Mutant p53 melanoma cell lines respond differently to CP-31398-induced apoptosis

by

CLEMENT KAR-MING HO

B.Sc., The University of British Columbia, 2000

B.Ed., The University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

April 2005

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ABSTRACT

p53, a tumour suppressor gene, is considered the guardian of genome which governs several important biological functions including cell cycle arrest, DNA repair and apoptosis. Under normal conditions, *p53* is tightly regulated and constitutively expressed at a low level. In human cancers, *p53* is one of the most frequently mutated genes with over 50% of all human malignancies containing an altered *p53* gene.

Many strategies have been developed in light of restoring wild-type *p53* functions to altered *p53*. Recently, a small pharmacological compound, CP-31398, was found through random screening to have the ability to promote proper *p53* protein folding, activate *p53* transcription of downstream targets, and slow tumor growth in mice. Additionally, CP-31398 was found to be able to convert mutant *p53* to wild-type conformation in several cell lines.

In this study, we sought to examine if CP-31398 can revert all mutant *p53* proteins to wild-type function. We studied a series of apoptotic responses to CP-31398 in three melanoma cell lines with various *p53* mutation status. Upon a moderate dose of CP-31398 treatment ($15\ \mu\text{g mL}^{-1}$), only the wild-type *p53* MMRU and the single *p53* point mutation MeWo cells exhibited apoptosis. Another melanoma cell line, Sk-mel-110, containing multiple *p53* mutations did not exhibit apoptosis. Despite CP-31398 enhanced overall *p53* protein level, its ability to promote proper folding of *p53* protein was limited to CP-31398 sensitive MMRU and MeWo cells. Although all cell lines showed an increased p21^{Waf1} transcription, only sensitive cells showed an increased Bax and PUMA transcription, an altered mitochondrial membrane potential, followed by the

release of cytochrome *c*, and cleaved caspases-9 and -3. We also demonstrated that Apaf-1, Fas and Fas-ligand were not involved in CP-31398 mediated apoptosis.

In Summary, our results suggest that the ability of CP-31398 to revert mutant p53 proteins to wild-type conformation may be correlated to *p53* mutation status. Mutant p53 reactivation by small molecules has an evident potential for the discovery of effective and specific anti-cancer drugs. To investigate *p53* mutation-dependent in response to CP-31398, further studies are required to explore the possibility of utilizing this compound as an anti-cancer therapeutic agent.

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LIST OF ABBREVIATIONS

Apaf-1	apoptotic protease activating factor-1
BER	Base excision repair
BH3	Bcl-2 homology 3
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
DBD	DNA binding domain
DED	Death effector domain
DISC	Death inducing Signalling Complex
DR5	Death receptor 5
EC ₅₀	Effective concentration 50%
EtBr	Ethidium bromide
FADD	Fas-associated Death Domain
GADD45	Growth arrest and DNA damage protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horseradish peroxidase
Mdm2	Mouse double minute 2
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
p53R2	p53-inducible ribonucleotide reductase small subunit 2 homolog
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pgp	P-glycoprotein
PI	Propidium iodide
PIG	p53-inducible genes
Pu	Purine
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
Py	Pyrimidine
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SRB	Sulforhodamine B
TBS	Tris buffered saline
TCA	Trichloroacetate
TNFR1	Tumour necrosis factor receptor type 1
TRAIL	TNF-related apoptosis-inducing ligand
UV	Ultraviolet
UVR	Ultraviolet radiation

ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr. Gang Li, for granting me the opportunity to explore and emerge myself in this exciting world of scientific research. His intellectual stimulation, encouragement and occasion to attend international conferences have allowed me to see the diversity and the depth of basic science can reach. To Dr. Vincent Ho, Dr. William Jia, Dr. Paul Rennie, and Dr. Chris Ong, I truly appreciate your input and involvement in my committee. Your suggestions and feedbacks have widened my understanding and scope of research work tremendously. As for the members of the Li lab, you have become my second family while we spent countless hours working side by side. Without your kindness, thoughtfulness, respect and friendship, I would not be able to endure all those long hours at my bench top persevering through my experiments. Above all, I am privileged to have the continuous support, unconditional love and ceaseless understanding from my parents, my brother, and my friends time after time. You have given me the strength to strive so that I can give a hundred and ten percent in everything I do. For that, thank you from the bottom of my heart.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Melanoma

1.1.1 Introduction

The skin is the largest organ covering the entire surface of the body consisting of elements of ectodermal, mesodermal and endodermal origin. One of the main functions of the skin is protection. It protects the body from external factors such as bacteria and surroundings. The skin contains secretions that can kill bacteria and the melanin provides a chemical pigment defense against ultraviolet (UV) light that can damage skin cells.

The skin can be divided into three major layers: epidermis, dermis, and the subcutaneous layer. The epidermis is the thin outer layer of the skin, which can be further divided into three sub-layers. The stratum corneum or horny layer, which contains mostly dead keratinocytes, protects the skin from harmful elements. Below this is a layer of living keratinocytes or squamous cells, which help provide the skin with what it needs to protect the rest of the body. The basal layer is the inner layer of the epidermis which contains basal cells. Basal cells continually divide, forming new keratinocytes and replacing the old ones that are shed from the skin's surface. The epidermis also contains melanocytes, which are cells that produce a brown-black skin pigment called melanin that determines skin and hair coloring. In fact, it is the melanin that helps protect against the damaging rays of the sun. The dermis is the middle layer of the skin which contains blood and lymph vessels, hair follicles, sweat glands, as well as pain and touch receptors. The subcutaneous layer is the deepest layer of skin consisting of a network of collagen and fat cells, which helps

conserve the heat of the body while protecting other organs from injury by acting as a shock absorber.

Such a diverse group of tissues gives rise to a wide variety of benign and malignant tumours that contribute to the most common cancer among all type of cancers (Boni *et al.*, 2002). In 2003 alone, over 80,000 cases of skin cancer were diagnosed in Canada, in which 76,000 cases were non-melanoma skin cancer including basal cell carcinoma and squamous cell carcinoma, both of which originate from keratinocytes, and 4,250 cases were melanoma which originates from melanocytes (National Cancer Institute of Canada: Canadian Cancer Statistics 2004, Toronto, Canada, 2004).

Although malignant melanoma contributes about 5% of all skin cancer cases, its incidence and mortality rate have been rising steadily in the past few decades. A stunning report indicated that the probability of an American developing melanoma during his/her lifetime leaped from one in 1,500 in 1960, to one in 68 in 2000, and is projected to increase to 1 in 50 by the year 2010 (Rigel *et al.*, 1996; Rigel, 2002; Dunlap *et al.*, 2004). The development of melanoma has been associated with intermittent acute sunlight exposure causing severe sunburns particularly during childhood or adolescence (Holman and Armstrong, 1984; Kricker *et al.*, 1995). It is characterised by skin lesions or abnormal growth that the appearance changes over time. Besides a mixture of colours in one lesion, the borders are often irregular and asymmetrical.

1.1.2 Aetiology of melanoma

Melanoma is derived from an uncontrolled proliferation of transformed pigment-producing melanocytes. The major environmental factor for the development of cutaneous melanoma

is believed to be UV radiation (UVR) (Romerdaahl *et al.*, 1988; Atillasoy *et al.*, 1998). UVR causes DNA damage in the forms of cyclobutane pyrimidine dimers and/or (6-4) photoproducts (Alcalay *et al.*, 1990). The development of melanoma can be linked to intermittent over-exposure to UVR causing painful sunburns particularly in the childhood (Holman and Armstrong, 1984). In fact more than 80% of melanoma cases in Australia are estimated to be triggered by over sun exposure (Armstrong and Krickler, 1993).

Patients with malignant melanoma have a poor prognosis even when treated with a combination of therapies. Advanced melanoma has been associated with one of the highest mortality rates with a 5-year patients' survival rate less than 10% (Roses *et al.*, 1991b). One of the reasons is due to the highly metastatic nature of malignant melanoma. Melanoma cells infiltrate from the skin and spread rapidly to other organs such as lung, liver, brain, bone and small intestine. The escalating high mortality rate is further compounded by the highly resistant nature of malignant melanoma to traditional radiation and chemotherapy (Garbe, 1993; Schadendorf *et al.*, 1994; Serrone and Hersey, 1999; Helmbach *et al.*, 2001). Currently, no effective treatment is available once melanoma metastasizes.

Although the molecular mechanisms involved in melanoma and chemoresistance have not been identified, it is assumed that inhibition or dysregulation of apoptosis leads to chemoresistance (Serrone and Hersey, 1999). Unlike non-melanoma skin cancers, p53 mutation is rare: less than 5% in primary melanoma, and approximately 20% in metastatic melanoma (Hussein *et al.*, 2003). The most common known genetic alterations is the deletion of chromosomal region 9q21, which harbours tumour suppressors p16^{INK4a} and p14^{ARF}, detected in about 50% in melanoma (Healy *et al.*, 1996; Rizos *et al.*, 2001). Loss

of p16^{INK4a} instead of p14^{ARF} appears to be a more consistent target in general (FitzGerald *et al.*, 1996). Inactivation of p16^{INK4a}, an inhibitor of cyclin-dependent kinase 4 (CDK4), could lead to the hyperactivity of retinoblastoma (Rb) and E2F-1 transcription factor; thus stimulating cell cycle progression. The decreased protein expression of p14^{ARF} from benign melanocytic nevi to metastatic melanoma suggests that inactivation of p14^{ARF} may be an important event in melanoma progression (Dobrowolski *et al.*, 2002). Moreover, the loss of function of p14^{ARF}, an inhibitor of Mdm2, may disrupt p53 protein stability (de Gruijl *et al.*, 2001).

Recently, *Apaf-1* (apoptotic protease activating factor-1) has also been linked to the development of melanoma (Soengas *et al.*, 2001). *Apaf-1* is a pro-apoptotic adaptor protein that complexes with cytochrome *c* and pro-caspase 9 to form apoptosome that mediates apoptosis. Allelic deletion of *Apaf-1* is observed in 40% of malignant melanoma cases (Hussein *et al.*, 2003). Furthermore, a loss of *Apaf-1* expression has been associated with chemoresistance in metastatic melanoma when cancerous cells escape from p53-dependent apoptosis (Soengas *et al.*, 2001).

1.2 p53 – the guardian of genome

1.2.1 Gene and protein structures

The p53 gene was discovered in the late 1970s and mapped to chromosome 17p13.1 in humans that spans 20 kilobases with 11 exons. It is a cellular phosphoprotein with a relative molecular mass of approximately 53kDa; hence, the p53 protein was so named (Lane and Crawford, 1979). Further work established that p53 is present at low levels in normal cells and higher levels in tumours and tumour derived cell lines. Initial findings

even suggested that increased levels of p53 might contribute to cancer. Gene transfer studies further demonstrated that *p53* functioned as an oncogene in *in vitro* experiments (Eliyahu *et al.*, 1984; Lane and Benchimol, 1990). However, subsequent studies confirmed that p53 was in reality a tumour-suppressor gene. The first definitive evidence for this conjecture came from the studies of human tumours (Baker *et al.*, 1989).

p53 protein is a sequence-specific transcription factor that modifies the transcription of an ever growing large number of genes (Fields and Jang, 1990; Farmer *et al.*, 1992; el-Deiry, 1998). This protein could be divided into various domains structurally and functionally (Figure 1.1). The transcriptional activation (TA) domain, which recruits basal transcriptional machinery required for transcribing new mRNA, consists of the first 42 amino acid residues at the N-terminus (Unger *et al.*, 1992). A proline-rich (P) domain consisting of residues 61 to 94 follows the transcriptional domain. The DNA binding domain (DBD) is made up of residues 102 to 292 (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993). The p53 consensus binding site consists of two copies of a 10-bp motif, 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0-13 bp (el-Deiry *et al.*, 1992). The tetramerization (TD) domain consisting of residues 324-355. This domain is required for p53 protein oligomerization and DNA binding (Iwabuchi *et al.*, 1993; Wang *et al.*, 1994). Finally, at the C-terminus are residues 363 to 393 consisting of basic amino acids that make up a negative regulatory (R) domain.

Figure 1.1 A schematic representation of the p53 protein. TA: transactivation domain, P: proline-rich domain, DBD: DNA binding domain, TD: tetramerization domain, and R: regulatory domain.

1.2.2 Regulation and post-translational modification of p53

Under normal conditions, p53 protein is constitutively expressed in almost all cell types. Due to its rapid turnover rate and its relatively short half-life of less than 30 minutes, the overall concentration inside a cell at any time is usually low. p53 protein is frequently in a latent form that lacks the ability to bind DNA or transactivate other genes.

One of the p53 regulators is Mdm2 (mouse double minute 2) oncoprotein (Barak *et al.*, 1993; Wu *et al.*, 1993). Under normal conditions, an increase in p53 protein level causes an elevated expression of Mdm2, which in turn targets p53 proteins for degradation. This downstream effector of p53 forms a negative feedback loop by binding to the transactivation domain of p53 (Momand *et al.*, 1992; Oliner *et al.*, 1993; Chen *et al.*, 1995). By shuttling p53 from the nucleus to the cytoplasm, Mdm2 mediates p53 turnover (Freedman and Levine, 1998; Tao and Levine, 1999a) and targets p53 for degradation via the ubiquitin-26S proteasome (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997; Fuchs *et al.*, 1998).

Another protein involved in the regulation of p53 via Mdm2 is the tumour suppressor p14^{ARF}, which is encoded by an alternative reading frame in the *CDKN2A/INK4a-ARF* locus. p14^{ARF} binds Mdm2 and directly inhibits Mdm2 activity without preventing the Mdm2-p53 interaction. It also prevents the ubiquitin ligase activity of Mdm2 to inhibit p53 degradation, thus increasing the half-life and activity of p53 (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Honda and Yasuda, 1999). p14^{ARF} has the ability to relocate Mdm2 to the nucleolus to prevent nuclear export of p53 (Tao and Levine, 1999b; Weber *et al.*, 1999) and to promote Mdm2 degradation (Zhang *et al.*, 1998).

1.2.3 p53 and cell cycle arrest

The p53 protein is a tumour suppressor involved in many cellular processes. Although it may yet be found to have other functions, the p53 protein has been shown to function as a transcriptional regulatory protein (el-Deiry, 1998; Vogelstein *et al.*, 2000). p53 activates transcription of a number of genes with roles in the control of cell cycle, including *p21^{Waf1}* (which encodes a regulator of CDK activity) (el-Deiry *et al.*, 1993), *GADD45* (a growth-arrest DNA damage-inducible gene) (Kastan *et al.*, 1992), Mdm2 (a negative regulator of p53 protein), and 14-3-3 σ (a regulator of G₂/M progression) (Hermeking *et al.*, 1997).

p21^{Waf1} activates both G₁ and G₂ cell cycle arrest by binding to a number of cyclin-CDK complexes involved in cell cycle transitions to inhibit kinase activity (Harper *et al.*, 1993; Sherr and Roberts, 1995; Fotedar *et al.*, 1996; Nakanishi *et al.*, 1997; Bates *et al.*, 1998; Dulic *et al.*, 1998; Niculescu *et al.*, 1998). G₁ CDKs are involved in the phosphorylation regulation of the Rb tumour suppressor protein. When unphosphorylated, Rb protein binds to E2F transcription factors to inhibit the activation of E2F-responsive target genes that are involved in the promotion of proliferation (Chellappan *et al.*, 1991; DeGregori *et al.*, 1995). Phosphorylation of Rb by G₁ CDK complexes allows E2F to transactivate genes required for progression through the cell cycle (Sherr and Roberts, 1995). Recently, *p21^{Waf1}* has been shown to directly bind to the E2F subunits and inactivate its transcriptional activity, independent of CDK regulation of Rb-E2F interaction (Delavaine and La Thangue, 1999).

p53 is also involved in both nucleotide excision repair which repairs UV-induced DNA damage such as pyrimidine dimers, and base excision repair which removes damaged bases through alkylating agents, oxygen-free radicals or hydrolysis (Ford and

Hanawalt, 1995; Wani *et al.*, 1999; Offer *et al.*, 2001; Zhou *et al.*, 2001). p53 transactivates DNA repair genes, such as *GADD45* and *p53R2*, a ribonucleotide reductase gene (Smith *et al.*, 1994; Nakano *et al.*, 2000; Tanaka *et al.*, 2000). Furthermore, the C-terminus of p53 binds directly to different forms of DNA damage, such as single-stranded DNA, ends of double-strand breaks, and DNA insertion or deletion mismatches.

1.2.4 p53 and apoptosis

Besides cell cycle arrest and DNA repair, p53 participates in the regulation of apoptosis or programmed cell death as well. Apoptosis can be triggered by a variety of physiological signals that is closely regulated in highly conserved molecular pathways. It occurs under normal conditions especially during embryonic development, metamorphosis, differentiation, and general cell turnover. Two well-characterized apoptotic pathways exist: the intrinsic or mitochondrial, and the extrinsic or death receptor pathways. Numerous studies have implicated p53 as an important protein required in apoptosis in experimental models *in vitro* (Lotem and Sachs, 1993; Lowe *et al.*, 1993). p53-dependent apoptosis has also been observed *in vivo* (Symonds *et al.*, 1994; Ziegler *et al.*, 1994; Tron *et al.*, 1998b).

p53 has been shown to take part in the mitochondrial-mediated apoptotic pathway (Shen and White, 2001) through regulation of members of the mitochondrial proteins, such as Bax and PUMA (Miyashita *et al.*, 1994a; Miyashita *et al.*, 1994b; Miyashita and Reed, 1995; Green and Reed, 1998). The homodimerization and heterodimerization of these proteins can lead to a change in the mitochondrial membrane potential and the sequential release of cytochrome *c* from the intermembrane space of the mitochondria into the

cytoplasm (Oltvai *et al.*, 1993; Liu *et al.*, 1996; Jurgensmeier *et al.*, 1998; Pastorino *et al.*, 1998; Rosse *et al.*, 1998; Shimizu *et al.*, 1999). Released cytochrome *c* can then bind to the cytoplasmic scaffolding protein Apaf-1, further causing an ATP/dATP-dependent conformational change that allows Apaf-1 to bind to the pro-domain of pro-caspase-9. An apoptosome is formed and pro-caspase-9 is activated through self-cleavage (Li *et al.*, 1997b; Rodriguez and Lazebnik, 1999; Saleh *et al.*, 1999; Stennicke *et al.*, 1999; Zou *et al.*, 1999; Jiang and Wang, 2000). Caspase-9 is an initiator caspase that is part of a cysteine-dependent aspartate-directed protease family. Caspases are synthesized as inactive zymogens and activated by proteolysis (Earnshaw *et al.*, 1999). An active caspase-9 can further initiate a cascade of proteolytic activation of other downstream effector caspases, first by cleaving pro-caspases-3 and -7, followed by pro-caspase-6 cleavage by active caspase-3.

These activated caspases represent the final event in the chain reaction of apoptosis. Cellular substrates including structural proteins, such as lamin B and actin, signalling proteins, and regulators of DNA replication or transcription are cleaved (Rosen and Casciola-Rosen, 1997). The morphological characteristics of apoptosis manifested by these molecular pathways include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (Wyllie *et al.*, 1980).

p53 has recently been shown to be involved in other aspects of the mitochondrial pathway. p53 can transcriptionally regulate PUMA (p53 upregulated modulator of apoptosis) (Nakano and Vousden, 2001; Yu *et al.*, 2001), and Apaf-1 (Moroni *et al.*, 2001; Rozenfeld-Granot *et al.*, 2002). PUMA has a BH3 or Bcl-2 homology 3 domain that mediates its interaction with Bcl-2 family member proteins, which can be localized to the

mitochondria, and induces cytochrome *c* release and apoptosis (Nakano and Vousden, 2001; Yu *et al.*, 2001). In addition, p53 increases the expression of PIGs (p53-inducible genes), which are believed to encode proteins that generate or respond to oxidative stress (Polyak *et al.*, 1997). These proteins may be involved in the production of reactive oxygen species (ROS) that lead to alterations of the mitochondrial membrane and release of cytochrome *c* or other polypeptides into the cytoplasm. Interestingly, a recent study showed that p53 itself localizes to the mitochondria prior to changes in mitochondrial membrane potential, cytochrome *c* release and pro-caspase-3 activation during p53-dependent apoptosis (Marchenko *et al.*, 2000).

Finally, p53 has been suggested to be involved in the Fas (also known as APO-1 or CD95) death receptor-mediated apoptotic pathway (Nagata, 1997; Ashkenazi and Dixit, 1998). Upon binding to its natural ligand, Fas ligand (Fas-L), the Fas receptor trimerizes and recruits the adaptor FADD (Fas-associated Death Domain) to its cytoplasmic death domain (Chinnaiyan *et al.*, 1995). FADD then recruits the initiator caspase, caspase-8, to the DISC (Death Inducing Signalling Complex) via its DED (Death Effector Domain) (Muzio *et al.*, 1996; Medema *et al.*, 1997). Caspase-8 is activated by autocatalytic cleavage (Muzio *et al.*, 1998), followed by activation of downstream executioner caspases, such as pro-caspases-3, -6 and -7, and apoptosis (Hirata *et al.*, 1998). The Fas pathway has been shown to be involved in p53-dependent apoptosis in response to various stimuli in some systems (Owen-Schaub *et al.*, 1995; Muller *et al.*, 1997; Bennett *et al.*, 1998; Muller *et al.*, 1998a; Muller *et al.*, 1998b; Fukazawa *et al.*, 1999; Hara *et al.*, 2000; Embree-Ku *et al.*, 2002; Lorenzo *et al.*, 2002). Others have shown that p53 can induce apoptosis independently of the Fas pathway (Fuchs *et al.*, 1997; Reinke and Lozano, 1997). p53-

dependent apoptosis may involve other members of the death receptor family, such as TNFR1 (tumour necrosis factor receptor type 1) (Inagaki-Ohara *et al.*, 2001) and the TRAIL (TNF-related apoptosis-inducing ligand) death receptor KILLER/DR5 (Wu *et al.*, 1997; Wu *et al.*, 1999).

1.2.5 p53 mutation in human cancers

Current studies suggest that the loss of normal p53 function is associated with cell transformation *in vitro* (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Baker *et al.*, 1990) and neoplasms development *in vivo* (Donehower *et al.*, 1992; Symonds *et al.*, 1994). In sporadic cancers in human, p53 has been shown to be one of the most frequently mutated genes (Hollstein *et al.*, 1991; Hollstein *et al.*, 1996). In fact, over 50% of human malignancies in epithelial, mesenchymal, haematopoietic, lymphoid, and nervous origin analyzed to date were shown to contain an altered p53 gene (Hollstein *et al.*, 1996; Soussi, 2000). Inborn germline mutations in the human p53 gene can result in Li-Fraumeni syndrome, which is a hereditary cancer susceptibility syndrome leading to a predisposition to sarcomas, lymphomas, breast, brain and other tumours at an early age (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). In animal model, p53 homozygous and hemizygous knockout mice have a near normal development, but with a number of genetic alterations, including gene amplification and abnormal chromosome numbers. These mice are also more prone to develop multiple tumours at a young age (Donehower *et al.*, 1992; Fukasawa *et al.*, 1997). Overall, inactivation of the p53 pathway seems to be a common mechanism in the development of cancer.

Most mutations in the *p53* gene are missense mutations producing a stable yet non-functional protein. Some mutations even lead to a gain of oncogenic function or dominant negative phenotype over wild-type p53 through heterotetramerization (Dittmer *et al.*, 1993; Hachiya *et al.*, 1994; Brachmann *et al.*, 1996; de Vries *et al.*, 2002). Mutant p53 protein folds into conformations that are not recognized by PAb1620 (Milner and Medcalf, 1990), a conformation-specific monoclonal antibody that recognizes only the wild-type p53 conformation. Due to the fact that most *p53* mutations are found within the core DNA binding domain (Cho *et al.*, 1994; Hollstein *et al.*, 1996), the transcribed protein usually cannot bind to DNA and has impaired transactivation function. This suggests that p53 DNA binding and transcriptional activation are critical in the response to DNA damage and other various p53 functions (Pietenpol *et al.*, 1994). In some cancers with unaltered *p53* genes, p53 may be inactivated indirectly through binding to viral proteins. Others may have gene products upstream or downstream of p53 altered.

1.2.6 p53 and chemotherapy

Currently many different forms of chemotherapy are available. They target various biochemical sites such as thymidylate synthase inhibitors, microtubule damage, topoisomerase inhibitors, and DNA crosslinking agents. The most common ones are by far DNA-damaging agents that generally destroy cancer cells by inducing apoptosis (Barry *et al.*, 1990; Schmitt and Lowe, 1999; Kaufmann and Earnshaw, 2000) through a p53-dependent mechanism (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Li *et al.*, 1998; Hayakawa *et al.*, 2000). In response to DNA damage induced by cytotoxic drugs, p53 protein level and functional activity increase, and thereby induces cell cycle arrest or apoptosis (Kastan *et*

al., 1991; Clarke *et al.*, 1993; Tishler *et al.*, 1993; Chernov and Stark, 1997). In general, cells with *p53* mutations have been shown to be more resistant to chemotherapy (Fan *et al.*, 1994; Lowe *et al.*, 1994; Nabeya *et al.*, 1995; Righetti *et al.*, 1996).

1.3 CP-31398 – a p53 stabilizing compound

1.3.1 Chemical structure of CP-31398

In approximately 50% of all human tumours, *p53* tumour suppressor gene can be inactivated by a single point mutation. Such a high mutation frequency indicates a strong selection for loss of normal *p53* function during tumourigenesis. Foster and colleagues (Foster *et al.*, 1999) screened a library of 100,000 synthetic compounds in order to identify small molecules that can rescue mutant *p53* and could stabilize the native conformation of the wild-type *p53* core domain upon thermal denaturation. Samples were heated in the presence of compounds and *p53* conformation was assessed by the conformation-specific antibodies PAb1620 and PAb240 that recognize the folded and unfolded conformations of *p53*, respectively.

The most promising molecule, CP-31398 is a compound that consists of a polycyclic hydrophobic group (R1) joined to an ionizable group (R2, usually an amine) by a linker of a specific length (Figure 1.2). The R1 and R2 groups of the active compounds are believed to make bivalent contact with the *p53* protein at two adjacent sites. The linker orients these two groups on the *p53* protein to enhance the stability of the active conformation (Foster *et al.*, 1999).

FIGURE 1.2 Chemical structure of p53-stabilizing compound, CP-31398. The compound consists of a hydrophobic group (R1, polycyclic) joined to an ionizable group (R2, often an amine) by a propyl-length linker.

1.3.2 Interaction between CP-31398 and cellular proteins

CP-31398 enhanced the stability of Ala-173 and His-273 mutant p53 core domains *in vitro*, restored native conformation and transcriptional transactivation activity to Ala-173 mutant p53 in living cells, and caused the induction of the p53 target gene p21^{Waf1}. Moreover, CP-31398 inhibited the growth of human melanoma and colon carcinoma cells carrying mutated p53 at position 249 or 241, respectively, in nude mice. No toxicity was observed at the therapeutic doses *in vivo* by the first report on this compound (Foster *et al.*, 1999).

At present, the exact mechanism of CP-31398 remains controversial. The compound could interact directly with already synthesized p53 or mediate p53 folding during biosynthesis. The first attempt to detect physical interactions between p53 and CP-31398 was unsuccessful. NMR did not show that CP-31398 binds to the core DNA binding domain of either recombinant wild-type or mutant p53. Rather, gel shift assays and NMR indicated that the compound binds DNA. Some experimental evidence suggests that CP-31398 can affect the folding of newly synthesized p53 only, which could explain the negative NMR results (Rippin *et al.*, 2002).

Further studies confirmed that CP-31398 treatment causes the induction of p53 target genes like p21^{Waf1}, Mdm2, Bax and Killer/DR5 (Takimoto *et al.*, 2002). CP-31398 also induced apoptosis in HCT116 cells carrying wild-type p53, but not in the isogenic p53-null line in which wild-type p53 has been knocked out. The compound caused an increased wild-type p53 levels and activated some p53 target genes such as p21^{Waf1} and Bax in HCT116 cells, but p21^{Waf1} upregulation was observed to a lesser extent in p53-null HCT116 cells (Luu *et al.*, 2002).

In support of the initial study, another group found that CP-31398 restored the DNA binding activity of mutant p53 (Takimoto *et al.*, 2002). CP-31398 upregulated and stabilized p53 protein levels in wild-type p53 expressing cell lines. It also induced apoptosis in six out of nine cell lines, with two of them also experienced G₁ cell cycle arrest.

CP-31398-mediated upregulation of wild-type p53 levels and transactivation of downstream p53 targets could simply because of the induction of DNA damage, as NMR studies have indicated that the compound is a DNA intercalator. Yet, p53 phosphorylation at Ser-15 and Ser-20, which is often associated with DNA damage, has not been observed after treatment with CP-31398 (Wang *et al.*, 2003). Instead, they showed that CP-31398 inhibited the ubiquitination of wild-type p53, which may explain its ability to induce p53 accumulation. Since a solid conclusion cannot be drawn from previous studies, more work is essential to further explore the possibility of this compound as a therapeutic agent.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines and cell culture

Three human melanoma cell lines with known p53 status were used for this study. The MMRU cell line was a kind gift from Dr. H.R. Byers (Boston University School of Medicine, Boston, MA) (Byers *et al.*, 1991); the MeWo and Sk-mel-110 cell lines were kind gifts of Dr. A.P. Albino (Memorial Sloan-Kettering Cancer Centre, New York, NY). The p53 status in these melanoma cell lines has been determined by DNA sequencing: MMRU contain wild-type p53 (Li *et al.*, 1995), while MeWo and Sk-mel-110 contain mutant p53 (Albino *et al.*, 1994). Melanoma cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin at 37°C (in a humidified atmosphere of 5% carbon dioxide).

2.1.2 Reagents, enzymes, and chemicals

Proteinase K and agarose were obtained from Invitrogen. The CP-31398 compound (Pfizer, Groton, CT) was dissolved in deionized distilled water (ddH₂O), filtered and stored at 4°C. All other chemicals were purchased from Sigma-Aldrich (Mississauga, ON).

2.2 Methods

2.2.1 Cell survival assay

Melanoma cells were seeded in 24-well plates at 4×10^5 cells per well and treated with CP-31398 at 80% confluency. Cell survival was determined with sulforhodamine B (SRB) (Sigma-Aldrich, Mississauga, ON, Canada) assay as described previously (Skehan *et al.*, 1990). SRB is a pink aminoxanthene dye with two sulfonic groups that binds to basic amino acid residues of proteins in cells fixed with trichloroacetate (TCA). This assay can provide a sensitive index of cellular protein content that is suitable for screening the number of adhering cells. After treating cells with CP-31398, growth medium was removed from each well and cells were fixed with 500 μ L of 10% TCA for 1 h at 4°C. Cells were then washed with distilled water for five times and excess water was removed. After air-dried overnight, cells were stained with 500 μ L of 0.4% SRB (w/v in 1% acetic acid) for 30 min at room temperature followed by four times of 1% acetic acid washes. Excess acetic acid was removed and cells were air-dried overnight. To solubilize the bound dye, 500 μ L of 10 mM Tris (pH 10.5) was added to each well. Following 20 min on a shaker, spectrophotometric readings were obtained at 550 nm for 100 μ L aliquot from each well.

2.2.2 Propidium iodide staining

Melanoma cells were seeded in 24-well plates at 4×10^5 cells per well and treated with CP-31398 at 80% confluency. Immediately after the treatment, cells pellets were collected by trypsinization followed by centrifugation at 2,000g for 5 min. Cell pellets were then resuspended in 1 mL of hypotonic fluorochrome buffer (0.1% Triton X-100, 0.1% Sodium

citrate, 25 $\mu\text{g mL}^{-1}$ RNase A, and 50 $\mu\text{g mL}^{-1}$ propidium iodide). Cells were then incubated at 4°C for 1 h, examined by a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Mississauga, ON) and analyzed with EXPO32 ADC analysis software to determine the percentage of cells that consist of different amount of DNA. Stained cells were also visualized under a fluorescent microscope (Zeiss, Chester, VA). Fluorescent microscopic images were taken with a cooled mono 12-bit Retiga-Ex camera (Q-imaging, Burnaby, BC, Canada) using the Northern Eclipse microscopy image analysis software (Empix Imaging, Mississauga, ON, Canada).

2.2.3 DNA fragmentation assay

Melanoma cells were grown in 100-mm plates and treated with CP-31398 at 80% confluency. Cells were collected and the number of cells was equalized after each treatment. Harvested cells were resuspended in 500 μL of proteinase K lysis buffer (20 mM calcium chloride, 0.5% SDS, 10 mM Tris-HCl, and 5 mM EDTA at pH 8.0) containing 1 mg mL^{-1} proteinase K (Invitrogen,) and incubated at 54°C with gentle agitation for 2 h. DNA was then extracted with phenol:chloroform (Sigma-Aldrich), ethanol precipitated, and resuspended at 37°C for 30 min in 20 μL of elution buffer (10 mM Tris-HCl and 0.5 mM EDTA [pH 9.0]). DNA was then analyzed on a 2% agarose gel for DNA fragmentation. The gels were stained with 0.5 $\mu\text{g mL}^{-1}$ of ethidium bromide and photographed under UV light with the gel print system (Biophotonics, Ann Arbor, MI).

2.2.4 Immunofluorescent staining

Melanoma cells were grown on glass coverslips in 35-mm plates and treated with CP-31398 at 80% confluency. Immediately after treatment, cells were washed twice with phosphate buffered saline (PBS) for 30 s each followed by 10 min of fixation and extraction with 2 mL of Fix/ex solution (8% paraformaldehyde, 4% sucrose in PBS [pH 7.2]) at room temperature. Cells were then washed three times 5 min each with PBS. In order to eliminate free aldehyde groups, cells were incubated with 1 mg mL⁻¹ of sodium borohydride (NaBH₄) in PBS three times 5 min each. Fixed cells on coverslips were then inverted onto 50 µL of 1:25 dilution of p53 antibody DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or PAb1620 (Oncogene, Boston, MA) in a humidifying chamber for 1 h. Cells were washed three times 5 min each with PBS. Subsequently, cells were incubated in 50 µL of secondary antibody at 1:100 dilution for 1 h followed by three washes with PBS for 5 min each. Cells were counter-stained with Hoescht 33258 before fixing on coverslips and mounting onto slides with Permount (Fisher Scientific, Nepean, ON, Canada) and visualized under a fluorescent microscope.

2.2.5 Mitochondrial transmembrane potential detection (MitoCapture™)

The MitoCapture™ Apoptosis Detection Kit (Calbiochem, San Diego, CA) provides a fluorescent-based method for distinguishing the healthy and apoptotic cells by detecting the changes in the mitochondrial membrane potential. The assay was performed according to the manufacturer's specifications. Melanoma cells were grown in 35-mm plates and treated with CP-31398 at 80% confluency. Following the drug treatment, 1x10⁶ cells per sample was resuspended in 1 mL of diluted MitoCapture™ cationic dye and incubated in a

5% CO₂ incubator at 37°C for 20 min. Cells were then centrifuged at 500g for 5 min and the cell pellets were resuspended in 1 mL of pre-warmed incubation buffer. Immediately afterwards, melanoma cells were observed under a fluorescence microscope. MitoCapture™ reagent that was aggregated in the mitochondria of healthy cells generates a red fluorescence. In apoptotic cells, MitoCapture reagent cannot accumulate in the mitochondria and remains in the cytoplasm, generating a green fluorescence.

2.2.6 Western blot analysis

Melanoma cells were grown in 100-mm plates and treated at 80% confluency. Immediately after treatment, cells were harvested and subjected to Western blot analysis as described (Towbin *et al.*, 1979; Burnette, 1981). Briefly, cells were harvested and lysed on ice for 10 min with 50 µL of Lysis buffer [1.0% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, and 50 mM HEPES (pH 7.5)] containing a mixture of freshly prepared protease inhibitors (100 µg mL⁻¹ phenylmethylsulfonyl fluoride, 1 µg mL⁻¹ aprotinin, 1 µg mL⁻¹ leupeptin, and 1 µg mL⁻¹ pepstatin A). Cell pellets were obtained by spinning at 16,000g in an Eppendorf microfuge for 10 min at 4°C. Supernatant was then transferred to a new tube and the pellet was discarded. The protein concentration was determined using Bradford assay (Bio-Rad, Mississauga, ON, Canada) at A 280. 50 µg per lane of protein was separated by either 8% or 12% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were immersed in blocking buffer [5.0% bovine serum albumin (BSA), 0.05% Tween 20, and Tris buffered saline (TBS) (pH 7.2)] blocked overnight. Membrane was then incubated with primary antibody diluted in TBS with 5.0% BSA for 60 min at room temperature. After three times

ten min each of washing in TBS with 0.05% Tween 20, membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 45 min at room temperature. After another three times ten min each of washing in TBS with 0.05% Tween 20, signals were detected with enhanced chemiluminescence detection kit (Amersham Biosciences, Baie d'Urfe, QC, Canada). Protein expressions on Western blots were quantified by densitometry with Quantity One software (Bio-Rad, Mississauga, ON, Canada). The fold-induction or reduction was corrected for differences in the actin loading control. Antibodies used for Western blotting were p53 (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3, Apaf-1, β -actin (BD Pharmingen, Mississauga, ON, Canada), and caspase-9 (Calbiochem, San Diego, CA).

2.2.7 Subcellular fractionation

Melanoma cells were grown in 100-mm plates, treated with CP-31398 at 80% confluency, and the subcellular fractionation was performed as described previously (Kim *et al.*, 2001) with minor modifications. Treated cells were harvested on ice and resuspended in 500 μ L buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 250 mM sucrose) containing a mixture of freshly prepared protease inhibitors (100 μ g mL^{-1} phenylmethylsulfonyl fluoride, 1 μ g mL^{-1} aprotinin, 1 μ g mL^{-1} leupeptin, and 1 μ g mL^{-1} pepstatin A). Resuspended cells were then passed through a 26-gauge needle 10 times to lyse cells. Unbroken cells, large fractured plasma membrane, and nuclei were removed by centrifugation at 1,000g at 4°C for 10 min. The resulting supernatant was then further centrifuged at 10,000g at 4°C for 20 min. The pelleted mitochondrial fraction was washed

with buffer A once and then solubilized in 50 μ L of TNC buffer (10 mM Tris-acetate, 0.5% NP-40, and 5 mM CaCl_2) containing a mixture of freshly prepared protease inhibitors as described above. The cytosol fraction was generated by centrifugation of the supernatant at 100,000g at 4°C for 60 min. The protein concentration was determined using Bradford assay (Bio-Rad). 50 μ g per lane of protein was separated by 15% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. After overnight blocking with the blocking buffer (5.0% bovine serum albumin, 0.05% Tween 20, and Tris buffered saline (TBS) [pH 7.2]), PVDF membranes were probed with anti-cytochrome *c* (BD Pharmingen) antibodies and detected with enhanced chemiluminescence detection kit (Amersham Biosciences).

2.2.8 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Melanoma cells were grown in 100-mm plates and treated with CP-31398 at 80% confluency, followed by total RNA extraction according to the manufacturer's specifications using the TRIzol™ Reagent (Invitrogen). Extracted RNA was quantified by UV spectrophotometry (Pharmacia Biotech, Cambridge, England). First-strand cDNA was prepared from 3 μ g of total RNA reverse-transcribed into cDNA using the Omniscript RT-kit (Qiagen Inc., Mississauga, ON, Canada). A mix containing 10x RT buffer, 5 mM dNTP mix and 10 μ M of oligo-dT primer (Invitrogen) to a total volume of 20 μ L. The RT mix was incubated at 37°C for 60 min followed by an inactivation at 93°C for 5 min.

For semi-quantitative PCR, 2 μ L of the first strand reaction were used for the total volume of 25 μ L PCR reaction. Each reaction contained 10x PCR buffer (200 mM Tris-

HCl [pH 8.4], 500 mM KCl, 50 mM MgCl₂, 10 mM dNTP mix, 25 µM of specific primers, and 5 U µL⁻¹ of Taq DNA polymerase) (Qiagen).

Specific primers are as followed:

Bax forward, 5'-AGCGGCGGTGAT GGACGGGTC-3',

Bax reverse, 5'-TCCAAGGCAGCTGGGGCCTCA-3' (608 bp);

p21^{Waf1} forward, 5'-ATGTCAGAACCGGCTGGG-3',

p21^{Waf1} reverse, 5'-TTAGGGCTTCCTCTTGA-3' (477 bp);

PUMA forward, 5'-TGTAGAGGAGACAGGAATCCACGG-3',

PUMA reverse, 5'-AGGCACCTAATTGGGCTCCATCTC-3' (311 bp);

GAPDH forward, 5'-CCACCCATGGCAAATTCCATGGCA-3',

GAPDH reverse, 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (270 bp).

The PCR mix was amplified as follows: 1) initial denaturation at 95°C for 3 min, 2) denaturation at 95°C for 1 min, 3) annealing at 52°C (*GAPDH*), 55°C (*p21^{Waf1}*), or 58°C (*Bax* and *PUMA*) for 30 s, 4) polymerization at 72°C for 30 s, 5) repeat steps two to four for the indicated number of cycles (30 for *GAPDH* and *p21^{Waf1}*, or 35 cycles for *Bax* and *PUMA*), and 6) final extension and polymerization at 72°C for 3 min. Samples were then electrophoresed on 2% agarose gels containing 0.5 µg mL⁻¹ ethidium bromide, visualized under UV light and photographed with the gel print system. The levels of mRNA expression were quantified by densitometry using the Quantity One software (Bio-Rad). The fold-induction was corrected for differences in the GAPDH internal loading control.

CHAPTER 3. DIFFERENTIAL RESPONSES OF MELANOMA CELL LINES WITH VARIOUS P53 MUTATIONAL STATUSES TO CP-31398-INDUCED APOPTOSIS

3.1 Rationale and Hypothesis

Metastatic melanoma is one of the most aggressive cancers with its death rate continuing to climb. Although surgical excision of tumours might provide some benefit by alleviating pain and enhancing the quality of life, advanced melanoma that has spread to internal organs often cannot be cured. Currently, the only treatment option for metastatic melanoma is chemotherapy but the response rate is very low because of resistance to conventional drugs (Garbe, 1993; Helmbach *et al.*, 2001). The exact resistance mechanisms vary depending on the mechanistic action of different drugs.

In some drug-resistant cancer cell lines, the level of intracellular level of drugs is reduced because of an increased efflux by P-glycoprotein (P-gp) (Gottesman and Pastan, 1993). This might be essential because in order for an anticancer drug to have an effect on cancer cells, a significant concentration must be reached inside a cell. In primary and metastatic melanoma, as well as in melanoma cell lines, however, no significant melanoma-specific correlation can be drawn between P-gp expression level and response to chemotherapy in melanoma patients (Schadendorf *et al.*, 1995). As a result, P-gp is not considered as a major drug resistance mediator in human melanoma cells.

Some anticancer agents, such as etoposide, target topoisomerase after entering a cell. Topoisomerase is a nuclear enzyme that participates in DNA transcription and recombination during mitosis. In fact, increased activity of topoisomerase II has been shown to correlate with etoposide resistance in melanoma (Lage *et al.*, 2000).

Other chemotherapeutic agents, such as cisplatin, act through induction of apoptosis. Their reduced efficiency in melanoma probably relates to defective apoptotic machinery when compared to other malignant cell types (Kaufmann and Earnshaw, 2000). Furthermore, the ability of anticancer agents to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies. Thus, inhibition of caspase activation may be an important factor in chemoresistance.

p53 is a widely-studied tumour suppressor that has the ability to trigger cell-cycle arrest and apoptosis in response to diverse stress stimuli. Under cellular stress, such as hypoxia, DNA damage, ionizing radiation, exposure to anticancer drugs, and oncogene activation, the p53 protein is rapidly accumulated in a cell (Ko and Prives, 1996; Giaccia and Kastan, 1998; Sherr, 1998; Asker *et al.*, 1999). This increase in p53 protein level can further transactivate its downstream target genes through specific DNA binding. Depending on the extent of DNA damage, p53 can either bring about cell cycle arrest in an effort to repair the damage, or induce apoptosis when the damage is too severe (Li *et al.*, 1997a; Tron *et al.*, 1998a). Apoptosis, or programmed cell death, is a tightly regulated self-protective mechanism in order to eliminate unnecessary or severely damaged cells.

More than half of all human tumours contain *p53* mutations and majority of the mutations are located in the DNA binding domain (Beroud and Soussi, 1998). The prevalent disruption of the specific DNA binding function of p53 by gene mutation

suggests that this function is critical for p53-mediated tumour suppression. Many conventional chemotherapeutic agents exploit this p53-dependent apoptotic pathway through DNA damaging events to destroy cancerous cells (Li *et al.*, 2000). In fact, tumours carrying mutant *p53* are often more resistant to chemotherapy than those carrying wild-type *p53* (Levine, 1997). Additionally, studies using the more complicated genetic background of tumour-derived cell lines found a high dependency of most clinically established anticancer drugs on functional p53 (Lowe *et al.*, 1994; Lowe, 1995; Weinstein *et al.*, 1997).

Conventional therapeutic agents amplify stress signals impelling damaged cells to self-destruct. Unfortunately, treatment fails when the cancer inactivates tumour-suppressor pathways. In most malignancies, mutation in *p53* leads to a shut down of the p53 tumour suppressor signaling pathway (Bullock and Fersht, 2001). Various strategies have been explored to restore mutant p53 to wild-type function (Selivanova *et al.*, 1998). A class of small pharmacological compounds has been identified in a random screening of over 100,000 synthetic molecules for its ability to promote the stable active conformation of wild-type p53. This class of small-molecule compounds can increase the activity of p53 and induce apoptosis in tumour cells (Foster *et al.*, 1999). Among all, CP-31398 showed the strongest ability to stabilize the wild-type p53 protein conformation against thermal denaturation. It was further shown that CP-31398 has a remarkable capacity to restore mutant p53 to behave like wild-type p53 and induce apoptosis in a number of cancer cell lines including melanoma cells (Foster *et al.*, 1999; Luu *et al.*, 2002; Rippin *et al.*, 2002; Takimoto *et al.*, 2002). Indeed, tumour cells are likely to be particularly sensitive to p53 reactivation. Some mutant p53 proteins tend to accumulate at high levels in tumour cells,

mainly due to inability of mutant p53 to transactivate Mdm2, whose product induces p53 degradation. Thus, reactivation of abundant mutant p53 may trigger a massive apoptotic response in tumour cells, whereas normal cells that express minute levels of p53 should not be affected (Bykov *et al.*, 2002).

While Foster and colleagues (1999) claimed that CP-31398 has the ability to rescue all mutant p53 to wild-type p53 activity, Tanner and Barberis (2004) argued that CP-31398 could not stimulate any mutant p53 to restore any detectable p53 activity. These contradictory results prompted us to further look into the apoptotic events caused by CP-31398. Moreover, seeing that Tanner and Barberis (2004) focused only on the transcription level of a single p53 response element, p21^{Waf1}, through luciferase activity, we further investigated the effect of CP-31398 from the apoptotic machinery perspective. More importantly, as metastatic melanoma responds poorly to traditional treatment methods, we sought to explore the possibility of using this new small molecule drug as a treatment for melanoma cells with various p53 mutation statuses. Noticing a differential response of melanoma cell lines to CP-31398 after initial screening, we hypothesized that the ability of CP-31398 to revert mutant p53 to wild-type p53 conformation may not be universal.

3.2 Results

3.2.1 Effects of CP-31398 on survival of human melanoma cells

Three human melanoma cell lines with different *p53* statuses were used for all the experiments. MMRU cells carry a wild-type *p53* gene while MeWo and Sk-mel-110 cells carry mutant *p53* genes (Table 3.1). In order to determine if CP-31398 has the ability to modulate cell survival in all three melanoma cell lines, SRB cell survival assay was performed after CP-31398 treatment. Fig. 3.1 shows the dose-dependent survival rate of melanoma cells after 20 h of CP-31398 treatment. The EC_{50} (effective concentration 50%) of CP-31398 for MMRU and MeWo was determined to be $15 \mu\text{g mL}^{-1}$ and this concentration was used for subsequent experiments. Fig. 3.2 shows the time-dependent survival rate of melanoma cell lines with or without $15 \mu\text{g mL}^{-1}$ of CP-31398 for 0, 16 or 24 h. Both MMRU and MeWo cells responded well upon exposure to CP-31398. In MMRU cells, survival rate dropped to 51% and 30% after 16 and 24 h of CP-31398 treatment, respectively ($P < 0.05$). Similar trend was observed in MeWo cells with the survival rate reduced to 53% and 27% after 16 and 24 h of CP-31398 treatment, respectively ($P < 0.05$). In Sk-mel-110, however, survival rate did not change significantly at a moderate concentration. It only decreased to 97% and 91% after 16 and 24 h of CP-31398 treatment, respectively. These results indicate that CP-31398 has the ability to modulate the survival rate in some human melanoma cell lines.

Table 3.1 Description of p53 mutation status found in melanocytic lesions.

Cell line	p53 status	Exon	Codon	Nucleotide substitution	Amino acid substitution	Reference
MMRU	Wild type	N/A	N/A	N/A	N/A	(Li <i>et al.</i> , 1995)
MeWo	Mutant	10	341	CC → TT	phe → stop	(Albino <i>et al.</i> , 1994)
Sk-mel-110	Mutant	4	75	CCT → CCC	pro → pro	(Albino <i>et al.</i> , 1994)
		4	76	GCA → ACA	ala → thr	
		4	104	CAG → CAC	gln → his	
		5	155	ACC → AGC	thr → ser	
		8	289	CTC → TTC	leu → phe	
		8	296	CAC → TAC	his → cys	

N/A = Not applicable

Figure 3.1 Survival of human melanoma cells after CP-31398 treatment is dose-dependent. Cell survival by SRB assay of MMRU, MeWo and Sk-mel-110 cells treated with various doses of CP-31398 for 20 h. Spectrophotometric readings were obtained and mean values and standard deviations for three independent experiments were calculated.

Figure 3.2 Alteration in survival of human melanoma cells after CP-31398 treatment. Cell survival by SRB assay of MMRU, MeWo and Sk-mel-110 cells treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 16 or 24 h. These experiments were repeated three times with similar results.

3.2.2 Differential responses of mutant p53 melanoma cell lines to CP-31398-induced apoptosis

Condensation and fragmentation of DNA is a hallmark of apoptosis. The DNA ladder assay was used to confirm that CP-31398-induced cell death is a result of apoptosis, not necrosis. DNA was extracted from CP-31398-treated cells and analyzed on an agarose gel. Fig. 3.3 shows extensive DNA fragmentation occurs in CP-31398 treated MMRU and MeWo cells but not in Sk-mel-110 cells or the untreated cells. Propidium iodide (PI) staining was also performed on fixed melanoma cells in order to examine the cellular morphological changes after 16 h of CP-31398 treatment. Arrows in Fig. 3.4 show condensed nucleus and evident apoptotic bodies in CP-31398-treated MMRU and MeWo cells, but not in Sk-mel-110 cells or the untreated control cells.

In addition to the morphological changes as a result of programmed cell death, fragmented hypo-diploid DNA can be visualized as a sub-G₁ peak on a DNA cell cycle histogram (Butt *et al.*, 2000). All melanoma cells with or without CP-31398 treatment were stained with propidium iodide and then analyzed by flow cytometry (Fig. 3.5). An increase of sub-G₁ cell percentage in both MMRU and MeWo further confirmed the decreased cell survival observed in SRB assay after CP-31398 treatment (Fig. 3.2). Since CP-31398 has been shown to have the ability to stabilize p53 (Foster *et al.*, 1999), the differential response observed between MMRU, MeWo and Sk-mel-110 cells may well be due to the various mutation statuses of these melanoma cell lines (Table 3.1).

Figure 3.3 CP-31398 induces DNA fragmentation in human melanoma cells. MMRU, MeWo and Sk-mel-110 cells were treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 24 h. DNA was extracted, separated on a 2% agarose gel, stained with 0.5 $\mu\text{g mL}^{-1}$ of ethidium bromide, and visualized under UV light.

Figure 3.4 CP-31398 induces apoptosis in human melanoma cells. MMRU, MeWo and Sk-mel-110 cells were treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 16 h, stained with propidium iodide, and visualized under a fluorescent microscope. Arrows indicate apoptotic bodies. Magnification, 200x. These experiments were performed three times with similar results.

Figure 3.5 CP-31398 increases sub-G₁ cell population in human melanoma cells. MMRU, MeWo and Sk-mel-110 cells were treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 16 or 24 h, stained with propidium iodide, and analyzed by flow cytometry. The experiment was performed three times with similar results and the representative data was shown.

3.2.3 Restoration and stabilization of p53 wild-type conformation

Since p53 is involved in apoptosis (Li *et al.*, 1997a; Tron *et al.*, 1998a) and CP-31398 has been shown to have the ability to stabilize p53 (Foster *et al.*, 1999), the level of p53 protein after CP-31398 treatment was examined. With the observed differential response to CP-31398 in melanoma cell lines, we performed Western blot analysis to determine if CP-31398 treatment affects the level of p53 protein levels. All three melanoma cell lines showed a time-dependent increase in the overall p53 protein levels although MMRU and MeWo cells showed a more significant increase after CP-31398 treatment than non-responsive Sk-mel-110 cells (Fig. 3.6). Our observation is consistent with the p53 stabilizing effect of this synthetic compound which was shown to block ubiquitination and degradation of p53 as previous described (Foster *et al.*, 1999; Wang *et al.*, 2003).

The level of p53 protein alone may not necessarily correlate to an increased p53 activity because active p53 conformation must be acquired in order for p53 to function (Hupp *et al.*, 2000). We therefore employed immunofluorescent staining to study the expression level of total and active conformation of p53 protein *in vivo*. All melanoma cells were immunofluorescent stained 16 h after 15 $\mu\text{g mL}^{-1}$ CP-31398 treatment and examined under a fluorescent microscope. An upregulation of overall p53 protein levels was detected by a mouse monoclonal anti-p53 antibody DO-1 in all three melanoma cell lines. DO-1 antibody recognizes both active and inactive conformations of wild-type and mutant p53 (Fig. 3.7) further confirming the results from Western blot analysis (Fig. 3.6). We also used another mouse monoclonal p53 antibody PAb-1620, which is a conformation specific antibody that recognizes the stabilized, non-denatured, active DNA binding domain in p53 protein (Gamble and Milner, 1988; Foster *et al.*, 1999). We showed that

CP-31398 treatment resulted in a significant increase in the level of active conformation of p53 protein in MMRU and MeWo cells (Fig. 3.7). In contrary, PAb-1620 antibody did not show any positive fluorescence staining in Sk-mel-110 cells. The inability of CP-31398 to rescue mutant p53 into an active and functional p53 in Sk-mel-110 cells may render the resistance to CP-31398-induced apoptosis.

Figure 3.6 Effect of CP-31398 on the overall expression of p53 proteins in human melanoma cells. Western blot analysis using anti-p53 antibody (DO-1) was performed on proteins extracted from MMRU, MeWo and Sk-mel-110 cells treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 0, 8, or 16 h. β -actin was used as a loading control.

Figure 3.7 Effect of CP-31398 on the expression of p53 proteins in human melanoma cells. Immunofluorescent staining using anti-p53 antibodies (DO-1 and PAb-1620) was performed on MMRU, MeWo and Sk-mel-110 cells after treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 16 h. Cells were counter-stained with Hoescht 33248 and overlay on the immunofluorescent pictures. These experiments were performed three times with similar results. Magnification, 200x.

3.2.4 Activation of p53 transcriptional activity

To determine if the transcriptional activity of p53 protein is increased after CP-31398 treatment, semi-quantitative RT-PCR of several p53 downstream targets was performed. p21^{Waf1} has the ability to inhibit cyclin-kinase activity and is tightly regulated at the transcriptional level by p53. It serves as an effector of p53 cell cycle control and mediates p53 suppression of tumour cell growth. We observed an upregulation of p21^{Waf1} by RT-PCR (Fig. 3.8) after CP-31398 treatment in all melanoma cell lines regardless of the response to CP-31398, which is consistent with the previous observation (Takimoto *et al.*, 2002).

Another p53 downstream target is Bax, which belongs to the Bcl-2 family apoptotic proteins (Miyashita *et al.*, 1994a; Miyashita *et al.*, 1994b; Miyashita and Reed, 1995). A time-dependent upregulation of Bax mRNA level was observed in both MMRU and MeWo cells when compared to the untreated control (Fig. 3.8). In contrast, Sk-mel-110 cells showed very little difference in Bax expression after CP-31398 treatment, most likely due to the absence of a functional p53 (Wang *et al.*, 1997).

PUMA (p53 upregulated mediator of apoptosis) is a BH3-only mitochondrial protein that is induced in cells following p53 activation. Antisense inhibition of PUMA expression could reduce the apoptotic response to p53, suggesting that PUMA plays an important role in mediating p53-induced cell death in some cell lines (Nakano and Vousden, 2001). We showed a time-dependent increase of PUMA mRNA level in MMRU and MeWo cells (Fig. 3.8). Similar to Bax mRNA level, Sk-mel-110 cells showed very little difference in PUMA mRNA expression after CP-31398 treatment. PUMA protein interacts with other Bcl-2 like proteins and promotes mitochondrial translocation and

Figure 3.8 Effect of CP-31398 on p21^{Waf1}, Bax and PUMA mRNA levels. Semi-quantitative RT-PCR of p21^{Waf1}, Bax and PUMA mRNA level was performed on RNA extracted from MMRU, MeWo and Sk-mel-110 cells treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 0, 8, or 16 h. GAPDH was used as an internal loading control. The experiment was performed three times with similar results and the representative data was shown.

multimerization of Bax (Yu *et al.*, 2003). These results suggested that the increased expression of PUMA and Bax may play a pivotal role in determining the outcome of p53-dependent apoptosis.

3.2.5 Alteration of mitochondrial transmembrane potential

Apoptosis can be caused by many different stimuli, such as cytotoxic drugs and γ -irradiation. An early stage event in apoptosis is the loss of membrane potential in mitochondria ($\Delta\Psi(M)$). This change can be regulated by p53 (Li *et al.*, 1999; Shimizu *et al.*, 1999; Zhang *et al.*, 1999; Karpinich *et al.*, 2002). Furthermore, increased Bax transcription results in mitochondrial membrane permeability change and the release of cytochrome *c* from the mitochondria into cytosol (Shimizu *et al.*, 1999).

To determine if CP-31398 induces a change in mitochondrial membrane potential during apoptosis, melanoma cells were treated with 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 16 h and then stained with a cationic dye using the MitoCaptureTM Apoptosis Detection Kit. This cationic dye accumulates and aggregates in the mitochondria in healthy cells and emits a red fluorescence. In apoptotic cells, the dye remains in the cytoplasm due to the change in mitochondrial membrane potential and emits a green fluorescence. These stained cells were visualized under a fluorescent microscope. Fig. 3.9A shows that untreated cells stained red in all three cell lines. After 16 h of CP-31398 treatment, only CP-31398 sensitive cells MMRU and MeWo showed a green fluorescent staining (apoptotic cells). Fig. 3.9B showed MMRU and MeWo cells had a significantly higher percentage of green-stained apoptotic cells (from 6% to 95%, $P < 0.005$, and from 6% to 97%, $P < 0.005$, respectively) than Sk-mel-110 cells after CP-31398 treatment (from 5% to 7%). These

Figure 3.9 Alteration of the mitochondrial membrane potential after CP-31398 treatment. MMRU, MeWo and Sk-mel-110 cells were treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 0 or 16 h, and stained with MitoCapture™ solution. **A)** Fluorescent microscopic photographs of control and CP-31398-treated (16 h) MMRU, MeWo and Sk-mel-110 cells. Red fluorescence represents live cells, while green fluorescence specifies apoptotic cells with altered mitochondrial membrane potential. Magnification, 200x. **B)** Quantification of the percentage of apoptotic cells based on the changes in the mitochondrial membrane potential. At least 500 cells from 5 random fields were counted.

A

B

data suggest that CP-31398 induced apoptosis by altering mitochondrial membrane potential.

3.2.6 Induction of cytochrome *c* release

A change in mitochondrial membrane potential is usually coupled with cytochrome *c* release from mitochondria into cytosol (Cryns and Yuan, 1998). Subcellular fractionation and Western blotting were performed to determine if indeed cytochrome *c* was released from mitochondria to cytosol in melanoma cells after treatment with $15\ \mu\text{g mL}^{-1}$ of CP-31398. Our results indicated that the cytochrome *c* level only decreased in the mitochondria and increased in the cytosol in CP-31398 sensitive MMRU and MeWo cells, while little cytochrome *c* release occurred in Sk-mel-110 cells (Fig. 3.10).

Figure 3.10 Effect of CP-31398 on cytochrome *c* release. Subcellular fractionation and Western blot analysis were performed on proteins extracted from MMRU, MeWo and Sk-mel-110 cells treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 16 h. Antibody against cytochrome *c* was used. β -actin was used as a loading control for the cytosolic fraction.

3.2.7 CP-31398 does not induce Apaf-1 expression

Once released from mitochondria, cytochrome *c* often binds to Apaf-1 and activates procaspases. Previous studies have shown that p53 can directly regulate Apaf-1 mRNA and protein levels during apoptosis (Robles *et al.*, 2001). After exposure to DNA-damaging agents, it has been shown that wild-type p53 can induce Apaf-1 mRNA and protein expression (Soengas *et al.*, 2001). Others have shown that metastatic melanomas often lose Apaf-1 (Roses *et al.*, 1991a) and Apaf-1 negative melanomas were invariably chemoresistant. Our Western blot analysis showed that Apaf-1 protein was expressed but the level did not change extensively in all three melanoma cell lines after CP-31398 treatment (Fig. 3.11), suggesting that p53 did not regulate Apaf-1 protein level in these cells during CP-31398-mediated apoptosis.

3.2.8 Activation of Caspases-9 and -3

Released cytochrome *c* often facilitates the interaction between Apaf-1 and caspase-9, resulting in the activation of caspase-9 and downstream effector caspases such as caspase-3 and -7 (Kannan *et al.*, 2001; Moroni *et al.*, 2001). To determine if the mitochondrial or caspase-9 pathway is activated in CP-31398-induced apoptosis, we analyzed the cleavage of caspase-9 by Western blotting. Fig. 3.11 shows that the pro-caspase-9 level is dramatically reduced after CP-31398 treatment in both MMRU and MeWo cells, while little change was observed in Sk-mel-110 cells. Since activated caspase-9 can cleave caspases-3 and -7, we further examined the pro-caspase-3 level after CP-31398 treatment. We found that CP-31398 reduced pro-caspase-3 level substantially in both MMRU and MeWo cells, while negligible changes in Sk-mel-110 cells were observed (Fig. 3.11).

Figure 3.11 Effect of CP-31398 on the expression of Apaf-1, pro-caspases-9 and -3. CP-31398 reduced pro-caspases-9 and -3 protein levels but not Apaf-1. MMRU, MeWo and Sk-mel-110 cells were treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 0, 8, or 16 h. Proteins were extracted and subjected to Western blot analysis using anti-caspase-9, anti-caspase-3, and anti-Apaf-1 antibodies. β -actin was used as a loading control.

3.2.9 CP-31398-induced apoptosis does not involve Fas receptor or Fas-ligand

Since p53 is involved in both the mitochondrial- and death receptor-mediated apoptosis pathways, we investigated if Fas death receptor pathway was involved in CP-31398-mediated apoptosis in human melanoma cells. We showed that neither Fas nor Fas-ligand (Fas-L) protein level was changed considerably (Fig. 3.12), indicating that Fas death receptor pathway probably did not participate in CP-31398 mediated apoptosis.

Figure 3.12 CP-31398 did not alter the expression of Fas and Fas-ligand proteins. MMRU, MeWo and Sk-mel-110 cells were treated with 0 or 15 $\mu\text{g mL}^{-1}$ of CP-31398 for 0, 8, or 16 h. Proteins were extracted and subjected to Western blot analysis using anti-Fas or anti-Fas-ligand (Fas-L) antibody was performed on proteins extracted in. β -actin was used as a loading control.

3.3 Discussion

In approximately 50% of all human tumours, the *p53* tumour suppressor gene is inactivated by point mutations. Such a high mutation frequency indicates a strong selection for loss of normal *p53* function during tumourigenesis (Soussi, 2000). One way to introduce wild-type *p53* gene into tumour cells is through viral mediated vectors such as adenovirus and retrovirus. Besides high toxicity, the use of viral delivery systems may involve an immuno-response to viral antigens (Walther and Stein, 2000). Another approach is to stabilize the *p53* protein in an active conformation which may help prolong its anti-tumour effects. A library of synthetic compounds was screened recently in an effort to identify small molecules that has the ability to stabilize the native conformation of *p53* and to rescue mutant *p53* (Foster *et al.*, 1999). Conformation-specific antibodies PAb1620 and PAb240, which recognize the folded and unfolded conformations of *p53* respectively, were used to evaluate the capability of compounds to stabilize the active conformation of the wild-type *p53* core domain upon thermal denaturation.

The most promising molecule, CP-31398, enhanced the stability of Ala-173 and His-273 mutant *p53* core domains *in vitro*. It restored native conformation and activated transcriptional activity to Ala-173 mutant *p53* in living cells, causing an induction of a *p53* target gene *p21^{Waf1}* (Foster *et al.*, 1999). Moreover, CP-31398 inhibited the growth of human colon carcinoma and melanoma cells carrying mutated *p53* at position 241 or 249, respectively, in nude mice. Additionally, no toxicity was observed at the therapeutic doses *in vivo* (Foster *et al.*, 1999; Bykov *et al.*, 2003).

At present, the exact mechanism of CP-31398 inhibition of tumour growth remains controversial. The capability of this compound to interact directly with already

synthesized p53 or mediate p53 folding during biosynthesis is still uncertain. The first attempt to detect physical interactions between p53 and CP-31398 was unsuccessful. Nuclear magnetic resonance (NMR) failed to show CP-31398 binds to the DNA binding core domain of either recombinant wild-type or mutant p53. Rather, gel shift assays and NMR indicated that the compound binds DNA. Some experimental evidence suggests that CP-31398 can affect the folding of newly synthesized p53 only, which could explain the negative NMR results (Rippin *et al.*, 2002).

CP-31398-induced apoptosis may contribute to its inhibitory effect on tumour growth. Our group used colon rectal cancer cells HCT116 that carries wild-type p53 and its isogenic p53-null cell line in which wild-type p53 has been knocked out. We found that CP-31398 only induced apoptosis in HCT116 cells that carries wild-type p53, but not in the p53-null line (Luu *et al.*, 2002). In addition, we found that CP-31398 enhances UVB induced apoptosis in MMRU melanoma cells by activating the p53-mitochondrial pathway (Luu and Li, 2002). Tumour cell lines that contain mutant p53 seem to respond differently to CP-31398-induced apoptosis. Takimoto and colleagues (2002) reported that after CP-31398 treatment mutant-p53 SW480 colon cancer cells underwent apoptosis. However, CP-31398 induced cell cycle arrest exclusively in mutant-p53 H460 lung cancer cells and DLD1 colon cancer cells.

Our results presented a differential survival rate in three melanoma cell lines after CP-31398 treatment. We first demonstrated that the survival of melanoma cell lines after 20 h of CP-31398 treatment is dose-dependent (Fig.3.1). When cells were treated with more than $30 \mu\text{g mL}^{-1}$ of CP-31398, all three cell lines underwent apoptosis. This might due to the reactive oxygen species induced by the toxicity of CP-31398 and induced p53-

independent apoptosis as others have previously reported (Wischhusen *et al.*, 2003). The EC_{50} (effective concentration 50%) of CP-31398 for MMRU and MeWo was determined to be $15 \mu\text{g mL}^{-1}$ and this concentration was used for subsequent experiments. At a moderate concentration, CP-31398 is only effective in wild-type p53 MMRU and single p53 mutant MeWo cells. These two cell lines showed a significant decrease in cell survival, while the Sk-mel-110 cell line which contains multiple p53 mutations had very little change in cell survival after CP-31398 treatment (Fig. 3.2). Programmed cell death was further confirmed in MMRU and MeWo, but not in Sk-mel-110 cells, by DNA laddering assay as apoptotic cells are characterized by DNA condensation and fragmentation (Fig. 3.3). Morphological changes can also be observed as membrane blebbing, nuclear condensation, DNA fragmentation, and prominent apoptotic bodies (Fig. 3.4).

These results triggered our interest in exploring the reasons behind the differential response to this new synthetic compound. Previous works have indicated that CP-31398 could stabilize both wild-type p53 and mutant p53 unanimously (Foster *et al.*, 1999; Hupp *et al.*, 2000; Rippin *et al.*, 2002). However, the ability of this compound to stabilize mutant p53 may depend on the location of p53 point mutation, which might account for the observed differential response in two mutant p53 melanoma cell lines that we used. We first confirmed the p53-stabilizing effect of CP-31398 by observing a gradual accumulation of overall p53 protein in all melanoma cell lines over a period of 16 h by Western blot analysis (Fig. 3.6). Immunofluorescent staining showed only MMRU and MeWo cells had an upregulation of p53 protein in active conformation after $15 \mu\text{g mL}^{-1}$ of CP-31398 treatment (Fig. 3.7). Although Sk-mel-110 cells also showed an increase in the

overall p53 protein level, p53 failed to retain an active conformation in this cell line as determined by immunofluorescent staining. In fact, it has been indicated that Sk-mel-110 carries a non-functional p53 gene product (Takimoto *et al.*, 2002); thus, our observation that the Sk-mel-110 cell line is resistant to CP-31398-induced apoptosis is consistent with the fact that it lacks a functional p53. It appears that both the location and the number of p53 mutations may determine the capability of CP-31398 to restore p53 active conformation and thus induce apoptosis. However, additional studies are required to further prove this notion.

As a transcriptional factor, p53 is capable to activate or repress its downstream genes in order to exert its biological and biochemical functions. One of the well-documented p53 downstream effectors is p21^{Waf1}, which is involved in cell cycle arrest (Yu *et al.*, 1998). Our result showed an enhanced p21^{Waf1} transcriptional activity in all three cell lines regardless of their sensitivity to CP-31398 (Fig 3.8), indicating that CP-31398 has the ability to enhance p21^{Waf1} transcription activity independent of p53 mutation status or sensitivity to CP-31398. Additionally, p21^{Waf1} transcription might be enhanced through a p53-independent pathway (DiGiuseppe *et al.*, 1995). The seemingly different results reported by Tanner and Barneris (2004) on the effect of CP-31398 for p21^{Waf1} promoter activity may simply due to luciferase assay they used, as in the control H1299 cells transfected with wild-type p53 the exposure of CP-31398 caused a reduction rather than an induction in the expression of the reporter gene. However, Foster and colleagues (1999) as well as this study using Western blotting and RT-PCR found that p21^{Waf1} expression is upregulated by CP-31398 regardless of the apoptotic response in H1299 lung carcinoma cells and melanoma cells, respectively.

In contrast, the apoptotic downstream transcriptional targets of p53 showed a correlation between CP-31398 and p53 mutation status. The delicate balance between pro-apoptotic targets (such as Bax and PUMA) and anti-apoptotic (such as Bcl-2 and Bcl-xL) factors seem to be the determinant if a cell undergoes apoptosis or not. One of these targets we looked at was the mitochondrial protein Bax. Studies have indicated that p53 induced apoptosis requires Bax to activate caspases-9 and -3 through mitochondrial cytochrome *c* release in human osteosarcoma Saos-2 cells (Narita *et al.*, 1998; Shimizu *et al.*, 1999). A number of studies have shown a relationship between p53 and yet another mitochondrial protein, PUMA, during apoptosis. PUMA promotes the translocation and multimerization of Bcl-2 family proteins, including Bax, in the mitochondria after binding to them. This is followed by a change in mitochondrial membrane potential, the release of cytochrome *c* from the mitochondria into the cytosol, and a cascade of caspase activation, resulting in programmed cell death (Nakano and Vousden, 2001; Yu *et al.*, 2003).

We noted an enhanced expression in both Bax and PUMA only in CP-31398 sensitive MMRU and MeWo, but not in CP-31398 resistant Sk-mel-110 cells (Fig. 3.8). This observation is consistent with the ability of CP-31398 to restore and stabilize p53 active conformation, activate transcription of other downstream targets, and induce apoptosis (Luu *et al.*, 2002). Sk-mel-110 cells on the other hand have consistently a relatively high background level of Bax and PUMA transcription indicating that other factors, which might due to a high level of anti-apoptotic proteins, contribute to its resistance to CP-31398 induced apoptosis.

We further demonstrated a series of apoptotic events from the alteration of mitochondrial membrane potential (Fig. 3.9), the release of cytochrome *c* from

mitochondria (Fig. 3.10), to the reduction in the levels of pro-caspases-9 and -3 (Fig. 3.11) in CP-31398 sensitive MMRU and MeWo cells. None of these changes were observed in CP-31398 resistant Sk-mel-110 cells. These findings are consistent with the previous studies showing that active p53 has the capacity to activate the Bax/mitochondrial/caspase-9 apoptosis pathway (Pearson *et al.*, 2000).

It is of interest to point out that the level of a p53 downstream scaffolding protein, Apaf-1, did not change in all three melanoma cell lines after CP-31398 treatment. This indicates that Apaf-1 was not involved in CP-31398 mediated apoptosis (Fig 3.11). Likewise, others have recently reported that activation of caspase-9 is independent of Apaf-1 expression in melanoma cells (Zanon *et al.*, 2004). In particular, Apaf-1 protein expression in Sk-mel-110 cells ruled out the possibility that resistance of this cell line to CP-31398 treatment was due to the loss of Apaf-1. Moreover, we showed that both Fas and Fas-ligand protein level did not change considerably in all three melanoma cell lines after CP-31398 regardless of their apoptotic responses (Fig. 3.12). This further confirms that the mitochondrial pathway instead of the Fas death receptor pathway is involved in CP-31398 induced apoptosis.

In conclusion, we demonstrated that after CP-31398 treatment, an increase in the stability of p53 protein in active conformation that correlates to apoptotic response. Overall, CP-31398 sensitive cell lines showed an increased transcription of Bax and PUMA, an altered mitochondrial membrane potential, a release of cytochrome *c* from the mitochondria into the cytosol, followed by the cleavage of pro-caspases-9 and -3. However, CP-31398 was not able to revert all mutant p53 proteins to wild-type conformation and induce apoptosis in all mutant p53 cell lines. Nonetheless, based on our

results, the ability for CP-31398 to induce apoptosis through the intrinsic mitochondrial pathway may well depend on the mutational status of p53. Mutant p53 reactivation by small molecules has an evident potential for the discovery of efficient and specific anti-cancer drugs. More studies are necessary to investigate *p53* mutation-dependent response to CP-31398 and explore the potential utilization of this compound as an anticancer agent.

CHAPTER 4. GENERAL CONCLUSIONS

4.1 Summary

p53 is a widely-studied tumour suppressor that has the ability to trigger cell-cycle arrest and apoptosis in response to diverse stress stimuli. Under cellular stress, such as hypoxia, DNA damage, ionizing radiation, exposure to anticancer drugs, and oncogene activation, the p53 protein is rapidly accumulated in a cell. This increase in p53 protein level can further transactivate its downstream target genes through specific DNA binding. Depending on the extent of DNA damage, p53 can either bring about cell cycle arrest in an effort to repair the damage, or induce apoptosis when the damage is too severe. Apoptosis, or programmed cell death, is a tightly regulated self-protective mechanism in order to eliminate unnecessary or severely damaged cells.

More than half of all human tumours contain p53 mutations and majority of the mutations are located in the DNA binding domain. The prevalent disruption of the specific DNA binding function of p53 by gene mutation suggests that this function is critical for p53-mediated tumour suppression. Many conventional chemotherapeutic agents exploit this p53-dependent apoptotic pathway through DNA damaging events to destroy cancerous cells. Conventional therapeutic agents amplify the stress signals, impelling damaged cells to self-destruct. Unfortunately, treatment fails when the cancer inactivates tumour-suppressor pathways. In most malignancies, mutation in *p53* leads to a shut down of the p53 tumour suppressor signaling pathway. Various strategies have been explored to restore mutant p53 to wild-type function. A class of small pharmacological peptide has been identified in a random screening. This class of small-molecule compounds can

promote the stable active conformation of wild-type p53, increase the activity of p53 and induce apoptosis in tumour cells. Among all, CP-31398 showed the strongest ability to stabilize the wild-type p53 protein conformation against thermal denaturation and has a remarkable capacity to restore mutant p53 to behave like wild-type p53 and induce apoptosis.

As metastatic melanoma responses poorly to traditional treatment methods, we sought to explore the possibility of using this new small molecule drug as a treatment for melanoma cells with various p53 mutation statuses. Noticing a differential response of melanoma cell lines to CP-31398 after initial screening, we hypothesized that the ability of CP-31398 to revert mutant p53 to wild-type p53 conformation may not be universal. Using three human melanoma cell lines, MMRU, which contains wild-type p53, and MeWo and Sk-mel-110, which carries mutant p53, we demonstrated that CP-31398 triggers apoptosis in some melanoma cell lines. Upon a moderate dose of CP-31398 treatment at EC_{50} ($15 \mu\text{g mL}^{-1}$), only MMRU (wild-type p53) and MeWo (single point mutation in the *p53* gene) cells exhibited apoptosis. With multiple p53 mutations, Sk-mel-110 cells did not respond at this moderate concentration. In spite of the fact that all three melanoma cell lines showed an increase in the overall p53 protein levels, only CP-31398 sensitive cell lines, MMRU and MeWo, exhibited a significantly detectable increase in active wild-type p53 conformation. Additionally, CP-31398 induced the expression of p53 downstream transcriptional targets including Bax and PUMA, altered the mitochondrial membrane potential, and enhanced the release of cytochrome *c* and activation of caspases-9 and -3 in CP-31398 sensitive cell lines, but not in multiple p53-mutation Sk-mel-110 cells.

4.2 Future Directions

With a steady increase in the incidence of melanoma, an effective treatment method is imminent. Although surgical removal of metastatic tumours may provide some benefit by easing pain, increasing the general quality of life, and lengthening survival, advanced melanoma that has spread to distant sites often cannot be cured.

In this thesis, we demonstrated that after CP-31398 treatment, the stability of p53 protein in active conformation increases which also correlates to apoptotic response. We showed that mutant p53 melanoma cell lines respond differently to CP-31398 induced apoptosis. Our results strongly suggest that CP-31398 may induce apoptosis in a p53-mutation specific manner. To test this hypothesis, isogenic cell line expressing either wild-type *p53* or a variety of mutant *p53* should be used. It would be beneficial, through site directed mutagenesis, to identify the key location in *p53* that is responsible for CP-31398 induced apoptosis.

Since p53 mutation is rare in melanoma (Hussein *et al.*, 2003) and we clearly showed that CP-31398 successfully induced apoptosis in a melanoma cell line expressing wild-type p53, MMRU, efforts should be made for this compound in clinical trials for treating melanoma patients. Previous study has established the low toxicity of CP-31398 in *in vivo* animal studies, which further favours the clinical trial (Foster *et al.*, 1999; Bykov *et al.*, 2003).

Besides skin cancer, CP-31398 may also be used for treating other type of cancers. For instance, our group and others have shown that this compound can induce apoptosis in HCT116 colon cancer cells (Luu *et al.*, 2002) and SW480 colon cancer cells (Takimoto *et al.*, 2002). In this thesis, we also showed that CP-31398 can induce apoptosis in Mewo

cell line, which contains a single point mutation. Other studies noted a similar observation in other cell lines that contain single point mutations (Foster *et al.*, 1999; Takimoto *et al.*, 2002). As over 50% of *p53* inactivation is due to single point mutation (Soussi, 2000), CP-31398 may hold potential to convert most mutant *p53* with single point mutation to wild-type conformation, and induce apoptosis. Mutant *p53* reactivation by small molecules has an evident potential for the discovery of effective and specific anti-cancer drugs. More studies are necessary to investigate *p53* mutation-dependent response to CP-31398 and explore the potential utilization of this compound as a potential anticancer therapeutic agent.

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