MOLECULAR MECHANISMS OF TUMOUR SUPPRESSION BY THE P53-STABILIZING COMPOUND CP-31398

by

Yvonne Luu

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

A thesis submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Medicine; Experimental Medicine Programme)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 2002

© Yvonne Luu, 2002
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Medicine

The University of British Columbia
Vancouver, Canada

Date November 5, 2002
ABSTRACT

The p53 tumour suppressor protein is considered the guardian of the genome and has a number of biological functions including cell cycle arrest, DNA repair, and apoptosis in response to various stresses, such as ultraviolet irradiation, serum deprivation, and exposure to anticancer drugs. The p53 gene is the most commonly mutated gene in human malignancies. Mutation in p53 leads to loss of tumour suppressive functions or gain of oncogenic functions, and ultimately cancer development. Many strategies are being developed to restore wild-type p53 function in cells with altered p53. CP-31398, a synthetic compound, was recently found to stabilize wild-type p53. Furthermore, CP-31398 rescued mutant p53 to enhance its transcriptional activity in vitro and in vivo and suppressed tumour growth in mice.

The objective of this study is to elucidate the molecular mechanisms mediated by CP-31398 under normal and stressed conditions. We first sought to determine the possible molecular mechanisms involved in CP-31398 suppression of tumour growth. Using two isogenic colon carcinoma cell lines with differing p53 status, we first confirmed the p53 stabilizing affect of CP-31398. We demonstrated that CP-31398 induced p53-dependent apoptosis and G2 arrest, as well as p53-independent G1 arrest. We found that CP-31398 upregulated transcription of the p53 downstream target gene, p21Waf1/Cip1. Furthermore, CP-31398 induced apoptosis through the mitochondrial/caspase-9 pathway. CP-31398 upregulated Bax and Bak mRNA and protein levels, altered mitochondrial membrane potential leading to the release of cytochrome c and activation of caspases-9 and -3.

Since ultraviolet-B irradiation in the sunlight is the primary environmental cause for malignant melanoma and p53 plays a crucial role in apoptosis, we then set out to determine if CP-31398 can enhance ultraviolet-B-induced apoptosis. We found that CP-31398 enhances ultraviolet-B-induced apoptosis in a wild-type p53 melanoma cell line, MMRU, by stabilizing
p53. The mitochondrial/caspase-9 pathway was found to be activated in CP-31398 enhancement of ultraviolet-B-induced apoptosis, via the upregulation of Bax, change in mitochondrial membrane potential, release of cytochrome c, and activation of caspase-9. Moreover, we found that CP-31398 enhanced cell death induced by ultraviolet-B in other wild-type (RPEP) and mutant p53 (PMWK) melanoma cell lines, but did not enhance cell death in the mutant melanoma cell line, SK-mel-110.

Melanoma is a chemoresistant cancer and studies have shown that p53 mutational status is a determinant for melanoma chemosensitivity. Since CP-31398 was shown to be able to rescue mutant p53 functions, we treated melanoma cells with CP-31398 together with the topoisomerase I inhibitor camptothecin which was shown to induce p53-dependent apoptosis, to determine if CP-31398 would enhance melanoma chemosensitivity. We found that CP-31398 did not enhance cell death induced by chemotherapeutic drugs in a wild-type (MMRU) and a mutant (SK-mel-110) p53 melanoma cell line. Pre-treatment of melanoma cells with CP-31398 also failed to enhance cell death induced by camptothecin. The inability of CP-31398 to enhance camptothecin-induced cell death could be explained by the fact that camptothecin-induced p53 accumulation is not enhanced after CP-31398 and camptothecin combination treatment. Furthermore, we demonstrated that CP-31398 did not enhance the sensitivity of MMRU cells to other chemotherapeutic drugs, such as vincristine and cisplatin.

In summary, we demonstrate that the p53-stabilizing compound CP-31398 is able to induce apoptosis via activating the mitochondrial/caspase-9 pathway. We hope that a better understanding of the molecular mechanisms of tumour suppression mediated by CP-31398 may lead to the establishment of this compound as a potential anticancer agent or the discovery of other similar synthetic compounds for cancer treatment.
# TABLE OF CONTENTS

Abstract ii

Table of Contents iv

List of Tables vii

List of Figures viii

Abbreviations x

Acknowledgements xi

CHAPTER 1 GENERAL INTRODUCTION 1

1.1 p53 Tumour Suppressor 1
   1.1.1 Gene and protein structure 1
   1.1.2 p53 regulation 3
   1.1.3 p53 function 5
   1.1.4 p53 mutation and cancer 10
   1.1.5 p53 and chemotherapy 11
1.2 p53 Stabilizing Compound, CP-31398 11
1.3 Melanoma 15
   1.3.1 Characteristics 15
   1.3.2 p53 and melanoma 16

CHAPTER 2 MATERIALS AND METHODS 18

2.1 Materials 18
   2.1.1 Cell lines and cell culture 18
   2.1.2 Reagents, enzymes, and chemicals 18
2.2 Methods 19
   2.2.1 Ultraviolet irradiation 19
   2.2.2 Cell survival assay 19
   2.2.3 DNA fragmentation assay 20
   2.2.4 Hoechst staining 20
   2.2.5 Flow cytometry 20
   2.2.6 Western blot analysis 21
   2.2.7 Immunofluorescent staining 22
   2.2.8 PARP detection 22
   2.2.9 Subcellular fractionation 23
   2.2.10 Mitochondrial transmembrane potential detection 23
   2.2.11 Reverse transcriptase (RT)-PCR 24
   2.2.12 Caspase inhibition assay 25
CHAPTER 3 CP-31398 INDUCES P53-DEPENDENT APOPTOSIS IN HUMAN COLON CARCINOMA CELL LINE HCT116

3.1 Rationale and Hypothesis 26
3.2 Results 27
3.2.1 CP-31398 induces p53-dependent apoptosis 27
3.2.2 CP-31398 induces cell cycle arrest 29
3.2.3 CP-31398 upregulates p53 protein and activates its transcriptional activity 32
3.2.4 CP-31398 alters mitochondrial transmembrane potential 37
3.2.5 CP-31398 induces cytochrome c release 38
3.2.6 CP-31398 activates caspases-9 and -3 38
3.3 Discussion 42
3.3.1 CP-31398 induces apoptosis by stabilizing p53 42
3.3.2 CP-31398 induces cell cycle arrest 43
3.3.3 CP-31398 enhances p53 transcriptional activity 44
3.3.4 CP-31398 induces the mitochondrial/caspase-9 pathway 44

CHAPTER 4 CP-31398 ENHANCES UVB-INDUCED APOPTOSIS OF MELANOMA CELL LINE MMRU 47

4.1 Rationale and Hypothesis 47
4.2 Results 48
4.2.1 CP-31398 upregulates p53 protein expression in melanoma cells 48
4.2.2 CP-31398 enhances UVB-induced apoptosis 48
4.2.3 CP-31398 upregulates p53 during UVB exposure 53
4.2.4 CP-31398 upregulates Bax and alters mitochondrial transmembrane potential 53
4.2.5 CP-31398 induces cytochrome c release after UVB exposure 55
4.2.6 CP-31398 activates caspases-9 and -3 55
4.2.7 CP-31398 reduces cell survival in other melanoma cell lines 59
4.3 Discussion 59

CHAPTER 5 CP-31398 DOES NOT ENHANCE CHEMOSENSITIVITY OF MELANOMA CELLS 63

5.1 Rationale and Hypothesis 63
5.2 Results 65
5.2.1 CP-31398 upregulates p53 protein expression in melanoma cells 65
5.2.2 CP-31398 does not enhance melanoma chemosensitivity 65
5.3 Discussion 71
5.3.1 CP-31398 does not have a role in chemosensitivity in human melanoma cell lines 71
CHAPTER 6 GENERAL CONCLUSIONS

6.1 Summary 75
6.2 Future Directions 76

REFERENCES 77
LIST OF TABLES

Table 1.1 Effect of CP-31398 on cell cycle distribution 31
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic representation of the p53 protein</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Chemical structure of p53-stabilizing compound, CP-31398</td>
<td>13</td>
</tr>
<tr>
<td>3.1</td>
<td>p53-dependent survival in HCT116 colon carcinoma cells after CP-31398 treatment</td>
<td>28</td>
</tr>
<tr>
<td>3.2</td>
<td>CP-31398 induced p53-dependent apoptosis is HCT116 cells</td>
<td>30</td>
</tr>
<tr>
<td>3.3</td>
<td>Effect of CP-31398 on the expression of p53, p21&lt;sup&gt;Waf1/Cip1&lt;/sup&gt;, and Bcl-2 family proteins</td>
<td>34</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of CP-31398 on Bax and Bak mRNA levels</td>
<td>36</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of CP-31398 on Bax and cytochrome c localization</td>
<td>39</td>
</tr>
<tr>
<td>3.6</td>
<td>CP-31398 alters the mitochondrial membrane potential</td>
<td>40</td>
</tr>
<tr>
<td>3.7</td>
<td>CP-31398 induces activation of caspases-9 and -3 and cleavage of PARP</td>
<td>41</td>
</tr>
<tr>
<td>4.1</td>
<td>Induction of p53 and survival of MMRU human melanoma cells after CP-31398 treatment</td>
<td>49</td>
</tr>
<tr>
<td>4.2</td>
<td>CP-31398 reduces cell survival after UVB exposure in MMRU cells</td>
<td>51</td>
</tr>
<tr>
<td>4.3</td>
<td>CP-31398 enhances UVB-induced apoptosis in MMRU cells</td>
<td>52</td>
</tr>
<tr>
<td>4.4</td>
<td>Induction of p53 in MMRU cells after UVB irradiation and CP-31398 treatment</td>
<td>54</td>
</tr>
<tr>
<td>4.5</td>
<td>CP-31398 increases Bax expression, alters the mitochondrial membrane potential, and induces cytochrome c release after UVB irradiation</td>
<td>56</td>
</tr>
<tr>
<td>4.6</td>
<td>CP-31398 induces activation of caspases-9 and -3 during UVB-induced apoptosis</td>
<td>58</td>
</tr>
<tr>
<td>4.7</td>
<td>CP-31398 upregulates p53 protein levels and reduces cell survival after UVB exposure in melanoma cells</td>
<td>60</td>
</tr>
<tr>
<td>5.1</td>
<td>Survival of SK-mel-110 human melanoma cells after CP-31398 treatment</td>
<td>66</td>
</tr>
<tr>
<td>5.2</td>
<td>Survival of MMRU and SK-mel-110 cells after CP-31398 and camptothecin (CPT) treatment</td>
<td>67</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>Induction of p53 in MMRU and SK-mel-110 cells after CP-31398 treatment</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>Survival of MMRU cells after pre-treatment with CP-31398, followed by treatment with CPT</td>
<td></td>
</tr>
<tr>
<td>Figure 5.5</td>
<td>Survival of SK-mel-110 cells after pre-treatment with CP-31398, followed by treatment with CPT</td>
<td></td>
</tr>
<tr>
<td>Figure 5.6</td>
<td>p53 protein levels in MMRU and SK-mel-110 cells treated with CP-31398 and/or CPT</td>
<td></td>
</tr>
<tr>
<td>Figure 5.7</td>
<td>Survival of MMRU cells after pre-treatment with CP-31398, followed by treatment with other chemotherapeutic drugs</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>Ara-c</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CIS</td>
<td>cisplatin</td>
</tr>
<tr>
<td>CM</td>
<td>cutaneous melanoma</td>
</tr>
<tr>
<td>CPT</td>
<td>camptothecin</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death Effector Domain</td>
</tr>
<tr>
<td>DISC</td>
<td>Death Inducing Signalling Complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated Death Domain</td>
</tr>
<tr>
<td>GADD45</td>
<td>growth arrest and DNA damage45</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>LFS</td>
<td>Li-Fraumeni syndrome</td>
</tr>
<tr>
<td>Mdm2</td>
<td>mouse double minute2</td>
</tr>
<tr>
<td>MM</td>
<td>malignant melanoma</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PIGs</td>
<td>p53-inducible genes</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RB</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SRB</td>
<td>sulforhodamine B</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetate</td>
</tr>
<tr>
<td>TNFR1</td>
<td>tumour necrosis factor receptor type 1</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VIN</td>
<td>vincristine</td>
</tr>
<tr>
<td>Z-VAD-FMK</td>
<td>benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Gang Li, for his intellectual stimulation and financial support. I would also like to thank my family, friends and members of the lab for their encouragement and support. To Drs. William Jia and Bill Salh, your suggestions and feedback are greatly appreciated.
CHAPTER 1. GENERAL INTRODUCTION

1.1 p53 Tumour Suppressor

1.1.1 Gene and protein structure

The p53 gene maps to chromosome 17p13.1 in humans, spans 20 kilobases and contains 11 exons. The coding region consists of exons 2-11, which encodes for a 393-amino acid, 53 kDa nuclear phosphoprotein. p53 protein is a sequence-specific transcription factor that activates and represses the transcription of a large and ever growing number of genes (Fields and Jang, 1990; Farmer et al., 1992; El-Deiry, 1998). The protein could be divided structurally and functionally into different domains (Figure 1.1). The transcriptional activation 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 domain is found following the transactivation domain, between residues 61 to 94. The DNA binding domain (DBD) of p53 consists of amino acid 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 the 10-bp motif, 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0-13 bp (El-Deiry et al., 1992). At the C-terminus, amino acid residues 324-355 make up the tetramerization domain, which is required for oligimerization and DNA binding. The tetramerization domain is used to form the tetrameric form of the protein consisting of a dimer with two β sheets and two α helices (Iwabuchi et al., 1993; Wang et al., 1994). Residues 363 to 393 consist of basic amino acids that make up a negative regulatory region at the C-terminus.
**Figure 1.1** Schematic representation of the p53 protein. TA: transactivation domain, P: proline-rich domain, DBD: DNA binding domain, TD: tetramerization domain, R: regulatory domain.
1.1.2 p53 regulation

In almost all cell types under normal conditions, p53 protein is constitutively expressed, but its concentration is low due to rapid turnover that results in a relatively short half-life of less than 30 minutes. In addition, p53 appears to be in a latent form that does not bind DNA well and cannot transactivate genes. The Mdm2 (mouse double minute2) oncoprotein, a downstream target of p53 (Barak et al., 1993; Wu et al., 1993), inhibits the p53 transactivation function by binding to the transactivation domain (Momand et al., 1992; Oilier et al., 1993; Chen et al., 1995b). Furthermore, Mdm2 mediates rapid turnover of p53 by shuttling p53 from the nucleus to the cytoplasm (Tao and Levine, 1999a; Freedman and Levine, 1998) to target it for degradation via the ubiquitin-26S proteosome (Haupt et al., 1997a; Kubbutat et al., 1997; Honda et al., 1997; Fuchs et al., 1998). Thus, a negative feedback loop results, whereby increased levels of p53 lead to increased expression of Mdm2, which targets p53 for degradation.

Another protein involved in the regulation of p53 via Mdm2 is the tumour suppressor p19ARF (p14ARF in humans), which is encoded by an alternative reading frame in the CDKN2A/INK4a-ARF locus. p19ARF binds Mdm2 and directly inhibits Mdm2 activity without preventing the Mdm2-p53 interaction. p19ARF 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). Moreover, p19ARF relocates Mdm2 to the nucleolus to prevent nuclear export of p53 (Tao and Levine, 1999b; Weber et al., 1999) and promotes Mdm2 degradation (Zhang et al., 1998). p19ARF is negatively regulated by p53 (Stott et al., 1998; Robertson and Jones, 1998).

p53 is known to be a crucial protein in cellular stress response. In response to physical or chemical DNA damaging stimuli such as ionizing irradiation, ultraviolet (UV) light, ribonucleotide depletion, anticancer drugs, and hypoxia, p53 protein is stabilized and its protein
level and transcriptional activity in cells is rapidly increased (Maltzman and Czyzyk, 1984; Haapajarvi et al., 1997; Nelson and Kastan, 1994; Linke et al., 1996). The p53 protein is rapidly accumulated through post-translational covalent and no-covalent modifications that reduce its turnover by preventing Mdm2 binding, and thus increasing its half-life and stability (Shieh et al., 1997; Unger et al. 1999; Ashcroft and Vousden, 1999). Covalent modifications, such as phosphorylation (by PI3K, ATM, ATR, Chk1, Chk2, DNA-PK, JNK, CK1, CAK, CKII), acetylation (by p300 and CBP) and sumolation, occur both at the N- and C-terminal domains of the protein (Meek, 1999; Jayaraman and Prives, 1999). For instance, the serine/threonine kinases Chk1 and Chk2 have been shown to phosphorylate serine 20 at the N-terminus of p53, in response to UV light and ionizing radiation, to prevent Mdm2-p53 interaction, thus leading to p53 stabilization (Unger et al., 1999; Hirao et al., 2000; Chehab et al., 2000; Shieh et al., 2000). Phosphorylation of p53 at the N-terminus on serines 15 (ATM and DNA-PK), 20, 33 (JNK1) and 37 (DNA-PK) and at the C-terminus on serines 392 (CKII), 315 (CDKs), and 371, 376 and 378 (PKC) increases p53 sequence-specific DNA binding, and consequently enhances the transactivation of its target genes (Jayaraman and Prives, 1999; Meek, 1999). Other modifications include addition or removal of glycosyl, ribose or ubiquitin. p53 protein in some systems requires a structural change, regulated by the basic C-terminal domain, to activate it for sequence-specific DNA binding. Post-translational modification of the C-terminal tail also alters the conformation of p53 and promotes p53 binding to DNA (Hupp et al., 1992; Jayaraman and Prives, 1995; Gu and Roeder, 1997; Sakaguchi et al., 1998). For example, upon DNA damage, serine 376 is dephosphorylated, creating a consensus binding site for 14-3-3 proteins, binding of which increases the DNA-binding activity of p53 (Waterman et al., 1998). DNA damage also leads to the acetylation of several lysine residues at the C-terminus of p53 to enhance sequence-specific DNA binding (Sakaguchi et al., 1998). N-terminal phosphorylation of p53 enhances its
binding to p300 (Lambert et al., 1998), which acetylates p53 on lysine 382 (Sakaguchi et al., 1998). Furthermore, activated p53 may also be retained in the nucleus to enhance its transcriptional activity (Fritsche et al., 1993; Zhang and Xiong, 2001).

1.1.3 p53 function

The p53 protein is a tumour suppressor involved in many cellular processes. Activated p53 functions as a transcription factor to regulate a vast number of downstream target genes involved in cell cycle arrest, DNA repair and apoptosis (El-Deiry, 1998; Zhao et al., 2000; Kannan et al., 2001). p53 also has a role in differentiation and development (Almog and Rotter, 1997), cellular senescence (Lundberg et al., 2000; Itahana et al., 2001), DNA replication, and maintenance of genomic stability (Liang et al., 2000; Fukasawa et al., 1996; Livingstone et al., 1992; Yin et al., 1992). Furthermore, p53 regulates genes involved in cytoskeletal functions and angiogenesis (Zhao et al., 2000; Dameron et al., 1994; Nishimori et al., 1997).

The cell cycle arrest function of p53 depends on its ability to function as a sequence-specific transcription factor (Crook et al., 1994; Pietenpol et al., 1994). p53 can induce both G1 and G2/M arrest through upregulation of p21^{Waf1/Cip1}, a cyclin-dependent kinase (CDK) inhibitor (El-Deiry et al., 1993, 1994; Harper et al., 1993; Xiong et al., 1993; Agarwal et al., 1995; Bunz et al., 1998). p53 also regulates the G2/M checkpoint of the cell cycle (Stewart et al., 1995; Agarwal et al., 1995), via induction of the 14-3-3\sigma, GADD45 (growth arrest and DNA damage45) and cyclin G genes (Hermeking et al., 1997; Wang et al., 1999; Shimizu et al., 1998). Induction of p53-dependent cell cycle arrest after DNA damage is required to allow time for DNA repair before replication or mitosis.

p21^{Waf1/Cip1} 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;
Nakanishi et al., 1997; Fotedar et al., 1996; Bates et al., 1998; Dulic et al., 1998; Niculescu et al., 1998; Sherr and Roberts, 1995). G1 CDKs are involved in the phosphorylation regulation of the retinoblastoma (Rb) tumour suppressor protein, which when unphosphorylated, binds to E2F transcription factors to inhibit the activation of E2F-responsive targets 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 transactive genes required for progression through the cell cycle (Sherr and Roberts, 1995). Recently, p21\textsuperscript{Waf1/Cip1} has been shown to directly bind to E2F subunits and inactivate the transcriptional activity of E2F, independently of CDK regulation of Rb-E2F interaction (Delavaine and La Thangue, 1999).

The C-terminal region of p21\textsuperscript{Waf1/Cip1} has been shown to bind to proliferating cell nuclear antigen (PCNA) (Chen et al., 1995a; Waga et al., 1994), an accessory factor for DNA polymerase activity in DNA replication and repair. p21\textsuperscript{Waf1/Cip1} binding to PCNA prevents PCNA-dependent DNA replication, but allows PCNA-dependent excision repair (Li et al., 1994; Shivji et al., 1994; Waga et al., 1994; Flores-Rozas et al., 1994). Thus, p53 upregulation of p21\textsuperscript{Waf1/Cip1} following DNA damage may have a role in DNA repair.

p53 is involved in both nucleotide excision repair which repairs UV-induced DNA damage such as pyrimidine dimers, and base excision repair which removes bases damaged by 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 genes that participate in DNA repair, such as GADD45 and p53R2, a ribonucleotide reductase gene (Smith et al., 1994; Nakano et al., 2000; Tanaka et al., 2000). GADD45 binds to PCNA and stimulates DNA excision repair (Smith et al., 1994). 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/deletion mismatches. p53 also associates with components of the repair machinery,
such as XPB/ERCC3, XPD/ERCC2 and p62, which are all part of the TFIIH protein complex, CSB, replication protein A, which is a single-stranded DNA binding protein, and Ref-1 (Wang et al., 1995; Albrechtsen et al., 1999).

p53 also participates in the regulation of apoptosis or programmed cell death, a type of cell death that involves highly regulated and conserved molecular pathways that are triggered by a variety of physiological signals. Apoptosis occurs under normal conditions during embryonic development, metamorphosis, differentiation, and general cell turnover. There are two well characterized apoptotic pathways, namely 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 (Lowe et al., 1993; Lotem and Sachs, 1993). p53-dependent apoptosis has also been observed in vivo (Symonds et al., 1994, Ziegler et al., 1994; Tron et al., 1998). The role of p53 in apoptosis involves both its transactivation function (Chao et al., 2000; Jimenez et al., 2000) and transactivation-independent mechanisms (Haupt et al., 1995, 1997b; Chen et al., 1996).

p53 has been shown to take part in the mitochondrial-mediated apoptotic pathway (Shen and White, 2001). Studies have shown that p53 regulates members of the Bcl-2 family, such as Bax and Bcl-2, pro- and anti-apoptotic proteins, respectively (Miyashita et al., 1994a,b; Miyashita and Reed, 1995; Green and Reed, 1998), which can undergo both homodimerization and heterodimerization. An increase in the ratio of Bax:Bcl-2 causes changes in the membrane potential of the mitochondria; consequently, cytochrome c and other polypeptides are released from the intermembrane space of the mitochondria into the cytoplasm (Liu et al., 1996; Rosse et al., 1998; Shimizu et al., 1999; Jurgensmeier et al., 1998; Pastorino et al., 1998; Oltvai et al., 1993). Once released, cytochrome c binds to the cytoplasmic scaffolding protein Apaf-1 (apoptotic protease activating factor-1), causing an ATP or dATP-dependent conformational
change that allows Apaf-1 to bind to the pro-domain of procaspase-9 to form the apoptosome and activate procaspase-9 by self-cleavage (Li P et al., 1997; Jiang and Wang, 2000; Saleh et al., 1999; Zou et al., 1999; Stennicke et al., 1999; Rodriguez and Lazebnik, 1999). Caspase-9 is an initiator caspase that is part of a family of cysteine-dependent aspartate-directed proteases that are synthesized as inactive zymogens and activated by proteolysis (Earnshaw et al., 1999). The active caspase-9 then initiates a cascade of proteolytic activation of other effector or downstream caspases, first by cleaving procaspase-3 and -7; activated caspase-3 then cleaves procaspase-6. Cleavage of a number of cellular substrates including structural proteins, such as lamin B and actin, signalling proteins, and regulators of DNA replication or transcription by activated caspases represent the final events in the chain reaction of apoptosis (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 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), Noxa (Oda E et al., 2000), and Apaf-1 (Moroni et al., 2001; Rozenfeld-Granot et al., 2002). PUMA has a BH3 or bcl-2 homology domain that mediates its interaction with bcl-2 family members, is localized to the mitochondria, and induces cytochrome c release and apoptosis (Nakano and Vousden, 2001; Yu et al., 2001). Noxa, a newly identified BH3-only member of Bcl-2 family, functions at the mitochondria and has been implicated in p53-mediated apoptosis (Oda E et al., 2000). Another protein regulated by p53 is p53AIP1, which localizes to the mitochondria and affects the mitochondrial membrane potential, leading to cell death (Oda K et al., 2000). A recently discovered p53 target gene, Peg3 or Pwl, has been shown to trigger Bax protein translocation from the cytoplasm to the mitochondria (Deng and
Wu, 2000). 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. Recently, a study showed that p53 itself localizes to the mitochondria prior to changes in mitochondrial membrane potential, cytochrome c release and procaspase-3 activation during p53-dependent apoptosis (Marchenko et al., 2000).

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 (FasL), 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 procaspase-3, -6 and -7, and apoptosis (Hirata et al., 1998). The Fas pathway has been shown to be involved in p53-dependent apoptosis in some systems and in response to various stimuli (Owen-Schaub et al., 1995; Fukazawa et al., 1999; Muller et al., 1998a,b; Muller et al., 1997; Hara et al., 2000; Bennett et al., 1998; Embree-Ku et al., 2002; Lorenzo et al., 2002). However, p53 also induces apoptosis independently of the Fas pathway (Fuchs et al., 1997; Reinke and Lozano 1997b). p53-dependent apoptosis can 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, 1999). Pidd, a death domain-containing protein capable of inducing apoptosis, has been shown to be a direct target of p53 (Lin et al., 2000).
1.1.4 p53 mutation and cancer

Current evidence suggests that the loss of normal p53 function is associated with cell transformation \textit{in vitro} (Eliyahu \textit{et al.}, 1989; Finlay \textit{et al.}, 1989; Baker \textit{et al.}, 1990) and development of neoplasms \textit{in vivo} (Donehower \textit{et al.}, 1992; Symonds \textit{et al.}, 1994). In human studies, \textit{p53} has been shown to be one of the most frequently mutated genes in sporadic cancer (Hollstein \textit{et al.}, 1991, 1996). More than 50\% of human malignancies of epithelial, mesenchymal, haematopoietic, lymphoid, and nervous origin analyzed to date were shown to contain an altered \textit{p53} gene (Soussi, 2000; Hollstein \textit{et al.}, 1996). Germline mutations in the \textit{p53} gene in humans result in the Li-Fraumeni syndrome (LFS), a hereditary cancer susceptibility syndrome leading to a predisposition to sarcomas, lymphomas, breast, brain and other tumours at a young age (Malkin \textit{et al}, 1990; Srivastava \textit{et al}, 1990). \textit{p53} homozygous and hemizygous knockout mice have a near normal development, but with a number of genetic alterations, such as gene amplification and abnormal chromosome numbers, and are prone to the development of multiple tumours at an early age (Donehower \textit{et al.}, 1992; Fukasawa \textit{et al.}, 1997). Taken together, inactivation of the \textit{p53} pathway seems to be a common mechanism in the development of cancer.

Most mutations in the \textit{p53} gene are missense that produce a stable, but non-functional protein. Some mutations lead to a gain of oncogenic function or dominant negative phenotype over wild-type \textit{p53} through heterotetramerization (Dittmer \textit{et al.}, 1993; Hachiya \textit{et al.}, 1994; Brachmann \textit{et al.}, 1996; de Vries \textit{et al.}, 2002). Mutant \textit{p53} protein folds into conformations that are not recognized by PAb1620 (Milner and Medcalf, 1990), a conformation-specific monoclonal antibody that recognizes the wild-type \textit{p53} conformation. Most \textit{p53} mutations are found within the DBD (Hollestein \textit{et al.}, 1996; Cho \textit{et al.}, 1994), resulting in a protein that cannot bind DNA and is impaired in its transactivation function. This suggests that \textit{p53} DNA binding and
transcriptional activation are important in the response to DNA damage and to tumour suppression by p53 (Pietenpol et al., 1994). In cancers with unaltered p53 genes, p53 is inactivated indirectly through binding to viral proteins, or the gene products upstream or downstream of p53 may be altered.

1.1.5 p53 and chemotherapy

Different forms of chemotherapy have a number of biochemical targets (e.g. thymidylate synthase inhibitors, microtubule damage, topoisomerase inhibitors, DNA crosslinking agents), but most are DNA-damaging agents and generally kill cancer cells by inducing apoptosis (Kaufmann and Earnshaw, 2000; Schmitt and Lowe, 1999; Barry et al., 1990) and through a p53-dependent mechanism (Li et al., 1998a; Lowe et al., 1993; Clarke et al., 1993; Hayakawa et al., 2000). Cells with p53 mutation have been shown to be more resistant to chemotherapy (Lowe et al., 1994; Fan et al., 1994; Righetti et al., 1996; Nabeya et al., 1995). In response to DNA damage induced by cytotoxic drugs, p53 protein levels and functional activity increase (Kastan et al., 1991; Chernov and Stark, 1997; Tishler et al., 1993). Activated p53 then induces cell cycle arrest or apoptosis (Kastan et al., 1991; Clarke et al., 1993).

1.2 p53 Stabilizing Compound, CP-31398

Since p53 protein has a very short half-life, compounds that stabilize the p53 protein conformation may help prolong its anti-tumour effects. In a recent study, Foster and colleagues (1999) screened more than 100,000 synthetic compounds for their ability to promote the stability of wild-type p53 DBD as well as that of full-length p53. Among this class of compounds, CP-31398 was found to stabilize the active DBD conformation of wild-type and mutant p53 protein upon heating, using the monoclonal antibody, mAb1620, which recognizes stabilized, non-
denatured, wild-type DBD. In addition, CP-31398 was shown to stabilize the active conformation of 175A mutant p53 transfected into p53-null H1299 lung carcinoma cells (Foster et al., 1999). The authors hypothesized that CP-31398 stabilizes the active conformation of the DBD of newly synthesized mutant p53, as the total level of p53 did not change after CP-31398 treatment and others have previously shown that small fractions of mutant p53 in cells retain the active conformation (Pavletich et al., 1993; Chen et al., 1993). Furthermore, intraperitoneal injection of CP-31398 into mice with subcutaneous tumours derived from H1299 transfectants resulted in increased levels of mutant p53 with active conformation. When H1299 cells were co-transfected with a p53-inducible luciferase reporter gene and a mutant p53 gene, CP-31398 increased the reporter gene expression by ~10-fold. CP-31398 also restored mutant p53 function in vivo by increasing reporter gene expression in tumours. p21<sup>Waf1/Cip1</sup>, a downstream effector of p53, was also shown to be upregulated by the mutant p53-transfected cells upon treatment with CP-31398 (Foster et al., 1999). Furthermore, twice daily injections of CP-31398 reduced tumour growth by at least 75% in mice with tumour xenografts containing mutant p53 (A375.S2 melanoma and DLD-1 colon carcinoma cell lines) (Foster et al., 1999). The data from this study provides solid evidence that CP-31398 exhibits tumour suppressive effects.

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 may be used to orient these two groups on the p53 protein and to enhance the stability of the active conformation.

At the start of our research, there were no other publications on CP-31398. Studies published recently on CP-31398 show some conflicting results. Rippin and colleagues (2002)
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 linker of a specific length.
recently showed that CP-31398 did not interact with wild-type or mutant R249S p53 core domain
in vitro and did not stabilize p53, contradictory to the results shown by Foster et al. (1999).
Rippin et al. (2002) found that CP-31398 intercalated with DNA, altered and destabilized the
DNA-p53 core domain complex and decreased sequence-specific DNA binding of wild-type and
mutant (His-273) p53. Furthermore, CP-31398 was toxic to several cell lines independently of
mutant p53. CP-31398 caused a small p53-dependent increase in Mdm2 expression and a larger
increase in Bax expression independently of p53. However, CP-31398 did stabilize the active
conformation of cells expressing His-175 mutant p53, which was inhibited by cycloheximide
suggesting that CP-31398 was stabilizing newly synthesized mutant p53 (Rippin et al., 2002).

In support of the initial study, Takimoto et al. (2002) found that CP-31398 restored the
DNA binding activity of mutant p53. CP-31398 upregulated and stabilized p53 protein levels in
wild-type p53 expressing cell lines. CP-31398 induced apoptosis in six out of nine cell lines,
while two underwent G1 cell cycle arrest. However, p53-null Saos2 cells were also sensitive to
killing by CP-31398, suggesting that CP-31398 induced p53-dependent and p53-independent cell
cycle arrest and apoptosis. p53 target genes, p21\textsuperscript{Waf1/Cip1} and KILLER/DR5, were upregulated by
CP-31398 in a p53-dependent and p53-independent manner. Interestingly, CP-31398 suppressed
the expression of Bax in almost all the cell lines, but when HCT116 Bax-/- cells were treated
with CP-31398, they underwent apoptosis with a delayed kinetics compared to the parental
HCT116 cells. Furthermore, CP-31398 upregulated non-p53 target genes and enhanced drug-
and TRAIL-mediated killing (Takimoto et al., 2002).

found that CP-31398 inhibited Mdm2-mediated ubiquitination of p53, but did not prevent the
physical association between Mdm2 and p53 in vivo. However, CP-31398 did not cause
phosphorylation of p53 at serine 20, which is often seen after treatment with DNA damaging
agents such as adriamycin. All the above studies show that CP-31398 has a number of biological functions, some of which do not involve p53.

1.3 Melanoma

1.3.1 Characteristics

The incidence and mortality rate of human malignant melanoma (MM) has been increasing steadily during the last few decades. In 1960, the probability of an American developing melanoma during his/her lifetime was expected to be one in 1500. In 2000, the probability increased to about one in 70 (Rigel et al., 1996). Malignant melanoma rapidly metastasizes to other organs such as lung, liver, brain, bone and small intestine and is highly resistant to radiation and chemotherapy (Schadendorf et al., 1994; Serrone and Hersey, 1999; Garbe, 1993; Helmbach et al., 2001); thus, patients with advanced MM have a very poor prognosis even with the use of common anticancer drugs. The chemoresistance characteristic of MM maybe intrinsic or develops as a result of use of cytostatic drugs (Garbe, 1993). The molecular mechanisms involved in melanoma and chemoresistance have not been identified, but it is assumed that inhibition or dysregulation of apoptosis leads to chemoresistance (Serrone and Hersey 1999).

Melanoma develops as the result of uncontrolled proliferation of transformed pigment-producing melanocytes. Melanoma occurs primarily in the skin, but also in other tissues that contain pigment cells. The major environmental factor for the development of cutaneous melanoma (CM) is UV irradiation (Romerdahl et al., 1988; Atillasoy et al., 1998), which causes DNA damage primarily in the forms of cyclobutane pyrimidine dimers and (6-4) photoproducts (Alcalay et al., 1990). The development of melanoma is related to intermittent over-exposure to UV causing sunburns and sun exposure in childhood, rather than the total accumulated UV dose
in a life-time (Holman and Armstrong, 1984). It is estimated that more than 80% of CM cases in Australia are due to sun exposure (Armstrong and Kricker, 1993). Hereditary CM is caused by germline mutations in CDKN2A (p16\textsuperscript{INK4a}) (Hussussian et al., 1994; Walker et al., 1995) or in the G1 cyclin-dependent kinase (CDK)-4, a target molecule inhibited by p16\textsuperscript{INK4a} (Zuo et al., 1996). The degree of sunlight exposure is important in both familial as well as non-familial disease as the penetrance of the CDKN2A mutation in familial CM is influenced by the degree of exposure to sunlight (Cannon-Albright et al., 1994).

1.3.2 p53 and melanoma

Resistance to chemotherapy is suggested to be associated with an inability to induce p53-dependent apoptosis as many chemotherapeutic agents have been shown to induce the p53 response. Mutations of the p53 gene are associated with poor prognosis, de novo or acquired resistance and relapse in many solid and haematopoitic malignancies (Lowe et al., 1994; Fan et al., 1994; Righetti et al., 1996; Nabeya et al., 1995), suggesting that the chemoresistant phenotype of melanoma may be due to mutations in the p53 gene. We have previously shown that melanoma cell lines with wild-type p53 are more sensitive to chemotherapy-induced apoptosis than mutant p53 cell lines, suggesting that chemotherapy-induced apoptosis in melanoma cells is p53-dependent (Li et al., 1998a, 2000). p53 mutations, however, are very rare in melanoma in contrast to all other types of skin cancer, with a frequency of 20-30% in metastatic melanoma and cultured melanoma cell lines (Hartmann et al., 1996; Albino et al., 1994; Sparrow et al., 1995). However, many groups only analyze exons 5 to 8 for point mutations, giving rise to a potential for an underestimation of the mutation frequency in melanoma. In addition, melanoma cells typically express high levels of wild-type p53 protein
(Weiss et al., 1993, 1995; Albino et al., 1994; Montano et al., 1994; Lassam et al., 1993; McGregor et al., 1993; Sparrow et al., 1995; Hartmann et al., 1996).

The current belief is that the p53 response to DNA damage in melanoma cells may be compromised due to abrogation of the pathways leading to the activation of p53 (Satyamoorthy et al., 2000; Rizos et al., 2001a,b) or the transactivation and tumour suppressive functions of p53 may be impaired (Bae et al., 1996; Parmar et al., 2000; Soengas et al., 2001). For instance, Satyamoorthy et al. (2000) showed that wild-type p53 in some human melanoma cell lines are not activated to induce cycle arrest or apoptosis in response to DNA damage, perhaps due to the absence of dephosphorylation of serine 376 in the C-terminus of p53, which normally increases the functional activity of p53 following DNA damage. Mutations that affect ARF can affect activation of p53, thus not requiring the mutational loss of p53 itself. In melanoma kindreds with INK4a/ARF mutations functionally impairing ARF, activation of the p53 pathway is affected (Rizos et al., 2001a,b). In a mouse model for melanoma generated in INK4a/ARF-deficient background (Chin et al., 1997), p53 mutations are absent. Bae et al., 1996 showed that many wild-type p53 melanoma cell lines were unable to induce GADD45 after exposure to ionizing radiation. Parmar et al. (2002) found that melanoma cell lines with wild-type p53 had reduced efficiency in inducing cell cycle arrest following irradiation. Apaf-1, a downstream target of p53, is often lost in metastatic melanoma, leading to an inability to activate p53-dependent apoptosis (Soengas et al., 2001).
CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines and cell culture

Human colon carcinoma cells, HCT116+/- and HCT116-/- (a kind gift of Dr. B. Vogelstein), were maintained in McCoy 5A medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, ON), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Four melanoma cell lines with known p53 status were used: The MMRU, RPEP and PMWK cell lines were kind gifts from Dr. H.R. Byers (Boston University School of Medicine, Boston, MA) (Byers et al., 1991); the SK-mel-110 cell line was a kind gift of Dr. A.P. Albino (Memorial Sloan-Kettering Cancer Center, New York, NY). The p53 status in the melanoma cell lines has been determined by DNA sequencing: MMRU and RPEP contain wild-type p53 (Li et al., 1995b), while PMWK and SK-mel-110 contain mutant p53 (Li et al., 1995b; Bae et al., 1996). Melanoma cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

2.1.2 Reagents, enzymes, and chemicals

Proteinase K and agarose were obtained from Invitrogen (Mississauga, ON). Chemicals such as camptothecin (CPT), vincristine (VIN) and cisplatin (CIS) were obtained from Sigma-Aldrich (Mississauga, ON). Stock solutions were prepared in dimethysulfoxide (DMSO) for CPT, methanol for VIN, ethanol for CIS, and all were stored at -20°C. The CP-31398 compound
(Pfizer, Groton, CT) was dissolved in deionized distilled water (ddH₂O) and stored at 4°C. All other chemicals were purchased from Sigma-Aldrich.

2.2 Methods

2.2.1 Ultraviolet irradiation
Medium was removed and the cells (at 80% confluency) were exposed to UVB (290-320 nm) using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ). The petri-dish cover was left on to filter possible UVC emissions from the UVB bulb. Medium was replaced and cells were incubated in a 5% CO₂ incubator at 37°C for 24 h after UVB irradiation.

2.2.2 Cell survival assay
Cells were plated into 24-well plates at 4x10⁵/well, grown for 24 h and treated at 80% confluency. Cell survival was determined with the sulforhodamine B (SRB) (Sigma-Aldrich) assay as described previously (Skehan et al., 1990; Li et al., 1998). SRB is a pink aminoxanthene dye with two sulfonic groups that binds to basic amino acid residues in proteins in cells fixed with trichloroacetate (TCA). SRB provides a sensitive index of cellular protein content. Briefly, after treatment, the medium was removed and the cells were fixed with 300 µl of 10% TCA for 1 h at 4°C. The cells were then washed five times with tap water and the excess water removed by flicking. The cells were air-dried and then stained with 300 µl of 0.4% SRB (w/v in 1% acetic acid) for 30 min at room temperature, washed four times with 1% acetic acid, and air-dried. The cells were then incubated with 500 µl of 10 mM Tris (pH 10.5) on a shaker for 20 min to solubilize the bound dye. Spectrophotometric readings were then taken at 550 nm for 100 µl aliquots.
2.2.3 DNA fragmentation assay

Cells were grown in 60-mm plates and treated at 80% confluency. After treatment, detached and attached cells were collected and pelleted by centrifugation. The cells were then lysed by incubating with 500 μl of lysis buffer containing 100 mM NaCl, 20 mM EDTA, 50 mM Tris-Cl (pH 8.0), 0.5% SDS, and 1 mg/ml proteinase K at 54°C with gentle agitation for 2 h or until all solid material was digested. DNA was extracted with phenol:chloroform and ethanol precipitated. DNA was resuspended in 20 μl of distilled H2O containing 50 μg/ml RNase A, incubated for 30 min at 37°C, and analyzed on a 2% agarose gel for DNA fragmentation. The gels were stained with 0.5 μg/ml ethidium bromide (EtBr) and photographed under UV light with a Gel Print System (Biophotonics, Ann Arbor, MI).

2.2.4 Hoechst staining

Cells were grown on coverslips in 35-mm plates and treated at 80% confluency. After treatment, cells were washed three times with 1 ml of phosphate buffered saline (PBS), fixed with 1.5 ml of 1:1 acetone:methanol for 10 min at -20°C and allowed to air dry. Cells were then stained with 1.5 ml of 10 μM of Hoechst 33258 (in PBS) for 10 min in the dark at room temperature. After staining, cells were washed three times with PBS for 2 min each, followed by a quick H2O wash and air-dried. Coverslips were mounted onto slides and visualized under a fluorescent microscope (Nikon, Tokyo, Japan) for apoptotic bodies.

2.2.5 Flow cytometry

Cells were grown in 24-well plates and treated at 80% confluency. Cells were collected by trypsinization and pelleted by centrifugation at 2,000g for 5 min. Next, cell pellets were resuspended in 1 ml of hypotonic fluorochrome buffer [0.1% Triton X-100, 0.1% sodium citrate,
25 μg/ml RNase A, and 50 μg/ml propidium iodide (PI). After incubation at 4°C overnight, the samples were then analyzed by flow cytometry to determine the percentage of subdiploid DNA. Samples were ran on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Mississauga, ON) and analyzed with EXPO32 ADC Analysis software.

### 2.2.6 Western blot analysis

Cells were grown in 100-mm plates, treated at 80% confluency, harvested and lysed in 70 μl of triple detergent buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% NaN₃, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The samples were then sonicated, incubated on ice for 20 min, and centrifuged at 12,000g for 10 min at 4°C. The supernatants were collected and a Bradford assay was performed to determine the protein concentration. Proteins (100 μg/lane) were separated on 12% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes. The PVDF was incubated with 5% BSA (bovine serum albumin) and primary antisera for 1 h at room temperature. Blots were washed three times in PBS with 0.04% Tween-20 for 5 min each then incubated with horseradish peroxidase (HRP)-conjugated secondary antisera for 1 h at room temperature. Signals were detected with SuperSignal ECL (Pierce, Rockford, IL). Antibodies used for Western blotting were p53 (DO-1) and Bax (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3, p21^Waf1/Cip1, Apaf-1 and β-actin (BD Pharmingen, Mississauga, ON, Canada), Bcl-2 and Bak (StressGen, Victoria, BC, Canada), and caspase-9 (Calbiochem, San Diego, CA). Protein expression on Western blots were quantified by densitometry using the Quantity One software (Bio-Rad, Mississauga, ON). The fold-induction or reduction were corrected for differences in the actin loading control.
2.2.7 Immunofluorescent staining

Cells were grown on coverslips in 35-mm plates and treated at 80% confluency. After treatment, cells were washed twice with PBS for 30 sec. Cells were then fixed and extracted with 2 ml of fix/ex solution [4% paraformaldehyde, 0.25% glutaraldehyde, 0.2% Triton X-100 in 1X PEM buffer (40 mM PIPES, 2.5 mM EGTA, 0.5 mM MgCl$_2$)] for 10 min at room temperature. Cells were then washed three times with PBS for 5 min each, incubated three times 5 min each with 1 mg/ml sodium borohydride (NaBH$_4$) in PBS to eliminate free aldehyde groups, and washed three times with PBS for 5 min each. Coverslips containing fixed cells were then inverted onto 50 µl of 1:25 dilution of p53 DO-1 or PAb1620 (Oncogene, Boston, MA, USA) antibodies and incubated in a humid chamber for 45 min, followed by three washes with PBS for 5 min each. Coverslips were then incubated with secondary antibody (1:100 dilution) for 45 min as previously described and washed three times with PBS for 5 min each. Finally, coverslips were incubated with 10 µM of Hoescht 33258 for 10 min, washed three times with PBS for 5 min each and mounted onto slides with mounting medium. Slides were then visualized under a fluorescent microscope.

2.2.8 PARP detection

Cells were grown in 60-mm plates and treated at 80% confluency. Cell lysates were obtained by resuspending cell pellets in 30 µl of lysis buffer (62.5 mM Tris-HCl [pH 6.8], 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% beta-mercaptoethanol, 100 µg/ml phenylmethlysulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A), sonicating for 15 sec, and incubating at 65°C for 15 min. The lysates were then incubated with 100 µg/ml of DNase I at 25°C for 30 min and then at 65°C for 10 min to solubilize. The samples
were separated on 8% SDS-polyacrylamide gels, blotted onto PVDF, and probed with an anti-PARP antibody (Calbiochem).

2.2.9 Subcellular fractionation

Cells were grown in 100-mm plates and treated at 80% confluency, followed by subcellular fractionation as described previously (Kim et al., 2001), which minor modifications. Briefly, cells were harvested after treatment and resuspended in 500 µl of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose) containing a mixture of protease inhibitors (100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A). Cell suspensions were then passed through a 26-gauge needle five times to lyse cells. Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifugation at 1000g at 4°C for 10 min. The resulting supernatant was centrifuged at 10,000g at 4°C for 20 min. The pellet fraction (mitochondria) was washed with buffer A and then solubilized in 50 µl of TNC buffer (10 mM Tris-acetate, pH 8.0, 0.5% NP-40, 5 mM CaCl₂) containing protease inhibitors. The cytosol was then generated by centrifugation of the supernatant at 100,000g at 4°C for 1 h. Protein concentrations were determined by a Bradford assay. Samples were then separated on 15% SDS-polyacrylamide gels, blotted onto PVDF, and probed with anti-cytochrome c (BD Pharmingen) and anti-Bax (N-20) (Santa Cruz Biotechnology) antibodies.

2.2.10 Mitochondrial transmembrane potential detection

Disruption of the mitochondrial transmembrane potential was detected using a MitoCapture™ Apoptosis Detection Kit (Calbiochem). The assay was performed according to the manufacturer’s specifications. Briefly, cells were grown on coverslips in 35-mm plates and
treated at 80% confluency. Following treatment, the medium was removed and the cells were incubated with 2 ml of diluted MitoCapture™ solution at 37°C in a 5% CO₂ incubator for 15 min. After incubation, the dye solution was removed and the cells were washed twice with 1 ml of the pre-warmed incubation buffer. The cells were then observed immediately under a fluorescent microscope.

2.2.11 Reverse transcriptase (RT)-PCR

Total RNA was isolated using the TRIzol™ Reagent (Invitrogen™ Life Technologies Inc., Burlington, ON, Canada) following the manufacturer’s instructions and quantitated by UV spectrophotometry. First-strand cDNA was prepared from 2 μg of total RNA reverse-transcribed into cDNA using the Omniscript RT kit from Qiagen Inc. (Mississauga, ON, Canada). A mix containing 10X RT buffer, 5 mM dNTP mix (5 mM each of dATP, dGTP, dCTP and dTTP), and 10 μM of oligo-dT primer (Promega) in a total volume of 20 μL was incubated at 37°C for 1 h. The reaction was then inactivated by heating at 93°C for 5 min. For semi-quantitative PCR, 2 μL of the first strand reaction were used for the 25 μL of PCR reaction contained, 10X PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 50 mM MgCl₂, 10 mM dNTP Mix, 5 Units/μL of the Taq DNA polymerase (Qiagen), and 25 μmol/μL of the specific primers:

**Bax** forward, 5’-CAGCTCTGAGCAGATCATGAAGACA-3’,

**Bax** reverse, 5’-GCCCATCTTCTTCCAGATGTTGAGC-3’ (535 bp);

**Bak** forward, 5’-ACTACAGGTGAAGGCTCTCACC-3’,

**Bak** reverse, 5’-CAATAGAGAAGGCACTGTCACC-3’ (332 bp);

**GAPDH** forward, 5’-CTCATGACCACAGTCCATGCCATC-3’,

**GAPDH** reverse, 5’-CTGCTTCACCACCTTCTTGATGTC-3’ (270 bp).

Amplification was carried out as follows: 1) initial denaturation at 95°C for 3 min, 2)
denaturation at 95°C for 30 sec, 3) annealing at 60°C (Bax) or 55°C (Bak) for 30 sec, 4) polymerization at 72°C for 30 sec, 5) repeat step 2 to step 4 for the indicated cycles (28 for Bax or 25 for Bak), and 6) final polymerization at 72°C for 3 min. Samples were then electrophoresed on 1% agarose gels containing 0.5 µg/ml EtBr and visualized under UV light as described in section 2.2.3. mRNA expression levels were quantified by densitometry using the Quantity One software (Bio-Rad). The fold-induction or reduction were corrected for differences in the GAPDH internal loading control.

2.2.12 Caspase inhibition assay

Cells were grown in 24-well plates and pre-treated for 1 h with 50 µM of the broad-based caspase inhibitor, benzylloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) (Calbiochem), followed by the addition of 20 µg/ml of CP-31398 for 24 h. Cell survival was determined by the SRB assay as described in section 2.2.2.
CHAPTER 3. CP-31398 INDUCES P53-DEPENDENT APOPTOSIS IN HUMAN COLON CARCINOMA CELL LINE HCT116

3.1 Rationale and Hypothesis

p53 is a tumour suppressor protein. Wild-type p53 inhibits transformation of primary rat embryo fibroblasts by E1A and ras oncogenes or mutant p53 and ras (Finlay et al., 1989; Eliyahu et al., 1988; Hinds et al., 1989). Wild-type p53 suppresses the induction of transformed foci by oncogenes, while mutant p53 does not have this inhibitory effect (Eliyahu et al., 1989). Furthermore, wild-type p53 can suppress growth of different types of tumour cells such as human colorectal carcinoma cells (Baker et al., 1990), osteosarcoma cells (Diller et al., 1990; Chen et al., 1990) and human glioblastoma cells (Mercer et al., 1990).

The ability of p53 to induce apoptosis is an important characteristic of its tumour suppressive function (Symonds et al., 1994). In vivo, p53 suppresses tumour growth by inducing apoptosis, while the absence of p53 leads to the absence of apoptosis and a faster rate of tumour development (Symonds et al., 1994; Howes et al., 1994; Pan and Griep, 1994; Morgenbesser et al., 1994). The importance of the apoptotic activity of p53 for its role as a tumour suppressor is supported by further studies which show that some naturally occurring tumour-derived p53 mutants are specifically defective in the induction of apoptosis, but retain the ability to induce growth arrest (Ryan and Vousden, 1998; Rowan et al., 1996). Loss of effectors of p53-mediated apoptosis, such as Bax, Apaf-1 and caspase-9, promote oncogenic transformation and tumour development (Yin et al., 1997; Soengas et al., 1999). Thus, loss of the apoptotic activity of p53 is crucial for eliminating its tumour suppressor function.

p53 also participates in several other biochemical pathways that are important in tumour suppression, such as transcription, DNA repair, genomic stability and cell-cycle control.
Activated p53 transactivates downstream genes whose products may be involved in tumour suppression, such as $p21^{Waf1/Cip1}$ and GADD45, both of which can inhibit cell growth (El-Deiry et al., 1993; Zhan et al., 1994). Inactivation of p53 leads to increased mutation frequency due to impaired DNA repair and genomic instability, resulting in gene amplification, aneuploidy, and chromosomal aberrations and ultimately the development of tumours (Donehower et al., 1992, Fukasawa et al., 1997, Livingstone et al., 1992, Yin et al., 1992).

The molecular mechanisms by which CP-31398 inhibits tumour growth in mice are currently unknown. Since p53 is involved in apoptosis, we hypothesize that CP-31398 may inhibit tumour growth by inducing p53-dependent apoptosis. If CP-31398 does stabilize p53 to induce apoptosis, we plan to determine if the mitochondrial-mediated pathway is involved.

3.2 Results

3.2.1 CP-31398 induces p53-dependent apoptosis

Two isogenic human colon carcinoma cell lines, HCT116+/+ containing wild-type p53 gene and HCT116-/- lacking the p53 gene, were used to determine if CP-31398 induces p53-dependent apoptosis. Firstly, Western analysis was performed to confirm that the p53 protein is expressed in HCT116+/+ but not in HCT116-/- cells (Figure 3.1A). To determine if CP-31398 reduces cell survival, HCT116+/+ and HCT116-/- cells were treated with various concentrations of CP-31398 for 24 h, followed by the SRB cell survival assay. The amount of SRB dye that remains bound to the surface of the fixed cells is a determinant of cell survival. As shown in Figure 3.1B, for each concentration of CP-31398 used, more dye was visibly bound to the HCT116-/- than the HCT116+/+ cells. After treatment with 10, 15, and 20 μg/ml of CP-31398, the survival rates in the HCT116+/+ cells were 66%, 27%, and 21% compared to 95%, 91%, and 80% in the
Figure 3.1 p53-dependent survival of HCT116 colon carcinoma cells after CP-31398 treatment. A. p53 protein expression in HCT116+/+ and HCT116-/- cells by Western blot analysis. B. Cell survival by SRB assay of HCT116+/+ and HCT116-/- cells treated with 0, 10, 15 or 20 µg/ml of CP-31398 for 24 h. Photographs were taken with a Nikon camera. C. Spectrophotometric readings of SRB staining from B. These experiments were performed at least three times and the results presented here are representative of each experiment.
HCT116-/- cells (P<0.01, P<0.00001, and P<0.00001, t-test), respectively (Figure 3.1C). These results indicate that CP-31398 reduces cell survival in p53-dependent manner, although CP-31398 does exhibit some p53-independent toxicity at higher doses.

Fragmentation of DNA, a hallmark of apoptosis, results in a hypodiploid DNA content that can be visualized as a pre-G1 peak on a DNA cell cycle histogram (Butt et al., 2000). To confirm the results of the SRB assay, HCT116 cells were treated with CP-31398, stained with propidium iodide (PI) and the pre-G1 population analyzed by flow cytometry. The results demonstrate that the subdiploid population was 49% in HCT116+/+ cells, compared to only 11% in HCT116-/- cells, after treatment with 10 µg/ml of CP-31398 for 24 h (P<0.001) (Figure 3.2A). 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. Figure 3.2B shows that more DNA fragmentation occurs in the HCT116+/+ than the HCT116-/- cells while the untreated cells do not exhibit any DNA fragmentation. Next, Hoechst staining was performed to assess cellular morphological changes after CP-31398 treatment. Figure 3.2C shows that apoptotic bodies are evident in CP-31398-treated HCT116+/+ cells, but not in the control cells. Collectively, these data show that CP-31398 induces p53-dependent apoptosis in HCT116 colon carcinoma cells.

3.2.2 CP-31398 induces cell cycle arrest

To determine if CP-31398 induces cell cycle arrest, flow cytometry was used to analyze the cell cycle distribution after CP-31398 treatment. Table 1.1 shows that both G1 and S phase populations were reduced (53.8% vs 38.7% and 17.1% vs 6.3%, respectively) in HCT116+/+ cells, while the G2/M phase population was significantly increased (29.1% vs 55%, P<0.001) after treatment with CP-31398, suggesting that a p53-dependent G2/M arrest is associated with
**Figure 3.2** CP-31398 induces p53-dependent apoptosis in HCT116 cells. A. HCT116+/+ and HCT116-/- cells were treated with 0 or 10 μg/ml of CP-31398 for 24 h, stained with PI, and the pre-G1 population was determined by flow cytometry. B. HCT116+/+ and HCT116-/- cells were treated with CP-31398 at 0 or 20 μg/ml for 24 h. DNA was extracted, separated on a 2% agarose gel, and stained with 0.5 μg/ml ethidium bromide. C. HCT116+/+ cells were treated with 0 (a) or 20 μg/ml (b) of CP-31398 for 24 h and stained with Hoechst. Arrows indicates apoptotic bodies. These experiments were performed at least three times and the results presented here are representative of each experiment.
Table 1.1 Effect of CP-31398 on cell cycle distribution. HCT116+/+ and HCT116-/- cells were treated with CP-31398 at 10 µg/ml for 24 h and analyzed for cell cycle distribution by PI staining and flow cytometry. Values are means ± S.D. for three independent experiments. These experiments were performed at least three times and the results presented here are representative of each experiment.

<table>
<thead>
<tr>
<th>% cell population</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCT116+/+</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>53.8 ± 1.3</td>
<td>17.1 ± 0.6</td>
<td>29.1 ± 0.5</td>
</tr>
<tr>
<td>CP-31398</td>
<td>38.7 ± 2.1</td>
<td>6.3 ± 1.5</td>
<td>55.0 ± 3.3</td>
</tr>
<tr>
<td><strong>HCT116-/-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>38.9 ± 1.3</td>
<td>23.4 ± 1.2</td>
<td>37.7 ± 2.3</td>
</tr>
<tr>
<td>CP-31398</td>
<td>50.0 ± 2.6</td>
<td>9.7 ± 1.7</td>
<td>40.3 ± 1.7</td>
</tr>
</tbody>
</table>
apoptosis in HCT116+/+ cells. In HCT116-/- cells, an increase of G1 (38.9% vs 50.0%, P<0.0005) and a decrease of S-phase population (23.4% vs 9.7%, P<0.0001) after CP-31398 treatment indicated a G1 phase arrest.

3.2.3 CP-31398 upregulates p53 protein and activates its transcriptional activity

Western blot analysis was performed to determine the p53 protein levels after CP-31398 treatment. As expected, HCT116-/- did not have any p53 expression before and after CP-31398 treatment since the p53 gene was knocked out in this cell line. However, there was a dose-dependent increase in p53 protein levels after treatment with 10, 15 and 20 μg/ml of CP-31398 for 24 h in HCT116+/+ cells (Figure 3.3A), which is consistent with the p53 stabilizing effect of this compound described by Foster et al. (1999). Immunofluorescent staining of HCT116+/+ cells showed that treatment with 20 μg/ml of CP-31398 led to an upregulation of the overall p53 protein levels as detected by the DO-1 antibody, which detects both the active and inactive conformations of wild-type and mutant p53 (Figure 3.3B), confirming the Western blot analysis (Figure 3.3A). Furthermore, CP-31398 treatment resulted in an increase in the portion of p53 that have the active conformation as detected by the PAb1620 antibody, a conformation specific antibody that recognizes stabilized, non-denatured, active DBD (Figure 3.3B).

To determine if the transcriptional activity of p53 protein is increased after CP-31398 treatment, Western blotting of p53 downstream targets was performed. The transcriptional activity of p53 protein is greatly increased in HCT116+/+ cells after CP-31398 treatment since there was a 45-fold induction of p21Waf1/Cip1 (Figure 3.3C), a direct downstream target of p53 (El-Deiry et al., 1993). An induction of p21Waf1/Cip1 to a smaller degree in HCT116-/- cells suggests that CP-31398 also induces p53-independent activation of p21Waf1/Cip1 (Figure 3.3C).
Bcl-2 family proteins involved in apoptosis, such as Bax and Bcl-2, are also known to be downstream targets of p53 (Miyashita et al., 1994a,b; Miyashita and Reed, 1995). To further investigate the p53 downstream effectors involved in CP-31398-induced apoptosis, we analyzed the expression levels of Bax and Bcl-2 proteins. Little change in the expression of Bcl-2 was observed after CP-31398 treatment in both cell lines (Figure 3.3C). Bax expression, however, increased 4-fold in the HCT116+/+ cells, with a minor increase in the HCT116-/- cells. Western analysis also showed an upregulation of Bak, another pro-apoptotic member of the Bcl-2 family, in HCT116+/+ and HCT116-/- cells after treatment with CP-31398 for 24 h. Bak, like Bax, is believed to induce apoptosis by heterodimerizing with and inactivating Bcl-2 and Bcl-XL in order to alter the mitochondrial membrane potential and induce cytochrome c release (Shimizu et al., 1999; Narita et al., 1998).

The upregulation of Bax protein is due to an upregulation at the mRNA level, as semi-quantitative RT-PCR showed that treatment of HCT116+/+ cells with increasing concentrations of CP-31398 leads to a dose-dependent upregulation of Bax mRNA compared to the untreated control (Figure 3.4A). In contrast, there is not much change in the Bax mRNA levels in the HCT116-/- cells, most likely due to the absence of p53 (Figure 3.4A). Bak mRNA was also upregulated to a small extent in HCT116+/+ after treatment with CP-31398 (Figure 3.4B).

A few studies have shown the link between p53 activation and upregulation and/or translocation of Bax to the mitochondria, leading to cytochrome c release and apoptosis (Cai et al., 2001; Karpinich et al., 2002). For instance, etoposide (topoisomerase II inhibitor) treatment of L929 fibroblasts leads to p53 phosphorylation and activation, which increases Bax synthesis and translocation to the mitochondria (Karpinich et al., 2002). To determine if Bax translocation is involved in CP-31398-induced apoptosis, we performed subcellular fraction and Western blotting on HCT116 cells treated with 0, 10 or 20 μg/ml of CP-31398 for 24 h. Figure 3.5 shows
Figure 3.3 Effect of CP-31398 on the expression of p53, p21\textsuperscript{Wafl/Cip1}, and Bcl-2 family proteins.  
A. Western analysis using anti-p53 antibody was performed on proteins extracted from HCT116+/+ and HCT116-/- cells treated with 0, 10, 15 or 20 \( \mu \text{g/ml} \) of CP-31398 for 24 h. \( \beta \)-actin was used as loading control.  
B. Immunofluorescent staining using anti-p53 antibodies (DO-1 and PAb1620) was performed on HCT116+/+ cells treated with 0 or 20 \( \mu \text{g/ml} \) of CP-31398 for 16 h.  
C. Western analysis was performed on proteins extracted from HCT116+/+ and HCT116-/- cells treated with 0 or 20 \( \mu \text{g/ml} \) of CP-31398 for 24 h. Antibodies against p21\textsuperscript{Wafl/Cip1}, Bax, Bak and Bcl-2 were used. \( \beta \)-actin was used as a loading control. These experiments were performed at least twice and the results presented here are representative of each experiment.
### A

<table>
<thead>
<tr>
<th></th>
<th>HCT116+/+</th>
<th></th>
<th>HCT116/-</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 10 15 20</td>
<td>0 10 15 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 1.7 2.5 6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

**Negative control**

- 0 µg/ml
- 20 µg/ml

- DO-1
- PAAb1620

### C

<table>
<thead>
<tr>
<th></th>
<th>HCT116+/+</th>
<th></th>
<th>HCT116/-</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 20</td>
<td>0 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 45.0</td>
<td>1.0 9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 3.7</td>
<td>1.0 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 6.2</td>
<td>1.0 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 1.1</td>
<td>1.0 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p21</td>
<td>Bax</td>
<td>Bak</td>
<td>Bcl-2</td>
</tr>
<tr>
<td></td>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4 Effect of CP-31398 on Bax and Bak mRNA levels. Semi-quantitative RT-PCR of Bax (A) and Bak (B) mRNA levels was performed on RNA extracted from HCT116+/+ and HCT116-/- cells treated with 0, 10, 15 or 20 µg/ml of CP-31398 for 24 h. GAPDH was used as an internal loading control. These experiments were performed at least three times and the results presented here are representative of each experiment.
that the Bax protein levels increased proportionally in the mitochondrial and cytosolic fractions after treatment with CP-31398 in HCT116+/+ cells, suggesting that Bax induction rather than translocation is responsible for CP-31398-induced apoptosis. Little or no change in Bax expression and translocation were observed in HCT116-/- cells after CP-31398 treatment.

Previous studies have shown that p53 can directly regulate Apaf-1 mRNA and protein levels during apoptosis (Kannan et al., 2001; Moroni et al., 2001). Fortin et al. (2001) showed that p53 upregulates Apaf-1 mRNA levels transcriptionally and protein levels during neuronal cell death. Robles et al. (2001) showed that wild-type p53 induced Apaf-1 mRNA and protein expression and apoptosis after exposure of cells to DNA-damaging agents. Apaf-1 protein levels, however, were not changed after CP-31398 treatment in HCT116+/+ or HCT116-/- cells (Figure 3.5), suggesting that p53 does not regulate Apaf-1 in these cells during CP-31398-induced apoptosis.

3.2.4 CP-31398 alters mitochondrial transmembrane potential

The mitochondrial membrane potential (ΔΨm) is decreased in the early stages of apoptosis induced by many types of stimuli and this change can be regulated by p53 (Li et al., 1999). For instance, cytosine arabinoside (Ara-c) induced p53-dependent apoptosis in cerebellar granule cells leads to a decrease in mitochondrial membrane potential (Zhang et al., 1999). Furthermore, increased Bax expression results in mitochondrial membrane permeability change and the release of cytochrome c from the mitochondria into the cytoplasm (Karpinich et al., 2000; Shimizu et al., 1999). To determine if CP-31398 induces a change in membrane potential during apoptosis, HCT116+/+ and HCT116-/- cells were treated with 20 μg/ml of CP-31398 for 12 or 24 h and then stained with a cationic dye using the MitoCapture™ Apoptosis Detection Kit. The cationic dye accumulates and aggregates in the mitochondria in healthy cells and emits
an orange-red fluorescence, while in apoptotic cells it remains in the cytoplasm due to the change in mitochondrial membrane potential and emits a green fluorescence. Stained cells were then visualized under a fluorescent microscope. Figure 3.6A shows that control cells stained orange-red while green fluorescent staining (apoptotic cells) was observed after 24 h CP-31398 treatment. HCT116+/+ had a significantly higher percentage of green-stained apoptotic cells than HCT116-/- cells after 12 h and 24 h CP-31398 treatment (32.4% and 55.6% vs 4.6% and 17.2%, P<0.0005 and P<0.0001, respectively) (Figure 3.6B). These data suggest that CP-31398 induces apoptosis by altering mitochondrial membrane potential in a time and p53-dependent manner.

3.2.5 CP-31398 induces cytochrome c release

Subcellular fractionation and Western blotting was performed to determine if cytochrome c is released in HCT116 cells after treatment with 0, 10 or 20 μg/ml of CP-31398. Our results indicated that the cytochrome c level decreased in the mitochondria and increased in the cytosol in HCT116+/+ cells after CP-31398 treatment while little cytochrome c release occurred in HCT116-/- cells (Figure 3.5).

3.2.6 CP-31398 activates caspases-9 and -3

Activation of the mitochondrial apoptotic pathway involves the release of cytochrome c from the mitochondria into the cytoplasm (Shimizu et al., 1999). Released cytochrome c 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 (Cryns and Yuan, 1998). To determine if the mitochondrial/caspase-9 pathway is activated in CP-31398-induced apoptosis, we analyzed the cleavage of caspase-9, caspase-3, and PARP by Western blot. Figure 3.7A shows that the
**Figure 3.5** Effect of CP-31398 on Bax and cytochrome c localization. Subcellular fractionation and Western analysis were performed on proteins extracted from HCT116+/+ and HCT116-/- cells treated with 0, 10 or 20 µg/ml of CP-31398 for 24 h. Antibodies against Bax, cytochrome c, and Apaf-1 were used. β-actin was used as a loading control for the cytosolic fraction. These experiments were performed at least three times and the results presented here are representative of each experiment.

<table>
<thead>
<tr>
<th></th>
<th>HCT116+/+</th>
<th></th>
<th>HCT116-/-</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5.6</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>20</td>
<td>8.2</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Bax (C)</td>
<td></td>
<td>Bax (C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.9</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>11.9</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Cyto C (C)</td>
<td></td>
<td>Cyto C (C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Cyto C (M)</td>
<td></td>
<td>Cyto C (M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>Apaf-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>β-actin</td>
<td></td>
<td>β-actin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3.6** CP-31398 alters the mitochondrial membrane potential. HCT116+/+ and HCT116-/- cells were treated with 0 or 20 µg/ml of CP-31398 for 12 or 24 h, and stained with MitoCapture™ solution. A. Microphotographs of control and CP-31398-treated (24 h) HCT116+/+ (a and b) and HCT116-/- (c and d) cells. Orange-red fluorescence represents live cells, while green fluorescence designates apoptotic cells. Magnification: 400X. 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. These experiments were performed at least three times and the results presented here are representative of each experiment.
Figure 3.7 CP-31398 induces activation of caspases-9 and -3 and cleavage of PARP. A. HCT116+/+ and HCT116-/- cells were treated with 0 or 20 μg/ml of CP-31398 for 24 h. Proteins were extracted and subjected to Western analysis using anti-caspase-9, anti-caspase-3, and anti-PARP antibodies. β-actin was used as a loading control. B. HCT116+/+ cells were pretreated with 50 μM caspase inhibitor Z-VAD-FMK for 1 h, followed by treatment with 0 or 20 μg/ml CP-31398 for 24 h. Cell survival was determined by SRB assay. These experiments were performed at least twice and the results presented here are representative of each experiment.
majority of the procaspase-9 is cleaved after CP-31398 treatment in HCT116+/+ cells, while little cleavage products were observed in HCT116-/- cells. Since activated caspase-9 can cleave caspase-3 and -7, we examined the procaspase-3 levels after CP-31398 treatment. We found that CP-31398 reduced procaspase-3 levels more in HCT116+/+ cells than in HCT116-/- cells (Figure 3.7A). Finally, we detected the cleavage of poly(ADP-ribose) polymerase (PARP), a known substrate for activated caspase-3 (Rosen and Casciola-Rosen, 1997), in CP-31398-treated HCT116+/+ cells, while little PARP cleavage occurred in HCT116-/- cells (Figure 3.7A).

To further confirm that CP-31398 induces apoptosis by activating caspases, we pre-treated HCT116+/+ cells with a broad-based caspase inhibitor, Z-VAD-FMK, prior to treatment with CP-31398 and determined the cell survival rate. Figure 3.7B shows that the caspase inhibitor significantly enhanced cell survival in CP-31398-treated HCT116+/+ cells, suggesting that caspases are being activated during CP-31398 induced apoptosis. Collectively, these data show that CP-31398 activates caspases-9 and -3, resulting in the cleavage of PARP.

3.3 Discussion

3.3.1 CP-31398 induces apoptosis by stabilizing p53

The synthetic compound CP-31398 was shown to inhibit the growth of tumour xenograph in mice (Foster et al., 1999). However, the issue of how CP-31398 exerts tumour suppression remains to be addressed. The results presented here demonstrate that CP-31398 induces p53-dependent apoptosis in the human colon carcinoma cell line, HCT116+/+, which contains wild-type p53 (Figure 3.2). The isogenic cell line, HCT116-/-, which lacks a functional p53 gene product, shows resistance to CP-31398 treatment (Figure 3.1-3.2). CP-31398 was shown to stabilize the p53 protein in vitro and in vivo (Foster et al., 1999; Takimoto et al., 2002). Here,
we confirm the p53-stabilizing effect of CP-31398 by observing a 6-fold accumulation of p53 and an upregulation of the active p53 conformation in HCT116+/+ cells after treatment at a concentration of 20 μg/ml as determined by Western blot analysis and immunofluorescent staining, respectively (Figure 3.3).

3.3.2 CP-31398 induces cell cycle arrest

We also demonstrate that CP-31398-induced p53-dependent G2/M cell cycle arrest is associated with apoptosis (Table 1). Our results agree with other reports showing the involvement of p53 in G2/M arrest (Stewart et al., 1995; Agarwal et al., 1995) and the correlation between G2/M arrest and apoptosis after anticancer drug treatment. For instance, etoposide-induced G2/M arrest in leukemic cells occurs in parallel to caspase activation and apoptosis (Sleiman and Stewart, 2000). A benzazepine compound, BBL22, induced tumour selective G2/M cell cycle arrest, which correlated with induction of apoptosis in epithelial and hematological cells (Xia et al., 2000). Recently, a novel p53 target gene, MCG10, was found to induce apoptosis and trigger G2/M cell cycle arrest (Zhu and Chen, 2000). MCG10 may, therefore, be involved in CP-31398-induced apoptosis. Our results also confirm those by Takimoto et al. (2002), who found treatment with CP-31398 upregulated p21^{Waf1/Cip1} and induced p53-dependent and -independent cell cycle arrest.

It is of interest to note that CP-31398 also induces p53-independent p21^{Waf1/Cip1} activation. Though to a lesser extent than in HCT116+/+ cells, CP-31398 induced a 9-fold increase in p21^{Waf1/Cip1} expression in HCT116-/- cells (Figure 3.3), suggesting that CP-31398 has biochemical functions other than its ability to stabilize p53 protein. p53-independent p21^{Waf1/Cip1} activation is also observed in human cells after UV radiation and anticancer drug treatment (Haapajarvi et al., 1999; Chinery et al., 1997). More importantly, the increased p21^{Waf1/Cip1}
results in G1 arrest in HCT116-/- cells. Our results are consistent with a previous study that showed that the upregulation of p21\textsuperscript{Waf1/Cip1} and subsequent G0/G1 arrest in colonic carcinoma cells induced by butyrate \textit{in vitro} is p53-independent and requires an intact mitochondrial membrane potential (Heerdt \textit{et al.}, 1998). The ability to induce cell cycle arrest in p53-deficient cells by CP-31398 has biological importance. For instance, patients with abnormal p53 genes, such as Li-Fraumeni syndrome, are predisposed to the development of multiple cancers (Evans and Lozano, 1997), presumably due to lack of cell cycle arrest (Williams \textit{et al.}, 1997; Paules \textit{et al.}, 1995) and reduced DNA repair (Therrien \textit{et al.}, 1999; Abrahams \textit{et al.}, 1998) and apoptosis (Camplejohn \textit{et al.}, 1995; Goi \textit{et al.}, 1997). Since G1 arrest is an important cellular stress response, the ability of inducing G1 arrest by CP-31398 could suggest its potential use as an agent for chemoprevention.

3.3.3 CP-31398 enhances p53 transcriptional activity

p53 is a transcriptional factor that activates or represses its downstream genes to exert its biological and biochemical functions. Some of the well-known p53 downstream effectors include p21\textsuperscript{Waf1/Cip1}, GADD45, and Bax. CP-31398 dramatically enhances p53 transcriptional activity by upregulating p21\textsuperscript{Waf1/Cip1}, Bax and Bak in HCT116+/- cells (Figure 3.3-3.4). Bcl-2 expression was not altered by treatment with CP-31398 in HCT116 cells (Figure 3.4), suggesting Bcl-2 is not involved in CP-31398-induced p53-dependent apoptosis.

3.3.4 CP-31398 induces the mitochondrial/caspase-9 pathway

These studies have demonstrated that the p53-stabilizing compound CP-31398 induces apoptosis by upregulating Bax and Bak (Figure 3.3-3.4), altering the mitochondrial membrane potential (Figure 3.6) leading to the release of cytochrome \textit{c} (Figure 3.5), and consequently activating
caspases-9 and -3 (Figure 3.7). Both Bax and Bak have been shown to cause mitochondrial membrane change, cytochrome c release and activation of caspase-9 (Degenhardt et al., 2002; Nutt et al., 2002; Wang et al., 2001; Narita et al., 1998; Shimizu et al., 1999; Wang et al., 2002). Furthermore, our findings are consistent with the previous studies showing that p53 activates the Bax/mitochondrial/caspase-9 apoptosis pathway. Studies have indicated that Bcl-2 family proteins are involved in the p53-induced mitochondrial apoptosis pathway. Miyashita et al. (1994a,b) reported that p53 upregulates Bax and downregulates Bcl-2 expression. Adenoviral transfer of wild-type p53 into lung cancer cells leads to upregulation of Bax and Bak protein levels and apoptosis, with no changes in Bcl-2 and Bcl-X<sub>L</sub> protein levels (Pearson et al., 2000). p53-dependent cell death in neurons correlates with Bax-dependent caspase-3 activation (Cregan et al., 1999). p53-dependent apoptosis was also found to proceed through a pathway involving depolarization of the mitochondrial electropotential gradient (delta(psi)m) and the generation of ROS (Lee, 1998). Schuler et al. (2000) found that p53 requires Bax to activate caspases-9 and -3 through mitochondrial cytochrome c during the apoptosis process in human osteosarcoma Saos-2 cells.

A number of studies have also indicated a relationship between p53 and Bak during apoptosis. p53 was shown to induce Bak in another human colon cancer cell line DLD-1 (Bartke et al., 2001). Cisplatin induced an up-regulation of Bak in the wild-type p53-expressing human ovarian carcinoma line cell, A2780, but not in A2780/cp70 cells that do not have functional p53 (Jones et al., 1998). Human glioblastoma A172 cells treated with hydrogen peroxide triggered apoptosis and increased the expression of p53, Bak, p21<sup>Waf1/Cip1</sup> and GADD45 proteins (Kitamura et al., 1999), suggesting that apoptosis in glial cells caused by oxidative stress may be due to p53 induction of Bak. The wild-type form of the temperature-sensitive murine p53<sup>val1135</sup> introduced into glioma cells resulted in G2/M arrest and accumulation of Bak protein (Pohl et al.,
1999), consistent with Bak induction and G2/M arrest observed in CP-31398-treated HCT116+/

Other studies, however, have shown that Bak expression during apoptosis is independent of p53 status. Paclitaxel increased the levels of Bak in both A2780 and A2780/cp72 cells, suggesting that Bak and apoptosis may be induced by a p53-independent mechanism (Jones et al., 1998). 5-fluorouracil (5-FU)-induced apoptosis in human colon cancer cell lines resulted in increased Bak expression in both wild-type (LoVo) and mutant (DLD1) p53 containing cells lines (Nita et al., 1998). Overexpression of the Bak gene in p53-sensitive and p53-resistant mesothelioma cell lines by adenovirus-mediated Bak gene transfer induced apoptosis and decreased cell viability in both cell lines (Pataer et al., 2001). Interferon-gamma (IFN-γ) has been shown to directly induce Bak during p53-independent apoptosis in a human colon adenocarcinoma cell line (Ossina et al., 1997). The small increase in Bak protein expression and low level of apoptosis seen in the HCT116-/- cells may be due to a p53-independent mechanism similar to that seen in these previous studies.

Taken together, our results demonstrate that CP-31398 induces apoptosis by inducing Bax and Bak expression, altering the mitochondrial membrane potential, and activating caspases-9 and -3. What remains to be determined is whether or not CP-31398 is able to induce apoptosis in cancer cells containing mutant p53. The ability of CP-31398 to induce apoptosis in human cancer cells will potentially establish it as a chemotherapeutic agent.
4.1 Rationale and Hypothesis

Malignant melanoma is a highly chemoresistant disease and its incidence has been increasing rapidly during the last few decades. UV irradiation, which causes DNA damage (Alcalay et al., 1990), is the major environmental factor for the development of cutaneous melanoma. UV-induced DNA damage, if not repaired promptly, can lead to mutations that activate oncogenes or inactivate tumour suppressor genes, increased genetic instability and tumourigenesis. Thus, the ability to repair UV-induced DNA damage or remove cells with irreparable DNA damage is important in the prevention of skin cancer development.

p53 is a key regulator in the stress response to UV-induced DNA damage (Hall et al., 1993; Liu et al., 1994; Ponten et al., 1995; Zhan et al., 1993; Abrahams et al., 1995; Davenport et al., 1999). Studies have shown that the p53 protein levels and transcriptional activity increase after UV irradiation (Cotton and Spandau, 1997; Zhan et al., 1993), presumably due to post-translational modifications such as phosphorylation and acetylation that increase the half-life of the protein (Gu and Roeder, 1997; Maki and Howley, 1997). In response to UV-induced DNA damage, p53 initiates G1 cell cycle arrest by upregulating p21\(^{\text{Waf1/Cip1}}\) (Liu and Pelling, 1995). In addition, p53 enhances the repair of UV-damaged DNA (Ford and Hanawalt, 1997; Smith et al., 1995; Mirzayans et al., 1996; Li et al., 1996, 1997; Li and Ho, 1998). Alternatively, p53 is able to induce apoptosis following UV exposure (Ziegler et al., 1994; Henseleit et al., 1997; Li and Ho, 1998; Tron et al., 1998) in cells with severely damaged DNA.

p53 mutations are rare in melanoma cells, with a frequency between 15-30%. Furthermore, wild-type p53 protein levels are expressed at high levels in melanoma cells. It is
suggested that the p53 response to UV-induced DNA damage is impaired in melanoma cells due to the presence of non-functional wild-type p53 or defects in the p53 activation or downstream pathways. Since p53 plays an important role in UV-induced apoptosis, we hypothesize that CP-31398 may enhance UVB-induced apoptosis in wild-type melanoma cells by stabilizing p53.

4.2 Results

4.2.1 CP-31398 upregulates p53 protein expression in melanoma cells
To determine if CP-31398 enhances UVB-induced apoptosis, a dose response was performed to determine the dose of CP-31398 that would induce maximum p53 protein expression without toxicity to the cells. A human melanoma cell line containing wild-type p53 gene, MMRU, was treated with 0, 4, 8, or 10 μg/ml of CP-31398 for 24 h and Western analysis was performed to determine the levels of p53 protein. Figure 4.1A shows that a maximum increase in p53 protein was obtained after treatment with 8 μg/ml of CP-31398. To determine the dose of CP-31398 that is non-toxic, MMRU cells were treated with increasing concentrations of CP-31398 for 24 h, followed by the SRB assay to quantify the amount of cell survival. Figure 4.1B shows that CP-31398 induces dose-dependent decrease in cell survival in MMRU cells and that treatment with 8 μg/ml of CP-31398 results in very little cell death (4%). Since treatment with 8 μg/ml of CP-31398 leads to very little cell death and high p53 protein expression, this non-toxic dose was used in subsequent experiments.

4.2.2 CP-31398 enhances UVB-induced apoptosis
To determine if CP-31398 reduces cell survival after UVB exposure, MMRU cells were irradiated with UVB at 0, 40, 60 or 80 mJ/cm² followed by treatment with 0 or 8 μg/ml of
Figure 4.1 Induction of p53 and survival of MMRU human melanoma cells after CP-31398 treatment. A. p53 protein expression by Western blot analysis in MMRU cells treated with various concentrations of CP-31398 for 24 h. Anti-p53 DO-1 was used as primary antibody and β-actin as a loading control. B. Survival of CP-31398-treated MMRU cells by SRB staining. These experiments were performed at least three times and the results presented here are representative of each experiment.
CP-31398. Twenty-four hours after treatment, the SRB assay was performed to quantify the cell survival rate. Figure 4.2A shows that while CP-31398 did not reduce cell survival for non-irradiated cells, it significantly reduced cell survival in UVB-irradiated cells at all the doses used. The survival rate for cells treated with CP-31398 after 40, 60 or 80 mJ/cm\(^2\) of UVB irradiation were 56%, 47%, and 40%, compared to 84%, 71%, 65% for UVB irradiation alone, respectively (P<0.01, P<0.01, P<0.001, t-test). CP-31398 also reduced cell survival in a time-dependent manner, starting after 8 h of treatment (Figure 4.2B). More than 24 h of treatment with 8 µg/ml of CP-31398, however, is toxic to the cells. The survival rate for cells treated with CP-31398 after 40 mJ/cm\(^2\) of UVB irradiation for 24, 48 and 72 h were 40%, 36%, and 7%, compared to 77%, 82%, 77% for UVB irradiation alone, respectively (P<0.01, P<0.001, P<0.001, t-test).

To confirm the SRB cell survival results, MMRU cells were irradiated with UVB, treated with CP-31398, and stained with PI to assess the pre-G1 population. Figure 4.3A shows that UVB-irradiated cells treated with 8 µg/ml of CP-31398 had a significantly higher percentage of hypodiploid DNA population than treatment with UVB alone (57%, 63% and 67% vs 18%, 24% and 24%, at 40, 60 and 80 mJ/cm\(^2\), respectively) (P<0.0001, P<0.0001, P<0.0001, t-test).

The DNA ladder assay was performed to confirm that CP-31398 enhancement of UVB-induced cell death is a result of apoptosis and not necrosis. DNA was extracted from UVB and CP-31398-treated cells and analyzed on an agarose gel for DNA fragmentation and laddering. Figure 4.3B shows that more DNA fragmentation occurs in UVB-irradiated cells treated with CP-31398 compared to cells that were irradiated with UVB but not treated with CP-31398. As expected, treatment with the non-toxic dose of CP-31398 resulted in no DNA fragmentation. Collectively, these data show that CP-31398 enhances UVB-induced apoptosis in MMRU melanoma cells.
Figure 4.2 CP-31398 reduces cell survival after UVB exposure in MMRU cells. A. MMRU cells were irradiated with 0, 40, 60 or 80 mJ/cm² of UVB followed by treatment with 0 or 8 μg/ml of CP-31398 for 24 h. Cell survival was then determined by SRB assay. B. MMRU cells were irradiated with 0 or 40 mJ/cm² of UVB followed by treatment with 0 or 8 μg/ml of CP-31398 for various periods of time. Cell survival was then determined by SRB assay. These experiments were performed at least three times and the results presented here are representative of each experiment.
Figure 4.3 CP-31398 enhances UVB-induced apoptosis in MMRU cells. A. MMRU cells were irradiated with 0, 40, 60 or 80 mJ/cm² of UVB followed by treatment with 0 or 8 µg/ml of CP-31398 for 24 h. Cells were stained with propidium iodide and the pre-G1 population was determined by flow cytometry. B. MMRU cells were irradiated with 0, 40, 60 or 80 mJ/cm² of UVB followed by treatment with 0 or 8 µg/ml of CP-31398 for 24 h. DNA was extracted and analyzed on a 2% agarose gel. These experiments were performed at least three times and the results presented here are representative of each experiment.
4.2.3 CP-31398 upregulates p53 during UVB exposure

Western blot analysis showed that treatment of MMRU cells with CP-31398 or UVB alone upregulated p53 protein levels after various periods of time, with the largest upregulation after 8 h of treatment. However, combination treatment with UVB and CP-31398 leads to a larger increase in p53 protein levels than UVB or CP-31398 treatment alone at each time point (Figure 4.4).

4.2.4 CP-31398 upregulates Bax and alters mitochondrial transmembrane potential

Since wild-type p53 has been shown to regulate Bax (Miyashita et al., 1994a,b; Regula and Kirshenbaum, 2001) and Bax has been shown to be involved in UV-induced cell death (Cho et al., 2001; Ouhtit et al., 2000; Reinke and Lozano, 1997a), we sought to determine if CP-31398 can enhance the expression of Bax protein during UVB-induced apoptosis. Figure 4.5A shows that CP-31398 treatment following UVB exposure induced more Bax expression compared to UVB irradiation alone. Bcl-2 levels did not change after UVB exposure alone or after combination treatment with UVB and CP-31398 for 24 h.

The MitoCapture™ Apoptosis Detection Kit was used to determine if CP-31398 induces a change in mitochondrial membrane potential during UVB-induced apoptosis. MMRU cells were exposed to 0, 40 or 60 mJ/cm^2 of UVB, treated with 0 or 8 µg/ml of CP-31398 for 24 h and then stained with a cationic dye. Figure 4.5B shows that control cells stained orange-red while green fluorescent staining (apoptotic cells) was observed in UVB-irradiated cells. There were a significantly higher percentage of apoptotic cells in the UVB-irradiated and CP-31398-treated cells compared to the cells irradiated with UVB alone (49% vs 33%, 55% vs 40% at 40 or 60 mJ/cm^2, respectively) (P<0.05, P<0.05) (Figure 4.5C). These data suggest that CP-31398 enhances UVB-induced apoptosis by altering the mitochondrial membrane potential.
**Figure 4.4** Induction of p53 in MMRU cells after UVB irradiation and CP-31398 treatment. p53 protein expression by Western blot analysis in MMRU cells irradiated with 0 or 40 mJ/cm² of UVB followed by treatment with 0 or 8 μg/ml CP-31398 for various periods of time. Anti-p53 DO-1 was used as primary antibody and β-actin as a loading control. These experiments were performed at least twice and the results presented here are representative of each experiment.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>8</th>
<th>0</th>
<th>8</th>
<th>0</th>
<th>8</th>
<th>0</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CP-31398</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p53</td>
<td>1.0</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
<td>4.8</td>
<td>1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>β-actin</td>
<td>1.0</td>
<td>1.5</td>
<td>1.7</td>
<td>3.0</td>
<td>2.1</td>
<td>4.8</td>
<td>1.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

- 0 h
- 8 h
- 12 h
- 24 h
4.2.5 CP-31398 induces cytochrome c release after UVB exposure

Subcellular fractionation and Western blotting was performed to determine if cytochrome c is released during CP-31398 enhancement of UVB-induced apoptosis. Figure 4.5A shows that cytochrome c levels decrease in the mitochondrial and increase in the cytosolic fractions after treatment with UVB alone, suggesting that UVB is inducing cytochrome c release during apoptosis. More cytochrome c release occurred in MMRU cells after UVB and CP-31398 treatment compared to cells exposed to only UVB or CP-31398 (Figure 4.5A), suggesting that CP-31398 enhances the release of cytochrome c during UVB-induced apoptosis.

4.2.6 CP-31398 activates caspases-9 and -3

To investigate if the mitochondrial/caspase-9 pathway is activated in CP-31398 enhancement of UVB-induced apoptosis, the cleavage of caspases-9 and -3 was analyzed by Western blotting. Figure 4.6 shows that more procaspase-9 is cleaved in UVB-irradiated cells treated with CP-31398 compared to cells irradiated with UVB alone. Procaspase-3 levels were also reduced more in UVB-irradiated and CP-31398-treated cells than in the cells treated with UVB alone. Treatment with 8 μg/ml of CP-31398 alone resulted in levels of procaspase-9 and -3 that are similar to the untreated control as expected since treatment with CP-31398 at this dose does not induce a significant amount of cell death. Finally, we detected the cleavage of PARP in MMRU cells that were exposed to either UVB alone or UVB/CP-31398 combination treatment, confirming the activation of caspase-3 (Figure 4.6). Collectively, these data show that CP-31398 activates the mitochondrial apoptotic pathway after UVB exposure.
Figure 4.5 CP-31398 increases Bax expression, alters the mitochondrial membrane potential, and induces cytochrome c release after UVB irradiation. MMRU cells were irradiated with 0 or 40 mJ/cm² of UVB followed by treatment with 0, 8 or 10 μg/ml of CP-31398 for 24 h. A. Western analysis of Bax, Bcl-2 and cytochrome c levels with anti-Bax, anti-Bcl-2 and anti-cytochrome c antibodies. β-actin was used as a loading control. B. MMRU cells were irradiated with 0, 40 or 60 mJ/cm² of UVB followed by treatment with 0 or 8 μg/ml of CP-31398 for 24 h, and then stained with MitoCapture™ solution. Microphotographs of control, UVB irradiated (40 mJ/cm²), CP-31398-treated (8 μg/ml), and UVB irradiated (40 mJ/cm²) and CP-31398-treated (8 μg/ml) MMRU cells. Orange-red fluorescence represents live cells, while green fluorescence designates apoptotic cells. Magnification: 400X. C. 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. These experiments were performed at least three times and the results presented here are representative of each experiment.
A

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bax</td>
<td>1.0</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Cyto c (C)</td>
<td>1.0</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Cyto c (M)</td>
<td>1.0</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Control

CP-31398

UV

C

Cell apoptosis (%) vs. CP-31398 Treatment (h)

- 0 μg/ml, 0 mJ
- 8 μg/ml, 0 mJ
- 0 μg/ml, 40 mJ
- 8 μg/ml, 40 mJ
- 0 μg/ml, 60 mJ
- 8 μg/ml, 60 mJ
Figure 4.6 CP-31398 induces activation of caspases-9 and -3 during UVB-induced apoptosis. MMRU cells were irradiated with 0 or 40 mJ/cm² of UVB followed by treatment with 0 or 8 µg/ml of CP-31398 for 24 h. Proteins were extracted and subjected to Western analysis using anti-caspase-9, anti-caspase-3 and anti-PARP antibodies. β-actin was used as a loading control. These experiments were performed at least twice and the results presented here are representative of each experiment.
4.2.7 CP-31398 reduces cell survival in other melanoma cell lines

To determine if CP-31398 reduces cell survival after UVB exposure in other melanoma cell lines, wild-type p53 (RPEP) and mutant p53 (PMWK and SK-mel-110) melanoma cell lines were irradiated with UVB at 0, 40, 60 or 80 mJ/cm² followed by treatment with 0, 8 or 10 µg/ml of CP-31398. Twenty-four hours after treatment, the SRB assay was performed to quantify the cell survival rate. We found that CP-31398 upregulates p53 and enhances cell death induced by UVB in RPEP (Figure 4.7A) and PMWK (Figure 4.7B) cells. The survival rate for RPEP cells treated with 8 µg/ml of CP-31398 after 40, 60 or 80 mJ/cm² of UVB irradiation were 54%, 42%, and 37%, compared to 77%, 59%, 52% for UVB irradiation alone, respectively (P<0.01, P<0.05, P<0.05, t-test). The survival rate for PMWK cells treated with 8 µg/ml of CP-31398 after 40, 60 or 80 mJ/cm² of UVB irradiation were 71%, 17%, and 13%, compared to 79%, 49%, 37% for UVB irradiation alone, respectively (P<0.05, P<0.01, P<0.01, t-test). However, CP-31398 did not enhance cell death induced by UVB in SK-mel-110 cells, even though p53 protein levels were upregulated (Figure 4.7C), presumably due to the presence of multiple mutations in the DBD of p53 (Ablino et al., 1994) that cannot be stabilized.

4.3 Discussion

Since p53 plays an important role in the cellular stress response to UV irradiation, we sought to determine if CP-31398 could be used to stabilize the p53 conformation to enhance UVB-induced apoptosis. Our results demonstrate that CP-31398 induces p53 accumulation and enhances UVB-induced apoptosis in the human melanoma cell line, MMRU (Figure 4.1-4.4). We also found that CP-31398 enhances UVB-induced apoptosis by upregulating Bax protein levels, altering the mitochondrial membrane potential and activating caspases-9 and -3 after UVB exposure (Figure 4.5-4.6). Our results are consistent with previous studies showing the
**Figure 4.7** CP-31398 upregulates p53 protein levels and reduces cell survival after UVB exposure in melanoma cells. RPEP (A), PMWK (B) and SK-mel-110 (C) cells were treated with various concentrations of CP-31398 for 24 h. Western blot analysis was then performed using anti-p53 DO-1 as primary antibody and β-actin as a loading control. RPEP (A), PMWK (B) and SK-mel-110 (C) cells were irradiated with 0, 40, 60 or 80 mJ/cm\(^2\) of UVB followed by treatment with 0, 8 or 10 µg/ml of CP-31398 for 24 h. Cell survival was then determined by SRB assay. These experiments were performed at least twice and the results presented here are representative of each experiment.
involvement of p53 in the mitochondrial-mediated apoptotic pathway. Studies have shown that UV exposure of mouse skin induced p53 expression followed by increased Bax and decreased Bcl-2 expression and apoptosis (Ouhtit et al., 2000). UV exposure of rat embryo fibroblasts leads to increased Bax expression (Reinke and Lozano, 1997a). Moreover, adenoviral introduction of wild-type p53 into p53-null Saos-2 cells causes Bax-dependent cytochrome c release and activation of caspases (Schuler et al, 2000). Wild-type p53 caused an increase in Bax gene expression and apoptosis as a result of loss of mitochondrial transmembrane potential Delta(psi)m and cytochrome c release and increased caspase-3-like activity in ventricular myocytes (Regula and Kirshenbaum, 2001). Activation of caspase-9 has been shown to be involved in UV-induced apoptosis of keratinocytes (Sitailo et al., 2002).

UV irradiation of keratinocytes leads to an upregulation of Fas and FasL mRNA and protein (Leverkus et al., 1997). Furthermore, UV has been shown to induce apoptosis by directly activating Fas receptor clustering, recruiting FADD to the receptor and activating caspase-3 (Rehemtulla et al., 1997; Aragane et al., 1998). UV has also been shown to induce apoptosis by activating TNFR1 aggregation and activation of caspase-8 (Sheikh et al., 1998). As p53 has been shown to be involved in death receptor-mediated apoptosis, it would be worthwhile to investigate whether or not this apoptotic pathway is involved in CP-31398 enhancement of UVB-induced cell death.

p53 plays an important role in cellular stress response to UV exposure such as DNA repair and apoptosis, and has been shown to suppress UV-induced skin cancer development in animal models (Jiang et al., 1999). Transgenic mice with abnormal p53 function develop more squamous cell carcinomas than control mice after UV irradiation (Li et al., 1995a, 1998b). Furthermore, Kanjilal et al. (1993) found that all 11 of UV-induced murine skin cancers examined had mutations in the p53 gene, underlying the importance of p53 to the development of
UV-induced skin cancer. In humans, individuals with LFS have germ-line mutations in the \( p53 \) gene are highly susceptible to the development of many forms of cancer (Nichols et al., 2001; Evans and Lozano, 1997). Our findings of CP-31398 enhancement of UVB-induced apoptosis in melanoma cells suggests that this compound may be used to stabilize p53 or rescue mutant p53 to suppress the development of skin cancer after UV exposure. More studies are required to determine if CP-31398 can enhance UV-induced apoptosis in normal keratinocytes and melanocytes.
CHAPTER 5. CP-31398 DOES NOT ENHANCE CHEMOSENSITIVITY OF MELANOMA CELLS

5.1 Rationale and Hypothesis

In the past few years, tremendous efforts have been made to develop mechanisms to introduce the wild-type p53 gene into tumour cells for the purpose of inducing p53-dependent apoptosis in cells with a defective apoptotic response. Viral-mediated vectors, such as retrovirus, adenovirus or adeno-associated virus, and herpes simplex virus type-1, have recently been developed for gene delivery into cancer cells. Retroviral-mediated transfer of wild-type p53 into patients with non-small cell lung cancer resulted in apoptosis and tumour regression and stabilization (Roth et al., 1996). Adenoviral-mediated overexpression of wild-type p53 in melanoma cell lines results in apoptosis in vitro and inhibits melanoma tumour growth in vivo (Cirielli et al., 1995). Adenoviral-mediated transfer of wild-type p53 into mutant p53 tumour cell lines results in cytotoxicity due to apoptosis (Katayose et al., 1995; Blagosklonny and el-Deiry, 1996). Furthermore, adenoviral infection with wild-type p53 into tumour cells sensitizes these cells to killing by chemotherapeutic agents in vitro and in vivo (Blagosklonny and el-Deiry, 1996; Fujiwara et al., 1994; Nemunaitis et al., 2000). Viral delivery systems, however, have a number of disadvantages, including low infectivity, inefficiency of gene transfer, inability to control viral targeting, cell type-specific expression, and the duration of expression. Risks such as high toxicity, immunoresponses to viral antigens, and potential viral recombination are also involved in viral delivery systems (Walther and Stein, 2000).

Another approach is to use a synthetic compound corresponding to the C-terminal amino acid residues 361-382 of p53, which was found to activate wild-type and mutant p53 DNA binding and mutant p53 transcriptional activity. Furthermore, this peptide caused growth
inhibition and apoptosis in human tumour cell lines with wild-type and mutant p53 (Selivanova et al., 1997, 1999; Kim et al., 1999). In addition, amifostine has been shown to activate wild-type p53 and to restore the transcriptional activity to mutant p53 (Maurici et al., 2001). Leptomycin B, a small molecule nuclear export inhibitor, and actinomycin D treatment of cervical carcinoma cells reduces human papillomavirus E6-E7 mRNA, activates p53 transcriptional activity and induces p53-dependent apoptosis (Hietanen et al., 2000). Thus, the use of small synthetic compounds, such as CP-31398, to stabilize the p53 protein and activate its transcriptional activity has chemotherapeutic potential in addition to a number of advantages. Firstly, CP-31398 is a small molecule that can enter tumour cells easily. Secondly, when systemically administered in mice, CP-31398 has low toxicity (Foster et al, 1999). Thirdly, CP-31398 was shown to rescue newly synthesized mutant p53 and to restore its transcriptional activity, allowing the potential use in the treatment of tumours with mutant p53.

Since p53 plays an important role in apoptosis of melanoma cells induced by chemotherapeutic drugs (Li et al, 1998, 2000), we hypothesize that CP-31398 may have a role in chemosensitivity by stabilizing p53 to enhance apoptosis induced by chemotherapeutic agents. Furthermore, CP-31398 may be used to sensitize chemoresistant melanoma cells to drug killing by restoring p53 function in cells with wild-type p53 that are non-functional or in cells with mutant p53. We used both wild-type p53 melanoma cell line MMRU and mutant p53 melanoma cell line SK-mel-110 for this study.
5.2 Results

5.2.1 CP-31398 upregulates p53 protein expression in melanoma cells

Using Western blotting and the SRB cell survival assay, we previously showed that CP-31398 (8 μg/ml) can upregulate p53 protein levels without toxicity to MMRU cells (Figure 4.1). Treatment of SK-mel-110, a human melanoma cell line containing mutant p53, with 8 or 10 μg/ml of CP-31398 for 24 h also upregulated p53 without toxicity to these cells (Figure 4.7C, 5.1), as shown by Western blotting and SRB assay, respectively. Since treatment with 8 μg/ml of CP-31398 leads to very little cell death and high p53 protein expression in both cell lines, this non-toxic dose was used in subsequent experiments.

5.2.2 CP-31398 does not enhance melanoma chemosensitivity

To determine if CP-31398 has a role in chemosensitivity, MMRU and SK-mel-110 cells were treated with 0 or 8 μg/ml of CP-31398 and various concentrations of camptothecin (CPT), a topoisomerase I inhibitor, for 24 h. After treatment, the SRB assay was performed to quantify the cell survival rate. Figure 5.2 shows that CP-31398 did not enhance the cell death induced by CPT in either MMRU (A) or SK-mel-110 (B). Next, we hypothesized that pre-treating cells with CP-31398 to upregulate the p53 protein levels prior to treatment with chemotherapeutic drugs might enhance cell death; therefore, we treated MMRU and SK-mel-110 cells with 8 μg/ml of CP-31398 for various time points and performed Western blotting to determine the time of exposure to CP-31398 that induces high p53 protein expression. p53 protein expression was significantly upregulated after 8 or 12 h of CP-31398 treatment in both cell lines (Figure 5.3). MMRU and SK-mel-110 cells were then pre-treated with 8 μg/ml of CP-31398 for 8 or 12 h,
Figure 5.1  Survival of SK-mel-110 human melanoma cells after CP-31398 treatment. SK-mel-110 cells were treated with various concentrations of CP-31398 for 24 h. Survival of CP-31398-treated SK-mel-110 cells was determined by SRB staining. These experiments were performed at least three times and the results presented here are representative of each experiment.
Figure 5.2 Survival of MMRU and SK-mel-110 cells after CP-31398 and camptothecin (CPT) treatment. MMRU (A) and SK-mel-110 (B) cells were treated with 8 µg/ml of CP-31398 and various concentrations of CPT for 24 h. Survival of CP-31398 and CPT-treated cells was determined by SRB staining. These experiments were performed at least three times and the results presented here are representative of each experiment.
Figure 5.3  Induction of p53 in MMRU and SK-mel-110 cells after CP-31398 treatment. MMRU (A) and SK-mel-110 (B) cell were treated with 8 μg/ml of CP-31398 for various periods of time and Western blot analysis was performed to determine p53 protein levels. β-actin was used as a loading control. These experiments were performed at least twice and the results presented here are representative of each experiment.
Figure 5.4 Survival of MMRU cells after pre-treatment with CP-31398, followed by treatment with CPT. MMRU cells were pre-treated with 8 μg/ml of CP-31398 for 8 h (A) or 12 h (B, C), followed by treatment with CPT alone for 24 h or treatment with CP-31398 and CPT for 24 h. Survival of CP-31398 and CPT-treated cells was determined by SRB staining (A, B). Cells were stained with PI and the pre-G1 population was determined by flow cytometry (C). These experiments were performed at least three times and the results presented here are representative of each experiment.
Figure 5.5 Survival of SK-mel-110 cells after pre-treatment with CP-31398, followed by treatment with CPT. SK-mel-110 cells were pre-treated with 8 μg/ml of CP-31398 for 8 h (A) or 12 h (B), followed by treatment with CPT alone for 24 h or treatment with CP-31398 and CPT for 24 h. Survival of CP-31398 and CPT-treated cells was determined by SRB staining. These experiments were performed at least three times and the results presented here are representative of each experiment.
followed by treatment with CPT for 24 h. Pre-treatment with CP-31398 for 8 or 12 h did not enhance cell death induced by CPT in either MMRU (Figure 5.4) or SK-mel-110 (Figure 5.5) cells. However, treatment of MMRU and SK-mel-110 cells with CP-31398 for 32 or 36 h is toxic to the cells. PI staining and flow cytometry analysis of the pre-G1 population showed no enhancement of CPT-induced cell death by pre-treatment with CP-31398 in MMRU cells (Figure 5.4C), confirming the cell survival results. Western blotting showed that combination treatment with CP-31398 and CPT did not upregulate p53 protein levels more than CPT treatment alone for 24 h in both cell lines (Figure 5.6), suggesting that CP-31398 does not enhance p53 protein levels during CPT-induced cell death. In addition, pre-treatment of MMRU cell with CP-31398 did not enhance cell death induced by cisplatin (an alkylating agent) or vincristine (an antimitotic agent) (Figure 5.7), chemotherapeutic agents with different modes of action from CPT.

5.3 Discussion

5.3.1 CP-31398 does not have a role in chemosensitivity in human melanoma cell lines

The results show that CP-31398 upregulates p53 protein levels in both wild-type (MMRU) (Figure 4.1) and mutant (SK-mel-110) (Figure 4.7C) p53 melanoma cell lines, but does not sensitize these cells to CPT either by co-treatment (Figure 5.2) or pre-treatment (Figure 5.4, 5.5). Western blot analysis showed treatment with CPT alone significantly upregulated p53 protein levels and that pre-treatment with CP-31398, followed by treatment with CPT did not increase p53 levels further (Figure 5.6). The finding that CP-31398 did not synergistically increase p53 levels after CPT treatment may account for the lack of enhancement of CPT-induced cell death by CP-31398. Furthermore, pre-treatment with CP-31398 also did not enhance cell death induced by other chemotherapeutic drugs, such as vincristine and cisplatin (Figure 5.7).
Figure 5.6  p53 protein levels in MMRU and SK-mel-110 cells treated with CP-31398 and/or CPT.  A. Western analysis for p53 protein levels was performed on protein extracted from MMRU cells treated with 8 μg/ml of CP-31398 alone for 12 h, cells treated with 200 nM of CPT alone for 24 h, or cells pre-treated with 8 μg/ml of CP-31398 for 12 h, followed by treatment with 200 nM CPT for 24 h. Anti-p53 DO-1 was used as primary antibody and β-actin as a loading control. B. Western analysis for p53 protein levels was performed on protein extracted from SK-mel-110 cells treated with 8 μg/ml of CP-31398 alone for 12 h, cells treated with 800 nM of CPT alone for 24 h, or cells pre-treated with 8 μg/ml of CP-31398 for 12 h, followed by treatment with 800 nM CPT for 24 h. Anti-p53 DO-1 was used as primary antibody and β-actin as a loading control. These experiments were performed at least twice and the results presented here are representative of each experiment.
**Figure 5.7** Survival of MMRU cells after pre-treatment with CP-31398, followed by treatment with other chemotherapeutic drugs. MMRU cells were pre-treated with 8 μg/ml of CP-31398 for 12 h, followed by treatment with cisplatin (A) or vincristine (B) for 24 h. Cell survival was determined by SRB staining. These experiments were performed at least three times and the results presented here are representative of each experiment.
Our results are contradictory to those by Takimoto et al. (2002), who found that combination treatment of a wild-type lung cancer (H460) and a mutant colon cancer (DLD1) cell line with CP-31398 and chemotherapeutic agents (etoposide, adriamycin, cisplatin) enhanced apoptosis induced by the chemotherapeutic agents additively. Differences in cell lines and treatment conditions may account for the discrepancies. Firstly, we used melanoma cell lines, which may be more chemoresistant than other types of cancer cell lines. Secondly, Takimoto et al. (2002) used cell lines that underwent cell cycle arrest when treated with CP-31398 or chemotherapeutic agents alone but underwent apoptosis when treated with combination CP-31398 and drugs. The authors suggest that a secondary signal from the chemotherapeutic drugs may cause a switch from cell cycle arrest to apoptosis in the CP-31398-treated cells through CP-31398 augmentation of p53 function. Perhaps CPT treatment alone in melanoma cells caused sufficient apoptosis so that CP-31398 treatment has no augmentation affect. Although we show that CP-31398 does not sensitize melanoma cells to chemotherapy-induced cell death, CP-31398 still has the potential to be used in the sensitization of other types of cells to chemotherapy.

A few studies have shown a benefit to the use of two apoptotic inducing agents in the treatment of tumours. For instance, combination therapy with lovastatin and butyrate showed a synergistic anti-tumour affect involving apoptosis and p53 against murine Lewis lung carcinoma in vitro and in vivo (Giermasz et al., 2002). Combination treatment of human retinoblastoma Y79 cells with sodium phenylbutyrate and topotecan, a topoisomerase I inhibitor, also had a synergistic apoptotic effect (Calvaruso et al., 2001). Perhaps co- or pre-treatment with CP-31398 in combination with more than one chemotherapeutic agent may be beneficial in the treatment of cancer.
6.1 Summary

This work was initiated to elucidate the molecular mechanisms mediated by CP-31398. We found that CP-31398 stabilizes wild-type p53 to induce apoptosis, confirming the results of other studies (Foster et al., 1999; Takimoto et al., 2002). Apoptosis occurred at least in part by the mitochondrial pathway through the upregulation of Bax and Bak, change in mitochondrial membrane potential leading to the release of cytochrome c and activation of caspase-9. We next sought to determine if CP-31398 has a role in the p53 stress response to UVB irradiation and chemotherapy. We found that CP-31398 was able to enhance apoptosis induced by UVB in a wild-type p53 melanoma cell line. Apoptosis in these circumstances was found to also involve the mitochondrial pathway. Surprisingly, CP-31398 did not enhance cell death induced by chemotherapeutic drugs in either a wild-type or mutant p53 melanoma cell line, contrary to a recent study (Takimoto et al., 2002). Melanoma cell lines were chosen due to the chemoresistant phenotype of melanoma and the belief that wild-type p53 function in these cells may be impaired; thus, CP-31398 may be able to restore wild-type p53 function in these cells in stress situations.

Studies have shown that CP-31398 has many biological functions depending on the cell type and stimuli (Foster et al., Rippin et al., 2002; Takimoto et al., 2002). CP-31398 has been shown to induce cell cycle arrest and apoptosis, through p53-dependent and p53-independent mechanisms. CP-31398 is also involved in UVB- and chemotherapy-induced apoptosis in a cell type dependent manner. Furthermore, CP-31398 can enhance the transcription of p53 and non-p53 target genes. Taken together, determining the cellular responses to CP-31398 in different cellular contexts can establish it as a chemotherapeutic agent.
6.2 Future Directions

p53 is involved in both the mitochondrial- and death receptor-mediated apoptotic pathways. Furthermore, activation of the TNFR1/Fas death receptor pathway leads to cleavage of cytosolic BID to truncated tBID by caspase-8, which induces oligomerization of Bax and Bak, resulting in cytochrome c release (Wei et al., 2000, 2001; Luo et al., 1998), thus providing a link between the death receptor and mitochondrial pathway. It would be interesting to see if the death receptor pathway is involved in CP-31398-induced apoptosis and if cross-talk between the two pathways is involved. p53-dependent apoptosis also involves transactivation-independent mechanisms, which may also be involved in CP-31398-induced apoptosis. The pathway involved in apoptosis induced by CP-31398 in mutant p53 cells also remains to be elucidated. It would also be of interest to determine if CP-31398 has a role in other cellular processes that involve p53, such as DNA repair of UV-induced DNA damage or cellular senescence, and those that are independent of p53.

In light of the recent finding that CP-31398 intercalates into DNA, the p53-dependent apoptosis in HCT116+/+ cells and the enhancement of UVB-induced apoptosis in MMRU cells after CP-31398 treatment may be explained differently. CP-31398 may indirectly upregulate and stabilize p53 to induce apoptosis by intercalating into DNA and causing DNA damage. This hypothesis is yet to be tested and much remains to be learned about the molecular mechanisms mediated by CP-31398.
REFERENCES


Chernov MV, Stark GR. The p53 activation and apoptosis induced by DNA damage are reversibly inhibited by salicylate. *Oncogene* 1997, 14: 2503-10.


Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995, 81: 505-12.


Hupp TR, Meek DW, Midgley CA, Lane DP. Regulation of the specific DNA binding function of p53. *Cell* 1992, 71: 875-86.


Reinke V, Lozano G. The p53 targets mdm2 and Fas are not required as mediators of apoptosis in vivo. *Oncogene* 1997b, 15: 1527-34.


Rizos H, Darmanian AP, Holland EA, Mann GJ, Kefford RF. Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF. *J. Biol. Chem.* 2001a, 276: 41424-34.


Wang W and El-Deiry WS. CP-31398 stabilized wild-type p53 by inhibiting p53 ubiquitination without inducing serine 20 phosphorylation or reduced interaction with MDM2. AACR abstract #4137 2002.


