced apoptosis is probably p53 independent.

3.6 CKIs regulate cell cycle changes in MB apoptosis

As previously demonstrated, lovastatin arrested the cell cycle in G1, but manumycin A did not. The regulation of CKIs in lovastatin- and manumycin A-induced changes of MB cells was also investigated.

3.6.1 p16INK4 expression

Following 20 μM of lovastatin and manumycin A treatment, Western blotting showed that, compared to controls, p16INK4 expression exhibited no significant change during both compound treatments (p>0.1, ANOVA) (Figure 3.29), indicating that p16INK4 was not responsible for lovastatin induced cell cycle blocking, and that other CKIs expression may account for G1 arrest.

3.6.2 p21WAF1 expression

Western blotting showed that, compared to controls, 20 μM lovastatin induced significant increases of p21WAF1 expression in Daoy and UW228 (p<0.05), and 20 μM manumycin A resulted in obvious increases of p21WAF1 in Daoy and D341 Med (p<0.05) (Figure 3.30). There was no change of p21WAF1 protein in lovastatin-treated D341 Med, manumycin A-treated UW228 and both lovastatin- and manumycin A-treated D283 Med. These results suggested that p21WAF1 over-expression accounts for lovastatin-induced G1 arrest in Daoy and UW228. However, over-expression of p21WAF1 in manumycin A-treated Daoy and D341 Med did not result in cell cycle alteration (see above). The likely reason will be noted in discussion.

3.6.3 p27KIP1 expression

Treatment of each MB cell line with 20 μM lovastatin and manumycin A
Figure 3.29. Lovastatin and Manumycin A Induced Changes of \( p16 \) INK4 Expression.

Western blotting for \( p16 \) INK4 proteins. The relative \( p16 \) INK4 band density was normalized to lane 8 on each membrane after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. No change of \( p16 \) INK4 expression was seen in both 20 \( \mu \)M lovastatin and Manumycin A treatment for varying intervals. Time intervals for lanes: (1) Untreated x 24 hr; (2) Vehicle of manumycin A x 24 hr; (3) Manumycin A x 24 hr; (4) Manumycin A x 12 hr; (5) Untreated, x 36 hr for Daoy and UW228, x 72 hr for D283 Med and D341 Med; (6) Lovastatin, x 24 for Daoy and UW228, x 48 hr for D283 Med and D341 Med; (7) Lovastatin, x 36 hr for Daoy and UW228, x 72 hr for D283 Med and D341 Med; (8) Vehicle of lovastatin, x 36 hr for Daoy and UW228, x 72 hr for D283 Med and D341 Med. A.U.: arbitrary units.
Figure 3.30. Lovastatin and Manumycin A Induced Changes of p21 WAF1 Expression.

Western blotting for p21WAF1 proteins. The relative p21 WAF1 band density was normalized to lane 8 on each membrane after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. 12 hr (lane 4) and 24 hr (lane 3) treatment of 20 μM manumycin A induced p21WAF1 increase in Daoy and D341 Med, but no change in UW228 and D283 Med, compared to p21WAF1 levels in controls (lanes 1 and 2, which represent 24 hr cells of no treatment and treatment of manumycin A vehicle, respectively). p21WAF1 expression was increased in 24 hr (lane 6) and 36 hr (lane 7) treatment of lovastatin in Daoy and UW228, while no change in D283 Med and D341 Med (lane 6: x 48 hr, lane 7: x 72 hr). The baseline level of p21WAF1 is shown in lane 5 (untreated cells, Daoy and UW228 x 36 hr, D283 Med and D341 Med x 72 hr) and lane 8 (lovastatin vehicle treatment, Daoy and UW228 x 36 hr, D283 Med and D341 Med x 72 hr).

A.U.: arbitrary units. *: p<0.05, Student's T-test for the comparison with untreated controls (lanes 1 and 5).
resulted in increases of p27KIP1 protein with varying levels, as assessed by Western blotting (Figure 3.31). 20 μM manumycin A treatment induced obvious increases of p27 KIP1 in D283 Med and D341 Med (p<0.05), but slight increases in Daoy and in 12 hr treated UW228, compared to p27 KIP1 levels in controls (no treatment and manumycin A vehicle treatment). In all four test cell lines, p27 KIP1 proteins were induced to increase (p<0.05) by 20 μM lovastatin, compared to the baseline level of p27 KIP1 in untreated cells and lovastatin vehicle treated cells. These results suggest that p27KIP1 over-expression accounts for lovastatin-induced G1 arrest in D283 Med and D341 Med, and its over-expression plus the above p21WAF1 over-expression contributed to lovastatin-induced G1 arrest in Daoy and UW228. However, over-expression of p27KIP1 was not followed by cell cycle changes in all manumycin A-treated cell lines (see above and discussion).

Figure 3.31 also showed that bands with molecular weight of 21 kDa were detected by p27KIP1 Mab in all lovastatin- and manumycin A- treated cells. This band may be a subunit of p27KIP1, or it may be p21WAF1 since p27KIP1 shares N-terminal sequence homology with p21WAF1 (Toyoshima and Hunter, 1994).

3.7 Blocking G-protein function induces MB apoptosis

To assess the involvement of G-protein function in isoprenylation inhibitor-induced apoptosis, we used MPA. This agent depletes intracellular GTP by blocking the synthesis of GDP and GTP, thus inhibiting G-protein activation of downstream signaling cascades.

3.7.1 MPA-induced morphological changes

After 10 to 40 μM MPA treatment in D341 Med for 48 hr, as well as in Daoy, UW228 and D283 Med for 96 hr, morphological changes were observed in each cell line (Figure 3.32). For attached cell lines Daoy and UW228, some cell bodies became
Figure 3.31. Lovastatin and Manumycin A Induced Changes of p27 KIP1 Expression.
Western blotting for p27 KIP1 proteins. The relative p27 KIP1 band density was normalized to lane 8 on each membrane after densitometric analysis of the blots. Columns: means of two different experiments; bars: SEM. Lanes 1 and 2: 24 hr cells of no treatment and treatment of manumycin A vehicle, respectively; Lanes 3 and 4: 24 hr and 12 hr treatment of 20 μM manumycin A; Lane 5: untreated cells, Daoy and UW228 x 36 hr, D283 Med and D341 Med x 72 hr; Lane 6: 20 μM lovastatin treatment, Daoy and UW228 x 24 hr, D283 Med and D341 Med x 48 hr; Lane 7: 20 μM lovastatin treatment, Daoy and UW228 x 36 hr, D283 Med and D341 Med x 72 hr; Lane 8: lovastatin vehicle treatment, Daoy and UW228 x 36 hr, D283 Med and D341 Med x 72 hr. A.U.: arbitrary units. *: p<0.05, Student’s T-test for the comparison with untreated controls (lanes 1 and 5).
Figure 3.32. Morphologic Changes Induced by MPA.
The top row shows control cells (vehicle treatment), while the bottom row shows cells following exposure to 10 μM of MPA, after the stated time interval (phase contrast microscope). Note some extensive cell rounding and fragmentation of all test cell lines.
Daoy x 96 hr; UW228 x 96 hr; D283 Med x 96 hr; D341 Med x 48 hr.
shrunken, some cells became round and detached from flasks. For the partially attached cell lines D283 Med and D341 Med, some attachment on flask was lost and cell bodies shrank. Since D341 Med required shorter treatment for morphological changes, it was considered most sensitive to MPA.

3.7.2 MPA-induced differentiation

After the cultured cells on glass cover slips were treated with MPA, cell quantity on slips was obviously decreased. The inhibition of cell growth, and UW228 differentiation were observed with immunohistochemical assay for GFAP and NF (Figure 3.33). After 7 days of 10 to 100 μM MPA treatment, Daoy was GFAP negative or weakly positive, while some cells in the control were GFAP positive, indicating the inhibition of cell growth and protein synthesis. Both treated and untreated Daoy were NF negative; some treated UW228 cells were GFAP positive, while control was GFAP negative, indicating UW228 growth inhibition and differentiation. Both treated and untreated UW228 were NF weakly positive. Since D283 Med and D341 Med grow largely in attachment on glass cover slips, they can be used for immunohistochemical analysis. After 6 days of 10 to 100 μM MPA treatment, D283 Med was NF negative or weakly positive, while the control was NF positive, indicating the inhibition of cell growth and protein synthesis; both treated and untreated D283 Med were GFAP negative. After 5 days of 0.5 to 2 μM MPA treatment, D341 Med was NF negative, while control was NF positive, indicating the inhibition of cell growth and protein synthesis; both treated and untreated D341 Med were GFAP negative. ≥10 μM MPA induced detachment of D341 Med at this time point, confirming that D341 Med was more sensitive to MPA than Daoy, UW228 and D283 Med.

3.7.3 Effects of MPA on cell viability

Treatment of each MB cell line with 10-40 μM MPA resulted in marked declines
Figure 3.33. Immunohistochemical Detection of MPA Treated Medulloblastoma Cells.
The top row shows control cells, while the bottom row shows cells following exposure to MPA, after the stated time intervals. DAOY and UW228 were treated with 10 μM MPA for 7 days, D283 Med was treated with 10 μM MPA for 6 days, D341 Med was treated with 0.5 μM MPA for 5 days. Both Daoy and UW228 were detected with GFAP antibody. Both D283 Med and D341 Med were detected with NF antibody. Control Daoy shows GFAP positive, MPA treated Daoy shows GFAP negative. Control UW228 is GFAP negative, MPA treated UW228 is GFAP positive. Both D283 Med and D341 Med controls show NF positive, while both treated D283 Med and D341 Med are NF negative.
(p<0.01, two-way ANOVA (time, [MPA])) in viable cell quantity, as assessed by trypan blue exclusion (Figure 3.34). Control cells increased in quantity between 24 to 96 hr. 1 μM MPA had no apparent effect on cell quantity of Daoy and UW228 as well as D283 Med (p>0.1), but prevented the anticipated cell quantity increase of D341 Med (p<0.05). For Daoy and UW228 as well as D283 Med, 10 μM MPA abrogated the expected increase in viable cell quantity after 60 hr (p<0.05), while 40 μM MPA inhibited the increase of viable cell quantity sooner and thereafter the quantity of viable cells was lower after 60 hr. For D341 Med, 10 and 40 μM MPA abrogated the expected increase in viable cell quantity after only 24 hr (p<0.05), and 40 μM MPA diminished more viable cell quantity after 48 hr. Following exposure to 40 μM MPA for 96 hr, Daoy and UW228 as well as D283 Med showed reduction of viable cell quantity to 15%, 30% and 20% of passed quantity, respectively. 40 μM MPA administration for 48 hr in D341 Med, however, resulted in reduction of viable cell quantity to 10% passed quantity. When compared to the number of viable cells in control flasks at maximum time intervals, the effect of 40 μM MPA was to reduce the quantity of viable cells by 75-90%.

3.7.4 MPA-induced apoptosis

To characterize MPA induced changes in these cells, cellular DNA was analyzed using flow cytometry. 10 to 40 μM MPA induced apoptosis at similar time points (about 96 hr) in Daoy, UW228 and D283 Med cell lines (Table 3.5). However, for D341 Med, 10 to 40 μM of MPA needed only 36 to 48 hr to induce apoptosis (Table 3.5); moreover, abundant apoptosis was induced with only 1 μM MPA after 72 hr, reflecting the high sensitivity of D341 Med to MPA. The percentage of apoptosis increased with increasing dosages between 10 and 40 μM, and the percentage of apoptosis resulting from 40 μM of MPA treatment increased following lengthened treatment in each cell line (Table 3.6).

DNA fragmentation indicating apoptosis was observed in all cell lines after ≥ 10 μM treatment, although the time periods necessary varied between cell lines (Figure 3.35).
Figure 3.34. Cell Death Induced by MPA.
Viability of MB cells was determined by trypan blue exclusion, in untreated cells (0 μM) and after exposure to 1-40 μM MPA for up to 96 hr. D341 Med was more sensitive, while Daoy and UW228 as well as D283 Med showed negligible effects until 60 hr of treatment. Data points: Means of three experiments. Bars: 2xSEM
Table 3.5. Summary of MPA-Induced Apoptosis in Dose-Dependent Manner (flow cytometric analysis of three experiments, PI staining)

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<th>MPA Concentration</th>
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<td>10 μM</td>
<td>72.2 ± 5.6</td>
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<td>40 μM</td>
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Note: $r^2$: Pearson correlation coefficient squared. Data: Mean ± SD.

Table 3.6. Summary of MPA-Induced Apoptosis in Time-Dependent Manner (flow cytometric analysis of three experiments, PI staining)

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<th>40 μM MPA Treatment</th>
<th>Apoptosis Percentage</th>
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<td>36 hr</td>
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<td></td>
<td>D283 Med</td>
</tr>
<tr>
<td>0 hr</td>
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</tr>
<tr>
<td>24 hr</td>
<td>0</td>
</tr>
<tr>
<td>36 hr</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>48 hr</td>
<td>7.0 ± 2.7</td>
</tr>
<tr>
<td>72 hr</td>
<td>33.5 ± 5.3</td>
</tr>
<tr>
<td>96 hr</td>
<td>78.7 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>D341 Med</td>
</tr>
<tr>
<td>0 hr</td>
<td>0</td>
</tr>
<tr>
<td>24 hr</td>
<td>0</td>
</tr>
<tr>
<td>36 hr</td>
<td>0.62</td>
</tr>
<tr>
<td>48 hr</td>
<td>0.66</td>
</tr>
<tr>
<td>72 hr</td>
<td>0.66</td>
</tr>
<tr>
<td>96 hr</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Note: $r^2$: Pearson correlation coefficient squared. Data: Mean ± SD.

For 10 to 40 μM of MPA treatment, the appearance of DNA fragmentation in D341 Med was about 48 hr, while Daoy and UW228 as well as D283 Med needed about 96 hr, again reflecting the higher sensitivity of D341 Med.

Flow cytometry and DNA fragmentation showed that MPA induced apoptosis, but the percentage of apoptotic cells in UW228 was lower (Table 3.5, 3.6 and Figure 3.35), suggesting that other mechanisms, such as differentiation, may account for the reduction in cell numbers in UW228 in response to MPA.

3.7.5 MPA-induced cell cycle changes

Flow cytometric analysis showed that MPA treatment induced G1 cell cycle arrest and blocked S phase entry in each cell line. Cell cycle distribution in cultures
Figure 3.35. DNA Fragmentation Caused by MPA.
Ethidium bromide-stained agarose gels of MB DNA after treatment with varying concentrations of MPA for the stated time interval. All four cell lines show apoptotic DNA 'laddering'. Daoy x 96 hr; UW228 x 96 hr; D283 Med x 96 hr; D341 Med x 48 hr.
Lane concentrations: (1) control cells; (2) treated with vehicle; (3) 10 μM MPA; (4) 40 μM MPA; (M) marker.
exposed to 40 μM MPA at varying intervals is summarized in Table 3.7. Although cell cycle distribution was unchanged during the initial 12 to 24 hr of treatment, the proportion of cells in S phase decreased concomitant with an increase in G1-phase cells as the time of exposure to MPA increased. The percentage of cells in G2/M phase remained stable (p>0.1) in UW228 and D341 Med, while it was reduced (p<0.05) in Daoy and D283 Med. The effect of MPA in increasing the proportion of G1-phase cells and reducing the proportion of S-phase cells was statistically significant (p<0.01). MPA induced cell cycle change was similar to lovastatin but not manumycin A, suggesting that G-protein function is required for MB proliferation, but that dysfunction of farnesylated G-proteins alone is not sufficient to induce cell cycle arrest. The significance of this finding is further discussed below.

3.7.6 Specificity of MPA effects

Only guanosine supplementation could prevent MPA-induced morphological changes, while adenosine or deoxyguanosine did not, indicating that such changes are due to its specific effect of GTP depletion. Similarly, only guanosine but neither adenosine nor deoxyguanosine prevented MPA-induced apoptotic peak and cell cycle arrest in flow cytometric analysis (Table 3.8), again indicating that MPA-induced apoptosis and cell cycle arrest are due to its specific effect of GTP depletion.

For apoptotic DNA ‘laddering’ analysis on agarose gel, again only guanosine, but not adenosine or deoxyguanosine, prevented MPA-induced DNA fragmentation when these compounds were supplemented into MPA treatment (Figure 3.36), supporting that MPA-induced apoptosis is due to its specific effect of GTP depletion.

3.8 Synergistic action of Lovastatin, manumycin A and MPA

To test whether these compounds (lovastatin, manumycin A and MPA) have similar mechanisms, and that their effects result from interference with the same pathway, a
Table 3.7. Summary of Effects of MPA (40 μM) on the Cell Cycle (flow cytometric analysis of three experiments, PI staining)

<table>
<thead>
<tr>
<th>Distribution of Cells (%)</th>
<th>Day</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>24 (con)</td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td>53.7 ± 2.8</td>
<td>54.1 ± 3.9</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>36.0 ± 1.5</td>
<td>37.6 ± 5.2</td>
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<tr>
<td>G2</td>
<td></td>
<td>10.3 ± 3.6</td>
<td>8.3 ± 3.0</td>
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<table>
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<th>Time (hr)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>24 (con)</td>
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<tr>
<td>G1</td>
<td></td>
<td>57.6 ± 3.3</td>
<td>60.5 ± 1.6</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>33.7 ± 2.8</td>
<td>30.5 ± 3.3</td>
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<tr>
<td>G2</td>
<td></td>
<td>8.7 ± 1.5</td>
<td>9.0 ± 2.7</td>
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<table>
<thead>
<tr>
<th>Distribution of Cells (%)</th>
<th>Day</th>
<th>Time (hr)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>24 (con)</td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td>49.0 ± 2.0</td>
<td>48.6 ± 2.1</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>35.1 ± 1.6</td>
<td>34.1 ± 2.2</td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>15.9 ± 0.8</td>
<td>17.3 ± 2.1</td>
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<table>
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<td></td>
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<td>12 (con)</td>
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<tr>
<td>G1</td>
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<td>53.0 ± 3.9</td>
</tr>
<tr>
<td>S</td>
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<td>33.1 ± 5.5</td>
<td>38.9 ± 1.2</td>
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<tr>
<td>G2</td>
<td></td>
<td>8.1 ± 1.5</td>
<td>8.1 ± 2.8</td>
</tr>
</tbody>
</table>

Note: Data: Mean ± SD. con: control. *: p<0.05, **: p<0.01, Scheffe multiple range test for the comparison of MPA treatment and its control at same time point.
Figure 3.36. Effects of Nucleosides on MPA-induced DNA Fragmentation. Ethidium bromide-stained agarose gels of DNA. Daoy cells were treated with test agents for 94 hr. Lane 1, control cells; Lane 2, treated with vehicle; Lane 3, treated with 40 μM MPA; Lane 4, treated with 40 μM MPA plus 500 μM adenosine; Lane 5, treated with 40 μM MPA plus 500 μM guanosine; Lane 6, treated with 40 μM MPA plus 500 μM deoxyguanosine; M, marker. Similar results were obtained in UW228, D283 Med and D341 Med.
Table 3.8. Flow Cytometric Analysis of the Effects of Nucleosides on MPA-induced Apoptosis and Cell Cycle Changes (flow cytometric analysis of three experiments, PI staining)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MPA</th>
<th>MPA+A</th>
<th>MPA+dG</th>
<th>MPA+G</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>49.7±6.9</td>
<td>56.8±4.7</td>
<td>45.4±6.2</td>
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<td>0</td>
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<tr>
<td>Survival</td>
<td>50.3±6.9</td>
<td>43.2±4.7</td>
<td>54.6±6.2</td>
<td>100*</td>
<td>100</td>
</tr>
<tr>
<td>Distribution</td>
<td>G1 90.3±5.3</td>
<td>86.3±5.1</td>
<td>78.5±5.4</td>
<td>66.1±2.9*</td>
<td>65.1±2.4</td>
</tr>
<tr>
<td>of Surviving Cells</td>
<td>S 7.4±3.1</td>
<td>9.4±2.6</td>
<td>14.6±2.8</td>
<td>25.8±4.7*</td>
<td>24.7±2.0</td>
</tr>
<tr>
<td>G2 2.3±2.8</td>
<td>4.3±2.1</td>
<td>6.9±1.5</td>
<td>8.1±4.3</td>
<td>10.2±1.2</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Daoy cells were treated with test reagents for 96 hr. MPA: 40 μM; A: adenosine, 500 μM; dG: deoxyguanosine, 500 μM; G: Guanosine, 500 μM. Data: Mean ± SD. *: p<0.01, Student's T-test for the comparison with MPA treatment. Similar results were obtained in UW228, D283 Med and D341 Med.

synergy experiment was performed. The choice of drug concentrations was based on the following observations: 1) low concentration (<10 μM) lovastatin and manumycin A have weak effects to induce apoptosis (Macaulay et al., 1999; Wang and Macaulay, 1999); 2) 40 μM of MPA does not induce apoptosis within 48 hr in Daoy, UW228 or D283 Med; 3) 1 μM of MPA has weak effects to induce apoptosis within 48 hr in D341 Med. On the basis of these observations, 2 μM of lovastatin and manumycin A plus 40 μM of MPA were co-provided to Daoy, UW228 and D283 Med for 48 hr, and 2 μM of lovastatin and manumycin A plus 1 μM of MPA were co-supplied to D341 Med for 48 hr. After treatment, cells were analyzed by flow cytometry. As shown in Table 3.9, (1) lovastatin, manumycin A, and MPA synergistically induced the appearance of apoptosis (p<0.01, ANOVA) with varying percentages; and (2) compared to single compound treatment, lovastatin plus MPA synergistically accelerated G1 arrest of the cell cycle (p<0.05, ANOVA) in Daoy and D341 Med, and resulted in slight increase of G1 phase cell proportions in UW228 and D283 Med; lovastatin plus MPA also accelerated decrease of S phase cell proportions (p<0.05, ANOVA) in Daoy, D283 Med and D341 Med, and slight decrease in UW228; these compounds had no synergistic effects on G2 change and; (3) single compounds alone could not induce apoptosis. These results suggest that the
### Table 3.9. Synergistic Effects of Lovastatin, Manumycin A and MPA on Medulloblastoma Apoptosis  
(flow cytometric analysis of three experiments, PI staining)

<table>
<thead>
<tr>
<th></th>
<th>Cell No. (%)</th>
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<th>L</th>
<th>MA</th>
<th>MPA</th>
<th>Treatment</th>
</tr>
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<tbody>
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<td></td>
<td></td>
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<td>MA+MPA</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0.6±0.5</td>
<td>0.8±0.7</td>
<td>22.2±2.5</td>
<td>43.3±3.3</td>
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<tr>
<td>Survival</td>
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<td>100</td>
<td>99.4±0.5</td>
<td>99.2±0.7</td>
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<td>75.7±3.3</td>
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<td>Distribution</td>
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<td>51.5±2.1</td>
<td>64.8±2.2</td>
<td>63.9±1.6</td>
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<tr>
<td>of Surviving</td>
<td>S</td>
<td>34.3±0.8</td>
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<td>35.3±1.1</td>
<td>17.1±0.7</td>
<td>20.8±1.8</td>
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<td>Cells</td>
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<td>15.5±2.0</td>
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### UW228

<table>
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<td>Apoptosis</td>
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<td>5.7±2.7</td>
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<td>Survival</td>
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<td>75.5±1.5</td>
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<td>of Surviving</td>
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<td>19.4±2.9</td>
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<td>9.4±2.2</td>
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<tr>
<td>Cells</td>
<td>G2</td>
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<td>10.1±2.0</td>
<td>9.5±1.8</td>
<td>13.9±1.7</td>
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(continued)
Table 3.9. Synergistic Effects of Lovastatin, Manumycin A and MPA on MB Apoptosis (cont.)
(flow cytometric analysis of three experiments, PI staining)

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<th>MPA</th>
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<tr>
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<td>23.8 ± 2.0</td>
<td>25.9 ± 1.5</td>
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<td>100</td>
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<td>Distribution of Surviving Cells</td>
<td>G1</td>
<td>54.7 ± 3.9</td>
<td>56.9 ± 4.6</td>
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<td>23.7 ± 1.4</td>
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<td>G2</td>
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<tr>
<td>Apoptosis</td>
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<td>0</td>
<td>0</td>
<td>10.7 ± 1.9</td>
<td>58.6 ± 2.2</td>
<td>60.1 ± 2.6</td>
<td>64.0 ± 4.0</td>
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<td>100</td>
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<td>36.0 ± 4.0</td>
<td>30.2 ± 4.0</td>
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<td>Distribution of Surviving Cells</td>
<td>G1</td>
<td>57.2 ± 3.5</td>
<td>58.6 ± 1.4</td>
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<td>69.8 ± 5.0</td>
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<td>61.9 ± 3.2</td>
<td>80.8 ± 4.4</td>
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<td>23.5 ± 1.2</td>
<td>28.7 ± 1.7</td>
<td>16.3 ± 5.7</td>
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<tr>
<td></td>
<td>G2</td>
<td>7.7 ± 1.1</td>
<td>10.1 ± 4.1</td>
<td>10.7 ± 3.3</td>
<td>3.1 ± 2.6</td>
<td>4.3 ± 4.4</td>
<td>9.4 ± 1.5</td>
<td>2.9 ± 1.8</td>
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</table>

Note: All four cell lines were treated with test reagents for 48 hr. L: lovastatin, 2 μM; MA: manumycin A, 2 μM; MPA: 40 μM for Duoy, UW228 and D283 Med, 1 μM for D341 Med. Data: Mean ± SD.
actions of these compounds may block different site(s) on the same pathway to induce MB apoptosis and cell cycle arrest.

After each cell line was treated under the above conditions, DNA electrophoresis showed that lovastatin plus manumycin A, or lovastatin plus MPA, or lovastatin plus manumycin A and MPA, or manumycin A plus MPA, synergistically induced the appearance of apoptotic DNA fragmentation to varying degrees, although no apoptosis was shown with single compounds (Figure 3.37). These results also suggest that different site(s) on the same pathway was individually targeted by these compounds to induce MB apoptosis.

3.9 Effects of blocking protein isoprenylation on primary MB

To investigate the therapeutic potential of blocking protein isoprenylation on primary MB, fresh primary MBs were cultured in vitro and used to determine the effects of lovastatin and manumycin A treatment. Primary MB, 98-8, grew in vitro as adherent cells with some suspension, while primary MB, 98-2627, grew in vitro as adherent cells.

3.9.1 Effects of lovastatin on primary MB

After primary MBs were minced and cultured to subconfluency, varying concentrations of lovastatin were administered for varying time intervals. After 96 hr treatment of $\geq 10 \, \mu M$ lovastatin, some MB cells became floating, shrunken and rounded (Figure 3.38A), similar to lovastatin-induced changes of Daoy and UW228. DNA electrophoresis showed that 96 hr treatment of $\geq 10 \, \mu M$ lovastatin induced apoptotic DNA 'laddering' (Figure 3.38B). Figure 3.38B also shows that lovastatin-induced apoptosis of primary MB was time- and dose-dependent.

Cultures of primary MBs contained proliferating fibroblasts (confirmed by detecting fibroblast antigen with fibroblast antigen monoclonal antibody (Ab-1) from Oncogene Science Diagnostics, Cambridge, MA). To exclude the possibility that
Figure 3.37. Apoptotic DNA Fragmentation Synergistically Induced by Lovastatin, Manumycin A and MPA. Ethidium bromide-stained agarose gels of MB DNA after 48 hr treatment with varying reagents. The concentrations of test reagents are Lovastatin: 2 μM; manumycin A: 2 μM; MPA: 40 μM for Daoy, UW228 and D283 Med, or 1 μM for D341 Med.

Lanes: (1) Untreated; (2) Vehicle Treated; (3) Lovastatin; (4) Manumycin A; (5) MPA; (6) Manumycin A plus MPA; (7) Lovastatin plus manumycin A; (8) Lovastatin plus MPA; (9) Lovastatin plus manumycin A and MPA; (10) marker.
Figure 3.38. Lovastatin-induced Changes in Primary Medulloblastoma.
A) Morphological changes. The left panels are control primary medulloblastoma (98-8 and 98-2627) culture (96 hr). The right panels show lovastatin treated primary MB (96 hr). Note apoptotic bodies in right panels.
B) Apoptotic DNA fragmentation on ethidium bromide-stained agarose gels after primary medulloblastoma cultures were treated with lovastatin for indicated time intervals. Lanes of lovastatin treatment: (M): Marker; (1) 20 µM, 120 hr; (2) 20 µM, 72 hr; (3) 20 µM, 96 hr; (4) 40 µM, 96 hr; (5) 10 µM, 96 hr; (6) Vehicle, 96 hr; (7) Untreated, 96 hr.
lovastatin-induced apoptosis in primary MB cultures is due to the death of such cells, the normal human fibroblast cell MRC-5 was used to assess the effects of lovastatin treatment. Following about 96 hr treatment of ≥ 10 μM of lovastatin, MRC-5 showed similar morphological changes and apoptotic DNA fragmentation (Figure 3.39). Therefore it was not possible to state which cell type(s) in these tumors was susceptible to lovastatin from this experiment (but see below).

3.9.2 Effects of manumycin A on primary MB

Primary MB cultures and MRC-5 were also used for farnesylation inhibitor assessment using manumycin A. After 36 hr treatment of ≥ 10 μM manumycin A, primary MBs showed apoptotic morphological changes and DNA fragmentation (Figure 3.40A and B) in a time- and dose-dependent manner. Unlike the lovastatin result, however, MRC-5 failed to show apoptotic DNA fragmentation after 36 hr treatment of ≥ 10 μM manumycin A. Subtle morphological changes were observed as attached cell numbers were reduced (Figure 3.40C and D). These data indicate that blocking protein farnesylation induces apoptosis in primary MBs as the result MB cell death, not fibroblast death.
Figure 3.39. Lovastatin-induced Changes in MRC-5.
A) Morphological changes. The top panel is normal MRC-5; the bottom panel shows lovastatin treated MRC-5 (72 hr). Note apoptotic bodies in bottom panel.
B) Apoptotic DNA fragmentation on ethidium bromide-stained agarose gel after cells were treated with lovastatin for indicated time intervals. Lanes of lovastatin treatment: (M): Marker; (1) Vehicle, 96 hr; (2) 20 μM, 24 hr; (3) 20 μM, 48 hr; (4) 20 μM, 72 hr; (5) 20 μM, 96 hr; (6) Untreated, 96 hr.
Figure 3.40. Manumycin A-induced Changes in Primary Medulloblastoma and MRC-5.

A) and C) Morphological changes in primary medulloblastoma 98-2627 and MRC-5 respectively. Apoptotic bodies are seen in 20 µM manumycin A treated primary medulloblastoma (36 hr), while only slight morphological changes are seen in manumycin A treated MRC-5 (48 hr).

B) and D) DNA on ethidium bromide-stained agarose gels after medulloblastoma 98-2627 and MRC-5 were treated with manumycin A for indicated time intervals respectively. Apoptotic DNA fragmentation occurs in primary medulloblastoma, but not in MRC-5. Lanes of manumycin A treatment: (M): Marker; (1) Untreated, 48 hr; (2) Vehicle, 48 hr; (3) 20 µM, 48 hr; (4) 20 µM, 36 hr; (5) 20 µM, 24 hr; (6) 20 µM, 12 hr; (7) 5 µM, 48 hr.
4.0 DISCUSSION

4.1 Lovastatin-induced apoptosis in MB

MB arises from neuroepithelial precursors in the developing cerebellum, likely of granule cell lineage (Yokota et al., 1996). MB recapitulates some phenotypic characteristics of these cells, including high expression of HMG-CoA reductase (Macaulay et al., 1994). Although the significance of this expression in MB has not been clarified, it may well relate to the participation of mevalonate derivatives in cellular proliferation (Keyomarsi et al., 1991) and/or in functional differentiation of neural tissue (Dimitroulakos and Yeger, 1996). Mitotic activity in MB cell lines is reduced after treatment with lovastatin, an HMG-CoA reductase inhibitor; a similar phenomenon has been observed in D283 Med and Daoy upon exposure to phenylacetate. Of note, however, no evidence of an apoptotic response was seen with this agent (Stockhammer et al., 1995).

Apoptosis in the external germinal layer can be induced both in vivo (Ferrer et al., 1997) and in primary cultures of fetal cerebellar explants (Galli et al., 1995). In addition, some MBs express Bcl-2 (Heck et al., 1994; Macaulay et al., 1996), a proto-oncogene which may serve to prevent apoptosis in otherwise susceptible precursors. Thus, it is not surprising that MB cells may be induced to undergo apoptosis with appropriate stimulation in vitro (Fulda et al., 1997; Kenigsberg et al., 1997). The data compiled herein provide morphologic, flow cytometric and DNA evidence of extensive apoptosis after exposure of MB cell lines to lovastatin in vitro. The reliance of MB cells on the HMG-CoA reductase pathway, as evidenced by high expression of the enzyme (Macaulay et al., 1994), may explain the increased sensitivity to inhibition of this enzyme.
Initiation of apoptosis in MB cells by lovastatin likely requires mevalonate depletion, the product of the reaction catalyzed by HMG-CoA reductase (Goldstein and Brown, 1990). The results detailed above indicate that inhibition of mevalonate production is a critical step in lovastatin-triggered apoptosis (Wang and Macaulay, 1999a) (see below). The main products of the mevalonate pathway are cholesterol and non-steroidal derivatives such as farnesyl (Goldstein and Brown, 1990). However, the depletion of cholesterol is not likely to play an important role in the initiation of apoptosis, because the serum in culture medium probably provides adequate supplemental cholesterol.

The identity of the lovastatin-depleted product(s) (which is important for cellular proliferation) in the mevalonate pathway is unknown; a recent study has narrowed the search to a step proximal to the formation of isopentenyl diphosphate (Cuthbert and Lipsky, 1997). The proto-oncoprotein p21 Ras requires the addition of a farnesyl moiety to allow proper membrane localization (DeClue et al., 1991; Schaber et al., 1990; Vogt et al., 1996). Failure of p21 Ras farnesylation results in inability to trigger signal transduction pathways which may participate in cell proliferation, growth and differentiation (Gibbs and Oliff, 1997; Kohl et al., 1994). However, growth inhibitory effects of lovastatin have previously been shown to be independent of farnesylation (DeClue et al., 1991), and may instead be mediated through depletion of other non-steroidal derivatives such as geranylgeraniol (Crick et al., 1996; Vogt et al., 1996). Whether specific inhibition of farnesylation recapitulates the apoptotic effects of lovastatin on MB cell lines is discussed below. Overexpression of p21 Ras has been demonstrated in 25% of MB primary tumors (Macaulay et al., 1996) but not MB cell lines; a previous report of N-Ras activation in a MB cell line (Fults et al., 1989) was invalidated by the subsequent demonstration that the tumor of origin was actually a rhabdomyosarcoma (Stratton et al., 1989). Effector mechanisms of lovastatin-induced apoptosis have not previously been established, nor has the relationship of this phenomenon to other manipulations which may induce apoptosis of MB in vitro (Fulda et al., 1997; Kenigsberg et al., 1997).
The relative resistance of D283 Med and D341 Med to the effects of lovastatin are unexplained, but may be attributable to increased expression of HMG-CoA reductase after lovastatin treatment in these cell lines (Figure 3.14) (see below). Alternatively, the differences in morphological phenotype (partially attached vs. attached), abnormalities of the c-myc gene in D283 Med and D341 Med (Figure 3.8) (Bigner et al., 1990; Friedman et al., 1988), the longer doubling times, or other unknown factors may partially account for these findings.

The difference of c-myc gene expression among the four MB cell lines may affect their sensitivity to lovastatin. Expression of the myc transcription factors is important for cell proliferation (Green et al., 1994); myc has also been implicated in the induction of apoptosis under certain conditions which cause growth arrest, such as growth factor and serum deprivation (Askew et al., 1991; Evan et al., 1992; Hermeking and Eick, 1994). A number of reports have focused on the oncogenic activity of myc proteins. Myc interacts with the retinoblastoma protein (pRB) and is able to override pRB-induced cell cycle arrest. Cell proliferation is induced when myc is expressed in the presence of certain growth promoting cytokines such as IL-2. Overexpression of myc results in uncontrolled cell proliferation (Duffy, 1993). Synergy between c-myc and p21 Ras overexpression is suggested by findings which document cooperation between these two protooncogenes in the induction of S-phase (Leone et al., 1997).

In the experimental paradigm describe herein, cells were administered lovastatin in standard growth medium-containing serum. Thus, it is possible that overexpression of c-myc in D283 Med and D341 Med confers relative resistance to lovastatin-induced apoptosis. However, lovastatin-induced apoptosis in MB appears to be independent of C-myc protein level, because reducing c-myc expression with antisense oligonucleotides did not affect lovastatin-induced apoptosis (Figure 3.9). This conclusion is supported by findings which indicate that the effect of lovastatin on neuroblastoma appears to be independent of the level of N-myc expression (Dimitroulakos and Yeger, 1996).
4.2 Blocking mevalonate pathway accounts for MB apoptosis

Several studies have demonstrated the ability of lovastatin to inhibit proliferation of tumor cells in vitro and induce cell death via apoptosis in primary acute myeloid leukaemia, acute T-cell leukaemia, neuroblastoma, glioma, medulloblastoma, HL-60 promyelocytic cells (Newman et al., 1994; Bansal et al., 1989; Dimitroulakos and Yeger, 1996; Jones et al., 1994; Macaulay et al., 1999; Perez and Mollinedo, 1994), raising the possibility of using lovastatin clinically as an anti-tumor drug. However, systematic investigation into the mechanism of lovastatin-induced apoptosis has not been resolved.

Initiation of apoptosis in MB cells by lovastatin likely requires mevalonate depletion. Our data show alterations in HMG-CoA reductase expression which confirm that lovastatin inhibits mevalonate production. We have also compiled morphological, cell cycle and DNA fragmentation data which demonstrate that mevalonate administration overcomes lovastatin-induced apoptosis. Therefore, blocking mevalonate production is a critical step in the mechanism of lovastatin-induced apoptosis.

HMG-CoA reductase is controlled by several feedback-regulation mechanisms (Goldstein and Brown, 1990). Our data indicate that transcription of the HMG-CoA reductase gene is increased following lovastatin administration in vitro because of decreased mevalonate production. HMG-CoA reductase expression is maintained at normal levels when mevalonate and lovastatin are co-administered. However, this feedback regulation of HMG-CoA reductase appears to vary among the four MB cell lines tested, in that expression levels in Daoy were less affected than in D283 Med and D341 Med, while UW228 showed changes in expression only if pre-treated with lovastatin. This indicates that the capacity of the adherent cell lines to up-regulate HMG-CoA reductase may be limited, compared to the partially attached cell lines. We speculate that the limited capacity to up-regulate HMG-CoA reductase in the adherent cell lines may confer increased
sensitivity to lovastatin, since shorter treatment times were sufficient to induce apoptosis in Daoy and UW228 compared with D283 Med and D341 Med.

We have demonstrated that lovastatin-treated cells can be diverted from the apoptosis pathway by mevalonate administration, but only if mevalonate is provided within a critical time period. MB cells treated with lovastatin for time periods beyond this 'window of opportunity' were apparently committed to die. A number of signals involving a variety of distinct pathways appear to trigger a common apoptosis pathway, yielding characteristic morphologic changes, DNA laddering and a flow cytometric 'apoptosis peak'.

The common end stage for these different triggers appears to be the activation of caspases, intracellular proteases which play a critical role in the execution of apoptosis. Inactive precursors of caspases are cleaved at aspartate residues to become active, resulting in apoptosis (Munday et al., 1995; Nicholson et al., 1995; Sleath et al., 1990). This possibility that caspase cleavage characterizes lovastatin-induced apoptosis of MB cells is discussed below. Interfering directly with the function of caspases, with their precursors, or with other participants in the apoptotic cascade might avert cell death, and it is conceivable that mevalonate inhibition of lovastatin-induced apoptosis is simply the result of such a phenomenon. Although mevalonate is not known to inhibit caspases, the data presented here do not address this possibility directly. Instead, the question was approached by following the downstream pathways for which mevalonate is required.

Because blocking the production of mevalonate appears to be responsible for lovastatin-induced apoptosis, it follows that depletion of specific mevalonate derivatives must be implicated in this phenomenon. One important requirement may be the inhibition of isoprenylation of particular cellular proteins. A number of important signalling proteins require isoprenylation in order to localize to the cell membrane and interact with other components of various signal transduction pathways (Khosravi et al., 1992). Mutant p21 Ras (Hancock et al., 1989) and heterotrimeric G protein (Finegold et al., 1990) are well-
studied isoprenylated signaling proteins which contribute to tumor growth; however, whether the inhibition of isoprenylation of these or other signaling proteins is responsible for lovastatin-induced apoptosis is not clear (Wang and Macaulay, 1999).

4.3 Blocking protein farnesylation is responsible for MB apoptosis

Lovastatin-induced apoptosis and inhibition of proliferation has been demonstrated in our laboratory (Macaulay et al., 1999) and elsewhere (Bansal et al., 1989; Dimitroulakos and Yeger, 1996; Jones et al., 1994; Perez and Mollinedo, 1994; Sumi et al., 1992). Clarification of the precise mechanism of this phenomenon was a major goal of the work presented herein.

The mevalonate derivatives geranyl and farnesyl pyrophosphate are required for isoprenylation of a variety membrane-bound proteins, including several signal transduction proteins that influence cellular proliferation. Inhibition of HMG-CoA reductase by lovastatin reduces post-translational modification of such proteins, abrogating their mitogenic or oncogenic activity (Khosravi et al., 1992). Using p21 Ras as a marker for isoprenylation, we showed that lovastatin inhibits isoprenylation and induces apoptosis (Wang and Macaulay, 1999). Because proteins are isoprenylated by the addition of either a farnesyl moiety or a geranylgeranoil moiety, we attempted to determine the importance of farnesylated proteins. Detailed study of the properties of geranylgeranylated proteins is beyond the scope of this work.

Manumycin A was chosen to distinguish between protein farnesylation and geranylgeranylation. This antibiotic has been shown to have an IC50 for yeast FPTase of 5 μM, and for rat brain of 35 μM; the IC50 of manumycin A for bovine brain GGPTase is 180 μM (Hara et al., 1993). In humans, 30 μM manumycin A has no effect on protein geranylgeranylation (Nagase et al., 1996), so at low concentrations (10-20 μM), manumycin A selectively inhibits protein farnesylation.
Most detectable p21 Ras was unprocessed after manumycin A treatment of MB cell cultures, indicating successful inhibition of protein farnesylation. The small amount of p21 Ras that was processed may reflect incomplete inhibition; however, it is more likely attributable to persistent geranylgeranylation of N- and K-Ras (Mangues et al., 1998; Whyte et al., 1997).

We found that inhibition of protein farnesylation by manumycin A induced apoptosis of cultured MB cells. Moreover, the more rapid time course of manumycin A effects compared with lovastatin suggests that manumycin A's site of action is downstream from that of lovastatin. It is suspected that the depletion of the mevalonate 'pool' following lovastatin treatment occurs gradually, and varies between cell lines; in contrast, ongoing requirements for protein farnesylation make the cell more susceptible to manumycin A treatment (see below). It is therefore postulated that blocking protein farnesylation is a critical step forLovastatin-induced apoptosis. This is consistent with previous reports that manumycin A inhibits the growth of K- and N-Ras transformed cells (Hara et al., 1993; Nagase et al., 1996), and that the farnesyl transferase inhibitor L-744,832 inhibits tumor growth and induces apoptosis (Mangues et al., 1998). Therefore, protein farnesylation likely plays a pivotal role in MB cell survival. Notably, our results differ from those of Miquel et al. (1997), who showed in human adenocarcinoma cells that geranylgeranyl transferase I inhibitor GGTV-298, but not the farnesyl transferase inhibitor FTI-277, induced both G0-G1 block and apoptosis. Thus, responses of human cancer cells to these agents appears to depend on tumor subtype, and may possibly be idiosyncratic.

Among farnesylated proteins, the Ras family has been postulated to play a pivotal role in signal transduction and growth control. Isoprenylation, enabling localization to the inner side of the plasma membrane, is a critical requirement for p21 Ras activity. Functional p21 Ras cycles between active (GTP-bound) and inactive (GDP-bound) states, transducing growth signals from tyrosine kinase receptors to mitogen activated protein (MAP) kinases en route to the nucleus (Marshall, 1994). It was originally suggested that
G1 arrest and inhibition of tumor growth by lovastatin result from inhibition of Ras function (Keyomarsi et al., 1991), and certain tumor cells containing mutated K-Ras were shown to be quite sensitive to FPTase inhibitors (Kohl et al., 1994; Whyte et al., 1997). The observation that about 25% of MBs strongly express ras (Macaulay et al., 1996) and that about 10% of MBs have activating ras mutations (Iolascon et al., 1991) support the possibility that the Ras pathways may be important in the pathogenesis of MB.

However, the importance of Ras in FPTase inhibitor-induced apoptosis has also been cast into doubt. In our experiments, the relationship between inhibition of Ras processing and apoptosis is not absolute, since the proportion of unprocessed Ras is maximal at 10 μM manumycin A, while the percentage of apoptotic cells increases only with higher concentrations of this compound (Figure 3.16). Others have shown that N- and K-Ras remain membrane-associated by geranylgeranylation even after protein farnesylation is inhibited (Whyte et al., 1997), perhaps reflecting alternate processing. Furthermore, even human tumor cells without Ras mutations may be sensitive to FPTase inhibitors (Sepp-Lorenzino et al., 1995), and alternate processing of N-Ras does not entirely protect transformed cells from the effects of FPTase inhibition (Mangues et al., 1998). It is therefore likely that other farnesylated proteins, such as lamins A and B, Rap2, phosphorylase kinase, rhodopsin kinase, cGMP phosphodiesterase, the γ subunit of transducin, and especially the Rho proteins (Khosravi et al., 1998; Lebowitz et al., 1995) are more important than Ras proteins in FPTase inhibitor-induced apoptosis. Detailed investigation into this unresolved issue is beyond the scope of this work.

Although the relationship between inhibition of Ras processing and apoptosis is not absolute, whether changes in ras gene expression contribute to apoptosis in MB was not known because isoprenylation inhibitors down-regulate ras gene expression in yeast (Dimster et al., 1995). Semiquantitative RT-PCR for N-, K- and H-ras revealed that isoprenylation inhibitors do not affect either total ras gene or specific ras gene expression in MB. Thus, changes in ras gene expression are not implicated in isoprenylation inhibitor-
induced MB apoptosis. However, our results showed that N-ras gene expression in these MB cells is generally higher than K- or H-ras. High levels of N-ras gene expression in these MB cells may account for some Ras being processed after manumycin A treatment.

In our studies, clear differences were apparent when lovastatin- and manumycin A-induced apoptosis were compared. Manumycin A-induced apoptosis is more rapid than lovastatin-induced apoptosis in all cell lines. The uniform sensitivity to manumycin A among all cell lines contrasts with the relative resistance of D283 Med and D341 Med to lovastatin. The likely explanation is that lovastatin inhibition of farnesylation follows gradual depletion of the farnesyl pyrophosphate pool, whereas manumycin A acts by inhibiting FPTase directly. This hypothesis is supported by the differences in HMG-CoA reductase gene expression effected by the two compounds: lovastatin results in up-regulation (Figure 3.14), while manumycin A causes down-regulation (Figure 3.22). Up-regulation following lovastatin treatment is attributed to feedback through the cholesterol biosynthesis pathway. The manumycin A-induced decrease in HMG-CoA reductase mRNA may reflect an accumulation of unused farnesyl pyrophosphate, which may have been diverted to other branches of the mevalonate pathway such as cholesterol biosynthesis, leading to feedback down-regulation of gene expression.

Differences between the precise sites of action of lovastatin vs. manumycin A are also reflected in their differing effects on the cell cycle. Lovastatin-induced apoptosis is accompanied by G1 cell cycle arrest, while manumycin A has no effect on the cell cycle. This is consistent with the observation that the FPTase inhibitor L-744,832 blocks proliferation and induces apoptosis without accompanying cell cycle arrest (Sepp-Lorenzino and Rosen, 1998). A possible explanation may be that lovastatin, but not manumycin A, also blocks protein geranylgeranylation, which is suspected to be critical for the G1 to S phase transition (Vogt et al., 1996). The manumycin A-induced down-regulation of HMG-CoA reductase gene is unlikely to abolish geranylgeranylation, allowing surviving cells to enter the cell cycle unperturbed.
The rapidity of manumycin A-induced cell death raises the possibility of a mechanism distinct from that involved in lovastatin-induced apoptosis. However, we demonstrate that lovastatin- and manumycin A both activate caspase-3, indicating that the final events in both drug treatments are the same. Blocking farnesylation of signal transduction proteins, possibly including p21 Ras, is a critical step. Activation of caspase-3 in both lovastatin- and manumycin A-induced apoptosis is consistent with a recent finding that prenyltransferase inhibitors induce apoptosis in proliferating thyroid cells through a caspase-3-like protease-dependent mechanism (Vitale et al., 1999).

4.4 Blocking G-protein function accounts for MB apoptosis

Since lovastatin blocks the isoprenylation of proteins including G-proteins, which play important roles in signaling pathway for cell proliferation, growth and differentiation, it was postulated that the blocking of G-protein function may contribute to the mechanisms of lovastatin-induced MB growth inhibition and apoptosis.

In this study, we investigated this possibility using mycophenolic acid (MPA) to deplete intracellular GTP. This compound blocks the conversion of IMP to GMP, the precursor to the synthesis of GDP and GTP, and thus blocks G-protein activation of downstream signaling cascades in MB cell lines. Morphological, flow cytometric and DNA laddering data show that MPA inhibits MB cell proliferation, arrests the cell cycle in G1, and induces cell differentiation and apoptosis. These results are consistent with the hypothesis that interference with G-protein function contributes to lovastatin-induced MB apoptosis.

The ability of MPA to cause apoptosis has been confirmed by several research groups (Catapano et al., 1995; Li et al., 1998; Messina et al., 1998). It is clear that MPA-induced apoptosis is due to depletion of GTP but not ATP, as all changes induced by MPA were prevented virtually completely by co-incubation with guanosine but not adenosine. It is possible that reduction of dGTP plays a contributory role in apoptosis since a reduction
in GTP will be accompanied by a decrease in dGTP due to its interconversion by ribonucleotide reductase (Nguyen and Sadee, 1986) and reduction in dGTP may impair DNA synthesis (Nguyen and Sadee, 1986). However, reduction of dGTP does not play a main role in MPA-induced apoptosis, as 500 μM of exogenous deoxyguanosine co-incubation failed to prevent the effect of MPA on cell death; indeed, even 25 μM of exogenous deoxyguanosine restore the dGTP content of human leukemia cells (CEM) and mouse T-lymphoma (S-49) mutant cells (Huang and Plunkett, 1995; Nguyen and Sadee, 1986).

Lovastatin, manumycin A and MPA synergistically induced apoptosis, indicating that they act on the same pathway. The longer time course of MPA compared with manumycin A is attributed to the time required to deplete GTP stores. It is noted that D341 Med exhibited apoptosis sooner than other MB cell lines when low concentrations of MPA were applied, and in fact D341 Med remained sensitive to MPA even at 1 μM concentration, but Daoy, UW228 and D283 Med did not. The mechanism is unclear, but different isoforms of IMPDH may be involved. IMPDH exists in two isoforms, type I and type II (Carr et al., 1993; Hager et al., 1995). Type II isoform is 3.9-fold more sensitive to MPA than is the type I isoform (Carr et al., 1993; Hager et al., 1995). The selective inhibition of the type II IMPDH may explain the sensitivity of D341 Med to MPA. Other unknown mechanisms may also be operative, but investigation into this difference is beyond the scope of this treatise.

Physiological regulation may also determine the response to MPA. The percentage of apoptosis in UW228 is lower than other cell lines. This may be attributable to the apparent differentiation in UW228. In Daoy, D283 Med and D341 Med, growth inhibition results in the inhibition of protein synthesis, and thus no expression of the differentiation markers, NF and GFAP. Interestingly, Daoy is GFAP positive after 7 days of culture, which is different from the origin report that Daoy is GFAP negative (Jacobsen et al., 1985). The likely explanation is that the original report is performed on the cells
cultured less than 5 days, since it is GFAP negative or very weakly positive before 5 days of culture. Alternatively, culture conditions may be slightly different, or after numerous passages the cell line may have matured.

In MPA-induced MB apoptosis, after MPA depletes GTP, it is possible that the function of certain G-proteins that normally suppress apoptosis was inhibited; in support of this hypothesis, some G-proteins have been found to modulate apoptosis (Bobak et al., 1997; Ghosh et al., 1997; Lacal, 1997; Moorman et al., 1996; Yan et al., 1995). In addition, MPA also induced cell cycle arrest in G1, similar to lovastatin induced changes (Macaulay et al., 1999). The likely explanation is that GTP depletion alters the function of G-proteins which are normally geranylgeranylated, because protein geranylgeranylation is required for cycling cells to pass through G1 (Miquel et al., 1997; Vogt et al., 1996), and lovastatin blocks protein isoprenylation including geranylgeranylation. Although this reinforces the hypothesis that non-farnesylated G-proteins are affected by non-selective isoprenylation inhibitors such as lovastatin, this question was not directly addressed by and is beyond the scope of this work.

Altogether, our results imply that interference with the function of G-proteins by blocking isoprenylation contributes to lovastatin-induced MB apoptosis. However, it is not known what isoprenylated G-proteins are responsible for MB survival. Identification of these proteins is the topic of ongoing research in several laboratories.

4.5 Molecular regulation of isoprenylation inhibition-induced MB apoptosis

*bcl-2*, a negative regulator of cell death, has been shown to promote cell survival and to inhibit apoptosis induced by many types of stimuli in certain cells (Melino et al., 1994; Miyashita and Reed, 1992; Vaux et al., 1988; Zhong et al., 1993). It is possible that bcl-2 expression in some MB cells could prevent apoptosis. However, Bcl-2 expression is low in D341 Med, one of two MB cell lines which are relatively resistant to lovastatin. In lovastatin-induced apoptosis, Bcl-2 levels are constant until apoptosis is well
underway. Because of this delay, it is unlikely that bcl-2 expression is directly affected by lovastatin, and so the decrease in Bcl-2 protein level likely plays little role in the induction of apoptosis.

Bax, a bcl-2 related gene product, accelerates apoptosis, in that the ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus (Oltvai et al., 1993). However, bax expression is unchanged during lovastatin and manumycin A treatment, so it is unlikely to have a significant role in the response to these compounds. If Bax itself is not involved, other elements of the apoptotic cascade may be implicated. Some possibilities include: 1) Bax-like molecules which may also regulate the induction of apoptosis. 2) A physiological response to isoprenylation inhibition which results in Bcl-2 and/or Bax relocation inside cells, thus allowing the release of other molecules or proteins (e.g. cytochrome c), culminating in caspase activation. Indeed, whether Bax binds to Bcl-2 inside cells has become controversial (Adams and Cory, 1998), because the detergents used in cell lysis facilitate their association (Hsu and Youle, 1998; Hsu et al., 1997). 3) Ligation of the receptor CD95-like, which bypasses the Bcl-2-inhibitable step common to most stress pathways, leading to caspase-8 activation (Adams and Cory, 1998; Green and Reed, 1998; Newton et al., 1998; Smith et al., 1996; Strasser et al., 1995; Thornberry and Lazebnik, 1998)

p53 is an important mediator of apoptosis and G1 cell cycle arrest in the cellular response to ionizing radiation and other DNA damaging agents (Cohen et al., 1992; Kuerbitz et al., 1992). p53 upregulates bax expression, bax being identified as a p53-immediate early response gene (Miyashita and Reed, 1995). The expression of the bax gene thus serves as a useful marker for p53-dependent apoptosis, although it is not the only molecule responsible for p53-driven apoptosis (Adams and Cory, 1998; Grasso and Mercer, 1997; Liebermann et al., 1995; Yin et al., 1997). The level of Bax protein did not show any change in lovastatin and manumycin A treatment, although in this treatment p53 is up-regulated in UW228 and D341 Med. The p53 increase may reflect physiological
regulation, perhaps contributing to lovastatin-induced cell cycle arrest (see below). Thus, isoprenylation inhibition-induced apoptosis is probably p53-independent. To support this view, lovastatin does induce apoptosis in other tumor cell lines and primary tumors through p53-independent pathways (Vitale et al., 1999; Rao et al., 1998; Lee et al., 1998; Borner et al., 1995). However, p53 can also induce apoptosis without initiating de novo protein or RNA synthesis (Caelles et al., 1994; Wagner et al., 1994), and both wild-type and mutant p53 can promote exit from ionizing-radiation induced G2 arrest resulting in apoptosis (Guillouf et al., 1995; Guillouf et al., 1998). Direct protein-protein interactions involving p53 could represent the non-transactivational mechanism for p53 regulation of apoptosis. For example, p53 binds to the transcription/repair complex TFIIH, and thus inhibits the helicase activity of two of its subunits (XP-B and XP-D) (Wang et al., 1996; Wang et al., 1995). It is not clear whether this mechanism is involved in isoprenylation inhibition-induced apoptosis in MB.

p53 proteins were increased in UW228 and D341 Med after isoprenylation inhibition and may contribute to cell cycle arrest through inducing the expression of another of its targets, p21WAF1 (Grana and Reddy, 1995; Hall and Peters, 1996; Sherr, 1996). In UW228 cell line, p21WAF1 protein was increased in lovastatin treatment, consistent with the interaction between p53 and P21WAF1. However, isoprenylation inhibitors including lovastatin induce p53-independent cell cycle arrest in other experimental systems (Lee et al., 1998; Rao et al., 1998; Vogt et al., 1997), raising the possibility that other molecules may regulate p21WAF1 expression in MB cells. Indeed, p21WAF1 expression is also p53-independent in TGF-β induced cell cycle arrest (Grana and Reddy, 1995; Hall and Peters, 1996; Sherr, 1996). p27KIP1 expression, but not p16INK4, is also correlated with lovastatin-induced cell cycle arrest in all MB cell lines, consistent with several reports from other investigators (Gray-Bablin et al., 1997; Hengst and Reed, 1996; Poon et al., 1995; Rao et al., 1998).
Although p21WAF1 and p27KIP1 levels were increased after monumycin A treatment in MB cell lines, no cell cycle change was apparent. One possible explanation for this unexpected result is that monumycin A-induced apoptosis is so rapid and efficient that cycling cells have no opportunity to arrest before being driven into the apoptosis pathway.

4.6 Clinical potential of isoprenylation inhibition on MB

Despite the impressive efficiency of cell death achieved with lovastatin treatment of MB cell lines and cultured primary MB cells, and its rarely significant side effects (blurred vision, impotence, insomnia and myopathy) (Reynolds, 1996) in current treatment on patients with hypercholesterolemia, some caution must be exercised before extrapolating these data to patients with MB. First, it is not clear that HMG-CoA reductase inhibition alone will be sufficient to achieve significant cell death in vivo. However, cancer xenograft models in immunodeficient mice have been successful in providing evidence of lovastatin efficacy in other cancer systems (Sumi et al., 1992). Second, the bioavailability of oral lovastatin is limited by the significant first-pass hepatic clearance, and parenteral administration may be necessary to achieve effective CNS dosages (Desager and Horsmans, 1996; Sumi et al., 1992). Because lovastatin is lipophilic, it is capable of penetrating the blood-brain barrier in concentrations that may have pharmacologic effects (Botti et al., 1991). However, even if cell death can be triggered in vivo, required drug dosages in vitro may approach margins of safety (Desager and Horsmans, 1996). Our observation that Daoy cells retain some sensitivity to lovastatin even after repeated treatment/rescue cycles is encouraging. In addition, other potentiating or differentiating agents may be effective in concert with lovastatin (Dimitroulakos and Yeger, 1996, Sumi et al., 1992). Third, it is not known whether the administration of lovastatin may interfere with the efficacy of conventional anti-neoplastic therapies, if given simultaneously. Typically, such agents depend on DNA replication for their action, and the inhibition of mitosis by lovastatin may abrogate such effects. Fourth, lovastatin does induce apoptosis
in normal human cell MRC-5, but manumycin A does not. The specific target sites, and which drug is chosen, should be considered before the clinical use of isoprenylation inhibitors; indeed, bioassays of individual neoplasms samples may be required to determine susceptibility in each patient. Finally, permanent cell lines are difficult to establish from MB (Pietsch et al., 1994), and those that survive may be biologically different from primary tumors (Bigner et al., 1990). The profound tumoricidal effects on all four cell lines tested in these studies suggests that even those tumors which are capable of surviving \textit{in vitro} retain sensitivity. Significantly, in our experiment two cultured different phenotype primary MBs are sensitive to lovastatin and manumycin A \textit{in vitro}. These results suggest that different primary MBs may sensitive to isoprenylation inhibitors, and thus encourage further investigation of the use of isoprenylation inhibitors on MB. In addition, the dismal prognosis attached to recurrent MBs provides a patient population for whom such novel therapies may be the last resort (Torres et al., 1994). Ultimately, unravelling the mechanism of lovastatin-induced apoptosis may lead to more effective medical therapy for MB, reducing mortality and morbidity following conventional treatment.

4.7 Conclusions

Lovastatin, manumycin A and MPA induce apoptosis in MB cell lines. Potential molecular regulatory mechanisms were investigated in this study. The following conclusions were drawn on the basis of the results.

1) Blocking the mevalonate pathway does induce apoptosis and block cell proliferation in MB cell lines and primary MB cell cultures.

2) Blocking farnesylation of proteins, especially G-proteins, at least partially accounts for apoptosis induced by blocking the mevalonate pathway with lovastatin.

3) Isoprenylation inhibitor-induced apoptosis acts through a p53-independent, caspase-3 dependent mechanism.
4) Changes in p21 ras expression do not accompany apoptosis induced by these compounds.

5) There is no evidence that Bcl-2 and Bax regulate isoprenylation inhibition-induced apoptosis in MB cells.

6) Lovastatin-induced up-regulation of the CKIs p21WAF1 and P27KIP1 contributes to cell cycle arrest and cell growth inhibition.

7) Regulation of HMG-CoA reductase gene expression is perturbed.

8) Therefore, the results presented here support our hypotheses that 1) HMG-CoA reductase activity is critical to MB cell survival, and inhibition of HMG-CoA reductase reduces MB cell proliferation and induce apoptosis; 2) that protein farnesylation and G-protein function are also critical for MB cell growth and survival; 3) that gene expression, such as CDK inhibitor genes and HMG-CoA reductase, but not ras, bcl-2, bax, p53 and c-myc, mediates isoprenylation inhibition-induced apoptosis and/or inhibition of proliferation.
5.0 REFERENCES


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6.0 APPENDIX

6.1 Publication

From this study, the following papers were formed and published.


6.2 Solutions and Buffers

Acrylamide mixture (28%): 28% (w/v) acrylamide, 0.8% (w/v) N,N'-methylene-bisacrylamide.
Acrylamide mixture (30%): 30% (W/V) acrylamide, 1.62% (W/V) N,N’-methylene-bisacrylamide.

Alkaline phosphatase buffer (pH 9.5): 100 mM Tris base, 100 mM NaCl, 50 mM MgCl₂.

Alkaline phosphatase substrate solution: 44 µl NBT stock + 33 µl BCIP stock / 10 ml alkaline phosphatase buffer.

BCIP stock: 5% (W/V) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in absolute dimethylformamide.

Digestion buffer (pH 8.0): 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% (W/V) SDS, 0.1 mg/ml proteinase K.

Hybrisol solution: 50% (V/V) formamide, 10% (V/V) Dextran Sulfate, 1% (W/V) SDS and blocking reagents.

Lower-Tris buffer (separating gel buffer, pH 8.8): 1.5 M Tris base, 0.4% (W/V) SDS.

Lysis buffer (pH 8.0): 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.5% (W/V) SDS.

MOPS solution, 10 X: 0.2 M MOPS, 10 mM EDTA, 50 mM NaAc, pH 7.0.

NBT stock: 7.5% (W/V) p-nitro blue tetrazolium chloride (NBT) in 70% (V/V) dimethylformamide.

PCR buffer, 10X: 500 mM KCl, 100 mM Tris-HCl, pH 8.3.

Phosphate buffer: 50 mM NaH₂PO₄-NaOH, pH 6.5, 5 mM EDTA.

Phosphate-buffered saline (PBS, pH 7.2-7.4): 0.8% (W/V) NaCl, 0.02% (W/V) KCl, 0.02% KH₂PO₄, 0.22% (W/V) Na₂HPO₄·7H₂O.
RIPA lysis buffer (pH 8.0): 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (V/V) Nonidet P (NP)-40, 0.5% (W/V) deoxycholate (DOC), 0.1% (W/V) SDS.

RNA electrophoresis sample buffer: 1 x MOPS, 7% (V/V) formaldehyde, 50% (V/V) formamide, and 10% (V/V) RNA sample buffer dye mix.

RNA sample buffer dye mix: 50% (V/V) glycerol, 1 mM EDTA, pH 8.0, 0.25% (V/V) bromophenol blue, 0.25% (V/V) xylene cyanol.

SDS-PAGE sample buffer, 2X: 100 mM Tris-HCl, pH 6.8, 10% (V/V) 2-mercaptoethanol, 4% (W/V) SDS, 20% (V/V) glycerol, 0.2% (W/V) bromophenol blue.

Separating gel mixture (10%): 3.33 ml 30% acrylamide mixture, 2.5 ml lower-Tris buffer, 30 μl 10% (W/V) ammonium persulfate, 100 μl 10% (W/V) SDS, 10 μl N,N,N',N'-tetramethylene-ethylenediamine (TEMED), 4 ml ddH2O (total volume 10 ml).

SSC, 20X: 3.0 M NaCl, 0.3 M Na3Citrate-2H2O. Adjust pH to 7.0 with 1 M NaOH.

Stacking gel mixture (3%): 0.54 ml 28% Acrylamide mixture, 1.3 ml upper-Tris buffer, 22.5 μl 10% (W/V) ammonium persulfate, 50 μl 10% (W/V) SDS, 7.5 μl N,N,N',N'-tetramethylene-ethylenediamine (TEMED), 3.1 ml ddH2O (total volume 5 ml).

TAE buffer, 1X: 40 mM Tris-acetic acid, 1 mM EDTA.

TBS-Tween 20 (TBST, pH 8.0): 0.05% (V/V) Tween 20 in TBS.

TE buffer (pH 8.0): 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Tris-buffered saline (TBS, pH 8.0): 50 mM Tris base, 0.15 M NaCl.

Tris-glycine buffer (pH 8.3), 10X: 0.25 M Tris base, 1.9 M glycine.

Tris-glycine electrophoresis buffer (pH 8.3), 1X: 10% (V/V) 10X Tris-glycine buffer, pH 8.3, 0.1% (W/V) SDS.
**Trypan blue:** 0.08% (W/V) trypan blue, 0.85% (W/V) NaCl.

**Upper-Tris buffer (stacking gel buffer, pH 6.8):** 0.5 M Tris base, 0.4% (W/V) SDS.

**Western blotting transfer buffer:** 10% (V/V) 10X Tris-glycine buffer, 10% (V/V) methanol, 80% (V/V) ddH$_2$O.