2. Materials and Methods

2.1 Cell culture

Human U2OS osteosarcoma cells (ARF-null, p53 wild type), SAOS-2 osteosarcoma cells (ARF wild type, p53-null), HCT116 colorectal cancer cells (ARF-mutant, p53 wild type), WMM1175 melanoma cells (ARF-null, p53-null) (Becker *et al.*, 2005), NARF2/NARF2_E6 osteosarcoma cells, H1299 non-small cell lung cancer cells, HEK293T human embryonic kidney cells and WS1 primary human skin fibroblasts (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Trace Scientific, Sydney) supplemented with 10% fetal bovine serum (FBS) and glutamine. All cells were cultured in a 37°C incubator with 5% CO_2. NARF2 and NARF2_E6 cells are derived from U2OS osteosarcoma cells and were a kind gift from Dr Gordon Peters (Cancer Research UK, London Research) and have been described previously (Rodway *et al.*, 2004).

2.1.1 Creation of U2OS_ARF and WMM1175_ARF cell lines

The IPTG (isopropyl ß-D-thiogalactopyranoside)-inducible mammalian expression system (Lac-switch system; Stratagene, La Jolla, California) was used to obtain U2OS_ARF and WMM1175_ARF cell clones carrying the stably integrated *p14ARF* gene under IPTG-inducible expression control. Full length *p14ARF* cDNA was cloned into the *POPRSVICAT* expression plasmid, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, California), into a WMM1175 and U2OS clone expressing the

lac repressor. Transfected cells were selected with hygromycin (200µg/ml; Invitrogen) and geneticin (500µg/ml; Invitrogen).

2.2 IPTG Induction Experiments

Stable cell clones were seeded 24 h prior to induction, and were induced to express p14ARF with 1mM IPTG. To ensure media and IPTG were not exhausted during experiments running for six or nine days, a further 5mL of media and 1mM IPTG were added per 75cm^2 flask every three days. All experiments were performed at least twice in duplicate unless otherwise indicated and representative plots shown. Percentages ± standard deviation are given where appropriate.

2.3 Transient Cell Transfections

For cell cycle distribution analysis of transiently transfected cells, cultured cells (1 x 10^6) were seeded in T75 flasks and co-transfected with the *p14ARF-FLAG5b* or *pCMV-FLAG5b* plasmid (6µg) and *pCMVEGFP-spectrin* (2µg) (marker of transfection and kindly provided by Dr Tom Shenk, Howard Hughes Medical Research Institute, USA) using Lipofectamine 2000 (Invitrogen). For Bcl-2 transfections, U2OS_ARF cells (2 x 10^5) were seeded in a T25 and transfected with *pCMVEGFP-Bcl-2* (1µg) or *pCMVEGFP-spectrin* (1µg) using Lipofectamine 2000. After 24 h the media was replaced with fresh media containing the cytotoxic drug and PBS or 1mM IPTG and the cells analysed three days later. *pCMVEGFP-Bcl-2* was a kind gift of Dr Mariana Brocardo (Westmead Millennium Institute, Sydney).

DNA was prepared for transfections using midi- and maxi-plasmid kits (Jet Star, Genomed Inc., Bad Oeynhausen, Germany). Bacteria (XL-1 Blue) were grown in Super Broth (12 g/L tryptone; 14 g/L yeast; 5 mL/L glycerol containing 28 mM KH_2PO_4; 71.4 mM K_2HPO_4).

2.4 Cell cycle and apoptosis assays by flow cytometry

Adherent and floating cells were combined and sub-divided into samples for cell cycle, cell death and protein expression analysis. For cell cycle analysis, cells were fixed in 70% ethanol at 4°C for at least 1 h, washed in PBS and stained with propidium iodide (50ng/µl) (Sigma, St. Louis, Missouri) containing ribonuclease A (50ng/µl) (Worthington Biochemical Corp, Lakewood, New Jersey). DNA content from at least 2000 cells was analysed using ModFIT software (Verity Software, USA). Numbers of cells with sub-G1 content were determined using CellQuest software (Becton Dickinson, Franklin Lakes, New Jersey). To assess cell size, the forward scatter of at least 2000 unfixed cells was measured.

Annexin-V staining was performed using FITC- or APC- conjugated annexin-V as detailed by the manufacturer (BD Biosciences, Franklin Lakes, New Jersey).

JC-1 and CMX-Ros assays were performed by sedimenting harvested cells at 2000 rpm for 5 min on a desk-top centrifuge then resuspending the cells in warm complete DMEM containing 10µM JC-1 (Molecular Probes, Eugene, Oregon) or 100nM CMX-Ros (Mitotracker, Invitrogen). Cells were incubated at 37˚C for 20 or 30 min,

respectively in the dark, washed once in PBS, then resuspended in PBS and analysed by flow cytometry.

Where transfected cells were used, cells containing the transgene were selected by flow cytometry using EGFP-spectrin as a marker. Flow cytometry was performed on a FACSCanto or FACSCalibur (Becton Dickinson, Franklin Lakes, New Jersey) and results analysed using FACS Diva or Cell Quest Pro software (Becton Dickinson).

2.5 Western Blotting

Proteins were extracted for 1 h at 4°C using radio-immunoprecipitation (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) with protease inhibitors (Roche, Basel, Switzerland). The protein concentration was determined with the Dc Protein Assay Kit (Bio-Rad, Hercules, California). Proteins (30-50µg) were resolved on 12% or 15% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, Massachusetts) at 100V for 1h (wet transfer) or 15mA/cm^2 for 2 h (semi-dry transfer) in Bio-Rad transfer apparatus. Membranes were blocked with 5% skim milk powder in Tris-buffered saline (TBS). Membranes were probed with primary antibody diluted in 0.5% Tween/TBS (TTBS) for at least two h (Table 2.1), then subjected to three washes of five minutes each. Secondary antibodies were diluted 1:6000 in TTBS and applied for one hour at room temperature. The membranes were then washed four times in TTBS for 15-20 minutes each and developed with Western Lightning enhanced chemiluminescence solution (Perkin Elmer, Waltham, Massachusetts) using Kodak X-OMAT BT film.

Antibodies used to in immunostaining and western blots

Antibody	Clone	Company
Primary Antibodies		
ß-actin	AC-74	Sigma
Bax	B-9	Santa Cruz
Bcl-2	100	Santa Cruz
Bcl-xL[1]	H-5	Santa Cruz
Bid[1]	2002	Cell Signaling
Bim	H-191	Santa Cruz
Active caspase 3[1]	C92-605	BD-Pharmingen
Caspase 8 p20	D-8	Santa Cruz
Casp 9 p10[1]	F-7	Santa Cruz
E2F-1	KH20	Neomarkers
FAS	C-20	Santa Cruz
FASL	NOK-1	Santa Cruz
GADD45	H-165	Santa Cruz
HSP-70	NA	Affinity BioReagents
Mcl-1[1]	NA	Santa Cruz
NOXA	114c307	Calbiochem
p14ARF	DCS-240	Sigma
p14ARF	Polyclonal	Abcam
$p16^{INK4a}$	SC-467	Santa Cruz
p21	6B6	BP Phamingen
p53	FL-393	Santa Cruz
p53	C-19	Santa Cruz
PUMA	Polyclonal	Cell Signaling
Survivin	60.11	Novus Biologicals
Tubulin-A	236-10501	Molecular Probes
PARP[1]	Polyclonal	Cell Signaling
Secondary Antibodies		
α-mouse-HRP[2]		DAKO
α-rabbit-HRP[2]		DAKO
α-mouse-Texas Red	T6390	Molecular Probes
α-rabbit-FITC		Molecular Probes

[1]Antibody diluted in 1% skim milk powder/TTBS. [2]HRP, Horse radish peroxidase. NA, Not available

2.5.1 Western Blotting Buffers and Gels

TBS (Tris-buffered saline)

0.9% NaCl in 20mM Tris-HCl pH 7.4

TTBS (Tween/Tris-buffered saline)

0.5% Tween20 in TBS

SDS Running Buffer

25mM Tris, 250mM Glycine, 0.1% SDS

Transfer Buffer

25mM Tris, 250mM Glycine, 20% Methanol

5% Stacking gel

5% acrylamide:bis-acrylamide 29:1 (Amresco, Cleveland, Ohio), 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% APS (Ammonium persulfate), 0.1% TEMED (N,N,N',N'-Tetramethylethylenediamine)

12% Resolving gel

12% acrylamide:bis-acrylamide 29:1, 375mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, 0.1% TEMED

15% Resolving gel

15% acrylamide:bis-acrylamide 29:1, 350mM Tris-HCl pH 8.8, 0.1%SDS, 0.1% APS, 0.1% TEMED

2.6 Indirect immunofluorescence

Cultured cells (1 x 10^5) seeded on coverslips in six-well plates were washed in PBS and fixed in 3.7% formaldehyde and then permeabilised with 0.1% triton-X/PBS. Cells were immunostained for 50 min with monoclonal mouse anti-p14ARF antibody (Sigma) or rabbit anti-p53 (Santa Cruz, Santa Cruz, California) followed by a 50 min exposure to

Texas red-conjugated anti-mouse secondary IgG or FITC-conjugated anti-rabbit IgG (Molecular Probes). Nuclei were visualized by Hoechst 33258 (Sigma) staining (2μg/ml). Coverslips were mounted on glass slides using either vector-shield (Vector Laboratories) or a mounting solution of 3% N-propyl galate dissolved in a 1:1 solution of PBS/glycerol.

2.7 Cytotoxic and chemosensitivity assays

Unless otherwise indicated, for chemosensitivity assays the media was changed 24 h after seeding and replaced with fresh media containing the cytotoxic drug and PBS or 1mM IPTG to induce p14ARF expression. p14ARF accumulates in U2OS_ARF cells 8 to 10 hours after the addition of IPTG, and arrest is detectable approximately 16h post IPTG treatment (data not shown). After three days of incubation, floating and adherent cells were harvested for analysis. For cells treated with UVB, media was removed from 10cm round dishes and replaced with 100uL/cm^2 warm PBS. Cells were then subject to 60J/m^2 UVB emitted at 115nm by an Ultra-Lum UVB lamp (Ultra-Lum Inc. Carson, California), then the PBS removed and the cells allowed to recover in media containing PBS or 1mM IPTG for three days. UVB output was measured with an IL1700 Radiometer (International Light, Peabody, Massachusetts). For cells treated with Adriamycin, cells were exposed to media containing 1μM Adriamycin in the presence or absence of 1mM IPTG for 6 h. Adriamycin containing media was removed, cells rinsed with fresh media, and then incubated with media containing either PBS or 1mM IPTG for three days. For UVC-treatment, media was removed and cells irradiated at 254nm at the indicated dose using a Stratalinker 1800 (Stratagene).

Cytotoxic Reagents used in chemosensitivity screen.

Drug	Company
Adriamycin	Pfizer
Camptothecin	Calbiochem
Cisplatin	Pfizer
UVB 115nM	Ultra-Lum
Actinomycin D	Sigma
DRB	Calbiochem
Dacarbazine	Sigma
Temozolomide	Schering-Plough
Melphalan	Sigma
Sodium Butyrate	Sigma
Trichostatin A	Sigma
Valproic Acid	Sigma
Taxol	Sigma

2.8 Inhibitors of apoptosis pathways

Cells were seeded in 6 well plates ($9x10^4$) and 24 h later the media removed and replaced with fresh media containing the inhibitor or appropriate control substance (listed below). The cytotoxic drug and IPTG/PBS were added to the cells after th e appropriate pre-treatment time. Cells were harvested three days later. Positive controls to check effective inhibition were 30μM etoposide (Sigma) for BIP-V5 and for NOK-1; 2ng/mL recombinant FasL/TNFSF6 (R&D systems, Minneapolis, Minnesota) which was added to cells with 10μg/mL anti-6xHis cross-linking antibody (R&D systems).

List of inhibitors used

Inhibitor	Concentration	Pre-treatment length	Company
z-VAD-fmk, caspase inhibitor	50μM	3h	BD Pharmingen
Bax inhibiting peptide V5 (BIP-V5)	100μM	5h	Calbiochem
Bax inhibiting peptide negative control (BIP-NC)	100μM	5h	Calbiochem
Fas inhibiting NOK-1	400ng/mL	3h	Santacruz
IgG	400ng/mL	3h	Sigma

2.9 Generation of cell pools with gene knock-down using lentivirus

The U2OS_ARF^{si_p53}, U2OS_ARF^{si_p21} and U2OS_ARF^{si_Luc} cell lines were created from the U2OS_ARF cell line and are pools of U2OS_ARF cells with knockdown of p53, p21^{Waf1} or the control knockdown against the luciferase gene, and were generated by stable genomic integration of a short hairpin RNA (shRNA) expression vector (*pSIH-HI-coGFP*; System Biosciences) using a lentivirus system. The *pSIH-H1-coGFP* vector also encodes the Copepod green fluorescent protein (similar to regular EGFP but with brighter colour) as a reporter for transduced cells. The lentivirus expression system used to generate these cell pools was based on a third generation lentivirus system developed by Dull *et al* (Dull *et al.*, 1998). This retroviral system uses three essential genes from the HIV-1 virus, is replication incompetent and stably integrates the target gene into the genome of most human cell types with high efficiency.

2.9.1 Lentivirus Plasmids

Plasmids encoding the shRNA sequence were constructed by ligating the oligonucleotide sequence into the BamH1/EcoR1 site of the *pSIH1-H1-copGFP* vector and transforming into XL10-Gold bacteria (Stratagene). Plasmids were sequenced to confirm the sequence using the primers:

Fwd 5'-AATGTCTTTGGATTTGGGAATCTTAT-3'

Rev 5'-TGGTCTAACCAGAGAGACCCAGTA-3'

The *luciferase_siH_H1_coGFP* control plasmid was provided by System Biosciences. shRNA sequences (in capitals), with appropriate spacers were:

p53 (Brummelkamp *et al.*, 2002):

gatccGACTCCAGTGGTAATCTACcttcctgtcagaGTAGATTACCACTGGAGTCtttttg

$p21^{Waf1}$ #1 (Zhang *et al.*, 2004):

gatccGACCATGTGGACCTGTCACcttcctgtcagaGTGACAGGTCCACATGGTCtttttg

$p21^{Waf1}$ #2 (Nicke *et al.*, 2005):

gatccCTTCGACTTTGTCACCGAGcttcctgtcagaCTCGGTGACAAAGTCGAAGtttttg

2.9.2 Making Lentiviral Stocks

HEK 293T cells were seeded in 8 x T150s with $1x10^7$ per flask and 20mL of DMEM supplemented with 10% FCS and glutamine. 24 h after seeding, the transfection mixture was prepared by mixing 160µg each of the three viral plasmids *pRSV-REV*; *pMDg/VSVG*; *pMDg/pRRE* and 320µg of the shRNA *pSIH1-H1-copGFP* plasmid in a final volume of 10.8 mL 0.1 x TE then adding 1.2 mL of 2.5 M $CaCl_2$. 12 mL of 2 x HEPES was added dropwise to the solution whilst mixing constantly. The mixture was allowed to stand for 20 minutes then 3 mL added to each flask and the cells incubated for 6 h. The media was removed from cells and replaced with 15 mL Optimem supplemented with glutamine. Cells were incubated for 72 h to allow virus particles to be produced and released into the media.

2.9.3 Lentiviral Transfection Reagents

0.1 x TE Buffer: 100 mM Tris-HCl pH 8.0, 10 mM EDTA
2.5M $CaCl_2$
2 x HEPES Buffer: 280 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4 pH 7.1

2.9.4 Lentivirus harvesting

To harvest the virus, supernatant was collected from all flasks, filter sterilised through a 0.44µm filter, then concentrated in a Centricon Ultra 70 (Millipore) filtration unit with 100kDa cut-off by centrifugation at 3000 rpm. Concentrated virus stocks were collected from the Centricon unit by inverting and centrifuging at 2000 rpm for 5 min, then aliquoted into screw capped tubes and stored at -70˚C until use.

Virus titres were determined by transducing U2OS cells with a range of virus concentrations and analysing the percentage of green cells using flow cytometry after 48 h. The titre was determined using the following calculation:

Viral particles/mL= (% positive cells/100 x Number of cells seeded) x (1/volume of virus added in mL).

2.9.5 Transduction of U2OS_ARF cells with lentivirus

24 h before transduction, $1x10^6$ U2OS_ARF cells were seeded in a T75. The cells were transduced by adding the lentivirus at a multiplicity of infection (MOI) of 10 to DMEM supplemented with 10% FCS, glutamine and containing 8µg/mL polybrene (Sigma). After 20 min incubation, media was removed from the cells and replaced with media containing the virus. After 48 h, cells were passaged into a T150 and immediately transduced for a second time at the same MOI. Final transduction efficiency was confirmed to be over 95% as determined five days later by flow cytometry analysis of green cells. Western blotting was used to determine levels of p53 and $p21^{Wafl}$ following IPTG induction of p14ARF expression for 48 h.

2.10 Cell fractionations

To ensure high p14ARF expression, U2OS and U2OS_ARF cells were seeded in T150's at $2x10^6$ and transfected the next day with *p14ARF-FLAG5b* or treated with IPTG and 50μM DRB respectively. Cells were harvested after 48 h and fractionated into cytoplasmic and mitochondrial fractions. Fractionation experiments were kindly performed by Suzanah Phillips. Trypsinised cells were washed in PBS then resuspended in 1mL of Buffer 1 (10mM Hepes pH 7.9, 15mM KCl, 2mM $MgCl_2$, 0.1mM EDTA and protease inhibitors (Roche)). Following 10 minutes incubation on ice, NP-40 was added to a final concentration of 0.02% (v/v), the cells vortexed briefly then centrifuged at 1000xg for 5 minutes are 4˚C. The supernatant, which contains the cytosolic fraction was collected. The pellet, consisting of the mitochondrial and nuclear fractions was resuspended and washed in 500μL Buffer 1, then resuspended in Disruption buffer (Qproteome kit, QIAGEN). The lysate was drawn through a 25 gauge needle seven times and centrifuged at 1000×g for 10 min at 4˚C. The pellet, consisting of nuclear fraction and cell debris was resuspended in RIPA buffer with protease inhibitors (Roche) to extract proteins. Mitochondria were retrieved from the supernatant by centrifugation at 6000×g for 10 min at 4˚, followed by washing in Mitochondrial Storage Buffer (Qproteome kit, QIAGEN). Proteins were extracted from mitochondria using RIPA buffer.

2.11 Chapter 6 materials and methods

Materials and methods used in Chapter 6 are presented here and include materials and methods used by co-authors to generate the paper that resulted from this chapter. All methods used are presented together to facilitate understanding of the experimental design as a whole. The contribution of individual authors may be found in Appendix 1.

2.11.1 Patients

Fresh-frozen tumour excision biopsies were obtained from 30 consecutive consenting patients undergoing elective therapeutic ILI at the Sydney Melanoma Unit. These patients had not undergone prior systemic therapies. Catheters were inserted percutaneously into the axial artery and vein of the affected limb and a pneumatic tourniquet was inflated proximally. Melphalan (7.5 mg/litre of tissue) and actinomycin-D (75 μg/litre of tissue) were rapidly infused into the arterial catheter whilst the limb was isolated. The infusate was then circulated for 20-30 minutes by catheter using a syringe attached to a three-way tap. Finally the limb was flushed with saline before the tourniquet was released and the catheters were removed (Thompson *et al.*, 1998).

Patient details are shown in Table 6.1. All had lower limb skin metastases and were eligible for the study if at least one lesion greater than 10 mm3 in volume could be excised, leaving at least one other lesion in the same field that could be evaluated for response. Response was determined by standard RECIST criteria at four-week intervals until disease progression, recorded by digital photography and confirmed by independent review of these records.

2.11.2 RNA extraction and cDNA synthesis

The excised lesion was frozen immediately in liquid nitrogen. Samples were subsequently trimmed of surrounding tissue and RNA extracted using Trizol (Sigma) and purified using a RNeasy column (Qiagen). Samples with evidence of melanin contamination were cleaned by heating to 65°C for 5 minutes during Trizol extraction

or by using Bio-Spin P-30 Tris columns (Bio-Rad). cDNA was synthesised from 100ng-1µg of RNA using Superscript III (Invitrogen) with oligo-dT primers. No-Superscript III enzyme controls were performed to ensure there was no contaminating genomic DNA.

2.11.3 Real-time RT-PCR

Real-time RT-PCR was performed using a Corbett Rotorgene 3000 with Platinum SYBR Green qPCR Master Mix (Invitrogen) for MITF. For the remaining genes Taqman probes were used, the master mix used for $p16^{INK4a}$, 18S and hP0 was Platinum qPCR SuperMix-UDG (Invitrogen) and Brilliant qPCR Master Mix (Stratagene) for p14ARF. DMSO 3% was added to $p16^{INK4a}$ and p14ARF reactions and Mg2+ increased to 5mM in p14ARF reactions. Primers used were:

MITFm fwd:	CCGTCTCTCACTGGATTGGTG
MITFm rev:	GCTTGCTGTATGTGGTACTTGG
$p16^{INK4a}$ fwd:	GCCCAACGCACCGAATAG
$p16^{INK4a}$ rev:	ACGGGTCGGGTGAGAGTG
$p16^{INK4a}$ probe:	FAM6-TCATGATGATGGGCAGCGCC-TAMRA (A/B 450025)
p14ARF fwd:	CTACTGAGGAGCCAGCGTCT
p14ARF rev:	ACGGGTCGGGTGAGAGTG
p14ARF probe:	FAM6- TCATGATGATGGGCAGCGCC-TAMRA (A/B 450025)

Equal loading was ensured by adjusting samples to the same average expression levels for the ribosomal proteins human P0/36B4 and 18S. hP0 and 18S levels were measured by real-time RT-PCR using VIC-labelled PDAR probes (Applied Biosystems).

2.11.4 Mutation status

Mutation status of N-RAS codon 61 was determined by RT-PCR followed by sequencing. The region to be sequenced was amplified by PCR, cleaned up with ExoSapIT (USB Corporation, Cleveland, Ohio, USA), and sequenced on an ABI PRISM 3100 (Applied Biosystems). Primers used were: N-RAS fwd: CTG ACA ATC CAG CTA ATC and N-RAS rev: GTC TTT TAC TCG CTT AAT CTG.

Mutation status of B-RAF at codon 600 was determined by SSCP (single stranded conformational polymorphism) analysis. Briefly, a 228bp fragment of B-RAF spanning codon 600 was amplified by RT-PCR (forward primer: ATA GAT ATT GCA CGA CAG AC; reverse primer: ATC TTG CAT TCT GAT GAC TTC). 6µl of PCR product was placed in formamide/EDTA loading buffer and denatured at 95ºC for 10 minutes then placed on ice for 2 minutes. Samples were run on an SSCP gel (10% polyacrylamide gel containing 1x Tris-glycine buffer and 5% glycerol) for 3.5 h at 22ºC and then the gel was silver stained (reagents listed below). Samples containing mutations showed up as aberrant bands on the gel, and were sequenced to confirm the mutation.

Mutation status of PTEN cDNA was determined by sequencing overlapping RT-PCR products as described in (Liu and Kagan, 1999). Partial PTEN sequences were obtained for tumour samples 006 and 026 due to limited RNA.

All sequences were analysed by visual inspection and using ANGIS Biomanager (www.angis.org.au)

2.11.5 Statistical analysis

The data were analysed using SPSS for Windows 15.0. Two-tailed tests with a significance level of 5% were used throughout. $p16^{INK4a}$, p14ARF and MITFm expression levels were log transformed to approximate normality prior to analysis. Response was considered as a dichotomous outcome, with patients showing complete or partial response classified as responding and patients with stable disease or progressive disease classified as not responding. Logistic regression analysis was used to test for association between all variables and the presence of response. The odds ratios and their 95% confidence intervals were used to quantify the degree of association between the independent predictors of response identified using multiple logistic regression with backward stepwise variable selection. Spearman rank correlation was used to quantify the degree of association between $p16^{INK4a}$ and MITFm.

2.11.6 Cell culture

In cytotoxicity assays, cells were exposed to 1mM IPTG for 4 days, followed by the addition of media containing IPTG and drugs to a final concentration of 25nM actinomycin-D or 200μM Melphalan for a further 24h.

2.11.7 Cell cycle and apoptosis analysis

Adherent and floating cells were combined and sub-divided into samples for cell cycle, cell death and protein expression analysis. For cell cycle analysis, cells were fixed in 70% ethanol at 4°C for 1 h, washed in PBS and stained with propidium iodide (50ng/μl) containing ribonuclease A (50ng/μl). DNA content from at least 2000 cells was analysed using ModFIT software. Western blots were probed for $p16^{INK4a}$ and ß-actin.

2.11.8 Single stranded conformational polymorphism reagents

Formamide/EDTA loading buffer

95% formamide, 10mM EDTA, 0.025% bromophenol blue/xylene (Sigma)

Single stranded conformational polymorphism (SSCP) Gel

10% acrylamide:bis-acrylamide 37.5:1 (Amresco), 50mM Tris, 380mM glycine, 5% glycerol, 0.05% APS, 0.05% TEMED

2.11.9 Silver staining

The gel was immersed in fixative for 5 min, washed 3 x 2 min in milliQ water then immersed in staining solution for 20 min. The gel was washed briefly in milliQ water then developed in chilled (8-10˚C) developing solution until the bands were visible. The developing reaction was stopped by immersing the gel in stop solution at 4˚C for 5 min. Gels were dried under vacuum at 80˚C for 60 minutes and then photographed.

Fixative and stop solution

7.5% acetic acid

Staining Solution

Freshly prepared 0.1% silver nitrate, 0.15% formaldehyde.

Developing solution

3% sodium carbonate, 0.15% formaldehyde, 0.0002% sodium thiosulphate.

3. Enforced expression of p14ARF induces p53-dependent cell cycle arrest but not apoptosis

3.1 Introduction

Escape from apoptosis is an essential step in cancer progression and forms the foundation of a cancer cell's resistance to chemotherapy and radiotherapy (Hanahan and Weinberg, 2000). Accordingly, human tumours with high metastatic potential have a significantly increased resistance to apoptosis (Glinsky *et al.*, 1997; Sierra *et al.*, 2000). Melanoma is an extremely aggressive cancer that disseminates to multiple organs (Balch *et al.*, 2001b), displays low apoptotic indices, such as apoptotic cells and is resistant to current cancer therapies, with complete treatment response after chemotherapy rarely benefiting more than 20% of patients (Glinsky *et al.*, 1997; Grossman *et al.*, 1999; Soengas and Lowe, 2003; Staunton and Gaffney, 1995). The resistance of melanoma to chemotherapeutic drugs has been associated with activation of anti-apoptotic factors such as Bcl-2, Mcl-1 and Bcl-xL (Bush and Li, 2003; Heere-Ress *et al.*, 2002; Thallinger *et al.*, 2003; Zhuang *et al.*, 2007), inactivation of pro-apoptotic effectors including Apaf-1, Bax and Bak (Fecker *et al.*, 2006; Soengas *et al.*, 2001), and the reinforcement of survival signals by members of the MAPK pathway, RAS and BRAF (Houben *et al.*, 2004; Ugurel *et al.*, 2007). Remarkably, melanomas display a low frequency of p53 inactivation despite their extreme chemoresistance, and it is possible that p53 function is disabled in melanomas by inactivation of the upstream p53 regulator p14ARF. p14ARF is frequently altered in melanomas, is a highly penetrant melanoma predisposition gene (Harland *et al.*, 2005; Randerson-Moor *et al.*, 2001; Rizos *et al.*, 2001b) and its inactivation can functionally replace p53 loss during

melanomagenesis in murine models (Chin *et al.*, 1997; You *et al.*, 2002). The impact of p14ARF on cell survival and chemoresistance has not been thoroughly investigated, and in this chapter the influence of p14ARF accumulation on cell survival is explored.

ARF expression quenches inappropriate mitogenic signalling by forcing cells to undergo growth arrest or apoptosis, and this response depends on the cellular context. Murine studies have demonstrated that enforced p19ARF expression in MEFs induces cell cycle arrest (Quelle *et al.*, 1995), whereas cells overexpressing p19ARF together with E1A, Myc or RAS oncogenes undergo apoptosis (de Stanchina *et al.*, 1998; Russell *et al.*, 2002; Zindy *et al.*, 1998). Activation of p19ARF alone can also trigger apoptosis; the ectopic expression of p19ARF from adenoviral vectors induced apoptosis in p53-null MEFs, p53-intact human U2OS cells, and p53-deficient human SAOS-2 cells (Tsuji *et al.*, 2002). The C-terminal region of the murine ARF protein (amino acids 130-169) is important for p19ARF-mediated apoptosis, but this region is not conserved in human p14ARF (Matsuoka *et al.*, 2003). Thus, p14ARF may not be as potent an apoptotic regulator as its murine counterpart. This may explain the lack of extensive apoptosis in human diploid fibroblasts over-expressing Myc, despite obvious upregulation of p14ARF and p53 (Drayton *et al.*, 2003). Some reports have shown that adenoviral expression of p14ARF did not promote apoptosis in p53-null human cells, (Weber *et al.*, 2002; Yarbrough *et al.*, 2002) but induced cell death in p53-intact cells (Deng *et al.*, 2002; Kim *et al.*, 2004; Matsuoka *et al.*, 2003; Yang *et al.*, 2000; Yarbrough *et al.*, 2002). In contrast, another study demonstrated adenoviral expression of p14ARF-induced apoptosis independent of p53 (Hemmati *et al.*, 2002).

In order to clarify the apoptotic function of the human p14ARF tumour suppressor we established inducible expression of p14ARF in p53-intact and p53-deficient human cell lines. Using these cell models the impact of long-term p14ARF induction on cell proliferation and cell death was thoroughly examined. In addition we investigated the role of transient p14ARF expression in normal human fibroblasts and human tumour cells. Our data show that expression of p14ARF alone promoted rapid and potent cell cycle arrest in a p53-dependent manner, but did not induce cell apoptosis.

3.2 Results

3.2.1 Inducible p14ARF promotes cell cycle arrest, but not apoptosis

To investigate the cell cycle regulatory role of p14ARF we established stable p14ARF-inducible clones in the p53-intact U2OS osteosarcoma cell line using an IPTG-inducible expression system. In this system repression of the p14ARF transgene by the lac repressor is relieved by addition of IPTG to the media. A number of clones were tested for p14ARF expression and cell cycle arrest in response to IPTG induction. All clones that showed inducible accumulation of p14ARF via western analysis showed nucleolar ARF distribution, and potent G1 cell cycle arrest that involved p53 stabilisation and $p21^{Waf1}$ induction. The U20S_ARF cell clone was selected for the majority of experiments, as it consistently showed the highest percentage of cells expressing p14ARF (above 80%). The inducible expression of p14ARF was assessed using IPTG concentrations ranging from 0.1mM to 10mM. Maximal expression of p14ARF was detected over this full range (Figure 3.1A) with potent G1 cell cycle arrest 48 h post induction (Figure 3.1B). A concentration of 1mM IPTG was chosen for subsequent experiments, as this low concentration was sufficient for full p14ARF induction and

suitable for long-term experiments. This dose of IPTG had no effect on the cell cycle distribution or survival of the parental U2OS_lac17 cell line, which expresses only the lac repressor, and was used to derive the U2OS_ARF cell clone (Figure 3.1C).

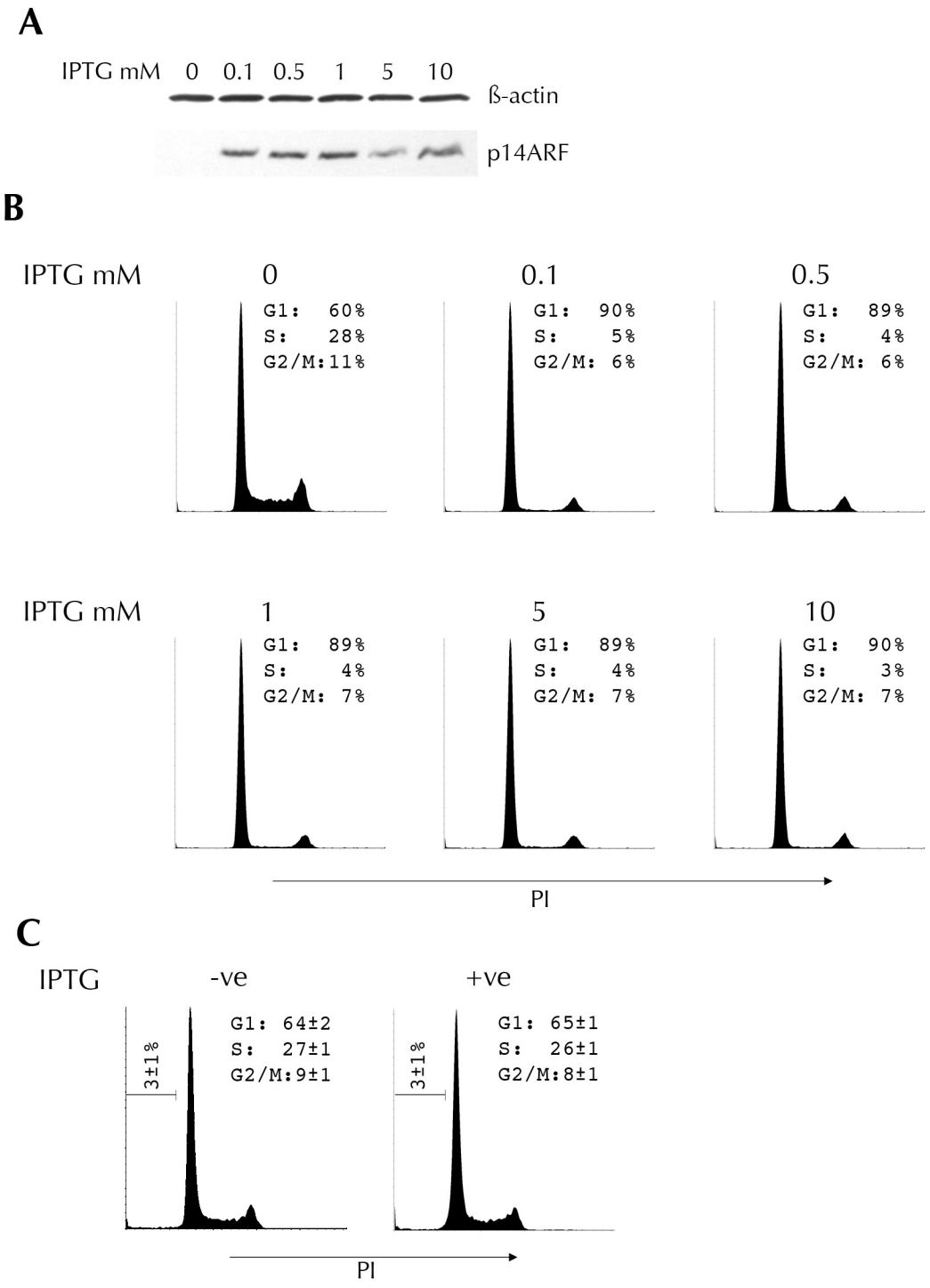


Figure 3.1 Induction of p14ARFexpression by IPTG in U2OS_ARF cells

(A) Expression of p14ARF was determined by western blotting in U2OS_ARF cells after 48 h exposure to a range of IPTG concentrations. Expression of p14ARF was observed in cells treated with 0.1-10mM IPTG. ß-actin was used as a protein loading control.

(B) Flow cytometry was used to examine cell cycle distribution of U2OS_ARF cells 48 h after treatment with a range of IPTG concentrations. DNA content of the cell nuclei was determined using propidium iodide (PI) staining. Single measurements were used.

(C) The influence of 1mM IPTG on the cell cycle distribution of the U2OS_lac17 parental cell line, which only expresses the lac repressor, was determined by flow cytometric analysis of PI stained nuclei, three days post treatment.

To evaluate the influence of p14ARF accumulation on the proliferation and survival of the U2OS_ARF cells, p14ARF expression was induced with 1mM IPTG. Three days post induction these cells behaved as expected, with strong nucleolar expression of p14ARF, increased nuclear p53 (Figure 3.2A), increased expression of the p53-transcriptional target $p21^{Waf1}$ (Figure 3.2B) and potent G1-phase cell cycle arrest with no increase in the proportion of sub-G1 apoptotic cells (Figure 3.2C). Both floating and adherent cells were collected for all measurements of apoptosis using flow cytometry or western blotting to ensure detached cells were also analysed.

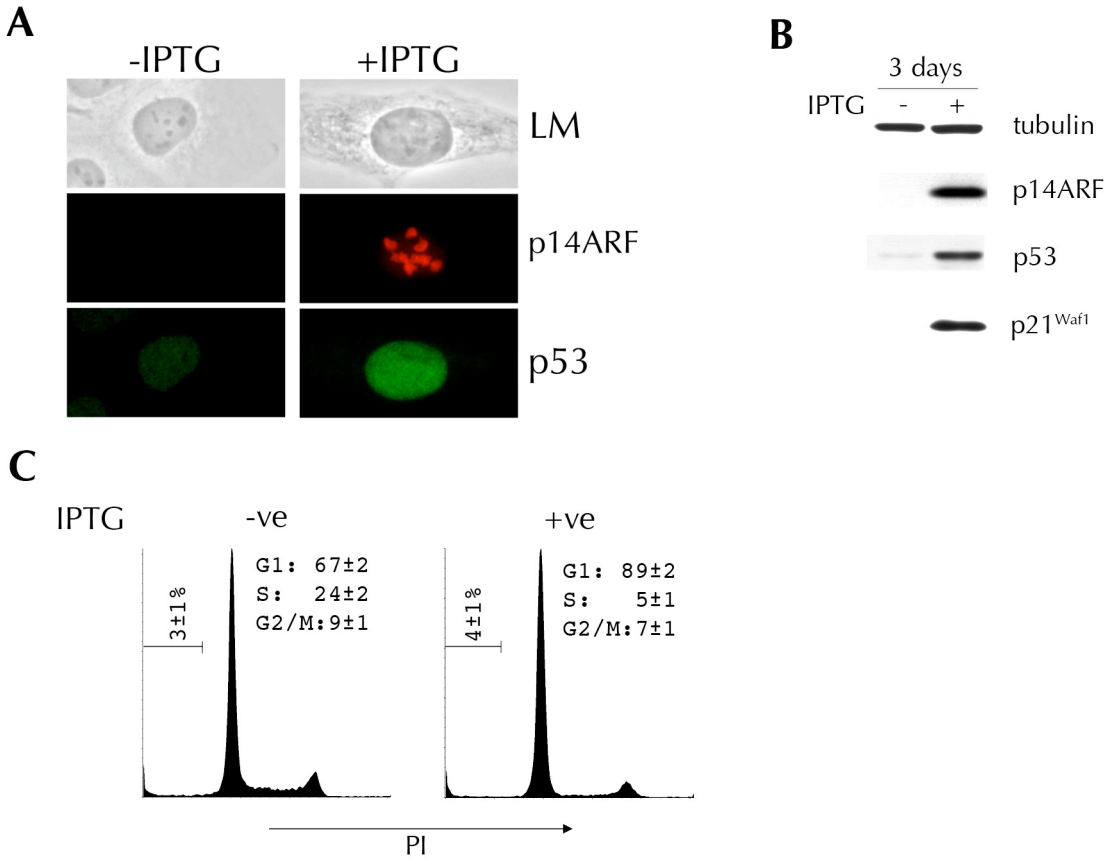


Figure 3.2 Induced p14ARF expression activates p53 in U2OS_ARF cells

(A) The nucleolar accumulation of p14ARF in IPTG treated U2OS_ARF cells was detected by immunofluorescence. Elevation of p53 in the nucleus as a result of p14ARF expression is also shown. LM, light microscopy.

(B) Expression of p14ARF, p53, $p21^{Waf1}$ and tubulin was determined three days after treatment of U2OS_ARF cells with 1mM IPTG (+) or PBS (-).

(C) Flow cytometry was used to examine cell cycle distribution of U2OS_ARF cells three days after treatment with 1mM IPTG. DNA content of the cell nuclei was determined using propidium iodide (PI) staining.

We also confirmed the suitability of U2OS_ARF cells for apoptotic studies by irradiating these cells with low levels (10 J/m^2) of UVC, which efficiently induces caspase dependent apoptosis (Choi *et al.*, 2000; Nishigaki *et al.*, 1999). Flow cytometric analysis of U2OS_ARF cells, three days post-irradiation, confirmed that UVC exposure induced significant apoptosis. Specifically, the sub-G1 population increased from 6±1% to 51±6% (Figure 3.3A) and the proportion of annexin-V positive, apoptotic cells increased from 9±1% to 34±5% (Figure 3.3B).

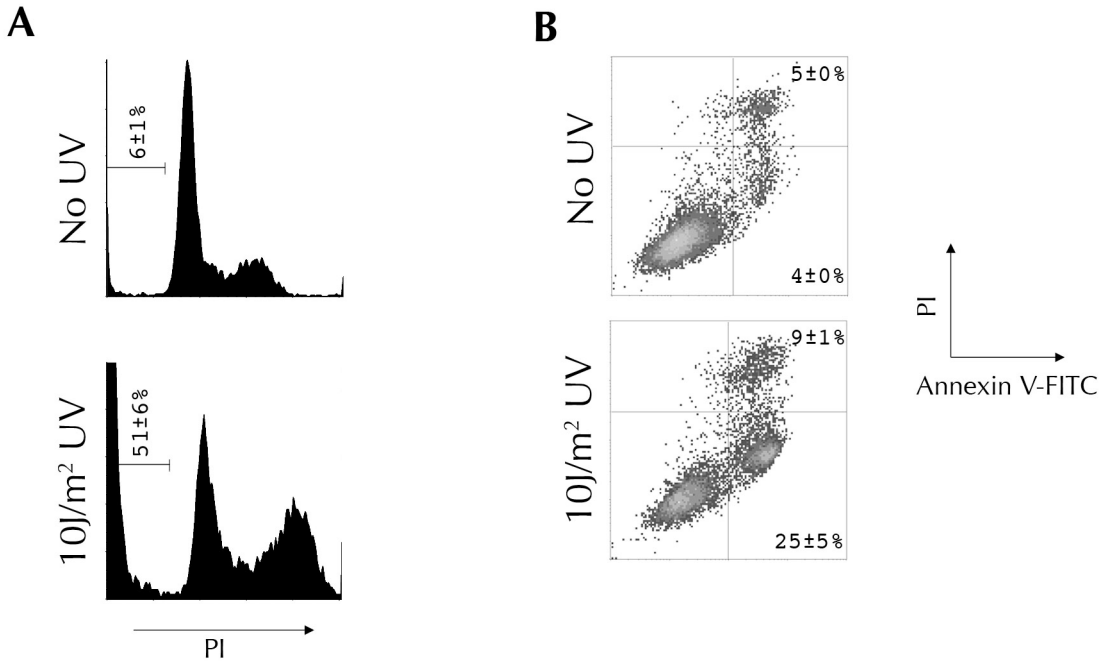


Figure 3.3 Analysis of apoptosis in the U2OS_ARF cells
(A) The apoptotic response of the U2OS_ARF cell line was examined three days after exposure to 10 J/m^2 UVC irradiation. The cell cycle distribution of propidium iodide (PI) stained nuclei was examined by flow cytometry. Cells with a DNA content less than 2N are apoptotic.
(B) Dual colour flow cytometric annexin-V analysis for apoptosis of U2OS_ARF cells was examined three days after exposure to 10 J/m^2 UVC irradiation. Cells that are annexin-V positive and PI-negative are in early apoptosis and cells positive for both annexin-V and PI dye are in the late stages of apoptosis.

ARF expression has been shown to cause cell cycle arrest that eventually progresses to apoptosis after five to seven days (Eymin *et al.*, 2003; Sekaric *et al.*, 2007; Yang *et al.*, 2000). To examine the impact of p14ARF accumulation over a longer time course the

expression of p14ARF was induced with 1mM IPTG for up to nine days. As shown in Figure 3.4A, strong nucleolar expression of p14ARF was detected and maintained in approximately 80% of U2OS_ARF cells up to nine days post induction. Addition of Hoechst 33258 nuclear stain showed that very few ARF expressing cells displayed the characteristic nuclear condensation associated with apoptotic cells (less than 5%), even though the accumulation of p14ARF led to the long-term stabilisation and activation of p53 as determined by the induction and maintenance of the p53-transcriptional target $p21^{Waf1}$ (Figure 3.4B).

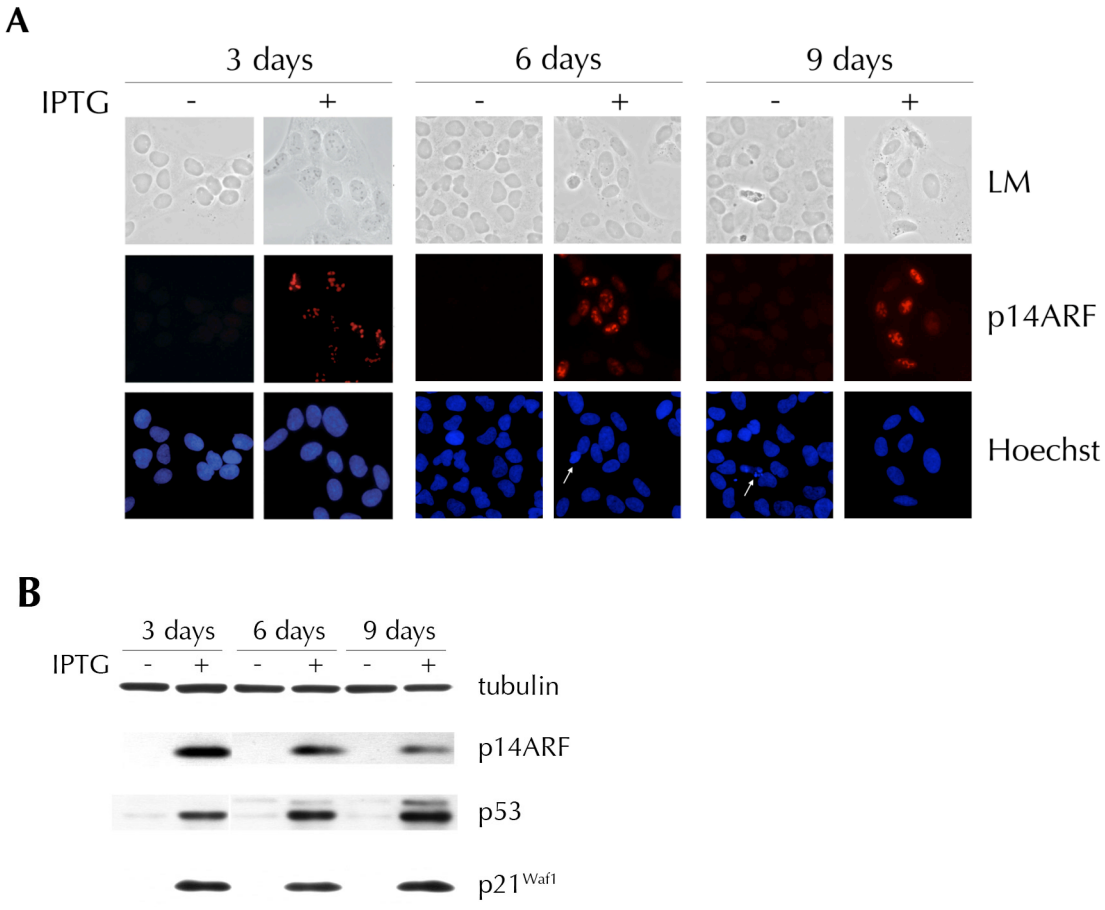


Figure 3.4 Induced p14ARF expression maintains activated p53

(A) The nucleolar accumulation of p14ARF in U2OS_ARF cells was detected 3, 6 or 9 days post IPTG induction by immunofluorescence. Hoechst staining was used to visualise cell nuclei. Arrows highlight apoptotic cells with condensed or fragmented nuclei. LM, light microscopy.

(B) Expression of p14ARF, p53, $p21^{Waf1}$ and tubulin loading control was determined 3, 6 or 9 days after treatment of U2OS_ARF cells with 1mM IPTG (+) or PBS (-).

Considering that apoptotic cells often detach from tissue culture flasks and would not be retained for immuno-cytochemical analysis, we analysed the cell cycle distribution of both adhered and floating cells using flow cytometry. As shown previously, p14ARF induction promoted a potent G1 cell cycle arrest and S-phase inhibition that was maintained throughout the nine day IPTG treatment (Figure 3.5A). p14ARF-mediated arrest was less evident nine days after IPTG exposure, because the uninduced control U2OS_ARF cells had reached confluency. Although p14ARF-induction promoted growth arrest in U2OS_ARF cells, there was no increase in apoptosis, as measured by the sub-G1 population (Figure 3.5A). Only the control uninduced U2OS_ARF cells showed a significant increase in sub-G1 cell population nine days post induction, again a result of reaching confluency (Figure 3.5A). Similar data were obtained with annexin-V staining; there was no significant difference in the percentage of apoptotic cells between induced and uninduced U2OS_ARF cells over the nine day IPTG treatment period (Figure 3.5B).

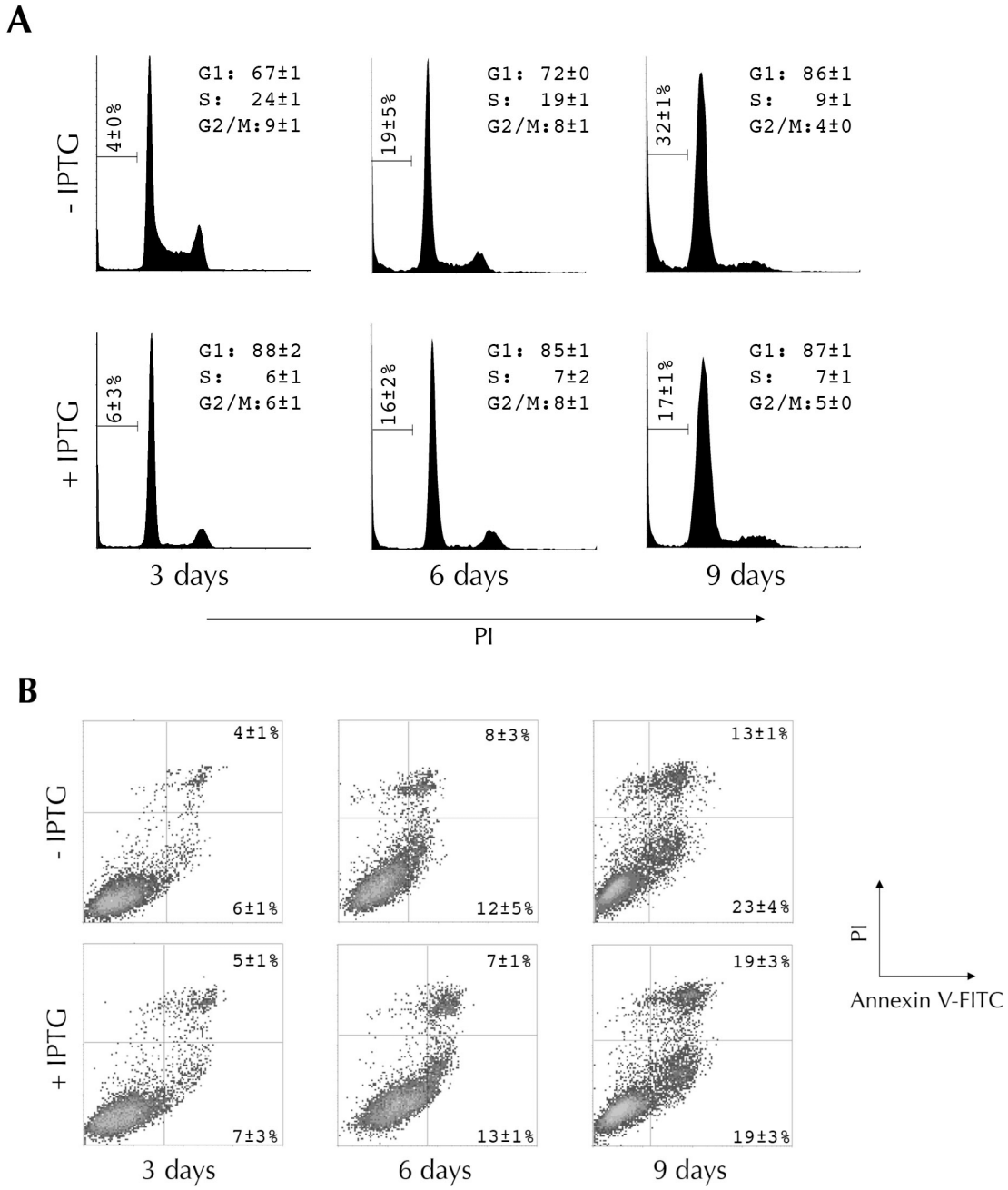


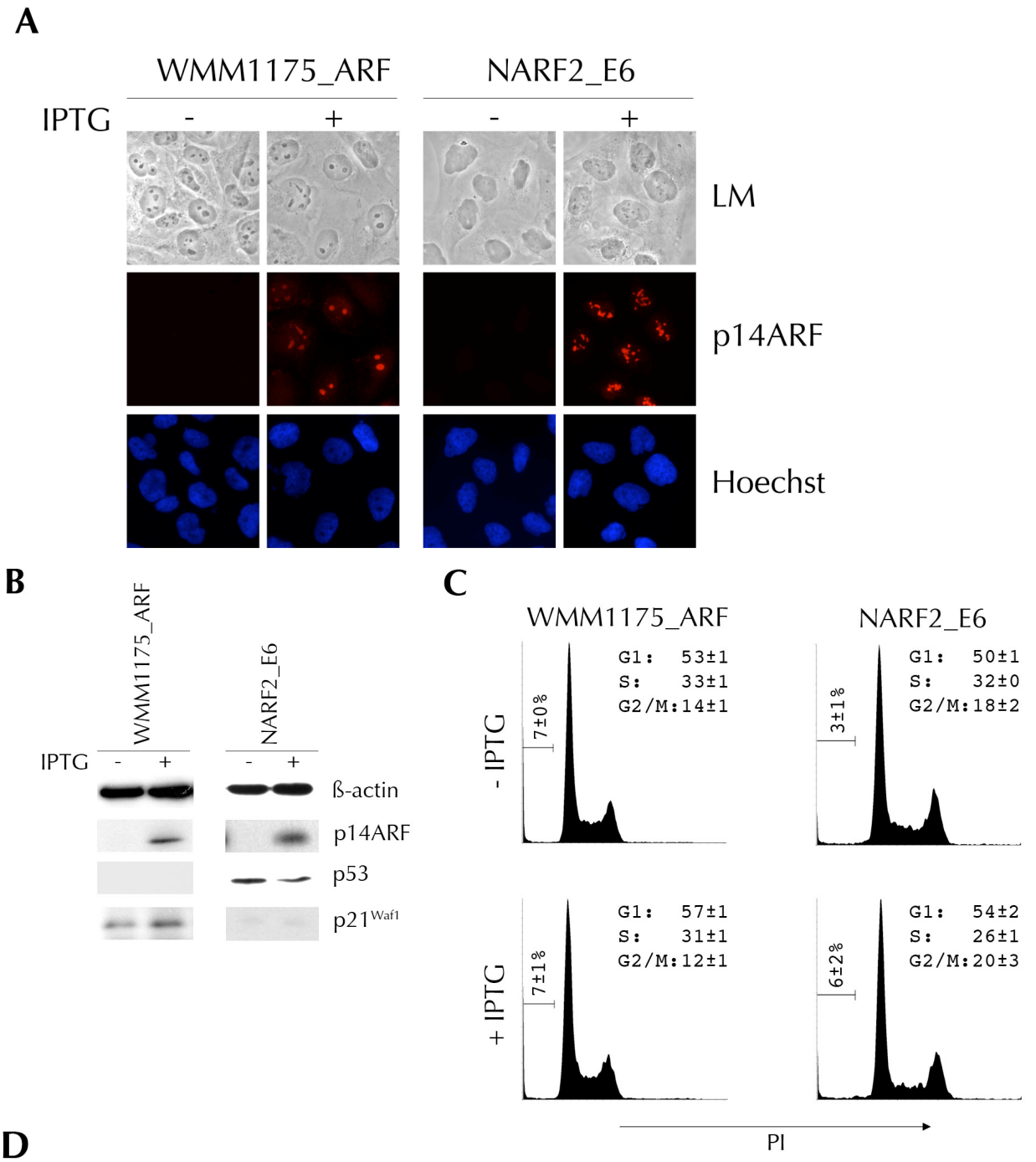
Figure 3.5 p14ARF induces G1 cell cycle arrest but not apoptosis

(A) The cell cycle distribution of U2OS_ARF cells was examined 3, 6 and 9 days post IPTG induction, using propidium iodide (PI) staining.

(B) Dual colour flow cytometric annexin-V analysis for apoptosis of U2OS_ARF cells treated with 1mM IPTG (+) or PBS (-), for 3, 6 or 9 days.

3.2.2 p14ARF requires intact p53 to inhibit cell cycle progression

To investigate whether p14ARF-induced cell cycle arrest was dependent on p53 we utilised the WMM1175_ARF melanoma and NARF2_E6 osteosarcoma cell lines (Becker *et al.*, 2001; Rodway *et al.*, 2004). The isogenic NARF2 and NARF2_E6 cells were generated by Dr Gordon Peters (London Research Institute, UK) and are derived from U2OS osteosarcoma cells, the NARF2_E6 cells constitutively express the human papilloma virus E6 ubiquitin ligase which degrades p53. All three cell lines express an IPTG-inducible form of nucleolar p14ARF (Figure 3.6A) but the WMM1175_ARF and the NARF2_E6 are functionally null for p53 (Figure 3.6B). The three-day induction of p14ARF in the WMM1175_ARF and NARF2_E6 cells did not induce growth arrest (Figure 3.6C) or significant cell death (Figures 3.6C and 3.6D). In contrast, induction of ARF in the isogenic NARF2 cells (Figure 3.7A), which are p53-intact with no constitutive E6 expression, promoted p53 accumulation (Figure 3.7B) and growth arrest in the G1 and G2 cell cycle phases (Figure 3.7C). The sub-G1 population increased from 4% to 16% after p14ARF was expressed for three days (Figure 3.7C), although there was no change in annexin-V positive cells (Figure 3.7D). The apparent increase in the proportion of sub-G1 NARF2 cells is most likely a result of their inherent instability. The isogenic NARF2 and NARF2_E6 cells are significantly larger in size with twice the DNA content of the parental U2OS cells (Figures 3.8A & B). Cells with increased ploidy are often unstable during culture (Castedo *et al.*, 2006), and we found that long term culturing of the NARF2 cells was associated with increased cell death (data not shown). This was not an issue with the U2OS_ARF cells, which were generated in our laboratory and appear similar to the parental U2OS cells in both size (Figure 3.8A) and DNA content (Figure 3.8B). The instability of the NARF2 cell lines did not permit long-term IPTG induction experiments.


A
WMM1175_ARF
NARF2_E6
IPTG
-
+
-
+
LM
p14ARF
Hoechst
B
WMM1175_ARF
NARF2_E6
IPTG
-
+
-
+
ß-actin
p14ARF
p53
p21Waf1
C
WMM1175_ARF
NARF2_E6
- IPTG
+ IPTG
7±0%
3±1%
7±1%
6±2%
G1: 53±1
S: 33±1
G2/M:14±1
G1: 50±1
S: 32±0
G2/M:18±2
G1: 57±1
S: 31±1
G2/M:12±1
G1: 54±2
S: 26±1
G2/M:20±3
PI
D
WMM1175_ARF
NARF2_E6
-IPTG
+IPTG
2±0%
3±1%
3±0%
3±0
5±1%
2±1%
4±1%
1±1%
PI
Annexin V-FITC

(Previous page)

Figure 3.6 Induced expression of p14ARF does not promote cell cycle arrest or apoptosis in the p53-null WMM1175_ARF melanoma cells or p53-inactivated NARF2_E6 cells

Expression of p14ARF was induced in p53-null WMM1175_ARF melanoma cells and E6 expressing NARF2_E6 osteosarcoma cells by addition of 1mM IPTG to the media for three days.

(A) The nucleolar accumulation of p14ARF detected by immunofluorescence. Hoechst staining was used to visualise cell nuclei. LM, light microscopy

(B) Western blot was used to determine expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin

(C) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining

(D) Apoptotic cells were also analysed by dual colour flow cytometric staining of annexin-V and PI exclusion

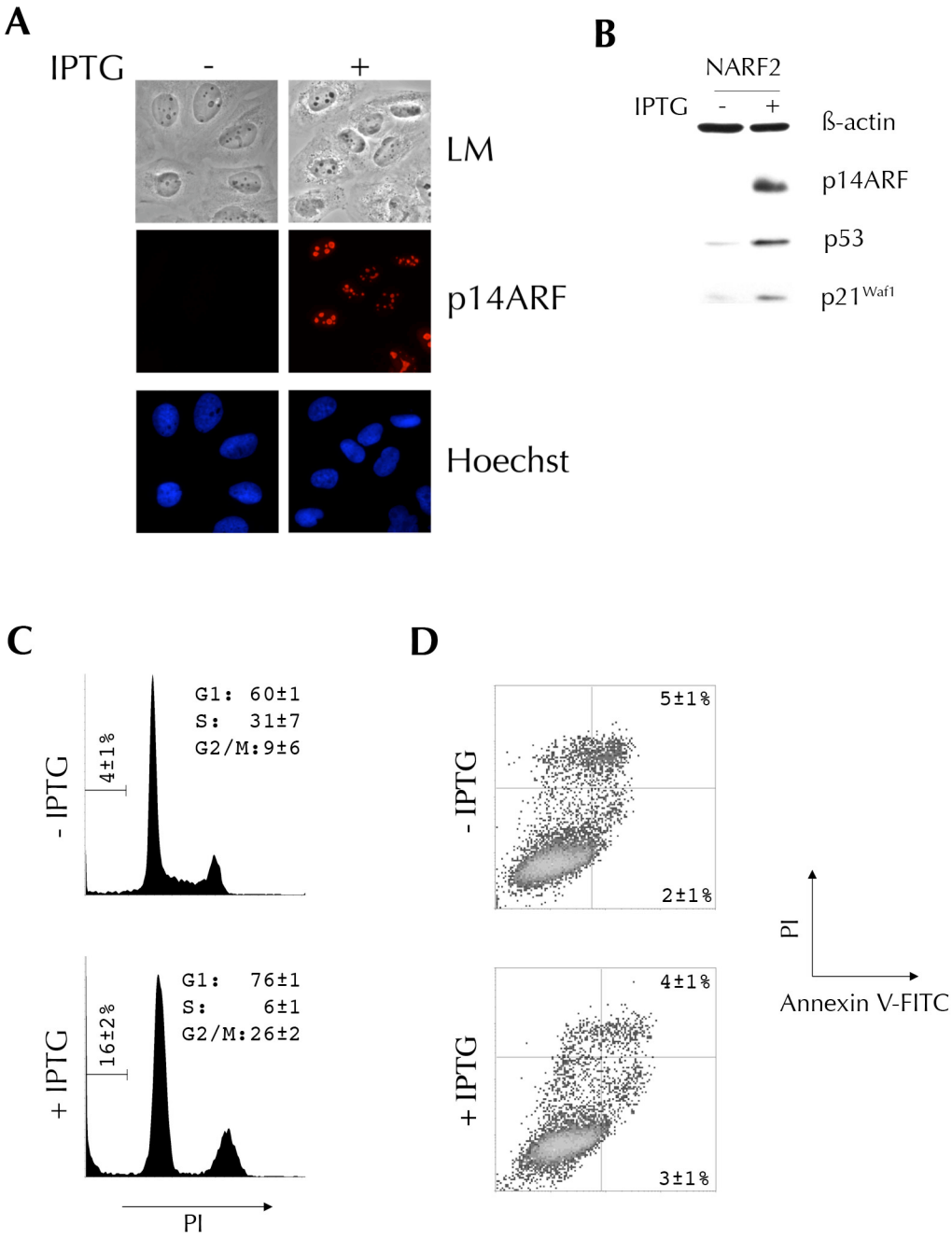


Figure 3.7 Induced expression of p14ARF does not lead to apoptosis in NARF2 cells

Expression of p14ARF was induced in p53 wild type NARF2 cells by addition of 1mM IPTG to the media for three days.

(A) The nucleolar accumulation of p14ARF detected by immunofluorescence. Hoechst staining was used to visualise cell nuclei. LM, light microscopy

(B) Western blot was used to determine expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin

(C) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining

(D) Apoptotic cells were also analysed by dual colour flow cytometric staining of annexin-V and PI exclusion.

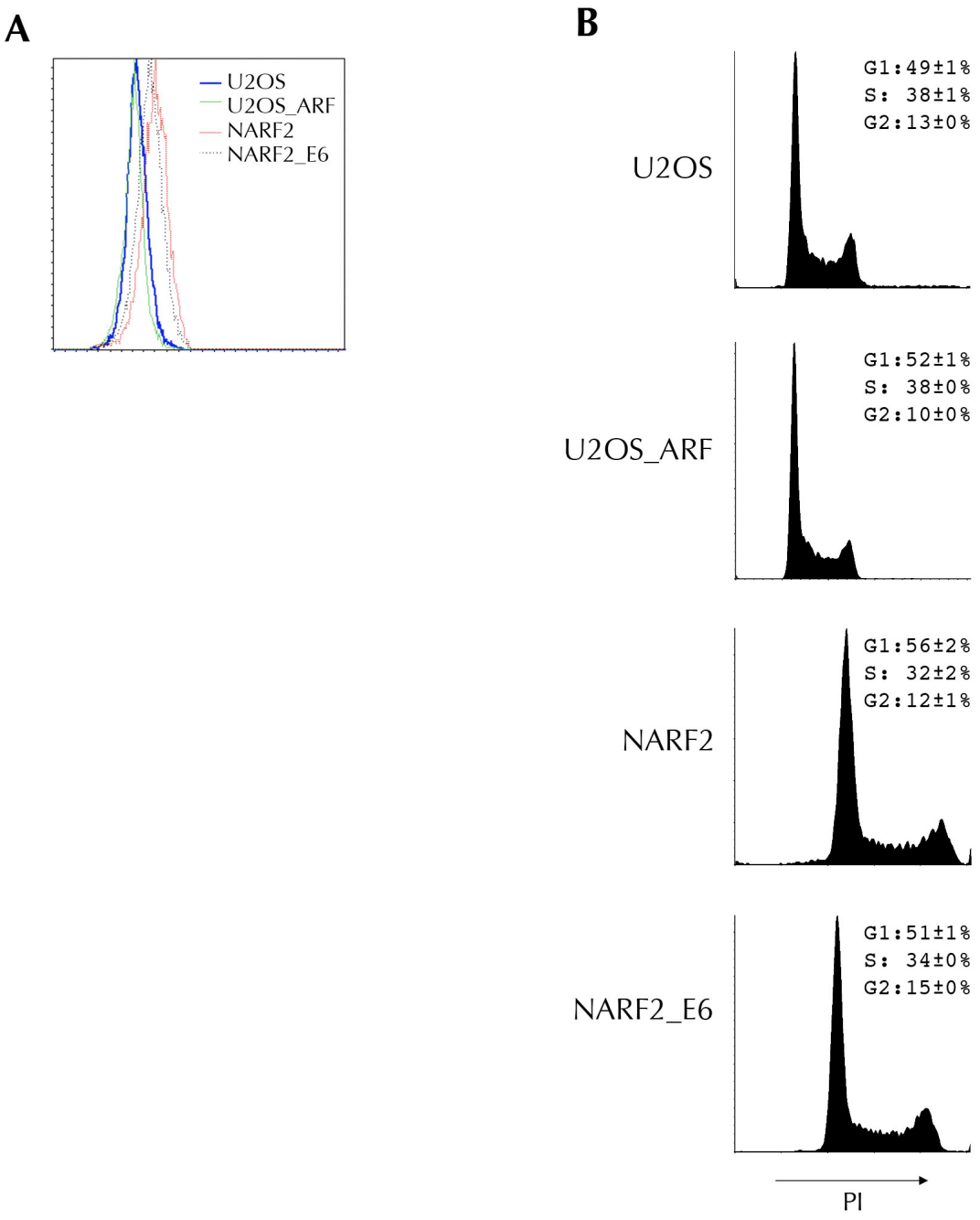


Figure 3.8 NARF2 and NARF2_E6 cells are tetraploid

(A) Flow cytometry was used to determine the size of NARF2 and NARF2_E6 cells were compared with the parental U2OS and the derived U2OS_ARF cell lines using forward scatter of unfixed cells. (B) The DNA content of NARF2 and NARF2_E6 cells were compared with the parental U2OS and the derived U2OS_ARF cell lines by flow cytometric analysis of propidium iodide (PI) stained nuclei 48 h after seeding. Instrument settings were kept the same for all samples.

3.2.3 Transient p14ARF expression does not promote apoptosis

We were able to perform longer-term induction of ARF in the p53-null WMM1175_ARF cells. In this melanoma line, accumulation of p14ARF over a nine day period did not increase the low levels of $p21^{Waf1}$ (Figure 3.9A), alter cell cycle distribution (Figure 3.9B) or the proportion of cells undergoing cell death (Figure 3.9C).

To exclude the possibility that genetic damage in our selected cell clones altered important apoptotic signalling pathways, we transiently introduced p14ARF into three other human cell lines. We transfected p53-null SAOS-2 osteosarcoma cells, which have been shown to undergo apoptosis in the presence of recombinant adenovirus expressing p14ARF (Hemmati *et al.*, 2002). In addition, we transiently introduced p14ARF into p53-intact HCT116 colon cancer cells and WS1 human primary skin fibroblasts. These cells were transfected with p14ARF and harvested 48 h later. Western blot analysis confirmed p14ARF expression in all transfected cells (Figure 3.10A). In the p53-null SAOS-2, no p53 was detected and levels of $p21^{Waf1}$ did not change. Transient expression of p14ARF in SAOS-2 cells caused a small increase in the G1 phase (less than 10%), but there was no significant change in apoptosis (Figure 3.10B). The transient expression of p14ARF in the p53-intact HCT116 cells caused an increase in the level of p53 and $p21^{Waf1}$, with the expected G1-phase arrest, but no p14ARF induced apoptosis (Figure 3.10B). Finally, ectopic expression of p14ARF had no impact on the cell cycle distribution of the slow growing primary human fibroblasts (94% of cells are already in G1 phase), and p14ARF expression did not induce cell death (Figure 3.10B).

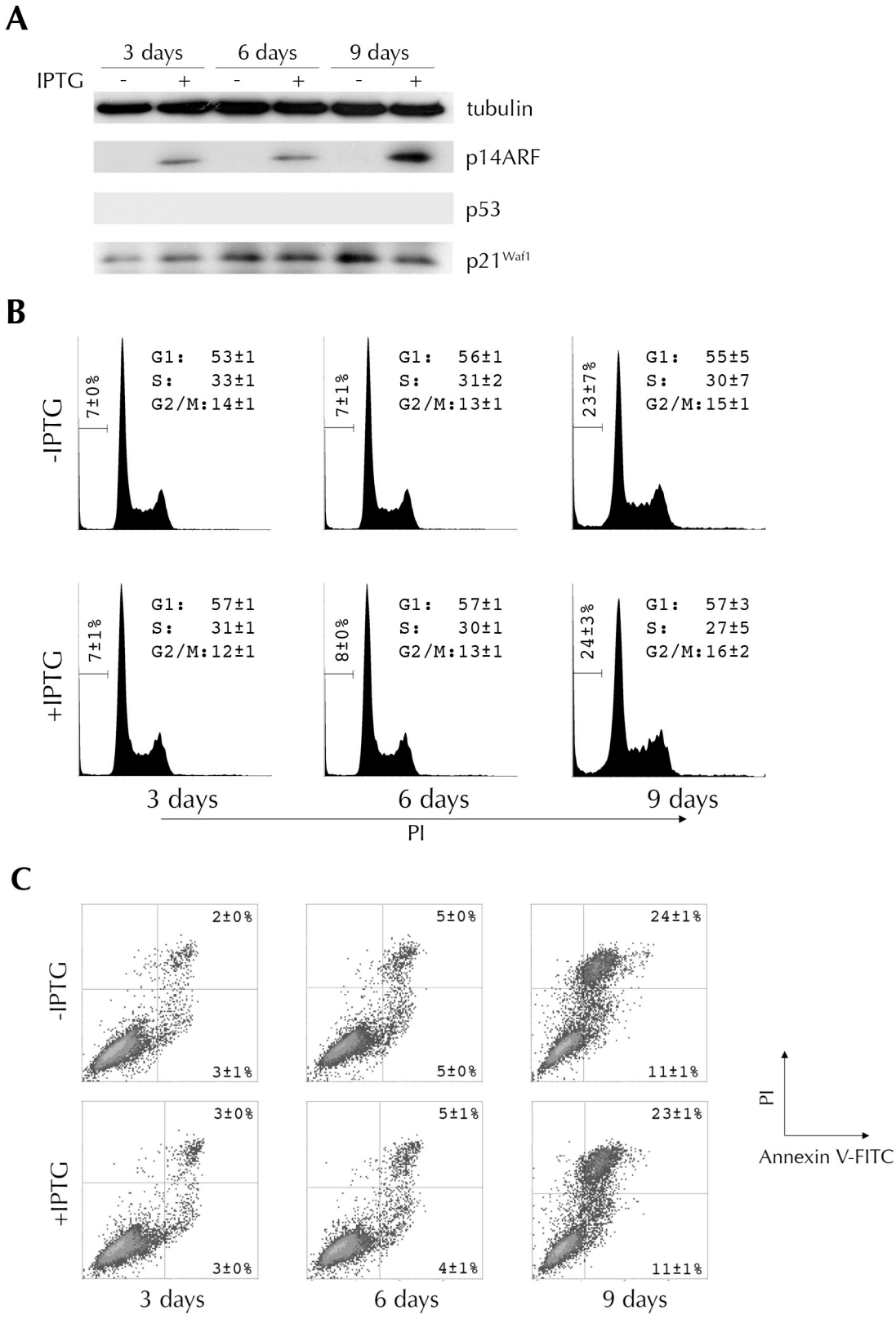
(Next page)

Figure 3.9 Long term expression of p14ARF does not promote cell cycle arrest or apoptosis in p53-null WMM1175_ARF melanoma cells

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and tubulin loading control was determined 3, 6 or 9 days after treatment of WMM1175_ARF cells with 1mM IPTG (+) or PBS (-).

(B) The cell cycle distribution of WMM1175_ARF cells was examined 3, 6 and 9 days post IPTG induction, using propidium iodide (PI) staining.

(C) Dual colour flow cytometric annexin-V analysis for apoptosis of U2OS_ARF cells treated with 1mM IPTG (+) or PBS (-), for 3, 6 or 9 days.


A
3 days
6 days
9 days
IPTG
- + - + - +
tubulin
p14ARF
p53
p21Waf1
B
-IPTG
+IPTG
7±0%
G1: 53±1
S: 33±1
G2/M:14±1
7±1%
G1: 56±1
S: 31±2
G2/M:13±1
23±7%
G1: 55±5
S: 30±7
G2/M:15±1
7±1%
G1: 57±1
S: 31±1
G2/M:12±1
8±0%
G1: 57±1
S: 30±1
G2/M:13±1
24±3%
G1: 57±3
S: 27±5
G2/M:16±2
3 days
6 days
9 days
PI
C
-IPTG
+IPTG
2±0%
3±1%
5±0%
5±0%
24±1%
11±1%
3±0%
3±0%
5±1%
4±1%
23±1%
11±1%
3 days
6 days
9 days
PI
Annexin V-FITC

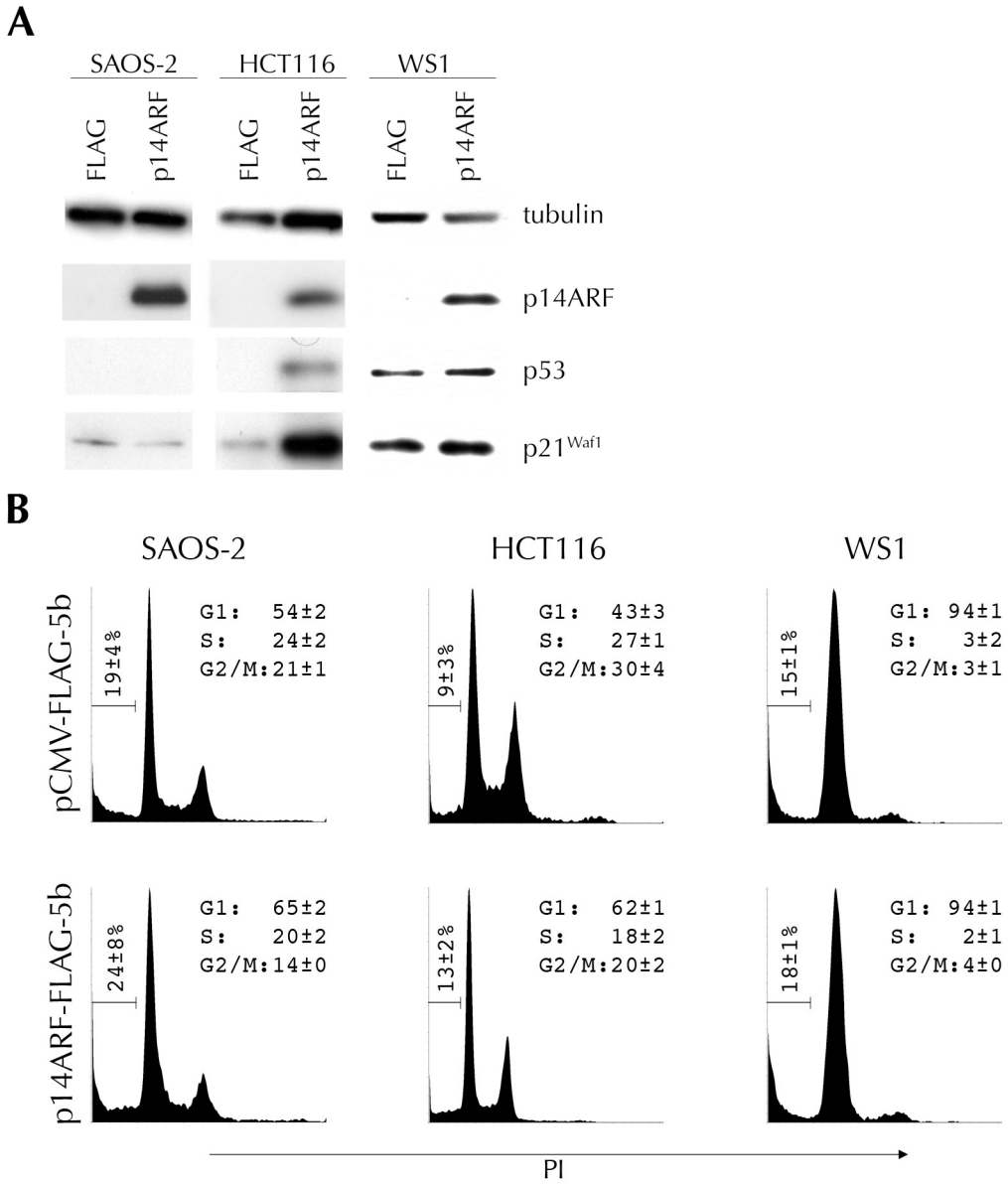


Figure 3.10 Transient p14ARF expression induces p53-dependent cell cycle arrest but not apoptosis in p53-intact or p53-deficient cells

SAOS-2, HCT116 and WS1 embryonic dermal fibroblast cells were transfected with the *p14ARF-FLAG* plasmid or *pCMV-FLAG* vector and *pCMVEGFP-spectrin*

(A) The expression of p14ARF-FLAG, p53, $p21^{Waf1}$ and tubulin was determined by western blotting, 48 h post transfection.

(B) The cell cycle distribution of cells expressing green fluorescent protein was determined, 48 h post transfection, using propidium iodide (PI) staining.

3.3 Discussion

Defining the tumour suppressor functions of human p14ARF has been complicated not only because this protein shares its genomic sequence with the *p16*^{INK4a} melanoma-predisposition gene, but also because much of the work on ARF has been performed on the murine homologue, which is not a reliable surrogate for human ARF. In particular, the mouse and human ARF proteins share only 49% amino acid homology, are differentially regulated (only mouse p19ARF is induced by replicative senescence (Brookes *et al.*, 2002) and RAS alone (Berkovich *et al.*, 2003)), and interact with different partners (only p19ARF binds pex19p (Sugihara *et al.*, 2001)). Of the two gene products derived from the *INK4a/ARF* locus, ARF appears to be the main tumour suppressor in mice, whereas p16^{INK4a} is the more important in humans. Further, point mutations affecting the human *INK4a/ARF* locus predominantly target p16^{INK4a} (Goldstein *et al.*, 2007; Sharpless and DePinho, 1999) and p14ARF-specific alterations are extremely rare (Fargnoli *et al.*, 1998; Rizos *et al.*, 2001b).

Another critical functional difference between the mouse and human ARF proteins may be their role in promoting apoptosis; whereas p19ARF alone can promote cell death, the impact of p14ARF on apoptosis was not clearly defined. Murine ARF contains a C-terminal apoptotic domain not shared by human ARF, suggesting p14ARF may not promote apoptosis to the same degree as its murine counterpart (Matsuoka *et al.*, 2003). Here, we have established that expression of p14ARF alone induces potent p53-dependent cell cycle arrest, but does not promote cell death in p53-null or p53-intact cells.

Although our data are supported by several reports (Eymin *et al.*, 2001; Weber *et al.*, 2002; Yarbrough *et al.*, 2002), many investigations have detected significant p14ARF-induced apoptosis. In these studies, adenoviral-mediated p14ARF expression induced dose-dependent cell death. In particular, the strong over-expression of p14ARF was capable of inducing apoptosis, whereas lower but easily detectable expression levels of p14ARF did not induce significant death (Deng *et al.*, 2002; Hemmati *et al.*, 2002; Kim *et al.*, 2004). It also appears that long periods of ectopic ARF expression are necessary to induce apoptosis. In two reports, both using ectopic adenoviral-mediated p14ARF expression in HCT116 cells, apoptosis was detected at three days, but not 24 h post infection - even though p14ARF induced cell cycle arrest at this earlier time point (Hemmati *et al.*, 2002; Hemmati *et al.*, 2005).

Perhaps the most revealing evidence regarding the apoptotic function of p14ARF comes from studies indicating that additional cellular stresses are required for p14ARF-induced apoptosis, such as cisplatin-induced DNA damage, serum withdrawal or exposure to interferons (Deng *et al.*, 2002; Nakazawa *et al.*, 2003; Sandoval *et al.*, 2004). Cellular stress may also help explain previous reports showing that adenoviral-mediated p14ARF expression induces apoptosis (Deng *et al.*, 2002; Hemmati *et al.*, 2002; Kim *et al.*, 2004). Adenoviral gene delivery methods independently induce cellular stress through the introduction of viral proteins designed to allow cell cycle progression and unscheduled DNA synthesis, as well as proteins which block the cell's normal response to the presence of excessive amounts of exogenous DNA (Brand *et al.*, 1999; Weitzman and Ornelles, 2005). These factors may synergise with ectopic p14ARF expression to induce apoptosis. The stable cell models we have used in this

study require very low levels of non-toxic IPTG to induce accumulation of p14ARF, and thus provide a more physiological model to analyse p14ARF functions.

Our data indicate that the primary response of cells to p14ARF accumulation is a potent and rapid p53-dependent cell cycle arrest. This is consistent with the senescent-like growth arrest induced in primary human cells by oncogenic stimuli that induce p14ARF, including RAS and E2F-1 (Berkovich *et al.*, 2003). Our results are also in agreement with Weber's study showing p14ARF mediated cell cycle arrest in HCT116 colon cancer cells is dependent on intact p53 signalling pathways (Weber *et al.*, 2002). The existence of p53-defective melanoma cell lines that express p14ARF is also consistent with p14ARF acting primarily via p53 (Daniotti *et al.*, 2004; Yang *et al.*, 2005). Furthermore loss of p53 and ARF are usually mutually exclusive events in tumours, strongly suggesting that ARF's principal role is p53-dependent (Bardeesy *et al.*, 2002; Fulci and al, 2000; Tannapfel *et al.*, 2001).

It has been proposed that p14ARF can sensitise cells to apoptosis, a process that has implications for cancer progression and response to anti-cancer therapy. Defining which agents cooperate with ARF to promote cell killing, and the mechanism by which this occurs, will provide a useful insight into chemoresistance in tumours, and are the subject of the next two chapters.

4. The role of p14ARF in cancer cell chemosensitivity

4.1 Introduction

The sensitivity of cancer cells to cytotoxic agents largely determines the success of systemic chemotherapy. Melanoma is notoriously resistant to chemotherapeutic agents and many drugs including nitrosoureas, taxanes, platinum-based drugs and vinca alkaloids have failed in large randomised studies (Chapman *et al.*, 1999; Creagan *et al.*, 1999; Eggermont and Kirkwood, 2004; Jungnelius *et al.*, 1998; Luikart *et al.*, 1984; Soengas and Lowe, 2003). This suggests that the drug resistance mechanisms active in melanoma cells are complex and inherent to the malignant behaviour of melanoma cells (Soengas and Lowe, 2003). Defining the molecular mechanisms of melanoma chemoresistance is the critical first step in identifying drug targets that can bypass defects in cell death programs.

While we have shown that p14ARF accumulation alone does not induce cell death, p14ARF may have a role in drug-induced apoptosis. This is an attractive concept because melanomas display a low frequency of p53 mutations despite their extreme chemoresistance. p53 is an important regulator of drug-induced apoptosis and mutations inactivating p53 have been linked to chemoresistance in many settings (Fan *et al.*, 1994; O'Connor *et al.*, 1997; Velculescu and El-Deiry, 1996). In melanomas, p53 function need not be directly disabled because they frequently carry alterations affecting p14ARF. In fact, studies using mouse models of melanoma have shown that disruption of p19ARF can functionally replace p53 loss during melanomagenesis (Chin *et al.*, 1997; You *et al.*, 2002). Analogous to human melanomas, tumours arising in these

mouse models express wild-type p53 (Chin *et al.*, 1997). Unfortunately, there is no data available on the sensitivity of these melanomas to cancer therapies.

Consistent with its role in the p53 pathway, there is evidence that ARF can sensitise cancer cells to death. For instance, enforced expression of p14ARF by adenovirus in p53-intact MCF-7 breast cancer cells sensitised cells to cisplatin but not taxol (Deng *et al.*, 2002). Taxol can cause death independently of p53 (Bacus *et al.*, 2001) suggesting p14ARF mediated chemosensitivity may involve p53. Additionally, glioma and lung cancer cells stably expressing p14ARF were rendered more sensitive to radiation, but only if they expressed wild type p53 (Gao *et al.*, 2001; Simon *et al.*, 2006). ARF did not sensitise cells to all cytotoxic insults; U-87MG glioma cells expressing p14ARF were no more sensitive to the microtubule poison vincristine or the nitrosourea BCNU (Simon *et al.*, 2006). The efficient induction of apoptosis in response to oncogene over-expression was also influenced by increased ARF in p53 positive MEFs (Pauklin *et al.*, 2005). Importantly, *INK4a/ARF* null (null for both *p19ARF* and $p16^{INK4a}$ genes) lymphomas that developed in the *Eμ-myc* transgenic mouse model (these animals constitutively express c-Myc in the B-cell lineage) displayed apoptotic defects and were markedly resistant to chemotherapy (Schmitt *et al.*, 1999). The *Eμ-myc* lymphomas never displayed coincident loss of both wild type *INK4a/ARF* and *p53* alleles, suggesting loss of both loci produced no additional advantage to these lymphomas.

There is some evidence that the ARF tumour suppressor modulates sensitivity to toxic agents independently of p53. Human SAOS-2 (p53-null) and U2OS (p53-intact) cells with inducible p14ARF expression underwent apoptosis in response to interferon-ß treatment, and this effect was not inhibited by expression of dominant-negative p53

(Sandoval *et al.*, 2004). Induction of p14ARF expression in p53 defective HT-1080 fibrosarcoma cells protected cells from death induced by the antifolates methotrexate, trimetrexate and raltitrexed via decreased dihydrofolate reductase levels and increased thymidine salvage (Magro *et al.*, 2004). ARF reduced dihydrofolate reductase levels by binding to the E2F-1 coactivator DP-1, leading to the inhibition of E2F-1 transcription (Datta *et al.*, 2002; Datta *et al.*, 2005). Thus, the functional inhibition of E2F-1 by ARF can modulate cell sensitivity to death in particular circumstances. Moreover the list of ARF binding partners is extensive and the functional significance for most ARF binding partners is poorly described. These binding partners offer potential p53-independent mechanisms for p14ARF-mediated chemosensitivity.

The ability of p14ARF to modulate the apoptotic response to cytotoxic agents is poorly defined, and no comprehensive screen of potential cooperating agents has been performed. In this chapter, the response of cancer cells to a series of cytotoxic drugs and p14ARF expression was investigated. In this extensive screen we show that p14ARF can enhance cell death in response to a subset of cytotoxic agents.

4.2 Results

4.2.1 Selection of cytotoxic agents

Having established that p14ARF induction potently activated the p53 pathway in U2OS_ARF cells, we analysed whether p14ARF induction altered drug-induced cell death. To investigate if p14ARF could sensitise cells to apoptosis, the U2OS_ARF cell line was exposed to 13 cytotoxic agents in the presence or absence of p14ARF induction. Cytotoxic agents were chosen to include drugs currently used in the

treatment of melanoma, drugs shown to chemosensitise with ARF in previous reports and other drugs to include various modes of action (Table 4.1). The osteosarcoma cell line U2OS_ARF has been used as a model as it does not express endogenous p14ARF but retains wild type p53 which is stabilised by p14ARF accumulation. Although a melanoma cell line would have been a preferable model, we were unable to successfully introduce the Lac-switch system into available melanoma cell lines that were p14ARF-null and p53 wild type.

U2OS_ARF cells were exposed to the cytotoxic agent in the presence or absence of 1mM IPTG. Cells were usually exposed to the drug for three days, except in the case of Adriamycin (doxorubicin), which was added to the media for six hours after which the media was replaced and the cells allowed to recover for three days, with or without IPTG. The shorter Adriamycin treatment was chosen partly to mimic *in vivo* treatment length and primarily because Adriamycin prevents correct propidium iodide staining of DNA for cell cycle analysis (Greene *et al.*, 1983; Krishan *et al.*, 1981). The concentration of each drug was adjusted to induce approximately 20% apoptosis in the absence of p14ARF to permit the detection of both increases and decreases in cell death in response to ARF induction.

The cytotoxic agents used in this study were prepared using a variety of solvents (PBS, DMSO, methanol and ethanol) as detailed by the manufacturers. To control for possible solvent effects, the U2OS_ARF cells were always treated with the same amount of solvent for each cytotoxic agent tested. The cell cycle distribution and annexin-V positivity were similar for all solvent controls and thus, for simplicity, a single figure represents the control results. As expected, three days of p14ARF induction alone

Table 4.1 Effect of p14ARF expression on drug sensitivity of U2OS_ARF cells.

Class	Drug	Action	Reason for selection	References
Topoisomerase inhibitors	Adriamycin (ADR)	Topoisomerase II inhibitor Generation of free radicals	Sensitivity to ADR is increased by ARF expression or p53 over-expression	(Blagosklonny and El-Deiry, 1998; Fornari *et al.*, 1994; Gallagher *et al.*, 2005; Guo-Chang and Chu-Tse, 2000)
	Camptothecin (CPT)	Topoisomerase I inhibitor.	Induces p53 dependent apoptosis in melanoma	(Christensen *et al.*, 2004; Li *et al.*, 2000)
DNA damage	Cisplatin	DNA binding, damage and inter/intra-strand crosslinks	Over-expression of ARF increases sensitivity of MCF-7 cells to cisplatin	(Deng *et al.*, 2002; Siddik, 2002)
	UVB	Dipyrimidine dimers 1-6 DNA photoproducts	Predicted to be responsible for the development of most melanomas	(Jhappan *et al.*, 2003; Kricker *et al.*, 2007)
Transcription inhibitors	Actinomycin D (ActD)	Inhibitor of RNA polymerases I and II	Used in isolated limb perfusion treatment of melanoma	(Yung *et al.*, 1992) (Thompson *et al.*, 1997)
	DRB	Inhibitor of RNA polymerase I Inhibits casein kinase II (CK2)	Disrupts nucleolus and inhibits rRNA synthesis and reduces stability of ARF binding partner NPM	(Chodosh *et al.*, 1989; David-Pfeuty *et al.*, 2001; Tawfic *et al.*, 1995; Yanofsky, 1988)
Alkylating agents	Dacarbazine (DTIC)	DNA alkylator	Primary drug used in systemic melanoma treatment	(Eggermont and Kirkwood, 2004)
	Temozolomide	DNA alkylator	DTIC analogue that doesn't require hepatic activation. Crosses blood/brain barrier.	(Middleton *et al.*, 2000)
	Melphalan	DNA alkylator Nitrogen mustard	Used in isolated limb perfusion treatment of melanoma	(Thompson *et al.*, 1997; Wikonkal *et al.*, 2003)
Histone Deacetylase inhibitors	Sodium Butyrate (NaB)	Short chain fatty acid inhibitor of histone deacetylase class 1 and IIa	Radio-sensitises melanoma. Therapeutic potential in leukaemia	(Bhalla, 2005; Munshi *et al.*, 2005)
	Trichostatin A (TSA)	Hydroxamic acid inhibitor of all histone deacetylase	Therapeutic potential and active against melanoma	(Bhalla, 2005; Bolden *et al.*, 2006; Peltonen *et al.*, 2005)
	Valproic acid (VPA)	Short chain fatty acid inhibitor of histone deacetylase class 1 and IIa	Similar structure and action to NaB.	(Bhalla, 2005)
Microtubule poisons	Taxol	Stabilises microtubules	p14ARF expression shown not to sensitise cells to Taxol	(Deng *et al.*, 2002; Schiff *et al.*, 1979; Torres and Horwitz, 1998)

resulted in G1 arrest and S-phase inhibition, with no change in the percentage of apoptotic sub-G1 or annexin-V cells (see Figure 4.1A & 4.1B).

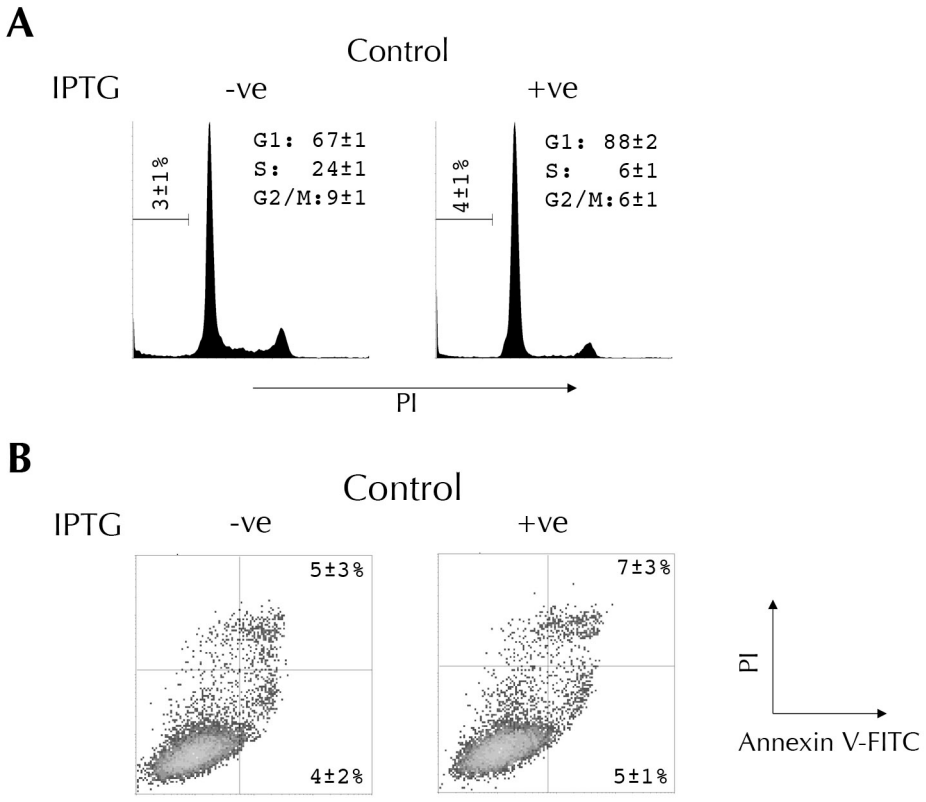


Figure 4.1 Analysis of cell cycle and apoptosis in the U2OS_ARF cells
(A) The cell cycle distribution of U2OS_ARF cells was examined three days post treatment with 0.2% DMSO carrier control and PBS (-) or p14ARF induction with 1mM IPTG (+). PI, propidium iodide.
(B) Dual colour flow cytometric annexin-V analysis for apoptosis of U2OS_ARF cells three days post treatment with 0.2% DMSO carrier control and PBS (-) or p14ARF induction with 1mM IPTG (+).

4.2.2 DNA binding agents and topoisomerase poisons

U2OS_ARF cells were treated with clinically important inhibitors of topoisomerase I and II, camptothecin or Adriamycin respectively, or the DNA binding and damaging agent cisplatin. Exposure of uninduced U2OS_ARF cells to 1μM Adriamycin for 6 hours followed by three days of recovery led to increased levels of p53 an increased levels of the p53 transcriptional target $p21^{Waf1}$ (Figure 4.2A). In the presence of IPTG, exposure to Adriamycin led to the accumulation of higher levels of p14ARF, with no

further increases in the level of p53, compared with the Adriamycin-only or IPTG-only treated cells. Nevertheless, the combination of p14ARF (IPTG treatment) and Adriamycin treatment induced significantly higher levels of apoptosis than Adriamycin treatment alone in the U2OS_ARF cells. In particular, the sub-G1 population increased from 25±1% to 37±3% (Figure 4.2B) and the proportion of annexin-V positive, apoptotic cells increased from 23±2% to 31±4% (Figure 4.2C).

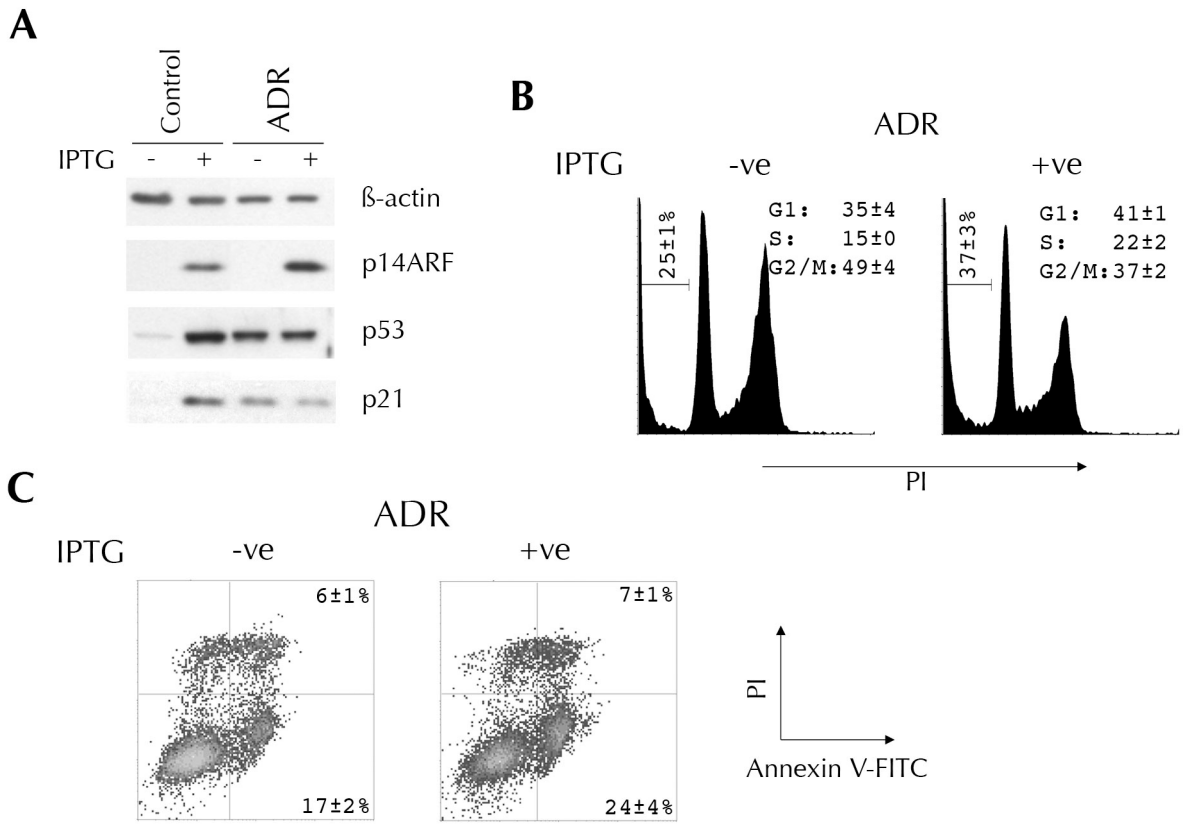


Figure 4.2 Expression of p14ARF sensitises cells to apoptosis in response to Adriamycin (ADR)

U2OS_ARF cells were harvested three days after 6h treatment with 1µM Adriamycin (ADR) and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.
(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.
(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.
(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

Treatment of U2OS_ARF cells with 0.7μM camptothecin increased levels of p53 and its target $p21^{Waf1}$ (Figure 4.3A) and promoted S-phase and G2/M accumulation (Figure 4.3B). Induction of p14ARF in the presence of camptothecin induced additional p53 and $p21^{Waf1}$ but did not sensitise U2OS_ARF cells to increased death in response to camptothecin treatment (Figure 4.3C).

As p14ARF expression has been shown to chemosensitise MCF-7 breast cancer cell to cisplatin (Deng *et al.*, 2002), we tested if p14ARF induction rendered U2OS_ARF cells more sensitive to this DNA damaging agent. Exposure of U2OS_ARF cells to 6μM cisplatin induced high p53 levels, but did not lead to $p21^{Waf1}$ upregulation (Figure 4.4A). In the presence of IPTG, treatment with cisplatin consistently led to the accumulation of higher levels of p14ARF, and increased levels of p53 but decreased levels of $p21^{Waf1}$, compared with the cisplatin only (Figure 4.4A). p14ARF expression decreased the level of apoptosis in response to cisplatin - specifically the sub-G1 population decreased slightly from 22±4% to 15±3%; Figure 4.4B) and total annexin-V positive cells from 25±5% to 16±2% (Figure 4.4C).

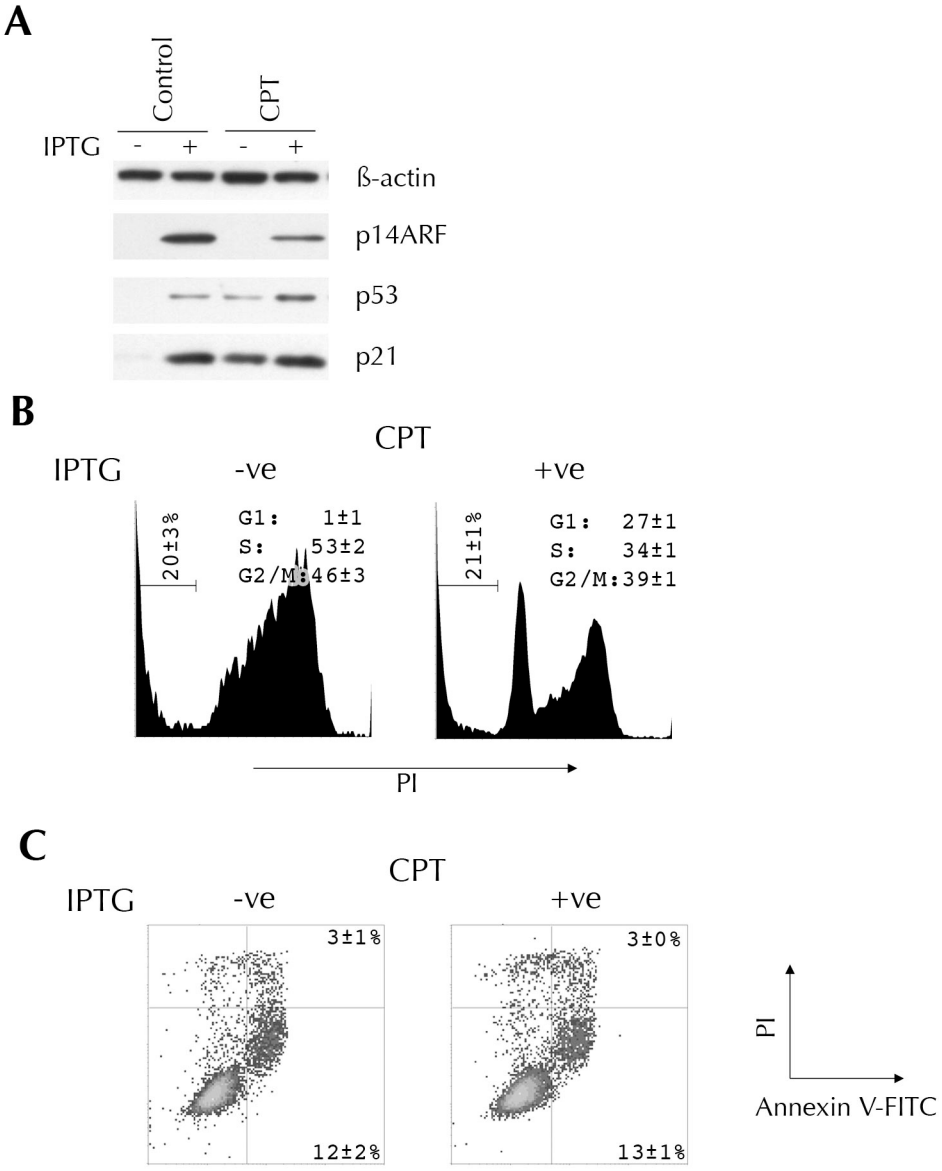


Figure 4.3 Expression of p14ARF does not sensitise cells to apoptosis in response to camptothecin (CPT)

U2OS_ARF cells were harvested three days after treatment with 0.7µM camptothecin and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

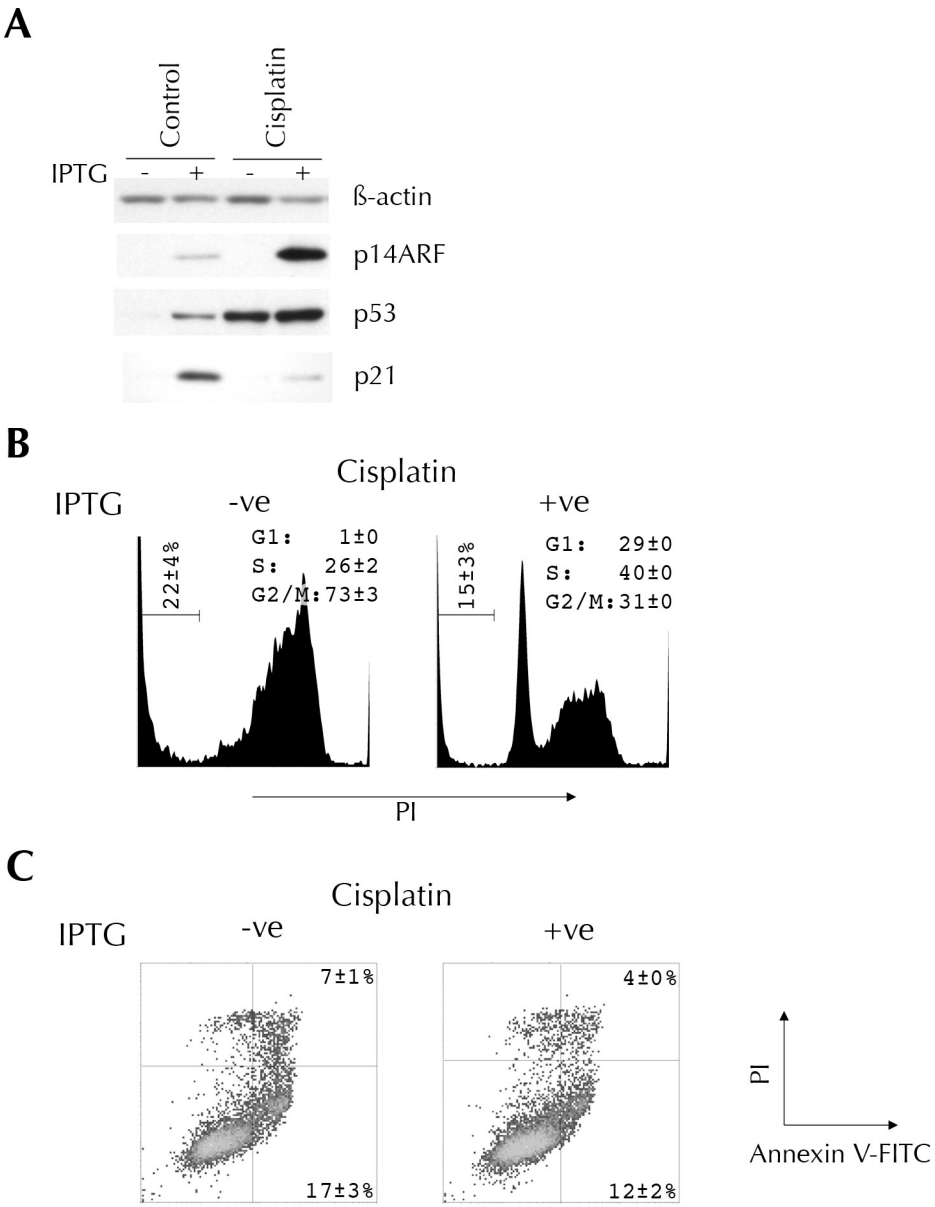


Figure 4.4 Expression of p14ARF reduces apoptosis in response to cisplatin

U2OS_ARF cells were harvested three days after treatment with 6µM cisplatin and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

4.2.3 p14ARF sensitises cells to apoptosis in response to UVB

Sun exposure is associated with an increased risk of melanoma and is predicted to be responsible for up to 97% of melanomas in Caucasian males in Queensland (Armstrong and Kricker, 1993; Armstrong and Kricker, 2001; Kricker *et al.*, 2007). UV radiation is the most damaging part of the sunlight spectrum and UVB radiation causes DNA damage by forming dipyrimidine dimers and 6-4 photoproducts (Jhappan *et al.*, 2003). To determine whether p14ARF expression sensitises U2OS_ARF cells to UVB mediated apoptosis, U2OS_ARF cells were treated with 60J/m^2 UVB followed by three days of recovery with IPTG induction. UVB exposure resulted in increased levels of p53 and $p21^{Waf1}$, but not to the levels achieved in IPTG only treated cells (Figure 4.5A). In cells exposed to IPTG and UVB the level of p14ARF accumulation was enhanced, and this was associated with further increases in the expression of p53 and $p21^{Waf1}$. Induction of p14ARF expression sensitised these cells to apoptosis in response to UVB, with the sub-G1 population increasing from 11±1% to 21±1% (Figure 4.5B) and annexin-V positive cells increasing from 25±2% to 36±4% (Figure 4.5C).

4.2.4 Inhibitors of transcription

Actinomycin D inhibits mRNA/rRNA transcription by inhibiting RNA polymerase I at low doses (<40nM) and both RNA polymerase I and RNA polymerase II at higher does (>800nM) (Haaf and Ward, 1996; Yung *et al.*, 1992) and is used to treat some cancers including rhabdomyosarcoma, Wilms tumour and in isolated limb perfusion for melanoma (Malogolowkin *et al.*, 2008; Thompson *et al.*, 1997; Thompson *et al.*, 1998; Walterhouse and Watson, 2007). Actinomycin D (7.5nM) treatment of U2OS_ARF cells for three days strongly induced both p53 and $p21^{Waf1}$ (Figure 4.6A) and, as

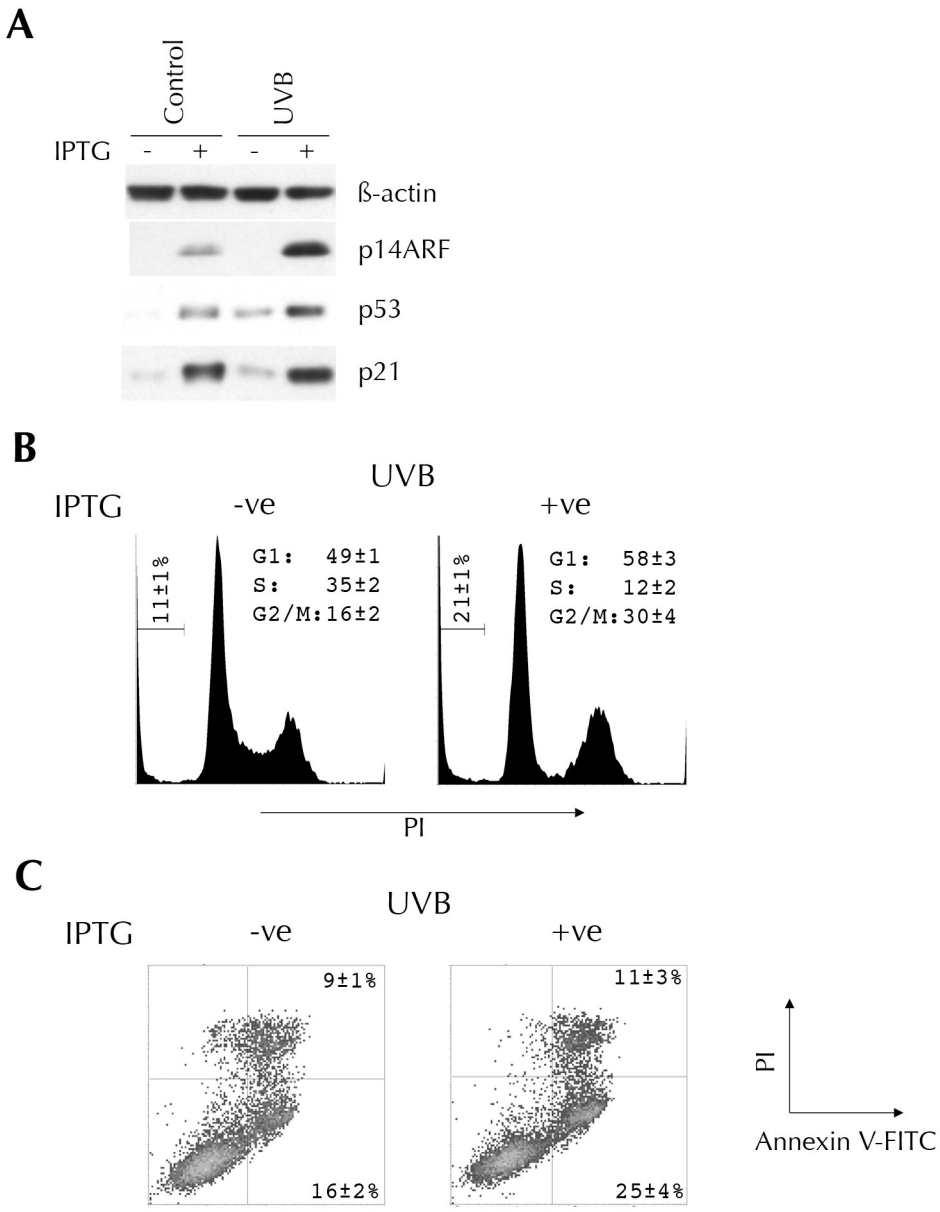


Figure 4.5 Expression of p14ARF sensitises cells to apoptosis in response to UVB radiation

U2OS_ARF cells were harvested three days after treatment with 60J/m^2 UVB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, p21^{Waf1} and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

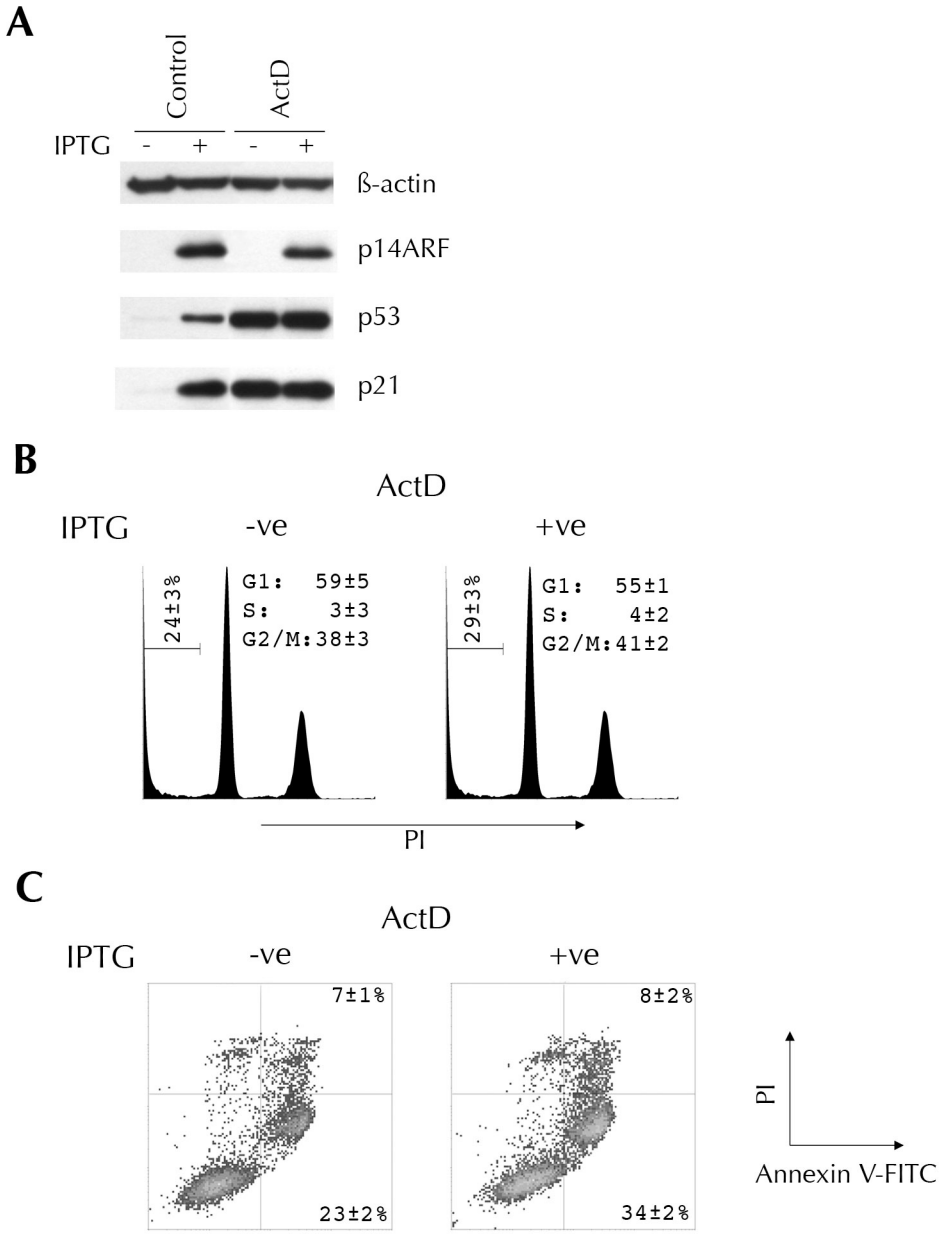


Figure 4.6 Expression of p14ARF does not sensitise cells to apoptosis in response to actinomycin D (ActD)

U2OS_ARF cells were harvested three days after treatment with 7.5nM actinomycin D and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

expected, reduced S-phase from 24±1% to 3±3% (Figure 4.6B). In actinomycin D exposed cells, induction of p14ARF expression lead to a small increase in the sub-G1 population of cells (24±3% vs 29±3%; Figure 4.6B) and a slight increase in the annexin-V positive population (30±3% vs 42±5%; Figure 4.6C), although there was no change in the already high levels of p53 and $p21^{Waf1}$ (Figure 4.6A). The slightly higher percentage of annexin-V positive cells compared to the sub-G1 population indicates that the U2OS_ARF cells exposed to actinomycin D (both with and without p14ARF induction) maybe in the early stages of apoptosis, but this was a minor proportion of the cells and was not supported by the sub-G1 data.

Exposure of U2OS_ARF cells to 50μM DRB (5,6 dichloro benzimidazole ribosode), an RNA synthesis and casein kinase II inhibitor, induced minor increases in the endogenous levels of p53 and $p21^{Waf1}$ (Figure 4.7A). In cells induced with IPTG, DRB exposure led to large increases in levels of p14ARF with no further increase in p53 accumulation and reduced levels of $p21^{Waf1}$, compared to the IPTG only treated cells. The combination of p14ARF induction and DRB promoted increased apoptosis to DRB treatment alone. Specifically, the sub-G1 population increased from 14±1% to 27±3% (Figure 4.7B) and annexin-V positive cells from 17±3% to 26±3% (Figure 4.7C). Although p14ARF induction usually promotes the accumulation of cells in the G1 phase of the cell cycle, in cells exposed to DRB, the induction of p14ARF promoted an increase in the proportion of cells in the G2/M phase, with G2/M population increasing from 20±2% to 54±4% (Figure 4.7B).

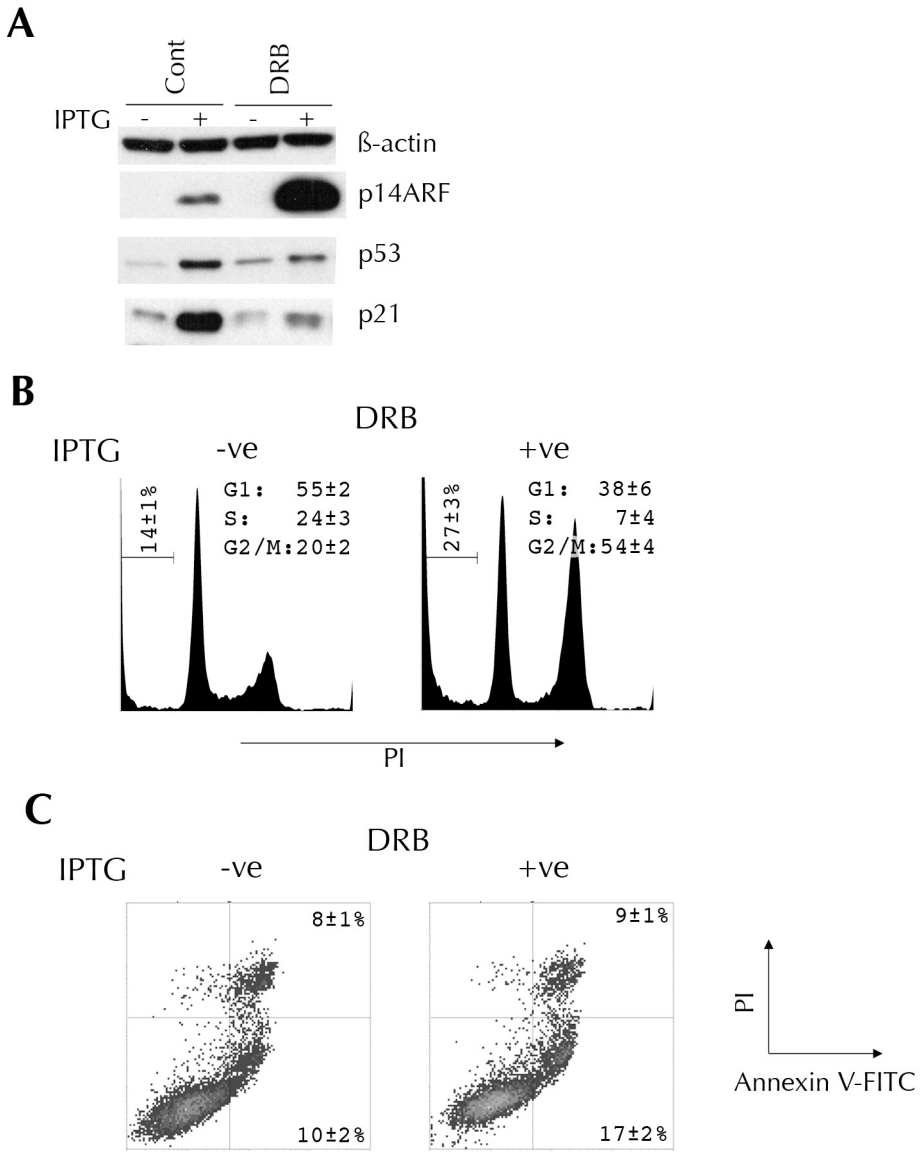


Figure 4.7 Expression of p14ARF sensitises cells to apoptosis in response to DRB

U2OS_ARF cells were harvested three days after treatment with 50µM DRB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

4.2.5 p14ARF does not chemosensitise U2OS_ARF cells to alkylating agents

As the alkylating agents dacarbazine (DTIC), temozolomide and melphalan are used in systemic and isolated limb perfusion treatment of melanoma, we tested if p14ARF sensitised cells to death in response to these agents. Treatment of U2OS_ARF cells with high concentrations of DTIC (1.5mM) induced p53 (Figure 4.8A) leading to cell death. The addition of IPTG did not augment the level of DTIC-induced death (Figure 4.8B & 4.8C). DTIC is a pro-drug that does not methylate DNA until it is converted to its active form (MTIC) by the liver. DTIC can be activated by white light (Metelmann and Von Hoff, 1983), which would have occurred to some extent during handling of the drug in our study. However light activation of DTIC for *in vitro* use is controversial due to the presence of cytotoxic photo-decomposition products and uncertainty of the exact products formed during photo-activation (Tentori and Graziani, 2004). Considering this, we obtained temozolomide (Schering-Plough), which is converted to MTIC in solution at physiological pH. Treatment of U2OS_ARF cells with 160 μM temozolomide alone resulted in less p53 accumulation compared to p14ARF-induced p53 accumulation, and temozolomide treatment did not lead to increased $p21^{Waf1}$ protein accumulation (Figure 4.9A). Temozolomide treatment caused a dramatic G2/M arrest in these cells and the addition of p14ARF expression did not alter the level of apoptosis or cell cycle distribution (Figure 4.9B & 4.9C).

Treatment of U2OS_ARF cells with the alkylating agent melphalan led to an increase in p53, with no associated increases in $p21^{Waf1}$ levels (Figure 4.10A). The combination of p14ARF induction and melphalan slightly enhanced p53 accumulation but $p21^{Waf1}$ protein levels remained low in comparison to the IPTG-only treated cells. p14ARF

induction in melphalan-treated cells increased the percentage of cells in the G1 phase from 1±1% to 15±7%, although it was hard to accurately fit the cell cycle histogram. While there was a small increase in sub-G1 population (22±3% to 30±5%, p-value = 0.03, two-tailed t-test) there was no change in the levels of apoptotic cells as measured by annexin-V staining (Figures 4.10B & 4.10C).

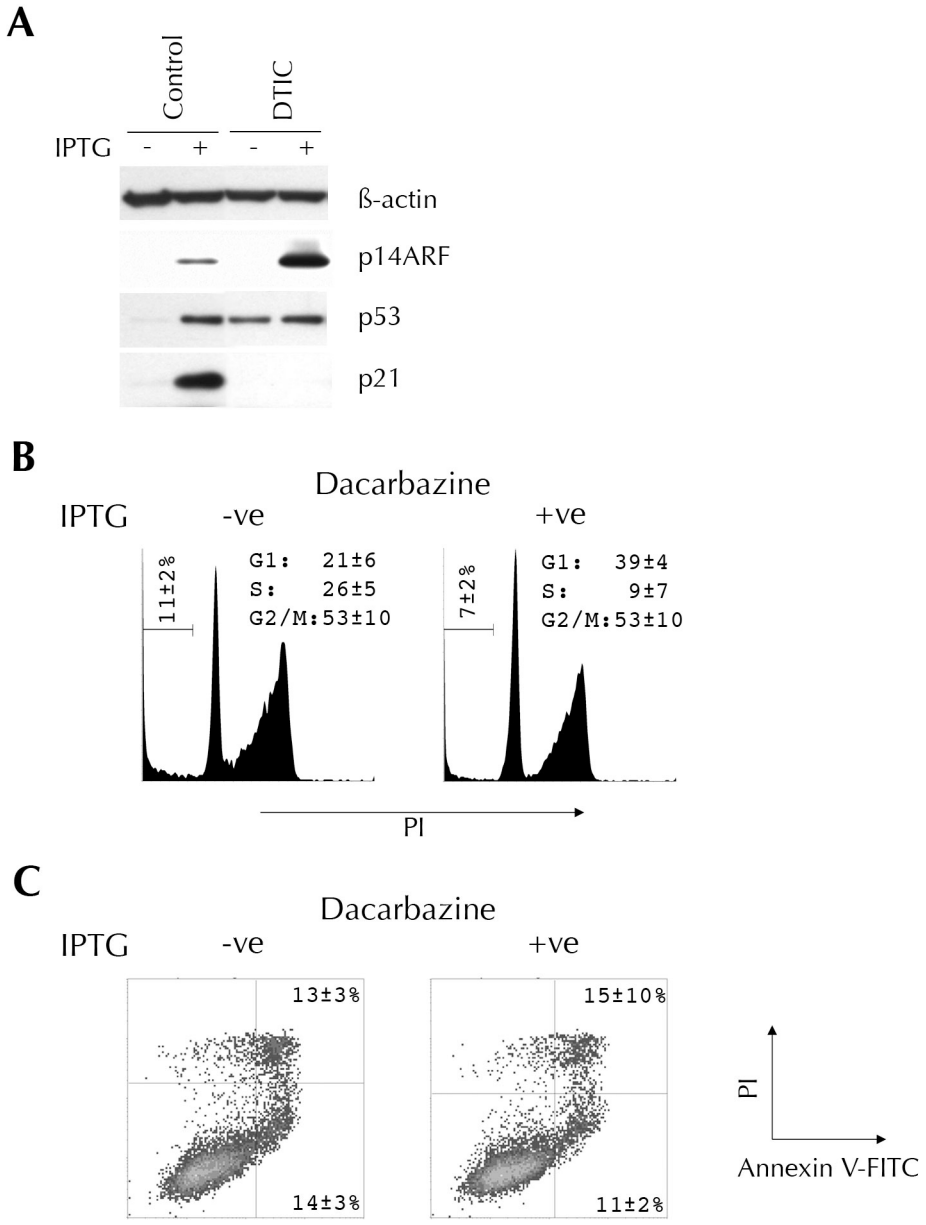


Figure 4.8 Expression of p14ARF does not sensitise cells to apoptosis in response to dacarbazine (DTIC)

U2OS_ARF cells were harvested three days after treatment with 1.5mM DTIC and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

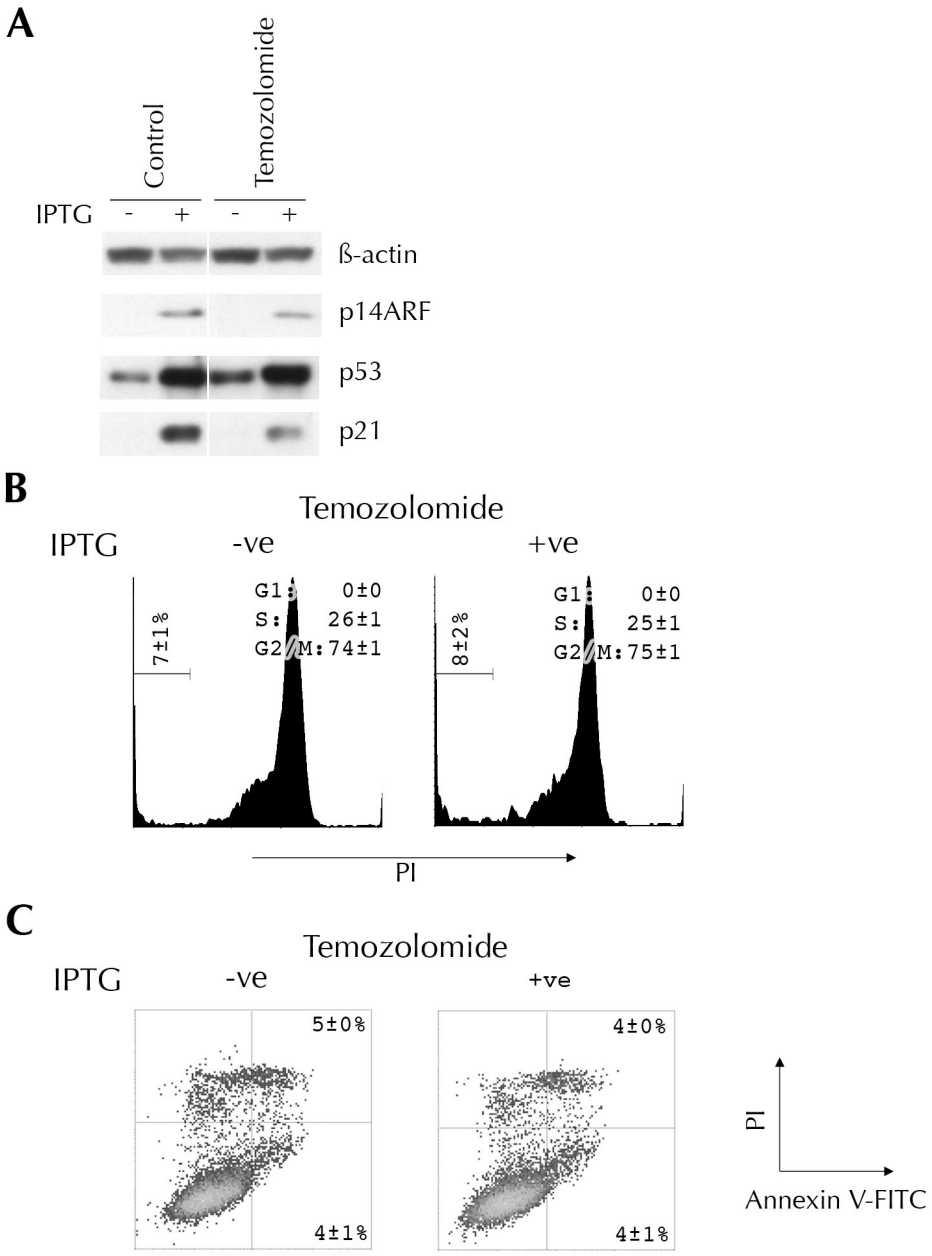


Figure 4.9 Expression of p14ARF does not sensitise cells to apoptosis in response to temozolomide

U2OS_ARF cells were harvested three days after treatment with 160µM temozolomide and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

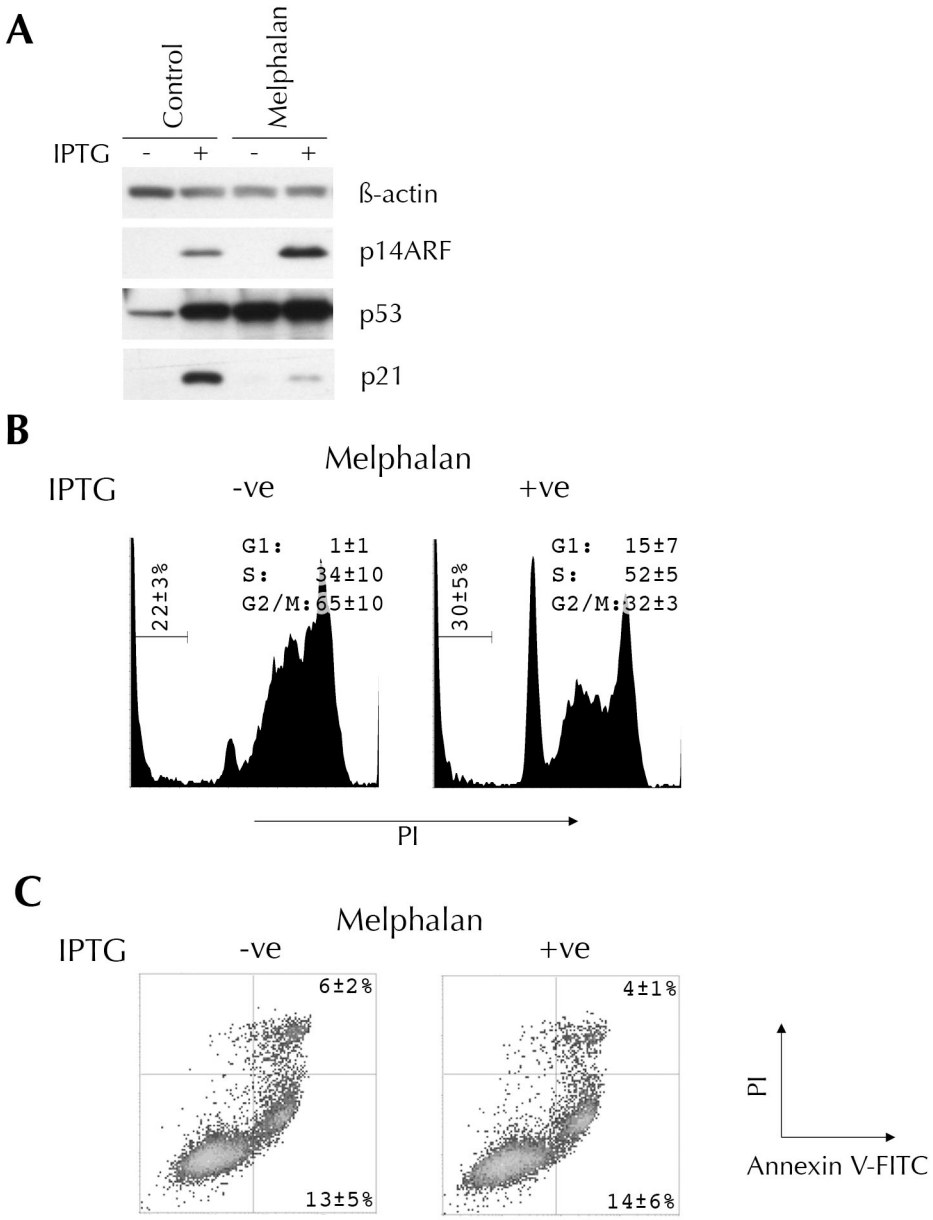


Figure 4.10 Expression of p14ARF sensitises cells to apoptosis in response to melphalan

U2OS_ARF cells were harvested three days after treatment with 100µM melphalan and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Wafl}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

4.2.6 p14ARF sensitises cells to death from histone deacetylase inhibitors

Histone acetylation and therefore chromatin structure is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Inhibitors of HDACs have been shown to cause growth arrest, differentiation and apoptosis in cancer cells, and show potential as therapeutic agents (Bolden *et al.*, 2006; Johnstone, 2002; Lindemann *et al.*, 2004).

To investigate if p14ARF expression could chemosensitise cells to HDAC inhibitors, U2OS_ARF cells were exposed to various HDAC inhibitors including sodium butyrate (NaB), trichostatin A (TSA) or valproic acid (VPA) in the presence or absence of IPTG. The $p21^{Waf1}$ promoter has NaB responsive elements (Xiao *et al.*, 1997) and as expected treatment of U2OS_ARF cells with 3mM NaB for three days induced strong $p21^{Waf1}$ expression in these cells with low levels of p53 (Figure 4.11A). In IPTG and NaB treated cells, levels of p53 and $p21^{Waf1}$ were similar to IPTG-only treated cells, although the level of p14ARF was slightly higher in the NaB plus IPTG exposed cells. NaB treated U2OS_ARF cells arrested in G1 and showed a significant decrease in the percentage of S-phase cells (Figure 4.11B). This G1 arrest was maintained once p14ARF expression was induced, and this was associated with increased apoptosis with the sub-G1 population increasing from 27±2% to 40±3% (Figure 4.11B) and the annexin-V positive population increasing from 29±2% to 37±2% (Figure 4.11C).

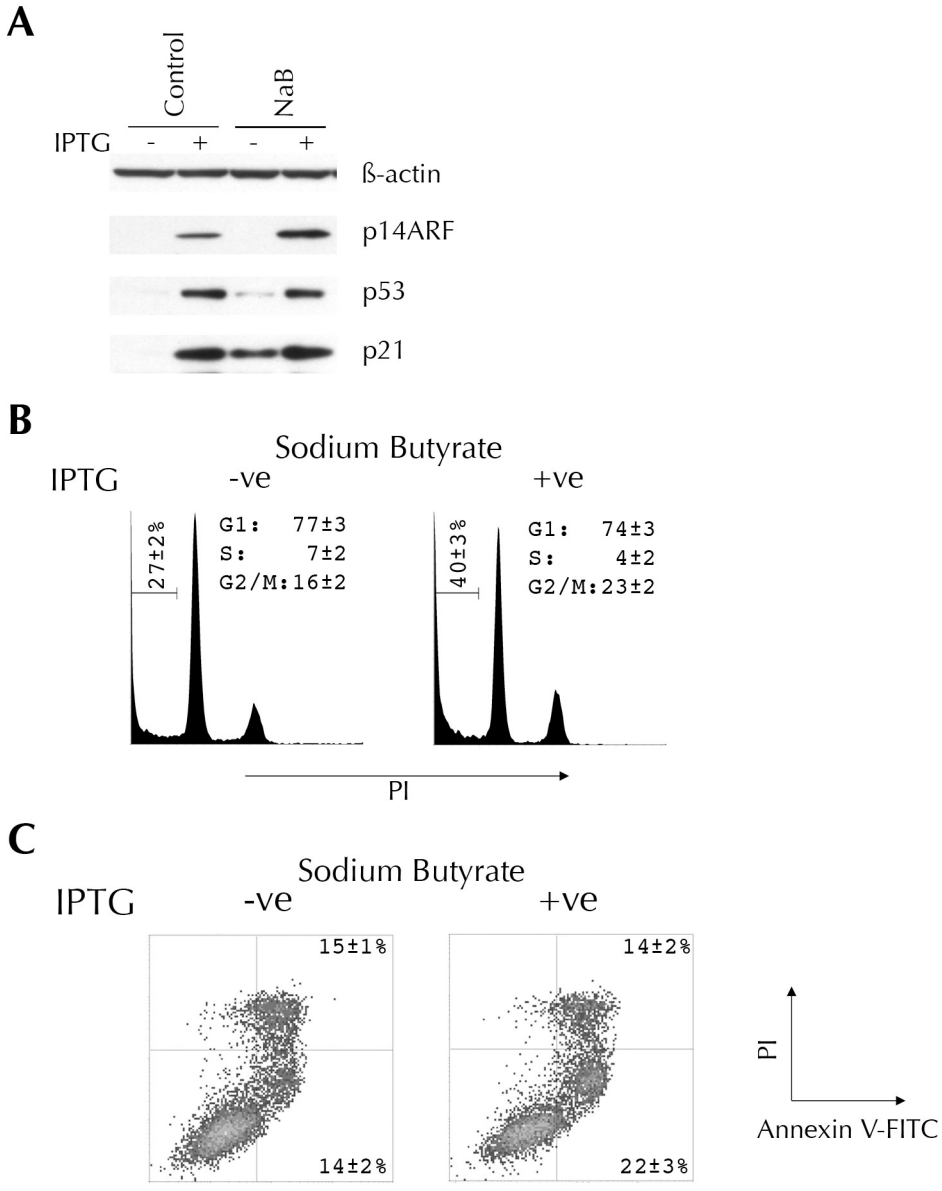


Figure 4.11 Expression of p14ARF sensitises cells to apoptosis in response to sodium butyrate (NaB)

U2OS_ARF cells were harvested three days after treatment with 3mM NaB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

The second histone deacetylase inhibitor tested was TSA, which has been shown to induce apoptosis and increase radiosensitivity in melanoma cell lines (Florenes *et al.*, 2004; Munshi *et al.*, 2005; Peltonen *et al.*, 2005). In cells induced to express p14ARF, 200nM TSA exposure led to further increases in the levels of p14ARF and p53, although levels of $p21^{Waf1}$ were not significantly changed from the p14ARF-induced control. TSA alone induced p53 and weakly increased levels of $p21^{Waf1}$ (Figure 4.12A). Enforced expression of p14ARF increased apoptosis in TSA exposed cells, raising the sub-G1 population from 25±6% to 47±8% (Figure 4.12B) and increasing total annexin-V positive cells from 32±3% to 49+5%, mainly due to an increase in early apoptotic (annexin-V positive, PI negative) cells from 23±3% to 40±5% (Figure 4.12C). Addition of IPTG to TSA treated cells also increased the G2/M population from 27±4% to 58±4% (Figure 4.12B).

As p14ARF expression chemosensitised cells to both NaB and TSA, we investigated a third histone deacetylase inhibitor to see if this function of p14ARF was common to this class of drugs. Accordingly, we treated cells with valproic acid, a histone deacetylase inhibitor with the same structural class and histone targets as NaB (Table 4.1). U2OS_ARF cells treated with VPA in the presence or absence of IPTG showed a similar pattern of cell cycle arrest and p53 and $p21^{Waf1}$ protein expression as cells treated with NaB (Figure 4.13A). However, p14ARF expression did not chemosensitise cells to apoptosis induced by VPA (Figures 4.13B & 4.13C). Differences in the effects of the three histone deacetylase inhibitors tested may explain why p14ARF sensitised cells to NaB and TSA but not VPA. For example VPA inhibits only class I histone deacetylases, while TSA is a pan HDAC inhibitor (Khan *et al.*, 2008), and VPA can stimulate growth of Ishikawa cells, while NaB and TSA do not (Rocha *et al.*, 2005b).

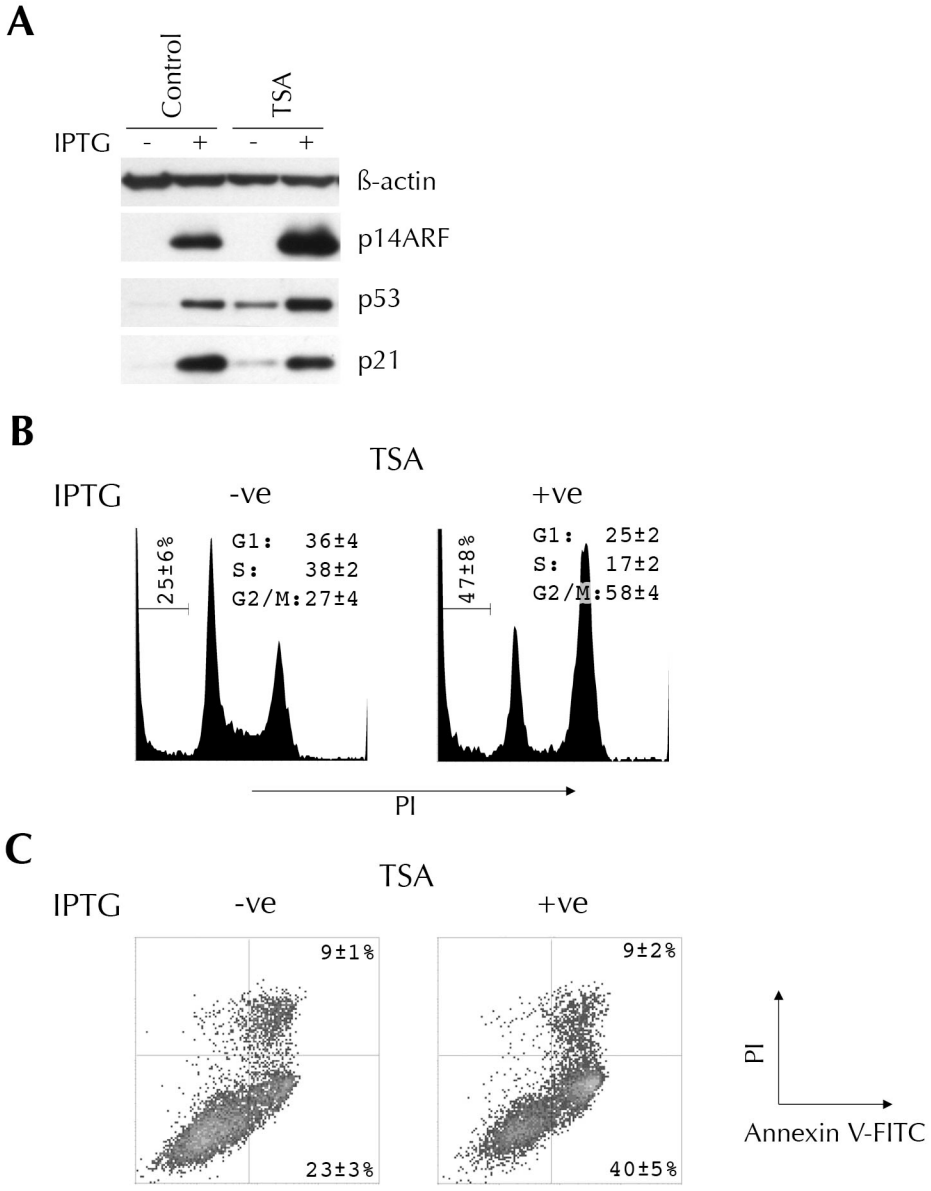


Figure 4.12 Expression of p14ARF sensitises cells to apoptosis in response to trichostatin A (TSA)

U2OS_ARF cells were harvested three days after treatment with 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

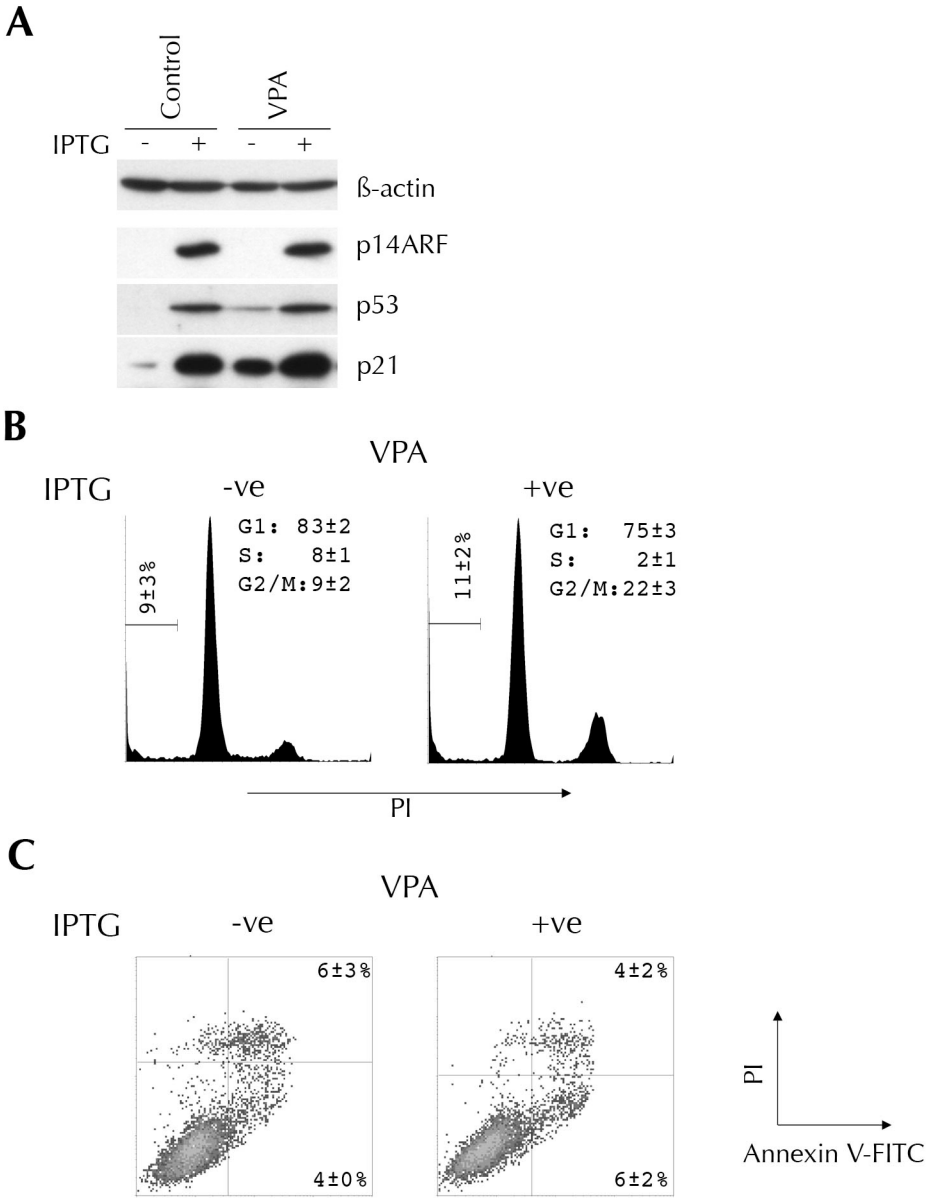


Figure 4.13 Expression of p14ARF does not sensitise cells to apoptosis in response to valproic acid (VPA)

U2OS_ARF cells were harvested three days after treatment with 4mM VPA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.

(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.

(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

4.2.7 p14ARF expression does not chemosensitise cells to taxol

Taxol is a cytotoxic agent that binds to the ß-subunit of tubulin and stabilises microtubules (Schiff *et al.*, 1979; Torres and Horwitz, 1998). Taxol stabilised p53 and increased $p21^{Waf1}$ levels in the U2OS_ARF cells (Figure 4.14A) and the induction of p14ARF did not promote further increases in p53 or $p21^{Waf1}$ levels. In agreement with others (Deng *et al.*, 2002) enforced expression of p14ARF did not increase apoptosis in response to taxol treatment as measured by the sub-G1 population (Figure 4.14B) or annexin-V positive cells (Figure 4.14C).

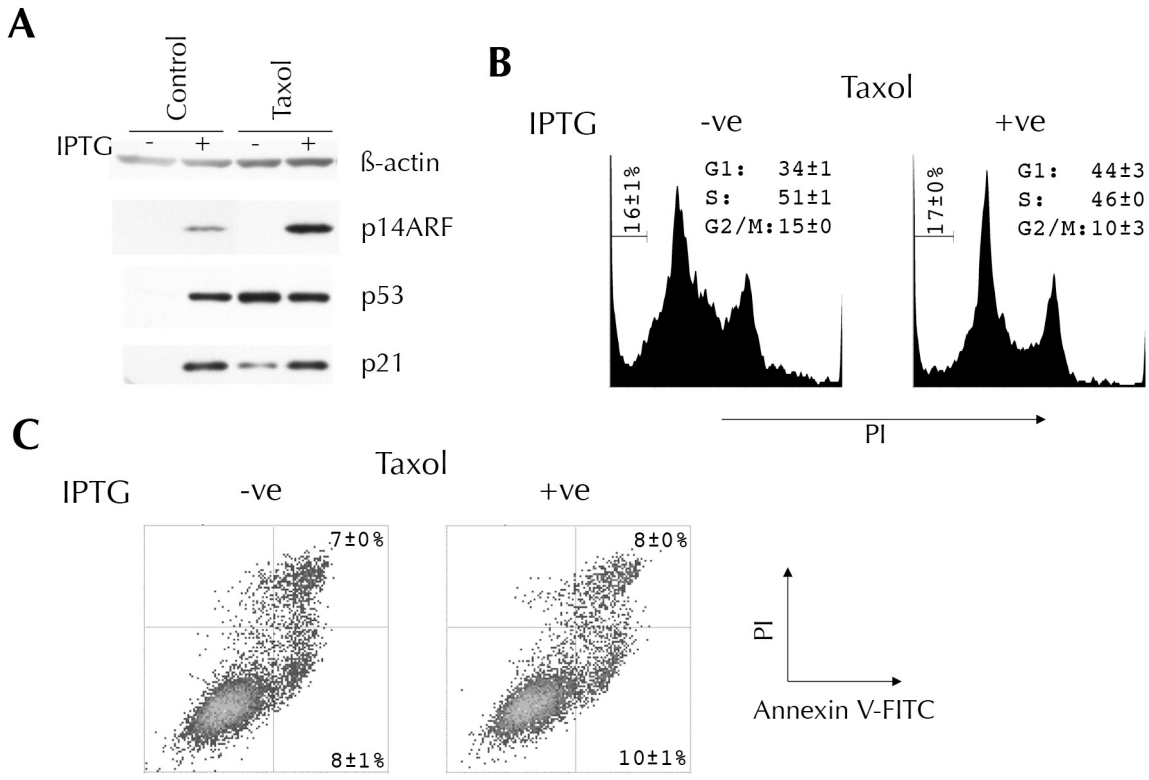


Figure 4.14 Expression of p14ARF does not sensitise cells to apoptosis in response to taxol

U2OS_ARF cells were harvested three days after treatment with 10nM taxol and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.
(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined by western blot.
(B) The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining.
(C) Apoptotic cells were assessed by dual colour flow cytometric annexin-V analysis.

4.2.8 IPTG does not contribute to chemosensitivity

Of the 13 cytotoxic agents screened for chemosensitisation with p14ARF in this work, only five (DRB, NaB, TSA, Adriamycin and UVB radiation) were found to co-operate with p14ARF in promoting cell death. DRB, NaB and TSA were subsequently chosen for further investigation, as they gave the largest increase in apoptosis in response to p14ARF induction (Chapter 5). To ensure IPTG itself did not increase chemosensitivity, the parental U2OS_lac17 cell line, which was used to derive the U2OS_ARF cell clone and expresses the lac repressor but not the *p14ARF* transgene, was treated with these three drugs in the presence or absence of 1mM IPTG. IPTG had no effect on levels of p53 or $p21^{Waf1}$ in U2OS_lac17 cells treated with DRB, NaB or TSA (Figure 4.15A), nor did IPTG affect the cell cycle (Figure 4.15B) or apoptosis (Figure 4.15C).

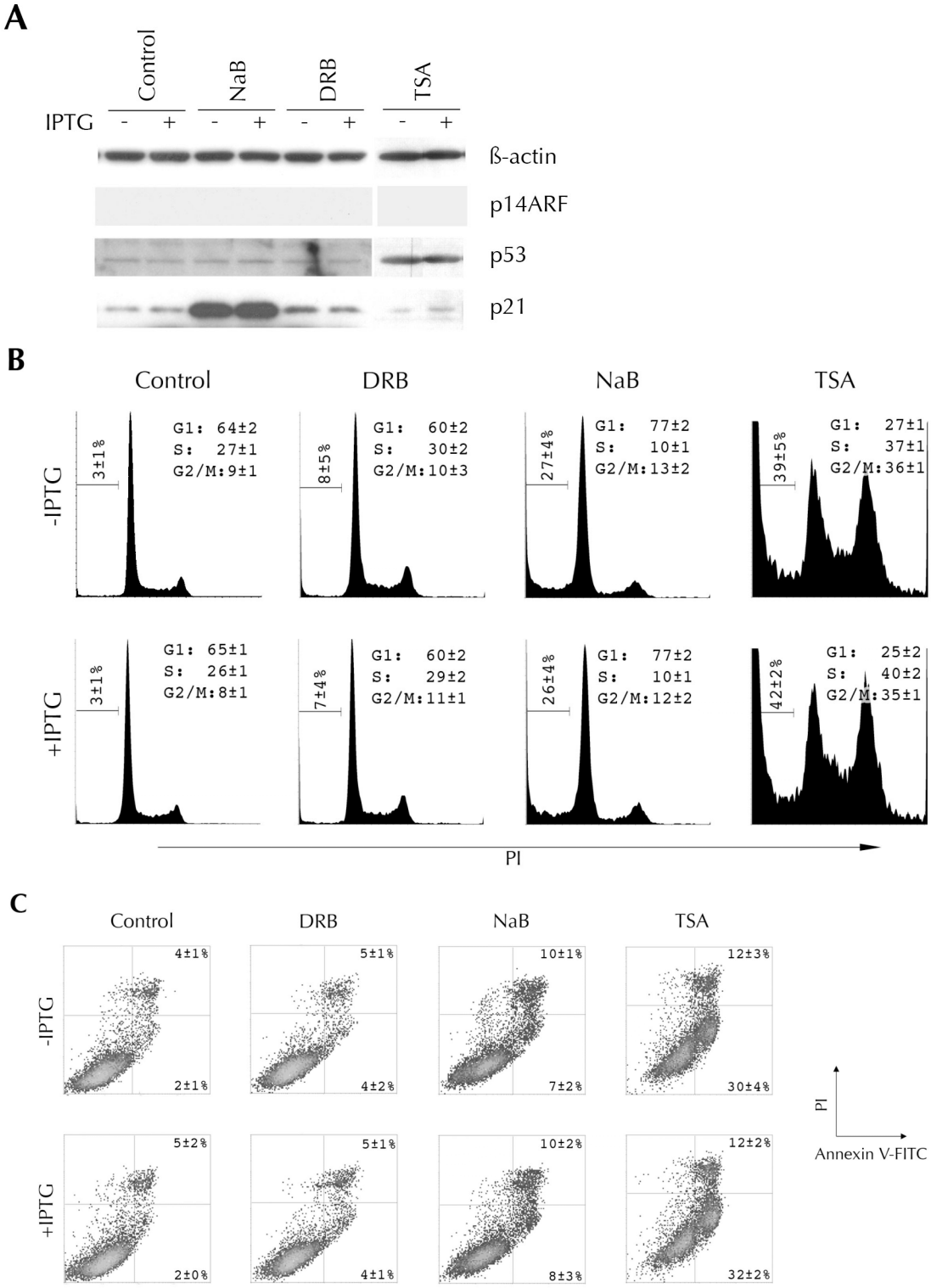


Figure 4.15 IPTG does not affect cell cycle or apoptosis in U2OS_lac 17 cells treated with DRB, sodium butyrate (NaB) or trichostatin A (TSA).

(A) Expression of p14ARF, p53, $p21^{Waf1}$ and ß-actin loading control was determined three days after treatment of U2OS_lac17 cells with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or IPTG (+).

(B) The cell cycle distribution of U2OS_lac17 cells was examined three days post 50µM DRB, 3mM NaB or 200nM TSA treatment and PBS (-) or IPTG (+), using propidium iodide (PI) staining.

(C) Dual colour flow cytometric annexin V analysis for apoptosis of U2OS_lac17 cells three days post treatment with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or IPTG (+).

4.2.9 Mechanism of DRB induced p14ARF protein levels.

Treatment of IPTG induced U2OS_ARF cells with DRB dramatically increased the amount of p14ARF protein compared to IPTG treatment alone (Figure 4.7A). To examine the basis of this increased ARF accumulation we initially analysed our protein extraction method. ARF is a nucleolar protein and some nucleolar proteins can be lost during extraction with RIPA buffer due to insolubility (our unpublished data). As DRB results in rapid and sustained nucleolar disruption (Haaf and Ward, 1996), with concomitant loss of ARF from the nucleolus (Figure 4.16A), it was possible that DRB treatment increased the efficiency of ARF extraction, rather than the total amount in the cell. However, extraction of p14ARF from induced U2OS_ARF cells (both DRB and control treated) was not increased by adding 6M urea to the RIPA buffer - which increases the extraction of insoluble nucleolar proteins (Figure 4.16B) (Rizos *et al.*, 2001a). This suggested that DRB led to an increase in the total amount of p14ARF protein.

The U2OS_ARF cell line only expresses p14ARF under the transgenic lac repressor promoter as the natural *INK4a/ARF* locus is hypermethylated (Park *et al.*, 2002). DRB did not de-methylate the natural *INK4a/ARF* locus as p14ARF protein was never detected in DRB treated U2OS_ARF cells that were not treated with IPTG (see Figure 4.7A). We then investigated if DRB could increase endogenous p14ARF levels in both SAOS-2 osteosarcoma and H1299 non-small cell lung cancer cells. Levels of endogenous p14ARF expressed by both cell lines was measured by western blot after 48 hours of exposure to 50μM DRB, a length of treatment sufficient to increase p14ARF levels in induced U2OS_ARF cells (data not shown). In both SAOS-2 and

H1299 cells, DRB did not increase p14ARF protein levels (Figure 4.16C). As expected, NaB and TSA did not have a significant effect on endogenous p14ARF either. To eliminate any possibility that the SAOS-2 and H1299 cancer cells harbour genetic alterations that prevent DRB impact on p14ARF expression, we treated WS-1 normal human embryonic fibroblasts with various levels of DRB for 48 hours. These cells express low levels of p14ARF that were not increased by exposure to DRB (Figure 4.16D).

As DRB did not increase p14ARF endogenous expression, we investigated whether the increase was due to the lac repressor system using WMM1175_ARF melanoma cells, which do not express endogenous p14ARF or p53 (Rizos *et al.*, 1999), but contain p14ARF under the same IPTG-inducible lac repressor system as U2OS_ARF cells (Gallagher *et al.*, 2005). As expected, treatment of WMM1175_ARF cells with 1mM IPTG for three days led to p14ARF expression, but no p53 or $p21^{Waf1}$ stabilisation. When IPTG induced cells were treated with DRB, p14ARF levels increased in a similar fashion to U2OS_ARF cells, suggesting that DRB only increases p14ARF levels under control of the lac expression system, and this increase is p53 independent (Figure 4.16E). TSA treatment of WMM1175_ARF cells was used as a control drug, and did not significantly change p14ARF levels. Considering that DRB's impact on p14ARF accumulation appears to be an artefact of the lac-switch system, we did not pursue the mechanism of this effect any further.

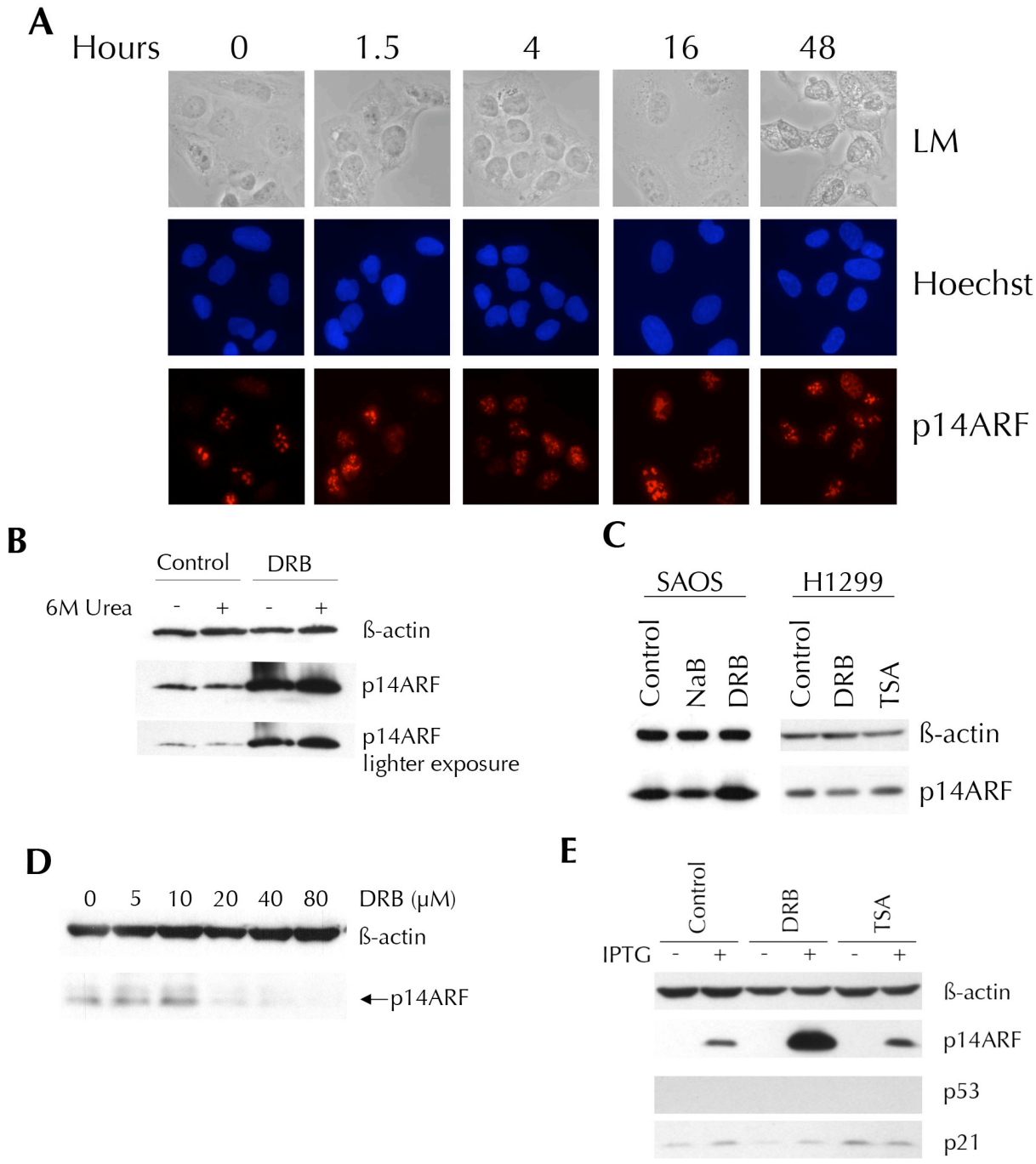


Figure 4.16 Analysis of the effect of DRB on p14ARF protein levels

(A) The nucleolar accumulation of p14ARF in IPTG treated U2OS_ARF cells was examined 0, 1.5, 4, 16 and 48 h after treatment with 50µM DRB by immunofluorescence. Hoechst staining was used to visualise cell nuclei. LM, light microscopy.

(B) The effect of 6M urea in the protein extraction buffer was tested in U2OS_ARF cells treated with 1mM IPTG and either 50µM DRB or DMSO (Control) was determined by western blotting. Recovery of p14ARF and ß-actin loading control was determined by western blot.

(C) Expression of endogenous p14ARF and ß-actin loading control was determined 48 hours after treatment of SAOS-2 and H1299 cells with 50µM DRB.

(D) Expression of endogenous p14ARF and ß-actin loading control was determined 48 hours after treatment of WS1 cells increasing amounts of DRB.

(E) Expression of p14ARF and ß-actin loading control was determined three days after treatment of WMM1175_ARF cells with 50µM DRB and PBS (-) or IPTG (+).

Table 4.2 Effect of p14ARF expression on drug sensitivity of U2OS_ARF cells.

Drug	**Dose**[a]	**IPTG**	**% sub-G1**	**% annexin-V positive**	**p53 levels**[c]	**p21^{Waf1} levels**[c]
Control	-	-	3±1	9±3	+	-
		+	4±1	13±2	+++	+++
ADR	1μM[b]	-	25±1	23±1	++	+
		+	37±3	31±4	++	+
Camptothecin	0.7μM	-	20±3	15±2	+++	+++
		+	21±1	16±1	++++	+++
Cisplatin	6μM	-	22±4	25±4	++++	+/-
		+	15±3	16±2	+++++	+
UVB	60J/m	-	11±1	25±2	++	+
		+	21±1	36±4	++++	+++
ActD	7.5nM	-	24±3	30±2	+++++	+++
		+	29±3	42±5	+++++	+++
DRB	50μM	-	14±1	17±3	+	+
		+	27±3	26±3	++	++
DTIC	1.5mM	-	11±2	27±1	++	-
		+	7±2	26±9	+++	-
Temozolomide	160μM	-	7±1	8±1	++	-
		+	8±2	7±1	+++	++
Melphalan	100μM	-	22±3	18±5	+++	-
		+	30±5	18±3	+++	+
NaB	3mM	-	27±2	29±2	+	+++
		+	40±3	37±2	+++	+++
TSA	200nM	-	25±6	32±3	++	+
		+	47±8	49±7	++++	+++
VPA	4mM	-	9±3	10±4	++	++
		+	11±2	10±1	+++	++++
Taxol	10nM	-	16±1	16±0	+++	+
		+	17±0	18±1	+++	+++

[a] U2OS_ARF cells were treated with indicated concentration of cytotoxic agent in the presence or absence of IPTG for three days.

[b] U2OS_ARF cells were treated with 1μM Adriamycin in the presence or absence of IPTG for 6 hours. The cells were then washed and incubated for a further three days in the presence or absence of IPTG.

[c] Level of p53 and p21^{Waf1} protein in cells compared to IPTG only treatmetn (+++).

4.3 Discussion

Many types of cancer, especially melanoma, are resistant to chemotherapy and modulating tumour chemosensitivity will be an important therapeutic approach in future cancer therapy. Drugs aimed at increasing the sensitivity of cells to death are already being tested in combination with traditional cytotoxic agents. For instance, the Bcl-2 anti-sense drug oblimersen, in combination with dacarbazine, was recently shown to increase overall survival in advanced melanoma patients without elevated baseline serum lactate dehydrogenase (Bedikian *et al.*, 2006). Additionally, novel drugs such as ABT-737 and GX15-070 that increase chemosensitivity by acting as BH3 mimetics also show therapeutic potential (Kang *et al.*, 2007; Nguyen *et al.*, 2007; Oltersdorf *et al.*, 2005; Witham *et al.*, 2007). Successful therapeutic development will depend on defining the genes that modulate drug-induced cell death. Here we show that p14ARF can increase the sensitivity of cells to death by a number of useful chemotherapeutic agents.

Of the 13 cytotoxic agents tested in our screen the induction of p14ARF increased sensitivity to only five of these agents (Table 4.2); p14ARF expression sensitised cells to the histone deacetylase inhibitors NaB and TSA, to the DNA damaging agents UVB and Adriamycin, and to the casein kinase and transcription inhibitor, DRB. From these data, it was not possible to predict the influence of ARF on drug-induced cell death. For instance, although ARF sensitised cells to two histone deacetylase inhibitors, it failed to synergise with the third HDAC inhibitor, VPA. Similarly, whereas most DNA damaging agents did not co-operate with ARF to promote cell death, UVB and Adriamycin did, although the type of damage these agents cause differs (Table 4.1).

There was also no evident association between ARF chemosensitisation and changes in cell cycle distribution, p53 accumulation, $p21^{Waf1}$ levels or p14ARF induction. The increased accumulation of p14ARF in response to DRB treatment is also unlikely to contribute to ARF's general role in drug-induced apoptosis, as it was not observed in the NaB, UV and TSA experiments and appears to be an artefact of our lac-switch cell model.

The mechanisms of p14ARF-induced apoptosis and chemosensitisation are not well established, but most likely occur via three alternative, but not necessarily independent, mechanisms. First, p14ARF suppression of HDM2 action allows increased accumulation of p53 in response to cytotoxic stress; second, p14ARF may re-direct the p53 response via post-translational modification from cell cycle arrest to apoptosis; and/or third, p14ARF may utilise a p53-independent pathway to promote drug-induced apoptosis, and this may involve novel ARF-binding proteins or novel ARF functions. The role of these potential mechanisms in our cell model are discussed below.

This first possible mechanism of p14ARF mediated chemosensitivity relates to the amount of p53 present in the cell, as opposed to any post-translational modifications. In the case of p53, "quantity" versus "quality" are difficult to separate as many modifications affect the stability of p53 and therefore its concentration in the cell. The total level of p53 in a cell - regardless of its state, is an important determinant of the transcriptional program that is activated. Some promoters are only activated in response to high levels of p53 (such as Bax, AIP1, and PIG3), and transcription from these weakly p53 responsive genes may change the p53 response from cell cycle arrest to apoptosis (Chen *et al.*, 1996; Kaeser and Iggo, 2002; Zhao *et al.*, 2000). Further

evidence for this effect is provided by gene dosage experiments that show ectopic expression of p53 in a variety of cancer cell lines leads to apoptosis in a dose dependant manner (Blagosklonny and el-Deiry, 1996; Blagosklonny and El-Deiry, 1998). Direct inhibition of HDM2 by p14ARF may allow a larger pool of p53 to accumulate in response to cytotoxic drugs - leading to increased chemosensitivity. This would be equivalent to the situation where ectopic expression of p53 confers increased chemosensitivity to a variety of DNA damaging drugs (Adriamycin, mitomycin C, cisplatin, etoposide, actinomycin D, CPT11), even though the drugs alone stabilise endogenous p53 (Blagosklonny and El-Deiry, 1998). In support of the hypothesis that p14ARF mediated chemosensitivity is simply due to increased levels of p53 protein, p53 levels were often higher in the drugs sensitised by p14ARF expression (for example UVB, NaB, TSA; Table 4.2). This was not always the case, however, and p14ARF induction also increased p53 levels in cells treated with some drugs that did not cooperate with p14ARF expression (such as temozolomide, cisplatin and valproic acid). Furthermore, although p14ARF induction sensitised cells to Adriamycin, these cells did not accumulate additional amounts of p53 (see Figure 4. 2A)

p14ARF may also impact on drug-induced cell death by promoting the post-translational modification of p53. The p53 protein can undergo a variety of post-translational modifications, including phosphorylation, acetylation, methylation, ubiquination and sumoylation, resulting in altered stability and transcriptional activity (Harris and Levine, 2005; Lavin and Gueven, 2006). For example, p53 may be phosphorylated at serine-15 and serine-20 by a number of cell stress receptors including ATM and Chk2. These phosphorylations result in increased stability and apoptosis, and mutations affecting these serine residues impair the ability of p53 to cause apoptosis

(Unger *et al.*, 1999). Direct modification of p53 by p14ARF would be a novel function of ARF. ARF can sumoylate a number of its binding partners, including HDM2 and E2F-1, HIF-1α, TBP-1 and $p120^{E4F}$, and when complexed with HDM2, ARF can also stimulate the sumoylation of p53 (Chen and Chen, 2003; Rizos *et al.*, 2005). Although p53 tagged with SUMO-1 has enhanced transcriptional activity (Rodriguez *et al.*, 1999b) we observed no evidence of sumoylated HDM2 or p53 in response to ARF induction in the U2OS_ARF cells (data not shown). p19ARF has been shown to induce acetylation of lysine residue 120 in p53 (Mellert *et al.*, 2007), a modification important for transcription of pro-apoptotic p53 targets such as Bax and PUMA (Sykes *et al.*, 2006). The mechanism by which this occurs is unknown, but acetylation of this same lysine residue is also induced by a number of cytotoxic agents - including UV, CPT and ADR (Sykes *et al.*, 2006). Thus, this is unlikely to be the mechanism behind p14ARF-mediated chemosensitivity as K120-p53 is acetylated by the cytotoxic reagent alone. p14ARF can also indirectly induce acetylation of p53 on K382 via the histone acyl transferase hAda3 (alteration/deficiency in activation) (Sekaric *et al.*, 2007), and ARF interaction with p53 binding partners (such as HDM2, Tip60 and NPM) could conceivably modulate p53 modification by other proteins.

Although modification of p53 is an attractive hypothesis to explain p14ARF mediated chemosensitivity, it should be noted that phosphorylation of p53 may be dispensable for its activation. Unphosphorylated p53 induced by ARF or nutlin, a small molecule inhibitor of HDM2, had the same transcriptional program as p53 phosphorylated on multiple residues as a result of doxorubicin and etoposide treatment (Jackson *et al.*, 2004; Thompson *et al.*, 2004). Additionally, acetylation is thought to have only a minor effect on p53 transcription (Lavin and Gueven, 2006).

Although several studies have implicated the importance of p53 in ARF mediated chemosensitisation (Gao *et al.*, 2001; Kim *et al.*, 2004; Simon *et al.*, 2006; Suzuki *et al.*, 2003), it remains possible that p14ARF alters cell death response in a p53-independent manner (Magro *et al.*, 2004). ARF mediated reduction of the protein tyrosine phosphatase, cell division cycle 25c (cdc25c) levels may deregulate the G2/M checkpoint, increasing sensitivity to apoptosis (Eymin *et al.*, 2006a; Eymin *et al.*, 2003; Normand *et al.*, 2005). The importance of the G2/M checkpoint in p14ARF mediated chemosensitivity is suggested by our observation that p14ARF plus drug often increased the G2/M population of cells.

Another candidate for p14ARF-mediated apoptosis is E2F-1, which is degraded by p14ARF induction in U2OS_ARF cells (Rizos *et al.*, 2007; Rizos *et al.*, 2005). Cellular response to E2F-1 is complicated - it can drive cell proliferation and in some settings can protect cells from apoptosis (Bandara *et al.*, 1997; Mason *et al.*, 2002; Wikonkal *et al.*, 2003). ARF binding, sumoylation and subsequent degradation of E2F-1 would abolish this protection. In most circumstances however, E2F-1 over-expression causes apoptosis (Bell and Ryan, 2003). Thus, ARF inhibition of E2F-1 transcription (Datta *et al.*, 2002; Datta *et al.*, 2005; Eymin *et al.*, 2001; Mason *et al.*, 2002; Rizos *et al.*, 2007) may actually protect cells from apoptosis as E2F-1 represses anti-apoptotic Mcl-1 (Croxton *et al.*, 2002) and transactivates the pro-apoptotic proteins ATM, apoptosis-stimulating p53 protein 1, apoptosis stimulating protein 2, junction-mediating regulatory protein and tumour protein 53-induced nuclear protein 1 (Berkovich and Ginsberg, 2003; Hershko *et al.*, 2005; Stanelle *et al.*, 2002). The complexities of

cellular response to the E2F-1 “double edged sword” (Stevens and La Thangue, 2003) make its involvement in p14ARF-mediated chemosensitivity hard to predict.

Our data confirm that p14ARF can increase chemosensitivity for a subset of drugs, *in vitro*. In the following chapters we describe potential mechanisms of ARF-induced cell death and explore whether p14ARF alters chemosensitivity *in vivo*, and if the status of p14ARF would be a useful predictor of melanoma chemoresistance.