



# Effects on normal fibroblasts and neuroblastoma cells of the activation of the p53 response by the nuclear export inhibitor leptomycin B

Philip Smart<sup>2</sup>, E Birgitte Lane<sup>2</sup>, David P Lane<sup>1</sup>, Carol Midgley<sup>1</sup>, Borek Vojtesek<sup>3</sup> and Sonia Lain<sup>\*1</sup>

<sup>1</sup>CRC Cell Transformation Group, Department of Biochemistry, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, UK; <sup>2</sup>CRC Cell Structure Research Group, Department of Anatomy and Physiology, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, UK; <sup>3</sup>Department of Cellular and Molecular Oncology, Masaryk Memorial Cancer Institute, Zluty Kopec 7, 656 53 Brno, Czech Republic

**p53 tumour suppressor protein levels and p53-dependent transcriptional activity have been recently shown to increase in cells treated with leptomycin B (LMB), an inhibitor of nuclear export. Experiments presented here show that LMB treatment leads to growth arrest and a senescence-like phenotype in human normal fibroblast cultures. This effect is reversible after removal of the drug and further passage by trypsinization. Instead, LMB has a strong cytotoxic effect on human neuroblastoma cell lines even at nanomolar concentrations. In both these cell types the effects of LMB are attenuated when the activity of the endogenous wild type p53 protein is abrogated by overexpression of a dominant negative p53 mutant. We conclude that the induction of the p53 response by LMB plays an important role in the effects of this drug on cultured cells.**

**Keywords:** leptomycin B; senescence; p53 response; normal fibroblasts; neuroblastoma

## Introduction

p53 transcriptional activity plays a key role in the mechanism by which mammalian cells undergo growth arrest in response to DNA damage, ribonucleotide depletion, hypoxia, heatshock, low pH and certain oncogenes (Bates and Vousden, 1999). It is believed that p53 induces growth arrest primarily through its role as a transcriptional activator of the *p21<sup>WAF1/CIP1</sup>* gene. Also, several lines of evidence strongly associate p53 with the establishment and maintenance of senescence (reviewed in Wynford-Thomas, 1999). Abrogation of p53 function leads either to a temporary or permanent escape from senescence. Furthermore both the protein level of p53 and *p21<sup>WAF1/CIP1</sup>* have been reported to be increased in senescent fibroblasts. Overexpression of wild type p53 can rapidly lead to the induction of senescence in tumour derived cells indicating a direct involvement of p53 in this process (Sugrue *et al.*, 1997; Wang *et al.*, 1998) while microinjection of antibodies that inactivate p53 is sufficient to both prevent and reverse senescence (Gire and Wynford-Thomas, 1998).

Accumulated p53 can also induce apoptosis in response to DNA damage (reviewed in Bates and Vousden, 1999). Activation of transcription by p53 also seems to be important for the apoptotic activity of this tumour suppressor. However, transcriptional activation by p53 may not be essential for p53-dependent apoptosis in every case and other activities of p53 may be required.

Leptomycin B (LMB) has recently been described to inhibit specific protein and RNA export from the nucleus to the cytoplasm. Several groups have recently shown that LMB interacts directly with CRM1, a receptor that mediates the export from the nucleus of viral and cellular proteins which contain leucine rich nuclear export signals (NES) (reviewed in Mattaj and Englemeier, 1998; Lain *et al.*, in press). Addition of LMB to the cell culture medium leads to the accumulation of p53 and of the products of p53 responsive genes in the nucleus of human normal fibroblasts and induces the transcription from a p53 dependent promoter in cultured cells (Lain *et al.*, 1999). This accumulation also occurs in tumour cell lines containing wild type p53 but not in cells that express mutant p53 or that are negative for this tumour suppressor (Freedman and Levine, 1998; Lain *et al.*, 1999). The description of a nuclear export signal in the HDM2 oncoprotein (Roth *et al.*, 1998), which mediates the rapid degradation of p53 (reviewed in Freedman and Levine, 1999), as well as in the p53 sequence (Stommel *et al.*, 1999) suggest that the export of p53 from the nucleus is mediated by the CRM1 exportin.

Although p53 is the gene most frequently mutated gene in human cancers (Kamb, 1995) a large number of carcinomas retain wild type p53. As LMB activates the p53 response we decided to compare the effects of this drug on normal fibroblasts and on cancerous cells containing wild type p53 activity. Three reasons led us to use human neuroblastoma cell lines for this purpose: (i) p53 mutations are not frequent in neuroblastoma (references in Ronca *et al.*, 1997); (ii) Neuroblastoma cells are very sensitive to the induction of the p53 activity (Ronca *et al.*, 1997); (iii) Neuroblastoma is one of the most common childhood cancers accounting for approximately 8% of all childhood malignancies (Melino *et al.*, 1997). In spite of the general responsiveness of these tumours to therapy, these harsh treatments impair normal development in children and increase the risk of new malignancies appearing later in life (Jenney, 1994) and therefore there is a clear need for less genotoxic therapeutic regimens.

\*Correspondence: S Lain  
Received 7 July 1999; revised 29 September 1999; accepted 29 September 1999

## Results

### *Effect of leptomycin B on the proliferation and metabolic activity of normal fibroblasts*

The aim of the first set of experiments was to test the effects of LMB on cell cycle progression of normal fibroblasts and compare it to the effect of UVC irradiation which also induces the p53 response. No cytopathic effects were observed in the LMB treated fibroblast cultures apart from the anticipated disappearance of mitotic figures. FACS analysis of exponentially growing HNFs after exposure to LMB showed a gross reduction in the number of cells synthesizing DNA (Figure 1a). Using another approach, HNFs were arrested in G0 by serum deprivation, released from starvation to allow their entry into G1 (Li *et al.*, 1996) and irradiated with UVC or treated with 2  $\mu$ m LMB. No significant cell loss was observed after either of these two treatments but both dramatically reduced the number of cells incorporating BrdU (Figure 1b). This result indicates that LMB, like UVC (de Laat *et al.*, 1996), prevents the entry of HNFs into the S phase of the cell cycle.

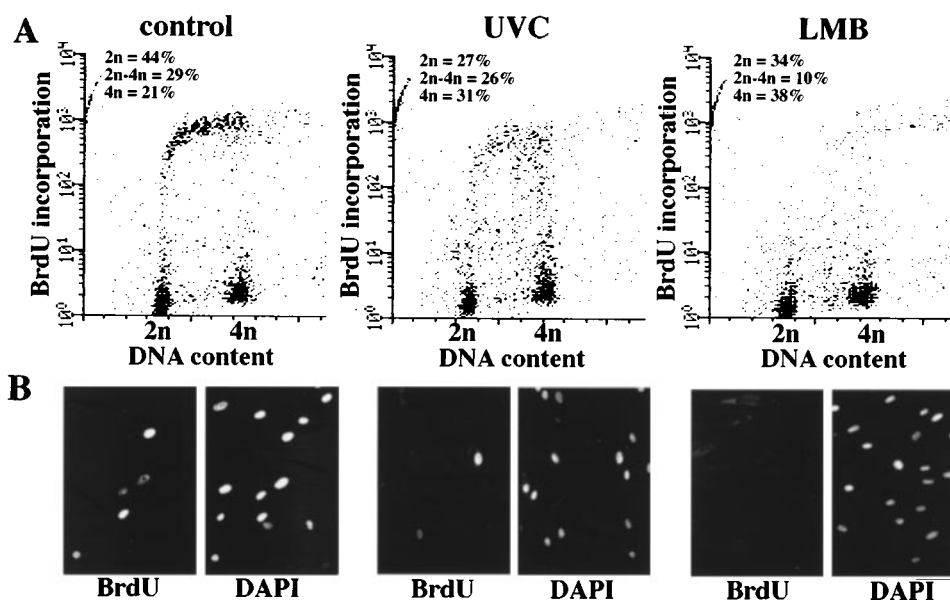
In order to assess whether the HNFs treated with LMB were still viable, we examined their metabolic activity by testing their MTT-reductase activity. When HNFs were treated for 72 h with 0.2, 2 or 20  $\mu$ m LMB a decrease of approximately 30% of the MTT-reductase activity was observed at all three concentrations (not shown). In order to test whether the effect of LMB in normal fibroblasts is primarily on cell proliferation or on cell survival we analysed the MTT-reductase activity in cultures treated with LMB and compared these values with the MTT-reductase activity of the cultures before treatment. The results shown in Table 1 were obtained using four different human fibroblast cell lines and show that LMB prevents normal growth but does not reduce the

MTT-reductase activity levels below that shown by the cultures at the start of the experiment. A similar analysis was performed using normal endothelial cells derived from bovine aorta (BAECs). A larger decrease in viability of this cell type was observed than was the case for human fibroblast cultures. Nevertheless, this decrease in the viability of the BAECs was not as pronounced as that obtained when neuroblastoma cell lines were analysed (see Table 2).

In order to test whether the effects of LMB on human primary fibroblasts depended on the function of p53 experiments were undertaken using a HNF derived cell line overexpressing a dominant negative truncated p53 (DFSF1-DDp53 cells). In agreement with previous

**Table 1** Time course analysis of the MTT-reductase activity in normal human fibroblast and DFSF1-DDp53 cultures treated with 200 nM LMB. The MTT-reductase activity at the time before treatment is considered as 100%

Cell line	Days in culture	% MTT-reductase activity - LMB	% MTT-reductase activity + LMB
DFSF1 passage 9	1	100 $\pm$ 4	98 $\pm$ 4.5
	2	110 $\pm$ 3	91 $\pm$ 9.5
	3	187 $\pm$ 4	112 $\pm$ 5
	4	179 $\pm$ 2	105 $\pm$ 2
GM38 passage 18	1	79 $\pm$ 13	84 $\pm$ 14
	2	114 $\pm$ 5	93 $\pm$ 3
	3	140 $\pm$ 4.5	107 $\pm$ 16
	4	150 $\pm$ 11	79 $\pm$ 5
MRC5 passage 22	1	100 $\pm$ 6	83 $\pm$ 2
	2	156 $\pm$ 14.5	101 $\pm$ 9
	3	237 $\pm$ 11	98 $\pm$ 13
	4	180 $\pm$ 16	76 $\pm$ 8
WI38 passage 22	1	133 $\pm$ 3	114 $\pm$ 1
	2	193 $\pm$ 10	145 $\pm$ 11
	3	216 $\pm$ 14	123 $\pm$ 11
	4	266 $\pm$ 16	100 $\pm$ 9
DFSF1-DDp53	1	158 $\pm$ 4	122 $\pm$ 8
	2	197 $\pm$ 10	118 $\pm$ 8.5
	3	207 $\pm$ 10	148 $\pm$ 9
	4	199 $\pm$ 3	123 $\pm$ 8.5



**Figure 1** (A) Proliferating HNFs (HFF1 passage 6) were left untreated, irradiated with 20 J/m<sup>2</sup> UVC or treated with 2  $\mu$ m LMB. After 18 h cells were pulse labelled with BrdU and subjected to FACS analysis. The proportion of cells in the cultures with different DNA contents is indicated. (B) HFF1 cells were serum starved for 2 days, allowed to enter G1 by incubation in 10% FCS containing medium for 7 h after which they were left untreated, irradiated with 20 J/m<sup>2</sup> UVC or treated with 2  $\mu$ m LMB. After 24 h they were pulse labelled with BrdU and stained with an anti-BrdU antibody. Nuclei were detected by DAPI staining

results (Ostermeyer *et al.*, 1996), although the levels of endogenous p53 were increased by the expression of this dominant negative mutant of p53, its function as a transcriptional activator seemed to be abrogated since the levels of the products of the two p53-responsive genes *HDM2* and *p21<sup>WAF1/CIP1</sup>* were not increased after LMB treatment of DFSF1-DDp53 cells (Figure 2a). Nevertheless, LMB still impaired cell growth of DFSF1-DDp53 cells (Table 1, Figure 2b). This may be due to an incomplete abrogation of the p53 functions in the DFSF1-DDp53 fibroblasts and/or to the effect of LMB on other cellular factors necessary for cell cycle progression.

Unlike the case of the p53 tumour suppressor, LMB had no effect on the levels of the p53 homologue p73 (Kaghad *et al.*, 1997). This indicates the existence of different mechanisms for the regulation of the levels of p53 and p73 as pointed out by other authors (reviewed in White and Prives, 1999).

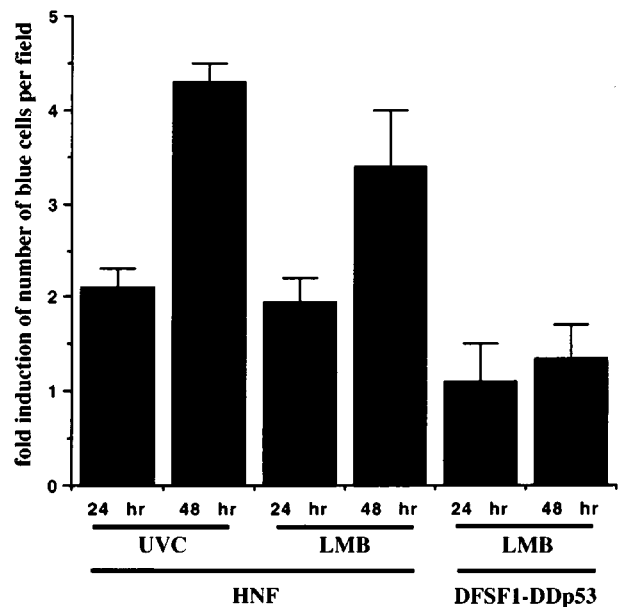
#### Treatment of human fibroblasts with UVC or leptomycin B induces a phenotype associated with premature senescence

Since UVC and LMB did not largely affect the metabolic activity of human fibroblasts, we tested whether the growth arrest induced by these agents was accompanied by the induction of markers associated with cellular senescence. A widely used marker of senescence, both *in vivo* and *in vitro*, is the onset of a  $\beta$ -galactosidase activity detectable at pH6 (Dimri *et al.*, 1995). When subconfluent human primary fibroblast cultures were subjected to 20 J/m<sup>2</sup> UVC we observed an increase in the number of cells expressing this senescence associated marker (Figure 3). A similar result was obtained when cells were treated with LMB (Figure 3). No significant differences in the level of induction of the SA- $\beta$ -Gal activity by UVC or LMB were observed with primary fibroblasts of different passage number, although the overall percentage of SA- $\beta$ -Gal positive cells increased with passage number as previously described (Dimri *et al.*, 1995). However, unlike with the parental cell line, LMB did not induce an increase in the number of cells showing the SA- $\beta$ -Gal activity in DFSF1-DDp53 cultures (Figure 3),

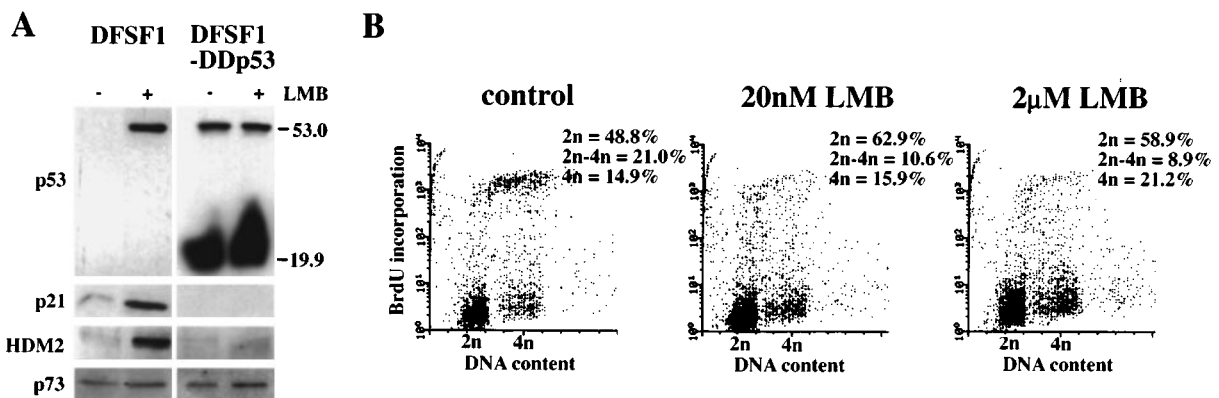
**Table 2** MMT reductase activity in cultures treated with 200 nM or 20 nM

Cell lines	% MTT reductase activity	
	-LMB	+LMB
DFSF1 passage 9	174.0 ± 11.1	146.8 ± 1.9
	174.0 ± 11.1	145.9 ± 3.3*
BAEC passage 10	174.8 ± 6.5	49.8 ± 2.9
	174.8 ± 6.5	59.3 ± 2.0*
SKNSH	207.8 ± 13.4	26.6 ± 2.0*
SHSY5Y	243.0 ± 7.6	18.1 ± 3.4*

(\*) LMB for 84 h. The MTT-reductase activity of the cultures at the time before treatment is considered as 100%



**Figure 3** Different HNF cultures were left untreated, irradiated with 20 J/m<sup>2</sup> UVC or treated with 2  $\mu$ M LMB. Twenty-four or 48 h after they were fixed and assayed for the SA- $\beta$ -Gal activity. The average number of blue cells per field was counted and divided by the average number of blue cells per field in untreated cultures. These results show the average from six independent experiments using different HNF lines at different passage numbers (5–25) and cultured at different densities. Approximately 500 cells were counted in each experiment. In a similar assay, DFSF1-DDp53 cells were treated with 2  $\mu$ M LMB and tested for the SA- $\beta$ -Gal activity. These results show the average from three independent experiments counting approximately 1000 cells for each treatment

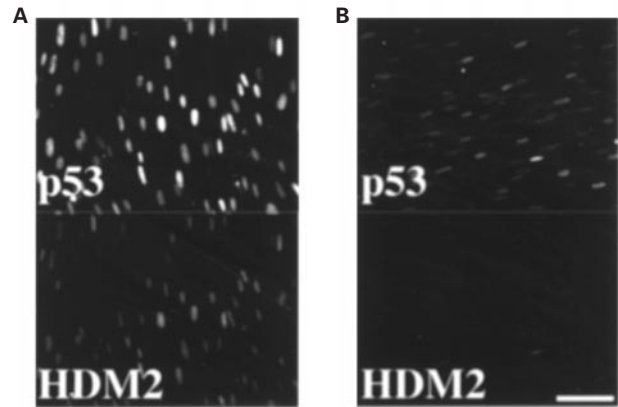


**Figure 2** (A) DFSF1 or DFSF1-DDp53 cells were left untreated or treated with 0.2  $\mu$ M LMB for 24 h. Whole cell extracts were analysed by Western blot using a mixture of the CM1 and CM5 rabbit sera against the endogenous human p53 (upper band) and the truncated dominant-negative murine p53 (lower band), respectively (top panel), with a mixture of the mAb1 and 118 mouse monoclonal antibodies against p21<sup>WAF1/CIP1</sup> (second panel), with the 4B2 mouse monoclonal antibody against HDM2 (third panel) or with a rabbit polyclonal antibody against full length human (bottom panel). (B) DFSF1-DDp53 cells were left untreated or treated with 20 nM or 2  $\mu$ M LMB. After 24 h cells were pulse labelled with BrdU and subjected to FACS analysis. The proportion of cells in the cultures with different DNA contents is indicated

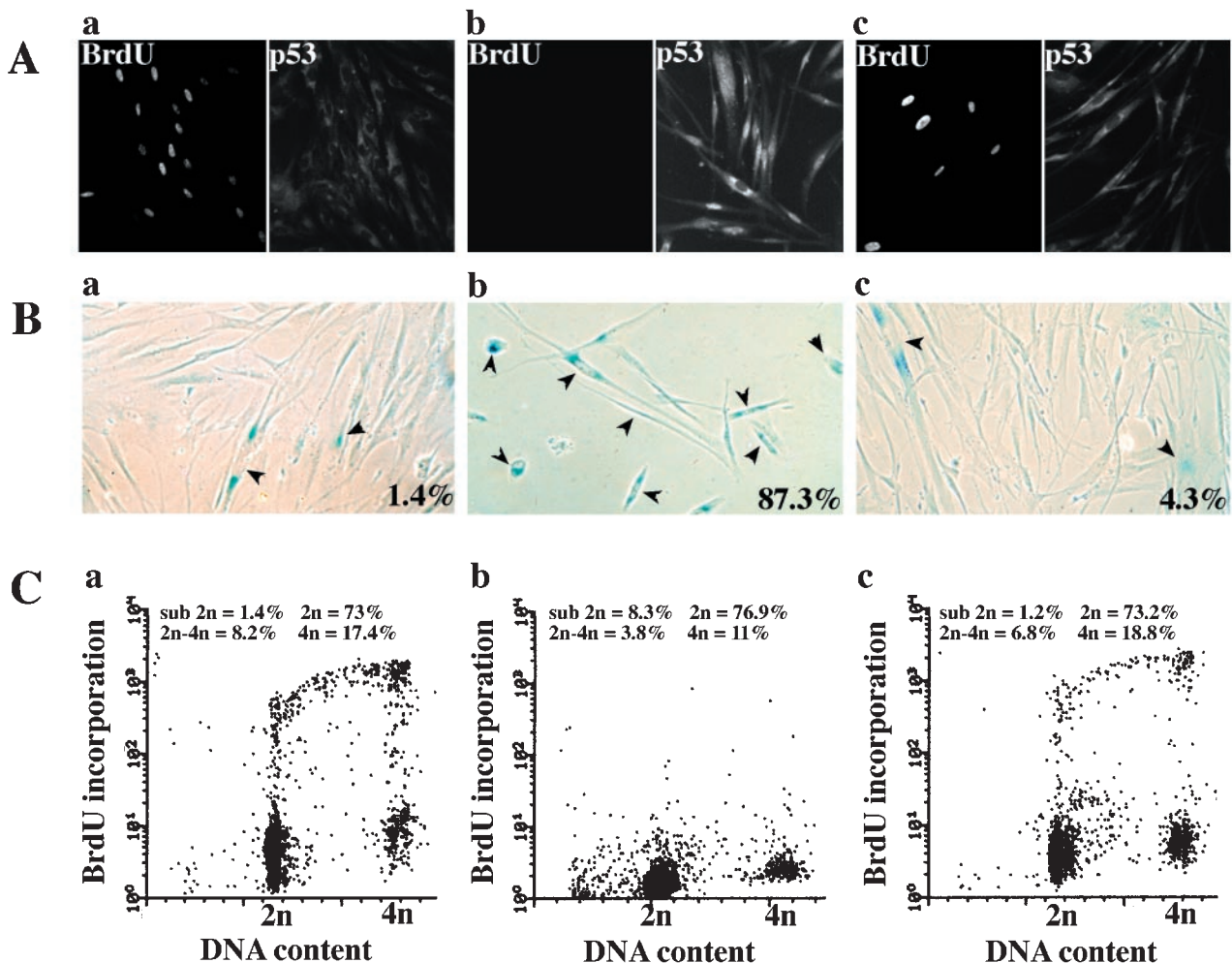
suggesting that the appearance of this senescence associated phenotype in response to LMB is dependent on p53 function. Consistent with this conclusion, LMB did not induce the SA- $\beta$ -Gal activity in SAOS2 cells overexpressing a transcriptionally inactive mutant of p53, while overexpression of wild type p53 in these cells was accompanied by the induction of this senescence associated marker (not shown).

*Reversibility of the effects of leptomycin B on HNFs*

To test the reversibility of the effects of LMB on human fibroblasts experiments were carried out using highly confluent HNF cultures to avoid the possibility that after withdrawal of the drug the progeny of highly proliferating cells less sensitive to the growth arrest induced by LMB could account for most of the cells in the culture. BrdU incorporation assays confirmed that virtually no cells in the cultures were able to synthesize DNA before the treatment with LMB (not shown). These cultures were then exposed for 3 days to 2  $\mu$ M



**Figure 4** Confluent HFF1 cells were treated with 2  $\mu$ M LMB for a total of 6 days (A) or exposed for 3 days to 2  $\mu$ M LMB after which the drug containing medium was replaced with DMEM / 10% FCS and cells were cultured for 3 more days (B). Cells were stained with a mixture of the 4B2 mouse monoclonal antibody against HDM2 and the CM1 rabbit serum against p53. Bar = 100  $\mu$ m



**Figure 5** Confluent HFF1 cultures were subjected to 3 days of continuous treatment with 2  $\mu$ M LMB after which they were allowed to recover from the treatment for another 3 days (c). In control experiments, cells were either left untreated (a) or were treated with 2  $\mu$ M LMB for 6 days (b). Cultures were trypsinized, diluted and seeded on to new plates and allowed to settle for 7 h. At this time, BrdU was added to the medium and cells were further cultured for 8 h, when they were fixed and stained with a mixture of the mouse monoclonal antibody against BrdU and the CM1 rabbit serum against p53 (A). The same type of cultures were also tested for SA- $\beta$ -Gal activity (B). The percentage of blue cells (indicated by arrows) in these cultures is shown. In parallel experiments, untreated cultures (a), cultures continuously treated with LMB (b) or cultures recovered from LMB treatment (c) were allowed reach confluence and split by trypsinization. Twenty-four hours after the cells had settled, they were pulse labelled with BrdU for 2 h and analysed by FACS (C). The proportion of cells in the cultures with different DNA contents is indicated

LMB after which the drug containing medium was replaced with fresh DMEM/10% FCS and cells were cultured for 3 more days. At this time after withdrawal of the drug, p53 and HDM2 staining in the nucleus decreased (Figure 4).

We then tested whether the HNFs could re-enter S phase after release from the LMB treatment. This was not possible by simply removing LMB. However, when HNF cultures were allowed to recover from LMB treatment and then trypsinized, diluted and seeded on to new plates some of the cells in the cultures could resume DNA synthesis (Figure 5A, panel c). This was accompanied by a decrease in the proportion of cells expressing the SA- $\beta$ -Gal activity (Figure 5B, panel c). Furthermore, after several days, the cells in these cultures proliferated (Figure 5C, panel c) and reached confluence. In contrast, cells derived from the cultures continuously treated with LMB for 6 days also adhered and spread after they were trypsinized and plated in the presence of LMB, but no replicating cells could be detected in these cultures (Figure 5C, panel b).

*Effect of LMB on the viability of human neuroblastoma cells*

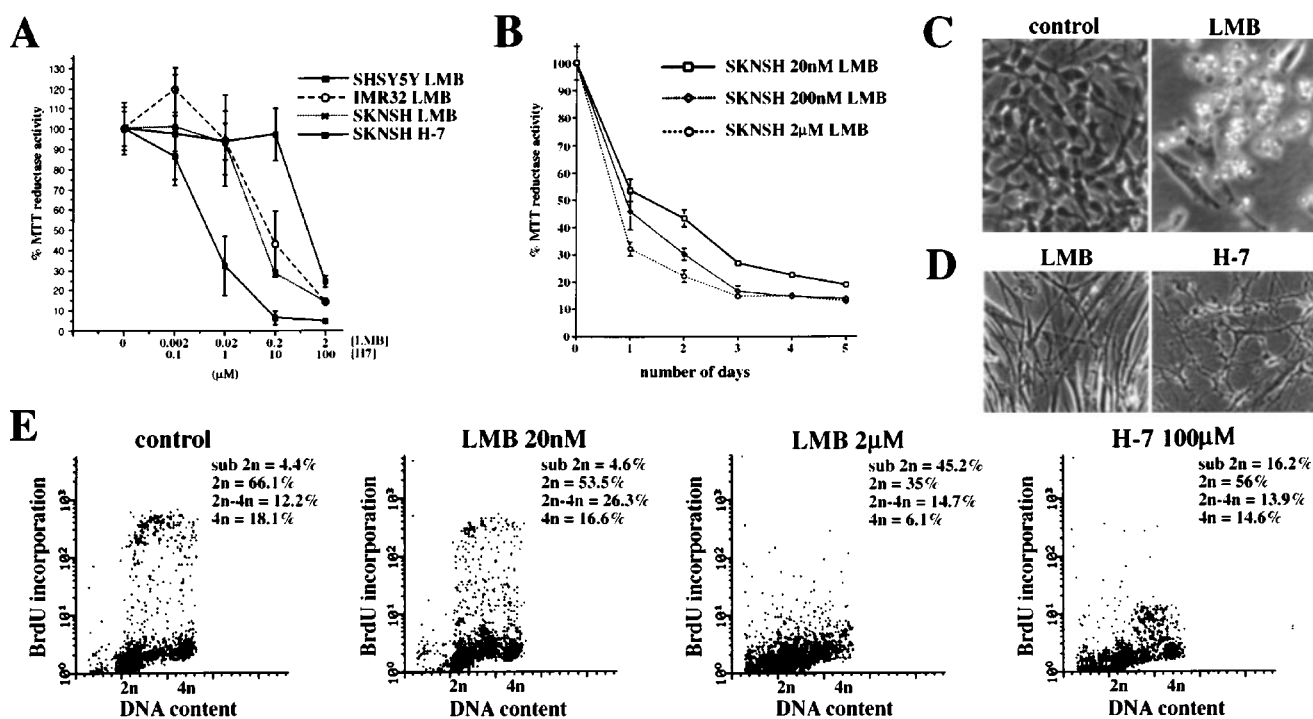
Several human neuroblastoma cell lines have been shown to express increased wild type p53 activity upon treatment with H-7, a potent serine/threonine protein kinase inhibitor (Ronca *et al.*, 1997). This induction was accompanied by a substantial decrease in the survival of neuroblastoma cells as compared with that of cell lines defective for wild type p53. When we added LMB to neuroblastoma cell cultures, we also

observed a considerable decrease in metabolic activity (Table 2, Figure 6A,B). This effect of LMB was accompanied by the detachment of cells from the culture plates (Figure 6C). LMB was able to reduce the viability of these p53 positive tumour cell lines even when added at only nanomolar concentrations. FACS analysis of SKNSH neuroblastoma cells pulse labelled with BrdU showed that these cells were unable to synthesize DNA after they were exposed to LMB. A significant proportion of cells with a sub-G1 DNA content appeared in the cultures treated with 2 mM LMB (Figure 6E).

To compare the effects of LMB and H-7 further, we tested the activity of this protein kinase inhibitor on HNFs. Unlike in the case of the LMB, the morphology of the H-7 treated HNFs was clearly altered within 24 h of treatment (Figure 6D). These results indicate that this export inhibitor is not only considerably more effective than H-7 in reducing survival of wild type p53-containing neuroblastoma cell lines but also has a milder effect than H-7 on the morphology of human primary fibroblasts.

*Overexpression of a dominant negative p53 fragment hampers the effect of leptomycin B on neuroblastoma cells*

The wild type p53 expressed in neuroblastoma cells was described to be primarily located in the cytoplasm (Moll *et al.*, 1995, 1996) and therefore the inhibition of nuclear export could be a rational way of promoting the function of p53 in these cells. However, we have not been able to observe a substantial and reliable

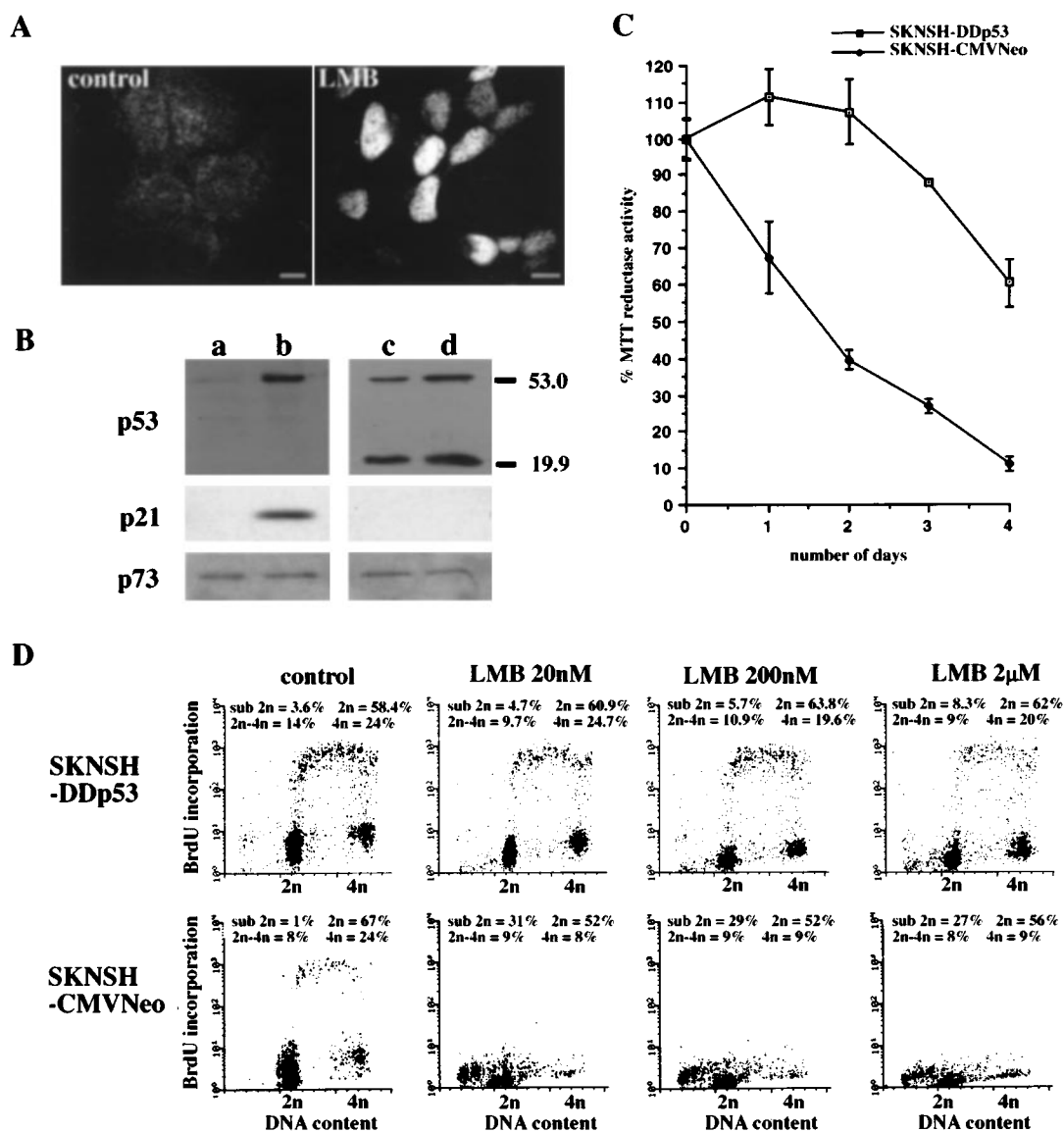


**Figure 6** (A) Analysis of the MTT-reductase activity in cultures of three human neuroblastoma cell lines (SKNSH, IMR32, and SHSY5Y) exposed to different concentrations of LMB for 24 h and of SKNSH neuroblastoma cells exposed to three different concentrations of LMB. (B) Time course analysis of the MTT-reductase activity in cultures of SKNSH cells exposed to three doses of LMB. (C) Morphology of untreated SKNSH cultures or of the same cultures exposed to 2 nM LMB for 48 h. (D) Morphology of HNFs exposed to 2  $\mu$ M LMB or 100  $\mu$ M H-7 for a period of 48 h. (E) FACS analysis of BrdU labelled SKNSH neuroblastoma cell cultures before and after exposure to 20 nM LMB, 2  $\mu$ M LMB, or 100  $\mu$ M H-7 for a period of 48 h. The proportion of cells in the cultures with different DNA contents is indicated

staining of the p53 antigen in the cytoplasm of neuroblastoma cells using a wide panel of antibodies (DO1, DO2, DO11, DO12, pAb421, CM1) (not shown). The only antibody against p53 that allowed us to detect a significant staining in the cytoplasm is the mouse monoclonal antibody 1801, which is known to cross-react with a cytoplasmic protein unrelated to p53 (Drane *et al.*, 1997).

p53 accumulation in the nucleus of SKNSH by LMB treatment was previously reported Stommel *et al.* (1999). However, it should be noted that these authors used the 1801 antibody which according to the data discussed above may not be sufficiently specific for p53. Nevertheless, using a wide panel of antibodies against p53 we also concluded that LMB induces an increase in the levels of p53 and its

accumulation in the nucleus of SKNSH neuroblastoma cells (Figure 7A). In order to test the role of p53 in the cytotoxic effect of LMB in neuroblastoma cells we created a SKNSH derived cell line permanently expressing a dominant negative mutant of murine p53 (SKNSH-DDp53 cells). Treatment with LMB increased the levels of p53 and of the product of the p53 responsive gene *p21<sup>WAF1/CIP1</sup>* in the SKNSH-CMVNeo control cells (Figure 7b). As in the case of the DFSF-DDp53 cell line, the levels of endogenous p53 were also increased by the expression of the dominant negative mutant of murine p53 in the SKNSH-DDp53 cells. However, the function of p53 as a transcriptional activator seemed to be abrogated since the levels of *p21<sup>WAF1/CIP1</sup>* in SKNSH-DDp53 cells were not responsive to the LMB treatment. As in



**Figure 7** (A) Detection of p53 by immunofluorescence staining with CM1 rabbit serum in untreated SKNSH cells or in the same cells treated with 200 nM LMB for 24 h. (B) Western blot analysis of SKNSH-CMVNeo cell extracts (lanes a and b) and SKNSH-DDp53 cell extracts (lanes c and d) with a mixture of the CM1 and CM5 rabbit sera against the endogenous human p53 (upper band) and the truncated dominant-negative murine p53 (lower band), respectively (upper panel), with the mAb1 antibody against *p21<sup>WAF1/CIP1</sup>* (middle panel) or with a rabbit serum against an N-terminal peptide of human p73 (bottom panel). Cells were left untreated (lanes a and c) or treated with 200 nM LMB for 24 h (lanes b and d). (C) Time course analysis of the MTT reductase activity of SKNSH-CMVNeo or SKNSH-DDp53 cells exposed to 0, 20 nM, 200 nM LMB, and 2  $\mu$ M LMB for 48 h. (D) FACS analysis of SKNSH-CMVNeo or SKNSH-DDp53 cells exposed to 0, 20 nM, 200 nM LMB, and 2  $\mu$ M LMB for 48 h. The proportion of cells in the cultures with different DNA contents is indicated

DFSF1 primary fibroblasts, the LMB treatment had no effects on the levels of p73 (Figure 7b).

The SKNSH-DDp53 cell line was more resistant to treatment with LMB (48 h exposure) than the control cells as determined by MTT-reductase activity assay and FACS analysis (Figure 7C and D). Longer exposure to LMB (Figure 7C) also decreased the viability of SKNSH-DDp53 cells. This effect of LMB on SKNSH-DDp53 cells after long exposures is probably related to the inhibition by LMB of the export from the nucleus of factors other than p53 or to an incomplete abolition of the p53 function in these cells.

The truncated p53 dominant negative mutant was also introduced in cells expressing inactive p53. These cells were as sensitive to the effects of LMB as the corresponding control cells (not shown). This result is consistent with the idea that the difference in the susceptibility to LMB between the neuroblastoma SKNSH-CMVNeo control cells and the SKNSH-DDp53 cells is due to the abrogation of the p53 function in the latter.

## Discussion

The search for non-genotoxic activators of the p53 tumour suppressor function is widely thought to be a promising approach in the development of new low risk strategies to treat cancers. One reasonable approach to achieve this is to inhibit the function of the factors that mediate the export of p53 from the nucleus to the cytoplasm. Addition of the nuclear export inhibitor LMB increases p53 levels and induces its accumulation in the nucleus of cells containing wild type p53 and activates p53-dependent expression of a reporter gene in cultured cells (Freedman and Levine, 1998; Lain *et al.*, 1999).

In order to test the effects of LMB on the p53 function in a normal cellular context we started our studies using human primary fibroblast cultures. In agreement with a previous report using primary rat fibroblasts (Yoshida *et al.*, 1990), here we show that LMB inhibits cell cycle progression in cultured human fibroblasts. Abrogation of the p53 function in these cells indicated that this effect of LMB may not be solely dependent on intact p53 function. Although LMB had no obvious cytopathic effect on primary fibroblast cultures it increased the number of cells expressing the SA- $\beta$ -gal activity. Several reports have demonstrated that the onset of senescence in normal cells in culture is dependent on the presence of intact p53 function (see above). In accordance with these observations, the inhibition of p53 function in these cells abrogated the induction of the SA- $\beta$ -gal activity by LMB.

Although it was generally accepted that once senescence is established reinitiation of DNA synthesis requires the loss of multiple suppressor pathways, a recent report (Gire and Wynford-Thomas, 1998) demonstrates that it is possible to reverse the senescence phenotype by interfering with the p53 function. We have shown that the withdrawal of LMB from the culture medium is sufficient to decrease the amount of p53 in the nucleus. However, and unlike in the experiments performed by Gire and Wynford-Thomas (1998) with highly specific antibodies against

p53, cells recovering from the LMB treatment, which also affects the localization of proteins other than p53, needed to be trypsinized in order to be capable of re-entering the cell cycle and lowering their SA- $\beta$ -Gal activity.

In contrast to the relatively mild and reversible effects of LMB at micromolar concentrations on HNFs, addition of LMB at nanomolar concentrations was enough to induce substantial cytotoxicity on p53 positive human neuroblastoma cell lines and had an intermediate effect on the viability of bovine aortic endothelial cells. The potent protein kinase inhibitor H-7 has been shown to have a similar effect on neuroblastoma cells (Ronca *et al.*, 1997). However, H-7 only had a significant effect when added at concentrations above 20  $\mu$ M. Furthermore, H-7 had clearly more dramatic effects on the morphology of HNFs than LMB, even when LMB was added at micromolar concentrations, more than 100 times the concentration required to induce a massive cytopathic effect in neuroblastoma cell cultures. We were also able to show that LMB induced the accumulation and localization of p53 in the nucleus of neuroblastoma cells and that, at least in part, the cytopathic effect of LMB in these cells is due to p53 or a p53-like function. The molecular mechanisms that confer the p53-mediated high susceptibility of neuroblastoma cells to the LMB treatment, are currently being analysed. The comparison of the effects of LMB on normal and tumour cells derived from the same cell type would be ideal for this purpose.

In order to test whether the p53 homologue protein p73 contributes to the effects of LMB in cultured cells, we analysed whether the treatment with this export inhibitor alters the levels of p73. Since this was shown not to be the case in either DFSF1 fibroblasts or in SKNSH neuroblastoma cells, we suggest that p73 may not play a major role in the effects of LMB on the proliferation and survival of these cells. However, it cannot be excluded that LMB does not have an effect on the activation of this p53 homologue.

Although further studies are required to establish the mechanism responsible for the effect of LMB on the function of p53, the results presented here combined with those in other reports (Freedman and Levine, 1998; Lain *et al.*, 1999; Ronca *et al.*, 1997; Stommel *et al.*, 1999) have served to gain insight into the importance for cell growth and transformation of the regulatory mechanisms governing protein export from the nucleus. The relevance of these studies is also sustained by the recently described role of the activator of the p53 function p14<sup>ARF</sup> as a nuclear export inhibitor (Zhang and Xiong, 1999).

LMB has been shown to reduce the growth of a range of tumours *in vitro* and in mouse model systems (Leopold *et al.*, 1984; Roberts *et al.*, 1986; Tunac *et al.*, 1985). However, after a phase I trial, its use in humans as an anticancer treatment was discouraged as it induced malaise and anorexia (Newlands *et al.*, 1996). Nevertheless, hints of clinical activity were seen in four out of 41 patients tested in this study. This study indicates that LMB or even less toxic variants of LMB (Kuhnt *et al.*, 1998) may not prove to be useful in the treatment of tumours probably due to their unwanted effects on a wide range of proteins, their higher toxicity on normal cells other than fibroblasts or due to their

pharmacological properties. However, we think it is reasonable to suggest that the development of ways to inhibit the nuclear export of p53 specifically is an appropriate strategy to potentiate the effect of antitumour therapies based on the induction or restoration of the p53 tumour suppressor function.

## Materials and methods

### Antibodies and reagents

The anti-p53 CM1 and CM5 rabbit sera were described in Midgley *et al.*, 1992, 1995. Mouse monoclonal anti-BrdU was purchased from Becton Dickinson. The mouse monoclonal antibodies 4B2 and 118 directed against MDM2 and p21<sup>WAF1/CIP1</sup> are described in Chen *et al.* (1993) and in Fredersdorf *et al.* (1996), respectively. Mouse monoclonal mAb1 anti-p21 antibody (Ab-1) was purchased from Oncogene Science. Anti-p73 rabbit sera were raised using full length human p73 $\alpha$  expressed in bacteria and purified as described in Midgley *et al.* (1992) or a peptide spanning amino acids 9 through 24 of human p73.

LMB was a gift from Patrick Chene and Ying Wang (Novartis) and H7 was purchased from Sigma.

### Cells and culture conditions

Normal human dermal foreskin fibroblasts DFSF1 and HFF1 are described in Lain *et al.*, 1999. GM38, MRC5 and WI38 human fibroblasts and SKNSH, SHSY5Y and IMR32 neuroblastoma cell lines were obtained from the ECACC. The DFSF1-DDp53 and SKNSH-DDp53 cell lines were derived by transfection of passage 9 DFSF1 primary fibroblasts or SKNSH neuroblastoma cells with plasmid pCMVNeop53DD which expresses a truncated mouse p53 including amino acid residues 1–14 and 302–390 under the control of the CMV promoter (Shaulian *et al.*, 1995). The control cell line SKNSH-CMV-Neo was derived by transfecting SKNSH cells with pCMV-Neo. Stable cell lines were selected at 1 mg/ml G418.

## References

- Bates S and Vousden KH. (1999). *Cell. Mol. Life Sci.*, **55**, 28–37.
- Chen J, Marechal V and Levine AJ. (1993). *Mol. Cell. Biol.*, **13**, 4107–4114.
- de Laat A, van Tilburg M, van der Leun JC, van Vloten WA and de Gruijl FR. (1996). *Photochem. Photobiol.*, **63**, 492–497.
- Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M and Campisi J. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 9363–9367.
- Drane P, Barel M, Balbo M and Frade R. (1997). *Oncogene*, **15**, 3013–3024.
- Fahraeus R, Paramio JM, Ball KL, Lain S and Lane DP. (1996). *Curr. Biol.*, **6**, 84–91.
- Fredersdorf S, Milne AW, Hall PA and Lu X. (1996). *Am. J. Pathol.*, **148**, 825–835.
- Freedman DA and Levine AJ. (1998). *Mol. Cell. Biol.*, **18**, 7288–7293.
- Freedman DA and Levine AJ. (1999). *Cell. Mol. Life Sci.*, **55**, 96–107.
- Gire V and Wynford-Thomas D. (1998). *Mol. Cell. Biol.*, **18**, 1611–1621.
- Jenney M. (1994). *Lancet*, **344**, 210–211.

### Flow cytometry analysis

Flow cytometry analysis of cells was performed as described previously (Renzing *et al.*, 1996). The cells were labelled with 30  $\mu$ M BrdU for 30 min unless otherwise stated. Analysis of BrdU incorporation was then carried out on a Becton Dickinson FACScan using Lysis II software.

### Senescence-associated $\beta$ -galactosidase (SA- $\beta$ -Gal) staining

Staining was carried out essentially as described previously (Dimri *et al.*, 1995). The number of cells expressing SA- $\beta$ -Gal and the total number of cells were counted using a  $\times 16$  Zeiss lens. For all experiments between 500 and 1500 cells were counted.

### Immunofluorescence staining

Cells were grown on Permanox chamber slides (Nunc) and stained as described in Lain *et al.* (1999). BrdU incorporation was analysed as described by Fahraeus *et al.* (1995).

### MTT-reductase assay

Drug or UVC induced cytotoxicity was examined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-reductase assay performed as described in Ronca *et al.* (1997). The plates were read at 550 nm on a Dynatech plate reader. The assays were performed in triplicate and the mean and standard deviations were calculated.

## Acknowledgements

We wish to thank Ian Ellis for his help in the culture of HNFs, Sakari Heitanen and Jean-Christophe Bourdon for proofreading of the manuscript. P Smart was recipient of a studentship from the Medical Research Campaign. S Lain and C Midgley are recipients of postdoctoral fellowships from the Cancer Research Campaign. B Vojtesek was partly supported by GA CR No.:312/99/1550. DP Lane is a Gibb fellow of the Cancer Research Campaign. This work was funded by the Cancer Research Campaign grants SP2059/0102 and SP060/0102.

- Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalou P, Lelias JM, Dumont X, Ferrara P, McKeon F and Caput D. (1997). *Cell*, **90**, 809–819.
- Kamb A. (1995). *TIGS*, **11**, 136–140.
- Kuhnt M, Bitsch F, Ponelle M, Sanglier J.-J, Wang Y and Wolff B. (1998). *Appl. Environ. Microbiol.*, **64**, 714–720.
- Lain S, Midgley CM, Sparks A, Lane EB and Lane DP. (1999). *Exp. Cell Res.*, **248**, 457–472.
- Lain S, Xirodimas D and Lane DP. *Exp. Cell Res.*, in press.
- Leopold WR, Shillis JL, Mertus AE, Nelson JM, Roberts BJ and Jackson RC. (1984). *Cancer Res.*, **44**, 1928–1932.
- Li R, Hannon GJ, Beach D and Stillman B. (1996). *Curr. Biol.*, **6**, 189–199.
- Mattaj JW and Englmeier L. (1998). *Annu. Rev. Biochem.*, **67**, 265–306.
- Melino G, Annicchiarico-Petruzzelli M, Lovat P, Farrace MG and Piacentini M. (1997). *Apoptosis and Cancer* (Book), pp. 222–244.
- Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane D and Barnes DM. (1992). *J. Cell. Sci.*, **101**, 183–189.
- Midgley CA, Owens B, Briscoe CV, Thomas DB, Lane DP and Hall PA. (1995). *J. Cell. Sci.*, **108**, 1843–1848.



- Moll UM, Laquaglia M, Benard J and Riou G. (1995). *Proc. Natl. Acad. Sci.*, **92**, 4407–4411.
- Moll UM, Ostermeyer AG, Haladay R, Winkfield B, Frazier M and Zambetti G. (1996). *Mol. Cell Biol.*, **16**, 1126–1137.
- Newlands ES, Rustin GJS and Brampton MH. (1996). *Br. J. Can.*, **74**, 648–649.
- Ostermeyer AG, Runko E, Winkfield B, Ahn B and Moll UM. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 15190–15194.
- Renzing J, Hansen S and Lane DP. (1996). *J. Cell. Sci.*, **109**, 1105–1112.
- Roberts BJ, Hamelehle KL, Selbolt JS and Leopold WR. (1986). *Cancer Chemother. Pharmacol.*, **16**, 95–101.
- Ronca F, Chan S-L and Yu VC. (1997). *J. Biol. Chem.*, **272**, 4252–4260.
- Roth J, Dobbstein M, Freedman DA, Shenk T and Levine AJ. (1998). *EMBO J.*, **17**, 554–564.
- Shaulian E, Haviv I, Shaul Y and Oren M. (1995). *Oncogene*, **10**, 671–680.
- Stade K, Ford CS, Guthrie C and Weis K. (1997). *Cell*, **90**, 1041–1050.
- Stommel JM, Marchenko ND, Jimenez GS, Moll UM, Hope TJ and Wahl GM. (1999). *EMBO J.*, **18**, 1660–1672.
- Sugrue MM, Shin DY, Lee SW and Aaronson SA. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 9648–9653.
- Tunac JB, Graham BD, Dobson WE and Lenzini MD. (1985). *J. Antibiotics*, **38**, 460–465.
- Wang Y, Blandino G, Oren M and Givol D. (1998). *Oncogene*, **17**, 1923–1930.
- White E and Prives C. (1999). *Nature*, **399**, 734–735, 737.
- Wynford-Thomas D. (1999). *J. Pathol.*, **187**, 100–111.
- Yoshida M, Nishikawa M, Nishi K, Abe K, Horinouchi S and Beppu T. (1990). *Exp. Cell Res.*, **187**, 150–156.
- Zhang YP and Xiong Y. (1999). *Mol. Cell.*, **3**, 579–591.