5. Mechanisms of p14ARF induced chemosensitivity

5.1 Introduction

In the previous chapter we demonstrated that p14ARF increases the sensitivity of U2OS_ARF cells to certain cytotoxic insults. Defining the mechanism(s) involved in p14ARF-mediated chemosensitivity may identify new drug targets as well as highlight the clinical settings in which p14ARF-induced chemosensitivity may be important.

The increase in chemosensitivity following p14ARF induction was associated with increased DNA fragmentation and phosphotidyl-serine exposure (as measured by increased sub-G1 content and annexin-V staining, respectively). These two features are markers of a classic apoptotic response, rather than necrosis or other forms of cell death (Clarke, 1990). The central mediators of apoptosis are a series of cysteine proteases, caspases, with initiator caspases (including caspase 2, 8 9 and 10) cleaving and activating effector caspases. The effector caspases (caspase 3, 6 and 7) cleave substrates such as the inhibitor of caspase-activated DNase (iCAD) and poly(ADP-ribose) polymerase (PARP), leading to the phenotypic changes characteristic of apoptosis, including chromatin condensation, exposure of phosphatidyl-serine on the plasma membrane and DNA fragmentation (Clarke, 1990).

Initiation of apoptosis can occur via the intrinsic or extrinsic pathways. The extrinsic pathway activates caspase 8 and is initiated by death receptors such as fibroblast-associated receptor (Fas), tumour necrosis factor receptors (TNFR1/2) and TNF-related apoptosis inducing ligand receptor (TRAIL-R1). The intrinsic pathway is activated by

mitochondrial outer membrane permeabilisation, allowing release of pro-apoptotic proteins such as cytochrome c, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI (SMAC/DIABLO) and apoptosis inducing factor (AIF) into the cytoplasm. Mitochondrial permeabilisation occurs when pores form in the mitochondrial outer membrane. These pores are formed via the assembly of pro-apoptotic Bcl-2-associated X protein (Bax) and Bcl-2 antagonist killer 1 protein (Bak) (Figure 5.1).

p53 is a key regulator of the intrinsic and extrinsic apoptotic pathways; p53 can transcriptionally activate several pro-apoptotic Bcl-2 family proteins, such as Bax, NOXA and PUMA, as well as death receptors such as Fas and the death domain containing protein PIDD (pro-apoptotic interacting domain), and also Fas ligand (FasL). Consequently, stabilisation of p53 by p14ARF may provide general pro-apoptotic signals to the cell via p53-dependent transactivation of PUMA, NOXA, Bax, Fas and FasL. Accordingly, p14ARF expression increased radio- and chemosensitivity in p53-intact cell lines (Gao *et al.*, 2001; Simon *et al.*, 2006). The impact of p53 on apoptosis is complex however, as p53 may preferentially transactivate pro-arrest rather than pro-apoptotic targets (eg $p21^{Waf1}$, GADD45, 14-3-3σ). The transcriptional program activated by p53 is influenced by post-translational modification of p53 and presence of co-stimulatory molecules such as hCas and hematopoietic zinc finger protein (Das *et al.*, 2007; Tanaka *et al.*, 2007; Xu, 2003).

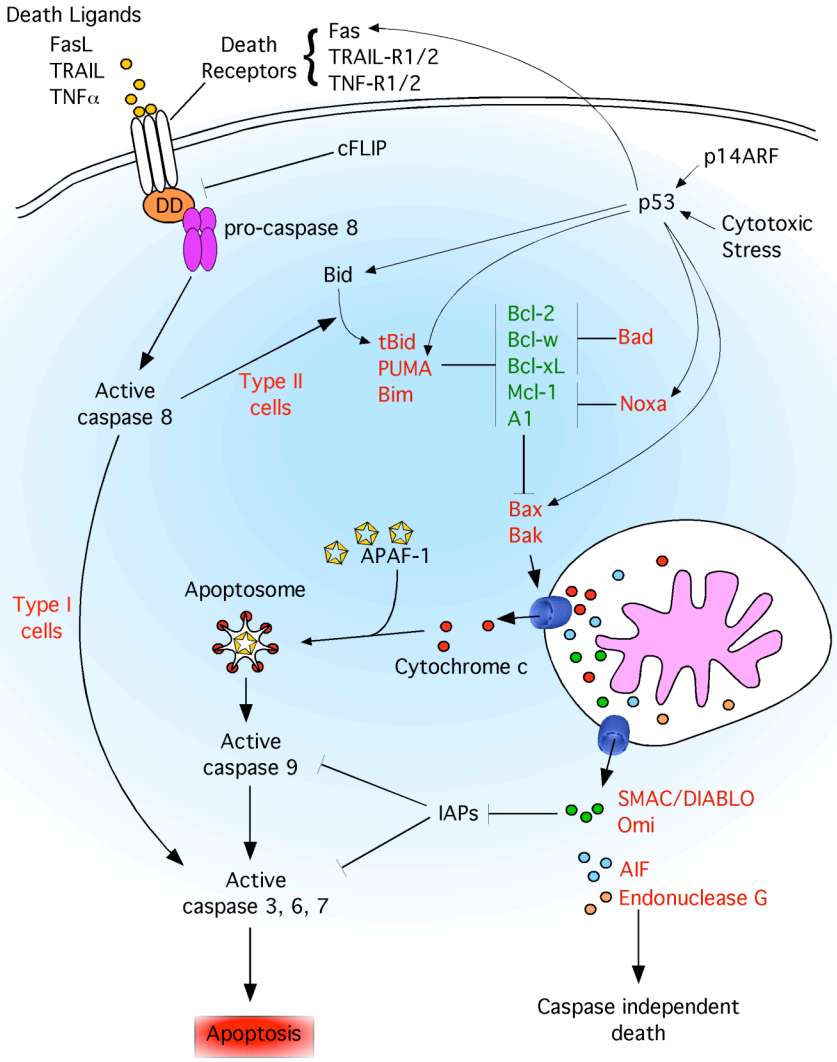
An alternate mechanism by which ARF may chemosensitise cells involves the short isoform of ARF known as smARF (short mitochondrial ARF) (Reef *et al.*, 2006).

Ectopic expression of smARF reduces mitochondrial membrane potential without causing cytochrome c release or caspase activation. smARF induces autophagic cell death in a p53- and Bcl-2-independent manner and is a short-lived product derived from the internal initiation of translation at Met45 (Reef *et al.*, 2006) (reviewed in (Reef and Kimchi, 2006)). smARF lacks the amino-terminal ARF nucleolar targeting domain and localises to the mitochondria, where it interacts with and is stabilised by the mitochondrial protein, p32 (Reef *et al.*, 2007).

We investigated a number of key apoptotic effectors to determine the mechanism underlying p14ARF-mediated chemosensitivity. Our data show that p14ARF-mediated chemosensitivity is p53- and caspase-dependent, and is reliant on loss of mitochondrial potential. Inhibition of individual components of some apoptotic pathways showed that p14ARF-mediated cell death was not strictly dependent on Bax or Fas. Moreover, we found no evidence that smARF contributed to chemosensitisation by p14ARF in our cell models. These results reinforce the importance of p53 and caspases in the apoptotic programs facilitated by ARF.

Figure 5.1 Overview of apoptosis pathways

Anti-apoptotic Bcl-2 family members are in green text. Pro-apoptotic Bcl-2 proteins and mitochondrial proteins are in red text. This figure is reproduced from chapter 1. DD; death domain.



5.2 Results

To elucidate the mechanism responsible for p14ARF-mediated chemosensitivity, we focused on the effect of ARF expression on three separate cytotoxic agents - DRB, NaB and TSA. These drugs were selected because p14ARF expression significantly enhanced their killing capacity (see chapter 4).

5.2.1 p14ARF mediated chemosensitivity is p53-dependent

Considering that p14ARF-mediated cell cycle arrest requires p53, and p53 is a well-established apoptotic regulator, the role of p53 on p14ARF-mediated chemosensitivity was investigated. As shown in the previous chapter, p53 is stabilised in response to DRB, NaB or TSA treatment of U2OS_ARF cells (Figure 5.2). As expected, induction of p14ARF expression alone also induced the accumulation of p53, to levels seen in the drug treated cells (Figure 5.2). These high levels of p53 were transcriptionally active as determined by increases in the levels of the p53 targets $p21^{Waf1}$ and, to a lesser extent, GADD45 (Figure 5.2).

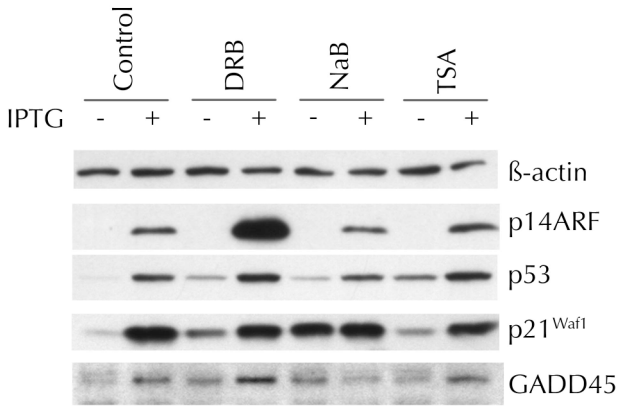


Figure 5.2 Expression of p53, $p21^{Waf1}$, and GADD45 in U2OS_ARF cells treated with cytotoxic drugs and IPTG

Expression of p14ARF, p53, $p21^{Waf1}$ GADD45 and ß-actin loading control was determined three days after treatment of U2OS_ARF cells with 50μM DRB, 3mM NaB or 200nM TSA and PBS (-) or IPTG (+).

To investigate if p14ARF required p53 to increase chemosensitivity we initially compared the isogenic NARF2 (p53-intact) and NARF2_E6 (p53-defective) osteosarcoma cell lines. Both these cell lines express IPTG inducible p14ARF but in the NARF2_E6 cell line, p53 is degraded by constitutive expression of the human papilloma virus E6 ubiquitin ligase (Rodway *et al.*, 2004).

To ensure p14ARF expression enhanced chemosensitivity in the NARF2 cells, the cells were exposed to DRB, NaB or TSA or a DMSO carrier control for 72 hours in the presence or absence of IPTG. As expected, the NARF2 cells behaved as our U2OS_ARF cells in response to IPTG and drugs (Figure 5.3, 5.4A & B). In particular, exposure of NARF2 cells to IPTG resulted in strong expression of p14ARF, and accumulation of p53 and $p21^{Waf1}$ (Figure 5.3) with concomitant cell cycle arrest (Figure 5.4A). IPTG treatment of NARF2_E6 cells also resulted in strong accumulation of p14ARF in control and drug treated cells, but the low levels of p53 found in these cells did not increase with ARF expression and there was no change in the low levels of $p21^{Waf1}$ (Figure 5.3). These data confirm that p53 was effectively inactivated by constitutive E6 expression in these cells.

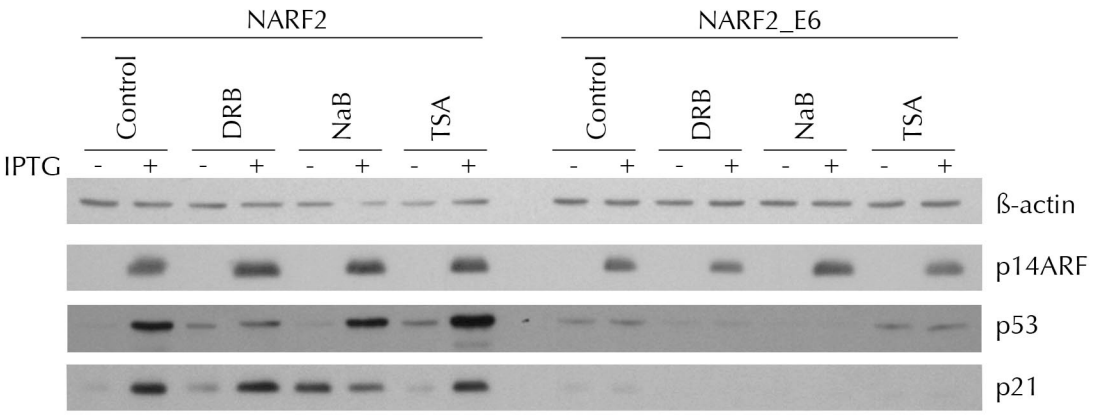


Figure 5.3 Expression of p14ARF, p53, p21^{Waf1} in NARF2 and NARF2_E6 cells in response to cytotoxic drugs and p14ARF induction

NARF2 and NARF2_E6 cells were treated for three days with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. p53 is inactivated in NARF2_E6 cells by the E6 protein. Western blot was used to determine expression of p14ARF, p53, p21^{Waf1} and ß-actin loading control.

Accumulation of p14ARF in the p53-intact NARF2 cells increased cell death in response to the three cytotoxic drugs used. Specifically, the addition of IPTG increased the sub-G1 NARF2 population when cells were treated with DRB (11±2% to 30±6%), NaB (18±2% to 32±4%) and TSA (59±12 to 77±8%) (Figure 5.4A). Annexin-V staining confirmed that p14ARF induction increased levels of apoptosis in response to DRB, NaB and TSA (Figure 5.4B). Further, as was the case in our U2OS_ARF cells, the induction of p14ARF also increased the G2/M population cells treated with DRB (26±3% to 45±2%), NaB (13±4 to 37±1%) and TSA (24±6 to 45±4%) (Figure 5.4A).

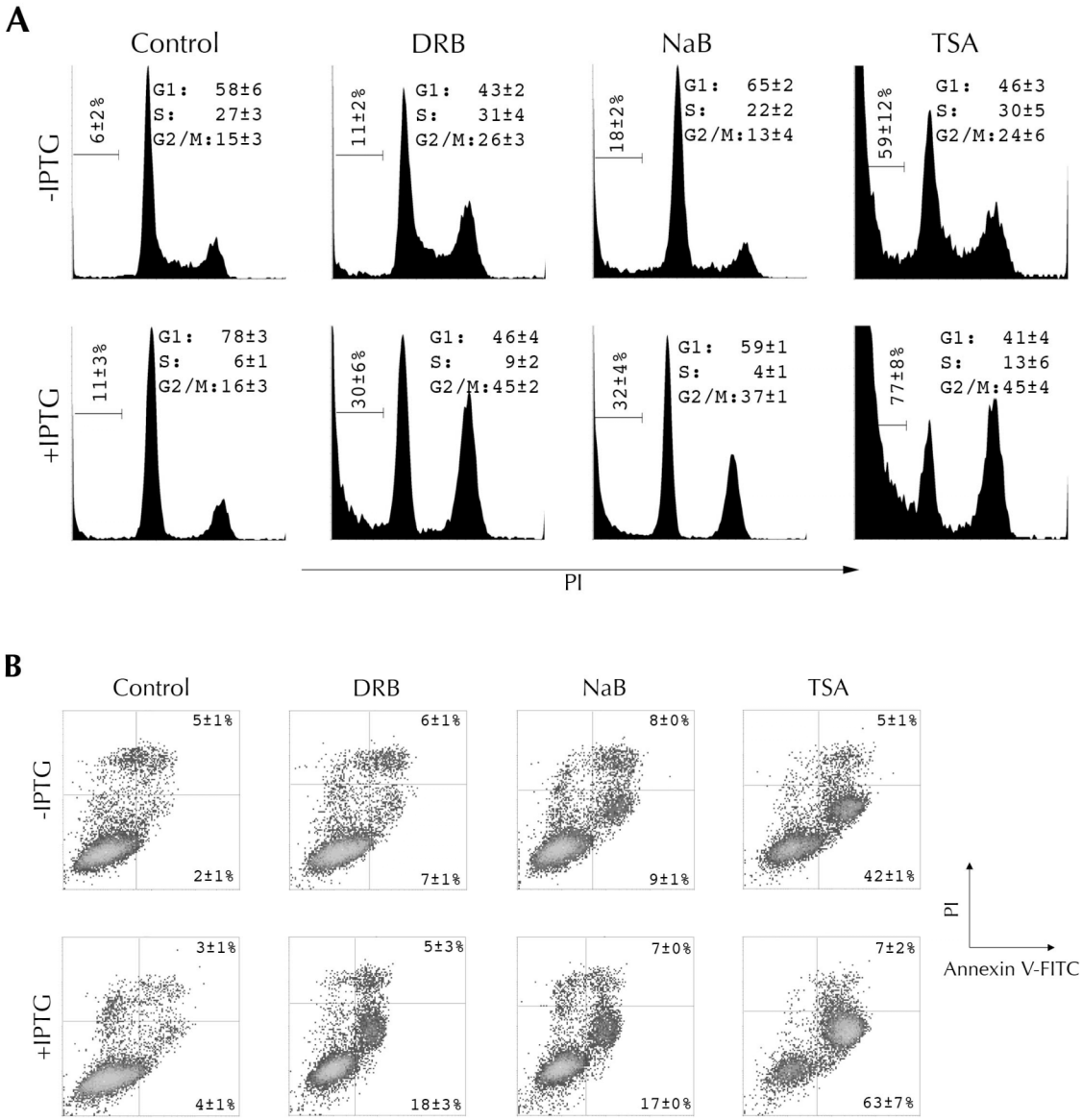


Figure 5.4 Expression of p14ARF sensitises NARF2 cells to apoptosis in response to cytotoxic drugs

NARF2 cells were treated for three days with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

(A) The cell cycle distribution and sub-G1 population of NARF2 cells was examined using propidium iodide (PI) staining.

(B) Apoptotic NARF2 cells were also analysed by dual colour flow cytometric staining of annexin-V and PI exclusion.

In contrast to the NARF2 cells, induction of p14ARF in NARF2_E6 cells did not increase chemosensitivity. For instance, induction of p14ARF expression did not increase the sub-G1 population of NARF2_E6 cells treated with DRB (8±2% to 10±2%), NaB (19±3% to 17±4%) or TSA (57±3% to 56±5%) (Figure 5.5A). Additionally, the annexin-V positive population was not increased by p14ARF induction in cells treated with DRB (8±3% to 10±3%), NaB (20±2% to 26±2%) or TSA (61±1% to 64±6%) (Figure 5.5B). Further, p14ARF accumulation did not lead to potent G1 arrest in these cells, although there was a slight decrease in S-phase cells in control (27±2% to 23±2%) and DRB treated cells (36±1 to 31±1%) and, surprisingly, an increase in S-phase in NaB treated cells (16±2% to 27±2%) (Figure 5.5A).

Although the NARF2 and NARF2_E6 are isogenic cell clones, differing in their p53 status, they have increased ploidy (Figures 3.8A & B) which can affect cell response to cytotoxic insult (Castedo *et al.*, 2006) and the constitutive expression of E6 has p53-independent effects, including increased telomerase activity and enhanced vascular endothelial growth factor transcription (Clere *et al.*, 2007; Veldman *et al.*, 2003). To avoid non-specific E6 effects, we utilised a lentivirus system to stably and specifically silence p53 expression in the U2OS_ARF cell line. U2OS_ARF cells were infected with lentivirus expressing a short-hairpin-RNA (shRNA) molecule targeting the p53 transcript (U2OS_ARF^{si_p53}) or the luciferase transcript (U2OS_ARF^{si_Luc}) as a negative control. The co-expression of GFP confirmed that over 95% of cells stably expressed the shRNA molecules (data not shown).

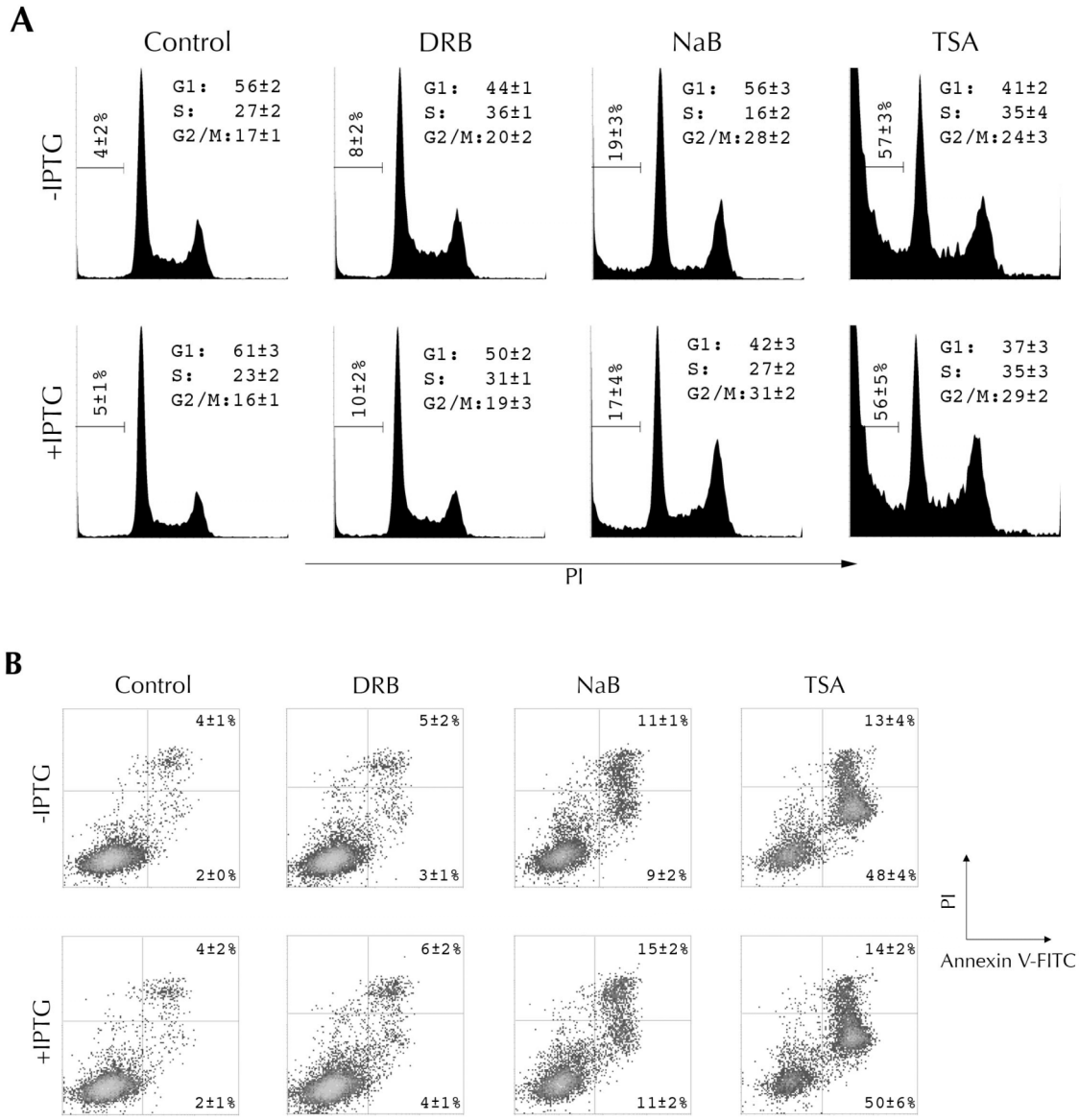


Figure 5.5 Expression of p14ARF does not sensitise NARF2_E6 cells to apoptosis in response to cytotoxic drugs

NARF2_E6 cells were treated for three days with 50μM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. p53 is inactivated in NARF2_E6 cells by the E6 protein.

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

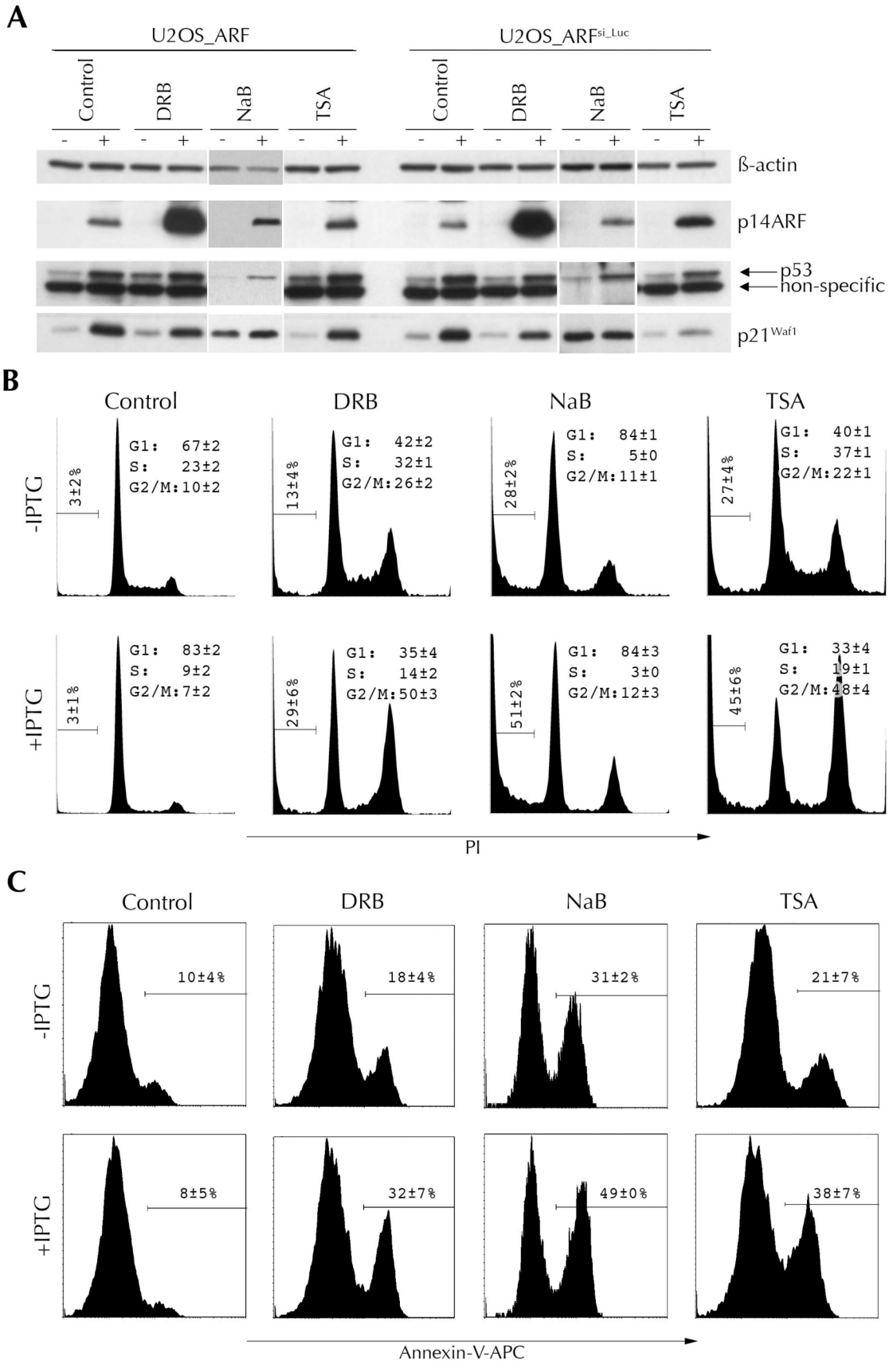
(B) Apoptotic cells were also analysed by dual colour flow cytometric staining of annexin-V and PI exclusion.

U2OS_ARF^{si_Luc} cells were treated with DRB, NaB or TSA in the presence or absence of IPTG and protein expression, cell cycle distribution and annexin-V positive cells were analysed. To analyse chemosensitisation using annexin-V staining, U2OS_ARF^{si_Luc} cells were initially fixed for 10 minutes in 2% paraformaldehyde to inactivate any residual lentiviral particles and annexin-V positive cells were quantitated. Paraformalehyde fixation prevented co-staining with propidium iodide, thus only one parameter could be analysed and the annexin-V results are presented as histograms rather than the usual 2-D dot plots. As expected, levels of p14ARF, p53 and p21^{Waf1} were induced in the control U2OS_ARF^{si_Luc} cells as in the parental U2OS_ARF cell line (Figure 5.6A). Cell cycle and apoptotic response of U2OS_ARF^{si_Luc} cells was also similar to the parental U2OS_ARF cells. In particular, induction of p14ARF expression resulted in potent G1 arrest, no change in apoptotic sub-G1 cells or annexin-V positive cells (Figure 5.6B & 5.6C). As expected, cell death in response to cytotoxic drugs was also increased upon ARF induction in U2OS_ARF^{si_Luc} cells; the sub-G1 population increased in U2OS_ARF^{si_Luc} cells treated with DRB (13±4% to 29±6%), NaB (28±2% to 51±2%) and TSA (27±4% to 45±6%) (Figure 5.6B).

As mentioned above, annexin-V analysis of lentiviral infected cells is presented as 2D histograms, and it was evident that ARF induction increased the annexin-V positive population of U2OS_ARF^{si_Luc} cells treated with DRB (18±4% to 32±7%), NaB (31±2% to 49±0%) and TSA (21±7% to 38±7%) (Figure 5.6C).

To confirm p14ARF increases chemosensitivity in a p53 dependent manner we applied a lentivirus expressing a p53-specific shRNA to create the U2OS_ARF^{si_p53} cell line. Silencing of p53 was very effective in these cells; p53 accumulation was barely

detectable and induction of $p21^{Waf1}$ was not evident in response to p14ARF induction (Figure 5.7A). While DRB treatment increased p14ARF levels in the parental U2OS_ARF cells, the increase was not as prominent in the $U2OS\_ARF^{si\_p53}$ cells, and in contrast to U2OS_ARF cells, NaB and TSA treatment lowered p14ARF protein levels. This effect is presumably related to the lacswitch system of expression, as neither DRB, NaB nor TSA affected p14ARF accumulation in the Saos-2 and H1299 cells, that express endogenous p14ARF (see Figure 4.16C). Low levels of $p21^{Waf1}$ were detected in response to NaB treatment. This is not unexpected as the $p21^{Waf1}$ promoter has NaB responsive elements, that are less responsive in the absence of p53 (Xiao *et al.*, 1997). As expected, induction of p14ARF did not affect the cell cycle distribution in the $U2OS\_ARF^{si\_p53}$ cells (Figure 5.7B). More importantly, p14ARF induction did not chemosensitise the $U2OS\_ARF^{si\_p53}$ cells to DRB, TSA or NaB treatment. In particular, no change was noted in sub-G1 population following IPTG induction of cells treated with carrier control (3±1% to 3±2%), DRB (16±4% to 17±4%), NaB (27±3% to 27±4%) or TSA (27±4% to 27±4%) (Figure 5.7B). Nor did ARF induction change the proportion of annexin-V positive $U2OS\_ARF^{si\_p53}$ cells treated with control (9±5% to 10±4%), DRB (19±5% to 19±6%), NaB (32±3% to 32±2%) or TSA (34±4% to 34±6%) (Figure 5.7C).


A
U2OS_ARF
U2OS_ARFsi_Luc
Control
DRB
NaB
TSA
ß-actin
p14ARF
p53
hon-specific
p21Waf1
B
Control
DRB
NaB
TSA
-IPTG
+IPTG
G1: 67±2
S: 23±2
G2/M: 10±2
3±2%
G1: 42±2
S: 32±1
G2/M: 26±2
13±4%
G1: 84±1
S: 5±0
G2/M: 11±1
28±2%
G1: 40±1
S: 37±1
G2/M: 22±1
27±4%
G1: 83±2
S: 9±2
G2/M: 7±2
3±1%
G1: 35±4
S: 14±2
G2/M: 50±3
29±6%
G1: 84±3
S: 3±0
G2/M: 12±3
51±2%
G1: 33±4
S: 19±1
G2/M: 48±4
45±6%
PI
C
Control
DRB
NaB
TSA
-IPTG
+IPTG
10±4%
18±4%
31±2%
21±7%
8±5%
32±7%
49±0%
38±7%
Annexin-V-APC

(Previous Page)

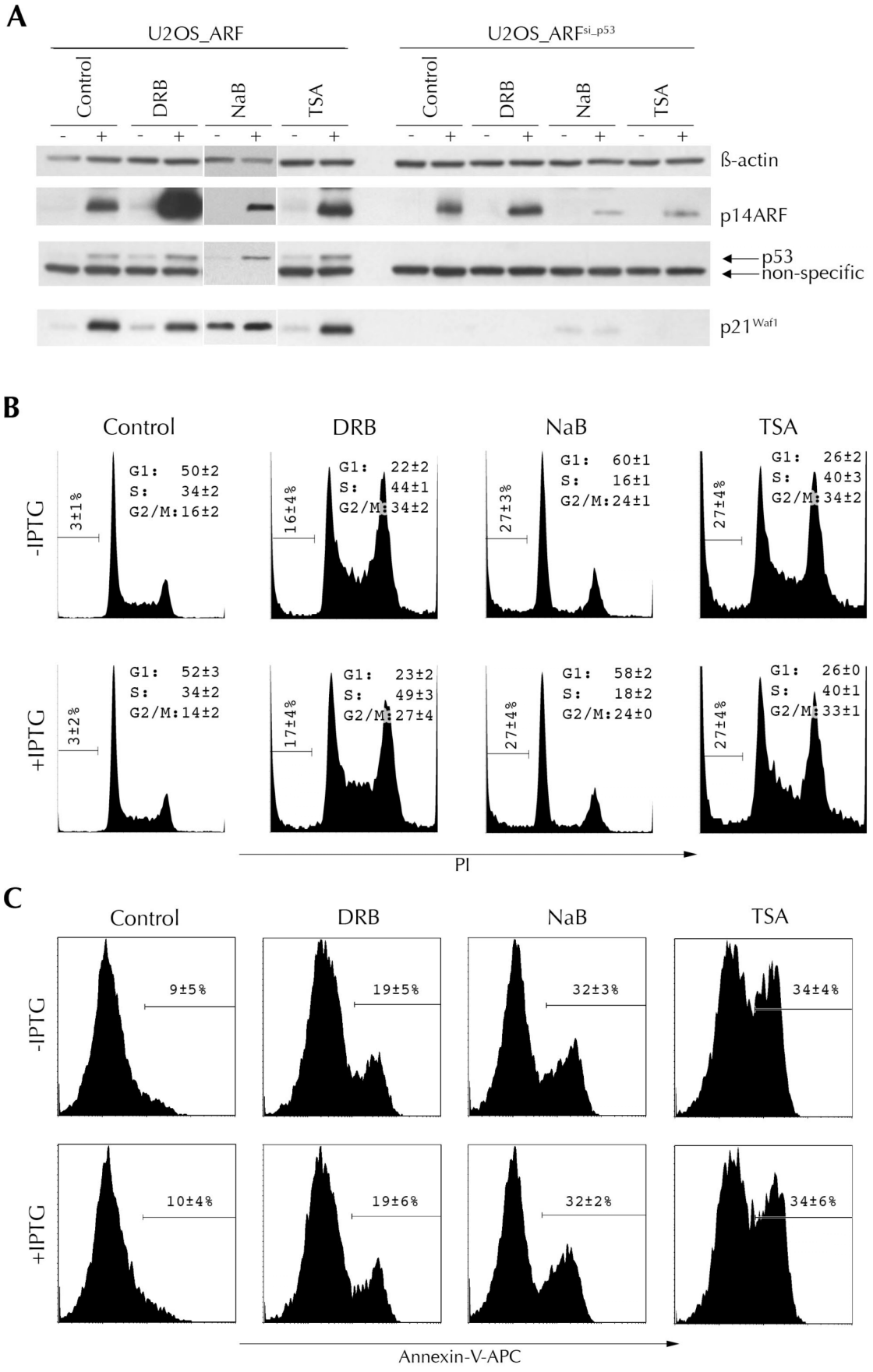
Figure 5.6 Expression of p14ARF sensitises U2OS_ARF^{si_Luc} cells to apoptosis in response to cytotoxic drugs

U2OS_ARF and U2OS_ARF^{si_Luc} cells were treated for three days with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.
(A) Western blot was used to compare expression of p14ARF, p53, p21^{Waf1} and ß-actin loading control.
(B) The cell cycle distribution and sub-G1 population of U2OS_ARF^{si_Luc} cells was examined using propidium iodide (PI) staining.
(C) Apoptotic U2OS_ARF^{si_Luc} cells were also analysed by flow cytometric staining of annexin-V.

(Next Page)

Figure 5.7 Expression of p14ARF does not sensitise U2OS_ARF^{si_p53} cells to apoptosis in response to cytotoxic drugs

U2OS_ARF and U2OS_ARF^{si_p53} cells were treated for three days with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. p53 is knocked down in U2OS_ARF^{si_p53} cells by RNA interference
(A) Western blot was used to compare expression of p14ARF, p53, p21^{Waf1} and ß-actin loading control.
(B) The cell cycle distribution and sub-G1 population of U2OS_ARF^{si_p53} cells was examined using propidium iodide (PI) staining
(C) Apoptotic U2OS_ARF^{si_p53} cells were also analysed by flow cytometric staining of annexin-V.


A
U2OS_ARF
U2OS_ARFsi_p53
Control
DRB
NaB
TSA
- + - + - + - +
ß-actin
p14ARF
p53
non-specific
p21Waf1
B
Control
DRB
NaB
TSA
-IPTG
+IPTG
G1: 50±2
S: 34±2
G2/M:16±2
3±1%
G1: 22±2
S: 44±1
G2/M:34±2
16±4%
G1: 60±1
S: 16±1
G2/M:24±1
27±3%
G1: 26±2
S: 40±3
G2/M:34±2
27±4%
G1: 52±3
S: 34±2
G2/M:14±2
3±2%
G1: 23±2
S: 49±3
G2/M:27±4
17±4%
G1: 58±2
S: 18±2
G2/M:24±0
27±4%
G1: 26±0
S: 40±1
G2/M:33±1
27±4%
PI
C
Control
DRB
NaB
TSA
-IPTG
+IPTG
9±5%
19±5%
32±3%
34±4%
10±4%
19±6%
32±2%
34±6%
Annexin-V-APC

5.2.2 Impact of p21^{Waf1} on ARF induced chemosensitivity

As p14ARF mediated chemosensitivity was p53 dependent, we investigated whether the key p53 target that inhibits cell cycle, p21^{Waf1}, was involved in p14ARF-mediated apoptosis. The cyclin dependent kinase inhibitor p21^{Waf1} is essential in ARF and p53-mediated G1 arrest (Hemmati *et al.*, 2005) and also inhibits apoptosis via cell cycle arrest and indirect inhibition of caspase activity (Sohn *et al.*, 2006; Waldman *et al.*, 1995; Wendt *et al.*, 2006). Additionally, loss of p21^{Waf1} via homozygous deletion in HCT116 colorectal cancer cells increases apoptosis in response to adenovirally-expressed p14ARF, as measured by DNA content, annexin-V positivity and caspase activation (Hemmati *et al.*, 2005). To investigate if p21^{Waf1} was a component of ARF-mediated chemosensitivity we used the lentivirus system to generate a pool of U2OS_ARF cells with silenced p21^{Waf1}. Two previously published p21^{Waf1} shRNA constructs were made that targeted different regions of the p21^{Waf1} mRNA, but despite infecting over 99% of cells as demonstrated by GFP expression, they did not efficiently reduce p21^{Waf1} expression levels (data not shown). Transducing U2OS_ARF cells with a mixture of these lentiviral constructs resulted in p21^{Waf1} knockdown of approximately 50%, and a pool of U2OS_ARF^{si_p21Waf1} cells was generated using this method (Figure 5.8A). These cells were treated with DRB, NaB or TSA, in the presence or absence of IPTG, and analysed three days later. Unfortunately, even with a 50% knockdown in p21^{Waf1} levels, the induction of p14ARF expression still induced G1 arrest (IPTG treatment increased cells in G1 from 69±0% to 81±2%) that was equivalent to the arrest induced in the parental U2OS_ARF cell and lentiviral control U2OS_ARF^{si_Luc} cells. Not surprisingly p14ARF-mediated chemosensitivity was also maintained, with induction of p14ARF expression approximately doubling in sub-G1 population of DRB,

NaB and TSA treated cells (Figure 5.8B & 5.8C), an increase that is similar to that seen in the control U2OS_ARF^{si_Luc} cells.

(Next page)

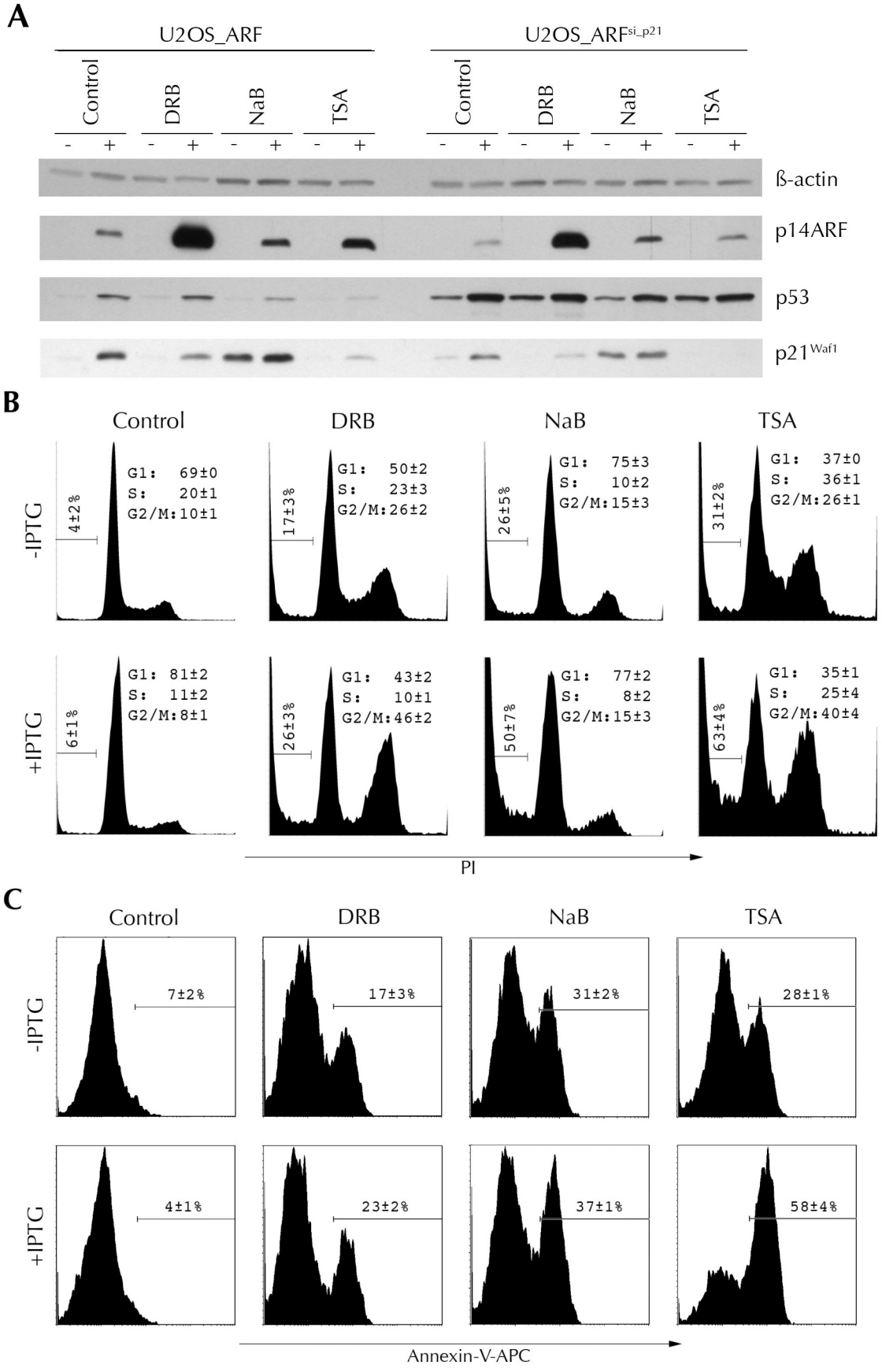
Figure 5.8 Expression of p14ARF sensitises U2OS_ARF^{si_p21} cells to apoptosis in response to cytotoxic drugs

U2OS_ARF and U2OS_ARF^{si_p21} cells were treated for three days with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. p21^{Waf1} is partially knocked down in U2OS_ARF^{si_p21} cells by RNA interference.

(A) Western blot was used to compare expression of p14ARF, p53, p21^{Waf1} and ß-actin loading control.

(B) The cell cycle distribution and sub-G1 population of U2OS_ARF^{si_p21} cells was examined using propidium iodide (PI) staining.

(C) Apoptotic U2OS_ARF^{si_p21} cells were also analysed by flow cytometric staining of annexin-V.


A
U2OS_ARF
U2OS_ARFsi_p21
Control
DRB
NaB
TSA
- + - + - + - + - + - + - + - +
ß-actin
p14ARF
p53
p21Waf1
B
Control
DRB
NaB
TSA
-IPTG
+IPTG
4±2%
G1: 69±0
S: 20±1
G2/M:10±1
17±3%
G1: 50±2
S: 23±3
G2/M:26±2
26±5%
G1: 75±3
S: 10±2
G2/M:15±3
31±2%
G1: 37±0
S: 36±1
G2/M:26±1
6±1%
G1: 81±2
S: 11±2
G2/M:8±1
26±3%
G1: 43±2
S: 10±1
G2/M:46±2
50±7%
G1: 77±2
S: 8±2
G2/M:15±3
63±4%
G1: 35±1
S: 25±4
G2/M:40±4
PI
C
Control
DRB
NaB
TSA
-IPTG
+IPTG
7±2%
17±3%
31±2%
28±1%
4±1%
23±2%
37±1%
58±4%
Annexin-V-APC

5.2.3 p14ARF does not sensitise U2OS_ARF cells to apoptosis in the presence of the pan-caspase inhibitor z-VAD-fmk.

The increased annexin-V population and cleavage of genomic DNA as a result of p14ARF induction (observed as an increase in sub-G1 population of propidium iodide stained nuclei) in drug treated U2OS_ARF cells suggested the involvement of caspase-mediated apoptosis. To determine if p14ARF mediated chemosensitivity was caspase dependent, U2OS_ARF cells were pre-treated with z-VAD-fmk, a pan-caspase inhibitor that irreversibly binds to the catalytic site of caspases (Medina-Palazon *et al.*, 2004). After 3 h of treatment with 50μM z-VAD-fmk, DRB, NaB or TSA was added, along with IPTG or PBS control and the cells analysed 72 hours later. Treatment of U2OS_ARF cells with this caspase inhibitor resulted in slight S-phase reduction (27±0% to 17±0%) and G1 arrest (64±1% to 73±1%) and decreased the low percentage of sub-G1 cells (5±2% to 1±0% of cells) and annexin-V positive cells (Figure 5.9A & 5.9B). Similarly, treatment of U2OS_ARF cells with z-VAD-fmk prevented apoptosis in response to DRB, TSA or NaB regardless of IPTG-induced p14ARF expression. For example, the annexin-V positive population of DRB treated U2OS_ARF cells remained at 11±1% (without IPTG) and 10±2% (with IPTG) (Figure 5.10B). z-VAD-fmk treatment increased the G1 population in uninduced DRB treated cells from 63±2% to 84±0% and in induced cells from 45±3% to 66±5% (Figure 5.10A), presumably as suppression of caspase activity can lead to changes in cell cycle distribution (Jorquera and Tanguay, 1999; Piekarz *et al.*, 2004). The apoptotic sub-G1 population of DRB treated cells was also reduced by z-VAD-fmk to 1±0% (without IPTG) and 4±1% (with IPTG) (Figure 5.10A). Similarly, results demonstrating the requirement of caspase activation for p14ARF-mediated chemosensitisation were obtained for NaB and TSA (Figure 5.11A, 5.11B, 5.12A & 5.12B).

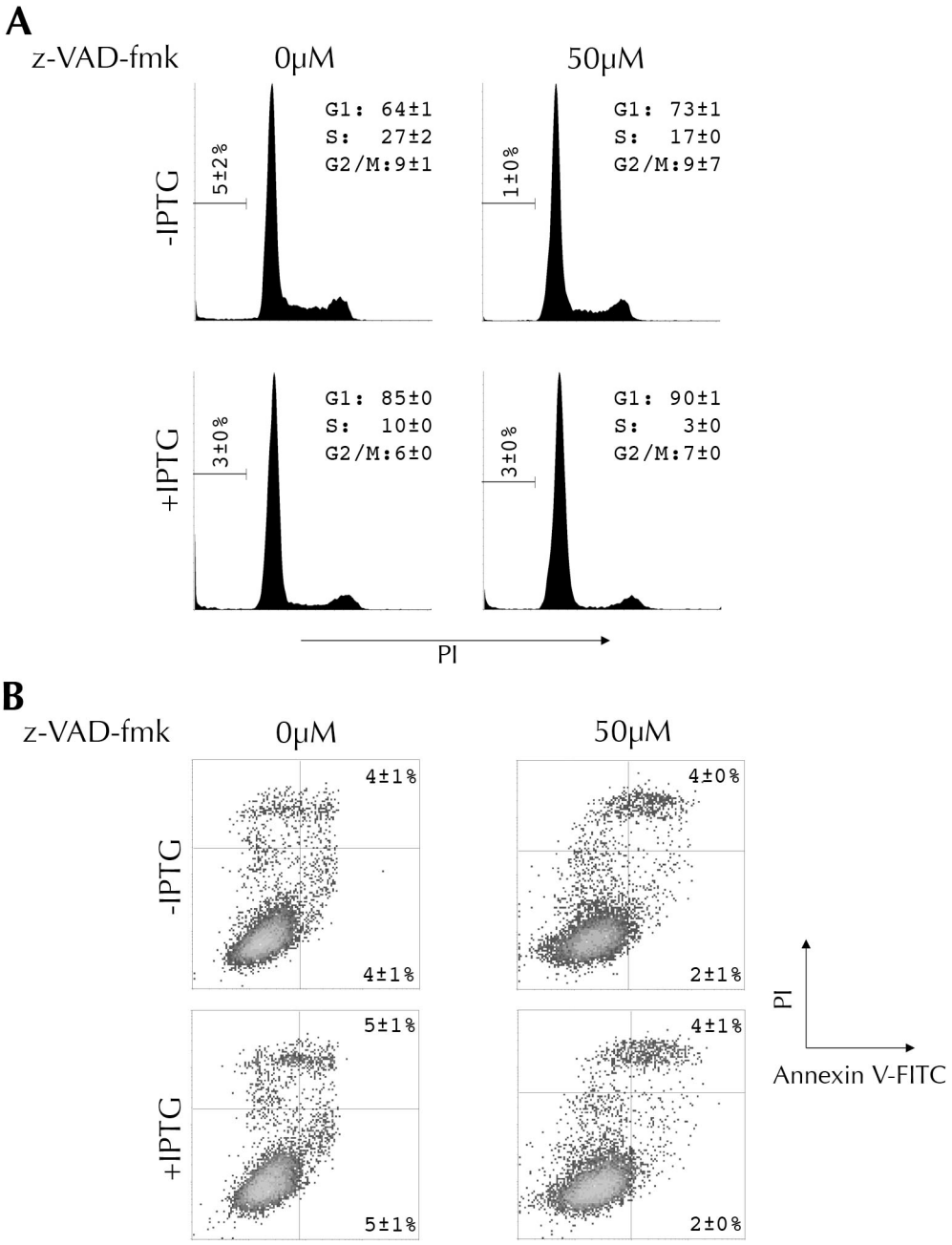


Figure 5.9 Analysis of the effect of pan-caspase inhibition on U2OS_ARF cells

U2OS_ARF cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk then treated for three days with 0.1% DMSO carrier control and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

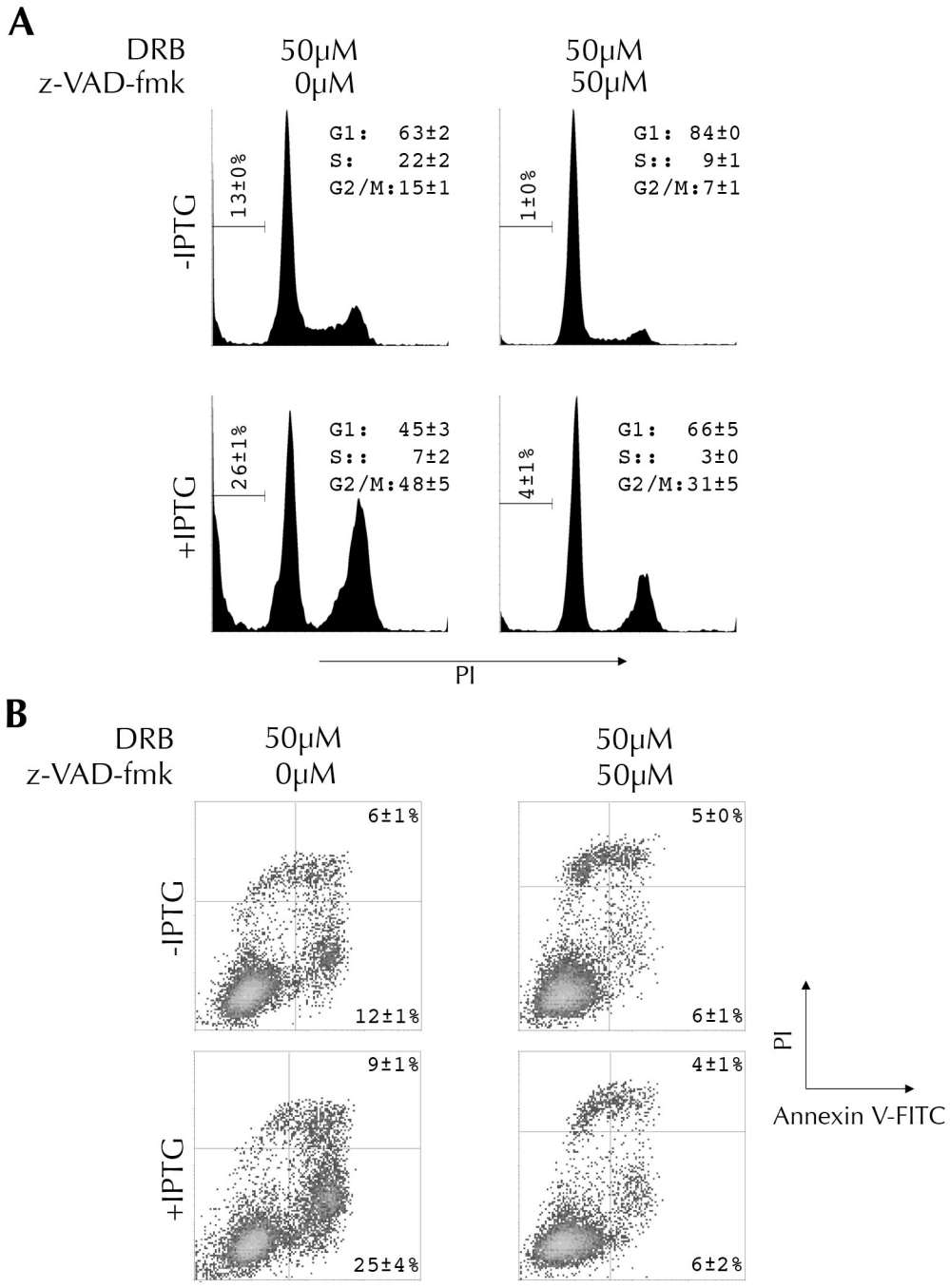


Figure 5.10 Pan-caspase inhibition prevents apoptosis and chemosensitivity induced by p14ARF expression in DRB treated U2OS_ARF cells

U2OS_ARF cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk or 0.1% DMSO carrier control, then treated for three days with 50µM DRB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

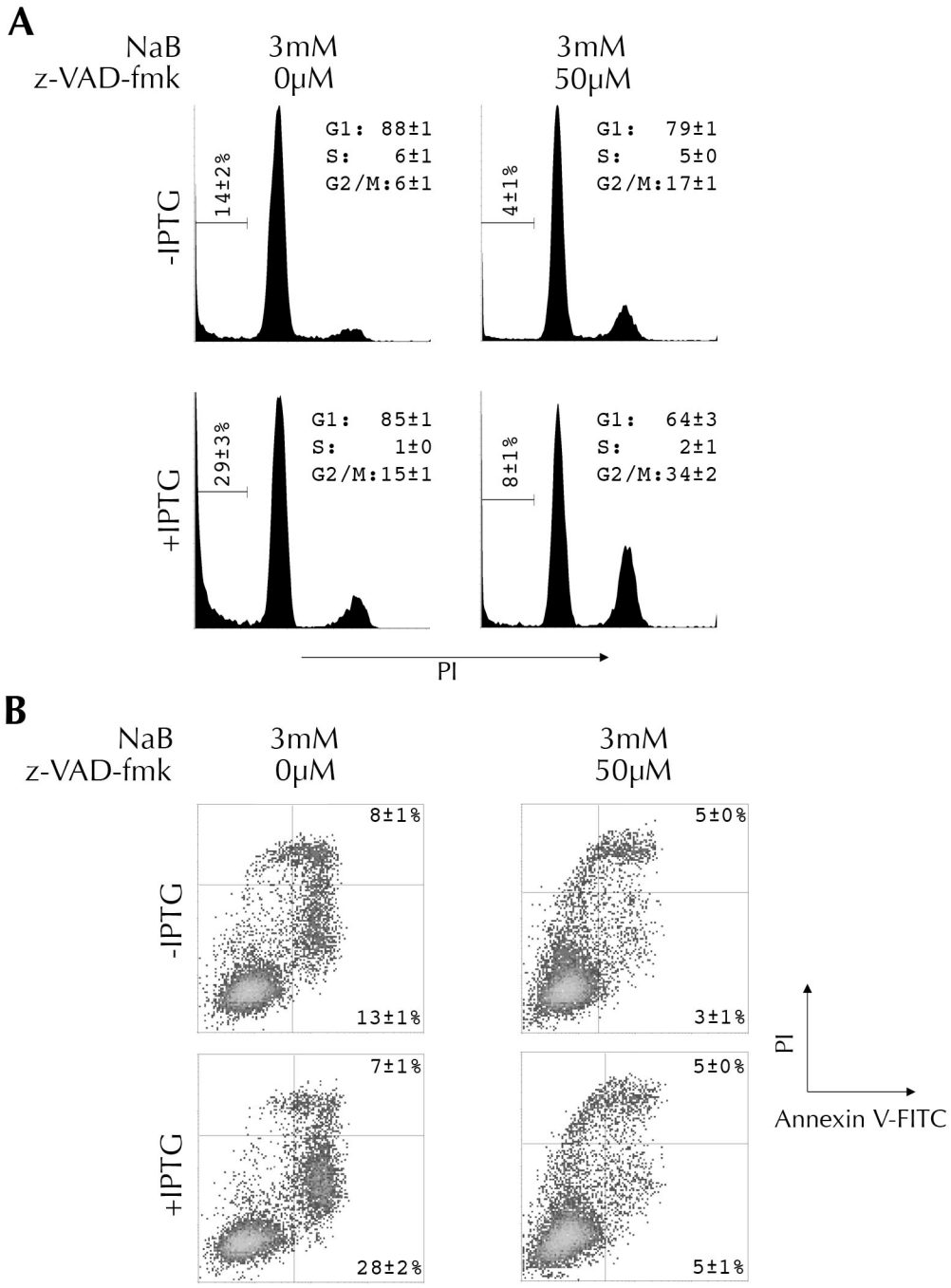


Figure 5.11 Pan-caspase inhibition prevents apoptosis and chemosensitivity induced by p14ARF expression in sodium butyrate (NaB) treated U2OS_ARF cells

U2OS_ARF cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk or 0.1% DMSO carrier control, then treated for three days with 3mM NaB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

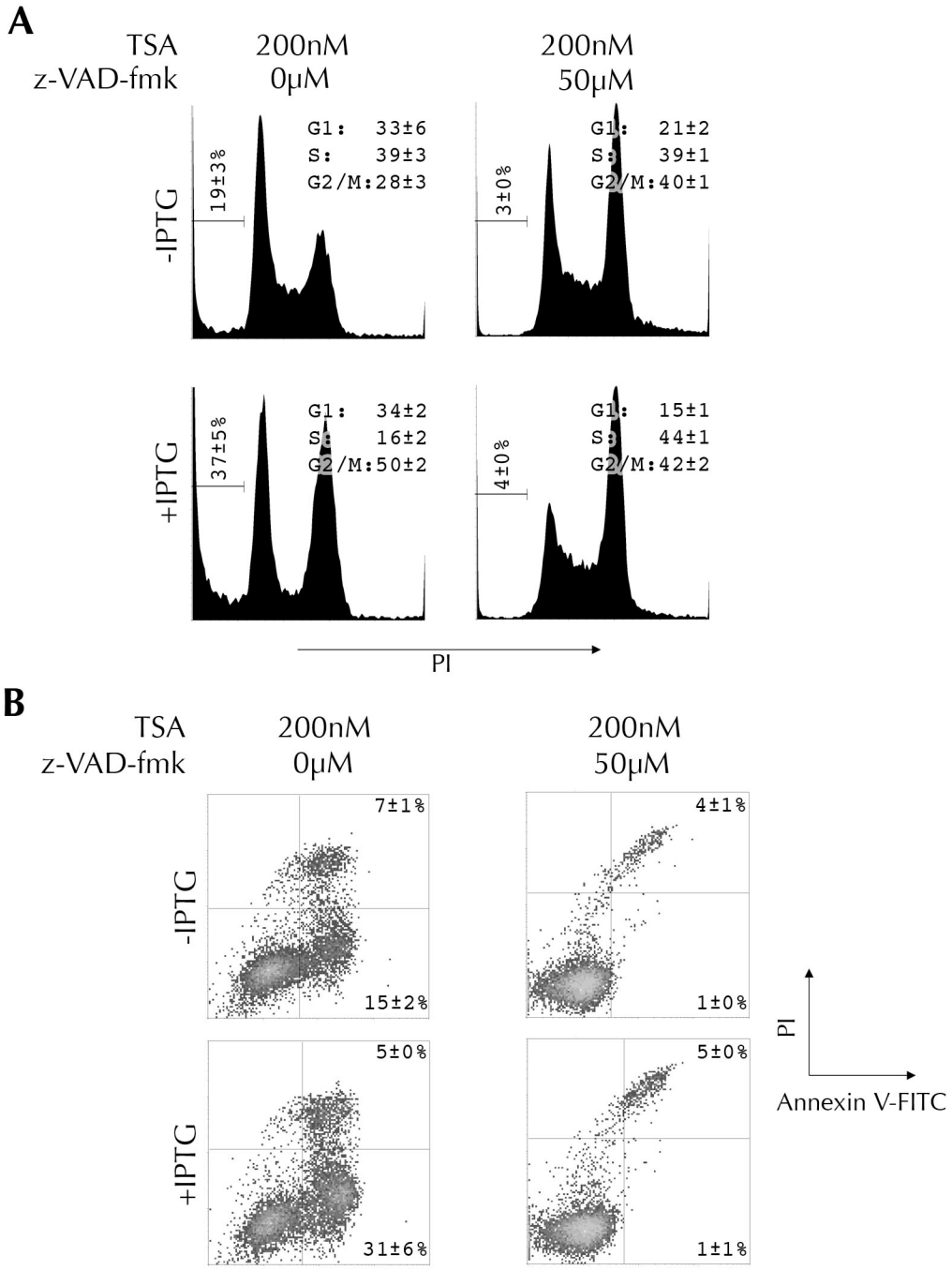


Figure 5.12 Pan-caspase inhibition prevents apoptosis and chemosensitivity induced by p14ARF expression in trichostatin A (TSA) treated U2OS_ARF cells

U2OS_ARF cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk or 0.1% DMSO carrier control, then treated for three days with 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

5.2.4 Active caspases are not detected in U2OS_ARF cells treated with cytotoxic drugs and induced to express p14ARF

As z-VAD-fmk prevented ARF-mediated chemosensitivity, we investigated changes in caspase 3, 8 and 9 as well as PARP, a target of caspase 3 proteolytic activity. U2OS_ARF cells were treated with DRB, NaB or TSA in the presence or absence of IPTG to induce p14ARF expression. Three days later cells were harvested and subject to western blotting. To detect cleaved caspase 3 we used an antibody that specifically detects active, cleaved caspase 3. Surprisingly, activated caspase 3 was not detected in induced or uninduced U2OS_ARF cells treated with DRB, NaB, TSA or carrier control (Figure 5.13). Detection of activated caspase 3 in a caspase positive control (U2OS_ARF cells 24 hours post 80J/m^2 UVC treatment) demonstrated the antibody could detect activated caspase 3 in these cells. Similarly, we did not detect cleaved caspase 8 or 9, even though the cleaved form of these proteins could be detected in UVC-treated U2OS_ARF cells (Figure 5.13). To investigate alterations in downstream targets of effector caspases, we used a PARP antibody that detects both full length and the 85kDa cleaved fragment of PARP. Full length PARP was not significantly lost in drug treated U2OS_ARF cells, regardless of the presence of p14ARF (Figure 5.13). Levels of cleaved PARP increased in U2OS_ARF cells treated with DRB and NaB, but were very low compared to levels of full length PARP. ARF induction did not affect the levels of PARP cleavage.

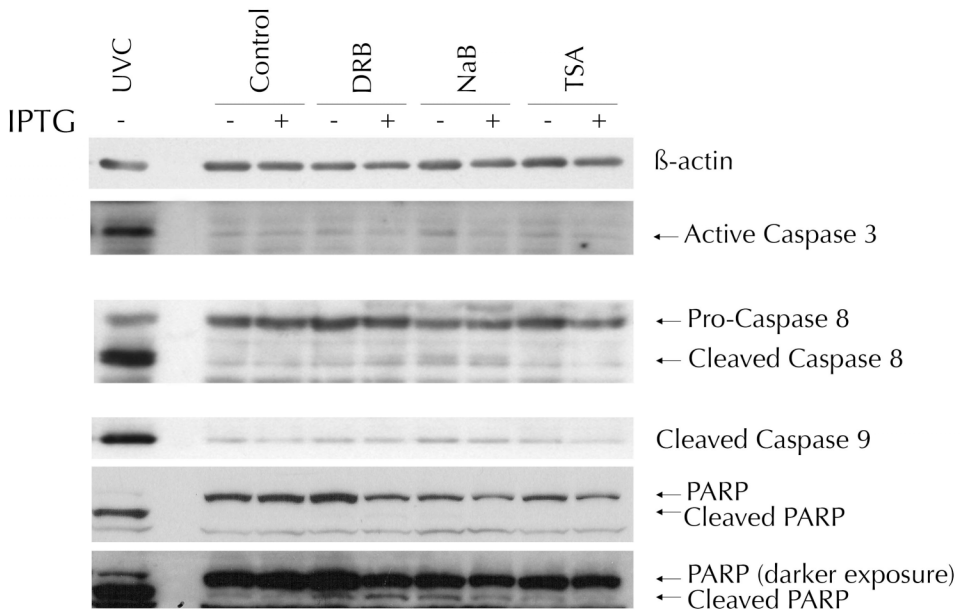


Figure 5.13 p14ARF induction in cytotoxic drug treated U2OS_ARF cells does not lead to activation of caspase 3, 8 or 9

Expression of active caspase 9, caspase 8, active caspase 3, PARP and ß-actin loading control was determined three days after treatment of U2OS_ARF cells with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. Arrows indicate the full length and cleaved protein bands where relevant.

5.2.5 p14ARF induced chemosensitivity is associated with mitochondrial depolarisation

Pore formation in the outer mitochondrial membrane as a result of Bax or Bak activation, and subsequent mitochondrial outer membrane permeability is a crucial step in the activation of the intrinsic apoptotic pathway. Pore formation allows release of cytochrome c, SMAC/DIABLO and AIF into the cytosol, which enable caspase dependent and caspase independent cell death to occur. Additionally, p53 increases transcription of a number of pro-apoptotic Bcl-2 members that facilitate activation of Bax and Bak, including PUMA, Noxa, Bid and Bax itself (Chipuk and Green, 2006). With this in mind, we determined if p14ARF mediated chemosensitivity is associated

with mitochondrial depolarisation by using JC-1, a cell permeable stain that fluoresces red in intact mitochondria but fluoresces increasingly green and less red in depolarised mitochondria. While mitochondrial depolarisation is not a direct measure of Bax/Bak pore formation, mitochondrial outer membrane permeabilisation is usually associated with permanent loss of mitochondrial membrane potential ($\Delta\Psi_m$) (Heiskanen *et al.*, 1999), and thus loss of $\Delta\Psi_m$ is used as a surrogate marker for mitochondrial outer membrane permeabilisation

U2OS_ARF cells were treated with DRB, NaB, TSA or DMSO carrier control in the presence or absence of IPTG, and mitochondrial potential measured by JC-1 staining after 72 hours. IPTG treatment had no effect on cells that were not exposed to cytotoxic drugs, with 10±1% of uninduced cells exhibiting depolarised mitochondria, compared to 10±2% of IPTG treated cells (Figure 5.14). In DRB treated cells, 15±1% of cells had depolarised mitochondria, which was increased to 30±3% by p14ARF induction (Figure 5.14). Enforced expression of p14ARF also increased mitochondrial depolarisation in NaB treated cells from 43±2% to 53±1% and TSA treated cells from 33±4% to 60±3% (Figure 5.14), demonstrating p14ARF mediated chemosensitivity is associated with mitochondrial outer membrane permeabilisation.

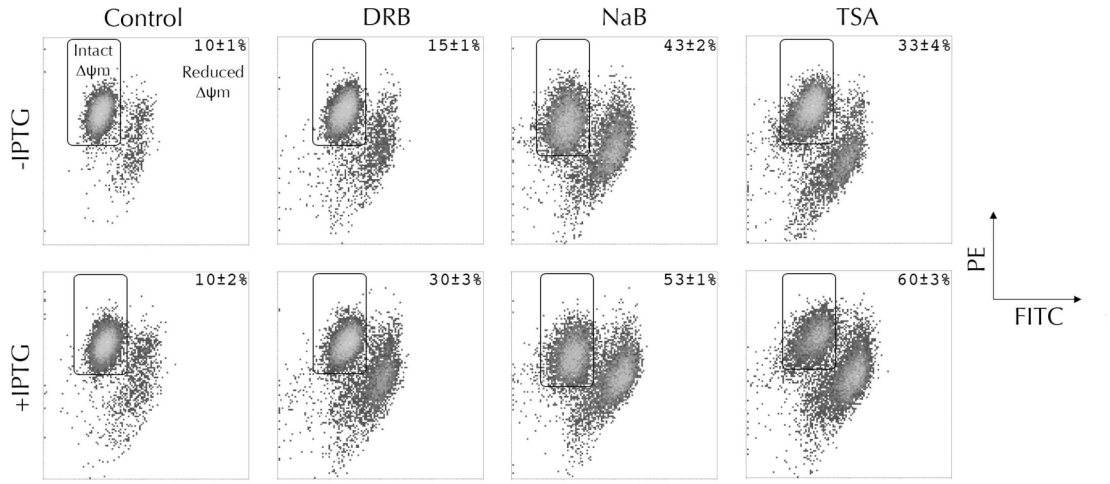


Figure 5.14 p14ARF induction increases mitochondrial depolarisation in cytotoxic drug treated U2OS_ARF cells

JC-1 staining was used to determine U2OS_ARF cells with intact (boxed) or depolarised mitochondria, three days after treatment with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. The percentage of depolarised cells is indicated.

5.2.6 Changes in Bcl-2 family members

As p14ARF mediated chemosensitivity was associated with mitochondrial depolarisation, we investigate the levels of a number of Bcl-2 family proteins, which control the initiation of pore formation in the outer mitochondrial membrane. We analysed expression level of several members of the Bcl-2 family, including pro-apoptotic members Bax, Bak, Bid, Noxa, PUMA, and anti-apoptotic Bcl-2, Bcl-x_L and Mcl-1 (Figure 5.15).

Induction of p14ARF expression decreased Bax levels slightly in drug treated and untreated cells. The reduction in Bax following ARF induction of drug treated cells was unexpected, especially given Bax is a transcriptional target of p53 (Miyashita and Reed, 1995). One explanation is Bax may be cleaved, generating an 18kDa C-terminal

fragment that potently promotes apoptosis (Gao and Dou, 2000; Wood *et al.*, 1998). However we did not detect the cleaved form of Bax, despite using an antibody (Bax clone B9, Santa Cruz Biotechnology, Santa Cruz, CA) that can detect activated cleaved Bax (Gao and Dou, 2000). Levels of Bak were not changed by drug treatment or p14ARF induction (Figure 5.15).

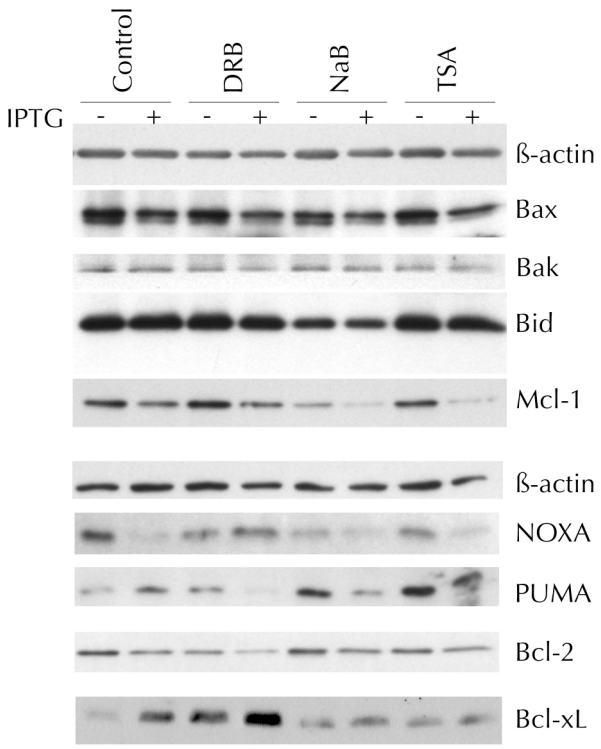


Figure 5.15 Analysis of Bcl-2 family proteins in U2OS_ARF cells treated with cytotoxic drugs and IPTG

Western blot was used to determine levels of Bcl-2 family proteins in U2OS_ARF cells three days after treatment with 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. Top ß-actin was probed as a loading control for Bax, Bak, Bid and Mcl-1. Middle ß-actin was probed as a loading control for NOXA, PUMA, Bcl-2 and Bcl-x_L,

The active form of the BH-3 only protein Bid (truncated Bid, tBid) is generated by caspase 8 cleavage, thus linking the extrinsic and intrinsic apoptotic pathways. Levels of full length, inactive Bid were unchanged by p14ARF induction or DRB and TSA

treatment, although they were reduced in NaB treated cells (Figure 5.15). Truncated Bid was not detected in any treatment, suggesting that NaB may reduce overall Bid levels, rather than promote Bid cleavage.

The pro-apoptotic proteins Noxa, PUMA are p53 targets and promote apoptosis by inhibiting anti-apoptotic Bcl-2 members. Contrary to expectations, Noxa levels were reduced by ARF induction in U2OS_ARF cells, and ARF caused a slight reduction or no detectable change in Noxa levels in cells treated with DRB, NaB or TSA (Figure 5.15). In contrast, PUMA accumulation was enhanced in response to p14ARF induction, but decreased in cells expressing ARF and treated with DRB or NaB. PUMA levels remained high in TSA treated cells in the presence of ARF.

Accumulation of the anti-apoptotic Bcl-2 was decreased by p14ARF induction alone and in cells expressing ARF and treated with DRB, NaB or TSA. Similarly, levels of anti-apoptotic Mcl-1 also decreased as a result of p14ARF induction in untreated and drug treated cells. Mcl-1 was also decreased by NaB treatment alone, but not by DRB or TSA treatment. Induction of p14ARF increased levels of the anti-apoptotic Bcl-x_L protein. Levels of Bcl-x_L increased in DRB treated cells and were further increased by ARF induction. Bcl-x_L levels were low in U2OS_ARF cells treated with NaB or TSA and were not altered by ARF expression in these cells (Figure 5.15).

Changes in the level of Bcl-2 family proteins is summarised in Table 5.1.

Table 5.1 p14ARF-induced changes in the protein level of Bcl-2 family members.

	ARF	Drug	Drug+ARF
Pro-apoptotic			
Noxa	↓	↔	↓
PUMA	↑	↑	↓/↑[1]
Bax	↓	↔	↓
Bak	↔	↔	↔
Bid	↔	↔/↓[2]	↔/↓[2]
Anti-apoptotic			
Bcl-2	↓	↔	↓
Mcl-1	↓	↔/↓[2]	↓
Bcl-x_L	↑	↑/↔[3]	↑↑/↔[3]

↓ protein expression decreased, ↑protein expression increased, ↔protein expression was unchanged

1 TSA and ARF increased PUMA levels

2 NaB decreased Bid and Mcl-1 expression

3 NaB or TSA treated cells did not show altered Bcl-x_L levels

5.2.7 *Loss of mitochondrial outer membrane potential is required for increased sensitivity to apoptosis caused by p14ARF induction*

We then investigated if increased mitochondrial depolarisation was required for p14ARF-mediated chemosensitivity. As both Bax and Bak may cooperate to cause mitochondrial outer membrane permeability, we blocked activation of both proteins by ectopically expressing EGFP-tagged Bcl-2, which sequesters BH-3 only proteins, preventing Bax and Bak oligomerisation and the formation of the mitochondrial permeability pore (Cheng *et al.*, 2001).

We used CMX-Ros to ensure Bcl-2 expression prevented mitochondrial outer membrane permeabilisation. CMX-Ros is a lipophilic, cationic dye that is concentrated in the mitochondria due to the negative matrix charge in the same manner as JC-1. Unfortunately JC-1 could not be used, due to fluorescent interference from the EGFP-tagged Bcl-2 molecule. Cells with depolarised mitochondria do not absorb as much

CMX-Ros and can be detected by flow cytometry as a population with reduced fluorescence.

U2OS_ARF cells transfected with Bcl-2_EGFP or the control spectrin_EGFP showed a similar CMX-Ros staining, indicating intact mitochondria and this was not affected by p14ARF induction (Figure 5.16A). As expected these cells also had a low percentage of apoptotic cells as measured by the sub-G1 population and annexin-V positive population (Figure 5.16B & C). Treatment of U2OS_ARF cells with 3mM NaB resulted in mitochondrial depolarisation, as indicated by reduced CMX-Ros uptake (Figure 5.17A). The mitochondria were protected by the overexpression of Bcl-2, which also reduced the percentage of sub-G1 cells from 43±1% to 26±4% and annexin-V positive cells from 38±4% to 21±2% (Figure 5.17B & 5.17C). Complete inhibition of apoptosis was not observed, as has been previously reported in cells treated with a low dose of a histone deacetylase inhibitor for extended periods (Lucas *et al.*, 2004). As expected, induction of p14ARF expression increased mitochondrial depolarisation in NaB treated cells but the loss of $\Delta\Psi_m$ was largely prevented by expression of Bcl-2_EGFP (Figure 5.17A). In cells expressing Bcl-2 and treated with NaB, p14ARF induction did not affect the levels of cell death, with uninduced/induced cells having a sub-G1 population of 26±4% vs. 26±2%, and annexin-V positive population of 21±3% vs. 26±2%. These data indicate that mitochondrial outer membrane permeabilisation is a crucial step for p14ARF-induced chemosensitivity.

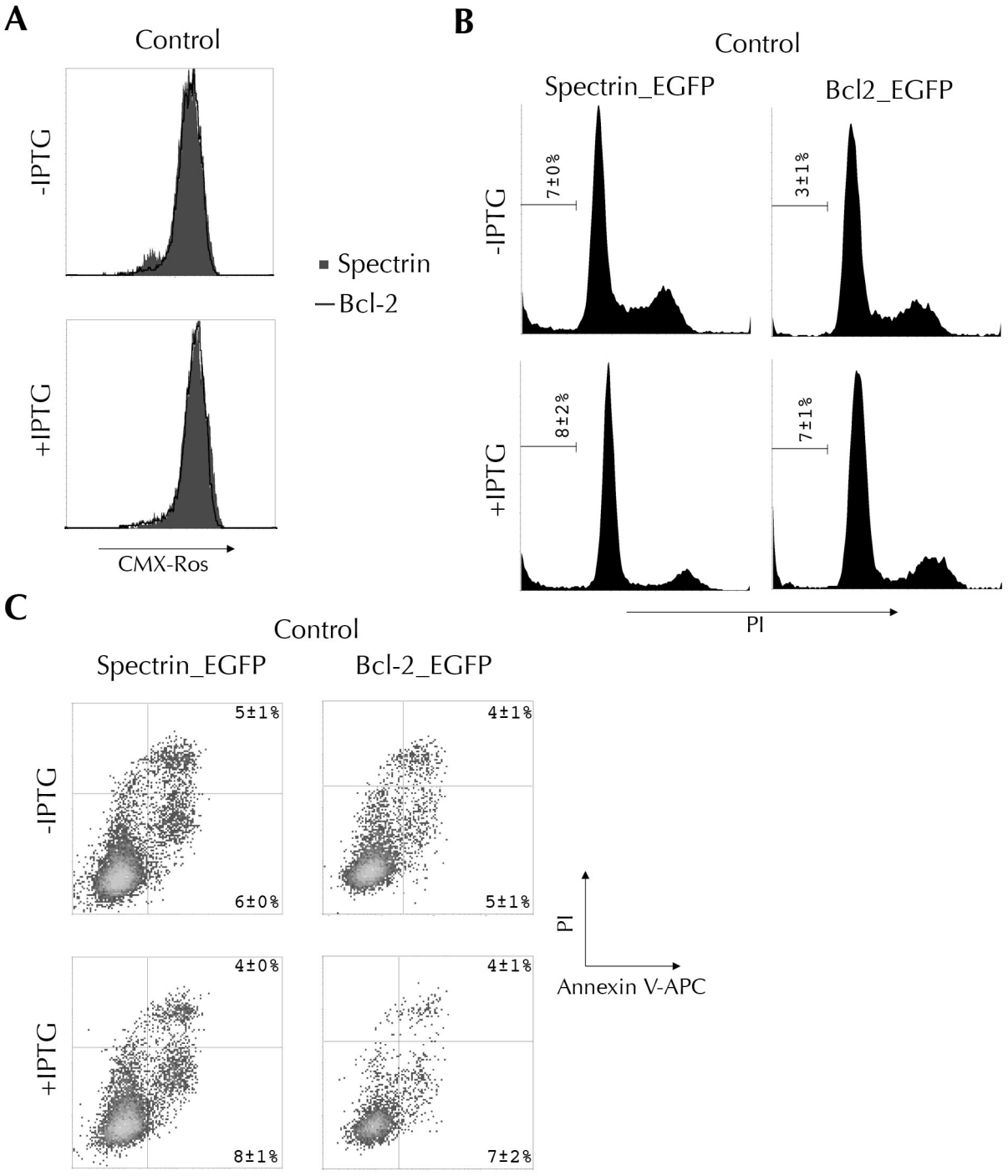


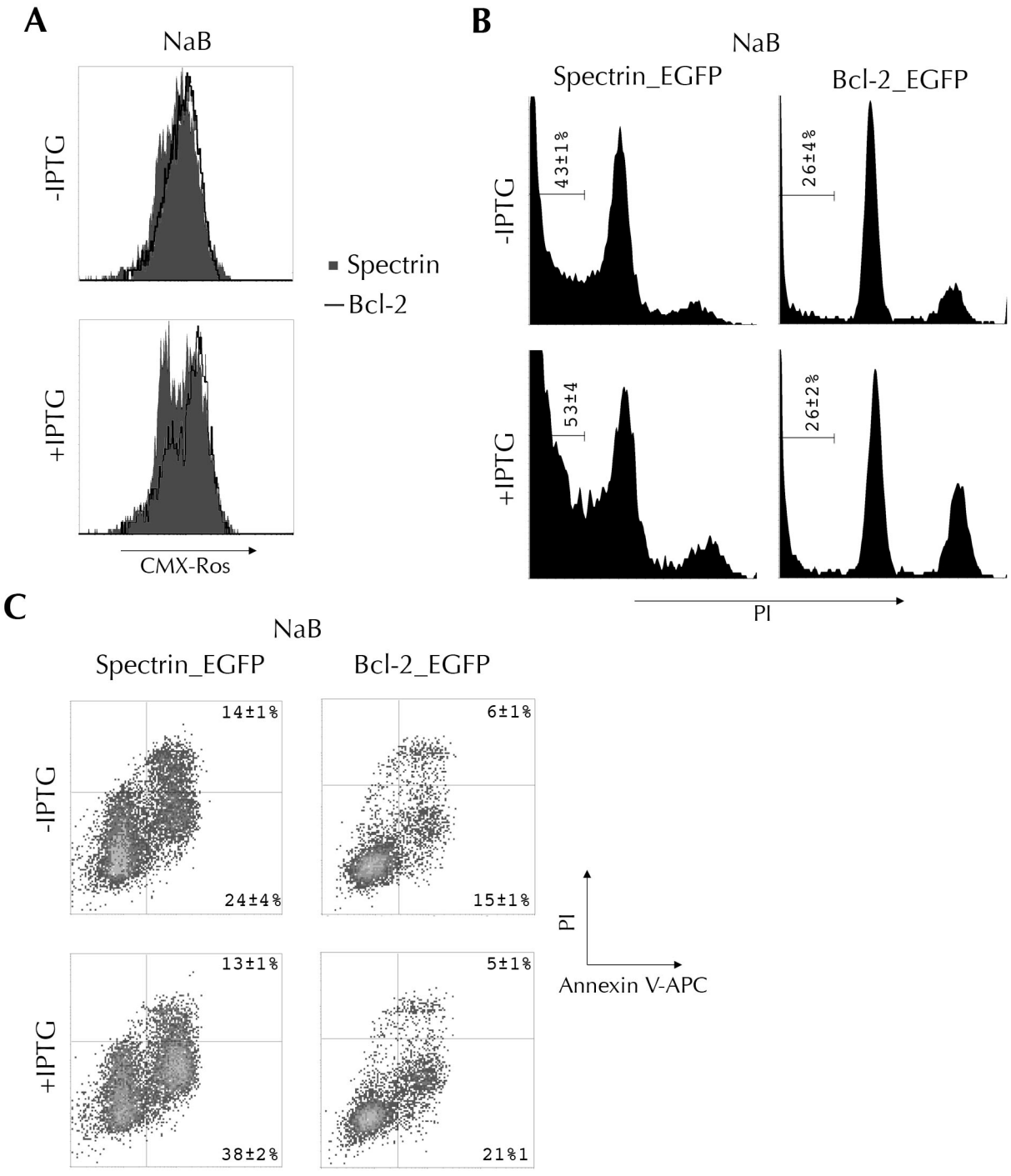
Figure 5.16 Analysis of the effect of Bcl-2 over expression in U2OS_ARF cells

U2OS_ARF cells were transfected with Bcl-2_EGFP or control Spectrin_EGFP. After 24 hours incubation, cells were treated for three days with PBS carrier control and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. Green cells expressing the transgene were selected by flow cytometry.

(A) Mitochondrial depolarisation was assessed by CMX-Ros staining. Loss of CMX-Ros fluorescence indicates mitochondrial depolarisation. Bcl-2_EGFP transfected cells is overlayed on control, spectrin_EGFP expressing cells.

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

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


A
NaB
-IPTG
+IPTG
Spectrin
Bcl-2
CMX-Ros
B
NaB
Spectrin_EGFP
Bcl-2_EGFP
-IPTG
+IPTG
43±1%
26±4%
53±4
26±2%
PI
C
NaB
Spectrin_EGFP
Bcl-2_EGFP
-IPTG
+IPTG
14±1%
24±4%
6±1%
15±1%
13±1%
38±2%
5±1%
21%1
PI
Annexin V-APC

(Previous page)

Figure 5.17 Bcl-2 over-expression prevents p14ARF-mediated mitochondrial depolarisation and chemosensitivity in sodium butyrate treated U2OS_ARF cells

U2OS_ARF cells were transfected with Bcl-2_EGFP or control Spectrin_EGFP. After 24 hours incubation, cells were treated for three days with 3mM sodium butyrate (NaB) and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. Green cells were selected by flow cytometry.

(A) Mitochondrial depolarisation was assessed by CMX-Ros staining. Loss of CMX-Ros fluorescence indicates mitochondrial depolarisation. Bcl-2_EGFP transfected cells are overlayed on control, spectrin_EGFP expressing cells.

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

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

5.2.8 p14ARF does not require Bax to sensitise cells to apoptosis

As the increased death resulting from p14ARF induction in drug treated cells was dependent on loss of mitochondrial potential, we investigated if Bax, an important mediator of mitochondrial depolarisation, was essential for this process. Although Bax levels decreased slightly as a result if p14ARF induction in drug treated cells (Figure 5.15), it is the conformation of Bax, rather than its expression that regulates its activity (Lalier et al., 2007; Miyashita and Reed, 1995). Additionally, loss of Bax alters the tumour spectrum of ARF-null mice, resulting in tumours uncommon in Bax+/+/ARF-/- mice such as osteosarcoma and hemangiosarcoma, as well as multiple primary tumours (Eischen et al., 2002). Furthermore, Bax is required for apoptosis caused by ectopic p19ARF expression, and Bax loss abrogates apoptosis caused by adenovirally-expressed p14ARF in human HCT116 colorectal cells (Hemmati *et al.*, 2006; Suzuki *et al.*, 2003).

To examine if p14ARF increased chemosensitivity is Bax-dependent, Bax activity was inhibited by adding the Bax inhibiting peptide V5 (BIP-V5) to U2OS_ARF cells. After five hours of pre-incubation, cells were exposed to DRB, NaB or TSA in the presence

or absence of IPTG and cell cycle and annexin-V staining analysed by flow cytometry after three days.

To confirm the Bax inhibitor was effective, U2OS_ARF cells were treated with BIP-V5 or the negative peptide control (BIP-NC) for 5 hours, then etoposide added and cells incubated for 72 hours. Etoposide killing of U2OS is partly Bax dependent (Taylor *et al.*, 2000) and BIP-V5 reduced apoptosis, with the sub-G1 population decreasing from 25±1% to 12±2% and the percentage of annexin-V positive cells decreasing from 26±3% to 18±2% (Figure 5.18 A & B).

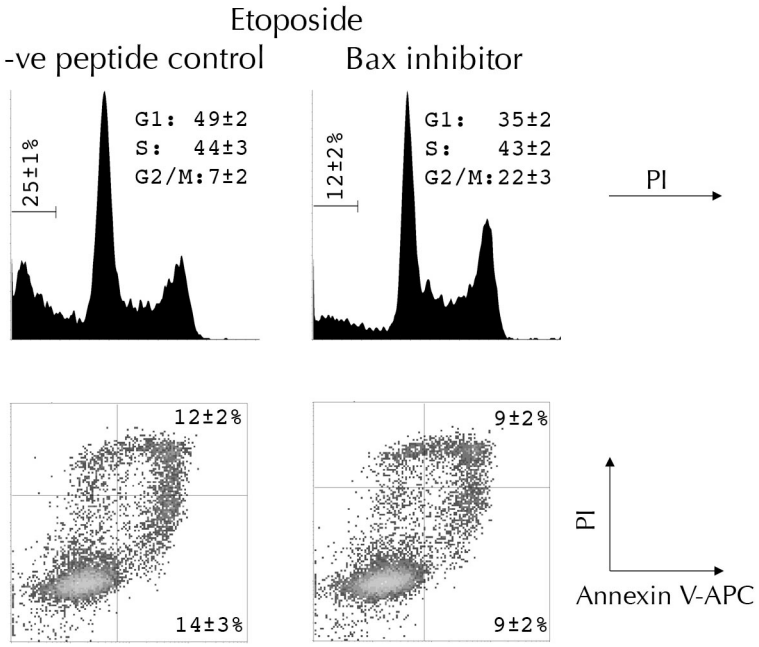


Figure 5.18 The Bax inhibitor BIP-V5 reduces apoptosis caused by etoposide
U2OS_ARF cells were pre-treated with the Bax inhibiting peptide BIP-V5 or control peptide BIP-NC then treated with 30µM etoposide for three days. The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining. Apoptotic cells were also analysed by dual colour flow cytometric staining of annexin-V and PI exclusion .

BIP-V5 had no effect on the cell cycle distribution or apoptosis of uninduced or induced U2OS_ARF cells, as measured by PI and annexin-V staining (Figure 5.19A & B). In cells treated with DRB, NaB or TSA, the Bax inhibitor reduced the total levels of apoptosis but did not prevent IPTG induction from increasing the level of apoptosis. Although the Bax inhibitor reduced overall levels of apoptosis, this was not associated with a change in cell cycle distribution. Specifically, in DRB plus BIP-NC treated cells, induction of IPTG increased the sub-G1 population from 15±2% to 26±3%, and annexin-V positive cells from 12±3 to 27±1%, while in DRB plus BIP-V5 treated cells IPTG changed the sub-G1 population from 13±4% to 19±2%, and annexin-V positive cells from 10±2% to 17±1% (Figure 5.20A & B). BIP-V5 acted in a similar fashion in cells treated with NaB (Figure 5.21A & B) and TSA (Figure 5.22A & B), reducing overall levels of apoptosis but not preventing p14ARF-mediated chemosensitivity. Thus, although Bax contributes to death as a result of drug treatment, with our without ARF induction, it is not essential for p14ARF-mediated chemosensitivity.

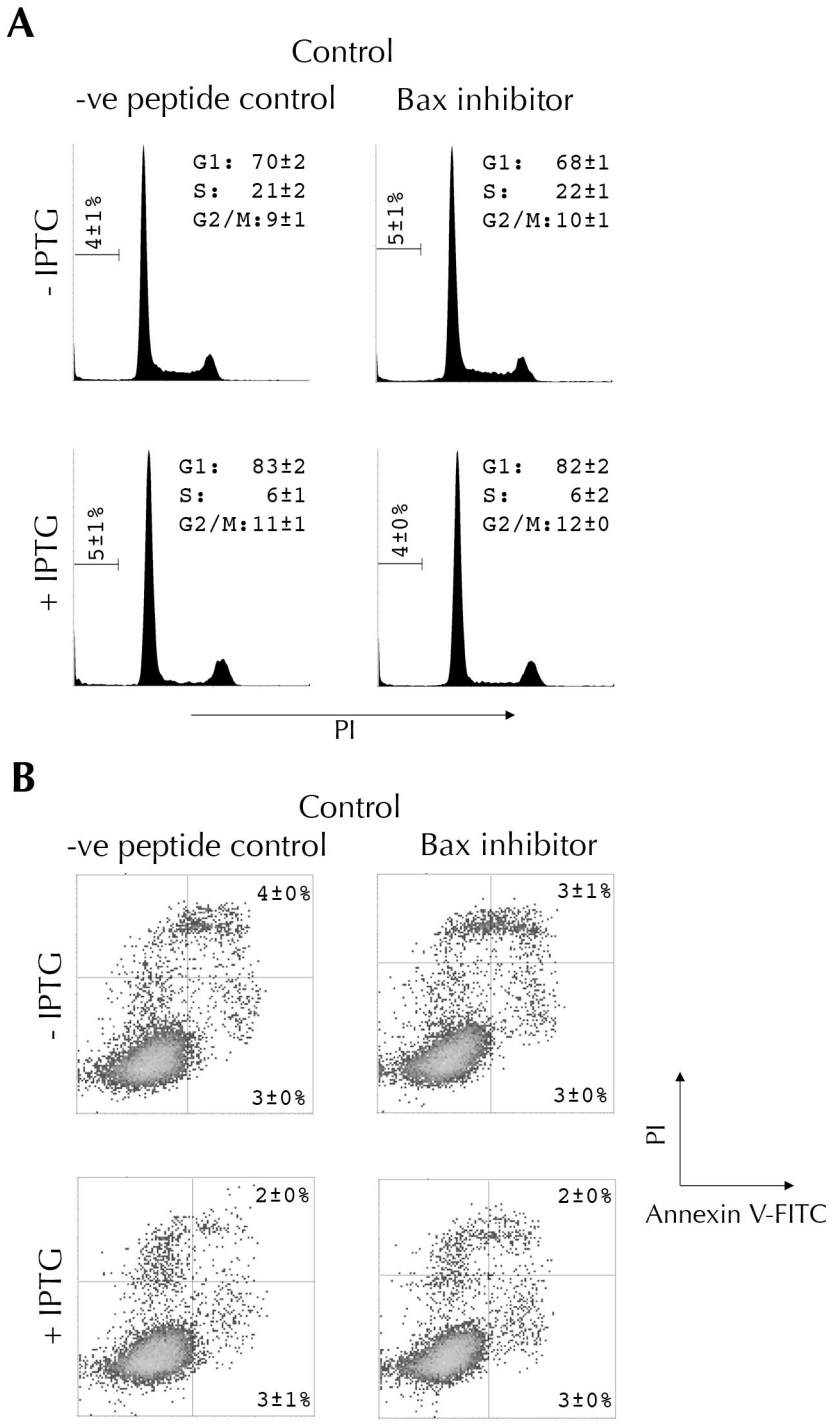


Figure 5.19 Analysis of the effect of Bax inhibition in U2OS_ARF cells

U2OS_ARF cells were pre-treated with the Bax inhibiting peptide BIP-V5 or control peptide BIP-NC then treated for three days with DMSO carrier control and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

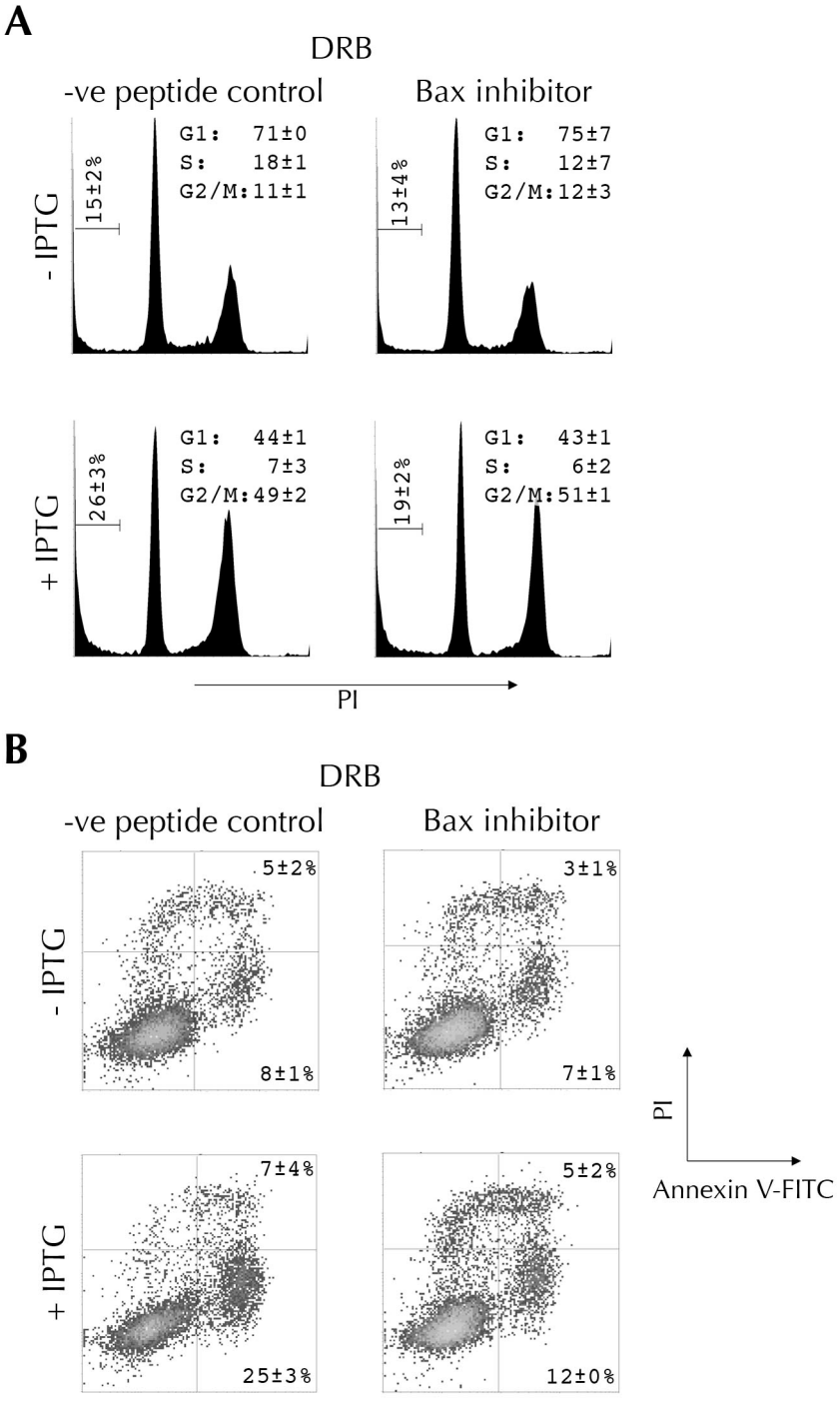


Figure 5.20 Bax inhibition does not prevent p14ARF increasing sensitivity of U2OS_ARF cells to DRB

U2OS_ARF cells were pre-treated with the Bax inhibiting peptide BIP-V5 or control peptide BIP-NC then treated for three days with 50µM DRB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

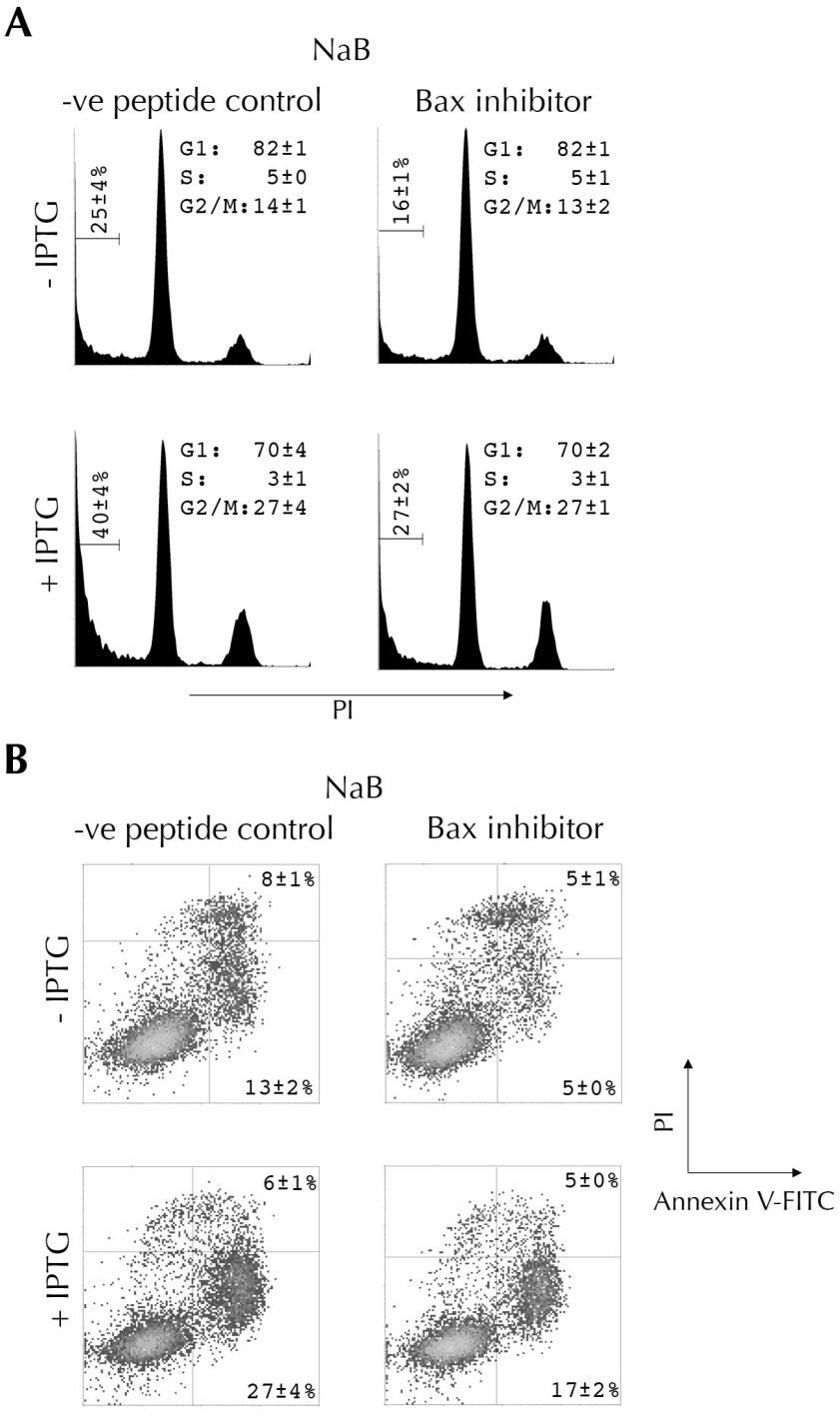


Figure 5.21 Bax inhibition does not prevent p14ARF increasing sensitivity of U2OS_ARF cells to sodium butyrate (NaB)

U2OS_ARF cells were pre-treated with the Bax inhibiting peptide BIP-V5 or control peptide BIP-NC then treated for three days with 3mM NaB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

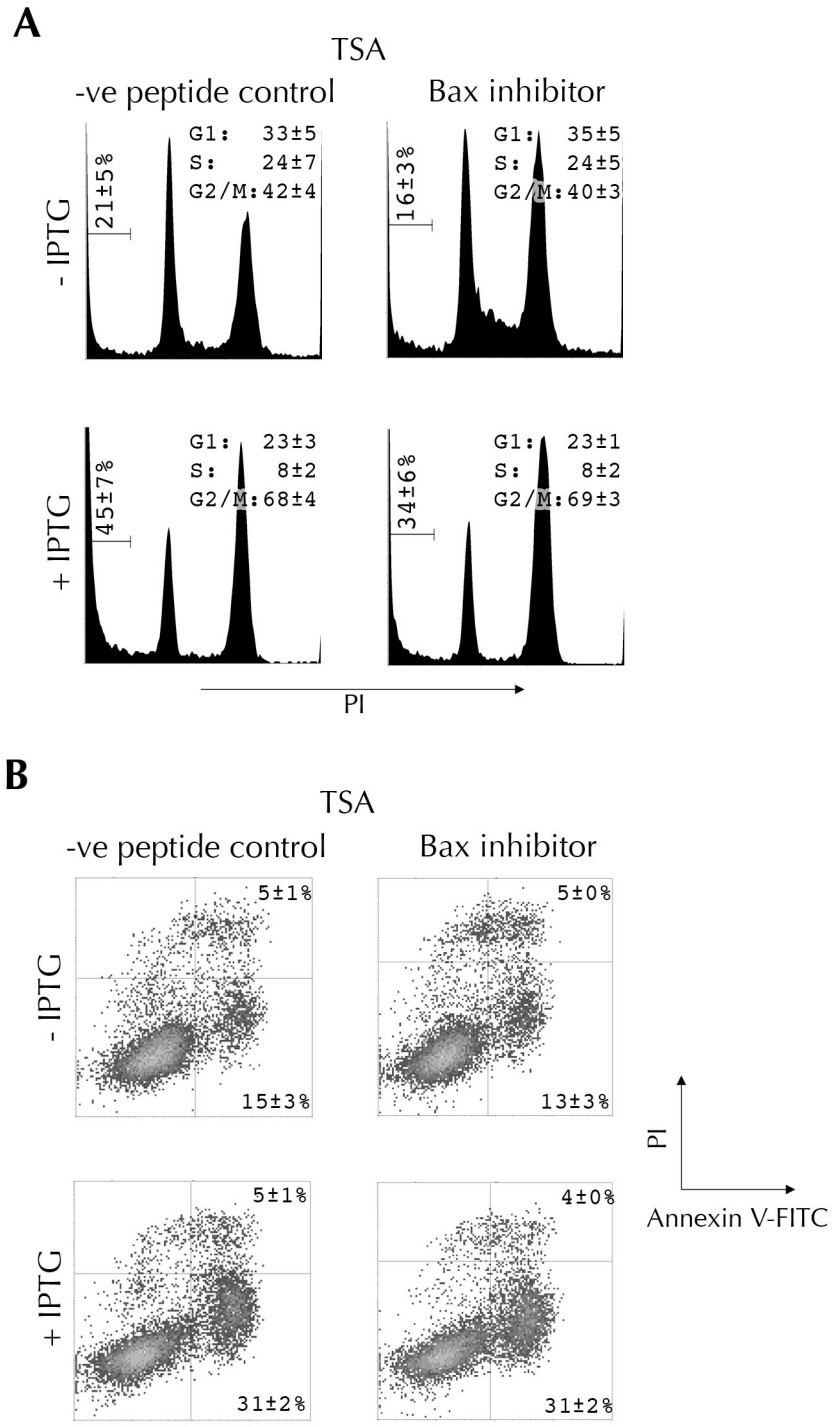


Figure 5.22 Bax inhibition does not prevent p14ARF increasing sensitivity of U2OS_ARF cells to trichostatin A (TSA)

U2OS_ARF cells were pre-treated with the Bax inhibiting peptide BIP-V5 or control peptide BIP-NC then treated for three days with 200nM TSA and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

5.2.9 p14ARF requires p53 to increase mitochondrial permeabilisation

As both p53 and mitochondrial depolarisation were required for p14ARF-mediated chemosensitivity, we investigated whether p53 was acting upstream of the mitochondria. We treated the p53 depleted U2OS_ARF^{si_p53} cells (see Figure 5.7A) and control U2OS_ARF^{si_Luc} cells with NaB in the presence or absence of IPTG and measured mitochondrial depolarisation with CMX-Ros, which was suitable to use in these GFP expressing cells. As expected p14ARF induction of NaB treated cells increased the population of cells with depolarised mitochondria (Figure 5.23A). However ARF did not affect mitochondrial depolarisation in NaB treated U2OS_AR^{si_p53} cells, demonstrating p14ARF requires p53 to increase mitochondrial depolarisation (Figure 5.23B).

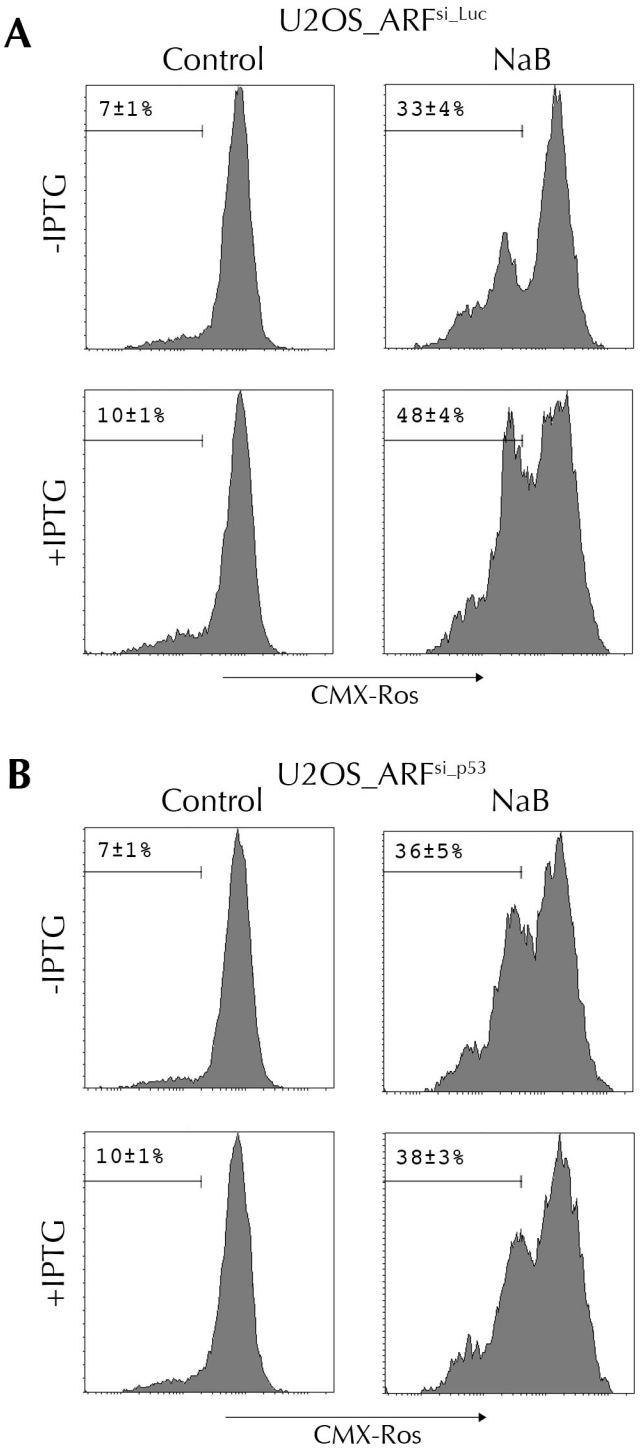


Figure 5.23 p53 is required for p14ARF to increase mitochondrial depolarisation in sodium butyrate (NaB) treated cells

U2OS_ARF^{si_Luc} and U2OS_ARF^{si_p53} cells were treated for 3 days with 3mM NaB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. p53 is ablated in U2OS_ARF^{si_p53} cells by RNA interference.

(A) Mitochondrial depolarisation in U2OS_ARF^{si_Luc} cells was assessed by CMX-Ros staining. Loss of CMX-Ros fluorescence indicates mitochondrial depolarisation.

(B) Mitochondrial depolarisation in U2OS_ARF^{si_p53} cells was assessed by CMX-Ros staining.

5.2.9 z-VAD-fmk does not inhibit loss of mitochondrial depolarisation induced by ARF expression

Although active caspases were not detected by western blotting, the pan-caspase inhibitor z-VAD-fmk prevented cell death induced by DRB, NaB and TSA, with or without p14ARF expression. To investigate if z-VAD-fmk protected these cells by preventing mitochondrial depolarisation, we pre-treated U2OS_ARF cells with 50μM z-VAD-fmk, then exposed them to NaB in the presence or absence of IPTG to induce p14ARF expression. Mitochondrial depolarisation was measured by CMX-Ros staining after three days of incubation. z-VAD-fmk treatment did not prevent p14ARF accumulation from increasing mitochondrial depolarisation (Figure 5.24), although these cells displayed a very low sub-G1 and annexin-V population (similar to Figure 5.11A & B). Thus p14ARF does not require caspases to promote mitochondrial depolarisation, but the apoptotic morphology that results is suppressed by z-VAD-fmk.

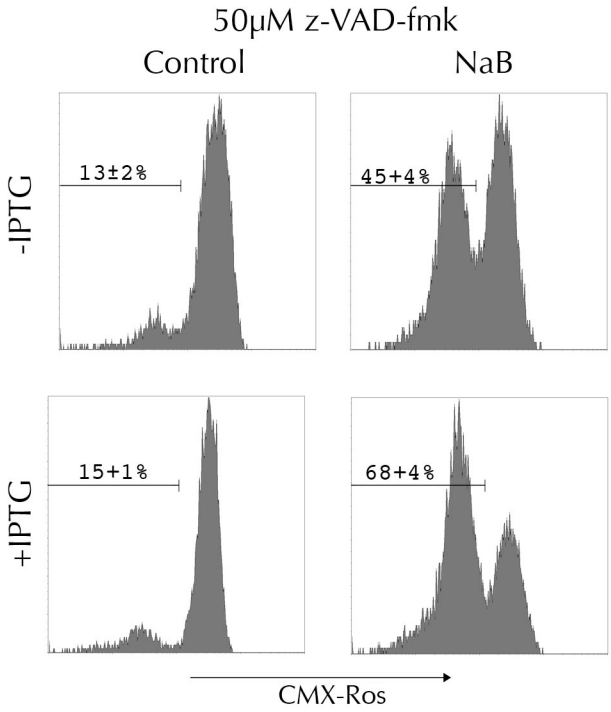


Figure 5.24 Pan-caspase inhibition does not prevent p14ARF from increasing mitochondrial depolarisation in sodium butyrate cells

U2OS_ARF cells were pre-treated with the pan-caspase inhibitor z-VAD-fmk then exposed for three days to 3mM sodium butyrate (NaB) and PBS (-) or 1mM IPTG (+) to induce p14ARF expression. Mitochondrial depolarisation was assessed by CMX-Ros staining. Loss of CMX-Ros fluorescence indicates mitochondrial depolarisation.

5.2.10 p14ARF does not require Fas activation to sensitise cells to apoptosis

p14ARF required p53, caspase activity and induction of mitochondrial permeability to increase sensitivity of cells to apoptosis caused by drugs. There are many p53 targets that can contribute to loss of mitochondrial outer membrane potential, making targeting of upstream effectors of the mitochondrial permeability shift difficult. We chose to investigate one such effector, Fas, as it is a p53 target and apoptosis caused by adenoviral expression of p14ARF in U2OS is partly mediated by Fas (Kim *et al.*, 2004). Induction of p14ARF increased Fas levels dramatically in IPTG-treated cells (Figure 5.25). DRB and TSA treatment of U2OS_ARF cells increased the amount of Fas, but

p14ARF induction did not alter the Fas levels in these cells. NaB caused a substantial decrease in Fas levels and p14ARF induction did not alter the low Fas levels in NaB treated cells (Figure 5.25). Levels of Fas ligand (FasL) were not altered by p14ARF induction, but were increased slightly by NaB treatment (Figure 5.25).

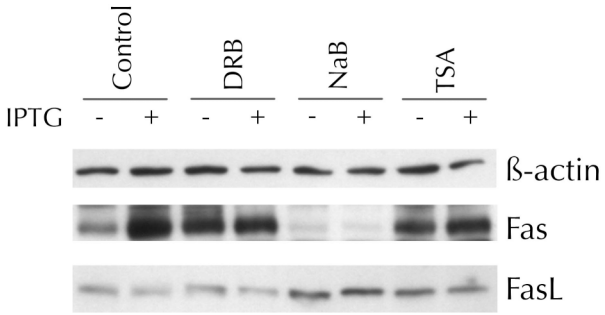


Figure 5.25 Expression of Fas and FasL in U2OS_ARF cells treated with cytotoxic drugs and IPTG

Western blot assessment of Fas and FasL in U2OS_ARF cells after three days exposure to 50µM DRB, 3mM NaB or 200nM TSA and PBS (-) or IPTG (+) to induce p14ARF expression.

As Fas levels were increased by p14ARF accumulation, we investigated if p14ARF mediated chemosensitivity was Fas dependent by utilising NOK-1, an antibody that binds FasL and prevents Fas mediated apoptosis. To test the effectiveness of NOK-1, we treated U2OS_ARF cells with Fas-Ligand (FasL), after pre-treatment of NOK-1 or IgG control. FasL treatment of U2OS_ARF cells lead to apoptosis, with a sub-G1 population of 24±2% and 34±3% annexin-V positive cells (Figure 5.26). NOK-1 treatment successfully inhibited FasL mediated apoptosis, reducing the sub-G1 percentage to 4±1% and the annexin-V positive population to 7±1% (Figure 5.26).

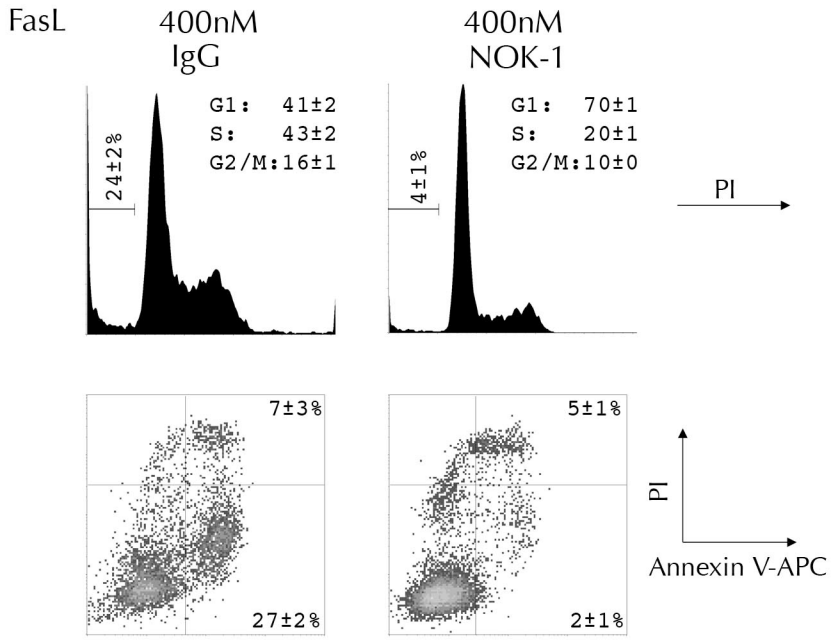


Figure 5.26 The Fas inhibitor NOK-1 prevents Fas mediated apoptosis
To demonstrate the effectiveness of Fas inhibition, U2OS_ARF cells were pre-treated with the Fas inhibiting antibody NOK-1 then treated with recombinant Fas Ligand (FasL) for two days. IgG was used as an isotype matched control for the NOK-1 antibody. The cell cycle distribution and sub-G1 population was examined using propidium iodide (PI) staining. Apoptotic cells were also analysed by dual colour flow cytometric staining of annexin-V and PI exclusion.

NOK-1 treatment of U2OS_ARF cells did not change cell cycle or apoptosis of cells compared to control IgG treated cells (Figure 5.27A & B). In the presence of IgG, IPTG increased the sub-G1 population of DRB treated cells from 10±2% to 22±0%, while in NOK-1 treated cells the sub-G1 population increased from 10±1% to 23±3% (Figure 5.28A). The NOK-1 inhibitor did not reduce apoptosis in U2OS_ARF cells treated with NaB (Figure 5.29A & B). Uninduced and induced cells treated with IgG and NaB had a sub-G1 population of 15±1% and 30±2%, and an annexin-V positive population of 22±2% and 35±2%. In cells treated with NOK-1 inhibitor and NaB, induction of p14ARF expression increased the sub-G1 population from 19±3% to 34±1% and annexin-V positive cells from 32±4% to 42±3%, demonstrating that p14ARF mediated apoptosis is not Fas dependent (Figure 5.29A & B).

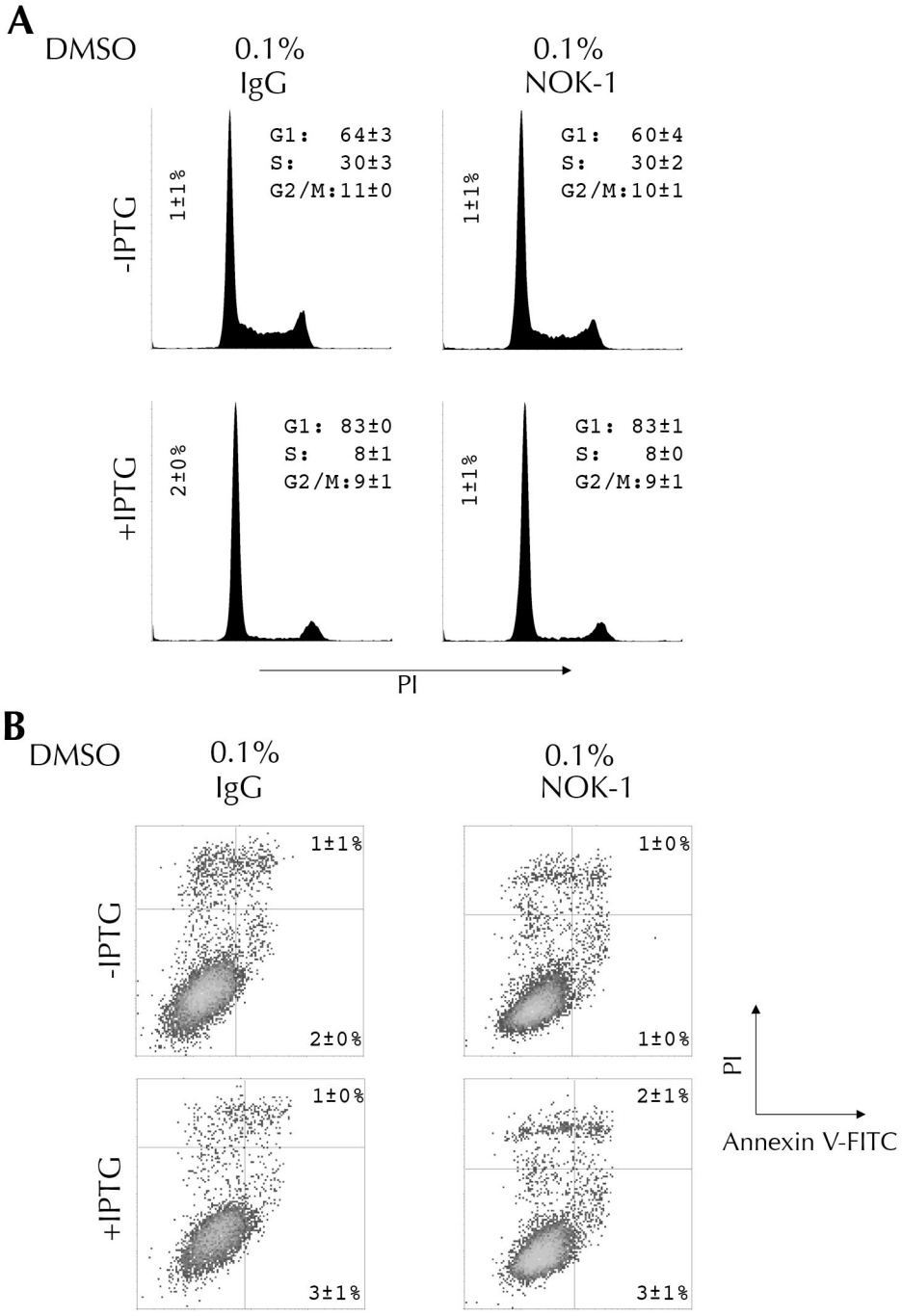


Figure 5.27 Analysis of the effect of Fas inhibition in U2OS_ARF cells

U2OS_ARF cells were pre-treated with the Fas inhibiting antibody NOK-1 or control IgG then treated for three days with DMSO carrier control and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

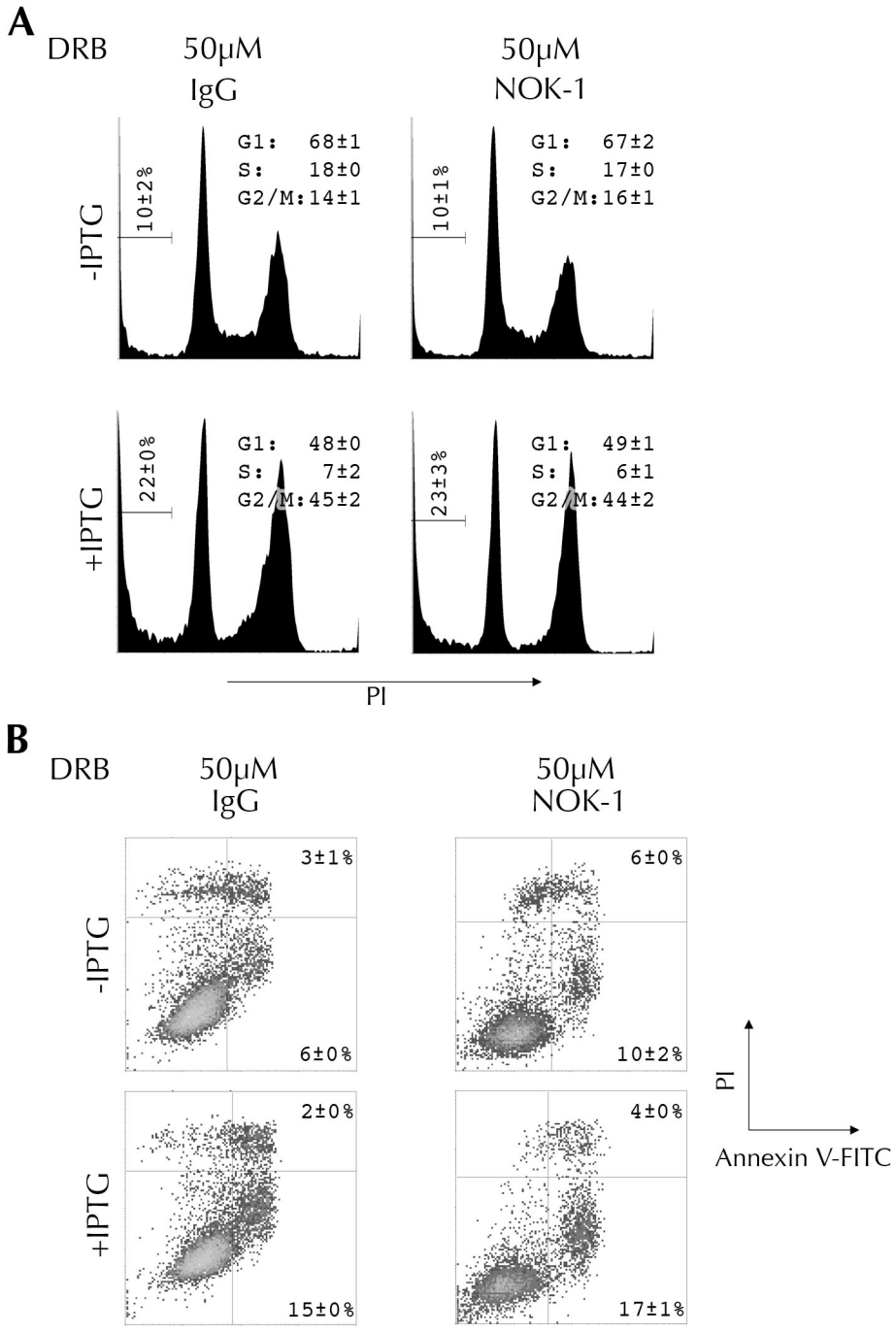


Figure 5.28 Fas inhibition does not prevent p14ARF increasing sensitivity of U2OS_ARF cells to DRB

U2OS_ARF cells were pre-treated with the Fas inhibiting antibody NOK-1 or control IgG then treated for three days with 50µM DRB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

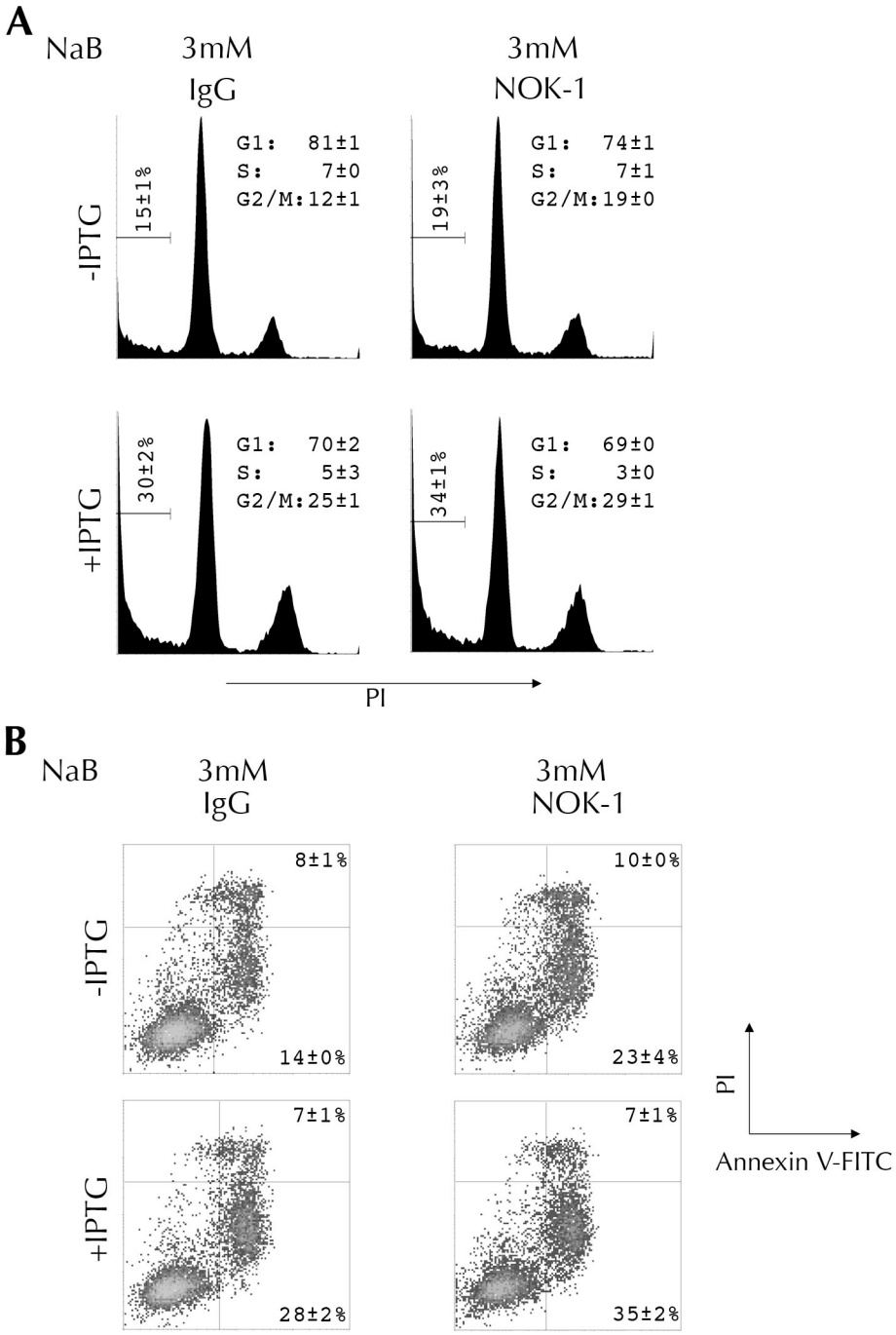


Figure 5.29 Fas inhibition does not prevent p14ARF increasing sensitivity of U2OS_ARF cells to sodium butyrate (NaB)

U2OS_ARF cells were pre-treated with the Fas inhibiting antibody NOK-1 or control IgG then treated for three days with 3mM NaB and PBS (-) or 1mM IPTG (+) to induce p14ARF expression.

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

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

5.2.11 p14ARF mediated chemosensitivity is not associated with smARF or mitochondrial p53

The smARF protein is formed as the result of internal initiation from the Met codon 48 of human p14ARF mRNA (Met 45 in mouse p19ARF) (Reef *et al.*, 2006). smARF localises to the mitochondria, where it associates with p32 and causes loss of mitochondrial membrane potential, autophagy and cell death (Reef *et al.*, 2006). Although smARF killing of cells is independent of p53, and not inhibited by z-VAD-fmk or Bcl-2 over-expression, we investigated if smARF contributed to p14ARF mediated chemosensitivity. As expression levels of p14ARF in the U2OS_ARF cell line is low, and smARF levels are typically much less than p14ARF levels, we transiently transfected U2OS cells with p14ARF to ensure high expression level of ARF, and smARF. To further increase the chances of detecting smARF, cells were divided into their nuclear, cytoplasmic and mitochondrial fractions, and protein fractions separated on a SDS-PAGE gel and subject to immuno-blotting. Blotting for mitochondrial HSP-70 and tubulin confirmed that the mitochondrial fractions were pure (Figure 5.30). Despite our attempts to increase smARF detection, we did not observe smARF in any cellular fraction (Figure 5.30). As p14ARF induction in DRB treated U2OS_ARF cells resulted in increased chemosensitivity and also much higher levels of p14ARF accumulation, we also subject these cells to fraction and immuno-blotting. Again, we did not detect the smARF protein in these cells, suggesting that smARF plays no role in p14ARF-mediated chemosensitivity (Figure 5.30). The same fractions were also probed for p53. p53 has been reported to move to the mitochondrial, where it antagonises Bcl-2 and Bcl-x_L and causes mitochondrial depolarisation and cytochrome c release (Mihara *et al.*, 2003). p14ARF mediated chemosensitivity was p53-dependent and associated

with mitochondrial depolarisation, raising the possibility that p53 translocation to the mitochondria may play a role. However p53 was never detected in the mitochondria, and was only found in the nuclear fraction (Figure 5.30). Therefore p53 did not move to the mitochondria as a result of p14ARF over-expression or during p14ARF induced chemosensitivity.

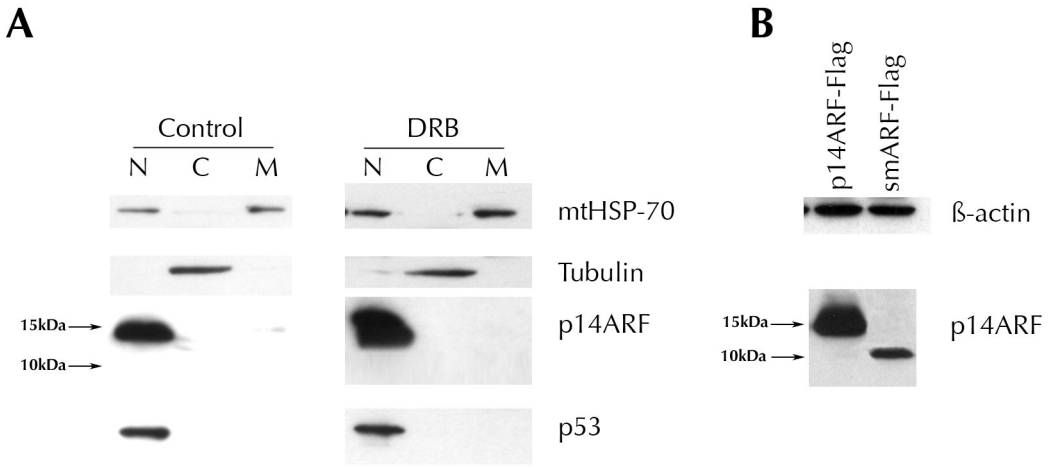


Figure 5.30 smARF and mitochondrial p53 do not contribute to p14ARF mediated chemosensitivity

(A) U2OS cells were transfected with p14ARF (Control) or U2OS_ARF cells were treated with 50µM DRB and 1mM IPTG to induce p14ARF expression (DRB) for 48 hours and cells separated into nuclear (N), cytoplasmic (C) and mitochondrial (M) fractions. Neither p14ARF, smARF nor p53 was detected in mitochondrial fractions. smARF migrates are approximately 9kDa. mtHSP70 and tubulin were used to as markers of mitochondrial and cytoplasmic fractions, respectively.

(B) A western blot was used to demonstrate that the p14ARF antibody can detect ectopic smARF. U2OS cells transfected with Flag tagged smARF or p14ARF as a positive control, and harvested after 24 hours. smARF-Flag migrates at approximately 10kDa. ß-actin is used to demonstrate equal loading.

5.3 Discussion

Apoptosis is an important cellular program regulating tissue homeostasis and tumourigenesis. All cancers circumvent apoptosis to a certain extent during tumorigenesis, and some cancers, including melanoma, are so resistant to apoptosis, that a wide-range of chemotherapies are ineffective. Increasing the sensitivity of these cancers to apoptosis is necessary for effective systemic treatments and the cellular pathways that modulate chemosensitivity are being actively analysed. In this study we show that expression of p14ARF sensitises cells to apoptosis via pathways that depend upon p53, caspase activation and mitochondrial depolarisation. Individual inhibition of Bax or Fas did not eliminate the ability of p14ARF to increase apoptosis, showing that there are a number of complimentary effectors downstream of p53 stabilisation.

p14ARF caused a p53-dependent depolarisation of mitochondria and while caspase inhibition did not prevent mitochondrial depolarisation, it did prevent apoptosis. Mitochondrial outer membrane permeabilisation can occur independently of caspases, and even in the presence of high concentrations of z-VAD-fmk, cytochrome c and SMAC/DIABLO are released from mitochondria in response to a variety of stimuli such as UVB, staurosporine and cisplatin (Dussmann *et al.*, 2003; Rehm *et al.*, 2003; Zhou *et al.*, 2005). While the increase in depolarisation caused by z-VAD-fmk was unexpected, z-VAD-fmk may increase kinetics of SMA/DIABLO release from permeable mitochondria, accelerating mitochondrial depolarisation (Rehm *et al.*, 2003). Interestingly, while z-VAD-fmk prevented the apoptotic phenotype, activation of caspases 3, 8 and 9 was not observed, nor was significant PARP cleavage. The paradigm of intrinsic pathway activation involves cytochrome c release, caspase 9

activation and caspase 3 cleavage and activation (Zimmermann *et al.*, 2001). Although, alternate apoptosis-regulatory pathways exist, the downstream activation of either caspase 9 or caspase 3 usually occurs. For example, once released from the mitochondria, SMAC/DIABLO and Omi, bind and disable IAPs including survivin and xIAP (Rehm *et al.*, 2003) and activate effector caspases 6 and 7. These active caspases would be expected to cleave caspase 3, 8 and 9 however, even as a late event during apoptosis. Thus, it is most likely that active caspase 3 or caspase 9 was present in our cell model but below detectable levels possibly because of apoptotic asynchrony and low apoptotic stimuli provided by the relatively long (three day) experimental time-frame we used.

Accumulation of ARF in the presence of DRB, NaB and TSA resulted in readily detectable apoptotic cell death without the stereotypical cleavage of PARP (see Figure 5.13). The presence of immunoreactive intact PARP (a substrate of caspase 3) suggests a defective pathway activating caspase 3 in U20S_ARF cells, and implies that ARF-mediated apoptosis does not require caspase 3, in this cell model. Accordingly we also detected minimal caspase 3 cleavage and activation (see Figure 5.13). Thus, although U20S_ARF cell death was totally inhibited by the caspase inhibitor, z-VAD-fmk, this does not appear to involve caspase 3 or the concomitant cleavage of PARP. A similar caspase 3-independent apoptotic programme, with no evidence of PARP cleavage, has also been reported in testicular germ cell tumour cell lines ((Burger *et al.*, 1999)). Whether, activation of additional caspase members compensate for the impaired caspase 3 activation remains to be elucidated.

Despite generating and testing two previously published $p21^{Waf1}$-specific shRNA molecules, we were unable to achieve effective knockdown of $p21^{Waf1}$ levels when using these molecules in a lentivirus expression vector (Nicke *et al.*, 2005; Zhang *et al.*, 2004). The combination of two $p21^{Waf1}$-shRNA molecules produced only minimal silencing that was not effective enough to reduce p14ARF-induced cell cycle arrest. Moreover, the use of siRNA molecules to specifically target $p21^{Waf1}$ was not feasible, as the transfection efficiency routinely achieved using the U20S_ARF cell clone was only 30% and would not have permitted analysis of the whole cell population. Thus, although we and others have shown that ARF-mediated cell cycle inhibition is largely dependent on $p21^{Waf1}$ (Hemmati *et al.*, 2005; Normand *et al.*, 2005), we are unable to make any conclusions regarding the role of $p21^{Waf1}$ in regulating ARF-induced chemosensitivity.

Our results reinforce the central role that p53 plays in ARF functions; p14ARF mediated cell cycle arrest, chemosensitivity and mitochondrial depolarisation are all p53-dependent. This is supported by other studies showing that p14ARF functions via the p53 pathway (Weber *et al.*, 2002) and can increase chemo- and radiosensitivity only in p53-intact cell lines (Gao *et al.*, 2001; Simon *et al.*, 2006).

Although, most melanomas retain functional p53 (Albino *et al.*, 1994; Kaleem *et al.*, 2000; Lubbe *et al.*, 1994), the use of ARF analogues to enhance this pathway may not be an effective treatment strategy. Melanomas are resistant to killing by p53-dependent mechanisms due to alterations in downstream mediators of apoptosis, such as over expression of Mcl-1 and xIAP, downregulation of APAF-1 and production of p53 splice variants that modulate p53 transcriptional response (Avery-Kiejda *et al.*, 2008; Soengas

et al., 2001; Zhang *et al.*, 2001; Zhuang *et al.*, 2007). These alterations may negate the p14ARF-induced activation of the p53 pathway and while this may be discouraging, direct targeting of critical downstream p53 effectors may provide new therapeutic opportunities. The combination of ARF mimetics and direct targeting of disabled p53 components may provide an especially effective approach to melanoma therapy. Genetic screening is adding to our knowledge of potential effectors. Loss or mutation of p14ARF alters the gene expression profile of melanomas with wild type p53, and pathways up-regulated in p14ARF containing melanomas such as the epidermal growth factor, platelet-derived growth factor and Wnt signalling pathways may contain new therapeutic targets (Packer *et al.*, 2007).

The mechanisms by which p14ARF influences p53 activity remain unresolved. Whether p53 transactivates pro-arrest or pro-apoptotic genes is influenced by various post-translational modification of p53, which can alter p53 conformation affinity to different promoters. For instance, phosphorylation of p53 at serine-46 is induced by DNA-damage signals and this phosphorylated form of p53 is an effective activator of the pro-apoptotic genes NOXA (Grossman *et al.*, 2003) and PUMA (reviewed in (Das *et al.*, 2008)). Additionally acetylation of lysine-120 of p53 by the MYST family of acetyl transferases increases transactivation of Bax and PUMA, but is dispensable for transcription of the non-apoptotic $p21^{Waf1}$ and *HDM2* genes (Sykes *et al.*, 2006). p53 function is also regulated by its many binding partners and these vary according to cell type and growth conditions. For instance, p300 and CREB binding protein (CBP) are transcriptional co-activators that bind, acetylate and activate p53 (Grossman, 2001). Hematopoietic zinc finger binds p53 and results in preferential transcription of pro-arrest genes (Das *et al.*, 2007).

In this study we did not identify an individual target down-stream of p53 that was essential for increased chemosensitivity. This is not surprising considering the redundancy available in the cell cycle and apoptotic pathways. The pro-apoptotic targets of p53 include Bax, Bid, APAF1, Fas, Killer/DR5, Noxa, p53AIP1, TP53 apoptosis effector, PIDD, PIGs, PUMA and insulin-like growth factor binding-protein 3; and in mice knock-out studies no single p53-induced product has been found to be responsible for p53-mediated apoptosis (reviewed in (Chipuk and Green, 2006; Slee *et al.*, 2004)). For example, Noxa loss did not alter sensitivity of mouse lymphocytes or thymocytes to cytotoxic agents, but decreased the sensitivity of MEFs and gastric epithelial cells to DNA damage (Shibue *et al.*, 2003; Villunger *et al.*, 2003). PUMA seems to play a wider role in p53-induced apoptosis; PUMA loss increased the resistance of thymocytes, lymphocytes and MEFs to genotoxic damage by etoposide and γ-irradiation (Villunger *et al.*, 2003). The induction of apoptosis by p53 targets also differs significantly between different tissues. For example, in mice irradiated with ionising radiation, Fas levels increase 5-fold in the spleen but do not increase in the liver or heart (Bouvard *et al.*, 2000). Thus, to determine the relative contribution of each p53 target in ARF-mediated apoptosis would require a detailed but global analytical approach, such as a proteomic or microarray screen.

Regardless of the precise p53-targets regulating ARF-mediated chemosensitisation, this study showed that the loss of mitochondrial outer membrane potential is the critical p53-dependent step in this program. There are a number of possible mechanisms by which ARF depolarises the mitochondria and the simplest one involves increased p53 expression. For instance, p53 repressors the transcription of the anti-apoptotic genes

Bcl-2 and *Mcl-1* and this would promote Bax-Bak pore formation in the mitochondrial outer membrane (Pietrzak and Puzianowska-Kuznicka, 2008; Wu *et al.*, 2001). Our data, however, indicate that p14ARF accumulation alone induced levels of p53 that were similar to the ARF plus drug combination, and yet ARF alone did not promote cell death. Furthermore p14ARF induction promoted higher p53 levels when combined with some drugs (such as TMZ, cisplatin and valproic acid) but it did not increase chemosensitivity to these drugs (see chapter 4). It is also possible that p53 is specifically modified by ARF, and the high levels of modified p53, in combination with specific stress signals, leads to an apoptotic p53-mediated transcriptional program. ARF can promote the sumoylation of p53, in the presence of HDM2 (Chen and Chen, 2003), and although the effect of this post-translational modification remains unresolved, it has been reported to enhance p53-dependent apoptosis (Kwek *et al.*, 2001; Lu and Yi, 2005; Melchior and Hengst, 2002; Muller *et al.*, 2000; Rodriguez *et al.*, 1999a). However, it should be noted that ARF-mediated p53-sumoylation has only been observed when ARF and hdm2 are overexpressed along with p53 (Chen and Chen, 2003) and we have never observed ARF-induced sumoylation of endogenous p53 in our stable U2OS_ARF cell line (data not shown).

An alternate hypothesis explaining ARF-mediated chemosensitivity is that ARF inhibition of HDM2 allows drug-stabilised p53 to engage in an expanded, pro-apoptotic transcriptional program (Figure 5.31). HDM2, while also acting to ubiquitinate and degrade p53, can bind the N-terminal transactivation domain of p53, compete for binding with p53 co-activators such as p300 and directly inhibit p53 transcription (Ito *et al.*, 2001; Kussie *et al.*, 1996; Oliner *et al.*, 1993; Wang *et al.*, 2001b). Additionally HDM2 promotes the degradation of p300 and inhibits activation of p53 by p300-

mediated acetylation (Jin *et al.*, 2002; Jin *et al.*, 2004). p53 that accumulates in response to ARF induction is not extensively phosphorylated or modified (Jackson *et al.*, 2004) and this p53 may preferentially transactivate cell cycle regulatory, rather than apoptotic targets. Certainly, there are detectable differences in p53-transactivation activity following treatment with adriamycin or induction of ARF (Jackson *et al.*, 2004). In cells treated with cytotoxic drugs, p53 is heavily modified by phosphate and acetyl groups, and although this p53 has the capacity to potently transactivate apoptotic regulatory genes, it is kept in check by HDM2 in a negative feedback loop. This loop is disengaged in the presence of ARF, which inhibits HDM2 and allows p53 to activate gene transcription. In fact, p53 is a more effective transcriptional regulator in the presence of ARF (Huang *et al.*, 2003).

In this model, ARF-mediated chemosensitivity would not require further increases in p53 levels or additional post-translational modifications. In fact, a similar model has been previously suggested (Korgaonkar *et al.*, 2002; Midgley *et al.*, 2000), and it has been shown that cells accumulating high levels of HDM2 show lower induction of pro-apoptotic p53 targets, while retaining effective induction of cell cycle inhibitory targets (Ohkubo *et al.*, 2006). As a result, cells with higher levels of HDM2 demonstrated lower levels of apoptosis in response to DNA damaging agents.

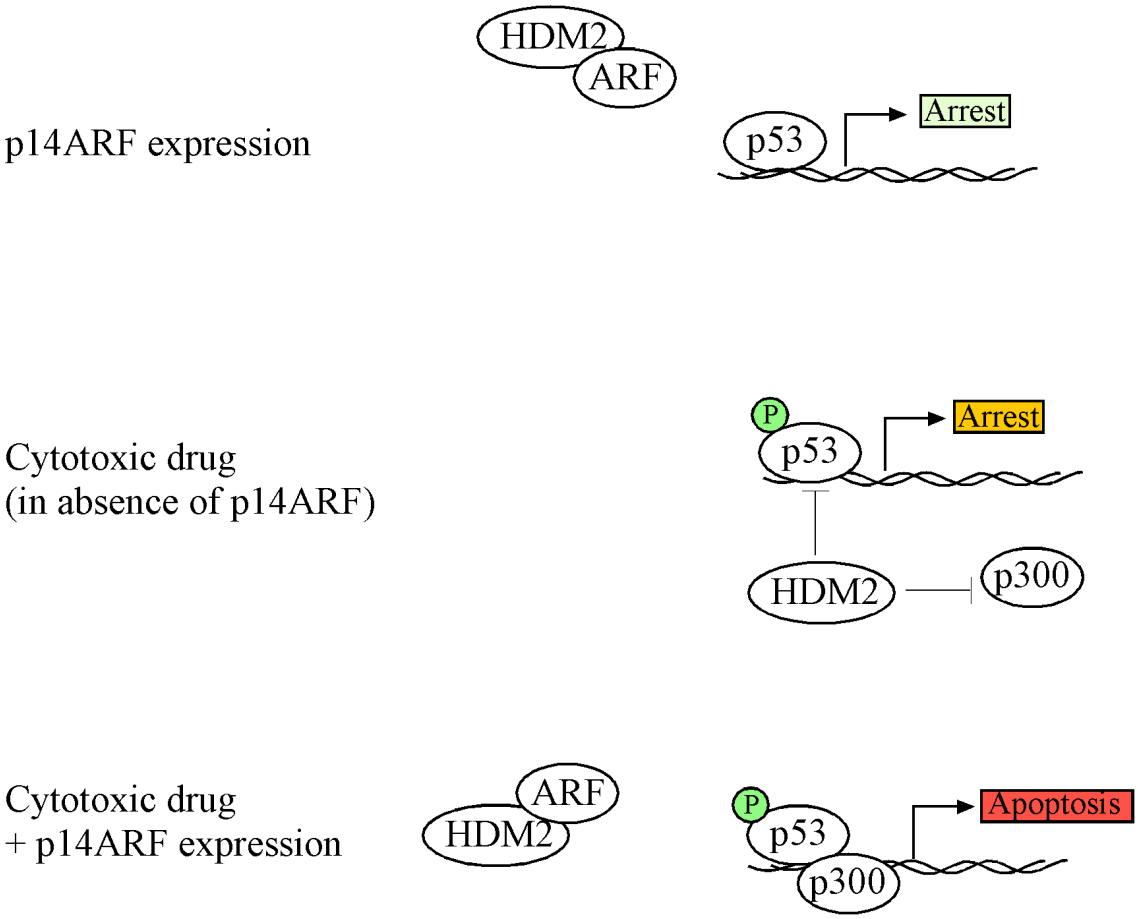


Figure 5.31 Proposed model showing the co-operative impact of p14ARF induction and cytotoxic drugs on p53-mediated cell death

p53 accumulated in response to ARF is relatively unmodified and transactivates cell cycle arrest genes. Although cytotoxic drugs stabilise p53 by inducing its post-translational modification, efficient p53-dependent transactivation is prevented by HDM2 competing for p53 co-activator p300 and other protein partners. The introduction of ARF relieves this repression, and a stabilised p53 interacts with transactivation partners to efficiently induce the expression of pro-apoptotic genes and cell death.

In conclusion, we have confirmed that p14ARF-cell cycle inhibitory and –apoptotic functions require p53. We also determined that loss of mitochondrial integrity was involved in ARF-mediated chemosensitivity, and although this also involved the p53 tumour suppressor, we did not define any single p53 target that controlled this process. Thus, the mechanism of p14ARF-mediated chemosensitivity requires further investigation, as does its importance in melanoma. The next chapter investigates whether p14ARF expression is associated with increased chemosensitivity of melanoma tumours *in vivo*.

6. p14ARF expression does not predict melanoma chemosensitivity *in vivo*

Publications arising from this chapter:

Activated N-RAS and $p16^{INK4a}$ expression are independent predictors of chemosensitivity in melanoma tumours

Stuart J Gallagher, John F. Thompson, James Indsto, Lyndee L Scurr, Bo-Fu Gao, Ruth Dunleavey, Graham J Mann, Richard F Kefford, Helen Rizos

Submitted: Journal of Clinical Oncology. March 2008.

6.1 Author contribution statement

This chapter consists of work recently submitted to Journal of Clinical Oncology. I gratefully acknowledge the contribution of the co-authors to this work. Author contribution statements from co-authors can be found in appendix 1. As first author on this paper, I state that I made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

6.2 Introduction

Metastatic cutaneous melanoma has a poor prognosis with two-year survival of less than 30% in patients with visceral involvement (Balch *et al.*, 2001b). This reflects the marked resistance of the disease to cytotoxic drugs. Single agent treatment with the alkylating agent dacarbazine (5-(3,3-dimethyl-l-triazeno)-imidazole-4-carboxamide, "DTIC") remains standard best systemic therapy for metastatic melanoma (Eggermont and Kirkwood, 2004). Although overall response rates to dacarbazine are less than 15% (Avril *et al.*, 2004; Middleton *et al.*, 2000), certain patients have highly sensitive tumours and rare complete remissions are often sustained (Coates and Segelov, 1994). To date there are few studies of *in vivo* molecular determinants of chemosensitive melanomas, as distinct from studies on cultured cell lines, and the mechanisms involved in melanoma chemoresistance have not yet been elucidated.

We sought molecular correlates of chemosensitivity in a unique cohort of prospectively acquired tumour samples from patients with metastatic melanoma receiving isolated limb infusion (ILI) chemotherapy (Thompson *et al.*, 1998). ILI is a complex and technically demanding procedure performed as a palliative procedure for extensive inoperable loco-regional recurrence in a limb, usually from multiple in-transit seeding in cutaneous lymphatic vessels. A tourniquet is applied to isolate the circulation of the affected limb, allowing very high doses of cytotoxic drugs to be administered locally, while minimizing toxic systemic effects (Thompson *et al.*, 1998). ILI is only performed in a few centres around the world on highly selected patients, and although cohort size will always be limited, it provides a unique platform for the study of chemosensitivity in melanoma. Firstly, unlike the majority of clinical situations, pre-treatment fresh-

frozen tumour samples are readily available. Secondly, response rates to ILI are >50% (Thompson *et al.*, 1998), allowing greater statistical power to assess molecular correlates of response than in systemic chemotherapy, where response rates are <15% (Thompson *et al.*, 2005).

We selected for this analysis key candidate genes known to be important in melanomagenesis and also linked to the regulation of chemosensitivity (Thompson *et al.*, 2005). In particular, we analysed the expression of MITFm and the p14ARF and $p16^{INK4a}$ melanoma tumour suppressor genes and the status of the B-RAF and N-RAS melanoma oncogenes and the PTEN tumour suppressor.

The *INK4a/ARF* locus on chromosome 9p is the most frequently deleted region in established melanoma (Curtin *et al.*, 2005) and is inherited in mutated form in approximately 39% of melanoma prone families (Goldstein *et al.*, 2007). *INK4a/ARF* encodes two tumour suppressor proteins, p14ARF and $p16^{INK4a}$, both of which have been shown to enhance the chemosensitivity of human cancer cells (Gallagher *et al.*, 2005; Simon *et al.*, 2006; Williams *et al.*, 2006; Yuan *et al.*, 2007). p14ARF accumulates in response to oncogenic stimuli and stabilises p53, leading to cell cycle arrest or apoptosis (reviewed in (Sherr, 2006)). The $p16^{INK4a}$ protein activates the retinoblastoma pathway by inhibiting CDK4 and 6 (Serrano *et al.*, 1993), leading to cell cycle arrest and, in some instances, cell death (Ausserlechner *et al.*, 2005). Loss of both p14ARF and $p16^{INK4a}$ is associated with melanoma progression (Dobrowolski *et al.*, 2002).

The microphthalmia-associated transcription factor, MITF, regulates development and differentiation of melanocytes, is expressed in most melanocytes (Koyanagi *et al.*, 2006) and is deregulated in melanoma. Increased levels of MITF may contribute to melanoma progression as MITF induces expression of the anti-apoptotic molecule, Bcl-2 (McGill *et al.*, 2002) and MITF ablation sensitises melanoma cells to cytotoxic drugs (Garraway *et al.*, 2005). MITF amplification is more frequent in metastatic melanoma and correlates with decreased patient survival (Garraway *et al.*, 2005). In normal melanocytes the MITF transcription factor transcriptionally activates $p16^{INK4a}$ to promote cell cycle arrest (Loercher *et al.*, 2005) and, as expected, MITF amplification is accompanied by $p16^{INK4a}$ inactivation in melanoma cell lines (Garraway *et al.*, 2005).

Activating N-RAS and B-RAF mutations are the most common oncogenic mutations in melanoma, indicating the importance of the RAS-RAF-MEK-ERK MAP kinase pathway in melanoma progression. Up to 80% of benign nevi (Pollock *et al.*, 2003) and 25-66% of melanomas contain activating B-RAF mutations (Davies *et al.*, 2002; Houben *et al.*, 2004). Activating N-RAS mutations are less common, occurring in 5-30% of melanoma (Houben *et al.*, 2004; Kumar *et al.*, 2003a; Omholt *et al.*, 2002). Activated B-RAF may also co-operate with loss of the PTEN tumour suppressor in promoting melanoma development. PTEN attenuates phosphoinositide 3-kinase (PI(3)K/AKT) signalling and the simultaneous activation of B-RAF and loss of PTEN simulate N-RAS activation to promote melanoma development (Tsao *et al.*, 2004). The high frequency of N-RAS and B-RAF mutations in melanoma, and the fact that these mutations have been correlated with poor prognosis (Houben *et al.*, 2004) indicates that they are potential therapeutic targets and yet there have been few studies investigating N-RAS/B-RAF status and melanoma response to common chemotherapeutic agents.

6.3 Results

6.3.1 Patient response

Fresh-frozen tumour biopsy samples from 30 melanoma patients who subsequently underwent ILI were analysed. Tumour response to ILI was classified as responsive, which included tumours showing complete response (n=5) and partial response (n=16), and non-responsive, which incorporated stable disease (n=7) and disease that progressed (n=2) (Table 6.1).

Table 6.1 Details of patients treated by isolated limb infusion with melphalan and actinomycin D

	Responders	**Non-responders**
Male/Female	5/16	2/7
Median age (range)	75 (48-93)	78 (46-86)
Best response (no.)	CR 5 PR 16	SD 7 PD 2
Mean time to best response	57 days	N/A
Mean time to PD in ILI field	272 days (from 13 responders)	94 days (from 2 PD and 5 SD)

CR-complete response, PR-partial response, SD-stable disease, PD-progressive disease, N/A-not applicable.

6.3.2 N-RAS and B-RAF are frequently mutated in melanoma

The vast majority of N-RAS and B-RAF mutations in melanoma occur at codon 61 (Q61K and Q61R) and codon 600 (V600E and V600K) respectively (Houben *et al.*, 2004; Poynter *et al.*, 2006). We analysed tumour samples for mutations affecting these codons using SSCP (Figure 6.1) and sequencing (Figure 6.2). Of the 30 tumours, ten (33%) showed mutation in N-RAS codon 61 and thirteen (43%) contained mutations

altering B-RAF codon 600 (Table 6.2). As expected, activation of N-RAS and B-RAF in these melanoma tumours was mutually exclusive and all mutations were heterozygous (see Figure 6.2).

Figure 6.1 SSCP analysis of a B-RAF RT-PCR fragment encompassing codon 600
Wild type (wt) control *B-RAF* PCR product was generated from the WMM1215 melanoma cell line and the *B-RAF*^{V600E} mutant control was amplified from a *B-RAF*^{V600E} expression plasmid. Amplified *B-RAF* cDNA from tumour samples are shown in lanes 3-7 (tumour samples 026, 003, 027, 044, 004 respectively).

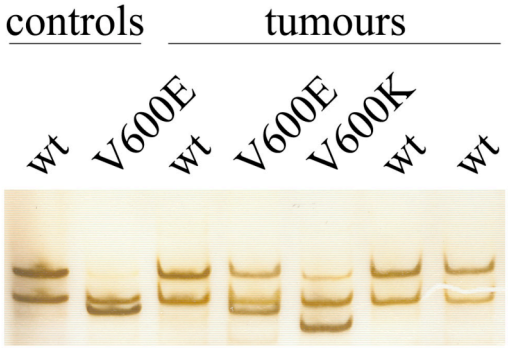


Figure 6.2 Sequence analyses of amplified B-RAF and N-RAS transcripts
The nucleotide and codon changes encoded are shown on the left of each sequence. Numbering is based on Genbank accession NM_004333 (B-RAF) and NM_002524 (N-RAS). wt, wild type sequence.

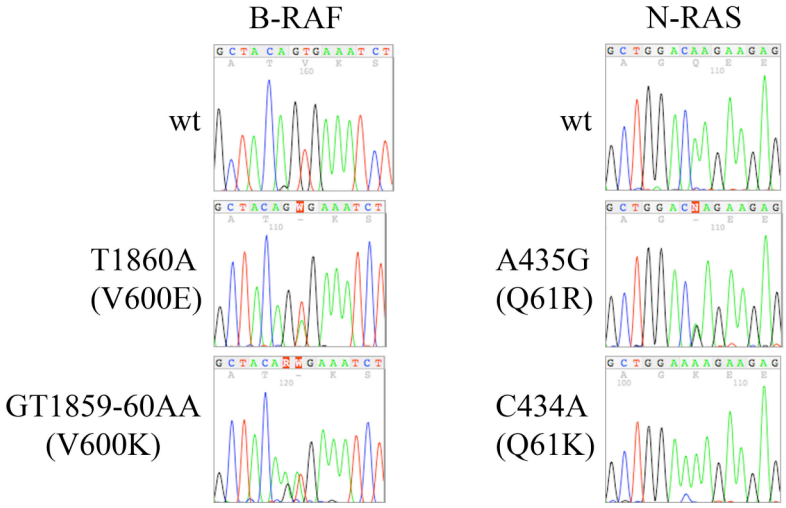


Table 6.2 Mutation status of 30 melanomas.

Mutation	Nucleotide	Number
N-RAS^{Q61R}	A435G	8
N-RAS^{Q61K}	C434A	1
N-RAS^{Q61K}	AC434-35TA	1
B-RAF^{V600E}	T1860A	9
B-RAF^{V600K}	GT1859-60AA	4
No mutation		7
Total		30

6.3.3 Expression analysis of p16^{INK4a}, p14ARF and MITF

There was considerable variation in p14ARF and p16^{INK4a} transcript expression (Figure 6.3). The majority of tumours (approximately 60%) displayed very low expression of both p14ARF and p16^{INK4a}. In most cases the expression of these transcripts was comparable, which is expected considering that these genes share genomic sequence. Only tumours 004, 030 and 041 showed discordant levels of the p16^{INK4a} and p14ARF transcripts. The expression of MITF was also variable with only four tumours (008, 045, 006, 025) displaying very low levels of this transcript (Figure 6.3).

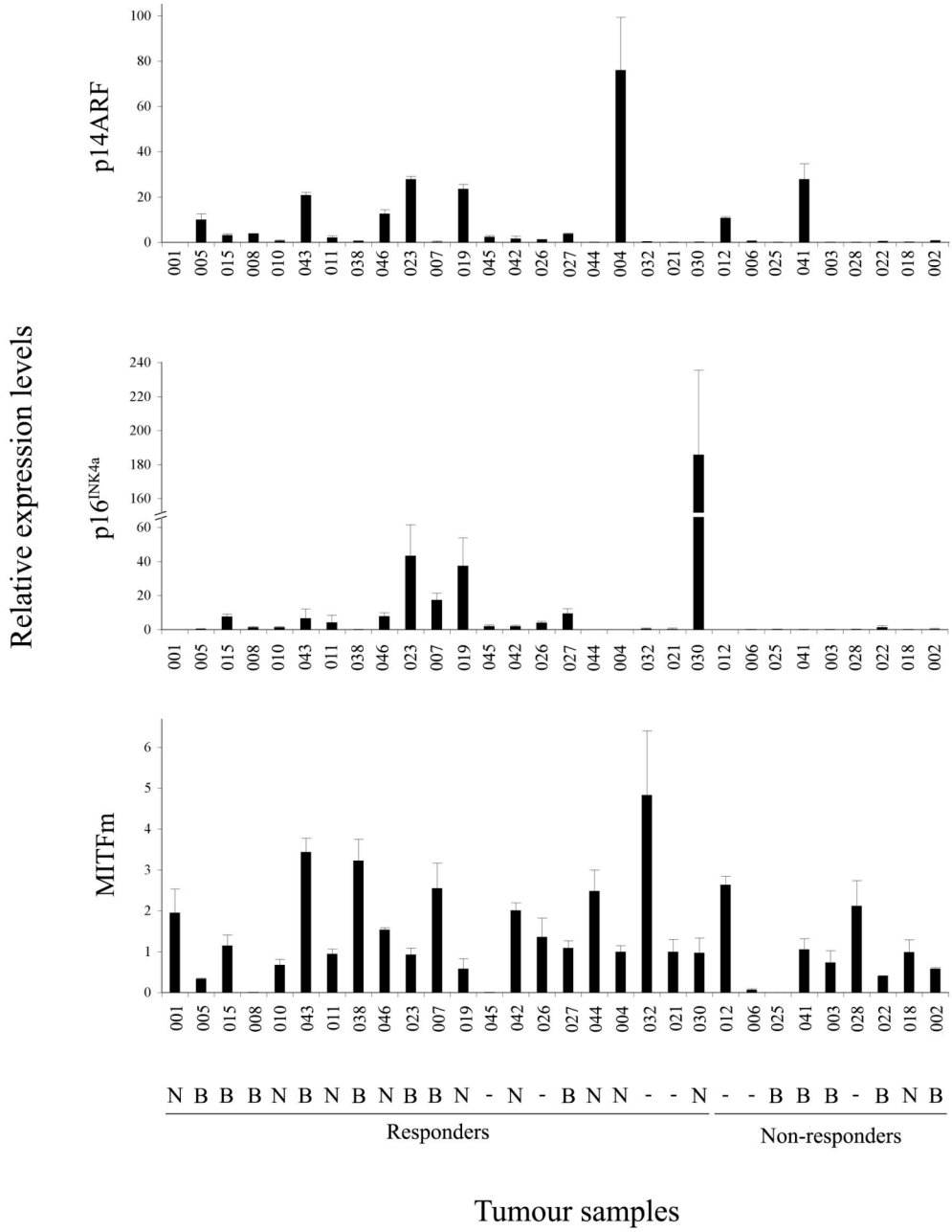


Figure 6.3 Relative expression levels of p14ARF, p16INK4a and MITFm

Relative expression levels of p14ARF, $p16^{INK4a}$ and MITFm in melanoma tumours was determined using quantitative real time RT-PCR. The mutation status of *B-RAF* and *N-RAS* for each tumour is also shown (N=mutant *N-RAS*, B=mutant *B-RAF*, -=wild type *N-RAS* and *B-RAF*).

6.3.4 N-RAS mutation and $p16^{INK4a}$ expression correlate with better response

We investigated whether the presence of N-RAS or B-RAF mutation or the expression level of $p16^{INK4a}$, p14ARF or MITF predicted response to chemotherapeutic drugs. The

independent predictors of response in this tumour set were presence of N-RAS mutation and high log transformed $p16^{INK4a}$ expression (Table 6.3). B-RAF mutation and expression level of p14ARF and MITF did not correlate with response. There was no correlation between $p16^{INK4a}$ and MITF expression levels nor did the presence of B-RAF mutation correlate with MITF expression (data not shown).

Table 6.3 Best fitting multiple logistic regression model of response together with adjusted Odds ratios and their 95% confidence intervals for independent predictors of response.

	B	S.E.	p-value	Odds Ratio	95% C.I. for OR	
					Lower	Upper
N-RAS mutation	3.31	1.68	.049	27.3	1.0	736.2
log $p16^{INK4a}$	.78	.35	.027	2.2	1.1	4.3
Constant	-3.92	1.94	.044	.02		

S.E., standard error; C.I., confidence interval; OR, odds ratio: B, regression coefficient.

6.3.5 Mutations affecting the PTEN tumour suppressor are not common in melanoma

Considering that oncogenic mutations affecting N-RAS and B-RAF were not equivalent in predicting melanoma response, it seemed likely that the MAP kinase signalling cascade, which is activated by both N-RAS and B-RAF, may not significantly influence treatment response. The main signalling cascade differentially activated by N-RAS and B-RAF is the PI(3)K)/AKT pathway and the integrity of this pathway was analysed by screening the PTEN tumour suppressor, which attenuates PI(3)K signalling. As expected, PTEN mutations were not common in our panel of melanomas, and were identified in only three tumours (003, 005 and 045). The 045 tumour, which was wild type for both N-RAS and B-RAF, expressed wild type PTEN and PTEN with the G44G (C1163T) silent amino acid change (data not shown). Tumour 003 expressed $B\text{-}RAF^{V600E}$ and carried the homozygous PTEN mutant I253N (T1789A) and tumour 005

expressed oncogenic B-RAF^{V600K} and was heterozygous for the PTEN P213S (C1668T) alteration (Figure 6.4).

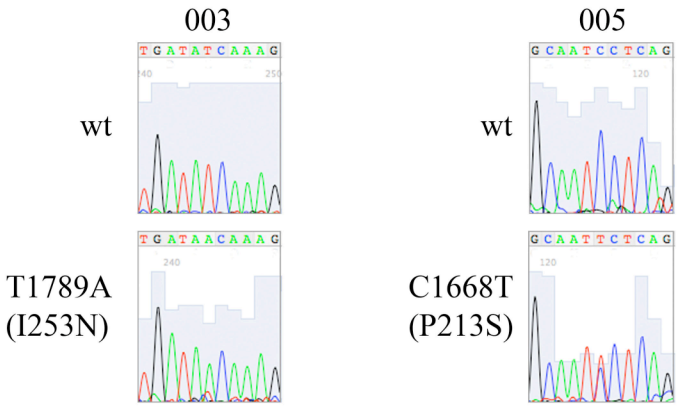


Figure 6.4 Sequence analyses of amplified PTEN transcripts derived from tumour samples 003 and 005

Sequences. The nucleotide and codon changes encoded are shown on the left of each sequence. Numbering is based on accession NM_000314. wt, wild type sequence.

6.3.6 p16^{INK4a} expression induces melanoma cell death

To investigate the impact of p16^{INK4a} expression on melanoma cell survival we utilised a stable p16^{INK4a}-inducible melanoma line. Expression of p16^{INK4a} in this WMM1175_p16^{INK4a} cell clone was induced with 1mM IPTG over a five day period (Figure 6.5A). As shown in Figure 6.5B, induction of p16^{INK4a} led to potent G1 cell cycle arrest and cell death, as determined by the increased sub-G1 population. Accumulation of p16^{INK4a} also sensitised WMM1175_p16^{INK4a} cells to death in response to the cytotoxic drugs melphalan and actinomycin-D. In particular, the sub-G1 population increased from 18% in the presence of p16^{INK4a} alone, to 26% with the addition of actinomycin-D (Figure 6.5C) and 30% in the presence of p16^{INK4a} and melphalan (Figure 6.5D).

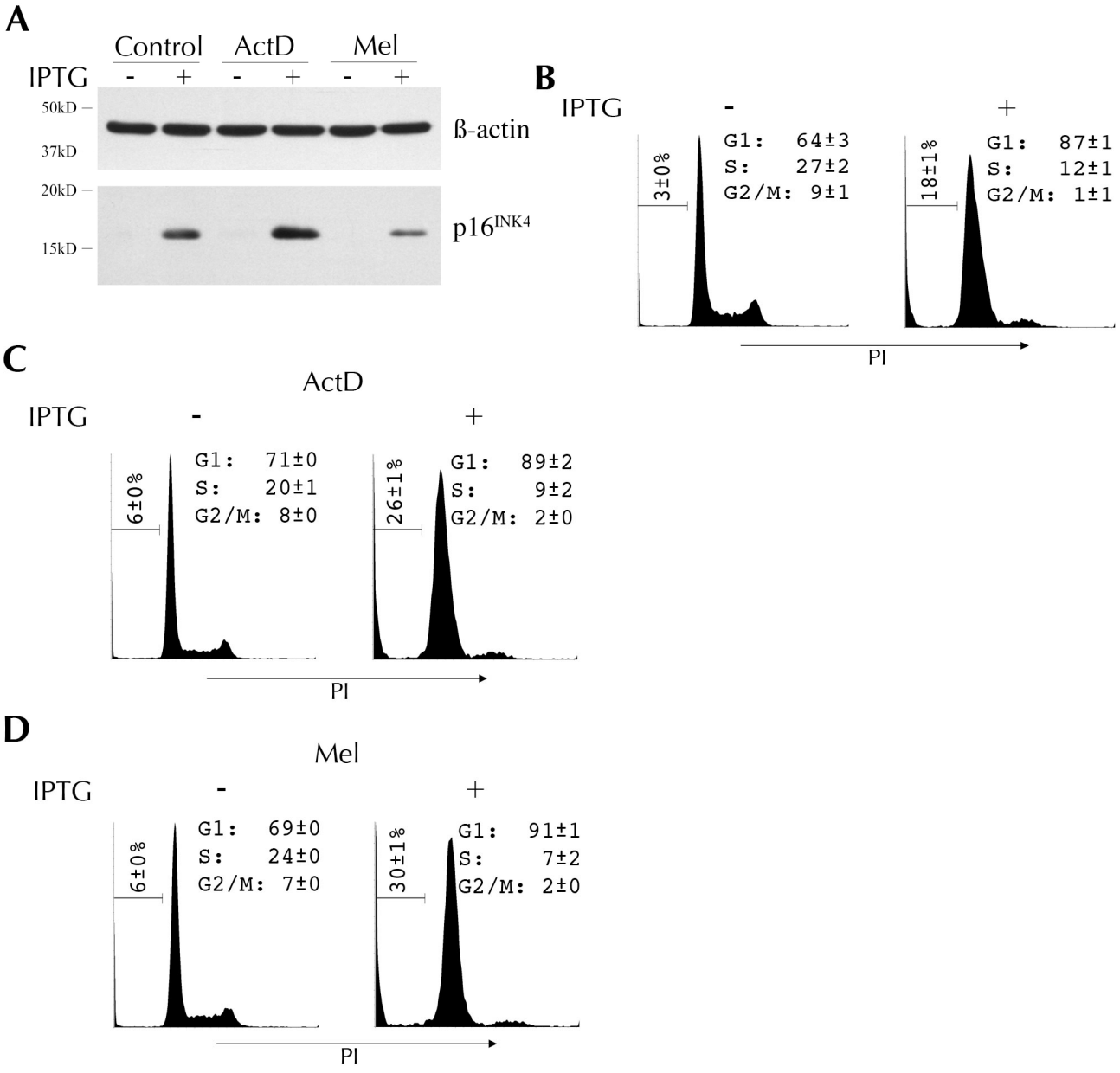


Figure 6.5 Induced expression of p16^{INK4a} promotes arrest, apoptosis and sensitises cells to cell death

(A) WMM1175_p16^{INK4a} melanoma cells were induced to express p16^{INK4a} with the addition of 1mM IPTG to the media for 4 days, then treated with IPTG and actinomycin-D (ActD), melphalan (Mel) or carrier control for 24 hours. Detection of ß-actin was used to demonstrate equal loading.

(B) WMM1175_p16^{INK4a} cells were incubated with media containing 1mM IPTG for 4 days then treated with control (ethanol) and IPTG for 24 hours. The cell cycle distribution of PI stained nuclei was examined by flow cytometry.

(C) 4-days post induction, WMM175_p16^{INK4a} cells were exposed to 25nM actinomycin-D and IPTG for 24h and cell cycle distribution examined.

(D) 4-days post induction, WMM175_p16^{INK4a} cells were exposed to 200µM melphalan and IPTG for 24h and cell cycle distribution examined.

6.4 Discussion

In this study we investigated the *in vivo* response of melanoma metastases to cytotoxic drugs administered by ILI to determine if response correlated with the expression of $p16^{INK4a}$, p14ARF and MITFm and the mutation status of N-RAS, B-RAF and PTEN. These gene products are central to the pathways commonly altered in melanoma progression and thus have potential interaction with mechanisms of cytotoxicity. We have shown that high $p16^{INK4a}$ expression and presence of mutant active N-RAS correlate with chemotherapeutic response of melanoma tumours. The fact that melanomas commonly lack $p16^{INK4a}$ and carry active B-RAF, rather than N-RAS may contribute to chemo-resistance.

The spectrum and high prevalence of B-RAF and N-RAS mutations (23/30) found in our melanoma samples is similar to other studies (Davies *et al.*, 2002; Houben *et al.*, 2004; Kumar *et al.*, 2003a). N-RAS and B-RAF mutations were mutually exclusive, as previously reported (Davies *et al.*, 2002), presumably since both these gene products signal through the MAP kinase pathway. The differential role of B-RAF and N-RAS in predicting melanoma chemosensitivity (this study) and survival (Ugurel *et al.*, 2007) may involve the PI(3)K/AKT pathway. Unlike B-RAF, N-RAS activates the PI(3)K/AKT pathway and several studies have implicated activation of the PI(3)K pathway as another crucial event in the progression of melanoma (Denoyelle *et al.*, 2006). In particular, RAS activation of the PI(3)K/AKT pathway controls the activation of endoplasmic reticulum stress response (Denoyelle *et al.*, 2006), which can activate cytoprotective effects in melanomas (Chen *et al.*, 2007). B-RAF and RAS also differ in their capacity to promote growth arrest in normal human cells (Chudnovsky *et al.*,

2005) and in their ability to cooperate with pRb and p53 loss to produce invasive melanocytic neoplasia (Tsao *et al.*, 2004). Finally, microarray studies show melanomas with an activating N-RAS mutation have different gene expression patterns to those with activating B-RAF mutations (Pavey *et al.*, 2004), and tumours containing N-RAS^{Q61R} are less dependent on MEK then tumours with B-RAF^{V600E} (Solit *et al.*, 2006).

It has been proposed that the combined activation of B-RAF and loss of PTEN simulates oncogenic N-RAS activity to simultaneously activate the MAP kinase and PI(3)K pathways. Accordingly, melanomas rarely express oncogenic N-RAS and altered PTEN (Tsao *et al.*, 2000), whereas they frequently carry oncogenic B-RAF and mutated PTEN (Tsao *et al.*, 2004). In our sample set, only two melanomas carried missense PTEN mutations. These mutations (P213S and I253N) presumably inactivate PTEN as they are located in the PTEN lipid-binding domain and have been identified in a human glioma and endometrial cancer (Mutter *et al.*, 2000; Zhou *et al.*, 1999). The two tumours with PTEN loss also expressed oncogenic forms of B-RAF, but patient response did not relate to PTEN status; 005 showed complete response whereas 003 had stable disease, post therapy. Considering the low frequency of PTEN alterations detected in melanomas, a significantly larger set of melanoma tumour samples needs to be analysed in order to accurately define the contribution of PTEN and the PI(3)K pathway to melanoma chemosensitivity. This is particularly important as the combination of B-RAF inhibitors with PI(3)K inhibitors have been shown co-operate in preventing melanoma cell proliferation and consistently enhanced melanoma chemosensitivity and suppressed invasive tumour growth (Meier *et al.*, 2007).

Our set of melanoma tumour samples displayed heterogeneity in MITF expression with no apparent correlation with chemosensitivity. Inhibition of MITF function in melanoma may trigger CDK2-mediated growth arrest (Du *et al.*, 2004) or apoptosis through bcl-2 downregulation (McGill *et al.*, 2002). Accordingly, MITF loss has been shown to sensitise melanoma cells to the cytotoxic agents, cisplatin and docetaxel (Garraway *et al.*, 2005). Alternatively, increased MITF expression has been shown to induce cell cycle arrest by activating the expression of the CDK inhibitor $p16^{INK4a}$ (Loercher *et al.*, 2005). We observed no association between MITF and $p16^{INK4a}$ expression in our melanoma tumours (data not shown).

MITF activity is also regulated by the MAP kinase pathway. Activation of MAP kinase signalling phosphorylates MITF, which simultaneously increases its transactivation potential and targets it for degradation via the 26S proteasome (McGill *et al.*, 2006; Wellbrock and Marais, 2005). Thus, MITF amplification, which is found in 20-30% of melanomas, is often accompanied by B-RAF mutation and $p16^{INK4a}$ loss (Garraway *et al.*, 2005). However, we did not see any correlation between MITF and B-RAF expression levels in this study (data not shown).

The ectopic expression of p14ARF enhances chemosensitivity in human tumour cell lines (Gallagher *et al.*, 2005) and p14ARF expression has been associated with improved prognosis in several cancers, including acute myeloid leukemias (Kwong *et al.*, 2005; Muller-Tidow *et al.*, 2004). However, no association between p14ARF expression and response was seen in our panel of melanoma tumours. Further, in a recent microarray study of melanoma tumours, p14ARF expression was not associated with survival (Winnepenninckx *et al.*, 2006). Although, p14ARF is frequently lost in

human melanomas, this usually occurs in combination with $p16^{INK4a}$ loss. Moreover, the majority of mutations affecting the *INK4a/ARF* locus target $p16^{INK4a}$ for inactivation (Sherr, 2001), indicating that, in humans, $p16^{INK4a}$ is the critical tumour suppressor encoded by this genomic sequence.

Several studies have shown that $p16^{INK4a}$ loss or deregulation correlates with poor prognosis in human cancers such as melanoma, leukaemia, colon and renal cancers (Mihic-Probst *et al.*, 2006; Ota *et al.*, 2006). While there is no necessary biological connection between poor prognosis and chemoresistance, it was possible that the high frequency of loss of $p16^{INK4A}$ in melanoma was a direct contributor to treatment failure. Consistent with this hypothesis, we found that retention of $p16^{INK4a}$ expression was associated with melanoma chemosensitivity. We also found that accumulation of $p16^{INK4a}$ promoted the death of melanoma cells in culture. More importantly, $p16^{INK4a}$ co-operated with the cytotoxic drugs melphalan and actinomycin-D to enhance melanoma cell death (see Figure 5). The mechanism by which $p16^{INK4a}$ expression promotes cell death in response to drugs requires investigation. This is especially relevant as melanoma cells have usually lost $p16^{INK4a}$ and display an intrinsic resistance to drug-induced cell death.

In summary, this study shows that $p16^{INK4a}$ expression and activation of N-RAS correlate with *in vivo* response of metastatic melanoma exposed to high doses of locally administered cytotoxic drugs. It will be interesting to determine whether these two molecules also influence the response of melanoma metastases exposed to lower doses of chemotherapy given systemically. Although *in vivo* studies on the chemosensitivity of metastatic melanoma are hampered by poor response rates and would require a much

larger sample size they would yield valuable data regarding the mechanisms involved in melanoma chemoresistance This study reinforces the importance of the *INK4a/ARF* locus and highlights the PI(3)k/AKT pathway, in the regulation and execution of apoptosis in melanoma and suggests directions to improve its responsiveness to conventional chemotherapeutic agents.

7. Conclusions

p14ARF is a critical tumour suppressor in melanoma; p14ARF is frequently inactivated in melanoma tumours and germline mutations that specifically target p14ARF confer a high risk of melanoma predisposition. The primary function of p14ARF is to relay oncogenic signals to the p53 cell cycle regulatory pathway and to rapidly induce cell cycle arrest. The strict p53-dependency of p14ARF function is further demonstrated by our work showing that p14ARF can chemosensitise tumour cells in a p53-dependent manner. The ability of p14ARF to enhance chemosensitivity was not evident in our cohort of melanoma patients and p14ARF expression did not predict response to high doses of actinomycin D and melphalan. Although melanomas usually retain functional p53, the downstream effectors may be altered or the induction of ARF expression (eg via abnormal proliferative signals) may be required to activate the p53 pathway.

Accordingly, p14ARF induction enhanced the response of our U2OS cell model to a set of cytotoxic drugs, by activating and possibly altering the transactivational program of p53. The mechanism(s) of ARF action on p53 are not well described and this requires further investigation, as it is clear from our data that simply inhibiting HDM2 to increase p53 levels is not adequate for ARF-mediated chemosensitisation – compare p53 and cell death levels in ARF-expressing cells treated with drugs temozolomide and camptothecin in Figures 4.3 & 4.9 (A, B & C). Thus, it is possible that p14ARF alters the function of the stabilised p53 either by i) directly inducing p53 post-translational modifications, such as sumoylation, ii) interfering with multiple p53 binding partners such as NPM, ARF-BP1 and HDM2 or iii) promoting p53 post-translational

modification indirectly via interaction with proteins such as ATR and hADA3. Elucidation of the mechanism(s) of p14ARF action on p53 would provide valuable information regarding the role of ARF in melanoma development and chemoresistance.

In addition to defining the role of p14ARF, it would be useful to identify the downstream p53 targets that regulate apoptosis in response to p14ARF accumulation. Targeting these genes directly may prove beneficial in treating melanoma, as they would bypass upstream alterations within the ARF-p53 pathway. The identification of genes and proteins with altered expression in the presence of ARF would require a global analytical approach of melanoma cell models and melanoma tumours. A similar approach has already confirmed that over 1000 probe sets were differentially expressed between p14ARF wild type and mutant melanoma cell lines (Packer *et al.*, 2007). There is currently no data available on the importance of any of these differentially expressed genes in melanoma. Similarly, we have no information on the specific role of p14ARF in melanocytes, even though p14ARF inactivation predisposes specifically to this cancer. These are important gaps in our knowledge of ARF and we need to carefully consider the experiments and models required to clearly define how ARF functions to suppress melanoma formation.

8 Appendix 1

This appendix contains author contribution statements regarding work contained on Chapter 6.

Fax to: **Attn: Stuart Gallagher**
Fax: 02 9845 9102

Or Post to: **Stuart Gallagher**
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:
Defined project goals and assisted with methodology. Analysed PTEN sequences. Finalised manuscript text and figures, before submitting manuscript.

regards,

Name: Helen Rizos

Signature: 

Date: 12 March 2008

Fax to: **Attn: Stuart Gallagher**
Fax: 02 9845 9102

Or Post to: **Stuart Gallagher**
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

Clinical data summary

regards,

Name: Dr Bo Gao

Signature: [redacted]

Date: 17/03/2008

Fax to: **Attn: Stuart Gallagher**
Fax: 02 9845 9102

Or Post to: **Stuart Gallagher**
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

I helped Stuart Gallagher to design and optimize the real time PCR analysis for p14ARF and p16INK4a. I also sequenced the PTEN gene in all of the samples and was involved in discussions regarding the work published in this paper.

regards,

Name: Dr Lyndee Scurr

Signature: [redacted]

Date: 12-3-2008

Fax to: **Attn: Stuart Gallagher**
Fax: 02 9845 9102

Or Post to: **Stuart Gallagher**
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

Designed experiment and experimental protocol
Edited paper
Obtained grant funding

regards,

Name: _____

Signature _____

Date: _____

Fax: 02 9845 9102

Or Post to: Stuart Gallagher
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

Assisted in design of experimental and surgical protocol. Performed surgery.

regards,

Name: John F. Thompson

Signature: Not avalable at time of printing

Date:

Fax to: **Attn: Stuart Gallagher**
Fax: 02 9845 9102

Or Post to: **Stuart Gallagher**
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

Advice and training on RNA extraction from solid tumours, measurement of RNA yield and QC for various applications, including microarrays and RT PCR. Primer combinations for detection of mutations in NRAS and BRAF genes were supplied. Advice on the nature and frequency of RAS-RAF mutations in various melanocytic lesions given.

regards,

Name: James Indsto

Signature: 

Date: 25/3/08

Fax: 02 9845 9102

Or Post to: Stuart Gallagher
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

Patient recruitment and assisted in tumour collection.

regards,

Name: Ruth Dunleavey

Signature: Not avalable at time of printing

Date:

Fax to: **Attn: Stuart Gallagher**
Fax: 02 9845 9102

Or Post to: **Stuart Gallagher**
Westmead Millennium Institute
Westmead Institute for Cancer Research.
Darcy Rd, Westmead, 2145. NSW

I consent to Stuart Gallagher using the paper "Activated N-RAS and p16INK4a expression are independent predictors of chemosensitivity in melanoma tumours" in his PhD thesis. To my knowledge, Stuart Gallagher made cDNA from RNA extracted from the tumour samples, performed real time analysis, SSCP, PCR, gene sequencing and analysis (except PTEN) and drafted the paper.

My contribution to the paper was:

Contribution to design of the clinical study, supervision of the tissue collection and preparation of derived specimens, co-supervision of analysis of clinical data, and interpretation of results.

regards,

Name: Assoc Prof Graham Mann

Signature: 

Date: March 27, 2008

9. References

Adams JM, Huang DC, Strasser A, Willis S, Chen L, Wei A *et al* (2005). Subversion of the Bcl-2 life/death switch in cancer development and therapy. *Cold Spring Harb Symp Quant Biol* **70:** 469-77.

AIHW, AACR (2007). *Cancer in Australia: an overview, 2006.* AIHW: Canbera.

Albino AP, Vidal MJ, McNutt NS, Shea CR, Prieto VG, Nanus DM *et al* (1994). Mutation and expression of the p53 gene in human malignant melanoma. *Melanoma Res.* **4:** 35-45.

Allen JD, Zhang XD, Scott CL, Boyle GM, Hersey P, Strasser A (2005). Is Apaf-1 expression frequently abrogated in melanoma? *Cell Death Differ* **12:** 680-1.

Apicelli AJ, Maggi LB, Jr., Hirbe AC, Miceli AP, Olanich ME, Schulte-Winkeler CL *et al* (2008). A non-tumor suppressor role for basal p19ARF in maintaining nucleolar structure and function. *Mol Cell Biol* **28:** 1068-80.

Armstrong BK, Kricker A (1993). How much melanoma is caused by sun exposure? *Melanoma Res* **3:** 395-401.

Armstrong BK, Kricker A (2001). The epidemiology of UV induced skin cancer. *J Photochem Photobiol B* **63:** 8-18.

Ausserlechner MJ, Obexer P, Geley S, Kofler R (2005). G1 arrest by p16INK4A uncouples growth from cell cycle progression in leukemia cells with deregulated cyclin E and c-Myc expression. *Leukemia* **19:** 1051-7.

Avery-Kiejda KA, Zhang XD, Adams LJ, Scott RJ, Vojtesek B, Lane DP *et al* (2008). Small Molecular Weight Variants of p53 Are Expressed in Human Melanoma Cells and Are Induced by the DNA-Damaging Agent Cisplatin. *Clin Cancer Res*.

Avril MF, Aamdal S, Grob JJ, Hauschild A, Mohr P, Bonerandi JJ *et al* (2004). Fotemustine compared with dacarbazine in patients with disseminated malignant melanoma: a phase III study. *J Clin Oncol* **22:** 1118-25.

Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP *et al* (2001). Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. *Oncogene* **20:** 147-55.

Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG *et al* (2001a). Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* **19:** 3635-48.

Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N *et al* (2001b). Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* **19:** 3622-34.

Bandara LR, Girling R, La Thangue NB (1997). Apoptosis induced in mammalian cells by small peptides that functionally antagonize the Rb-regulated E2F transcription factor. *Nat Biotechnol* **15:** 896-901.

Bardeesy N, Morgan J, Sinha M, Signoretti S, Srivastava S, Loda M *et al* (2002). Obligate roles for p16(Ink4a) and p19(Arf)-p53 in the suppression of murine pancreatic neoplasia. *Mol Cell Biol* **22:** 635-43.

Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL *et al* (1998). p14ARF links the tumour suppressors RB and p53. *Nature* **395:** 124-5.

Becker TM, Ayub AL, Kefford RF, Mann GJ, Rizos H (2005). The melanoma-associated 24 base pair duplication in p16INK4a is functionally impaired. *Int J Cancer* **117:** 569-73.

Becker TM, Rizos H, Kefford RF, Mann GJ (2001). Functional impairment of melanoma-associated p16(INK4a) mutants in melanoma cells despite retention of cyclin-dependent kinase 4 binding. *Clin Cancer Res* **7:** 3282-8.

Beddingfield FC, 3rd (2003). The melanoma epidemic: res ipsa loquitur. *Oncologist* **8:** 459-65.

Bedikian AY, Millward M, Pehamberger H, Conry R, Gore M, Trefzer U *et al* (2006). Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. *J Clin Oncol* **24:** 4738-45.

Bell LA, Ryan KM (2003). Life and death decisions by E2F-1. *Cell Death Differ* **11:** 137-142.

Benanti JA, Williams DK, Robinson KL, Ozer HL, Galloway DA (2002). Induction of extracellular matrix-remodeling genes by the senescence-associated protein APA-1. *Mol Cell Biol* **22:** 7385-97.

Berkovich E, Ginsberg D (2003). ATM is a target for positive regulation by E2F-1. *Oncogene* **22:** 161-7.

Berkovich E, Lamed Y, Ginsberg D (2003). E2F and Ras synergize in transcriptionally activating p14ARF expression. *Cell Cycle* **2:** 127-33.

Bernard D, Martinez-Leal JF, Rizzo S, Martinez D, Hudson D, Visakorpi T *et al* (2005). CBX7 controls the growth of normal and tumor-derived prostate cells by repressing the Ink4a/Arf locus. *Oncogene* **24:** 5543-51.

Bertwistle D, Sugimoto M, Sherr CJ (2004). Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. *Mol Cell Biol* **24:** 985-96.

Berwick M, Orlow I, Hummer AJ, Armstrong BK, Kricker A, Marrett LD *et al* (2006). The prevalence of CDKN2A germ-line mutations and relative risk for cutaneous malignant melanoma: an international population-based study. *Cancer Epidemiol Biomarkers Prev* **15:** 1520-5.

Bhalla KN (2005). Epigenetic and chromatin modifiers as targeted therapy of hematologic malignancies. *J Clin Oncol* **23:** 3971-93.

Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B *et al* (2002). Geographical variation in the penetrance of CDKN2A mutations for melanoma. *J Natl Cancer Inst* **94:** 894-903.

Blagosklonny MV, el-Deiry WS (1996). In vitro evaluation of a p53-expressing adenovirus as an anti-cancer drug. *Int J Cancer* **67:** 386-92.

Blagosklonny MV, El-Deiry WS (1998). Acute overexpression of wt p53 facilitates anticancer drug-induced death of cancer and normal cells. *Int J Cancer* **75:** 933-40.

Boddy MN, Freemont PS, Borden KL (1994). The p53-associated protein MDM2 contains a newly characterized zinc-binding domain called the RING finger. *Trends Biochem Sci* **19:** 198-9.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA *et al* (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74:** 597-608.

Bolden JE, Peart MJ, Johnstone RW (2006). Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* **5:** 769-84.

Borg A, Sandberg T, Nilsson K, Johannsson O, Klinker M, Masback A *et al* (2000). High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. *J Natl Cancer Inst* **92:** 1260-6.

Bothner B, Lewis WS, DiGiammarino EL, Weber JD, Bothner SJ, Kriwacki RW (2001). Defining the molecular basis of Arf and Hdm2 interactions. *J Mol Biol* **314:** 263-77.

Bottger A, Bottger V, Garcia-Echeverria C, Chene P, Hochkeppel HK, Sampson W *et al* (1997). Molecular characterization of the hdm2-p53 interaction. *J Mol Biol* **269:** 744-56.

Bouvard V, Zaitchouk T, Vacher M, Duthu A, Canivet M, Choisy-Rossi C *et al* (2000). Tissue and cell-specific expression of the p53-target genes: bax, fas, mdm2 and waf1/p21, before and following ionising irradiation in mice. *Oncogene* **19:** 649-60.

Brady SN, Yu Y, Maggi LB, Jr., Weber JD (2004). ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway. *Mol Cell Biol* **24:** 9327-38.

Brand K, Klocke R, Possling A, Paul D, Strauss M (1999). Induction of apoptosis and G2/M arrest by infection with replication-deficient adenovirus at high multiplicity of infection. *Gene Ther* **6:** 1054-63.

Brookes S, Rowe J, Ruas M, Llanos S, Clark PA, Lomax M *et al* (2002). INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *Embo J* **21:** 2936-45.

Brummelkamp TR, Bernards R, Agami R (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550-3.

Brummelkamp TR, Kortlever RM, Lingbeek M, Trettel F, MacDonald ME, van Lohuizen M *et al* (2001). TBX-3, the gene mutated in Ulnar-Mammary Syndrome, is a negative regulator of p19ARF and inhibits senescence. *J Biol Chem* **8:** 6567-72.

Bullani RR, Huard B, Viard-Leveugle I, Byers HR, Irmler M, Saurat JH *et al* (2001). Selective expression of FLIP in malignant melanocytic skin lesions. *J Invest Dermatol* **117:** 360-4.

Bullani RR, Wehrli P, Viard-Leveugle I, Rimoldi D, Cerottini JC, Saurat JH *et al* (2002). Frequent downregulation of Fas (CD95) expression and function in melanoma. *Melanoma Res* **12:** 263-70.

Burger H, Nooter K, Boersma AW, van Wingerden KE, Looijenga LH, Jochemsen AG *et al* (1999). Distinct p53-independent apoptotic cell death signalling pathways in testicular germ cell tumour cell lines. *Int J Cancer* **81:** 620-8.

Bursch W (2001). The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ* **8:** 569-81.

Burton RC, Armstrong BK (1994). Recent incidence trends imply a nonmetastasizing form of invasive melanoma. *Melanoma Res* **4:** 107-13.

Bush JA, Li G (2003). The role of Bcl-2 family members in the progression of cutaneous melanoma. *Clin Exp Metastasis* **20:** 531-9.

Calabro V, Mansueto G, Santoro R, Gentilella A, Pollice A, Ghioni P *et al* (2004). Inhibition of p63 transcriptional activity by p14ARF: functional and physical link between human ARF tumor suppressor and a member of the p53 family. *Mol Cell Biol* **24:** 8529-40.

Castedo M, Coquelle A, Vivet S, Vitale I, Kauffmann A, Dessen P *et al* (2006). Apoptosis regulation in tetraploid cancer cells. *Embo J* **25:** 2584-95.

Chakraborty AK, Funasaka Y, Slominski A, Ermak G, Hwang J, Pawelek JM *et al* (1996). Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B. *Biochim Biophys Acta* **1313:** 130-8.

Chapman PB, Einhorn LH, Meyers ML, Saxman S, Destro AN, Panageas KS *et al* (1999). Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. *J Clin Oncol* **17:** 2745-51.

Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ, Borden EC (2004). Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. *Cell Death Differ* **11:** 915-23.

Chen D, Kon N, Li M, Zhang W, Qin J, Gu W (2005a). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell* **121:** 1071-83.

Chen L, Chen J (2003). MDM2-ARF complex regulates p53 sumoylation. *Oncogene* **22:** 5348-57.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG *et al* (2005b). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17:** 393-403.

Chen LH, Jiang CC, Kiejda KA, Wang YF, Thorne R, Zhang XD *et al* (2007). Thapsigargin Sensitizes Human Melanoma Cells to TRAIL-Induced Apoptosis by Up-Regulation of TRAIL-R2 through the Unfolded Protein Response. *Carcinogenesis*.

Chen X, Ko LJ, Jayaraman L, Prives C (1996). p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev* **10:** 2438-51.

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T *et al* (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* **8:** 705-11.

Chin L, Pomerantz J, Polsky D, Jacobson M, Cohen C, Cordon-Cardo C *et al* (1997). Cooperative effects of INK4a and ras in melanoma susceptibility in vivo. *Genes Dev* **11:** 2822-34.

Chipuk JE, Green DR (2006). Dissecting p53-dependent apoptosis. *Cell Death Differ* **13:** 994-1002.

Chodosh LA, Fire A, Samuels M, Sharp PA (1989). 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. *J Biol Chem* **264:** 2250-7.

Choi KH, Hama-Inaba H, Wang B, Haginoya K, Odaka T, Yamada T *et al* (2000). UVC-induced apoptosis in human epithelial tumor A431 cells: sequence of apoptotic changes and involvement of caspase (-8 and -3) cascade. *J Radiat Res (Tokyo)* **41:** 243-58.

Christensen MO, Krokowski RM, Barthelmes HU, Hock R, Boege F, Mielke C (2004). Distinct effects of topoisomerase I and RNA polymerase I inhibitors suggest a dual

mechanism of nucleolar/nucleoplasmic partitioning of topoisomerase I. *J Biol Chem* **279:** 21873-82.

Chua BT, Guo K, Li P (2000). Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *J Biol Chem* **275:** 5131-5.

Chudnovsky Y, Adams AE, Robbins PB, Lin Q, Khavari PA (2005). Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. *Nat Genet* **37:** 745-9.

Chwieralski CE, Welte T, Buhling F (2006). Cathepsin-regulated apoptosis. *Apoptosis* **11:** 143-9.

Cirman T, Oresic K, Mazovec GD, Turk V, Reed JC, Myers RM *et al* (2004). Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *J Biol Chem* **279:** 3578-87.

Clark PA, Llanos S, Peters G (2002). Multiple interacting domains contribute to p14ARF mediated inhibition of MDM2. *Oncogene* **21:** 4498-507.

Clarke PG (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol (Berl)* **181:** 195-213.

Clere N, Bermont L, Fauconnet S, Lascombe I, Saunier M, Vettoretti L *et al* (2007). The human papillomavirus type 18 E6 oncoprotein induces Vascular Endothelial Growth Factor 121 (VEGF121) transcription from the promoter through a p53-independent mechanism. *Exp Cell Res* **313:** 3239-50.

Coates AS, Segelov E (1994). Long term response to chemotherapy in patients with visceral metastatic melanoma. *Ann Oncol* **5:** 249-51.

Colombo E, Bonetti P, Lazzerini Denchi E, Martinelli P, Zamponi R, Marine JC *et al* (2005). Nucleophosmin is required for DNA integrity and p19Arf protein stability. *Mol Cell Biol* **25:** 8874-86.

Colombo E, Martinelli P, Zamponi R, Shing DC, Bonetti P, Luzi L *et al* (2006). Delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant. *Cancer Res* **66:** 3044-50.

Coory M, Baade P, Aitken J, Smithers M, McLeod GR, Ring I (2006). Trends for in situ and invasive melanoma in Queensland, Australia, 1982-2002. *Cancer Causes Control* **17:** 21-7.

Creagan ET, Suman VJ, Dalton RJ, Pitot HC, Long HJ, Veeder MH *et al* (1999). Phase III clinical trial of the combination of cisplatin, dacarbazine, and carmustine with or without tamoxifen in patients with advanced malignant melanoma. *J Clin Oncol* **17:** 1884-90.

Croxton R, Ma Y, Song L, Haura EB, Cress WD (2002). Direct repression of the Mcl-1 promoter by E2F1. *Oncogene* **21:** 1359-69.

Curtin JA, Busam K, Pinkel D, Bastian BC (2006). Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* **24:** 4340-6.

Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H *et al* (2005). Distinct sets of genetic alterations in melanoma. *N Engl J Med* **353:** 2135-47.

Dai DL, Martinka M, Bush JA, Li G (2004). Reduced Apaf-1 expression in human cutaneous melanomas. *Br J Cancer* **91:** 1089-95.

Damalas A, Kahan S, Shtutman M, Ben-Ze'ev A, Oren M (2001). Deregulated ß-catenin induces a p53- and ARF-dependent growth arrest and cooperates with Ras in transformation. *EMBO J* **20:** 4912-4922.

Daniotti M, Oggionni M, Ranzani T, Vallacchi V, Campi V, Di Stasi D *et al* (2004). BRAF alterations are associated with complex mutational profiles in malignant melanoma. *Oncogene* **23:** 5968-77.

Das S, Boswell SA, Aaronson SA, Lee SW (2008). P53 promoter selection: choosing between life and death. *Cell Cycle* **7:** 154-7.

Das S, Raj L, Zhao B, Kimura Y, Bernstein A, Aaronson SA *et al* (2007). Hzf Determines cell survival upon genotoxic stress by modulating p53 transactivation. *Cell* **130:** 624-37.

Datta A, Nag A, Raychaudhuri P (2002). Differential regulation of E2F1, DP1 and the E2F1/DP1 complex by ARF. *Mol Cell Biol* **22:** 8398-8408.

Datta A, Sen J, Hagen J, Korgaonkar CK, Caffrey M, Quelle DE *et al* (2005). ARF directly binds DP1: interaction with DP1 coincides with the G1 arrest function of ARF. *Mol Cell Biol* **25:** 8024-36.

Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N *et al* (2000). Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett* **476:** 118-23.

David-Pfeuty T, Nouvian-Dooghe Y, Sirri V, Roussel P, Hernandez-Verdun D (2001). Common and reversible regulation of wild-type p53 function and of ribosomal biogenesis by protein kinases in human cells. *Oncogene* **20:** 5951-63.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S *et al* (2002). Mutations of the BRAF gene in human cancer. *Nature* **417:** 949-54.

de Stanchina E, McCurrach ME, Zindy F, Shieh SY, Ferbeyre G, Samuelson AV *et al* (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev* **12:** 2434-42.

Deng X, Kim M, Vandier D, Jung YJ, Rikiyama T, Sgagias MK *et al* (2002). Recombinant adenovirus-mediated p14(ARF) overexpression sensitizes human breast cancer cells to cisplatin. *Biochem Biophys Res Commun* **296:** 792-8.

Denoyelle C, Abou-Rjaily G, Bezrookove V, Verhaegen M, Johnson TM, Fullen DR *et al* (2006). Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. *Nat Cell Biol* **8:** 1053-63.

Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *Embo J* **18:** 5242-51.

Deveraux QL, Reed JC (1999). IAP family proteins--suppressors of apoptosis. *Genes Dev* **13:** 239-52.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997). X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388:** 300-4.

DiGiammarino EL, Filippov I, Weber JD, Bothner B, Kriwacki RW (2001). Solution structure of the p53 regulatory domain of the p19Arf tumor suppressor protein. *Biochemistry* **40:** 2379-86.

Divito KA, Berger AJ, Camp RL, Dolled-Filhart M, Rimm DL, Kluger HM (2004). Automated quantitative analysis of tissue microarrays reveals an association between high Bcl-2 expression and improved outcome in melanoma. *Cancer Res* **64:** 8773-7.

Dobrowolski R, Hein R, Buettner R, Bosserhoff AK (2002). Loss of p14ARF expression in melanoma. *Arch Dermatol Res* **293:** 545-51.

Draus JM, Elliott MJ, Atienza C, Jr., Stilwell A, Wong SL, Dong Y *et al* (2001). p53 gene transfer does not enhance E2F-1-mediated apoptosis in human colon cancer cells. *Exp Mol Med* **33:** 209-19.

Drayton S, Rowe J, Jones R, Vatcheva R, Cuthbert-Heavens D, Marshall J *et al* (2003). Tumor suppressor p16INK4a determines sensitivity of human cells to transformation by cooperating cellular oncogenes. *Cancer Cell* **4:** 301-10.

Du J, Widlund HR, Horstmann MA, Ramaswamy S, Ross K, Huber WE *et al* (2004). Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. *Cancer Cell* **6:** 565-76.

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D *et al* (1998). A third generation lentivirus vector with a conditional packaging system. *Journal of Virology* **72:** 8463-8471.

Duro D, Bernard O, Della Valle V, Berger R, Larsen CJ (1995). A new type of p16INK4/MTS1 gene transcript expressed in B-cell malignancies. *Oncogene* **11:** 21-9.

Dussmann H, Kogel D, Rehm M, Prehn JH (2003). Mitochondrial membrane permeabilization and superoxide production during apoptosis. A single-cell analysis. *J Biol Chem* **278:** 12645-9.

Eggermont AM, Kirkwood JM (2004). Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years? *Eur J Cancer* **40:** 1825-36.

Eischen CM, Rehg JE, Korsmeyer SJ, Cleveland JL (2002). Loss of Bax alters tumor spectrum and tumor numbers in ARF-deficient mice. *Cancer Res* **62:** 2184-91.

Elliott MJ, Dong YB, Yang H, McMasters KM (2001). E2F-1 up-regulates c-Myc and p14(ARF) and induces apoptosis in colon cancer cells. *Clin Cancer Res* **7:** 3590-7.

Emanuel PO, Phelps RG, Mudgil A, Shafir M, Burstein DE (2008). Immunohistochemical detection of XIAP in melanoma. *J Cutan Pathol* **35:** 292-7.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391:** 43-50.

Eymin B, Claverie P, Salon C, Brambilla C, Brambilla E, Gazzeri S (2006a). p14(ARF) Triggers G(2) Arrest Through ERK-Mediated Cdc25C Phosphorylation, Ubiquitination and Proteasomal Degradation. *Cell Cycle* **5**.

Eymin B, Claverie P, Salon C, Leduc C, Col E, Brambilla E *et al* (2006b). p14ARF activates a Tip60-dependent and p53-independent ATM/ATR/CHK pathway in response to genotoxic stress. *Mol Cell Biol* **26:** 4339-50.

Eymin B, Karayan L, Seite P, Brambilla C, Brambilla E, Larsen CJ *et al* (2001). Human ARF binds E2F1 and inhibits its transcriptional activity. *Oncogene* **20:** 1033-41.

Eymin B, Leduc C, Coll JL, Brambilla E, Gazzeri S (2003). p14ARF induces G2 arrest and apoptosis independently of p53 leading to regression of tumours established in nude mice. *Oncogene* **22:** 1822-35.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* **148:** 2207-16.

Fakharzadeh SS, Trusko SP, George DL (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *Embo J* **10:** 1565-9.

Fan S, el-Deiry WS, Bae I, Freeman J, Jondle D, Bhatia K *et al* (1994). p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res* **54:** 5824-30.

Fargnoli MC, Chimenti S, Keller G, Soyer HP, Dal Pozzo V, Höfler H *et al* (1998). CDKN2a/p16^{INK4a} mutations and lack of p19ARF involvement in familial melanoma kindreds. *J Invest. Dermatol.* **111:** 1202-1206.

Farrow SN, White JH, Martinou I, Raven T, Pun KT, Grinham CJ *et al* (1995). Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature* **374:** 731-3.

Fatyol K, Szalay AA (2001). The p14ARF tumor suppressor protein facilitates nucleolar sequestration of hypoxia-inducible factor-1alpha (HIF-1alpha) and inhibits HIF-1-mediated transcription. *J Biol Chem* **276:** 28421-9.

Fecker LF, Geilen CC, Tchernev G, Trefzer U, Assaf C, Kurbanov BM *et al* (2006). Loss of proapoptotic Bcl-2-related multidomain proteins in primary melanomas is associated with poor prognosis. *J Invest Dermatol* **126:** 1366-71.

Fletcher JI, Huang DC (2008). Controlling the cell death mediators Bax and Bak: puzzles and conundrums. *Cell Cycle* **7:** 39-44.

Florenes VA, Oyjord T, Holm R, Skrede M, Borresen AL, Nesland JM *et al* (1994). TP53 allele loss, mutations and expression in malignant melanoma. *Br J Cancer* **69:** 253-9.

Florenes VA, Skrede M, Jorgensen K, Nesland JM (2004). Deacetylase inhibition in malignant melanomas: impact on cell cycle regulation and survival. *Melanoma Res* **14:** 173-81.

Fornari FA, Randolph JK, Yalowich JC, Ritke MK, Gewirtz DA (1994). Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells. *Mol Pharmacol* **45:** 649-56.

Fridman JS, Lowe SW (2003). Control of apoptosis by p53. *Oncogene* **22:** 9030-40.

Fulci G, al e (2000). p53 gene mutation and INK4a-ARF deletion appear to be two mutually exclusive events in human glioblastoma. *Oncogene* **19:** 3816-3822.

Gallagher S, Kefford RF, Rizos H (2005). Enforced expression of p14ARF induces p53-dependent cell cycle arrest but not apoptosis. *Cell Cycle* **4:** 465-72.

Gao G, Dou QP (2000). N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem* **80:** 53-72.

Gao N, Hu YD, Cao XY, Zhou J, Cao SL (2001). The exogenous wild-type p14ARF gene induces growth arrest and promotes hypersensitivity in human lung cancer cell lines. *J Cancer Res Clin Oncol* **127:** 359-367.

Garnett TO, Filippova M, Duerksen-Hughes PJ (2007). Bid is cleaved upstream of caspase-8 activation during TRAIL-mediated apoptosis in human osteosarcoma cells. *Apoptosis* **12:** 1299-315.

Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S *et al* (2005). Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* **436:** 117-22.

Gibson L, Holmgreen SP, Huang DC, Bernard O, Copeland NG, Jenkins NA *et al* (1996). bcl-w, a novel member of the bcl-2 family, promotes cell survival. *Oncogene* **13:** 665-75.

Gil J, Bernard D, Martinez D, Beach D (2004). Polycomb CBX7 has a unifying role in cellular lifespan. *Nat Cell Biol* **6:** 67-72.

Glinsky GV, Glinsky VV, Ivanova AB, Hueser CJ (1997). Apoptosis and metastasis: increased apoptosis resistance of metastatic cancer cells is associated with the profound deficiency of apoptosis execution mechanisms. *Cancer Lett* **115:** 185-93.

Goldstein AM, Chan M, Harland M, Gillanders EM, Hayward NK, Avril MF *et al* (2006). High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer Res* **66:** 9818-28.

Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT *et al* (2007). Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet* **44:** 99-106.

Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR (2000). The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol* **2:** 156-62.

Goll DE, Thompson VF, Li H, Wei W, Cong J (2003). The calpain system. *Physiol Rev* **83:** 731-801.

Gorden A, Osman I, Gai W, He D, Huang W, Davidson A *et al* (2003). Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. *Cancer Res* **63:** 3955-7.

Gozuacik D, Kimchi A (2007). Autophagy and cell death. *Curr Top Dev Biol* **78:** 217-45.

Gradilone A, Gazzaniga P, Ribuffo D, Scarpa S, Cigna E, Vasaturo F *et al* (2003). Survivin, bcl-2, bax, and bcl-X gene expression in sentinel lymph nodes from melanoma patients. *J Clin Oncol* **21:** 306-12.

Greene RF, Collins JM, Jenkins JF, Speyer JL, Myers CE (1983). Plasma pharmacokinetics of adriamycin and adriamycinol: implications for the design of in vitro experiments and treatment protocols. *Cancer Res* **43:** 3417-21.

Grossman D, McNiff JM, Li F, Altieri DC (1999). Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. *J Invest Dermatol* **113:** 1076-81.

Grossman SR (2001). p300/CBP/p53 interaction and regulation of the p53 response. *Eur J Biochem* **268:** 2773-8.

Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, Tagami H *et al* (2003). Polyubiquitination of p53 by a Ubiquitin Ligase Activity of p300. *Science* **300:** 342-4.

Guo-Chang F, Chu-Tse W (2000). Transfer of p14ARF gene in drug-resistant human breast cancer MCF-7/Adr cells inhibits proliferation and reduces doxorubicin resistance. *Cancer Lett* **158:** 203-10.

Gwosdz C, Scheckenbach K, Lieven O, Reifenberger J, Knopf A, Bier H *et al* (2006). Comprehensive analysis of the p53 status in mucosal and cutaneous melanomas. *Int J Cancer* **118:** 577-82.

Haaf T, Ward DC (1996). Inhibition of RNA polymerase II transcription causes chromatin decondensation, loss of nucleolar structure, and dispersion of chromosomal domains. *Exp Cell Res* **224:** 163-73.

Haber DA (1997). Splicing into senescence: the curious case of p16 and p19ARF. *Cell* **91:** 555-8.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell* **100:** 57-70.

Harland M, Taylor CF, Chambers PA, Kukalizch K, Randerson-Moor JA, Gruis NA *et al* (2005). A mutation hotspot at the p14ARF splice site. *Oncogene* **24:** 4604-4608.

Harris SL, Levine AJ (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* **24:** 2899-908.

Hasan MK, Yaguchi T, Sugihara T, Kumar PK, Taira K, Reddel RR *et al* (2002). CARF is a novel protein that cooperates with mouse p19ARF (human p14ARF) in activating p53. *J Biol Chem* **277:** 37765-70.

Haupt Y, Maya R, Kaza A, Oren M (1997). Mdm2 promotes the rapid degradation of p53. *Nature* **387:** 296-299.

Heere-Ress E, Thallinger C, Lucas T, Schlagbauer-Wadl H, Wacheck V, Monia BP *et al* (2002). Bcl-X(L) is a chemoresistance factor in human melanoma cells that can be inhibited by antisense therapy. *Int J Cancer* **99:** 29-34.

Heiskanen KM, Bhat MB, Wang HW, Ma J, Nieminen AL (1999). Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells. *J Biol Chem* **274:** 5654-8.

Hemmati PG, Gillissen B, von Haefen C, Wendt J, Starck L, Guner D *et al* (2002). Adenovirus-mediated overexpression of p14(ARF) induces p53 and Bax-independent apoptosis. *Oncogene* **21:** 3149-61.

Hemmati PG, Guner D, Gillissen B, Wendt J, von Haefen C, Chinnadurai G *et al* (2006). Bak functionally complements for loss of Bax during p14ARF-induced mitochondrial apoptosis in human cancer cells. *Oncogene* **25:** 6582-94.

Hemmati PG, Normand G, Verdoodt B, von Haefen C, Hasenjager A, Guner D *et al* (2005). Loss of p21 disrupts p14 ARF-induced G1 cell cycle arrest but augments p14 ARF-induced apoptosis in human carcinoma cells. *Oncogene* **24:** 4114-28.

Henderson C, Mizzau M, Paroni G, Maestro R, Schneider C, Brancolini C (2003). Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). *J Biol Chem* **278:** 12579-89.

Hershko T, Chaussepied M, Oren M, Ginsberg D (2005). Novel link between E2F and p53: proapoptotic cofactors of p53 are transcriptionally upregulated by E2F. *Cell Death Differ* **12:** 377-83.

Hewitt C, Lee Wu C, Evans G, Howell A, Elles RG, Jordan R *et al* (2002). Germline mutation of ARF in a melanoma kindred. *Hum Mol Genet* **11:** 1273-9.

Ho J, Benchimol S (2003). Transcriptional repression mediated by the p53 tumour suppressor. *Cell Death Differ* **10:** 404-8.

Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M (2002). Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* **277:** 3247-57.

Holland EA, Schmid H, Kefford RF, Mann GJ (1999). *CDKN2A* ($p16^{INK4a}$) and *CDK4* mutation analysis in 131 Australian melanoma probands: Effect of family history and multiple primary melanomas. *Genes Chromosomes Cancer* **25:** 339-348.

Honda R, Tanaka H, Yasuda H (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420:** 25-7.

Houben R, Becker JC, Kappel A, Terheyden P, Brocker EB, Goetz R *et al* (2004). Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. *J Carcinog* **3:** 6.

Howe HL, Wingo PA, Thun MJ, Ries LA, Rosenberg HM, Feigal EG *et al* (2001). Annual report to the nation on the status of cancer (1973 through 1998), featuring cancers with recent increasing trends. *J Natl Cancer Inst* **93:** 824-42.

Huang Y, Tyler T, Saadatmandi N, Lee C, Borgstrom P, Gjerset RA (2003). Enhanced Tumor Suppression by a p14ARF/p53 Bicistronic Adenovirus through Increased p53 Protein Translation and Stability. *Cancer Res* **63:** 3646-53.

Indsto JO, Kumar S, Wang L, Crotty KA, Arbuckle SM, Mann GJ (2007). Low prevalence of RAS-RAF-activating mutations in Spitz melanocytic nevi compared with other melanocytic lesions. *J Cutan Pathol* **34:** 448-55.

Inoue K, Roussel MF, Sherr CJ (1999). Induction of ARF tumor suppressor gene expression and cell cycle arrest by transcription factor DMP1. *Proc Natl Acad Sci U S A* **96:** 3993-8.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V *et al* (1997). Inhibition of death receptor signals by cellular FLIP. *Nature* **388:** 190-5.

Itahana K, Bhat KP, Jin A, Itahana Y, Hawke D, Kobayashi R *et al* (2003). Tumor suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation. *Mol Cell* **12:** 1151-64.

Ito A, Lai C-H, Zhao X, Saito S, Hamilton MH, Apella E *et al* (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by mdm2. *EMBO J* **20:** 1331-1340.

Jackson MW, Agarwal MK, Agarwal ML, Agarwal A, Stanhope-Baker P, Williams BR *et al* (2004). Limited role of N-terminal phosphoserine residues in the activation of transcription by p53. *Oncogene* **23:** 4477-87.

Jackson MW, Lindström MS, Berberich SJ (2001). MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. *Oncogene* **276:** 25336-25341.

Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* **397:** 164-8.

Jacobs JL, Keblusek P, Robanus-Maandag E, Kristel P, Lingbeek M, Nederlof PM *et al* (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19ARF) and is amplified in a subset of human breast cancers. *Nature Genetics* **26:** 291-299.

Janku F, Novotny J, Julis I, Julisova I, Pecen L, Tomancova V *et al* (2005). KIT receptor is expressed in more than 50% of early-stage malignant melanoma: a retrospective study of 261 patients. *Melanoma Res* **15:** 251-6.

Jemal A, Clegg LX, Ward E, Ries LA, Wu X, Jamison PM *et al* (2004). Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival. *Cancer* **101:** 3-27.

Jemal A, Devesa SS, Hartge P, Tucker MA (2001). Recent trends in cutaneous melanoma incidence among whites in the United States. *J Natl Cancer Inst* **93:** 678-83.

Jhappan C, Noonan FP, Merlino G (2003). Ultraviolet radiation and cutaneous malignant melanoma. *Oncogene* **22:** 3099-112.

Jin Y, Zeng SX, Dai MS, Yang XJ, Lu H (2002). MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation. *J Biol Chem* **277:** 30838-43.

Jin Y, Zeng SX, Lee H, Lu H (2004). MDM2 mediates p300/CREB-binding protein-associated factor ubiquitination and degradation. *J Biol Chem* **279:** 20035-43.

Johnstone RW (2002). Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov* **1:** 287-99.

Jorquera R, Tanguay RM (1999). Cyclin B-dependent kinase and caspase-1 activation precedes mitochondrial dysfunction in fumarylacetoacetate-induced apoptosis. *Faseb J* **13:** 2284-98.

Jungnelius U, Ringborg U, Aamdal S, Mattsson J, Stierner U, Ingvar C *et al* (1998). Dacarbazine-vindesine versus dacarbazine-vindesine-cisplatin in disseminated malignant melanoma. A randomised phase III trial. *Eur J Cancer* **34:** 1368-74.

Kaeser MD, Iggo RD (2002). Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. *Proc Natl Acad Sci U S A* **99:** 95-100.

Kaleem Z, Lind AC, Humphrey PA, Sueper RH, Swanson PE, Ritter JH *et al* (2000). Concurrent Ki-67 and p53 immunolabeling in cutaneous melanocytic neoplasms: an adjunct for recognition of the vertical growth phase in malignant melanomas? *Mod Pathol* **13:** 217-22.

Kalinichenko VV, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM *et al* (2004). Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. *Genes Dev* **18:** 830-50.

Kamijo T, Bodner S, van de Kamp E, Randle DH, Sherr CJ (1999). Tumor spectrum in ARF-deficient mice. *Cancer Res* **59:** 2217-22.

Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF, Sherr CJ (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc Natl Acad Sci U S A* **95:** 8292-7.

Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA *et al* (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91:** 649-59.

Kang MH, Kang YH, Szymanska B, Wilczynska-Kalak U, Sheard MA, Harned TM *et al* (2007). Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 in vitro and in vivo. *Blood* **110:** 2057-66.

Karayan L, Riou J-F, Séité P, Migeon J, Cantereau A, Larson C-J (2001). Human ARF protein interacts with Topoisomerase I and stimulates its activity. *Oncogene* **20:** 836-848.

Karst AM, Dai DL, Martinka M, Li G (2005). PUMA expression is significantly reduced in human cutaneous melanomas. *Oncogene* **24:** 1111-6.

Kerr JF, Wyllie AH, Currie AR (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:** 239-57.

Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N *et al* (2008). Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem J* **409:** 581-9.

Kichina JV, Rauth S, Das Gupta TK, Gudkov AV (2003). Melanoma cells can tolerate high levels of transcriptionally active endogenous p53 but are sensitive to retrovirus-transduced p53. *Oncogene* **22:** 4911-7.

Kim M, Sgagias M, Deng X, Jung YJ, Rikiyama T, Lee K *et al* (2004). Apoptosis induced by adenovirus-mediated p14(ARF) expression in U2OS osteosarcoma cells is associated with increased Fas expression. *Biochem Biophys Res Commun* **320:** 138-44.

Kim SH, Mitchell M, Fujii H, Llanos S, Peters G (2003). Absence of p16INK4a and truncation of ARF tumor suppressors in chickens. *Proc Natl Acad Sci U S A* **100:** 211-6.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH *et al* (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14:** 5579-88.

Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P *et al* (2001). Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem* **276:** 46639-46.

Korgaonkar C, Hagen J, Tompkins V, Frazier AA, Allamargot C, Quelle FW *et al* (2005). Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function. *Mol Cell Biol* **25:** 1258-71.

Korgaonkar C, Zhao L, Modestou M, Quelle DE (2002). ARF function does not require p53 stabilization or Mdm2 relocalization. *Mol Cell Biol* **22:** 196-206.

Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE, Oltvai ZN (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Semin Cancer Biol* **4:** 327-32.

Koyanagi K, O'Day SJ, Gonzalez R, Lewis K, Robinson WA, Amatruda TT *et al* (2006). Microphthalmia transcription factor as a molecular marker for circulating tumor cell detection in blood of melanoma patients. *Clin Cancer Res* **12:** 1137-43.

Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci U S A* **90:** 3516-20.

Kreuz S, Siegmund D, Scheurich P, Wajant H (2001). NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol Cell Biol* **21:** 3964-73.

Kricker A, Armstrong BK, Goumas C, Litchfield M, Begg CB, Hummer AJ *et al* (2007). Ambient UV, personal sun exposure and risk of multiple primary melanomas. *Cancer Causes Control* **18:** 295-304.

Krimpenfort P, Quon KC, Mooi WJ, Loonstra A, Berns A (2001). Loss of p16INK4a confers susceptibility to metastatic melanoma in mice. *Nature* **413:** 83-86.

Krishan A, Dutt K, Israel M, Ganapathi R (1981). Comparative effects of adriamycin and N-trifluoroacetyladriamycin-14-valerate on cell kinetics, chromosomal damage, and macromolecular synthesis in vitro. *Cancer Res* **41:** 2745-50.

Kumar R, Angelini S, Czene K, Sauroja I, Hahka-Kemppinen M, Pyrhonen S *et al* (2003a). BRAF mutations in metastatic melanoma: a possible association with clinical outcome. *Clin Cancer Res* **9:** 3362-8.

Kumar R, Angelini S, Hemminki K (2003b). Activating BRAF and N-Ras mutations in sporadic primary melanomas: an inverse association with allelic loss on chromosome 9. *Oncogene* **22:** 9217-24.

Kumar R, Angelini S, Snellman E, Hemminki K (2004). BRAF mutations are common somatic events in melanocytic nevi. *J Invest Dermatol* **122:** 342-8.

Kuo ML, den Besten W, Bertwistle D, Roussel MF, Sherr CJ (2004). N-terminal polyubiquitination and degradation of the Arf tumor suppressor. *Genes Dev* **18:** 1862-74.

Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ *et al* (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274:** 948-53.

Kwek SS, Derry J, Tyner AL, Shen Z, Gudkov AV (2001). Functional analysis and intracellular localization of p53 modified by SUMO-1. *Oncogene* **20:** 2587-99.

Kwong RA, Kalish LH, Nguyen TV, Kench JG, Bova RJ, Cole IE *et al* (2005). p14ARF protein expression is a predictor of both relapse and survival in squamous cell carcinoma of the anterior tongue. *Clin Cancer Res* **11:** 4107-16.

Lai Z, Ferry KV, Diamond MA, Wee KE, Kim YB, Ma J *et al* (2001). Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization. *J Biol Chem* **276:** 31357-67.

Lalier L, Cartron PF, Juin P, Nedelkina S, Manon S, Bechinger B *et al* (2007). Bax activation and mitochondrial insertion during apoptosis. *Apoptosis* **12:** 887-96.

Lane DP (1992). Cancer. p53, guardian of the genome. *Nature* **358:** 15-6.

Lavin MF, Gueven N (2006). The complexity of p53 stabilization and activation. *Cell Death Differ* **13:** 941-50.

Lee C, Smith BA, Bandyopadhyay K, Gjerset RA (2005). DNA damage disrupts the p14ARF-B23(nucleophosmin) interaction and triggers a transient subnuclear redistribution of p14ARF. *Cancer Res* **65:** 9834-42.

Leiter U, Schmid RM, Kaskel P, Peter RU, Krahn G (2000). Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. *Arch Dermatol Res* **292:** 225-32.

Lekmine F, Chang CK, Sethakorn N, Das Gupta TK, Salti GI (2007). Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. *Biochem Biophys Res Commun* **354:** 830-5.

Lens MB, Dawes M (2004). Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. *Br J Dermatol* **150:** 179-85.

Levine AJ (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88:** 323-31.

Li G, Bush JA, Ho VC (2000). p53-dependent apoptosis in melanoma cells after treatment with camptothecin. *J Invest Dermatol* **114:** 514-9.

Li H, Zhu H, Xu CJ, Yuan J (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94:** 491-501.

Li LY, Luo X, Wang X (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412:** 95-9.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES *et al* (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91:** 479-89.

Li Y, He L, Bruce A, Parihar K, Ingram A, Liu L *et al* (2006). p14ARF inhibits the growth of p53 deficient cells in a cell-specific manner. *Biochim Biophys Acta* **1763:** 787-96.

Lin EY, Orlofsky A, Berger MS, Prystowsky MB (1993). Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J Immunol* **151:** 1979-88.

Lindemann RK, Gabrielli B, Johnstone RW (2004). Histone-deacetylase inhibitors for the treatment of cancer. *Cell Cycle* **3:** 779-88.

Lindstrom MS, Klangby U, Inoue R, Pisa P, Wiman KG, Asker CE (2000). Immunolocalization of human p14(ARF) to the granular component of the interphase nucleolus. *Exp Cell Res* **256:** 400-10.

Lingbeek ME, Jacobs JJ, van Lohuizen M (2002). The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene p14ARF via a variant T-site in the initiator. *J Biol Chem* **277:** 26120-7.

Linggi B, Muller-Tidow C, van de Locht L, Hu M, Nip J, Serve H *et al* (2002). The t(8;21) fusion protein, AML1 ETO, specifically represses the transcription of the p14(ARF) tumor suppressor in acute myeloid leukemia. *Nat Med* **8:** 743-50.

Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G *et al* (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379:** 349-53.

Liu J, Kagan J (1999). Method to distinguish between the MMAC1/PTEN gene and its pseudogene in RT-PCR analysis of point mutations. *Biotechniques* **26:** 19-22, 24.

Liu T, Biddle D, Hanks AN, Brouha B, Yan H, Lee RM *et al* (2006). Activation of dual apoptotic pathways in human melanocytes and protection by survivin. *J Invest Dermatol* **126:** 2247-56.

Loercher AE, Tank EM, Delston RB, Harbour JW (2005). MITF links differentiation with cell cycle arrest in melanocytes by transcriptional activation of INK4A. *J Cell Biol* **168:** 35-40.

Lohrum MA, Ashcroft M, Kubbutat MH, Vousden KH (2000). Identification of a cryptic nucleolar-localization signal in MDM2. *Nat Cell Biol* **2:** 179-81.

Lohrum MA, Woods DB, Ludwig RL, Balint E, Vousden KH (2001). C-terminal ubiquitination of p53 contributes to nuclear export. *Mol Cell Biol* **21:** 8521-32.

Lu X, Yi J (2005). SUMO-1 enhancing the p53-induced HepG2 cell apoptosis. *J Huazhong Univ Sci Technolog Med Sci* **25:** 289-91.

Lubbe J, Reichel M, Burg G, Kleihues P (1994). Absence of p53 gene mutations in cutaneous melanoma. *J Invest Dermatol* **102:** 819-21.

Lucas DM, Davis ME, Parthun MR, Mone AP, Kitada S, Cunningham KD *et al* (2004). The histone deacetylase inhibitor MS-275 induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells. *Leukemia* **18:** 1207-14.

Luikart SD, Kennealey GT, Kirkwood JM (1984). Randomized phase III trial of vinblastine, bleomycin, and cis-dichlorodiammine-platinum versus dacarbazine in malignant melanoma. *J Clin Oncol* **2:** 164-8.

Maestro R, Dei Tos AP, Hamamori Y, Krasnokutsky S, Sartorelli V, Kedes L *et al* (1999). Twist is a potential oncogene that inhibits apoptosis. *Genes Dev* **13:** 2207-17.

Magro PG, Russo AJ, Li WW, Banerjee D, Bertino JR (2004). p14ARF expression increases dihydrofolate reductase degradation and paradoxically results in resistance to folate antagonists in cells with nonfunctional p53. *Cancer Res* **64:** 4338-45.

Maldonado JL, Fridlyand J, Patel H, Jain AN, Busam K, Kageshita T *et al* (2003). Determinants of BRAF mutations in primary melanomas. *J Natl Cancer Inst* **95:** 1878-90.

Malogolowkin M, Cotton CA, Green DM, Breslow NE, Perlman E, Miser J *et al* (2008). Treatment of Wilms tumor relapsing after initial treatment with vincristine, actinomycin D, and doxorubicin. A report from the National Wilms Tumor Study Group. *Pediatr Blood Cancer* **50:** 236-41.

Marks R (1999). Two decades of the public health approach to skin cancer control in Australia: why, how and where are we now? *Australas J Dermatol* **40:** 1-5.

Martin AG, Fearnhead HO (2002). Apocytochrome c blocks caspase-9 activation and Bax-induced apoptosis. *J Biol Chem* **277:** 50834-41.

Mason SL, Loughran O, La Thangue NB (2002). p14(ARF) regulates E2F activity. *Oncogene* **21:** 4220-30.

Matsuoka M, Kurita M, Sudo H, Mizumoto K, Nishimoto I, Ogata E (2003). Multiple domains of the mouse p19ARF tumor suppressor are involved in p53-independent apoptosis. *Biochem Biophys Res Commun* **301:** 1000-10.

McDermid I, AIWA, AACR, NCSG (2005). *Cancer incidence projections, Australia 2002 to 2011*. Australian Institute of Health and Welfare (AIWH), Australasian Association of Cancer Registeries (AACR) and the National Cancer Strategies Group (NCSG): Canberra.

McGill GG, Haq R, Nishimura EK, Fisher DE (2006). c-Met expression is regulated by Mitf in the melanocyte lineage. *J Biol Chem* **281:** 10365-73.

McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK *et al* (2002). Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell* **109:** 707-18.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH *et al* (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *Embo J* **16:** 2794-804.

Medina-Palazon C, Bernard E, Frost V, Morley S, Sinclair AJ (2004). KIPase activity is a novel caspase-like activity associated with cell proliferation. *Eur J Biochem* **271:** 2716-23.

Meier F, Busch S, Lasithiotakis K, Kulms D, Garbe C, Maczey E *et al* (2007). Combined targeting of MAPK and AKT signalling pathways is a promising strategy for melanoma treatment. *Br J Dermatol* **156:** 1204-13.

Melchior F (2000). SUMO-nonclassical ubiquitin. *Annu Rev Cell Dev Biol* **16:** 591-626.

Melchior F, Hengst L (2002). SUMO-1 and p53. *Cell Cycle* **1:** 245-9.

Mellert H, Sykes SM, Murphy ME, McMahon SB (2007). The ARF/oncogene pathway activates p53 acetylation within the DNA binding domain. *Cell Cycle* **6:** 1304-6.

Metelmann HR, Von Hoff DD (1983). In vitro activation of dacarbazine (DTIC) for a human tumor cloning system. *Int J Cell Cloning* **1:** 24-32.

Middleton MR, Grob JJ, Aaronson N, Fierlbeck G, Tilgen W, Seiter S *et al* (2000). Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *J Clin Oncol* **18:** 158-66.

Midgley CA, Desterro JM, Saville MK, Howard S, Sparks A, Hay RT *et al* (2000). An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. *Oncogene* **19:** 2312-23.

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P *et al* (2003). p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* **11:** 577-90.

Mihic-Probst D, Mnich CD, Oberholzer PA, Seifert B, Sasse B, Moch H *et al* (2006). p16 expression in primary malignant melanoma is associated with prognosis and lymph node status. *Int J Cancer* **118:** 2262-8.

Miller AJ, Mihm MC, Jr. (2006). Melanoma. *N Engl J Med* **355:** 51-65.

Millward MJ, Bedikian AY, Conry RM, Gore ME, Pehamberger HE, Sterry W *et al*. (2004). *ASCO*: New Orleans, pp 708.

Miyashita T, Reed JC (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80:** 293-9.

Mizushima N (2007). Autophagy: process and function. *Genes Dev* **21:** 2861-73.

Momand J, Zambetti GP, Olson DC, George D, Levine AJ (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69:** 1237-45.

Moore L, Venkatachalam S, Vogel H, Watt JC, Wu CL, Steinman H *et al* (2003). Cooperativity of p19ARF, Mdm2, and p53 in murine tumorigenesis. *Oncogene* **22:** 7831-7.

Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Nakayama K *et al* (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* **267:** 1506-10.

Muller S, Berger M, Lehembre F, Seeler JS, Haupt Y, Dejean A (2000). c-Jun and p53 activity is modulated by SUMO-1 modification. *J Biol Chem* **275:** 13321-9.

Muller-Tidow C, Metzelder SK, Buerger H, Packeisen J, Ganser A, Heil G *et al* (2004). Expression of the p14ARF tumor suppressor predicts survival in acute myeloid leukemia. *Leukemia* **18:** 720-6.

Munshi A, Kurland JF, Nishikawa T, Tanaka T, Hobbs ML, Tucker SL *et al* (2005). Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. *Clin Cancer Res* **11:** 4912-22.

Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA *et al* (2000). Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst* **92:** 924-30.

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM (1998). An induced proximity model for caspase-8 activation. *J Biol Chem* **273:** 2926-30.

Nakamura S, Roth JA, Mukhopadhyay T (2000). Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. *Mol Cell Biol* **20:** 9391-8.

Nakano K, Vousden KH (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7:** 683-94.

Nakazawa Y, Kamijo T, Koike K, Noda T (2003). ARF tumor suppressor induces mitochondria-dependent apoptosis by modulation of mitochondrial Bcl-2 family proteins. *J Biol Chem* **278:** 27888-95.

Nguyen M, Marcellus RC, Roulston A, Watson M, Serfass L, Murthy Madiraju SR *et al* (2007). Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A*.

Nicke B, Bastien J, Khanna SJ, Warne PH, Cowling V, Cook SJ *et al* (2005). Involvement of MINK, a Ste20 family kinase, in Ras oncogene-induced growth arrest in human ovarian surface epithelial cells. *Mol Cell* **20:** 673-85.

Nishigaki R, Mitani H, Tsuchida N, Shima A (1999). Effect of cyclobutane pyrimidine dimers on apoptosis induced by different wavelengths of UV. *Photochem Photobiol* **70:** 228-35.

Normand G, Hemmati PG, Verdoodt B, von Haefen C, Wendt J, Guner D *et al* (2005). p14ARF induces G2 cell cycle arrest in p53- and p21-deficient cells by down-regulating p34cdc2 kinase activity. *J Biol Chem* **280:** 7118-30.

O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S *et al* (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. *Embo J* **17:** 384-95.

O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M *et al* (1997). Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Res* **57:** 4285-300.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T *et al* (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288:** 1053-8.

Ohashi A, Funasaka Y, Ueda M, Ichihashi M (1996). c-KIT receptor expression in cutaneous malignant melanoma and benign melanotic naevi. *Melanoma Res* **6:** 25-30.

Ohkubo S, Tanaka T, Taya Y, Kitazato K, Prives C (2006). Excess HDM2 impacts cell cycle and apoptosis and has a selective effect on p53-dependent transcription. *J Biol Chem* **281:** 16943-50.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* **362:** 857-60.

Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA *et al* (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435:** 677-81.

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74:** 609-19.

Omholt K, Karsberg S, Platz A, Kanter L, Ringborg U, Hansson J (2002). Screening of N-ras codon 61 mutations in paired primary and metastatic cutaneous melanomas: mutations occur early and persist throughout tumor progression. *Clin Cancer Res* **8:** 3468-74.

Omholt K, Platz A, Kanter L, Ringborg U, Hansson J (2003). NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. *Clin Cancer Res* **9:** 6483-8.

Ota N, Kawakami K, Okuda T, Takehara A, Hiranuma C, Oyama K *et al* (2006). Prognostic significance of p16(INK4a) hypermethylation in non-small cell lung cancer is evident by quantitative DNA methylation analysis. *Anticancer Res* **26:** 3729-32.

Ozoren N, Kim K, Burns TF, Dicker DT, Moscioni AD, El-Deiry WS (2000). The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* **60:** 6259-65.

Packer LM, Pavey SJ, Boyle GM, Stark MS, Ayub AL, Rizos H *et al* (2007). Gene expression profiling in melanoma identifies novel downstream effectors of p14ARF. *Int J Cancer* **121:** 784-90.

Paliwal S, Pande S, Kovi RC, Sharpless NE, Bardeesy N, Grossman SR (2006). Targeting of C-terminal binding protein (CtBP) by ARF results in p53-independent apoptosis. *Mol Cell Biol* **26:** 2360-72.

Palmero I, Pantoja C, Serrano M (1998). p19ARF links the tumour suppressor p53 to Ras. *Nature* **395:** 125-6.

Parisi T, Pollice A, Cristofano AD, Calabro V, La Mantia G (2002a). Transcriptional regulation of the human tumour suppressor p14ARF by E2F1, E2F2, E2F3 and Sp-1 like factors. *Biocem Biophys Res Commun* **291:** 1138-1145.

Parisi T, Pollice A, Di Cristofano A, Calabro V, La Mantia G (2002b). Transcriptional regulation of the human tumor suppressor p14(ARF) by E2F1, E2F2, E2F3, and Sp1-like factors. *Biochem Biophys Res Commun* **291:** 1138-45.

Park YB, Park MJ, Kimura K, Shimizu K, Lee SH, Yokota J (2002). Alterations in the INK4a/ARF locus and their effects on the growth of human osteosarcoma cell lines. *Cancer Genet Cytogenet* **133:** 105-11.

Pauklin S, Kristjuhan A, Maimets T, Jaks V (2005). ARF and ATM/ATR cooperate in p53-mediated apoptosis upon oncogenic stress. *Biochem Biophys Res Commun* **334:** 386-94.

Pavey S, Johansson P, Packer L, Taylor J, Stark M, Pollock PM *et al* (2004). Microarray expression profiling in melanoma reveals a BRAF mutation signature. *Oncogene* **29:** 29.

Peltenburg LT, de Bruin EC, Meersma D, Smit NP, Schrier PI, Medema JP (2005). Expression and function of the apoptosis effector Apaf-1 in melanoma. *Cell Death Differ* **12:** 678-9.

Peltonen K, Kiviharju TM, Jarvinen PM, Ra R, Laiho M (2005). Melanoma cell lines are susceptible to histone deacetylase inhibitor TSA provoked cell cycle arrest and apoptosis. *Pigment Cell Res.* **18:** 196-202.

Piekarz RL, Robey RW, Zhan Z, Kayastha G, Sayah A, Abdeldaim AH *et al* (2004). T-cell lymphoma as a model for the use of histone deacetylase inhibitors in cancer therapy: impact of depsipeptide on molecular markers, therapeutic targets, and mechanisms of resistance. *Blood* **103:** 4636-43.

Pietrzak M, Puzianowska-Kuznicka M (2008). p53-dependent repression of the human MCL-1 gene encoding an anti-apoptotic member of the BCL-2 family: the role of Sp1 and of basic transcription factor binding sites in the MCL-1 promoter. *Biol Chem* **389:** 393-93.

Pollice A, Nasti V, Ronca R, Vivo M, Iacono ML, Calogero R *et al* (2004). Functional and Physical Interaction of the Human ARF Tumor Suppressor with TBP-1 (Tat-binding Protein-1). *J Biol Chem* **279:** 6345-6353.

Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM *et al* (2003). High frequency of BRAF mutations in nevi. *Nat Genet* **33:** 19-20.

Pollock PM, Pearson JV, Hayward NK (1996). Compilation of somatic mutations of the CDKN2 gene in human cancers: non-random distribution of base substitutions. *Genes Chromosomes Cancer* **15:** 77-88.

Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L *et al* (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92:** 713-23.

Porn-Ares MI, Samali A, Orrenius S (1998). Cleavage of the calpain inhibitor, calpastatin, during apoptosis. *Cell Death Differ* **5:** 1028-33.

Poynter JN, Elder JT, Fullen DR, Nair RP, Soengas MS, Johnson TM *et al* (2006). BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res* **16:** 267-73.

Qi Y, Gregory MA, Li Z, Brousal JP, West K, Hann SR (2004). p19ARF directly and differentially controls the functions of c-Myc independently of p53. *Nature* **431:** 712-7.

Quelle DE, Zindy F, Ashmun RA, Sherr CJ (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83:** 993-1000.

Radfar A, Unnikrishnan I, Lee HW, DePinho RA, Rosenberg N (1998). p19(Arf) induces p53-dependent apoptosis during abelson virus-mediated pre-B cell transformation. *Proc Natl Acad Sci U S A* **95:** 13194-9.

Randerson-Moor JA, Harland M, Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J *et al* (2001). A germline deletion of $p14^{ARF}$ but not CDKN2A in a melanoma-neural system tumour syndrome family. *Human Mol. Genet.* **10:** 55-62.

Reef S, Kimchi A (2006). A smARF way to die: a novel short isoform of p19ARF is linked to autophagic cell death. *Autophagy* **2:** 328-30.

Reef S, Shifman O, Oren M, Kimchi A (2007). The autophagic inducer smARF interacts with and is stabilized by the mitochondrial p32 protein. *Oncogene* **26:** 6677-83.

Reef S, Zalckvar E, Shifman O, Bialik S, Sabanay H, Oren M *et al* (2006). A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death. *Mol Cell* **22:** 463-75.

Rehm M, Dussmann H, Prehn JH (2003). Real-time single cell analysis of Smac/DIABLO release during apoptosis. *J Cell Biol* **162:** 1031-43.

Riedl SJ, Li W, Chao Y, Schwarzenbacher R, Shi Y (2005). Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* **434:** 926-33.

Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ (2000). Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev* **14:** 23-7.

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

Rizos H, Darmanian AP, Indsto JO, Shannon JA, Kefford RF, Mann GJ (1999). Multiple abnormalities of the p16INK4a-pRb regulatory pathway in cultured melanoma cells. *Melanoma Res* **9:** 10-9.

Rizos H, Darmanian AP, Mann GJ, Kefford RF (2000). Two arginine rich domains in the p14ARF tumour suppressor mediate nucleolar localization. *Oncogene* **19:** 2978-85.

Rizos H, Diefenbach E, Badhwar P, Woodruff S, Becker TM, Rooney RJ *et al* (2003). Association of p14ARF with the p120E4F transcriptional repressor enhances cell cycle inhibition. *J Biol Chem* **278:** 4981-9.

Rizos H, McKenzie HA, Ayub AL, Woodruff S, Becker TM, Scurr LL *et al* (2006). Physical and functional interaction of the p14ARF tumor suppressor with ribosomes. *J Biol Chem* **281:** 38080-8.

Rizos H, Puig S, Badenas C, Malvehy J, Darmanian AP, Jimenez L *et al* (2001b). A melanoma-associated germline mutation in exon 1beta inactivates p14ARF. *Oncogene* **20:** 5543-7.

Rizos H, Scurr LL, Irvine M, Alling NJ, Kefford RF (2007). p14ARF regulates E2F-1 ubiquitination and degradation via a p53-dependent mechanism. *Cell Cycle* **6:** 1741-7.

Rizos H, Woodruff S, Kefford RF (2005). p14ARF interacts with the SUMO-conjugating enzyme Ubc9 and promotes the Sumoylation of its binding partners. *Cell Cycle* **4:** 597-603.

Robertson KD, Jones PA (1998). The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol Cell Biol* **18:** 6457-73.

Rocha S, Campbell KJ, Perkins ND (2003). p53- and Mdm2-independent repression of NF-kappa B transactivation by the ARF tumor suppressor. *Mol Cell* **12:** 15-25.

Rocha S, Garrett MD, Campbell KJ, Schumm K, Perkins ND (2005a). Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. *Embo J* **24:** 1157-69.

Rocha W, Sanchez R, Deschenes J, Auger A, Hebert E, White JH *et al* (2005b). Opposite effects of histone deacetylase inhibitors on glucocorticoid and estrogen signaling in human endometrial Ishikawa cells. *Mol Pharmacol* **68:** 1852-62.

Rodriguez MS, Desterro JM, Lain S, Midgley CA, Lane DP, Hay RT (1999a). SUMO-1 modification activates the transcriptional response of p53. *Embo J* **18:** 6455-61.

Rodriguez MS, Desterro JMP, Lain S, Midgley CA, Lane DP, Hay RT (1999b). SUMO-1 modification activates the transcriptional response of p53. *EMBO J* **18:** 6455-6461.

Rodway H, Llanos S, Rowe J, Peters G (2004). Stability of nucleolar versus non-nucleolar forms of human p14(ARF). *Oncogene* **23:** 6186-92.

Ross AJ, Amy SP, Mahar PL, Lindsten T, Knudson CM, Thompson CB *et al* (2001). BCLW mediates survival of postmitotic Sertoli cells by regulating BAX activity. *Dev Biol* **239:** 295-308.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J* **16:** 6914-25.

Rubbi CP, Milner J (2003). Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J* **22:** 6068-77.

Ruchaud S, Korfali N, Villa P, Kottke TJ, Dingwall C, Kaufmann SH *et al* (2002). Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. *Embo J* **21:** 1967-77.

Ruffolo SC, Shore GC (2003). BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. *J Biol Chem* **278:** 25039-45.

Russell JL, Powers JT, Rounbehler RJ, Rogers PM, Conti CJ, Johnson DG (2002). ARF differentially modulates apoptosis induced by E2F1 and Myc. *Mol Cell Biol* **22:** 1360-8.

Sakahira H, Enari M, Nagata S (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391:** 96-9.

Saldanha G, Potter L, Daforno P, Pringle JH (2006). Cutaneous melanoma subtypes show different BRAF and NRAS mutation frequencies. *Clin Cancer Res* **12:** 4499-505.

Saldanha G, Purnell D, Fletcher A, Potter L, Gillies A, Pringle JH (2004). High BRAF mutation frequency does not characterize all melanocytic tumor types. *Int J Cancer* **111:** 705-10.

Samraj AK, Sohn D, Schulze-Osthoff K, Schmitz I (2007). Loss of caspase-9 reveals its essential role for caspase-2 activation and mitochondrial membrane depolarization. *Mol Biol Cell* **18:** 84-93.

Sandoval R, Xue J, Pilkinton M, Salvi D, Kiyokawa H, Colamonici OR (2004). Different requirements for the cytostatic and apoptotic effects of type I interferons. Induction of apoptosis requires ARF but not p53 in osteosarcoma cell lines. *J Biol Chem* **279:** 32275-80.

Sasaki Y, Niu C, Makino R, Kudo C, Sun C, Watanabe H *et al* (2004). BRAF Point Mutations in Primary Melanoma Show Different Prevalences by Subtype. *J Invest Dermatol* **123:** 177-83.

Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, El-Deiry WS (2002). BID regulation by p53 contributes to chemosensitivity. *Nat Cell Biol* **4:** 842-9.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ *et al* (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17:** 1675-87.

Schauer E, Trautinger F, Kock A, Schwarz A, Bhardwaj R, Simon M *et al* (1994). Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes. *J Clin Invest* **93:** 2258-62.

Schiff PB, Fant J, Horwitz SB (1979). Promotion of microtubule assembly in vitro by taxol. *Nature* **277:** 665-7.

Schmitt CA, McCurrach ME, de Stanchina E, Wallace-Brodeur RR, Lowe SW (1999). INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev* **13:** 2670-7.

Sekaric P, Shamanin VA, Luo J, Androphy EJ (2007). hAda3 regulates p14ARF-induced p53 acetylation and senescence. *Oncogene* **26:** 6261-8.

Selzer E, Schlagbauer-Wadl H, Okamoto I, Pehamberger H, Potter R, Jansen B (1998). Expression of Bcl-2 family members in human melanocytes, in melanoma metastases and in melanoma cell lines. *Melanoma Res* **8:** 197-203.

Serrano M, Hannon GJ, Beach D (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366:** 704-7.

Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85:** 27-37.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88:** 593-602.

Shamanin VA, Androphy EJ (2004). Immortalization of human mammary epithelial cells is associated with inactivation of the p14ARF-p53 pathway. *Mol Cell Biol* **24:** 2144-52.

Sharpless NE, Bardeesy N, Lee K-H, Carrasco D, Castrillon DH, Aguirre AJ *et al* (2001). Loss of p16INK4a with retention of p19ARF predisposes mice to tumorigenesis. *Nature* **413:** 86-91.

Sharpless NE, DePinho RA (1999). The INK4A/ARF locus and its two gene products. *Curr Opin Genet Dev* **9:** 22-30.

Sharpless NE, Kannan K, Xu J, Bosenberg MW, Chin L (2003). Both products of the mouse Ink4a/Arf locus suppress melanoma formation in vivo. *Oncogene* **22:** 5055-9.

Sharpless NE, Ramsey MR, Balasubramanian P, Castrillon DH, DePinho RA (2004). The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene* **23:** 379-85.

Sherr CJ (1996). Cancer cell cycles. *Science* **274:** 1672-1677.

Sherr CJ (2001). The INK4a/ARF network in tumour suppression. *Nature Reviews* **2:** 731-737.

Sherr CJ (2006). Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer* **6:** 663-73.

Shibue T, Takeda K, Oda E, Tanaka H, Murasawa H, Takaoka A *et al* (2003). Integral role of Noxa in p53-mediated apoptotic response. *Genes Dev* **17:** 2233-8.

Shin MS, Park WS, Kim SY, Kim HS, Kang SJ, Song KY *et al* (1999). Alterations of Fas (Apo-1/CD95) gene in cutaneous malignant melanoma. *Am J Pathol* **154:** 1785-91.

Shvarts A, Brummelkamp TR, Scheeren F, Koh E, Daley GQ, Spits H *et al* (2002). A senescence rescue screen identifies BCL6 as an inhibitor of anti-proliferative p19(ARF)-p53 signaling. *Genes Dev* **16:** 681-6.

Siddik ZH (2002). Biochemical and molecular mechanisms of cisplatin resistance. *Cancer Treat Res* **112:** 263-84.

Sierra A, Castellsague X, Escobedo A, Lloveras B, Garcia-Ramirez M, Moreno A *et al* (2000). Bcl-2 with loss of apoptosis allows accumulation of genetic alterations: a pathway to metastatic progression in human breast cancer. *Int J Cancer* **89:** 142-7.

Simon M, Voss D, Park-Simon TW, Mahlberg R, Koster G (2006). Role of p16 and p14ARF in radio- and chemosensitivity of malignant gliomas. *Oncol Rep* **16:** 127-32.

Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD *et al* (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* **144:** 281-92.

Slee EA, O'Connor DJ, Lu X (2004). To die or not to die: how does p53 decide? *Oncogene* **23:** 2809-18.

Soddu S, Blandino G, Scardigli R, Martinelli R, Rizzo MG, Crescenzi M *et al* (1996). Wild-type p53 induces diverse effects in 32D cells expressing different oncogenes. *Mol Cell Biol* **16:** 487-95.

Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X *et al* (2001). Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* **409:** 207-11.

Soengas MS, Lowe SW (2003). Apoptosis and melanoma chemoresistance. *Oncogene* **22:** 3138-51.

Sohn D, Essmann F, Schulze-Osthoff K, Janicke RU (2006). p21 blocks irradiation-induced apoptosis downstream of mitochondria by inhibition of cyclin-dependent kinase-mediated caspase-9 activation. *Cancer Res* **66:** 11254-62.

Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A *et al* (2006). BRAF mutation predicts sensitivity to MEK inhibition. *Nature* **439:** 358-62.

Soufir N, Avril M-F, Chompret A, Demenais F, Bombled J, Spatz A *et al* (1998). Prevalence of *p16* and *CDK4* germline mutations in 48 melanoma-prone families in France. *Hum. Mol. Genet.* **7:** 209-216.

Sparrow LE, Soong R, Dawkins HJ, Iacopetta BJ, Heenan PJ (1995). p53 gene mutation and expression in naevi and melanomas. *Melanoma Res* **5:** 93-100.

Sreeramaneni R, Chaudhry A, McMahon M, Sherr CJ, Inoue K (2005). Ras-Raf-Arf signaling critically depends on the Dmp1 transcription factor. *Mol Cell Biol* **25:** 220-32.

Stanelle J, Stiewe T, Theseling CC, Peter M, Putzer BM (2002). Gene expression changes in response to E2F1 activation. *Nucleic Acids Res* **30:** 1859-67.

Staunton MJ, Gaffney EF (1995). Tumor type is a determinant of susceptibility to apoptosis. *Am J Clin Pathol* **103:** 300-7.

Stennicke HR, Salvesen GS (1999). Catalytic properties of the caspases. *Cell Death Differ* **6:** 1054-9.

Stevens C, La Thangue NB (2003). E2F and cell cycle control: a double-edged sword. *Arch Biochem Biophys* **412:** 157-69.

Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ *et al* (2001). Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem* **276:** 3149-57.

Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S *et al* (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *Embo J* **17:** 5001-14.

Sugihara T, Kaul SC, Kato J, Reddel RR, Nomura H, Wadhwa R (2001). Pex19p dampens the p19ARF-p53-p21WAF1 tumor suppressor pathway. *J Biol Chem* **276:** 18649-52.

Sugimoto M, Kuo ML, Roussel MF, Sherr CJ (2003). Nucleolar Arf tumor suppressor inhibits ribosomal RNA processing. *Mol Cell* **11:** 415-24.

Sui G, Affar el B, Shi Y, Brignone C, Wall NR, Yin P *et al* (2004). Yin Yang 1 is a negative regulator of p53. *Cell* **117:** 859-72.

Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M *et al* (2000). Two distinct pathways leading to nuclear apoptosis. *J Exp Med* **192:** 571-80.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM *et al* (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397:** 441-6.

Suzuki A, Kawano H, Hayashida M, Hayasaki Y, Tsutomi Y, Akahane K (2000). Procaspase 3/p21 complex formation to resist fas-mediated cell death is initiated as a result of the phosphorylation of p21 by protein kinase A. *Cell Death Differ* **7:** 721-8.

Suzuki A, Tsutomi Y, Akahane K, Araki T, Miura M (1998). Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene* **17:** 931-9.

Suzuki H, Kurita M, Mizumoto K, Nishimoto I, Ogata E, Matsuoka M (2003). p19ARF-induced p53-independent apoptosis largely occurs through BAX. *Biochem Biophys Res Commun* **312:** 1273-7.

Sykes SM, Mellert HS, Holbert MA, Li K, Marmorstein R, Lane WS *et al* (2006). Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell* **24:** 841-51.

Szklarczyk R, Heringa J, Pond SK, Nekrutenko A (2007). Rapid asymmetric evolution of a dual-coding tumor suppressor INK4a/ARF locus contradicts its function. *Proc Natl Acad Sci U S A* **104:** 12807-12.

Tago K, Chiocca S, Sherr CJ (2005). Sumoylation induced by the Arf tumor suppressor: a p53-independent function. *Proc Natl Acad Sci U S A* **102:** 7689-94.

Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS *et al* (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem* **273:** 7787-90.

Tanaka T, Ohkubo S, Tatsuno I, Prives C (2007). hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. *Cell* **130:** 638-50.

Tang L, Tron VA, Reed JC, Mah KJ, Krajewska M, Li G *et al* (1998). Expression of apoptosis regulators in cutaneous malignant melanoma. *Clin Cancer Res* **4:** 1865-71.

Tannapfel A, Busse C, Weinans L, Benicke M, Katalinic A, Geissler F *et al* (2001). INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas. *Oncogene* **20:** 7104-9.

Tao W, Levine AJ (1999). P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. *Proc Natl Acad Sci U S A* **96:** 6937-41.

Tawfic S, Olson MO, Ahmed K (1995). Role of protein phosphorylation in post-translational regulation of protein B23 during programmed cell death in the prostate gland. *J Biol Chem* **270:** 21009-15.

Taylor ST, Hickman JA, Dive C (2000). Epigenetic determinants of resistance to etoposide regulation of Bcl-X(L) and Bax by tumor microenvironmental factors. *J Natl Cancer Inst* **92:** 18-23.

Tentori L, Graziani G (2004). Correspondence re: DC Lev et al., Dacarbazine causes transcriptional up-regulation of interleukin 8 and vascular endothelial growth factor in melanoma cells: a possible escape mechanism from chemotherapy. Mol Cancer Ther, 2003;2(8):753-63. *Mol Cancer Ther* **3:** 383; author reply 383-4.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR *et al* (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81:** 801-9.

Thallinger C, Wolschek MF, Wacheck V, Maierhofer H, Gunsberg P, Polterauer P *et al* (2003). Mcl-1 antisense therapy chemosensitizes human melanoma in a SCID mouse xenotransplantation model. *J Invest Dermatol* **120:** 1081-6.

Thompson JF, Hunt JA, Shannon KF, Kam PC (1997). Frequency and duration of remission after isolated limb perfusion for melanoma. *Arch Surg* **132:** 903-7.

Thompson JF, Kam PC, Waugh RC, Harman CR (1998). Isolated limb infusion with cytotoxic agents: a simple alternative to isolated limb perfusion. *Semin Surg Oncol* **14:** 238-47.

Thompson JF, Scolyer RA, Kefford RF (2005). Cutaneous melanoma. *Lancet* **365:** 687-701.

Thompson T, Tovar C, Yang H, Carvajal D, Vu BT, Xu Q *et al* (2004). Phosphorylation of p53 on key serines is dispensable for transcriptional activation and apoptosis. *J Biol Chem* **279:** 53015-22.

Tompkins V, Hagen J, Zediak VP, Quelle DE (2006). Identification of novel ARF binding proteins by two-hybrid screening. *Cell Cycle* **5:** 641-6.

Torres K, Horwitz SB (1998). Mechanisms of Taxol-induced cell death are concentration dependent. *Cancer Res* **58:** 3620-6.

Tsao H, Goel V, Wu H, Yang G, Haluska FG (2004). Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. *J Invest Dermatol* **122:** 337-41.

Tsao H, Zhang X, Fowlkes K, Haluska FG (2000). Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines. *Cancer Res* **60:** 1800-4.

Tsuji K, Mizumoto K, Sudo H, Kouyama K, Ogata E, Matsuoka M (2002). p53-independent apoptosis is induced by the p19ARF tumor suppressor. *Biochem Biophys Res Commun* **295:** 621-9.

Turk B, Stoka V (2007). Protease signalling in cell death: caspases versus cysteine cathepsins. *FEBS Lett* **581:** 2761-7.

Twiddy D, Cohen GM, Macfarlane M, Cain K (2006). Caspase-7 is directly activated by the approximately 700-kDa apoptosome complex and is released as a stable XIAP-caspase-7 approximately 200-kDa complex. *J Biol Chem* **281:** 3876-88.

Ugurel S, Thirumaran RK, Bloethner S, Gast A, Sucker A, Mueller-Berghaus J *et al* (2007). B-RAF and N-RAS Mutations Are Preserved during Short Time In Vitro Propagation and Differentially Impact Prognosis. *PLoS ONE* **2:** e236.

Unger T, Sionov RV, Moallem E, Yee CL, Howley PM, Oren M *et al* (1999). Mutations in serines 15 and 20 of human p53 impair its apoptotic activity. *Oncogene* **18:** 3205-12.

Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL (1996). Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc Natl Acad Sci U S A* **93:** 4974-8.

Uren RT, Dewson G, Chen L, Coyne SC, Huang DC, Adams JM *et al* (2007). Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. *J Cell Biol* **177:** 277-87.

van Delft MF, Huang DC (2006). How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res* **16:** 203-13.

van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P (2003). Mitochondrial intermembrane proteins in cell death. *Biochem Biophys Res Commun* **304:** 487-97.

Vandenabeele P, Orrenius S, Zhivotovsky B (2005). Serine proteases and calpains fulfill important supporting roles in the apoptotic tragedy of the cellular opera. *Cell Death Differ* **12:** 1219-24.

Vaux DL, Cory S, Adams JM (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335:** 440-2.

Velculescu VE, El-Deiry WS (1996). Biological and clinical importance of the p53 tumor suppressor gene. *Clin Chem* **42:** 858-68.

Veldman T, Liu X, Yuan H, Schlegel R (2003). Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter. *Proc Natl Acad Sci U S A* **23:** 23.

Villunger A, Michalak EM, Coultas L, Mullauer F, Bock G, Ausserlechner MJ *et al* (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* **302:** 1036-8.

Vivo M, Calogero RA, Sansone F, Calabro V, Parisi T, Borrelli L *et al* (2001). The human tumor suppressor arf interacts with spinophilin/neurabin II, a type 1 protein-phosphatase-binding protein. *J Biol Chem* **276:** 14161-9.

Voorhoeve PM, Agami R (2003). The tumor-suppressive functions of the human INK4A locus. *Cancer Cell* **4:** 311-9.

Wajant H (2003). Death receptors. *Essays Biochem* **39:** 53-71.

Wajant H, Gerspach J, Pfizenmaier K (2005). Tumor therapeutics by design: targeting and activation of death receptors. *Cytokine Growth Factor Rev* **16:** 55-76.

Waldman T, Kinzler KW, Vogelstein B (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* **55:** 5187-90.

Walker GJ, Hussussian CJ, Flores JF, Glendening JM, Haluska FG, Dracopoli NC *et al* (1995). Mutations of the CDKN2/p16^{INK4} gene in Australian melanoma kindreds. *Hum. Mol. Gen.* **4:** 1845-1852.

Walterhouse D, Watson A (2007). Optimal management strategies for rhabdomyosarcoma in children. *Paediatr Drugs* **9:** 391-400.

Wang J, He X, Luo Y, Yarbrough WG (2006). A novel ARF-binding protein (LZAP) alters ARF regulation of HDM2. *Biochem J* **393:** 489-501.

Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996). BID: a novel BH3 domain-only death agonist. *Genes Dev* **10:** 2859-69.

Wang T, Kobayashi T, Takimoto R, Denes AE, Snyder EL, el-Deiry WS *et al* (2001a). hADA3 is required for p53 activity. *Embo J* **20:** 6404-13.

Wang X, Arooz T, Siu WY, Chiu CH, Lau A, Yamashita K *et al* (2001b). MDM2 and MDMX can interact differently with ARF and members of the p53 family. *FEBS Lett* **490:** 202-8.

Weber HO, Samuel T, Rauch P, Funk JO (2002). Human p14(ARF)-mediated cell cycle arrest strictly depends on intact p53 signaling pathways. *Oncogene* **21:** 3207-12.

Weber JD, Jeffers JR, Rehg JE, Randle DH, Lozano G, Roussel MF *et al* (2000a). p53-independent functions of the p19(ARF) tumor suppressor. *Genes Dev* **14:** 2358-65.

Weber JD, Kuo ML, Bothner B, DiGiammarino EL, Kriwacki RW, Roussel MF *et al* (2000b). Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex. *Mol Cell Biol* **20:** 2517-28.

Weber JD, Taylor LJ, Roussel MF, Sherr CJ, Bar-Sagi D (1999). Nucleolar Arf sequesters Mdm2 and activates p53. *Nat Cell Biol* **1:** 20-6.

Wei W, Hemmer RM, Sedivy JM (2001). Role of p14(ARF) in replicative and induced senescence of human fibroblasts. *Mol Cell Biol* **21:** 6748-57.

Weitzman MD, Ornelles DA (2005). Inactivating intracellular antiviral responses during adenovirus infection. *Oncogene* **24:** 7686-96.

Wellbrock C, Marais R (2005). Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. *J Cell Biol* **170:** 703-8.

Wendt J, Radetzki S, von Haefen C, Hemmati PG, Guner D, Schulze-Osthoff K *et al* (2006). Induction of p21CIP/WAF-1 and G2 arrest by ionizing irradiation impedes caspase-3-mediated apoptosis in human carcinoma cells. *Oncogene* **25:** 972-80.

Wikonkal NM, Remenyik E, Knezevic D, Zhang W, Liu M, Zhao H *et al* (2003). Inactivating E2f1 reverts apoptosis resistance and cancer sensitivity in Trp53-deficient mice. *Nat Cell Biol* **5:** 655-660.

Williams RT, Roussel MF, Sherr CJ (2006). Arf gene loss enhances oncogenicity and limits imatinib response in mouse models of Bcr-Abl-induced acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A* **103:** 6688-93.

Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI *et al* (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19:** 1294-305.

Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE *et al* (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315:** 856-9.

Winnepenninckx V, Lazar V, Michiels S, Dessen P, Stas M, Alonso SR *et al* (2006). Gene expression profiling of primary cutaneous melanoma and clinical outcome. *J Natl Cancer Inst* **98:** 472-82.

Witham J, Valenti MR, De-Haven-Brandon AK, Vidot S, Eccles SA, Kaye SB *et al* (2007). The Bcl-2/Bcl-XL Family Inhibitor ABT-737 Sensitizes Ovarian Cancer Cells to Carboplatin. *Clin Cancer Res* **13:** 7191-8.

Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Reed JC *et al* (1998). Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* **17:** 1069-78.

Woods YL, Xirodimas DP, Prescott AR, Sparks A, Lane DP, Saville MK (2004). p14 Arf promotes small ubiquitin-like modifier conjugation of Werners helicase. *J Biol Chem* **279:** 50157-66.

Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM (2001). Negative regulation of bcl-2 expression by p53 in hematopoietic cells. *Oncogene* **20:** 240-51.

www.cancerresearchuk.org. (2007). *Cancer Research UK, Vol. 2007*.

Xiao H, Hasegawa T, Miyaishi O, Ohkusu K, Isobe K (1997). Sodium butyrate induces NIH3T3 cells to senescence-like state and enhances promoter activity of p21WAF/CIP1 in p53-independent manner. *Biochem Biophys Res Commun* **237:** 457-60.

Xirodimas DP, Chisholm J, Desterro JM, Lane DP, Hay RT (2002). P14ARF promotes accumulation of SUMO-1 conjugated (H)Mdm2. *FEBS Lett* **528:** 207-11.

Xirodimas DP, Stephen CW, Lane DP (2001). Cocompartmentalization of p53 and Mdm2 is a major determinant for Mdm2-mediated degradation of p53. *Exp Cell Res* **270:** 66-77.

Xu SQ, El-Deiry WS (2000). p21(WAF1/CIP1) inhibits initiator caspase cleavage by TRAIL death receptor DR4. *Biochem Biophys Res Commun* **269:** 179-90.

Xu Y (2003). Regulation of p53 responses by post-translational modifications. *Cell Death Differ* **10:** 400-3.

Yamamura K, Kamada S, Ito S, Nakagawa K, Ichihashi M, Tsujimoto Y (1996). Accelerated disappearance of melanocytes in bcl-2-deficient mice. *Cancer Res* **56:** 3546-3550.

Yang, al e (1996). Bad, a heterodimeric partner for Bcl-xl and Bcl-2 displaces bax and promotes cell death. *Cell* **80:** 285-291.

Yang CT, You L, Yeh CC, Chang JW, Zhang F, McCormick F *et al* (2000). Adenovirus-mediated p14(ARF) gene transfer in human mesothelioma cells. *J Natl Cancer Inst* **92:** 636-41.

Yang G, Rajadurai A, Tsao H (2005). Recurrent patterns of dual RB and p53 pathway inactivation in melanoma. *J Invest Dermatol* **125:** 1242-51.

Yang Y, Li CC, Weissman AM (2004). Regulating the p53 system through ubiquitination. *Oncogene* **23:** 2096-106.

Yanofsky C (1988). Transcription attenuation. *J Biol Chem* **263:** 609-12.

Yarbrough WG, Bessho M, Zanation A, Bisi JE, Xiong Y (2002). Human tumor suppressor ARF impedes S-phase progression independent of p53. *Cancer Res* **62:** 1171-7.

Yazdi AS, Palmedo G, Flaig MJ, Puchta U, Reckwerth A, Rutten A *et al* (2003). Mutations of the BRAF gene in benign and malignant melanocytic lesions. *J Invest Dermatol* **121:** 1160-2.

You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R *et al* (2002). Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. *Proc Natl Acad Sci U S A* **99:** 1455-60.

Youle RJ, Strasser A (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* **9:** 47-59.

Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L (2003). PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci U S A* **100:** 1931-6.

Yu X, Acehan D, Menetret JF, Booth CR, Ludtke SJ, Riedl SJ *et al* (2005). A structure of the human apoptosome at 12.8 A resolution provides insights into this cell death platform. *Structure* **13:** 1725-35.

Yuan XW, Zhu XF, Huang XF, Sheng PY, He AS, Yang ZB *et al* (2007). p14ARF Sensitizes Human Osteosarcoma Cells to Cisplatin-Induced Apoptosis in a p53-Independent Manner. *Cancer Biol Ther* **6**.

Yung BY, Chang FJ, Bor AM, Lee ES (1992). Schedule-dependent effects of two consecutive, divided, low doses of actinomycin D on translocation of protein B23, inhibition of cell growth and RNA synthesis in HeLa cells. *Int J Cancer* **52:** 317-22.

Zhang XD, Zhang XY, Gray CP, Nguyen T, Hersey P (2001). Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of human melanoma is regulated by smac/DIABLO release from mitochondria. *Cancer Res* **61:** 7339-48.

Zhang Y, Xiong Y (1999). Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. *Mol Cell* **3:** 579-91.

Zhang Y, Xiong Y, Yarbrough WG (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92:** 725-34.

Zhang Z, Wang H, Li M, Agrawal S, Chen X, Zhang R (2004). MDM2 is a negative regulator of p21 WAF1/CIP1, independent of p53. *J Biol Chem* **3:** 3.

Zhao R, Gish K, Murphy M, Yin Y, Notterman D, Hoffman WH *et al* (2000). Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. *Genes Dev* **14:** 981-93.

Zhong Q, Gao W, Du F, Wang X (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* **121:** 1085-95.

Zhou LL, Zhou LY, Luo KQ, Chang DC (2005). Smac/DIABLO and cytochrome c are released from mitochondria through a similar mechanism during UV-induced apoptosis. *Apoptosis* **10:** 289-99.

Zhou XP, Li YJ, Hoang-Xuan K, Laurent-Puig P, Mokhtari K, Longy M *et al* (1999). Mutational analysis of the PTEN gene in gliomas: molecular and pathological correlations. *Int J Cancer* **84:** 150-4.

Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, Thompson JF *et al* (2007). Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. *Mod Pathol* **20:** 416-26.

Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, Thompson JF *et al* (2006). Progression in melanoma is associated with decreased expression of death receptors for tumor necrosis factor-related apoptosis-inducing ligand. *Hum Pathol* **37:** 1286-94.

Zimmermann KC, Bonzon C, Green DR (2001). The machinery of programmed cell death. *Pharmacol Ther* **92:** 57-70.

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ *et al* (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* **12:** 2424-33.