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MOLECULAR DETERMINANTS OF CELLULAR RESPONSE TO ANTICANCER AGENTS TREATMENT

Thesis submitted for the degree of Doctor of Philosophy at the Open

University

Discipline of Life Sciences

By

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To my wife Giovanna

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Contents	Page
Acknowledgements	3
Contents	4
List of Figures	9
List of Tables	12
Summary	13
Chapter 1 Introduction	16
1.1 Cancer and anticancer drug treatments	17
1.2 Cell cycle progression and checkpoints in normal cells	21
1.3 G1 checkpoint	28
1.3.1 The p53 gene and protein	29
1.3.1.1 Regulation of p53	32
1.3.1.2 Activation of p53	34
1.3.1.3 Functions of p53	39
1.3.2 The p53 homologue p73	42
1.3.2.1 Structure of the p73 gene	46
1.3.2.2 Regulation of p73	50
1.3.3 Interactions between p53 and p73	51
1.3.4 Mechanism of control of the G1 checkpoint mediated by p53 and p73	52
1.4 S phase checkpoint	54
1.5 G2 checkpoint	55

1.5.1 The checkpoint genes CHK1 and CHK2	61
1.5.1.1 CHK2	62
1.5.1.2 CHK1	65
1.6 Mitotic checkpoint	68
1.7 Modulation of checkpoints following DNA damage	69
1.8 Sensors of damage	72
1.9 Checkpoint defects in cancer cells	74
Chapter 2 Aims	78
Chapter 3 Materials and Methods	82
3.1 Culturing of cells	83
3.1.1 Maintenance of cells in culture	83
3.1.2 Long term storage in liquid nitrogen	84
3.1.3 Preparation of drug solutions	85
3.1.4 Cell growth inhibition induced by anticancer agents	86
3.2 Generation of cell clones with selected gene alterations	88
3.2.1 Preparation of constructs	88
3.2.2 Preparation of competent bacterial cells for transformation	90
3.2.3 Transformation of bacteria	92
3.2.4 Identification of recombinant clones	93
3.2.5 Purification of DNA from bacteria	94
3.2.6 DNA transfection	96
3.2.7 Isolation of clones stably expressing the gene of interest	97

3.3 Transient transfection and luciferase activity	98
3.4 Host cell reactivation assay	99
3.5 RNA analysis	101
3.5.1 Isolation of total RNA	101
3.5.1.1 Cesium Chloride method	101
3.5.1.2 Purification of RNA on columns	103
3.5.2 RT-PCR	103
3.5.3 Northern blotting analysis	107
3.5.3.1 Sample preparation and gel analysis	107
3.5.3.2 Blotting procedure	109
3.5.3.3 Preparation of probes	110
3.5.3.4 Hybridisation	111
3.5.4 Gene expression by microarray technology	112
3.6 Western blotting	114
3.6.1 Preparation of cellular proteins	114
3.6.1.1 Calibration curve preparation	115
3.6.1.2 Determination of protein concentration in cellular extracts	116
3.6.2 SDS-PAGE	116
3.6.3 Protein transfer and detection	118
3.7 Evaluation of kinase activity	120
3.8 Screening of genomic libraries	121

3.9 Flow cytometric analysis of cell cycle phases distribution	123
Chapter 4 Results	125
4.1 p53 and its effect on cellular response to anticancer agents	126
4.1.1 Introduction	126
4.1.2 p53 and DDP	127
4.1.3 p53 and taxol	135
4.1.4 Post-translational p53 modifications	138
4.1.5 p53 and “new drugs”	145
4.1.6 Discussion	147
4.2 p73 and its effect on cellular response to anticancer agents	149
4.2.1 Introduction	149
4.2.2 Characterization of stable p73-overexpressing clones	150
4.2.3 DNA repair capacity of the p73 overexpressing clones	159
4.2.4 Sensitivity of p73 overexpressing clones to several anticancer agents	162
4.2.5 Discussion	165
4.3 CHK1 and its effects on cellular response to anticancer agents	167
4.3.1 Introduction	167
4.3.2 The effect of overexpression of mutant CHK1 in human osteosarcoma cells	168
4.3.3 The effect of overexpression of mutant CHK1 in human colon cancer cells	172

4.3.4 The effect of CHK1 inhibition on cellular response to DDP	175
4.3.5 Discussion	178
4.4 CHK2 and its effects on cellular response to anticancer agents	180
4.4.1 Introduction	180
4.4.2 Generation and properties of cells overexpressing dominant negative CHK2	180
4.4.3 Discussion	184
4.5 Cross-talk between p53 and CHK1	186
4.5.1 Introduction	186
4.5.2 p53 status and CHK1 protein levels	186
4.5.3 Cell cycle analysis and CHK1 levels following DDP treatment	191
4.5.4 Mechanism of p53-induced downregulation of CHK1 levels	193
4.5.5 Isolation of CHK1 genomic sequences	197
4.5.6 Discussion	205
Chapter 5 General discussion	207
Chapter 6 References	215
Chapter 7 Appendices	252
7.1 List of abbreviations	253
7.1 List of publications	255

List of figures

<u>Figure</u>	<u>Page</u>
1.1 Schematic representation of the cell cycle phases	22
1.2 Cyclins and cyclin dependent kinases participating in cell cycle regulation in mammals	23
1.3 Cyclins and cdks during the cell cycle. I	26
1.4 Cyclins and cdks during the cell cycle. II	27
1.5 Structure and domains of the p53 protein	31
1.6 Post-translational modifications of p53	37
1.7 Schematic representation of the pathways induced by p53 following activation	38
1.8 p73 structure and homology between p53, p63 and p73	43
1.9 Structure of TA p73 and of DN p73	47
1.10 C-terminal splicing variants of p73	49
1.11 CHK1 and CHK2 phosphorylation of cdc25C	60
1.12 Structure of human CHK2 gene	63
1.13 Structure of human CHK1 gene	67
1.14 Pathways activated in mammalian cells upon stress induction	70
1.15 ATM and ATR-dependent pathways	73
4.1 Activity of DDP in HCT116 and HCT116/E6 cells	129
4.2 Activity of DDP in HCT116 p53 ^{-/-} and HCT116 p53 ^{+/+} cells	130

4.3 Induction of p53 and p21 following DDP treatment	131
4.4 Activity of DDP in HCT116 p21 ^{-/-} and HCT116 p21 ^{+/+} cells	133
4.5 Activity of DDP in A2780 and A2780/E6 cells	134
4.6 Activity of taxol in A2780 and A2780/E6 cells	136
4.7 Activity of taxol in HCT116 p53 ^{-/-} , p53 ^{+/+} , p21 ^{-/-} and p21 ^{+/+} cells	137
4.8 p53 phosphorylation following DDP or taxol treatment in HCT116 cells	139
4.9 p53 phosphorylation following DDP or taxol treatment in A2780 cells	141
4.10 Effect of wortmannin on DDP-induced phosphorylation of p53	143
4.11 Role of ATM and ATR kinases in p53 phosphorylation induced by DDP	144
4.12 Activity of roscovitine and TSA in HCT116 p53 ^{-/-} and HCT116 p53 ^{+/+} cells	146
4.13 In vitro growth of p73 overexpressing clones	152
4.14 Pseudocolor representation of gene expression profiles in A2780/p73.4 and A2780/p73.5 clones	153
4.15 Quantitative analysis of gene expression profiles in A2780/p73.4 and A2780/p73.5 clones	155
4.16 Northern blot analysis of NER genes in p73-overexpressing clones	158
4.17 DNA repair activity in A2780 parental and p73-overexpressing	

cells	160
4.18 Activity of DDP, UV, taxol and topotecan in p73-overexpressing clones	163
4.19 Activity of roscovitine and TSA in p73-overexpressing clones	164
4.20 Activity of DX and MNNG in U2OS, U2OS/CHK1 and U2OS/D130 cells	171
4.21 CHK1 protein levels in HCT116-derived clones measured by Western Blotting	173
4.22 In vitro growth of HCT116, HCT116/CHK1 and HCT116/D130 cells	174
4.23 Effect of UCN-01 on the cytotoxic activity of DDP	177
4.24 CHK2 mRNA expression in U2OS-derived clones	182
4.25 In vitro growth of clones overexpressing CHK2	183
4.26 Cytotoxicity of DDP in CHK2 overexpressing clones	185
4.27 CHK1 protein levels in HCT116 and HCT116/E6 cells treated with DDP	188
4.28 Quantitative analysis of the inhibition of CHK1 levels	189
4.29 CHK1 protein levels in HCT116 p53+/+ and HCT116 p53-/- cells treated with DDP	190
4.30 Cell cycle phase distribution in cells treated with DDP	192
4.31 CHK1 mRNA levels in HCT116 and HCT166/E6	195
4.32 CHK1 levels in U2OS cells treated with DDP	196
4.33 Autoradiograph of a genomic library spotted on filters and	

hybridized with a CHK1 cDNA probe	199
4.34 Southern blot of a genomic CHK1 clone	200
4.35 Sequence of the 864 bp CHK1 genomic fragment	203
4.36 Luciferase activity in cells transfected with the 864 bp CHK1 genomic fragment	204

List of tables

4.1 List of relevant genes overexpressed in p73-overexpressing clones	157
4.2 Activity of DDP against U2OS, U2OS/CHK1 and U2OS/D130 cells	169
4.3 Activity of DDP against HCT116, HCT116/CHK1 and HCT116/D130 cells	176

SUMMARY

The response of cancer cells to treatment with anticancer agents is mediated by several factors, among which the functionality of proteins participating in the control of cell cycle progression and genomic integrity is an important one. The studies reported here were aimed at understanding the role of crucial proteins, participating in key processes in normal cells, in determining cellular sensitivity towards the cytotoxicity of anticancer agents. Four different proteins have been investigated: p53, p73 and the two cell cycle checkpoint proteins CHK1 and CHK2.

P53 has been selected because of its undisputed role in tumor formation and development, for its high prevalence of mutations in human cancer and for its role in normal cells in response to different stimuli. P73 is a structural homologue of p53 which shares functions with p53 but in addition has other very distinct functions. The two checkpoint proteins CHK1 and CHK2 control cell cycle progression subsequent to DNA damage, particularly during the S and G2 phases of the cell cycle, and their importance is currently under intensive elucidation.

The studies have been conducted in isogenic cell systems, to minimize as much as possible interference by other alterations invariably present when two different cell types are considered. Using two widely used anticancer agents, cisplatin (DDP) and taxol, it was shown that for both drugs the presence of functional p53 was associated with resistance

to drug-induced cytotoxicity. These results have been obtained in different experimental systems of human cancer cells of epithelial origin such as the colon carcinoma cell line HCT116 and the ovarian cancer cell line A2780. With respect to p73, the work took advantage of the availability in the laboratory of two subclones overexpressing p73 derived from a human ovarian cancer cell line by transfection with the p73 alpha cDNA. These two clones over-express DNA repair genes, particularly those participating in the nucleotide excision repair (NER). These cells are prone to repair lesions recognized by NER. Due to this fact, the p73-overexpressing clones were less susceptible to treatment with DDP and UV irradiation, as both lesions are recognized by NER.

Other drugs, such as doxorubicin and topotecan inducing damage which is not repaired by NER, exhibited similar activities in parental and p73-overexpressing clones. For both CHK1 and CHK2, experiments which were performed using clones transfected with dominant negative mutants failed to show differences between transfected clones and parental cells in the cytotoxicity of DNA-damaging agents. However the use of inhibitors of these kinases resulted in increased activity of DDP, suggesting that both CHK1 and CHK2 may play a role in determining sensitivity of cancer cells to drugs, but that the dominant negative mutants in some way masked these effects.

The potential involvement of CHK1 in response to stress is underlined by evidence of a link between p53 and CHK1, implying that

they mutually regulate each other in a way which controls both the activation and the repression of checkpoint response following DNA damage.

1. INTRODUCTION

1.1 Cancer and anticancer drug treatments

Cancer still represents one of the leading causes of death in western countries.

The currently available strategies for the treatment of cancer include surgery, radiotherapy and chemotherapy. Surgery and radiotherapy have been proved to be moderately effective in the treatment of different tumor types. For the majority of tumors, chemotherapy, either following surgery or radiotherapy, remains the treatment of choice. The majority of the drugs so far used in clinical practice as anticancer agents are broadly acting antiproliferative, cytotoxic drugs (Workman 2001; Zhang 2002). The mechanism of action of these drugs is relatively unspecific, as their activity is generally directed against the synthesis, structure and function of DNA, or against mechanisms of cell division.

Such cytotoxic drugs have helped to bring about the considerable improvement in treatment outcome in the case of many cancers observed in the last 2 decades (Adjei 1999; Bengtson and Rigas 1999; Thigpen 2000; Worden and Kalemkerian 2000). There is no doubt that, for example, the introduction of cisplatin, a DNA-interacting agent, or taxanes, tubulin interfering drugs, has provided a dramatic improvement in morbidity and mortality over previously available therapies for patients suffering from cancer (Greco and Hainsworth 1999; Fossella 1999; du Bois et al. 1999; Belani 1999; Ozols 2000).

The use of these compounds, however, has drawbacks. These drawbacks include their high toxicity against normal cells, which is responsible for the relatively low therapeutic index of these compounds, the sometimes limited activity and the susceptibility of cancer cells to induction of drug resistance by these molecules. A huge amount of work has been focussed on the synthesis of new analogues of clinically active anticancer agents, with the aim to identify molecules overcoming some of the detrimental effects of these compounds.

The widely used anticancer agent cisplatin serves as an instructive example. Its major dose-limiting toxicity is nephrotoxicity (Meyer and Madias 1994; Alberts and Noel 1995). Different platinum containing analogues have been developed, which have shown reduced renal toxicity. One of these analogues, carboplatin is now in clinical use (Ozols 1992). Similarly, cisplatin or carboplatin have shown reduced activity in cancer cells with defects in genes involved in mismatch repair (MMR) (Fink et al. 1998; Durant et al. 1999). Since such defects are relatively frequent in human colon, gastric and endometrial cancers, research efforts are directed at the identification of molecules which are able to overcome this effect without loss of the potent antitumor activity of cisplatin or carboplatin.

Oxaliplatin, a 1,2 diaminocyclohexane containing derivative of cisplatin, has been reported in experimental systems to be equally active in cancer cells proficient or deficient in MMR (Raymond et al. 1998). This

property is obviously an advantage, and opens up the possibility to use such analogues in tumors with specific and defined genetic defects (Cvitkovic and Bekradda 1999; Wiseman et al. 1999).

In addition to the search of analogues of currently used anticancer agents, much effort has been devoted in recent years to the discovery of new anticancer agents with possibly more specific, tumor-targeted mechanisms of action. The selection and synthesis of these molecules is now possible thanks to a better understanding of the molecular pathways regulating the function of normal cells and to the results of molecular studies dedicated to the elucidation of the molecular mechanisms responsible for the development of neoplasia. The increasing knowledge of the molecular targets to be hit in cancer cells, will enable the selection of new targeted drugs to be used alone or in combination in the treatment of tumors with suitable molecular characteristics.

The approach of defining gene defects in tumors in order to select a specific target-oriented drug for therapy is valid for gene alterations which are directly related to the initiation and progression of a tumor. In this case targeting this defect is likely to induce specific killing of the cancer cell. There are examples of tumors, specially leukemias, with characteristic chromosomal translocation producing constantly activated kinases, for example the bcr-abl fusion protein, which can be specifically hit by drugs acting against the aberrantly activated target (O'Dwyer and Druker 2000; Druker 2002; Capdeville et al. 2002; Barbany et al. 2002). Nevertheless the

vast majority of malignancies present multiple gene defects which are not easily targeted by a single drug. If different mutations or gene alterations are present in the same tumor, the possibility to use combinations of target-oriented drugs is attractive. On the other hand, the knowledge of the gene and molecular pathway defects in tumors could also be used to select the best available conventional therapy for that specific tumor.

In this case, the increase in the knowledge of the molecular characteristics of a tumor needs to be associated with the knowledge of the action of anticancer agents and on the role of proteins known to be altered in tumors as determinants of their activity. This will be of help in selecting the best possible therapy with drugs with well established activity.

One of the areas which seems to be particularly attractive for the future selection of new molecules or new combinations of therapies is cell cycle regulation. It is in fact well established that cancer cells invariably harbor disrupted or aberrantly regulated pathways that mediate cell growth and cell death (Hall and Peters 1996; Sherr 2000; McDonald and El-Deiry 2001; Bunz 2001).

In normal cells there is a continuous balance between cell death and cell growth, which is finely regulated by proteins acting as checkpoints (Morgan 1995; Polymenis and Schmidt 1999; Kastan 2001; Bartek and Lukas 2001a). During the cell cycle, each step is rigorously controlled by a protein or by a group of proteins that verify that no alterations occur before proceeding to the next step. In contrast, malignant cells are often

characterised by the fact that this regulation is defective. Details of differences in cell cycle regulatory machinery between normal and malignant cells will be described in more detail in the following chapters.

1.2 Cell cycle progression and checkpoints in normal cells.

To divide and pass genetic material to daughter cells, a cell needs to replicate its DNA and to segregate the duplicated chromosomes thus formed. The phase in which DNA is replicated is called phase S (synthetic), while the division phase is called M phase (mitotic). Between the S and M phases, there are two gaps, G1 and G2, respectively, which are intermediate phases, in which a cell prepares itself for DNA synthesis (G1) or mitosis (G2) (Nurse 1994; O'Connell and Nurse 1994) (Fig. 1.1).

From a molecular point of view, the progression through the different phases of the cell cycle is mediated by a highly conserved family of protein kinases, called cyclin-dependent kinases (cdk) (Pines 1994; Doree and Galas 1994). These kinases need to be activated through the binding with a specific regulatory subunit, called cyclin (Morgan 1995; Lees 1995; Arellano and Moreno 1997; Pavletich 1999). The different cyclins identified so far have been given letters A to T, while the different cdk's are numbered 1 to 11 (Jesus and Ozon 1995) (Fig. 1.2). The cdk/cyclin complexes are universal cell cycle regulators, and each complex controls a specific transition between the different cell cycle

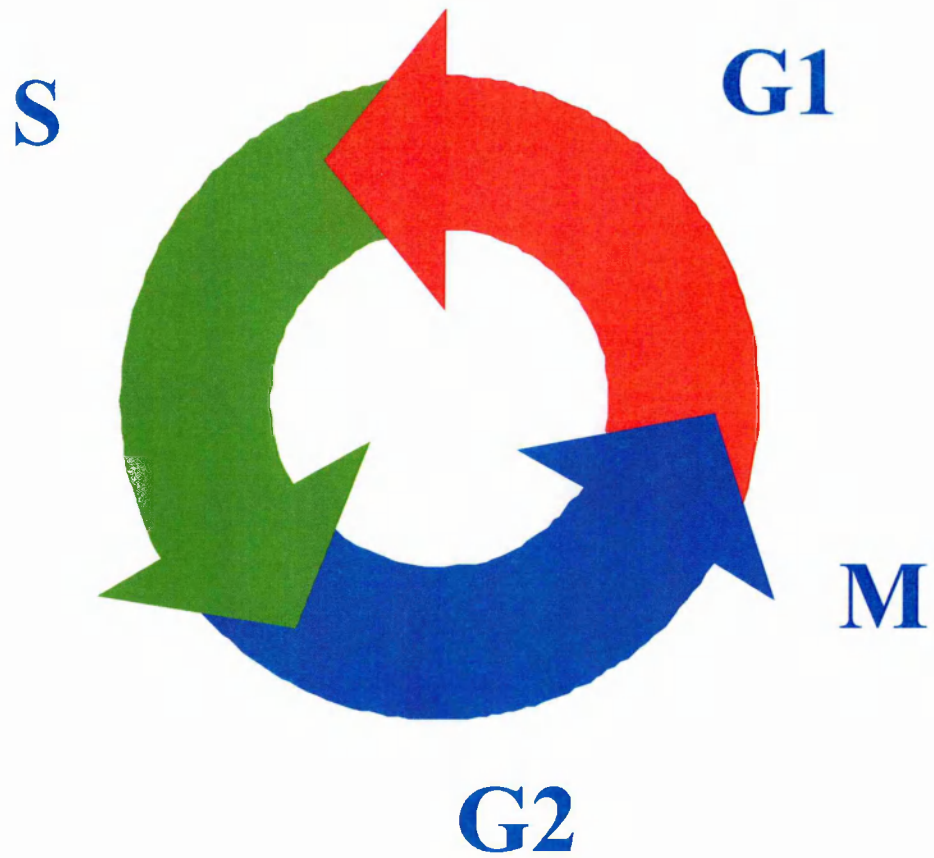


Figure 1.1

Schematic representation of the different phases of the cell cycle: synthesis (S), mitosis (M) gap 1 (G1) and gap 2 (G2).

CYCLINS

A
B1,B2
C
D1,D2,D3
E
F
G
H
I
T1,T2a, T2b

**CYCLIN-DEPENDENT
KINASES**

CDC2
CDK2
CDK3
CDK4
CDK5
CDK6
CDK7
CDK8
CDK9
CDK10
CDK 11

Figure 1.2

List of the known cyclins and cyclin dependent kinases participating in cell cycle regulation in mammals.

phases, although some redundancy exists. The cdk/cyclin complexes act by phosphorylating specific and distinct substrates which are mediators of their activity. Moreover, the activity of the complex between cdks and cyclins is further controlled by small proteins called cdk inhibitors (Elledge and Harper 1994; Harper 1997; Sherr and Roberts 1999), which can be divided into two groups, one of which works by directly inhibiting the catalytic function of the cdk/cyclin complex (p21, p27, p57), the other working by binding to the cyclin regulatory subunit and detaching it from the cdk (INK4 family) (Xiong et al. 1993; Carnero and Hannon 1998; Pavletich 1999). During the cell cycle, the levels of cdks are relatively constant, while cyclin levels fluctuate during the different phases (Morgan 1995; Arellano and Moreno 1997). The fluctuation of the levels is one of the reasons why these proteins have been called cyclins. A common molecular feature of cyclins is the presence in their aminoacidic sequence of the so called cyclin box, an approximately 100 amino acid motif which is responsible for the interaction of cyclins with cdks (Nugent et al. 1991). A further mechanism of regulation of cdk activity is through post-translational modification. As an example, *cdc2*, working mainly in G2 phase, is phosphorylated at threonine 161 by the cdk activating kinase CAK, and this modification results in activation of kinase activity (Rhind et al. 1997; Kaldis 1999). Two other modifications, i.e. phosphorylation at threonine 14 and at tyrosine 15, act as negative regulators of the kinase activity (Coleman and Dunphy 1994; Berry and Gould 1996; Fattaey and

Booher 1997). This negative regulation mechanism is relieved by the action of specific phosphatases of the CDC25 family, which comprises CDC25A, CDC25B and CDC25C (Jesus and Ozon 1995; Draetta and Eckstein 1997). In particular, the two phospho residues at threonine 14 and tyrosine 15 of *cdc2*, are specifically removed by CDC25C. Phosphorylations at threonine 14 and tyrosine 15 inhibit *cdc2* activity, and the removal of these two modifications is mandatory for the cell to proceed through mitosis (Berry and Gould 1996; Fattaey and Booher 1997; Zachariae 1999).

The different cdk complexes act in concert throughout the cell cycle. The activation of one cdk follows the inactivation of the preceding one (Figs 1.3 and 1.4).

All these processes are constantly monitored, and at the cross-road between one cell cycle phase and the next, there is a checkpoint that must ensure that all the processes have been correctly pursued (Nojima 1997; O'Connor 1997; Johnson and Walker 1999; Kastan 2001; Melo and Toczyski 2002). It has in fact to be carefully checked that, during S phase, alterations in duplication of the DNA do not occur, to avoid the segregation of aberrant genetic material to the daughter cells. Similarly, during mitosis, the correct division of the genetic material is mandatory. If any of these conditions are not fulfilled, the different checkpoint proteins become activated, inducing cell cycle arrest, and, if the entity of the lesion is repairable, the damage is removed or, if the lesion is unrepairable, programmed cell death is activated.

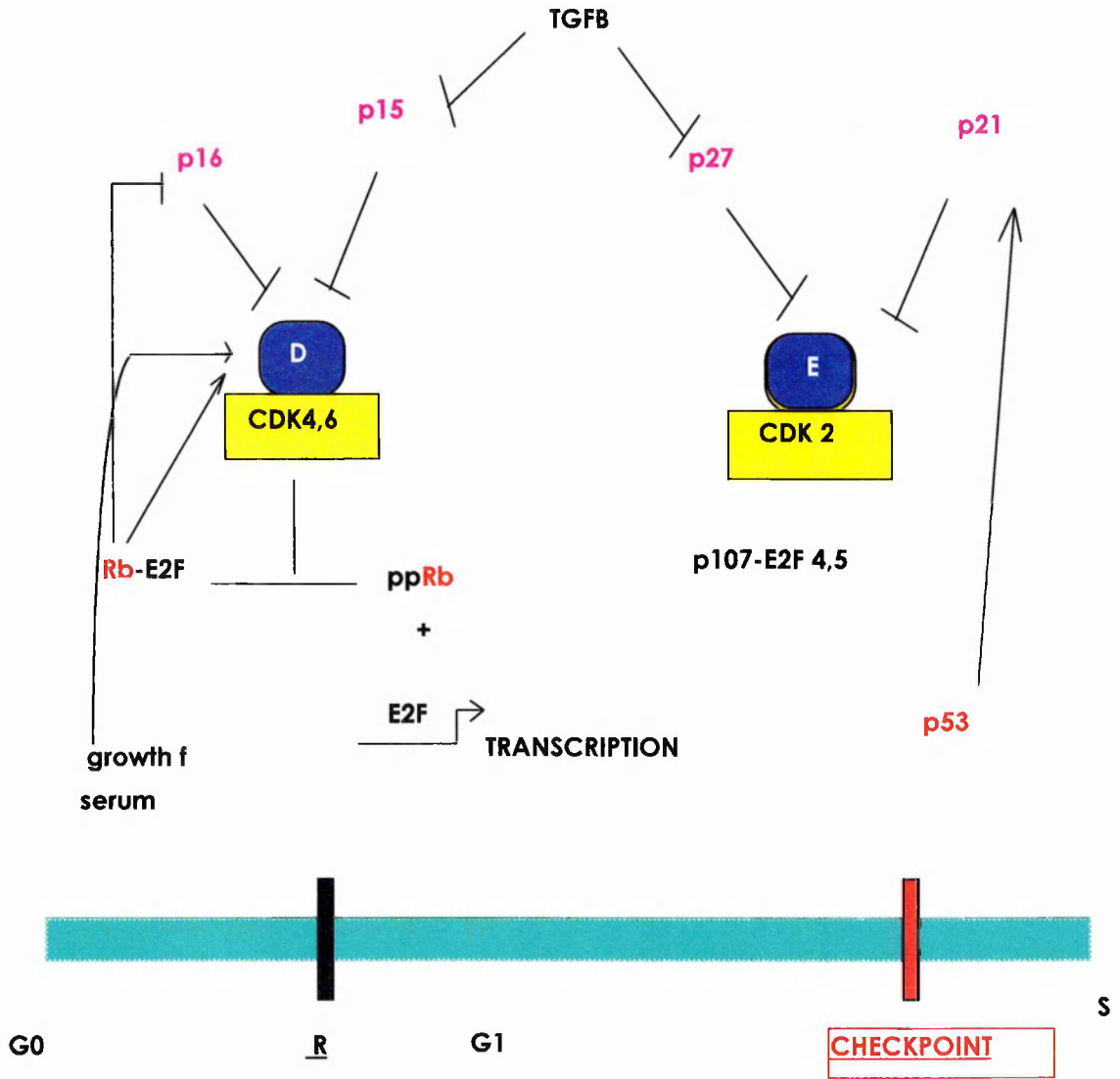


Figure 1.3

Temporal association between cyclins and cdk during the cell cycle. The scheme reports some important proteins participating in the passage between G₀, G₁ and S phases. R is the restriction point; Rb retinoblastoma. The G₁ checkpoint controlled by p53 is indicated.

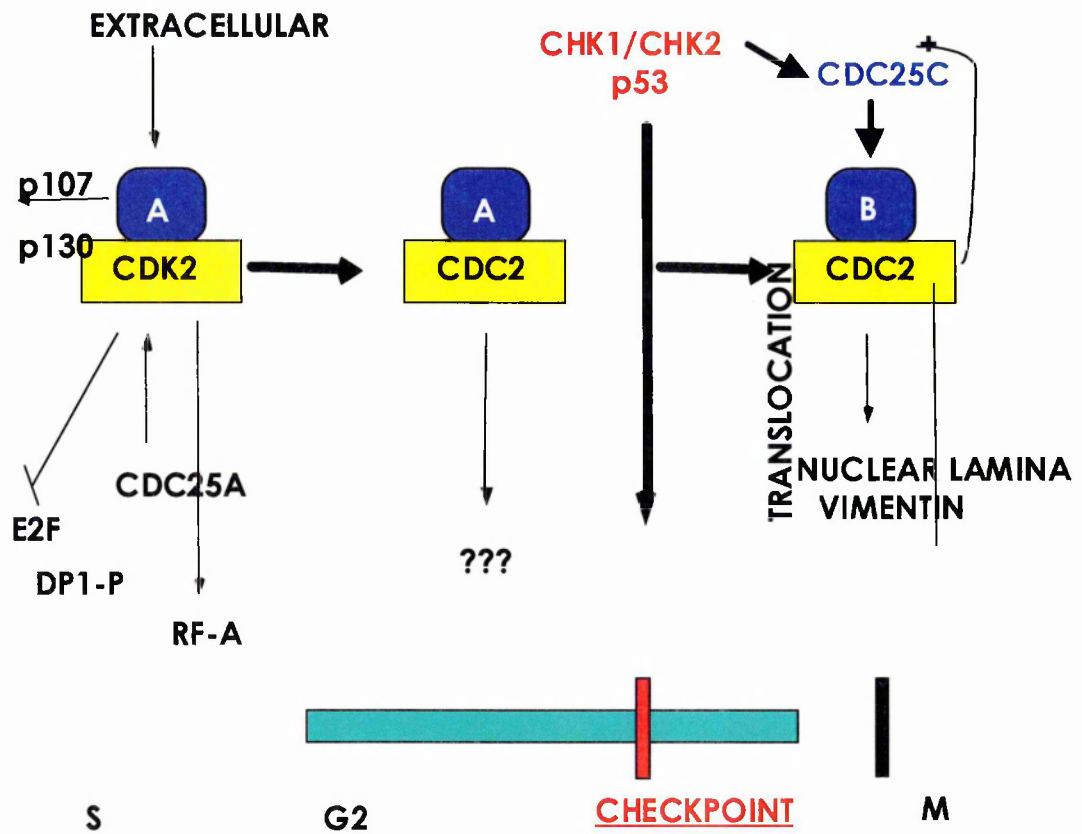


Figure 1.4

Temporal association between cyclins and cdks during the cell cycle. The scheme reports some important proteins participating in the passage between S, G2 and M phases. The G2 checkpoint controlled by CHK1 and CHK2 is indicated.

For each phase of the cell cycle a distinct checkpoint has been characterized, although often there are overlapping functions between the different proteins participating in the checkpoints.

1.3 G1 checkpoint

This mechanism of control is essential, considering that during the G1 phase cells embark on critical decisions such as commitment to replicate the DNA and to complete the cell division cycle. If sufficient proliferation stimuli are present, at the so called "restriction point" (Planas-Silva and Weinberg 1997) in late G1, there is the decision to be made to enter into S phase. This decision appears to be irreversible in normal unstressed cells (Bartek and Lukas 2001b; Bartek and Lukas 2001c). Even if cells have passed this point, genotoxic stress can still activate checkpoints which delay the further progress before S phase. At the restriction point there is the passage between a mitogen-dependent growth to a mitogen-independent growth.

During G1 phase, two temporally distinct checkpoints can be envisaged. A first, very rapid induction of G1 arrest does not need to be dependent on transcription and protein synthesis (Bartek and Lukas 2001b). Emerging evidence suggests that targeted ubiquitination of the phosphatase CDC25A, which normally abrogates the inhibitory phosphorylation of CDK2, is a rapid and efficient system to halt cell cycle

progression. The signal activating the proteasome-mediated degradation of CDC25A is the phosphorylation of the phosphatase by protein kinases CHK1 and CHK2 (Mailand et al. 2000; Falck et al. 2001; Bartek and Lukas 2001b).

The second G1 checkpoint is a late G1 arrest, which is transcription dependent. This checkpoint is mainly governed by the product of the tumor suppressor gene p53. This nuclear protein is normally present at very low levels in undamaged cells, because it is rapidly exported to the cytoplasm and degraded by the proteasome (Haupt et al. 1997). The signal mediating the nuclear-cytoplasmic transport of p53 is its binding with the protein mdm2 (Haupt et al. 1997; Honda et al. 1997). Upon damage, a very rapid accumulation of p53 is observable in the nucleus. This accumulation requires the detachment of mdm2, and it is likely to be a consequence of post translational modifications of p53, particularly phosphorylation at the N-terminus, the region which binds mdm2 (Shieh et al. 1997; Banin et al. 1998; Meek 1998).

1.3.1 The p53 gene and protein

The p53 gene has been discovered in the early 1980s as a gene product associated with the large T antigen. Initially thought to work as an oncogene, the crucial role of p53 in many different cellular functions became clear later on (Oren 1985; Oren and Prives 1996a; Levine 1997).

The p53 gene, mapping on chromosome 17, encodes for a protein of 393 aminoacids with an apparent molecular weight of 53 KDa. The cDNA encoding for p53 is highly conserved between different species. It consists of different domains (Fig 1.5) each characterized by a specific function (Haffner and Oren 1995). The central DNA-binding domain is crucial for the activity of p53. In fact, p53 acts mainly as a transcription factor and is able to recognize with relative specificity the DNA sequence 5' - PuPuPuCT/AT/AGPyPyPy- 3' (Levine 1997). The conservation through evolution of p53 is particularly evident in this region.

A second region, contained the first 60 aminoacids at the N-terminus, constitutes a transcriptional activation domain which is able to interact with the basal transcriptional machinery and positively regulates gene expression. The amino acids at position 13-23 of the human p53 protein are highly conserved in different species, indicating that this feature is another critical region of the protein. This region is in fact the one responsible for the binding to the two TFIID subunit, TATA-associated factors TAF70 and TAF31 (Lu and Levine 1995; Baptiste et al. 2002). Furthermore, this is the region in which the negative regulator of p53, mdm2, binds (Bottger et al. 1997). The crucial amino acids responsible for the binding of p53 to mdm2 have been identified thanks to the availability of the crystal structure of the N-terminal region of mdm2 with a p53-derived peptide containing aminoacids 13-29 (Kussie et al. 1996). This region has previously been shown by mutational analysis to be

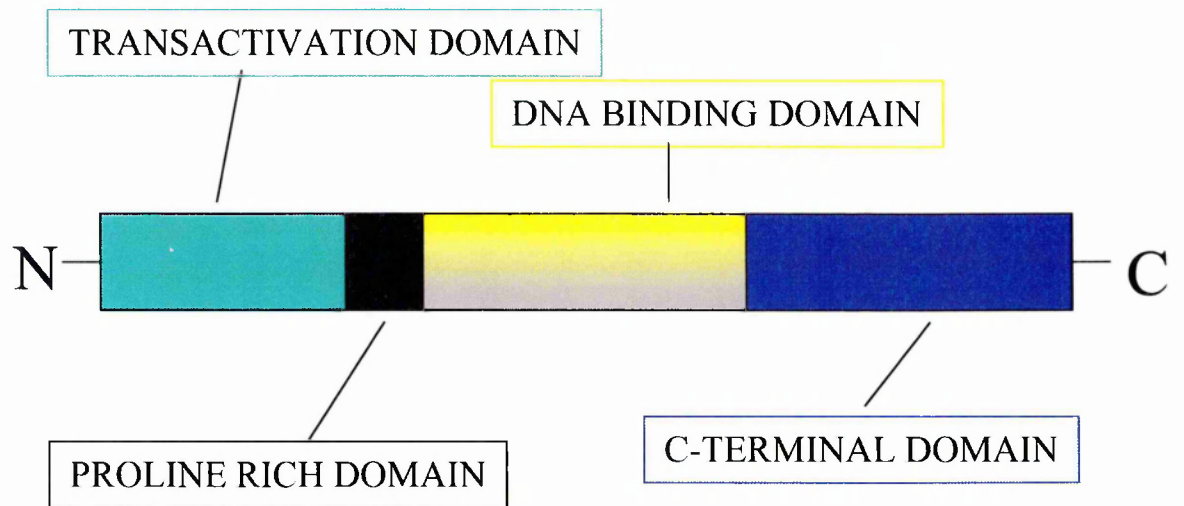


Figure 1.5
Structure and domains of the p53 protein.

potentially involved in mdm2 binding (Freedman et al. 1997). Interestingly, this part of the protein contains different residues which can undergo phosphorylation.

The third region, the C-terminal region, contains the residues important for oligomerization, as the native p53 protein is present as a tetramer (Oren 1985; Haffner and Oren 1995). The C-terminus contains another DNA binding domain that recognizes various forms of DNA not in a sequence-specific way, but rather in a structure-specific manner (Ahn and Prives 2001). This part of the molecule can bind with high affinity to a wide variety of DNA structures, including three- and four-way junctions, stem-loops, single stranded ends, insertion or deletion mismatches, irradiated DNA and DNA aggregates (McKinney and Prives 2002).

The p53 molecule is present in two conformationally distinct forms, one latent and the other active in sequence specific binding to DNA. One of the functions of the C-terminus of p53 is thought to be the ability to drive the molecule toward these two forms (Hupp and Lane 1994; Ahn and Prives 2001).

1.3.1.1 Regulation of p53

In normal, unstressed conditions, levels of p53 are very low. Immediately after a stress, it is induced, and its levels rapidly rise and accumulate at nuclear site (Vogelstein and Kinzler 1992; Ko and Prives 1996; Oren and Prives 1996a). This mechanism is mainly post-

translational, and one of the crucial steps is mediated by the interaction of p53 with its regulator mdm2. In normal conditions, in fact, p53 is bound by mdm2, and this binding is a signal leading to nuclear export and ubiquitination (Haupt et al. 1997; Michael and Oren 2002). Once ubiquitinated, p53 is degraded through the proteasome.

The mechanism involved in mdm2-dependent degradation of p53 has been partially elucidated. Mdm2 possesses activities of a ubiquitin ligase and is able to perform self-ubiquitination and ubiquitination of its substrates (Haupt et al. 1997; Buschmann et al. 2000). Mdm2 shows an E3 ubiquitin ligase activity towards p53 (Honda et al. 1997). The role of mdm2, however, is not only that of simply adding ubiquitin molecules to p53, but recent evidence indicates that there are consecutive regulated steps that occur before degradation of p53 occurs. In fact p53 ubiquitination occurs mainly in the nucleus, and p53 thus modified is then exported through an active mechanism controlled by mdm2 into the cytoplasm, where it is degraded by cytoplasmic proteasomes (Geyer et al. 2000; Boyd et al. 2000). To support this mechanism, mdm2 indeed contains in its structure nuclear localization and export signals which are necessary for its shuttling between nucleus and cytoplasm (Roth et al. 1998). It is thought that ubiquitination is required for nuclear export of p53 (Inoue et al. 2001; Michael and Oren 2002). In addition recent evidence indicates that mdm2 can participate in p53 degradation through its acidic domain, hence by a mechanism distinct from its E3 ligase activity (Argentini et al.

2001). The activity of mdm2 is controlled by post-translational modifications such as phosphorylation and sumoylation, which add additional levels of complexity to the picture. (Buschmann et al. 2000; Maya et al. 2001; Goldberg et al. 2002)

The importance of mdm2 in the homeostasis of p53 has become clear when attempts to generate knock out mice for mdm2 failed due to embryo lethality. This lethality was shown to be avoided by preparing mdm2 knock out mice in a p53 null background (Montes de Oca Luna et al. 1995). The reason why mdm2 mice did not proceed to birth is probably because levels of p53 are constantly high (lacking its main negative regulator), thus inducing growth arrest and/or apoptosis in all cells in the absence of stress induction (de Rozières et al. 2000).

1.3.1.2 Activation of p53

From all this evidence, it appears clear that activation of p53 following damage needs a starting point: the detachment of p53 itself from mdm2. Two distinct mechanisms have been so far described. One is related to the ability of p53 to undergo phosphorylation at the N-terminus following stress induction (Lane 1998). This phosphorylation is a signal leading to the release of mdm2 from p53 (Prives 1998). The residues in p53 important for interaction with mdm2 are mostly serine 15 and serine 20. These two sites can be phosphorylated in vitro by different kinases, including ATM, ATR, DNA-PK, CHK1 and CHK2 (Meek 1998; Kapoor

and Lozano 1998; Banin et al. 1998; Chehab et al. 2000; Shieh et al. 2000). The involvement of a specific kinase is dictated by the kind of damage the cell has received. Serine 15 of p53, for example is clearly phosphorylated by ATM following IR and by ATR following UV (Meek 1998; Lane 1998; Kapoor and Lozano 1998).

It has been reported that different anticancer agents are able to induce phosphorylation of p53 in cells growing in culture, indicating that this is the mechanism responsible also for anticancer agent-dependent activation of p53 (Knippschild et al. 1996; Shieh et al. 1997; Giaccia and Kastan 1998). Interestingly, different anticancer agents could be able to activate different kinases which differently phosphorylate p53 depending on the lesion the agents induce.

Another mechanism which mediates the detachment of p53 from mdm2 has been reported in cells activated by oncogenic transformation. In this case the mechanism leading to dissociation of mdm2 from p53 involves the p14arf protein (Eischen et al. 1999). This protein is encoded by the INK4a gene, the same gene encoding the cdk inhibitor p16; but, by using a different frame, a second protein product is synthesised which is completely different from p16 (Quelle et al. 1995). p14arf is able to bind mdm2 at residues different from those necessary for the interaction with p53. Once bound by p14arf, mdm2 is localized to nucleolar structures and sequestered (Pomerantz et al. 1998; Weber et al. 1999), thus resulting in a release of p53 and in a rise in its levels.

Besides the region important for binding to mdm2, p53 is phosphorylated at other N-terminal sites (Siliciano et al. 1997; Giaccia and Kastan 1998; Meek 1998). Moreover, the C-terminus also contains potentially phosphorylatable sites, which are indeed phosphorylated in cells (Oren and Prives 1996b; Lu et al. 1998) (Fig. 1.6). Different kinases responsible for these post-translational modifications have been identified, including Casein Kinase II (CKII), protein kinase C, cdks (Giaccia and Kastan 1998; Meek 1998). These modifications are thought to be important for stabilization of sequence specific DNA binding and possibly for determining the substrate specificity in transcriptional activation of downstream genes (Oren and Prives 1996b).

Phosphorylation is not the only post-translational modification of p53. It has been reported in fact that p53 is a substrate of histone acetyltransferases (HATs) and is efficiently acetylated at the C-terminus (Gu and Roeder 1997). These acetylations, occurring at lysine 320, 372, 373, 382 and 381 and mediated by the HATs P300 and pCAF were found in cells in response to various stimuli (Liu et al. 1999; Prives and Manley 2001). The acetylation of p53 does not seem to stimulate the sequence specific binding, but could be important for the recruitment of transcriptional co-activators or for p53 localization (Prives and Manley 2001), although this issue is yet to be defined.

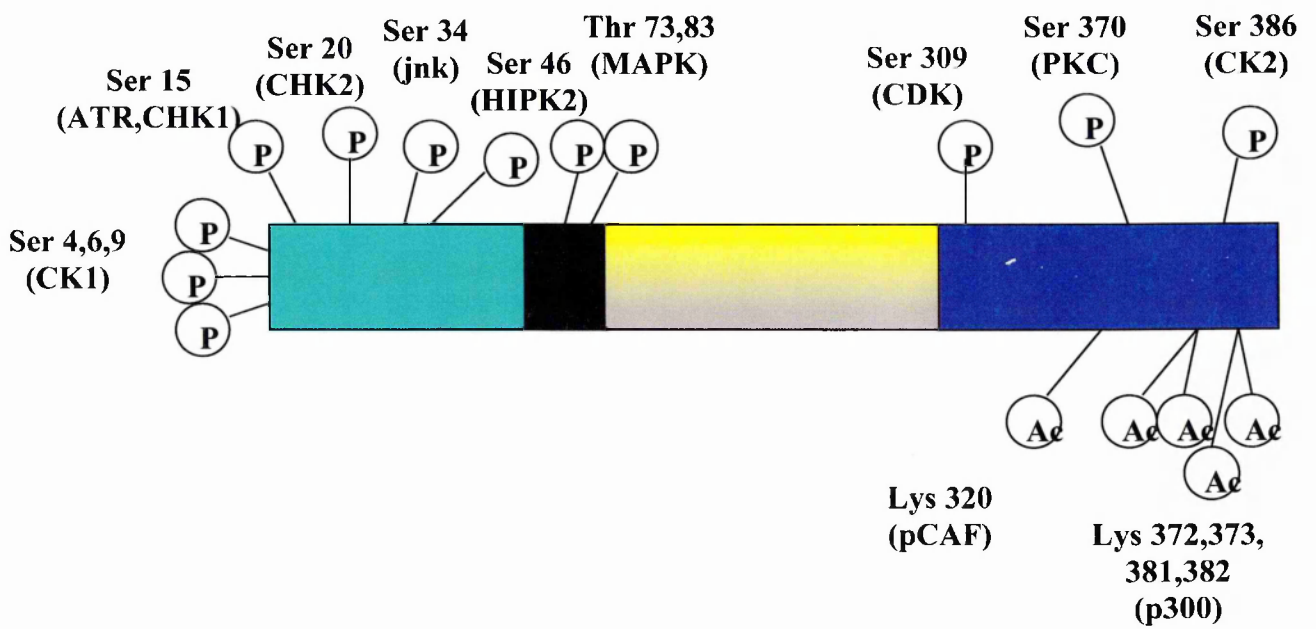


Figure 1.6
Post-translational modifications of p53.

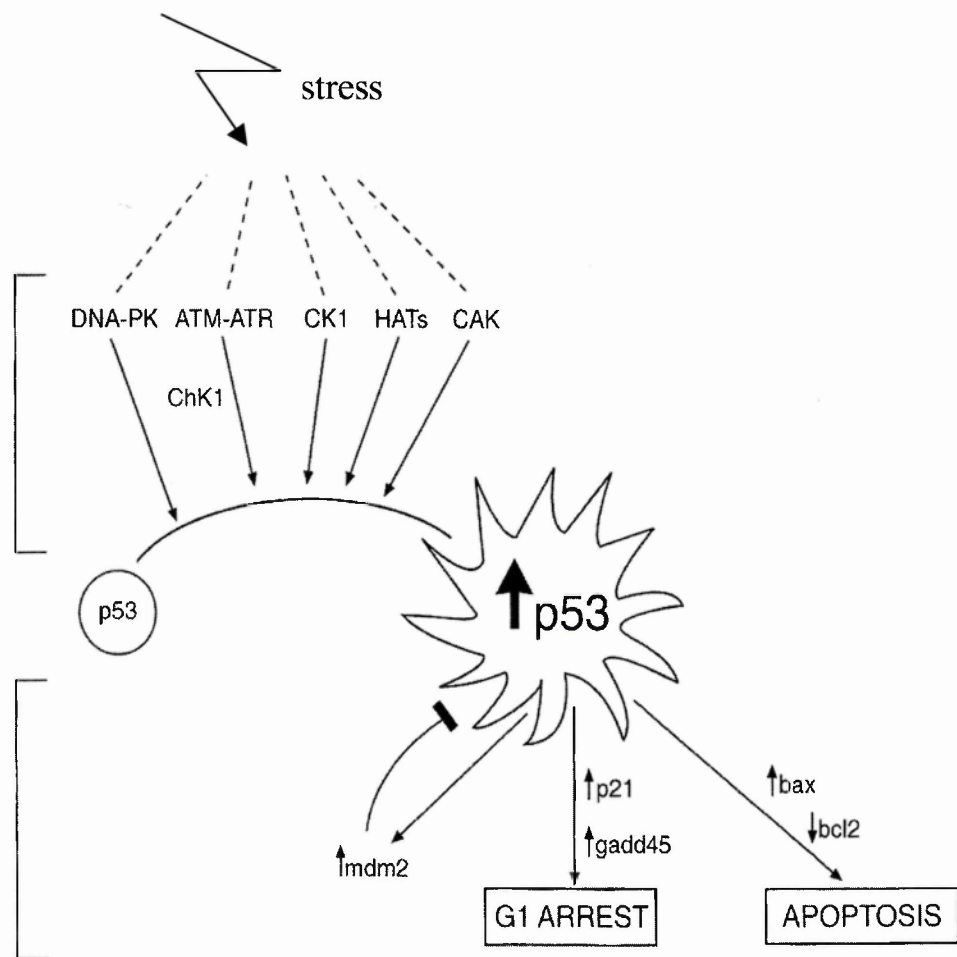


Figure 1.7

Schematic representation of the pathways activated by p53 following activation.

1.3.1.3 Function of p53

p53 exerts its activity mainly as transcription factor. Following activation, it activates the transcription of distinct classes of genes. Two important classes of genes activated by p53 are those involved in cell cycle regulation and in apoptosis. As a result, p53 induction can lead either to cell cycle arrest or to apoptosis (Bates and Vousden 1996; Ko and Prives 1996; Waldman et al. 1997a; Vogelstein et al. 2000) (Fig. 1.7). The critical decision between halting the cell cycle or activating cell death is dependent on the cell type and on the kind and extent of the damage induced (Ko and Prives 1996; Chen et al. 1996). This is conceptually rather simple: if the damage induced is sufficiently low to be managed by inducing cell cycle arrest to possibly allow the repair of the damage, then the cell cycle arrest is preferred. If the damage is thought to be unrepairable, the decision must be to induce apoptosis, in order to eliminate damaged cells which could pass aberrant material to daughter cells. Experiments have in fact shown that depending of the cell type or the extent of damage, p53 induces the activation of cell cycle-regulating or apoptosis-regulating genes. Two genes, p21 and bax, are among the most studied p53 downstream genes responsible for p53 induction of cell cycle arrest or apoptosis, respectively (el Deiry et al. 1993; Miyashita and Reed 1995). Many other genes, however, have been implicated in p53-mediated cell cycle arrest or apoptosis, including the 14-3-3 sigma protein, GADD45, FAS, Noxa, PUMA, the family of PIGs, AIP (Kastan et al. 1992; Hermeking et al. 1997;

Muller et al. 1998; Vogelstein et al. 2000; Oda et al. 2000a; Oda et al. 2000b; Yu et al. 2001; Nakano and Vousden 2001). Among the downstream p53 effectors, p21 and bax are probably the most important and most frequently studied, and they serve as paradigm of p53 ability to induce cell cycle arrest (p21) or apoptosis (bax). The p53 responsive elements present in the DNA regulatory sequences of either the p21 or the bax gene are different. The element present in the bax promoter, in particular, is less efficiently bound by p53 and, in transcription experiments using heterologous reporter genes, it has a weaker p53-transcriptional response compared to the analogous element in p21 (De Feudis et al. 2000). The relative propensity of p53 to activate p21 rather than bax could be one of the reasons why often p53-induced cell cycle arrest predominates over apoptosis. The critical decision between activating apoptotic or growth arrest inducing genes is however not only mediated by the sequence of the DNA element, but a growing body of evidence indicates that the different post-translational modifications of p53 are the key factors in determining the final decision. As an example, one of the p53 responsive genes involved in apoptosis, the AIP gene, is not efficiently transcribed, if p53 is not phosphorylated at residue serine 46, which probably induces a conformational change in p53 allowing efficient binding and transactivation ability for this specific gene (Oda et al. 2000b; Hofmann et al. 2002).

This type of evidence might also explain the discrepancies in the literature concerning the role of p53 as a determinant of cellular response to damage. In some cells it has been reported that the presence of an intact p53 is associated with resistance to induction of damage, while in other cells the opposite was found (Fan et al. 1995; Morgan and Kastan 1997; Debernardis et al. 1997).

Cells which are prone to activate mechanisms of apoptosis and in which the p53 activation is able to induce such cell death mechanism, will be more susceptible to damage if p53 is present, while in cells where the apoptotic response is compromised, the p53-dependent cell cycle effects will predominate.

The critical role of apoptotic versus cell cycle arrest response is underlined by the finding obtained using isogenic cell systems derived from the wild-type p53 expressing human cell line HCT116 in which the p21 or the bax gene have been inactivated through targeted homologous recombination (Waldman et al. 1997b; Zhang et al. 2000). Experiments with these cells have shown that inactivation of p21 was associated with an increased response to damage, and that bax removal was indeed associated with a decreased apoptotic response, yet both systems having a similar ability to respond to stress induction by activating p53 (Waldman et al. 1997b; Zhang et al. 2000). Results from our laboratory have shown that if p53 is forced to transcriptionally activate bax rather than p21, a massive apoptotic response and a marked increased response to treatment

with anticancer agents are found (De Feudis et al. 2000). In this cellular system, the amount of p53 following treatment did not change between parental and “bax-forced” cells, and levels of p21 in the two sublines were similar. What was really changing was the ratio between bax and p21 which was found important for triggering apoptotic response following anticancer agent treatment. Interestingly, these results have been confirmed in nude mice transplanted with these cells and treated in vivo with taxol (De Feudis et al. 2000). From these and other evidences, the role of p53 in determining cellular response to stress is hard to study without taking into account the upstream and downstream processes taking place.

Another way p53 exerts its activity is *via* transcriptional repression (Vogelstein and Kinzler 1992; Vogelstein et al. 2000). This process is another mechanism responsible for its activity. The molecular mechanisms responsible for transcriptional repression have been less well elucidated, the ability of p53 to interfere with transcription factor sp1 activity and binding to DNA has been discussed (Bargonetti and Manfredi 2002).

1.3.2 The p53 homologue p73

P53 was thought for a long time to be the only member of the p53 protein family. Recently two further members have been discovered, p73 and p63 (Kaghad et al. 1997; Kaelin 1999b; Yang et al. 2002). Both share a certain degree of homology with p53, particularly in the DNA-binding domain.

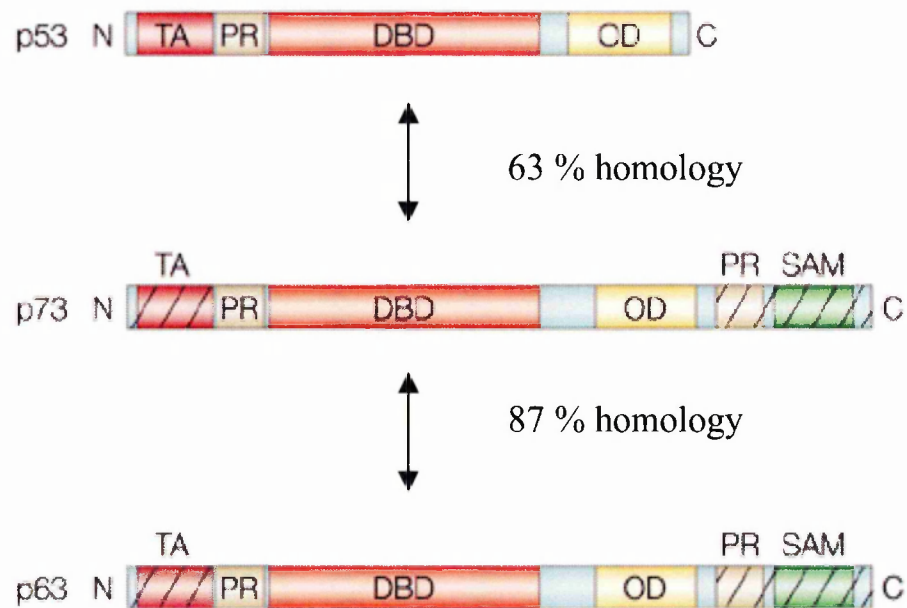


Figure 1.8
p73 structure and homology between p53, p63 and p73.

Human p73 has 65% sequence homology to human p53 in the DNA binding domain at amino acid level (Kaghad et al. 1997) (Fig. 1.8). As a consequence, p73 is able to bind to the same DNA sequences which are recognized by p53, and to activate transcription of p53-downstream genes (Smith et al. 1997). There is evidence however which suggests that there could be differences between p53 and p73 with respect to the pathway of activation of downstream genes. Certain genes are better substrate for p73 than p53 and vice versa (Zhu et al. 1998; Lee and La Thangue 1999). As an example, p73 is a stronger activator than p53 of genes such as GADD45, while p53 is stronger than p73 in activating the transcription of p21 (Lee and La Thangue 1999).

The p73 gene has been mapped in the region 1p36 of chromosome 1, a region frequently deleted in human tumors (Kaghad et al. 1997). This finding, together with the relative sequence homology with p53, led to the assumption that p73, like p53, could act as a tumor suppressor (Oren 1997). There are, however, some important differences between p53 and p73, which cast doubt on the contention that p73 is a tumor suppressor (Grob et al. 2002; Stiewe and Putzer 2002). In contrast to p53, the p73 gene is rarely mutated in human cancers (Kaelin 1999b; Moll et al. 2001). Different reports in the literature show that the levels of wild-type p73 in human tumors are higher than in the normal tissues from which they are derived. In some normal tissues the p73 gene has been reported to be imprinted, and only one allele is expressed (Kaghad et al. 1997; Stiewe and

Putzer 2002). In tumors both alleles can be expressed, again suggesting that the wild-type form of the protein can be overexpressed in tumors (Codegani et al. 1999; Nozaki et al. 2001; Novak et al. 2001). It has to be noted that there are conflicting results concerning the imprinting of p73, and evidence has been reported indicating that the gene is not imprinted in normal human tissues (Kaelin 1999a; Marin and Kaelin 2000). Nevertheless, even if it is questionable if human tumors overexpress a wild-type form in respect to normal tissues, it is clear that the levels of p73 are not decreased in tumors when compared to normal tissues. Another important difference between p53 and p73 is that mice, in which the p53 has been deleted, invariably develop tumors, whilst p73 knock out mice display strong neurological and immunological defects, but do not develop cancer (Kaelin 1999a; Yang et al. 2000; Moll et al. 2001).

The p73 protein differs from p53 also in the way in which it responds to stress induction. While p53 rapidly accumulates following damage *via* a protein stabilization-mediated post-translational mechanism, p73 is probably regulated at the transcriptional level (Levrero et al. 1999; De Laurenzi and Melino 2000). Moreover, its regulation is quantitatively much less important than that of p53. In fact, depending on the damage induced and the cellular context, the levels of p53 can be increased by a factor of 10-50, while the levels of p73 have been reported either not to increase or to increase by a factor of only 2-4.

1.3.2.1 Structure of the p73 gene

While p53 presents a relatively simple genomic structure, the p73 gene has a more complex structure. The gene consists of 14 exons and its coding region is comprised between exons 2 and 14 (Kaghad et al. 1997). It uses two different promoters for its transcription, one 5' upstream of the non coding exon 1 and the other one located in the large intron 3, 30 kb downstream to the first promoter (Yang et al. 2002). The use of these two distinct promoters leads to the synthesis of two proteins with sometimes opposite effects (Moll et al. 2001) (Fig. 1.9). The first promoter transcribes a protein harboring its intact and functional transactivation domain. When the transcription starts from the second, alternative promoter, the protein formed lacks the first amino acids containing the transactivation domain, and generates the so called DN forms (Yang and McKeon 2000; Moll et al. 2001). The proteins with the intact transactivation domain retain their ability to transactivate p53 responsive genes, while the DN form could act as a dominant negative protein, inhibiting either the full length p73 (TAp73) or other members of the family, and in particular p53 (Pozniak et al. 2000; Moll et al. 2001; Stiewe et al. 2002a). Studies on the relatively new DN forms are however at their inception, and further evidence is needed to better clarify the role of this particular isoform of p73. An additional level of complexity is added by the formation, through the use of the first promoter, of another transactivation-deficient form of p73, which is produced by alternative splicing *via* skipping of exon 2

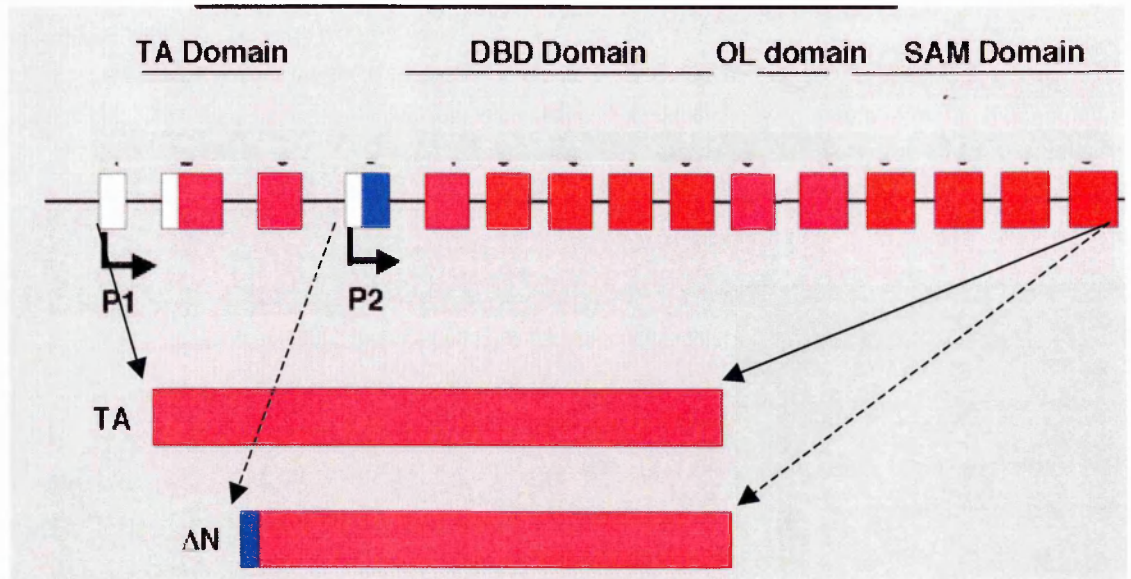


Figure 1.9
Structure of TA p73 and of ΔN p73.

(delta2p73) (Fillippovich et al. 2001).

It is interesting to note that the DN form is transcribed using a promoter that contains in its DNA sequence a putative p53 responsive element (Kartasheva et al. 2002; Vossio et al. 2002). It has been reported that this DNA sequence is indeed able to respond to a wild type form of p53 in vitro and in cells transfected with a fragment of this promoter fused to the luciferase gene. This finding suggests a mechanism by which p53 could regulate this specialised form of p73 (Vossio et al. 2002). If the data showing that the DN form of p73 is able to interact and block p53 activity are confirmed and extended to different cell types, the evidence that p53 can regulate one of its negative regulators is somewhat reminiscent of the interaction between p53 and its well-studied negative regulator mdm2 (Haupt et al. 1997).

Furthermore, p73 undergoes different C-terminal splicing routes leading to different isoforms termed alpha (which is the full length), beta, gamma, delta, epsilon and zeta (Kaghad et al. 1997; De Laurenzi et al. 1998; Zaika et al. 1999; De Laurenzi et al. 1999).

These isoforms arise through the skipping of one of several exons (Fig. 1.10). All the C-terminal isoforms maintain the DNA binding domain and the tetramerization domain. From a transcriptional activation point of view the different isoforms display different behaviour (Ueda et al. 1999). In particular gamma p73, which has a short C-terminus tail and structurally resembles p53, is a weaker transcriptional activator compared

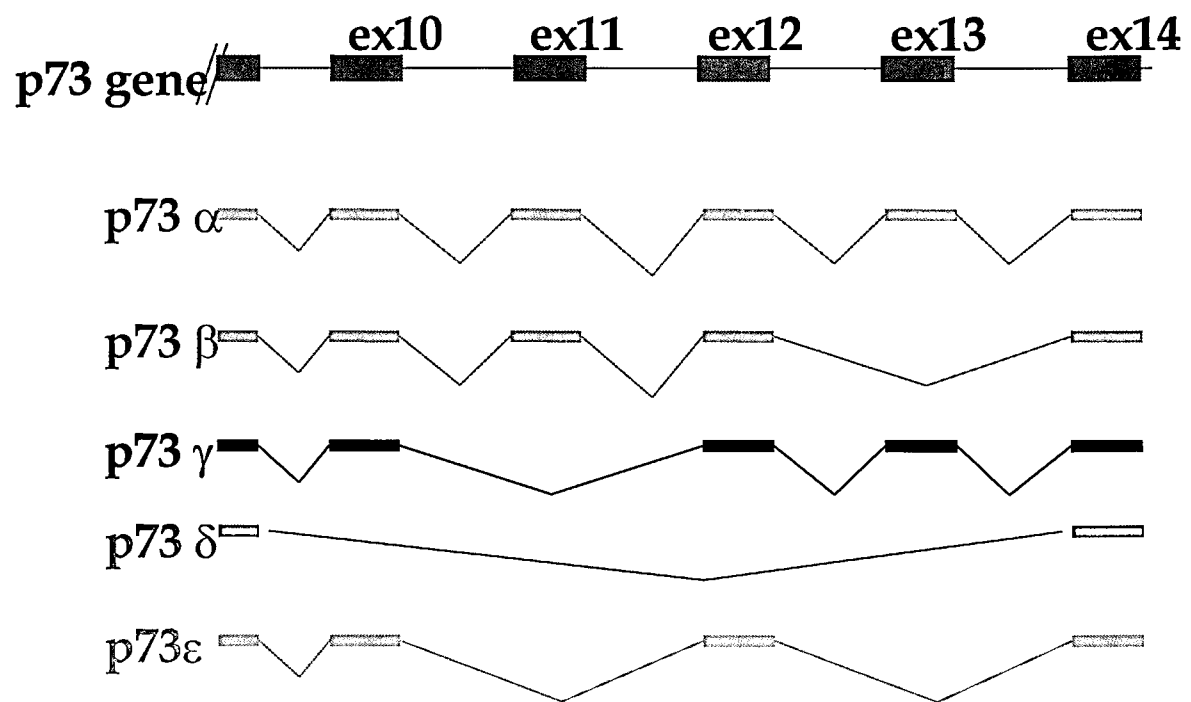


Figure 1.10
C-terminal splicing variants of p73

to the full length alpha isoform (Levrero et al. 1999; Moll et al. 2001). Preliminary reports indicate that the different C-terminal isoforms could have different specificity towards the downstream genes to be activated. The alpha p73 isoform contains an additional domain, the SAM domain, which is generally found in those proteins involved in development (Kaghad et al. 1997; De Laurenzi and Melino 2000). The presence of this domain, which has been confirmed by crystal structure to be a canonical one, would suggest the possibility of homo and hetero oligomerization with other SAM-containing proteins, although evidence obtained so far would cast doubt on this possibility (Wang et al. 2001).

1.3.2.2 Regulation of p73

While p53 is rapidly activated following stress induction, through a transcription-independent mechanism, levels of p73 have been found either to be unchanged or only increased marginally (Levrero et al. 1999; Vikhanskaya et al. 2000). The mechanisms leading to p73 activation following stress induction are likely to be mediated by cofactors. It has been shown for example that following DNA damage, c-Abl kinase is activated, which is able to directly interact with p73 and to phosphorylate p73 (Yuan et al. 1999; Gong et al. 1999; Agami et al. 1999). Phosphorylation of p73 is indeed seen following gamma irradiation in c-Abl expressing cells but not in c-abl-negative cells, and it seems to mediate the apoptotic response of p73 (Gong et al. 1999; Agami et al. 1999; Yuan et al. 1999). As

has been suggested for p53, p73 is acetylated following induction of damage (Costanzo et al. 2002), and this post-translational modification is probably important for the p73-dependent activation of downstream apoptotic genes (Costanzo et al. 2002).

Transcriptionally, p73 has been found to be regulated by the E2F1 factor (Levrero et al. 1999; Stiewe and Putzer 2000). In its promoter sequences there are multiple binding sites for E2F1 (Ding et al. 1999; Seelan et al. 2002). This fact would link the regulation of the expression of p73 to the cell cycle, E2F1 being released from retinoblastoma protein Rb in G1 phase to activate the transcription of genes necessary in S-phase (Nevins et al. 1991; Qin et al. 1995).

1.3.3 Interactions between p53 and p73

Sharing common DNA binding sites, p53 and p73 are likely to compete inside the cells for binding to DNA. Evidence has been reported that competition could happen between wt p53 and TAp73 (Ueda et al. 1999; Vikhanskaya et al. 2000) resulting, when TAp73 is overexpressed, in a attenuated p53 response to damage induction.

Direct complex formation inside the cell between wtp53 and TAp73 has never been shown. Interaction and complex formation between the different forms of wt p73 and mutated p53 or between DNp73 and wt p53 have been reported (Di Como et al. 1999; Strano et al. 2000; Gaiddon et al. 2001). Particularly intriguing is the observation that the physical

interaction between mutants p53 and p73 does not occur through the oligomerization domain, as could be expected, but rather through the DNA binding domain of mutants p53 and the DNA binding and oligomerization domains of p73 (Moll et al. 2001; Gaiddon et al. 2001). These possible interactions, when the result is antagonism of function, must be considered as additional ways to inactivate p53 or p73 inside the cells.

1.3.4 Mechanism of control of the G1 checkpoint mediated by p53 and p73

The molecular mechanism through which p53 exerts its activity of G1 phase checkpoint involves its ability to activate the transcription of the cell cycle inhibitor p21/waf1 (el Deiry et al. 1993). This small protein of 21 Kda acts as a cyclin dependent kinase inhibitor, with a broad spectrum of activity, being able to bind and inactivate the activity of cdks complexed with different cyclins (Xiong et al. 1993; Harper 1997). Its main activity, however, is toward G1/S cdks/cyclin complexes; and in vitro experiments have shown that the affinity of p21 for cdks acting during G1/S phases is higher than that for the G2 cdk cdc2 (Harper and Elledge 1996).

p21 was recognized as one of the first genes transactivated by p53, and it was shown that genomic sequences around the p21/waf1 gene contain a canonical p53 responsive element located 2.4 Kb upstream of the

starting coding sequence, which was able to respond to p53 in different experimental systems (el Deiry et al. 1993).

Although there is evidence that p21 transcription can be induced following DNA damage even in the absence of p53 (Vikhanskaya et al. 1995), its transcription is mostly controlled by p53, and cells lacking the DNA binding domain of p53 or presenting mutations in this domain, fail to activate p21 and have a strongly reduced ability to induce G1 arrest (Fan et al. 1995; Wahl et al. 1996).

p21 is able to form quaternary complexes in vitro and in the cell with PCNA, cdks and cyclins. When p21 complexes stoichiometrically with cdk and the cyclin complex, it completely abolishes cdk kinase activity, at least in vitro (Xiong et al. 1993). This mechanism of cell cycle arrest is different from that of other cdk inhibitors such as p16 and other members of the INK4 family, which instead bind directly and sequester the cdk (Sherr and Roberts 1999; Roussel 1999).

The importance of p21 in mediating p53-induced G1 arrest has been further demonstrated by the generation of somatic p21^{-/-} cells which lack the ability to induce G1 arrest following damage induction, even in the presence of a functional p53 (Waldman et al. 1997a).

1.4 S phase checkpoint

The S phase checkpoint is a transient phenomenon which delays the rate of DNA synthesis in response to DNA damage. Differently from the G1 and G2 checkpoints, this checkpoint lacks a maintenance component which helps to delay, but does not permanently arrest, cells with an incompletely duplicated genome (Rhind and Russell 2000a; Kastan 2001; Bartek and Lukas 2001b).

The existence of this checkpoint has been demonstrated in yeasts and in mammalian cells. The proteins participating in this checkpoint in mammalian cells include ATM, Nibrin and the recombinational repair protein Mre11 (Dasika et al. 1999; Petrini 2000; Rhind and Russell 2000a). These proteins are linked together since ATM has been shown to phosphorylate Nibrin and phosphorylated Nibrin is able to interact with Mre11 and Rad50 (Falck et al. 2002). This linkage, and the fact that a protein directly involved in DNA repair is required for the S-phase checkpoint, suggest that this particular checkpoint may actively regulate DNA damage repair during S phase. This suggestion would imply that the slow-down of DNA replication induced by the activation of the S-phase checkpoint not only provides time to repair, but hints at the existence of an active checkpoint-dependent association between replication, recombination and repair (Masai and Arai 2000; Rhind and Russell 2000a).

The activation of the S-phase checkpoint, as already pointed out, does not block the cell cycle, but only slows it down. DNA synthesis proceeds in the presence of DNA damage at a lower rate thus allowing the machinery to better deal with the DNA lesions that might be encountered. A number of lesions will remain and will subsequently activate the G2 checkpoint. A permanent arrest during DNA replication would be detrimental, because it would limit the amount of template for efficient repair by homologous recombination and could cause the re-start of DNA synthesis in areas where it was already started thus running the risk of over replication of partial genomic regions (Melo and Toczyski 2002; Falck et al. 2002).

1.5 G2 checkpoint

Looking at the data in the literature, it becomes evident that the cyclin dependent kinase cdc2 plays a central role in the progression from G2 phase to mitosis (O'Connor 1997; O'Connell et al. 2000). Important studies have been conducted in yeasts which had shown avenues to define DNA-damage checkpoint genes operating in this cell cycle phase. Many protein, in fact, have been shown to participate, at different levels, at this checkpoint (O'Connor 1997; O'Connell et al. 2000; Smits and Medema 2001; Bulavin et al. 2002). Among these, the p53 protein, the role of which

in the G1 checkpoint has been already examined, is also involved in the control of this cell cycle transition. (Taylor and Stark 2001)

Phosphorylation of tyrosine 15 residue of cdc2 is maintained during arrest of cells in the G2 phase following irradiation, either in yeast or in mammalian cells (Carr 1996). Keeping this residue phosphorylated is a way to prevent activation of the cyclin B/cdc2 complex associated with the inability of the cells to proceed out of G2 (O'Connell et al. 2000; Walworth 2000). As a proof of principle, introduction of a cdc2 variant with a mutant residue in position 15 resulted in the abrogation of G2 arrest in yeast (Smits and Medema 2001).

The inhibition of cdc2 activation is achievable in different ways. There can be signals inducing the activity of kinase(s) phosphorylating the inhibitory site tyrosine 15 (wee1), signals inducing the activation of specific phosphatases (CDC25) or signals binding and/or sequestering the cdc2/cyclin B complex (Tourret and McKeon 1996; O'Connell et al. 2000; Molinari 2000; Smits and Medema 2001).

Although p53 has been mainly involved in regulating the G1 checkpoint, it has also an important role in controlling the G2/M transition (Taylor and Stark 2001). p53 is able to activate the transcription of three distinct genes interfering with the activity of cdc2. It strongly activates the cdk inhibitor p21 which inhibits the cdc2 kinase activity, although with a much less efficiency compared to other cdks (el Deiry et al. 1993). P53 transcriptionally activates the GADD45 gene, which is able

to dissociate the complex between cdc2 and cyclin B (Wang et al. 1999; Zhan et al. 1999). GADD45, which has specific activity against the cdc2/cyclin B complex, does not efficiently inhibit the activity of the CDK2/cyclin E complex. This fact is likely to be the reason why its role in G1 arrest is negligible (Zhan et al. 1999). Immunoprecipitation studies showed that GADD45 associates with cdc2 and not with cyclin B, again indicating that it inhibits cdc2 activity by preventing its binding to cyclin B (Zhan et al. 1999).

The third p53 downstream gene inhibiting cdc2 activity is 14-3-3 sigma. This gene is particularly responsive to p53 in vitro (Hermeking et al. 1997). Its product, the protein 14-3-3 sigma, inhibits cdc2 activity with a mechanism distinct from those of p21 or GADD45. The cdc2/cyclin B complex needs to be present in the nucleus to exert its activity and to activate mitosis, through a mechanism involving the binding of importin alpha and beta. The 14-3-3 sigma protein is able to bind cdc2 and to anchor the complex cdc2/cyclin B in the cytoplasm (Chan et al. 1999; Taylor and Stark 2001). Prevention of nuclear translocation of cdc2/cyclin B is sufficient to halt the cells in G2.

An additional mechanism through which p53 can induce G2 arrest is through direct repression of cyclin B and cdc2 gene transcription. As already discussed (see chapter 1.3.1.3) p53 activates the transcription of different genes through direct binding to a recognition DNA sequence. It can also repress the transcription of different genes through several

distinct mechanisms, including quenching of transcription factors, binding to proteins, such as the histone acetyl transferase p300 necessary for transcriptional activation of certain genes. P53 can also interfere with sequence specific transcription factors (Vogelstein and Kinzler 1992; Levine 1997). Among the genes repressed by p53 is cyclin B1 (Innocente et al. 1999; Krause et al. 2000). The cyclin B1 promoter region responsible for these effects of p53 has been mapped (Krause et al. 2000). As a consequence of cyclin B1 repression, the levels of cyclin B1 protein decrease, which is followed by a decrease in cdc2 protein levels. Even for cdc2 there are data suggesting a direct transcriptional repression by p53, and a promoter region of the cdc2 gene has been mapped and shown to be susceptible to p53 (Yun et al. 1999; Taylor et al. 2001).

The other, and probably most important, mechanism which blocks cdc2 activity is by interference with the removal of the inhibitory phosphorylation at tyrosine 15 (and threonine 14). The dephosphorylation of these sites is crucial to allow the activation of the kinase cdc2, and it is mediated by the activity of the CDC25C phosphatase (Russell 1998; Smits and Medema 2001; Mondesert et al. 2002). CDC25C is normally localized in the cytoplasm and translocates to the nucleus just before mitosis (Russell 1998; Raleigh and O'Connell 2000). The mechanism responsible for translocation of CDC25C between cytoplasm and nucleus implicates the association with 14-3-3 proteins (not the sigma isoform) (Dalal et al. 1999; Lopez-Girona et al. 1999). When the phosphatase is bound to 14-3-3

proteins, it remains cytoplasmic and the dissociation is necessary for nuclear import. The CDC25C amino acidic region responsible for the binding to the 14-3-3 protein has been mapped, and mutations in this region are indeed able to abolish the cytoplasmic localization of CDC25C (Dalal et al. 1999). The region of CDC25C interacting with 14-3-3 proteins contains a phosphorylation site, serine 216, which has been shown to be indeed phosphorylated throughout interphase but not during mitosis (Ogg et al. 1994). The phosphorylation of serine 216 of CDC25 is necessary for the association of this phosphatase with the 14-3-3 protein. The kinase responsible for the phosphorylation of CDC25C on serine 216 during interphase has been cloned and named C-TAK1 (for Cdc Twentyfive C Associated protein Kinase) (Peng et al. 1998). C-TAK1 is ubiquitously expressed in the cytoplasm of human cells, where it would facilitate the phosphorylation of CDC25C while it is being synthesised in the cytoplasm (Dalal et al. 1999; Smits and Medema 2001). Altogether these results indicate that CDC25C can activate the cdc2/cyclinB complex only if it can translocate to the nucleus, and the nuclear translocation is possible only if CDC25C is not phosphorylated at serine 216 (Smits and Medema 2001).

Following stress induction, increased phosphorylation of CDC25C is observed (Fig. 1.11), which prevents activation of cdc2/cyclin B complex and arrests cells in G2 (Smits and Medema 2001).

The kinases responsible for the phosphorylation of the serine 216 residue induced by stress have been identified, and they are CHK1 and

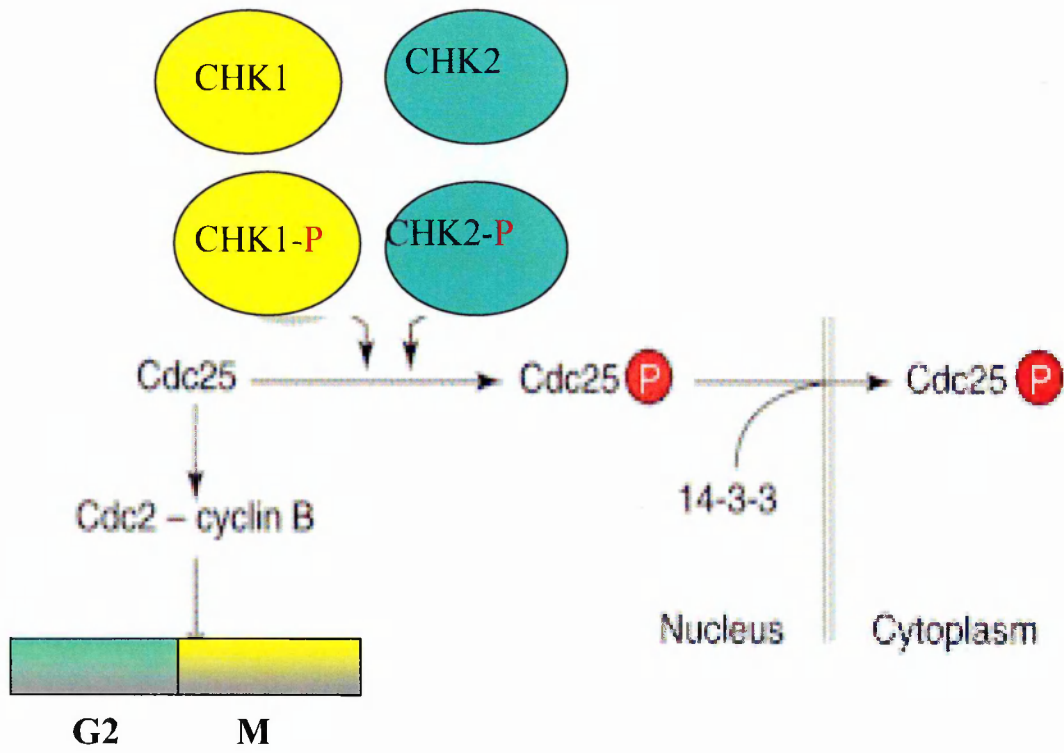


Figure 1.11

CHK1 and CHK2 dependent phosphorylation of cdc25C and regulation of cdc2/cyclin B complex activity.

CHK2 (Furnari et al. 1997; Sanchez et al. 1997). These two kinases are both able to phosphorylate CDC25C at serine 216 *in vitro*, although *in vivo* evidence would suggest that the major kinase responsible for this phosphorylation is CHK1 (Sanchez et al. 1997; Walworth 2001).

A further mechanism of control of G2/M transition is regulated by p38 MAP kinase (Bulavin et al. 2001). This kinase is activated following damage, particularly after UV radiation and, once activated, is able to phosphorylate different substrates including the phosphatase CDC25B. This phosphatase is important in the initiation of the G2/M checkpoint, rather than its maintenance (Bulavin et al. 2002). Phosphorylation of CDC25B increases the ability of the phosphatase to bind to the 14-3-3 protein. Studies with specific inhibitors of p38 showed a decrease in initiation of G2/M checkpoint following UV radiation (Bulavin et al. 2001). This mechanism has been clearly observed following UV radiation, but not with other types of damage suggesting that it could represent a "damage specific" checkpoint activation.

1.5.1 The checkpoint genes CHK1 and CHK2

CHK1 and CHK2 are two relatively recently discovered genes which are continuously gaining importance with respect to their role as controller of cell cycle progression, particularly in response to damage (Walworth 2000; Rhind and Russell 2000b). The two proteins encoded by the genes have been initially discovered in yeasts and later in mammals.

Although they share some common features, they have clearly distinct functions in controlling different phases of the cell cycle (Rhind and Russell 2000b).

1.5.1.1 CHK2

CHK2 belongs to a family whose founder, Rad53, was first identified in 1994 as a kinase involved in many checkpoint responses in budding yeast (Allen et al. 1994). Homologues of Rad53 were subsequently found in *Schizosaccharomyces pombe* (*cds1*) and in higher eukaryotes (Murakami and Okayama 1995; Brown et al. 1999). The overall structure of CHK2 proteins is similar in all eukaryotes (Fig. 1.12) with a degree of homology of the protein sequence across species that roughly reflects the evolutionary distance among the different organisms. Despite their overall structural homology and shared biological role as important transducers of cell cycle checkpoint signals, there are functional differences between the various CHK2 homologues. The mammalian CHK2 seems to respond primarily to the most lethal type of DNA damage, double strand breaks, which are caused by ionizing radiation and radiomimetic drugs (Terado et al. 1993).

The human CHK2 gene spans 50 kilobases of genomic DNA and contains 14 exons (Bartek et al. 2001). At protein level, the structure of the CHK2 kinases comprises several evolutionary conserved elements (Bartek et al. 2001): the SQ/TQ motif (located in the amino-terminal domain and

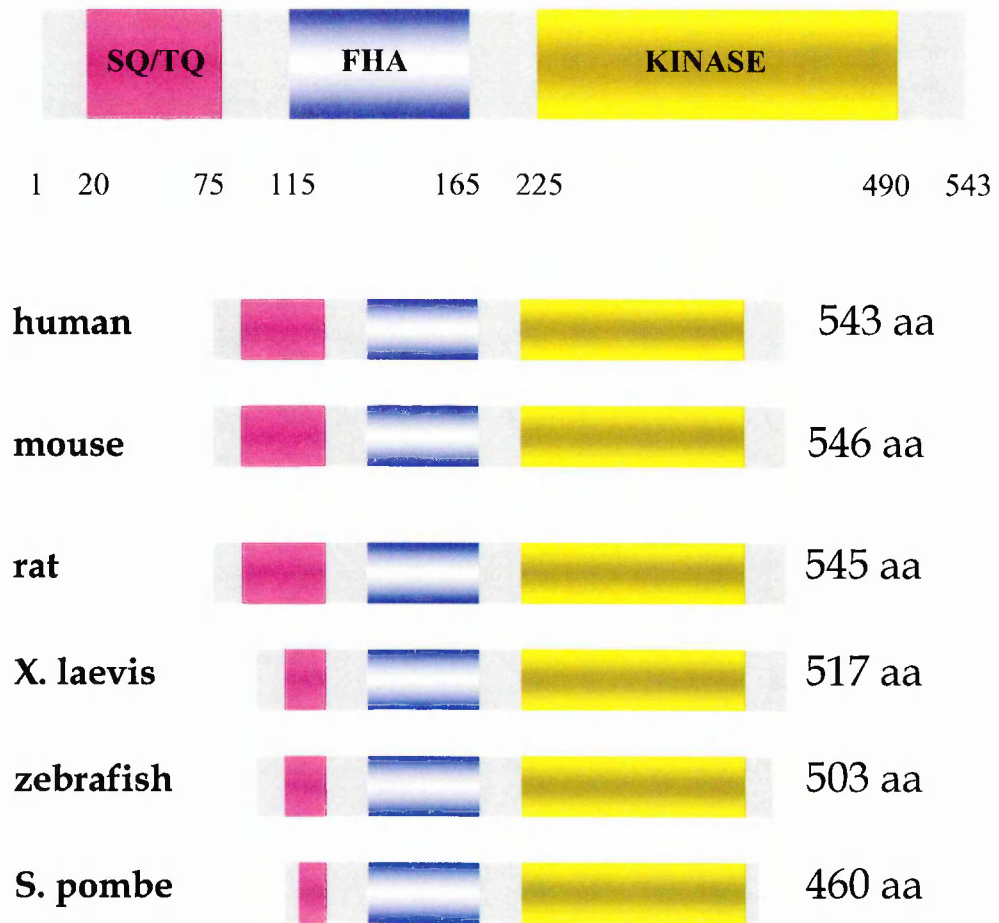


Figure 1.12

Structure of human CHK2 gene and homology with other eukaryotes. The SQ/TQ-rich domain, the FHA domain and the kinase domain are reported.

containing a series of seven serine or threonine residues followed by glutamine), the forkhead-associated domain - FHA - which seems to bind the phosphothreonine residues and to be involved in protein-protein interactions and the kinase domain, which occupies the entire carboxy terminal half of CHK2. An apparently unique feature of the human protein, not conserved in lower eukaryotes, is a c-Abl src homology domain without clear functional significance, at least as established so far.

In response to genotoxic damage, CHK2 is activated by ATM-dependent phosphorylation that targets its threonine 68 residue, which is a prerequisite for the subsequent activation step attributable to its autophosphorylation on the residues threonine 383 and threonine 387 in the activation loop of the kinase domain (Matsuoka et al. 1998; Lee and Chung 2001; Ahn et al. 2002). Once activated, CHK2 propagates the checkpoint signal to several pathways leading to cell cycle arrest (in G1, S and G2 phases), to activation of DNA repair and, in some cases, to activation of apoptosis (Bartek et al. 2001).

The checkpoint mammalian downstream effectors which have been demonstrated to be substrates of CHK2 *in vivo* include p53, BRCA1, CDC25A and CDC25B, all proteins the activation of which might explain the above mentioned downstream effects of CHK2 checkpoint activation, i.e cell cycle arrest, DNA repair or apoptosis (Bartek et al. 2001).

Immunocytochemical studies showed that in cultured mammalian cells CHK2 is predominantly a nuclear protein, this localization being

expected for a protein involved in the regulation of the DNA-damage checkpoint (Tominaga et al. 1999; Lee et al. 2000). Human neuronal cells constitute the only exception, in which CHK2 has been shown to have a predominantly cytoplasmic localization (Lukas et al. 2001). This localization is reminiscent of that of its upstream regulator, ATM (Barlow et al. 2000). It has been suggested that the ATM-CHK2 pathway in neurons might have a specialized cytoplasmic role related to protection of sensitive neurons against oxidative stress (Rotman and Shiloh 1997).

1.5.1.2 CHK1

The checkpoint kinase 1 (CHK1), initially identified in *Schizosaccharomyces pombe* (*S. Pombe*) is a G2/M checkpoint protein that is conserved throughout the eukaryotic kingdom.

The function of CHK1 has been studied both in *S. Pombe* and mammalian cells and found to be roughly similar. In *S. Pombe*, CHK1 is essential for cell cycle arrest following DNA damage (Lindsay et al. 1998). It is phosphorylated in response to DNA damage in a manner dependent on the function of several Rad gene products, including Rad3, a fission yeast homologue of the human protein ATM (Chen et al. 1999; Walworth 2001). Active CHK1 phosphorylates CDC25C, which provides a binding site for the 14-3-3 family protein Rad 24 and is thus exported out of the nucleus. As a result the cdc2 kinase is kept inactive (Chen et al. 1999). Moreover, CHK1 has been shown to phosphorylate the wee1 kinase in

vitro, implying that CHK1 might also facilitate G2 arrest through wee1, that inhibits cdc2 by phosphorylation at tyrosine 15 residue (Lee et al. 2001). As already discussed, an analogous checkpoint pathway involving ATM/ATR, CHK1 and CDC25C has been identified in mammalian cells.

The CHK1 gene has been mapped to chromosome 11 in the region 11p24 (Sanchez et al. 1997). The human protein encoded by the gene is a nuclear protein of 476 amino acids with a molecular size of 54 Kda (Fig. 1.13). The predicted human CHK1 protein is 29% identical and 44% similar to *S. pombe* CHK1, 40% identical and 56% similar to *C. elegans* CHK1, and 44% identical and 56% similar to *Drosophila* CHK1 (Sanchez et al. 1997).

Sequence analysis revealed a highly conserved N-terminal kinase domain (residues 1-265), a flexible linker region and a less conserved C-terminal region with undefined function. The crystal structure of the human CHK1 kinase domain revealed structural features important for kinase activity and substrate selectivity, suggesting that CHK1 becomes fully active upon substrate binding, and that its activity is not regulated by phosphorylation within the kinase domain (Chen et al. 2000).

CHK1, differently from CHK2, is an unstable protein (with a half-life of less than two hours) (Bartek et al. 2001) and its expression is restricted to the S and G2 phases of the cell cycle, suggesting that CHK1 might regulate the timing of mitosis by controlling the activity of CDC25 during the normal cell cycle (Kaneko et al. 1999).

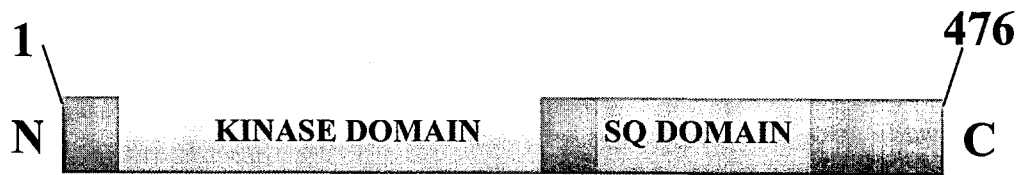


Figure 1.13

Structure of human CHK1 gene .

The kinase and SQ domains are indicated. N and C indicate the amino and carboxy terminal, respectively

CHK1 $-/-$ mice suffered an early embryonic death, indicating that CHK1 is essential for cell growth and differentiation at an early stage of development (Takai et al. 2000; Liu et al. 2000). ATR $-/-$ mice have also been reported to be characterised by similar early embryonic death and mitotic catastrophes, further suggesting a link between ATR and CHK1 (Brown and Baltimore 2000).

1.6 Mitotic checkpoint

Moving towards mitosis, cells must distribute their replicated genetic material evenly between the two daughter cells. In this phase microtubules organize into a bipolar spindle which segregates the duplicated chromosomes (Meier and Ahmed 2001). This process must be tightly controlled because mis-segregation of chromatids will lead to aneuploidy (Jallepalli and Lengauer 2001). In human cells, if centrosome separation (necessary for the bipolar spindle formation) does not occur, prometaphase is delayed and the mitotic checkpoint inhibits the chromatids separation until all the kinetochores are attached to the microtubules (Scolnick and Halazonetis 2000). Moreover, a further control checks the exit from mitosis and is able to block the network until completion of chromosome separation (Rudner and Murray 1996; Amon 1999). The crucial steps in the progression through mitosis are controlled by the disruption of the mitotic inhibitory proteins, a phenomenon

occurring when these proteins are ubiquitinated by APC/C (anaphase promoting complex /cyclosome) and targeted to the proteasome for degradation (Morgan 1999; Harper et al. 2002). In order to ubiquitinate the substrates, APC/C must be complexed with the protein cdc20 (Wassmann and Benezra 2001). The mitotic checkpoint directly inhibits the APC/C function (Wassmann and Benezra 2001). Genes able to signal and activate this checkpoint have been discovered and include the Mad and Bub genes which have been well characterized in *S. cerevisiae*. Mammalian homologues have been discovered and also found to play a role in mitotic checkpoint control (Li and Benezra 1996; Taylor et al. 1998).

1.7 Modulation of checkpoints following DNA damage

Following the induction of damage, the different checkpoints are activated and prepared for the cascade of events eventually leading to cell cycle arrest or apoptosis (Fig 1.14). Both p53 and CHK1/CHK2 need to be activated in order to start the complex pathways they govern. These checkpoint proteins are not present at the site of damage and must be downstream to factors which are able to identify the lesion and to “inform” the checkpoint proteins (Khanna et al. 2001; Abraham 2001).

In vitro experiments have clearly shown that both p53 and CHK1/CHK2 are post-translationally modified by a number of kinases. p53 is phosphorylated at many sites by different kinases including CKII,

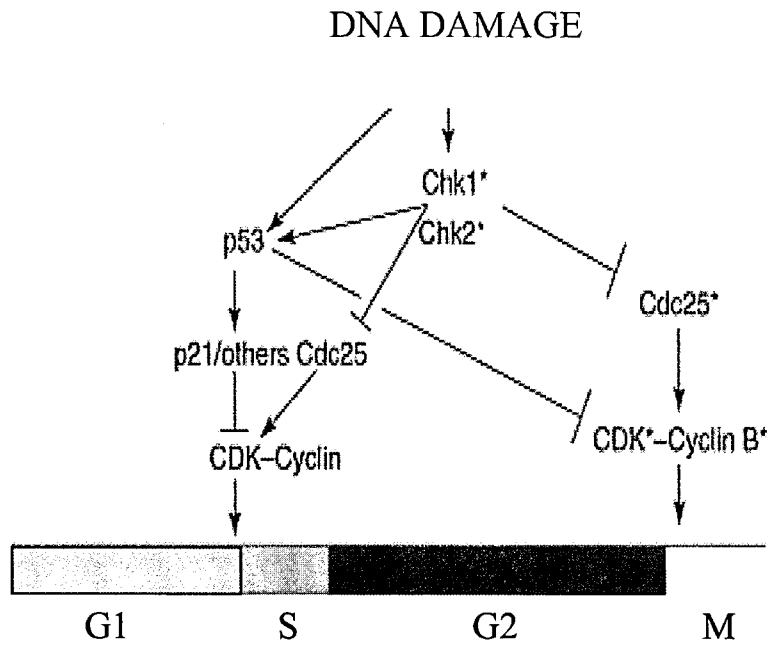


Figure 1.14
Pathways activation in mammalian cells following stress induction.

ATM, ATR, DNA-PK (see 1.3.1.2), while CHK1/CHK2 were shown to be phosphorylated by ATM and ATR (Sanchez et al. 1997; Abraham 2001; Tian et al. 2002).

The post-translational modifications of p53 and CHK1, and particularly their phosphorylation are necessary for their ability to work as checkpoint proteins. In fact studies with mutants lacking serine 15 or serine 20 phosphorylable sites clearly showed that the response to damage in these cells was defective (Fiscella et al. 1993; Unger et al. 1999). Similarly, inability to phosphorylate CHK1/CHK2 was associated with a strong reduction of their activity (Guo et al. 2000).

Interestingly the p53 sites at serine 15 and serine 20, which are particularly important for p53 activation, since they are in the region bound by the negative regulator mdm2, are also phosphorylable by CHK1/CHK2 (Prives 1998; Chehab et al. 2000; Hirao et al. 2000; Shieh et al. 2000). This finding indicates again how the cellular response to damage is finely controlled, and how the key proteins participating in mounting the response to damage are interconnected.

The activation of checkpoint proteins is therefore another crucial aspect that has to be considered when the checkpoint proteins activity is studied.

1.8 Sensors of damage

One of the major problems for a cell is to detect rapidly a lesion present in its DNA. The proteins able to quickly recognize the presence of damage and to signal the damage to checkpoint proteins are called “sensors” of the damage. The different sensor proteins must specifically recognize the kind of damage occurring in order to be able to activate the appropriate checkpoint pathway and also must be able to recognize very low levels of damage such as those which occur endogenously. The ATM/ATR protein kinases are the most studied sensors of damage in human cells (Lavin 1999; Khanna et al. 2001; Abraham 2001). Both belong to the family of phosphatidyl-inositol 3- kinase like protein kinases (Lavin et al. 1995; Durocher and Jackson 2001). They are able to be quickly recruited to sites of damages in DNA where they form complexes with other proteins such as the replication factor RF-C and PCNA (Shiloh 2001; Balajee and Geard 2001; Unsal-Kacmaz et al. 2002). The complex formation at sites of damage is a prerequisite for the activation of cellular response which is started by phosphorylation of checkpoint proteins (Lakin et al. 1999; Durocher and Jackson 2001; Shiloh 2001). Evidence in vitro and particularly in vivo shows that ATM/ATR are able to phosphorylate two of the checkpoint proteins discussed in this introduction, p53 and CHK1 (Hoekstra 1997; Tibbetts et al. 1999; Lakin et al. 1999). This step is crucial for the activation of both checkpoints and in fact cells lacking the ATM

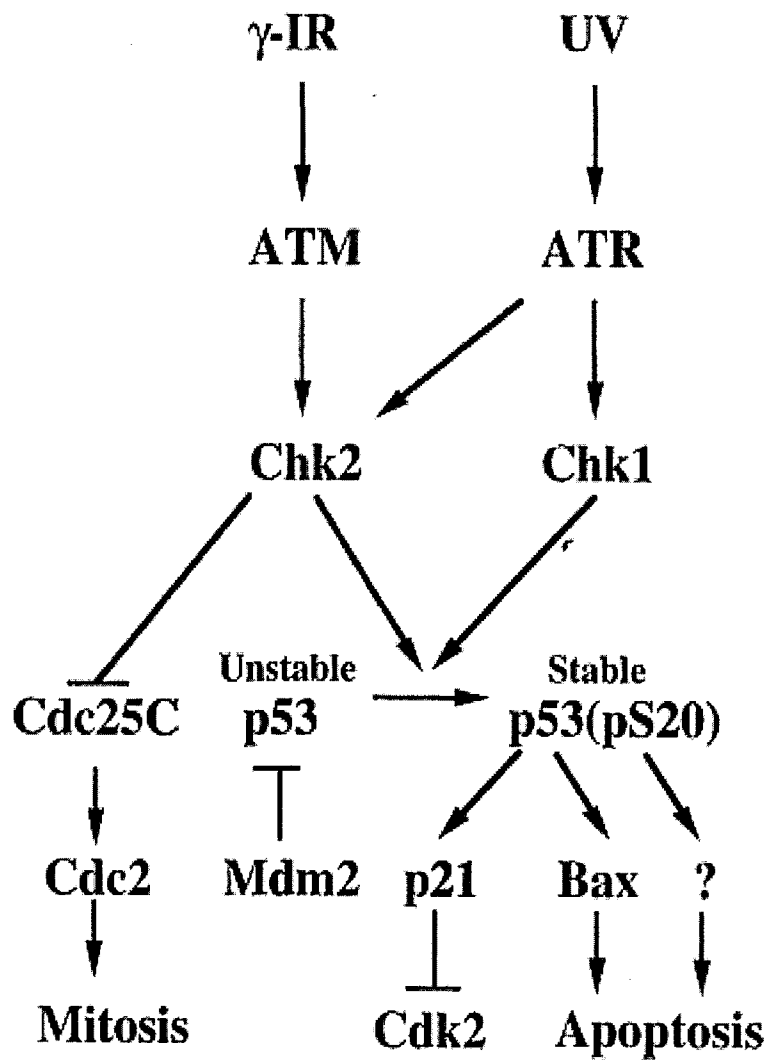


Figure 1.15
ATM and ATR-dependent pathways are activated by different stimuli.

BRCA1 is a breast cancer tumor suppressor gene encoding a protein containing a BRCT motif, generally found in many proteins implicated in DNA damage response and genome stability (Casey 1997; Bork et al. 1997). BRCA1 is phosphorylated upon DNA damage by ATM, ATR and CHK2 (Cortez et al. 1999; Tibbetts et al. 1999; Lee et al. 2000). BRCA1 has been shown to be essential for activating CHK1, and in fact activation of CHK1 kinase after exposure to ionizing radiation occurred only when the BRCA1 protein was expressed (Yarden et al. 2002).

1.9 Checkpoint defects in cancer cells

Having established the fundamental role of checkpoint proteins and checkpoint control mechanisms in normal cells, a lot of evidence indicates that in cancer cells these mechanisms are invariably defective. The inactivation of control mechanisms, including cell cycle control and apoptotic response, is often included in the mechanisms responsible for the transition of a normal cell to a cancer cell. As already discussed, the gene encoding p53 is the gene most frequently found mutated in human cancer, and the accumulation of mutations in this gene are particularly frequent in advanced tumors (Harris 1996; Hollstein et al. 1997). More than 90% of the mutations found in the p53 gene are in the evolutionary conserved central DNA binding domain, which further underlines the importance of transcriptional activity in p53 overall activity (Harris 1996). Besides gene

mutations and deletions, inactivation of p53 has been reported in human tumors by alternative mechanisms, including cytoplasmic sequestration (Moll et al. 1996), which blocks the potential activity of p53 as a transcriptional factor, and viral inactivation (Kessis et al. 1993; Crook et al. 1994). The E6 gene of the human papilloma virus HPV16, for example, which binds p53 and allows its rapid proteasome-dependent degradation, is thought to be responsible for the p53 inactivation in cervical cancer, where inactivation by gene mutation/deletion is less important (Ngan et al. 1994). Additionally, germline mutations in the p53 gene predispose to cancer, as borne out by Li-Fraumeni patients, in whom the inactivation of one p53 allele strongly predisposes to cancer (Davison et al. 1998; Chompret 2002).

The CHK1 and CHK2 genes have also been found mutated in cancer. Mutations of CHK2 have been reported in families with Li Fraumeni syndrome and in patients with hematopoietic vulval and breast tumors, all leading to inactivation of the protein (Bell et al. 1999; Lee et al. 2001; Reddy et al. 2002; Ingvarsson et al. 2002; Hangaishi et al. 2002).

Mutations in the CHK1 gene are much less frequent in human tumors than those found with respect to the CHK2 gene. The presence of a nucleotide stretch of nine consecutive nucleotides in the coding region of CHK1 led to the hypothesis that tumors with defects in mismatch repair and hence with microsatellite instability could accumulate mutations around this region. This hypothesis was indeed found to be correct. In

tumors of the colon and endometrium, in which microsatellite instability occurs frequently, insertion/deletion of one nucleotide in the coding region of CHK1 can be found (Bertoni et al. 1999; Vassileva et al. 2002; Furlan et al. 2002). The insertion/deletion of one nucleotide leads to a frameshift mutation that in the case of CHK1 results in protein truncation and inactivation (Bertoni et al. 1999). It is important to note that these mutations are always heterozygous and that one wt allele is always present. This situation differs from that pertaining to other genes susceptible to the presence of microsatellite instability, such as the bax gene, in which case inactivation of both allele can be found (Rampino et al. 1997). It is still unclear whether haploinsufficiency in CHK1 can have detrimental effects for checkpoint response, particularly because there are no cellular models representative of the clinical situation, which can be used as a tool to investigate this point. Many attempts to disrupt and abrogate the CHK1 gene in mammalian cells failed, and CHK1 knock out mice do not develop due to embryo lethality (Takai et al. 2000).

Finally, inactivation of DNA damage sensors have been reported in human pathological conditions. Germline defects in ATM gene are responsible for ataxia telangectasia, a neurological disorder which also predisposes to cancer, due to a decreased ability to properly activate the DNA damage response (Delia et al. 2000; Yan et al. 2000).

If the altered expression of checkpoint proteins in human cancer can be regarded as one of the possible mechanisms responsible for the

transformation of a normal cells into a cancer cell, we can take advantage of the different expression of these proteins between cancer cells and normal cells in order to study possible ways to increase the selectivity of anticancer agents. The lack of a proper cell cycle arrest following damage observable in certain cancer cells can be, for example, an advantage for those drugs able to activate an apoptotic response and for which a cell cycle arrest would be detrimental. Another attractive possibility would be to specifically target those factors which are aberrantly expressed in cancer cells. The combination of the knowledge of the molecular mechanisms responsible for the cellular response to stress and the effect of alterations of those proteins involved in the cellular response to a given drug could eventually help to optimise treatment with conventional drugs, design new combinations of conventional drugs with cellular response modulators and find new target-oriented drugs, with the final aim to improve selectivity and outcome of cancer treatment.

2. AIMS

During the last 2 decades the therapy of patients with cancer has clearly been improved implying alleviation of much human misery. Nevertheless there is still an urgent need to find new and better anticancer drugs, or new treatment schedules and combinations of currently used drugs, which offer high activity, exquisite specificity towards cancer cells, hence possess a high therapeutic index (see chapter 1.1 of the Introduction) .

Theoretically the ideal anticancer drug should be a drug which exerts its activity against a specific molecular target present in cancer, but not in normal cells, has favourable characteristics of absorption, a pharmacokinetic profile allowing concentrations of active drug to reach the target, and, last not least, allows being formulated in a suitably dosage form. The knowledge of important molecular targets in cancer cells is indispensable for the rational design of new molecules (see chapter 1.1).

i) The present studies were undertaken with the overall aim of characterising, in human cancer cells growing in culture, the roles which certain proteins play in the control of cell cycle progression in the context of the cellular response to treatment with classical anticancer agents.

The choice of protein which was to be studied with this aim in mind was based on the evidence that, almost invariably, cancer cells are characterised by aberrant regulation of the cell cycle, due to mutation or inactivation of proteins acting as checkpoints and normally functioning as guardians of genomic integrity (see chapter 1.9 of the Introduction). The studies were therefore focussed on 1. the tumor suppressor gene p53,

which is the gene that most frequently is found mutated in human cancer, 2. its recently discovered homolog p73, and 3. the checkpoint protein CHK1.

ii) To study properly the role of these proteins in the determination of cellular response to treatment, it was considered essential to generate and characterize appropriate cellular models. A substantial part of the work was therefore devoted to set up these systems and, in particular, to generate isogenic cellular systems, which have a similar genetic background and differ only in the presence, or expression, of the protein of interest.

iii) The cellular subclones selected with these particular characteristics were then studied in terms of phenotype and ability to grow, features which are also essential for the interpretation of drug-induced effects.

iv) For p73 and CHK1, which are relatively new genes, their regulation following damage has been taken into account. Information on the genomic structure and regulatory regions of the CHK1 gene has not been available at the inception of the work described here. Therefore, the isolation and characterization of these sequences has been another important objective of the work.

v) Once the regulatory region of the CHK1 gene was partially sequenced, two further aims of the project were to determine the

transcriptional regulation of CHK1 following DNA damage, and to identify the transcriptional factors possibly involved in this regulation.

vi) Another objective of the work was to study the differential activation of the N-terminal full length p73 and its N-terminal truncated form (DNp73) following DNA damage. It has been reported that these two isoforms originate from two distinct promoters which contain in their DNA sequences binding sites for different transcriptional factors. It can be hypothesised that different kinds of damage could lead to the differential activation of the two isoforms. Such differential activation is likely to be associated with different cellular responses due to different and sometimes opposite effects of the two isoforms.

vii) Finally, cross connections between the different checkpoint proteins and their reciprocal regulation, knowledge of which emanated from results obtained in the cellular systems used in the work described here or in previous studies, have been investigated. This aspect is particularly interesting considering that the different checkpoints, once activated following damage, and once the damage has been repaired, need to be reported to their basal level of activity (inactivated) in order to allow repaired cells to progress through the cell cycle. The complexity of the systems suggests a possible cross-talk and regulation of the different proteins involved in the checkpoints.

The study of the existence of such cross-talk and its preliminary molecular characterization have been two further aims of this work.

3. MATERIALS AND METHODS

3.1 Culturing of cells

3.1.1 Maintenance of cells in culture

All the cell culture procedures were carried out aseptically in laminar flow hoods. Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

The human cancer cell lines used in these studies were: the human ovarian carcinoma cell lines A2780 and SKOV-3, the human col carcinoma cell line HCT116, the human osteosarcoma cell line U2OS, the human lymphoblastoids cell lines IARC1663, AT11 and AT13.

Each cell line was maintained in its culture medium which was RPMI 1640 medium supplemented with 10% calf serum for A2780, SKOV-3, U2OS, IARC1663, AT11 and AT13 cells and ISCOVE's modified medium supplemented with 10% calf serum for HCT116. The different media were purchased from Sigma and contained all the mineral and supplements necessary for the growth of the cells except for the serum, which was added when needed.

The clones derived from the human colon-carcinoma cell line HCT-116, clone 40.16 (p53 +/+), clone 379.2 originated by targeted deletion of the p53 gene (p53 -/-) and clone p21-/- originated by targeted deletion of the p21 gene (p21 -/-) were kindly supplied by Dr. Vogelstein from Johns Hopkins University (Baltimore, MA, USA). The HCT116-derived clone in which p53 has been inactivated by transfection with the E6 protein of the

HPV16 virus was previously generated in the laboratory (Vikhanskaya et al. 1999).

Cells were passaged routinely before they reach confluence to maintain a logarithmic growth. The cells were renewed every four-five months of culture.

Procedures to detach and subculture cells were the same for all the cell lines used and consisted of two washes with warm sterile phosphate buffer saline (PBS) and detachment with a solution of 1X trypsin/EDTA (Mascia Brunelli). The trypsin activity was stopped by adding calf serum-containing medium. After centrifugation at 1200 rpm for 10 minutes, cells were resuspended in the appropriate medium, counted at the cell culture counter (Coulter Counter, ZM), and seeded at the desired density.

3.1.2 Long term storage in liquid nitrogen

To generate and maintain batches of cells, exponentially growing cell were washed twice with PBS and centrifuged at 1200 rpm for 10 minutes at room temperature. The cell pellet was resuspended in culture medium containing 50% of cryoprotective medium (Bio-Whittaker, Milan-Italy) and 20% of calf serum to a density of 5,000,000 cells/ml. Aliquots of 1 ml were kept on ice 30 minutes, cooled slowly for 3 h in nitrogen liquid stream and then immersed in liquid nitrogen. Cells were recovered from the cell bank by rapid thawing to 37 °C in a water bath, centrifuged at 1200 rpm for 10 minutes, resuspended in the appropriate culture medium and

transferred to a tissue culture flask. The day after the medium was removed and new, fresh medium was added.

3.1.3 Preparation of drug solutions

The drugs used in the experiments were cisplatinum (DDP, Sigma), taxol (obtained through the NCI), roscovitin (Sigma), doxorubicin (DX, Pharmacia, Nerviano Italy), methyl-nitro-nitrosoguanidine (MNNG, Sigma), topotecan (Sigma), UCN-01 (Sigma), wortmannin (Sigma) and trichostatin A (TSA, Sigma).

DDP was prepared fresh for any experiment by preparing a solution of 0.5 mg/ml in medium and allowing this solution to equilibrate with proteins present in the serum at 37°C for 30 minutes before treatments. Taxol was prepared as a 2 mM stock solution in DMSO and subsequently diluted in fresh medium the day of treatment. The stock solution was maintained at -20°C. MNNG, wortmannin, UCN-01, topotecan, roscovitine and TSA were also prepared as stock solutions in DMSO, at concentrations of 10-50 mM, stored at -20°C and freshly diluted in medium just before treatment. DX was prepared as a 1 mg/ml solution in water and stored at -20°C in the dark.

After treatment, the medium containing the drugs and the remaining solutions were properly discarded.

3.1.4 Cell growth inhibition induced by anticancer agents

The cytotoxic activity of anticancer agents was studied using two different methods. For A2780 and U20S cells, 100 μ l of cells were seeded in 96 wells plates at a density of 20.000 cells/ml and allowed to attach for at least 72 hours. A range of concentrations of each drug was used and each concentration was repeated in six replicate wells. Treatment was performed for one hour. For cell treatments, the stock solution of the drug was diluted in fresh medium to a concentration double the maximal selected. From this solution, 100 μ l were added to 100 μ l of medium containing cells to have the final desired concentration. Subsequent concentrations were obtained by serial dilution in medium. Untreated cells were incubated with an equivalent volume of fresh medium.

At the end of 1 hour incubation, the medium was aspirated and replaced with 200 microliters of PBS to wash the cells. The PBS was then aspirated and 100 microliters of fresh medium added. The cells were then left in the incubator at 37°C for further 72 hours.

The degree of growth inhibition induced by a drug was evaluated with the MTT assay. This assay is based on the ability of mitochondrial succinato dehydrogenase enzyme to metabolise the tetrazolium salts (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolio bromide). Basically, 20 μ l of a solution of MTT (6 mg/ml diluted in sterile PBS) were added to each well and the plate incubated for at least 4 hours at 37 °C in the dark. The medium was then aspirated and the insoluble salts resuspended with 100

microliter of a solution of 0.25 N HCl in isopropyl alcohol. The absorbance of each well was then read using a Titertek equipped with a filter for 550 nM. The absorbance value for each drug treated well was then compared with that of the untreated cells. The mean concentration inhibiting the growth by 50% (IC₅₀) was determined for each drug from at two-three independent experiments.

For HCT116 cells, the activity of the different compounds was assessed by measuring the inhibition of colony formation. Cells were seeded at a density ranging from 200 to 400 cells/ml in 6 wells-plates (2 ml per well). 24-48 hours after seeding the cells were treated with different concentrations of the drugs. In this case a 10 fold more concentrated solution of the drugs was prepared and 200 µl of this solution added to each well to give the final desired concentration. At the end of treatment, the medium was removed and the cells were incubated in drug-free medium for 10-12 days in 37°C incubator, to allow the formation of colonies. When in control cells the colonies were well visible under the microscope, the medium was aspirated and the wells washed with 1 ml of PBS. Then the colonies were stained with crystal violet by adding 1 ml of the commercially available solution, and, after extensive washings in water, with the final two washes in distilled water, the plates were air dried and the number of stained colonies counted by the Entry level Image System (Immagini & Computer, Italy). A background correction was made and the smallest control colony was taken as the minimum for

the establishment of the cut-off point. Each experiment was repeated at least twice and consisted of three replicated per point.

3.2 Generation of cell clones with selected gene alterations

3.2.1 Preparation of constructs

To transfer the gene of interest in the genome of host cells, the cDNA was excised from the plasmid of origin by restriction endonuclease digestion and subcloned in the appropriate expression vector. The expression vector used in our experiment was essentially the pCDNA3 produced by Invitrogen. The principal features of this plasmid are: the presence of a strong viral promoter (CMV) driving the transcription, a polylinker sequence containing the DNA recognition sequence for many restriction enzymes to facilitate the subcloning of the gene of interest, a bacterial resistance gene (ampicillin) for selection of recombinants and a eukaryotic resistance gene (neomycin) for selection of cells containing the plasmid. The procedure to subclone the gene of interest we used followed the general molecular biology techniques reported in Sambrook et al. (1989). In summary, the gene of interest is excised from the plasmid of origin by digestion with the appropriate restriction enzymes for 1 hour at 37°C in a buffer supplied with the enzyme. The entire reaction is loaded on 1% agarose gel to separate the cDNA insert from the plasmid. Agarose gel is

prepared by dissolving 1g of ULTRA pure agarose (Sigma) in 100 ml of 1x TAE buffer:

<u>TAE Buffer (50x)</u>	<u>final concentration</u>
242 g Tris base	2 M
100 ml EDTA 0.5M, pH 8	50 mM
57.1 ml glacial acid acetic	

to 1 l with deionised water. The solution was stored at room temperature.

The solution is heated in a microwave and boiled until all the agarose is dissolved. The solution is then cooled to approximately 50°C before pouring in a casting tray. The gel was then stained in a solution of 1 microgram/ml of ethidium bromide in TAE buffer for 30 minutes and the DNA bands visualized with a UV transilluminator.

When the two expected bands were present, the appropriate band containing the cDNA was excised from the gel and the DNA extracted from the agarose slice using the QIAgel kit (Qiagen) exactly following the manufacturer's instruction. The cDNA so obtained was stored at 4°C. The pCDNA-3 plasmid was digested with the same restriction enzymes used to excise the cDNA from the original plasmid. After digestion, the plasmid was treated with shrimp alkaline phosphatase at 37°C for 60 minutes to remove the 5' phosphates and to reduce the probability of the plasmid to recirculate without insert. The reaction was then heated at 70 °C to

inactivate the phosphatase. The linearized pCDNA3 plasmid was ligated with the excised cDNA insert in 20 microliters of a solution containing 1U of T4 DNA ligase (Promega) and 1X ligase buffer (30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP, supplied with the ligase) for 4 hours at room temperature. Five microliters of this solution were then used to transform competent bacteria.

3.2.2 Preparation of competent bacterial cells for transformation

Fifty ml of sterile bacterial cellular suspension (XL1-blue, Stratagene) were mixed, in a sterile 50 ml conical tube, with 10 ml of sterile LB medium:

<i>Luria-Bertani Broth (LB)</i>	<u><i>final concentration</i></u>
<i>10 g bacto-tryptone</i>	<i>1% (w/v)</i>
<i>5 g bacto-yeast extract</i>	<i>0.5% (w/v)</i>
<i>10 g NaCl</i>	<i>1% (w/v)</i>

to a final volume of 1 l with deionised water. The medium was then autoclaved for 15 min and the antibiotic of selection (for pCDNA3 derived plasmids, ampicillin at the final concentration of 50 µg/ml) was added once it had cooled to 55°C.

and allowed to grow in a 37°C heated shaking incubator (Folabo) at 225 rpm overnight. One ml of such liquid bacterial culture was then transferred into a sterile 500 ml bottle containing 100 ml of sterile LB

medium and the bottle was placed into the 37°C heated shaking incubator at 225 rpm.

To harvest bacterial cells in logarithmic growth phase, 2 hours later, 1 ml of liquid culture was transferred, under a laminar flow in a disposable cuvette and the absorbance at 600 nm wave-length was read on the spectrophotometer. *E. coli* concentration in the liquid culture was calculated by considering that 1 A_{600 nm} unit corresponds to about 8x log bacterial cells/ml. When the 600 nm absorbance reached A 0.3 units (corresponding to roughly 2.4 x 10⁸ cells/ml;) the bacterial suspension was transferred in two ice-cold sterile 50 ml conical tubes and the cell growth was stopped by placing the tubes on ice for 15 minutes. *E. coli* cells were pelleted by centrifugation at 3,000 rpm for 10 minutes at 4°C and, after the removal of LB medium, cell pellets were pooled in the same tube by gentle resuspension in 10 ml of ice-cold sterile 0.1 M CaCl₂ solution. After addition of 40 ml of ice-cold sterile 0.1 M CaCl₂ solution, bacterial suspension was incubated on ice for 30 minutes and subsequently centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was then removed and the cell pellet was carefully resuspended in 5 ml of an ice-cold sterile 0.1 M CaCl₂ containing 15% (v/v) glycerol (Sigma). This bacterial suspension was dispensed in 1.5 ml eppendorf tubes (400-500 µl aliquots for each tube) and kept at 4°C for 24 hours after which the tubes were quickly frozen in liquid nitrogen and stored at -80°C. The cells

maintained their competence for transfection for 1-2 months when kept at -80°C .

Fifty μl of freshly prepared competent cells were transformed with 50 ng of a DNA vector able to confer ampicillin resistance and $1/100,000$ (dilution factor, 10^5) of the bacterial suspension was plated as described in agar plates. Transformation efficiency, calculated on the basis of the formula $(\text{NUMBER OF COLONIES}) \times (10^3) \times (\text{DILUTION FACTOR}) / 50$ ng, was expressed as the number of colony forming units (CFU) per μg of plasmid DNA. Generally, 10^6 - 10^8 CFU per μg of plasmid DNA were indicative of a good preparation of competent bacterial cells.

3.2.3 Transformation of bacteria

In an ice-cold 10 ml Falcon tube (Falcon, Becton Dickinson), 50 μl of competent bacterial cells were diluted to a final volume of 100 μl with 0.1 M CaCl_2 and 20 μl of the ligation reaction (3.2.1) were then added. The mixture was gently mixed by tapping and the tube was chilled on ice for 30-40 minutes, incubated for 120 seconds at 42°C in a water bath and for 2 minutes again on ice. After a 5 minute incubation at room temperature, 900 μl of LB medium were added and tube was placed into a 37°C heated shaking incubator at 225 rpm for 1 hour. The tube was then centrifuged at 3,000 rpm for 5 minutes at room temperature, most of the supernatant LB removed and the bacterial cell pellet resuspended in the remaining fluid and spreaded into 90-mm dish (Corning-Costar Italia-Milan, Italy)

containing LB agar medium plus the antibiotic of selection: (*LB medium was prepared as previously described. Agar (1.5% w/v) (Life Technologies) was added before autoclaving and the solution poured on 90-mm dish (Corning-Costar, Milan-Italy) and allow to dry under sterile hood) and incubated overnight at 37°C without agitation.*

3.2.4 Identification of recombinant clones

Each colony, representing antibiotic-resistant/transformed growing bacterial cells, was picked-up with a sterile disposable loop, and spread to another agar plate containing LB agar plus selection antibiotic and incubated overnight at 37°C. Each colony was numbered and half of each was dissolved in a 1.5 ml eppendorf tube containing 20 µl of 1x STE:

<u>1x STE</u>	<u>final concentration</u>
0.2 ml EDTA 0.5 M	10 mM
0.2 ml TRIS.HCl pH 7.5 1 M	20 mM
0.2 ml NaCl 5M	100 mM

to a final volume of 10 ml of deionised water.

To this solution, an equal volume of phenol:chloroform (SIGMA) 1:1 was added. The tubes were vortexed and centrifuged for 2 min at 12000 rpm. 10 microliters of the upper, aqueous solution were transferred to another eppendorf tube containing 2 µl of 6x loading buffer:

<u>Loading Buffer (6x)</u>	<u>final concentration</u>
1ml EDTA 0.5 M	0.1 M
2.5 g sucrose	50% (w/v)
100 μ l bromophenol blue 10%	0.25% (w/v)
100 μ l xylene cyanol 10%	0.25% (w/v)

to a final volume of 5 ml of deionised water. 10 μ l of ethidium bromide (10 mg/ml) were then added and the solution was stored at 4°C.

and the entire solution loaded on 1% agarose gel. The gel was run for 1 hour at 100V, and DNA visualized under UV transilluminator. The samples containing bands migrating differently from control non-ligated plasmid, which was processed together with the ligated colonies, were considered positives. The remaining half of the spreaded colony of these positive clones was taken and dissolved in a 15 ml falcon tube containing 3 ml of liquid LB medium supplemented with antibiotic (ampicillin, 50 μ g/ml final concentration) and allowed to grow over night at 37°C with shaking at 225 rpm.

3.2.5 Purification of DNA from bacteria

Plasmidic DNA was purified from bacterial suspension using the miniprep system (Qiagen). The procedure, starting from 1.5 ml of bacterial suspension was exactly as described by the manufacturer. The recovered DNA, in 50 μ l of water, was subjected to restriction digestion with

appropriate restriction enzymes to verify that the insert was indeed present in the colony isolated. After digestion and separation on agarose gel, the fragments of DNA were visualized by using an UV transilluminator. The positive colonies containing the right insert were stored at -80°C after the addition, to 0.8 ml of the bacterial suspension used for the enzyme digestion, of 0.2 ml of ultra pure glycerol (Sigma). When necessary, the remaining DNA was used for confirmation by DNA sequencing, which was performed through custom sequencing services (through a core facility available at Mario Negri Bergamo, Bergamo, Italy). Once verified that the plasmid contained the right insert and sequenced, if necessary, a large scale preparation of DNA was performed, using the Qiagen midi preparation kit following the procedures reported in the instruction manual.

The DNA recovered from the midi preparation was quantified at the spectrophotometer by reading the absorbance at 260 nm and 280 nm. The quality of the DNA prepared was determined by the ratio between 260 nm and 280 nm absorbances, which should be 1.8

For quantification the extinction coefficient was used. The amount of DNA was calculated considering that a solution of 50 micrograms of DNA/ml would give a reading of 1 at 260 nm.

3.2.6 DNA-Transfection

Transfection of DNA in the different cell lines used was performed by using the calcium phosphate precipitate method. With this method, DNA, dissolved in sterile water is mixed with a solution of 2 M CaCl₂ (prepared by dissolving 14.7 g of CaCl₂ in 100 ml H₂O and filtering the solution with a syringe equipped with a 0.2 µm filter) to give a final concentration of 0.25 M CaCl₂ in a volume of 250 µl. In the meantime, for each transfection to be performed a 4 ml transparent tube (Falcon) is prepared by adding 250 µl of a 2x HEPES-Buffered Saline (HEBS 2x) :

<u>HEBS 2x</u>	<u>final concentration</u>
1.6g NaCl	280 mM
0.074g KCl	10 mM
0.027g Na ₂ HPO ₄ ·H ₂ O	1.5 mM
0.02g Dextrose	12 mM
1g HEPES	50 mM

The salts are dissolved in 50-70 ml of distilled water and the pH of the solution is then adjusted to 7.2 with 0.5 M NaOH and then brought to 100 ml with sterile water.

Then a 1ml sterile pipette fixed to an automatic pipettor is placed at the bottom of the tube containing the HEBS solution and the air forced inside the tube. While the solution is bubbling, the DNA-CaCl₂ solution is added dropwise. When all the DNA has been added, the tube is vortexed for 20

seconds and kept in a tube holder for 30 minutes. The solution is then added dropwise to the cells seeded in a 25 cm² flask and incubated 16 hours at 37°C.

The cells with the DNA-CaPO₄ solution are then visualized under the microscope to verify the presence of small precipitates of CaPO₄ on all the surface of the flask. Once verified this, the medium is removed and the cells extensively washed with PBS (three four times, with the PBS left on the cells 5 minutes to help in removing as much as possible the crystals). After washing, the cells are resuspended in complete medium.

3.2.7 Isolation of cell clones stably expressing the gene of interest

When clones of cells stably expressing the gene of interest have to be recovered, 48 hours after the end of transfection the medium is removed and the cells detached with trypsin EDTA solution. After counting, the cells are seeded in 9 mm plates at a density of 5000 cells/ml in medium containing the selection antibiotic (500 micrograms/ml of G418 for neomycin resistance gene containing plasmids, or 400 micrograms/ml of hygromycin, for plasmid containing the hygromycin resistance gene). At these antibiotic concentrations parental cells are killed and the only cells growing are likely to be those which have integrated in their DNA the transfected plasmid. The plates are then kept at 37°C and the medium renewed every two-three days. When colonies are formed, they are visualized under the microscope and isolated from the plate by using

plastic rings which are made adherent to the plate with vaselin. Once isolated, 20 microliters of a trypsin EDTA solution are added and the cells of the single colony detached and placed in a 24 wells plates containing 1 ml of medium plus selection antibiotic. The different clones picked up from the original plates are grown and passed in duplicated in 6 well plates. One plate is used to verify the presence of the gene of interest and the other is used to maintain the cell clone for further studies and for long term storage. Depending on the system available, the clones can be screened by western blotting or northern blotting, to verify the overexpression of the protein or of the mRNA of the gene of interest, respectively.

The clones overexpressing the gene inserted are then expanded and stored in different aliquots in liquid nitrogen.

3.3 Transient transfections and luciferase activity

Transient transfection experiments are performed following the same procedure described in 3.2.6. These experiments are performed to evaluate the promoter activity of DNA fragments subcloned in appropriate vectors. The constructs used utilize the non mammalian gene luciferase, which is inserted in a promoterless plasmid. For our experiments we have used the pGL2 vector from Promega, which contains the luciferase gene followed by a viral enhancer and a multiple cloning

site at its 5'. The transfection and transient expression of this plasmid in mammalian cells results in a very low level of luciferase (due to the absence of a promoter), which is detected in cell lysates using a commercially available kit (Dual luciferase system, Promega). The genomic fragments to be analyzed are subcloned in the multiple cloning sites of pGL2 vector using restriction enzymes-based ligations. Once verified the exact insertion and orientation of the fragment using the procedures reported in 3.2.5, the plasmids are transfected in mammalian cells growing in culture. 48-72 hours following transfection, the medium is removed and the cell processed as reported in the manual of instruction of the kit. The luciferase levels are measured in a luminometer and the values corrected for the expression of a control plasmid co-transfected with the plasmid under examination, which encodes for a renilla luciferase distinguishable from the fire-fly luciferase utilized in these experiments.

3.4 Host cell reactivation assay

This method is used to determine the ability of a cell to repair the damage induced in a plasmidic DNA. In the experiments reported here, the plasmids utilized are the pGL2 control vector (Promega) which is composed by a viral SV40 promoter followed by a luciferase gene, a polyadenylation signal and a viral enhancer and PG13-luc (kindly

supplied by Dr. Vogelstein) which is a pGL2-derived plasmid in which the luciferase gene is under the control of 13 copies of the p53 consensus binding site. When transfected in mammalian cells this vector allows the expression of luciferase driven by the SV40 viral promoter or by the p53-dependent promoter.

To measure the repair capability of a cell, the plasmid is damaged with DDP *in vitro*, by incubating 50 micrograms of the plasmid with 20 or 200 micromolar DDP for 2 hours at 37°C in final volume of 100 μ l of PBS. To remove unbound DDP, at the end of the reaction the plasmid DNA is precipitated by adding 10 μ l of 3M sodium acetate and two volumes of ethanol and centrifuging the solution at 12000 rpm for 20 minutes at 4°C. The pellet is washed in cold 70% ethanol (300 μ l), dried, resuspended in 100 μ l of water and quantified at the spectrophotometer. Equal amounts (5 μ g) of undamaged or damaged plasmid are then transfected in cultured cells using the calcium phosphate technique. 24 and 48 hours after transfection, the cells are harvested and the luciferase activity measured as described in chapter 3.3.

Again, as an internal control the undamaged renilla luciferase encoding plasmid is used. The luminometer values are then reported to the values obtained in undamaged plasmid-transfected cells and a percentage value of the repair in cells can be obtained.

3.5 RNA analysis

3.5.1 Isolation of total RNA

3.5.1.1 Cesium Chloride Method

Cells growing in culture to be analyzed for RNA expression were washed twice with ice-cold PBS and directly scraped in 1 ml of GTC:

<u>4 M Guanidine thiocyanate (GTC)</u>	<u>final</u>
<u>concentration</u>	
25 g guanidine thiocyanate	4 M
<i>Dilute in 30 ml of warm H₂O and then add:</i>	
367 mg sodium citrate	25 mM
<i>Adjust to pH to 7 with NaOH.</i>	
833 μ l sarcosyl 30%	0.5%
350 μ l β -mercapto-ethanol	0.1 M
<i>to a final volume of 50 ml with sterile water. Filter with Millipore HA 0.45 μm filters and store in the dark at room temperature.</i>	

Scraped cells were collected into a 15 ml polypropylene tube, and the DNA was destroyed by vigorously vortexing and by passing the solution through a disposable syringe equipped with a 18 g needle three-four times. The suspension was carefully layered on top of 2 ml of 5.7 M cesium chloride :

<u>5.7 M Cesium Chloride (CsCl)</u>	<u>final concentration</u>
95.99 g CsCl	5.7 M
830 μ l NaAc 3M, pH 5.4	25 mM

to a final volume of 100 ml with 0.1 M EDTA, pH 7.5. The solution is then autoclaved. The final density should be 1.7 g/l.

in polyallomer tubes (Beckman Polyallomer 13 x 51 mm). The tubes were then centrifuged using a swing rotor (SW40) at 48000 rpm (50000xg) for 16 hours at room temperature in a Beckman centrifuge. The supernatant was carefully removed and the upper section of the tube was cut off leaving only approximately 1 cm of the bottom which was immediately inverted to drain the remaining fluid. The purified total RNA stayed at the bottom of the tube. It was vigorously resuspended with two 100 μ l aliquots of ice-cold water and transferred to a 1.5-ml Eppendorf tube, then precipitated with NaAc (final concentration 0.3 M) and two volumes of 96% ice-cold ethanol. After at least 1 hour at -80°C , the tube was then centrifuged for 30 min at 4°C at 15000xg (Eppendorf centrifuge), and the pellet washed with 70% ice-cold ethanol. The pellet was dried in a speed-vacuum centrifuge and resuspended in 50-100 μ l of pure water. RNA concentration and purity was determined spectrophotometrically detecting the absorbance at 280 nm and 260 nm. Pure RNA should have a 260 nm/280nm absorbance ratio of 2.0. The RNA concentration was calculated considering that a

solution of 40 micrograms of RNA per ml has a 260 nm absorbance value of 1. Samples were then stored at -80°C .

3.5.1.2 Purification of RNA on columns

The SV total RNA isolation kit (Promega) was used for isolating total RNA as an alternative to the time consuming cesium chloride method when the amount of RNA to be recovered was not a limiting factor. In fact, with this method is possible to isolate pure RNA from few cells in a limited time (1-2 hours). The cells are lysed in a GTC containing solution which maintains the integrity of RNA while disrupting cells and dissolving cell components. RNA was then prepared following exactly the manufacture's instructions. The purified RNA is then eluted with a small volume of water (generally 50 microliters). An aliquot of this solution was then used to determine the RNA concentration are described above. Samples were stored at -80°C until use.

3.5.2 RT-PCR

Techniques based on the polymerase chain reaction (PCR) follow the principles in which a pair of primers (forward and reverse) define the region to be amplified. After denaturation (generally at 95°C), primers are allowed to anneal to their complementary strand by lowering the temperature to their optimal annealing temperature (AT) which is specific for each set of primers and is linked to the oligonucleotides sequence. AT

can be calculated on the basis of the following formula: $AT = 4x (G+C) + 2x (A+T)$.

An elongation step allows the Taq DNA polymerase to start the polymerisation reaction downstream of the 3' ends of the primers. These three steps are then repeated for "n" cycles to allow exponential amplification of the target sequence.

RT-PCR has been shown to be several thousand-fold more sensitive than traditional RNA blot techniques. The technique involves the transformation of RNA to cDNA strand, followed by PCR amplification. Amplification is measured by resolving products through agarose gel electrophoresis. Since by increasing the number of amplification steps the reaction reaches a plateau, when a semi-quantitative analysis is needed, is necessary to keep the cycles at a relatively low number to ensure sensitivity without reaching saturation. An internal control, in general a housekeeping gene is used when semiquantitative data are needed.

For RT-PCR analysis, in these experiment the RNA-PCR core KIT (Applied biosystems) was used:

1 µg of total RNA is retrotranscribed to cDNA in 0.5 ml eppendorf tubes, using random hexamers and MMLV reverse transcriptase enzyme. The reaction was performed at 23°C for 10 min, followed by 45 min at 42°C and 10 min at 99°C. Reaction products were kept at 4°C until the PCR steps.

PCR reaction master mix was made in a final volume of 25 μ l following the manufacturer's instructions. Forward and reverse primers were added to a final concentration of 500 nM. Primers were designed on the basis of the sequence reported in the gene-bank database. The "GeneFisher" software free available online (web address: <http://bibiserv.techfak.uni-bielefeld.de/genefisher/>) was used to design the best set of primers to be used for each gene. Synthesis of oligonucleotides used as primers was performed by Sigma.

A drop of mineral oil (Sigma) was layered on the top of the reaction and the mixture quickly spinned in a microfuge to recover all the fluids at the bottom of the tube and placed in DNA thermal cycler. Amplification conditions and cycle numbers were selected each time according to the AT of the pair of primers.

At the end of the amplification 10 μ l of the reaction (carefully avoiding the removal of the mineral oil) were placed in another eppendorf tube and mixed with 1/6th of the volume (2 μ l) of loading buffer :

<u>Loading Buffer</u>	<u>final concentration</u>
1ml EDTA 0.5 M	0.1 M
2.5 g sucrose	50% (w/v)
100 μ l bromophenol blue 10%	0.25% (w/v)
100 μ l xylene cyanol 10%	0.25% (w/v)

to a final volume of 5 ml of deionised water. 10 μ l of ethidium bromide (10 mg/ml) were then added and the solution was stored at 4°C.

The mixture was then loaded on agarose gels, prepared by dissolving 1 g of agarose in 100 ml of TAE 1x:

<u>TAE Buffer (50x)</u>	<u>final concentration</u>
242 g Tris base	2 M
100 ml EDTA 0.5M, pH 8	50 mM
57.1 ml glacial acid acetic	

to 1 l with deionised water. The solution was stored at room temperature.

The solution was boiled to completely dissolve the agarose and cooled at room temperature before pouring in an horizontal electrophoresis chamber.

Samples were electrophoresed at 100 V for approximately 30 min in TAE 1x buffer. DNA bands were visualised under UV light. The assessment of the expected DNA fragment length was achieved using molecular weight markers solutions (Fermentas) which were loaded in a lane parallel to that of the samples and electrophoresed together with the samples.

3.5.3 Northern blotting analysis

Northern blotting is electrophoresis and transfer of RNA to a membrane followed by hybridisation to a specific probe. It provides a measure of the levels of an RNA species within a cell.

3.5.3.1 Sample preparation and gel analysis

During gel electrophoresis RNA molecules must be completely unfolded by the addition of a denaturing agent. Formaldehyde denatures RNA by reacting with amine groups preventing the formation of G-C and A-T base pairs. A formaldehyde agarose gel was prepared in MOPS (Sigma) buffer following the scheme here reported:

<u>Formaldehyde Agarose Gel</u>	<u>final concentration</u>
0.8 g ultra pure agarose	1%
8 ml MOPS buffer 10x	1x
14.4 ml formaldehyde 37%	6.66%
57.6 ml water	

The agarose is dissolved in water by boiling. The other reagents are added when the gel reached approximately 60°C.

<i>MOPS Buffer (10x)</i>	<u><i>final concentration</i></u>
93 g MOPS	0.2 M
20.51 g NaAc	0.5 M
1.86 g EDTA	10 mM

to 500 ml with deionised water. The pH was adjusted to 7 with NaOH and the solution autoclaved.

The gel was poured in a chemical hood and let until solidified.

Equal amounts of RNA (generally 10 µg) were mixed with 3 volumes of a RNA denaturing solution prepared just prior to use following the receipt:

<u><i>RNA Denaturing Solution</i></u>	<u><i>final concentration</i></u>
2.5 ml deionised formamide	50%
0.5 ml MOPS 10x	1x
806 µl formaldehyde 37%	6%

to a final volume of 5 ml with sterile water.

Formamide is deionised to remove ionic contaminants which could hydrolyse RNA. To do this commercially available Formamide was added to mixed-bed resin (BioRad) (5 g resin/100 ml formamide) and stirred for at least 1 h in the dark. Formamide was filtered from the resin with Whatman paper, aliquoted and stored at -20°C.

RNA samples were denatured at 65°C for 15 min and quickly chilled on ice. Then 1/6th of the volume of loading buffer was added to the mixture immediately before loading.

The RNA loading buffer consists of:

	<u>final concentration</u>
500 μ l glycerol	50%
25 μ l bromophenol blue 10%	0.25%
475 μ l of water	

1 μ l of ethidium bromide (10 mg/ml) is added to 29 μ l of this solution. The solution is aliquoted and stored at -20°C.

After loading, the gel running was performed in MOPS buffer (1x) at constant voltage of 80 V, for 2-3 h. At the end of the run the gel was briefly visualised under UV-light to visualize the two distinct, abundant, ribosomal bands (18S and 28S) which are indicative of a good separation and absence of degradation. The RNA was then blotted onto a membrane.

3.5.3.2 Blotting procedure

A capillary blot was set up using a solution of 20 x SSC (175.3 g NaCl, 88.2 g sodium citrate in 1 l of water) as transfer buffer.

The wick was made up of 2 pieces of 3MM paper with each end dipping into the transfer solution. The gel was placed on the wick with wells facing down, and air bubbles were gently removed. A nylon membrane

(GeneScreen plus, NEN) of exactly the same size of the gel was soaked in 20xSSC for at least 10 minutes and carefully placed on top of the gel and gently squeezed to remove air bubbles. Three sheets of 3MM paper were then added over the membrane followed by kleenex towels. The blot system was left for 16-24 hours after which the membrane was removed, washed by immersion in deionised water, left to dry at room temperature for approximately 30 min and examined under UV light to mark the position of the RNA 18S and 28S fragments. The membrane was then baked at 80°C for 2 hours to remove any residual formaldehyde from the RNA, as this could affect the efficiency of the hybridisation. The blot was stored at 4°C until ready for hybridisation.

3.5.3.3 Preparation of probes for northern blot analysis

Probes suitable for determining the level of the message of the gene of interest were prepared either by directly labeling the entire plasmid containing the gene insert, by using the gene insert excised by the plasmid by restriction enzyme digestion or by using PCR amplified fragments. Whatever the starting material was, the radioactive probe was prepared using a klenow fragment of polymerase I - based method. A commercially available kit (Rediprime, Amersham) was used for this purpose. For labeling, 50 ng of DNA as starting material were used and all the steps described in the manual were followed using as radioactive nucleotide the ³²P-labeled dCTP (Amersham, 3000 Ci/mmol). Purification of the probe

from unlabelled nucleotide was performed using Sephadex G50 columns (Boehringer-Mannheim) exactly following the manufacturer instructions. The incorporated radioactivity detected by cherenkov counts and the probe was diluted to have a final concentration of 500000 cpm/ml of hybridisation solution.

3.5.3.4 Hybridisation

The membrane prepared as described in 3. was pre-hybridised for at least 4 hours at 42°C in a glass bottle containing 10-15 ml of hybridisation solution:

	<u>final concentration</u>
2 ml NaCl 5M	2 M
5 ml deionised formamide 100%	50%
2 ml dextran sulphate 50%	10% (w/v)
250 µl Salmon sperm DNA 10 mg/ml	100 µg /ml

The salmon sperm DNA was prepared by boiling the solution for 10 min, chilling on ice and storing in aliquots at -20°C.

The radiolabeled probe was denatured by boiling for 5 min and quickly chilling on ice. The glass bottle containing the membrane was removed from the hybridization oven and the probe directly added to the solution, carefully trying not to touch the membrane. The mixture was gently mixed and the incubation performed overnight at 42°C.

The membrane was washed twice at room temperature for 10 minutes with 2x SSC, followed by two 2 washes of 15 min each at 65°C with 2 x SSC + 1% SDS and a final wash in 2x SSC without SDS for 10 minutes.

The blot was kept humid by wrapping it in Saran wrap and either exposed to film at -80°C or exposed to a phosphorimager screen at room temperature for at least 5 h.

When necessary, the probe was stripped from the membrane by boiling the blot for 10 min in 0.5% (w/v) SDS solution. The blot was again kept humid, checked for radioactivity and either stored at 4°C or hybridised with another probe.

3.5.4 Gene expression by microarray technology

The gene expression profile in parental A2780 cells and in the two clones overexpressing p73 (A2780/p73.4 and A2780/p73.5) has been evaluated using the ATLAS gene expression system (Clontech BD, ATLAS human cancer filter set). Total RNA from the three different cells lines has been isolated from logarithmically growing cells using the SV total RNA system (Promega), see 3.5.1.2. Ten micrograms of total RNA were then retrotranscribed to cDNA in the presence of a radiolabeled nucleotide. The procedure was performed following the user manual's instructions.

In a 0.5 ml eppendorf tube 1µl of CDS primer mix (a mix of primers specific for each filter set) and 2 µl of total RNA (5 µg/ml) were mixed and incubated for 2 minutes at 70°C in a thermal cycler. The temperature was

then reduced to 50°C and the incubation continued for further 2 minutes. Then 8 µl of master mix, prepared by mixing 2 µl of 5x reaction buffer (250 mM Tris.HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂) 1 µl of dNTP Mix (5 mM each dCTP, dGTP, dTTP), 3.5 µl of ³²P-dATP (10 µCi/µl), 0.5 µl of DTT (100 mM) and 1 ml of MMLV reverse transcriptase, were added and the reaction incubated at 50°C for 25 minutes. At the end 1 ml of termination mix (0.1 M EDTA, 1mg/ml glycogen) was added and the probes purified on sephadex columns (3.5.3.3).

Atlas filters were prehybridized for 30 minutes with 10 ml of the solution supplied with the filters at 68°C. To this solution, 5-10 x 10⁶ cpm of probes were added and the hybridization performed at 68°C for 16 hours. Filters were then removed from the oven and washed as described in 3.5.3.4

The filters were immediately placed in srana wrap and exposed using a phosphoimager. The image imported from the phosphoimager was then analyzed with the ATLAS software using the grid relative to filters used (ATLAS human cancer). When analyzed, the differences in expression levels between p73 overexpressing clones and parental cells were considered significant if showing at least a two fold difference.

3.6 Western blotting

Western blot analysis involves separating proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), transferring them to nitro-cellulose electrophoretically and then using specific antibodies to detect the protein of interest.

3.6.1 Preparation of cellular proteins

Total proteins were extracted from cells growing in culture through a lysis method. Basically cell cultures were washed twice with ice-cold PBS and then scraped with a disposable scraper in 500 μ l of PBS. The solution was then centrifuged at 1200 rpm for 10 minutes and after centrifugation, the pellet was resuspended in 100 μ l of lysis buffer (for cells growing in suspension the pellet obtained after the second wash was directly resuspended in the lysis buffer):

<i>Lysis Buffer</i>	<u><i>final concentration</i></u>
<i>20 μl Triton X-100</i>	<i>1%</i>
<i>20 μl Tris 1M, pH 7.4</i>	<i>10 mM</i>
<i>60 μl NaCl 5 M</i>	<i>150 mM</i>
<i>8 μl leupeptin, 5 mg/ml</i>	<i>20 μg/ml</i>
<i>5 μl aprotinina 2 mg/ml</i>	<i>5 μg/ml</i>
<i>20 μl PMSF 100 mM</i>	<i>1 mM</i>

to a final volume of 2 ml with sterile water.

and incubated on ice for 30 minutes in an orbital shaker. After 10 min of centrifugation at 12000 x g at 4°C, cellular debris were pelleted and the total protein present in the supernatant was recovered and placed in a fresh Eppendorf tube (1.5 ml). An aliquot (10 µl) was used for determination of protein concentration (see section 3.6.1.2).

3.6.1.1 Calibration curve preparation

Solutions of bovine serum albumin (BSA) (ranging between 1 and 20 µg/200 µl) were prepared from a stock solution of BSA obtained by dissolving powdered BSA (Sigma) in water. In a 1.5 ml tube, 200 µl of each BSA solution were mixed with 600 µl of distilled water and 200 µl of BioRad protein assay dye (BioRad). In the blank sample, 800 µl of distilled water was mixed with 200 µl of BioRad Protein assay dye. Samples were rapidly transferred into disposable cuvettes (PBI International, Milan-Italy) and the absorbance at 595 nm was measured in the spectrophotometer. The absorbance value corresponding to the blank sample was subtracted from the values obtained in the BSA-containing samples. Each calibration sample was run in triplicate. The calibration curve obtained in such a way, allows extrapolation of the exact absorbance value corresponding to 1 µg of proteins present in the solution.

3.6.1.2 Determination of protein concentration in cellular extracts

Protein concentration in the total cellular extract was determined according to Bradford protocol.

The concentration of proteins in the samples was determined by mixing in a 1.5 ml tube 10 μ l of protein extract with 200 μ l of BioRad protein assay dye and distilled water in a final volume of 1 ml. Samples were processed as for the calibration curve and the amount of proteins calculated from the absorbance value corresponding to 1 μ g of proteins (obtained from the calibration curve).

3.6.2 SDS-PAGE

An aliquot (20 μ g) of protein of each sample was mixed with the same amount of 2x SDS loading buffer :

<i>SDS Loading Buffer (2x)</i>	<u><i>final concentration</i></u>
<i>200 μl Tris 1 M, pH 8.8</i>	<i>100 mM</i>
<i>400 μl DTT 1 M</i>	<i>200 mM</i>
<i>800 μl SDS 10%</i>	<i>4%</i>
<i>400 μl glycerol</i>	<i>20%</i>
<i>40 μl bromophenol blue 10%</i>	<i>0.2%</i>
<i>to a final volume of 2 ml with sterile water</i>	

and the mixture was boiled for 5 min. Samples were loaded onto 5% stacking gel and 12% separating gel:

<u>Stacking Gel</u>	<u>final concentration</u>
2.5 ml Tris-HCl 0.5 M, pH 6.8	125 mM
0.1 ml SDS 10% (w/v)	0.1% (w/v)
1.62 ml 30% acrylamide/bis 37.5:1	5% (w/v)
<i>To a final volume of 10 ml with deionised water.</i>	
50 μ l ammonium persulphate 10% (w/v)	0.05% (w/v)
5 μ l TEMED	

<u>Separating Gel</u>	<u>final concentration</u>
5.0 ml Tris-HCl 1.5 M, pH 8.8	750 mM
0.2 ml SDS 10% (w/v)	0.1% (w/v)
5.36 ml 30% acrylamide/bis 37.5: 1	8% (w/v)
<i>To a final volume of 10 ml with deionised water.</i>	
100 μ l ammonium persulphate 10% (w/v)	0.05% (w/v)
7.5 μ l TEMED	

Stacking and separating gels were prepared shortly before pouring. Ammonium persulphate catalyses polymerisation and TEMED accelerates the reaction and so these two reagents were added last.

Proteins were resolved on a minigel apparatus (BioRad) and run for 2 h at 50V in 1x TGE buffer:

<i>Running Buffer (TGE 5x)</i>	<u><i>final concentration</i></u>
15.15 g Tris base	25 mM
72 g glycine	192 mM
5 g SDS	0.1% (w/v)

to a final volume of 5 l with deionised water. The buffer was stored at room temperature.

Electrophoresis progress was followed using pre-stained molecular weight markers (14.3-200 KD), prepared following the manufacturer's instructions (BioRad).

3.6.3 Protein transfer and detection

The separated proteins were transferred onto nitro-cellulose (at 60V for 3 h) using BioRad Mini transfer blot equipment in 1x transfer buffer:

<i>Transfer Buffer 1x</i>	<u><i>final concentration</i></u>
24.2 g Tris Base	50 mM
28.5 g glycine	100 mM
4 ml SDS 10%	0.01%
800 ml methanol	20%

to a final volume of 4 l with deionised water. The solution was stored at room temperature.

Filters were stained with Ponceau red solution (Sigma) to check sample loading. Blots were placed in TBS-T 0.1% with 10% non-fat dried milk:

<i>TBS-T 0.1%</i>	<u><i>final concentration</i></u>
2.42 g Tris base	20 mM
8 g NaCl	137 mM
1 g Tween 20%	0.1% (w/v)

to a final volume of 1 l with deionised water. The pH was adjusted to 7.6 with concentrated HCl and stored at 4°C.

and shaken o/n at 4°C to block non-specific binding. All following procedures were carried out at room temperature on a shaker. Blots were exposed for 1 h at room temperature to the desired antibodies diluted to the optimal working solution in TBS-T 0.1%. After incubation, the blots were washed twice with TBS-T 0.1% and incubated with the appropriate horseradish-peroxidase linked anti-mouse or anti-rabbit IgG secondary antibody (Amersham) for 1 h using dilutions. Blots were washed as previously described, and detection was performed with enhanced chemiluminescent detection system (ECL, Amersham-Life Science). Briefly, the horseradish peroxidase acts as a catalyst for the oxidation of a

luminol substrate, which subsequently emits small but sustained quantities of light. This chemiluminescence is specifically enhanced allowing an image to be recorded on photosensitive film. Blots were exposed to film for different time ranging from 15" to 3 min and developed using X-o graph compact x-2 developer with Kodak GBX developer and fixer.

3.7 Evaluation of kinase activity

Activity of DNA-dependent protein kinase (DNA-PK) was determined using a commercially available kit (SignaTECT DNA-PK Assay System, Promega) following the instructions. Cell extracts were prepared as described in 3.6.1 and kinase activity determined in triplicate, either in absence or in the presence of double-strand DNA .

ATR activity was determined after immunoprecipitation with anti ATR antibodies. Briefly 100 μg of protein extracts (prepared as in 3.6.1) were incubated at 4°C with 1 μg of anti ATR antibody (SantaCruz Biotechnology) overnight with continuous, gentle rocking. To this solution, 50 μl of agarose beads (Santa Cruz Biotechnology) were added and the incubation continued for 1 hour. Agarose bound kinase was collected by centrifugation at 13000 rpm in an eppendorf tube for 5 min and washed twice with 500 μl of PBS. Kinase reaction was performed in 25 μl reaction containing: 1.5 μg of histone H1 (Sigma, used as substrate),

5 μ Ci of 32 P- γ ATP (Amersham), 1 μ M ATP, 0.5 mM DTT, 50mM Tris HCl, 10 mM MgCl₂. After 20 minutes incubation at 30°C, 25 μ l of 2x loading buffer (see 3.6.2) are added and the mixture heated at 90°C for 5 minutes, cooled on ice and loaded on 10% SDS-PAGE gels. The gel was run for 16 hours at 30 V after which the gel is removed and directly exposed to autoradiographic films to detect phosphorylated H1 histone. The intensity of the bands was determined using a densitometer (Immagini & Computer, Italy).

3.8 Screening of genomic libraries

Genomic clones containing the genomic CHK1 sequences were isolated by screening a genomic library spotted on filters obtained through the UK Human Genome Mapping Project Resource Centre (UK-HGMP-RC).

The human PAC library RPC11 has been constructed in the vector, pCYPAC2N. The source is a normal male blood donor, and the insert size is about 110 kb.

The library consists of approximately 120,000 clones which have been spotted on 22.2 x 22.2 cm Hybond N nylon membranes (Amersham). Each clone has been spotted twice to give 36,864 (18,432 x 2) spots on each membrane. 7 filters cover the whole library.

The 7 filters were prehybridized at 65°C in 25 ml of a solution containing 6x SSC, 100 μ g/ml of salmon sperm, 0.5% SDS and 5x Denhardt's solution

(a 50 x Denhart solution is prepared by dissolving 1 g of Ficoll 400, 1 g of polyvinylpyrrolidone and 1 g of BSA fraction V in 100 ml of water; the solution is then filtered and stored at -20°C) with gentle, continuous agitation. To this solution, radiolabeled CHK1 cDNA probe (prepared as reported in 3.5.4.3) was added and the incubation continued for further 16 hours at 65°C. The filters were then washed twice at room temperature in 2x SSC, followed by two 2 washes of 15 min each at 65°C with 2 x SSC + 1% SDS and a final wash in 2x SSC without SDS for 10 minutes and exposed to autoradiographic films. Once developed, the films were oriented with the grid supplied with the filters and the positive clones identified. This procedure was facilitated by the double spotting of each clone. The identified, positive clones, were requested to the UK-HGMP-RC and obtained as glycerol stock. From these stocks, the PAC clones were isolated using the Qiagen midi preparation kit following the procedures reported in the instruction manual. To isolate shorter genomic fragments containing the CHK1 gene, an aliquot of the genomic DNA isolated was digested with different restriction enzymes in their appropriate buffer, supplied together with the enzyme, for 16 hours at 37°C and the entire reaction loaded on 0.8% agarose gels. After 16 hours running at 30 V, the gel was visualized under UV and a picture collected for subsequent identification for the positive bands, blotted to a nylon membrane (Amersham) and hybridized with a radiolabeled CHK1 cDNA probe. Hybridization and washing conditions were the same used for the

screening of the genomic filters. The autoradiograph was compared to the ethidium bromide picture and the bands which hybridized with the probe identified. A second digestion was performed on the remaining genomic DNA maintaining exactly the same conditions previously used. Once separated on gel, the bands corresponding to the previously selected positive bands were excised and isolated using the QIAgel extraction kit (Qiagen). The band was ligated in pGL2 vector in the multiple cloning site using the same restriction enzyme used for the digestion of the plasmid and sequenced using the primers available from the manufacturer to detect the orientation. The clones containing sense and antisense constructs were further amplified and DNA isolated and used in transient transfection experiments for the determination of luciferase activity.

3.9 Flow cytometric analysis of cell cycle phases distribution

The experiments aimed at evaluating the effects of drugs on cell cycle phase distribution were performed by Eugenio Erba and his team in our department.

For this purpose they received the cells either from untreated or treated samples and the treatment conditions were essentially those reported for the evaluation of cytotoxic activity (as described in section 3.1.4).

To analyze cell cycle phase distribution, at the end of treatment or at the end of incubation in drug-free medium, the medium was removed by

aspiration and replaced with 5 ml of PBS. After a gentle wash, the PBS was removed and new PBS added. The cells were gently scraped in PBS and recovered by centrifugation at 1200 rpm for 10 minutes. The cells were fixed in ice-cold ethanol 70%, washed in PBS and resuspended in 2 ml of a solution containing 2.5 mg/ml of propidium iodide and 25 mg/ml of RNase and stained overnight in the dark at 4°C. At least 500.000 cells per sample were analyzed using the FACSORT system (Becton Dickinson). Analysis of cells' distribution in the different phases of the cell cycle was calculated following a previously published procedure (Broggini et al. 1991).

4.RESULTS

4.1 p53 and its effect on cellular response to anticancer agents

4.1.1 Introduction

p53 is one of the key proteins participating in the control of genomic integrity (Levine 1997; Vogelstein et al. 2000). In the majority of human tumors the gene is inactivated (Harris 1996; Hollstein et al. 1997). The lack of expression of a functional p53 in tumors, could be an advantage for those anticancer agents showing an increased activity towards cells with inactivated p53. To test the effect of p53 on the response of epithelial-derived cancer cells, isogenic cell systems differing only for the expression of p53 (or of p53-regulated genes such as p21) were obtained. These systems, in which two cell lines with the same genetic background are compared, are probably the best systems in which the “true” effect of p53 can be evaluated.

In the majority of the experiments reported in this section, the focus of attention was on comparing the in vitro growth inhibitory activity and the ability to activate checkpoint proteins of two widely used anticancer agents acting by different mechanisms of action, cisplatin (DDP) and taxol. They were selected not only for their widespread use in the clinical practice, but also as paradigms of drugs which differ in their ability to induce DNA damage. DDP does, in fact, induce multiple lesions at the level of DNA, including DNA strand breaks and DNA inter- and

intra- strand crosslinks, while taxol does not directly interact with DNA and acts by inhibiting microtubule depolymerization (Eastman 1983; Horwitz 1992).

Throughout the different experimental systems, directly generated in our laboratory or obtained by other laboratories, these two drugs were compared in isogenic cell pairs in terms of their in vitro cytotoxic activity and their ability to induce differential cell response. In some cases the different cell systems were also tested with other new promising anticancer drugs acting by yet unknown mechanisms or selected to hit specific molecular targets.

4.1.2 p53 and DDP

Initially the HCT116-derived cell system was used, which is defined by the parental colon cancer cell line and its sublines differing with respect to p53 status, to evaluate the effect of p53 expression on the cytotoxic activity of anticancer agents. This system is particularly interesting since two independent sublines were available in which p53 was inactivated by transfection with the E6 protein of the human papilloma virus HPV16 (leading to HCT116/E6) or by targeted homologous recombination (HCT116 p53-/-).

In both cases the activity of anticancer agents was tested by using the colony assay. Figure 4.1 shows the results obtained with DDP in HCT116 HCT116/E6 cells. The figure shows the percentage of inhibition of colony formation in the two cell lines treated with different concentrations of the drug for 1 hour. HCT116/E6 cells were more sensitive to treatment with DDP than parental cells. The IC₅₀, calculated from different experiments is approximately 5 μ M for parental cells and 15 μ M for E6-transfected cells. Similar results were obtained when cells, in which p53 had been inactivated at the genomic level, were used (figure 4.2). Here the cells without p53 were more sensitive to DDP at all concentrations tested. The calculated IC₅₀ values for HCT116 parental and HCT116 p53^{-/-} cells were 5-10 μ M and 20-25 μ M, respectively.

In these two independent systems, the expression of p53 and of p21, selected as an indicator of the activation of p53 downstream genes, was checked by Western blotting. The results are shown in figure 4.3. As expected, DDP was able to induce the expression of p53 in parental cells, but not in the p53-inactivated cells. The p53 downstream gene p21 was also nicely detectable in parental cells, but not in HCT116/E6 or HCT116 p53^{-/-} cells after DDP treatment. These results confirm not only that in both systems p53 was indeed undetectable, but also that there was no p53-dependent activation of downstream genes.

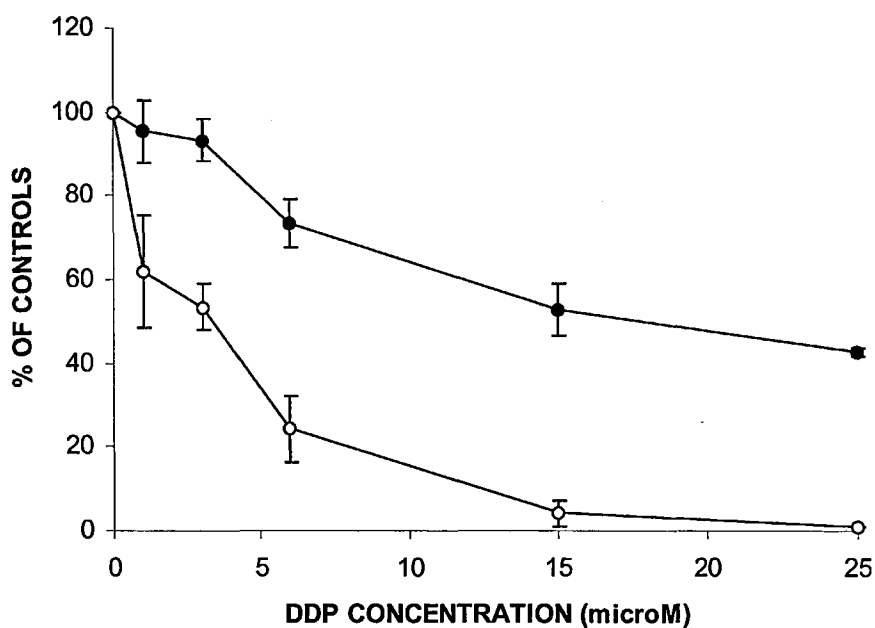


Figure 4.1

In vitro cytotoxic activity of DDP in HCT116 parental (●) and in HCT116/E6 cells (○). Cells were treated with different concentrations of DDP for 1 hour. At the end of treatment the drug was removed and the cells incubated in drug free medium for 14 days. The percentage of colonies in treated cells was calculated relative to untreated cells. The results are the mean \pm SD of three independent experiments each performed in triplicate.

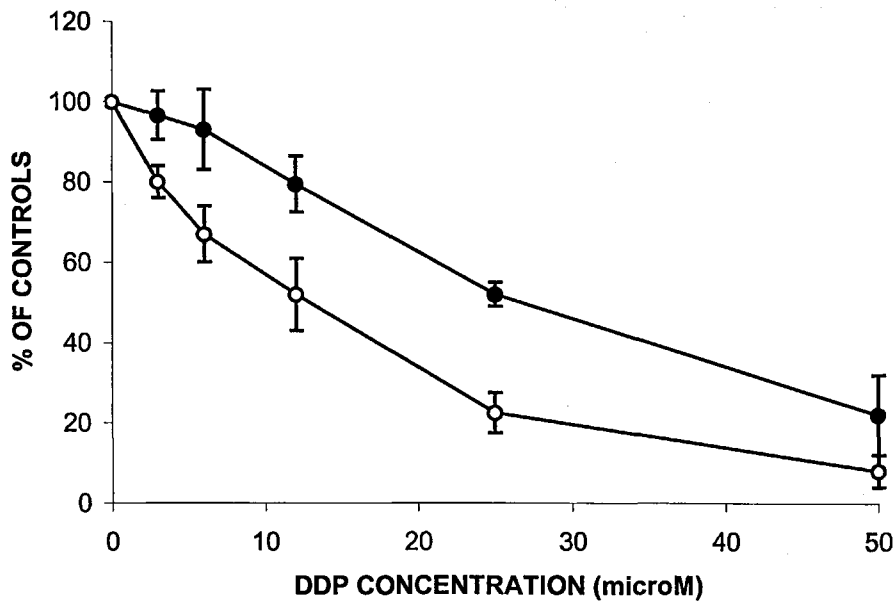


Figure 4.2

In vitro cytotoxicity of DDP in HCT116 p53 +/+ (○) and in HCT116 p53 -/- cells (●). Cells were treated with different concentrations of DDP for 1 hour. At the end of treatment the drug was removed and the cells incubated in drug free medium for 14 days. The percentage of colonies in treated cells was calculated relative to untreated cells. The results are the mean +/- SD of two experiments each consisting of three replicates.

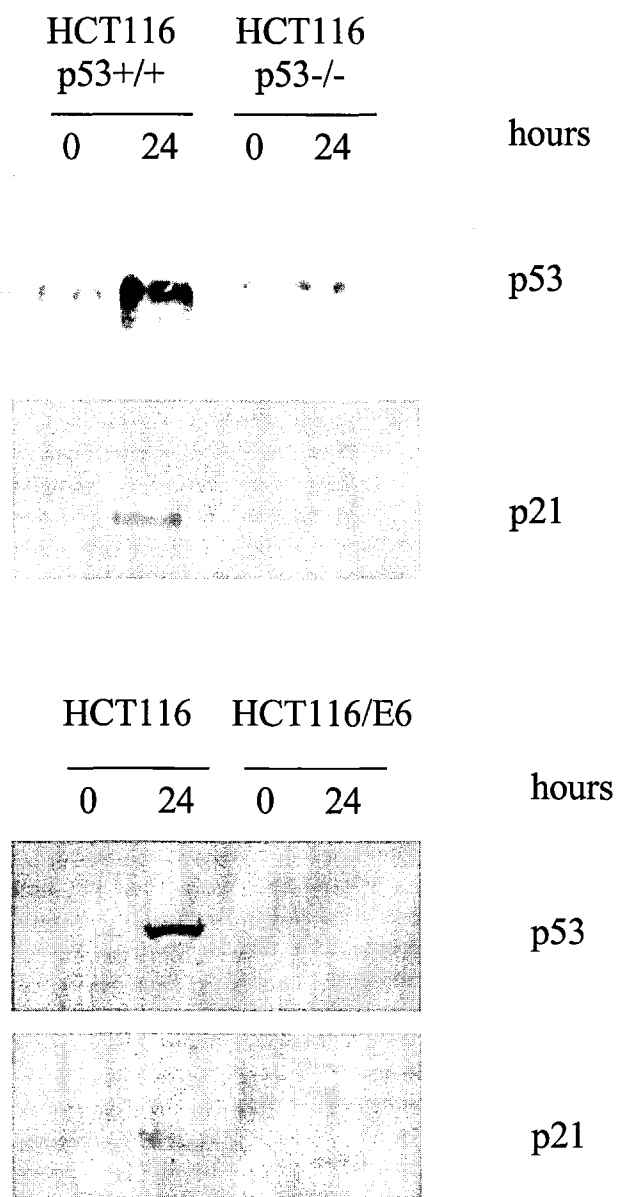


Figure 4.3

Representative western blot showing the effect of DDP treatment on p53 and p21 protein levels in HCT116 p53+/+, and in HCT116 p53-/- (upper panel) and in HCT116 and HCT116/E6 cells (lower panel).

Cells were treated with 25 μ M DDP for 1 hours and cellular extract taken after 24 hours incubation in drug-free medium.

The blot was hybridized with anti p53 and anti p21 antibodies.

To see whether the effect of p53 on DDP activity was due to the strong p53-induced activation of the cell cycle inhibitory gene p21 observed in these cells, we used a p21^{-/-} clone derived from parental HCT116 cells.

In these cells the p53 protein is normal and functional and it is the p21 gene which has been inactivated by targeted homologous recombination. The *in vitro* growth-inhibitory activity of DDP in parental and p21^{-/-} cells is shown in figure 4.4. Cells lacking p21 were more susceptible to DDP treatment, as were cells lacking p53. The ratio between the IC₅₀ deduced from the growth inhibition curves in parental and p21^{-/-} cells is approximately 2-3, similar to the ratio found between p53^{+/+} and p53^{-/-} cells.

These data have been confirmed in the system derived from the human ovarian carcinoma cell line A2780. In this genetic background the virally inactivated p53 containing cells were available, which were obtained in our laboratory using the same plasmid and methods used to select the HCT116/E6 clone. A2780 cells did not sustain targeted homologous recombination, as HCT116 cells did, and it was therefore not possible to inactivate the p53 gene genetically in these cells. In the A2780/E6 clone, (figure 4.5) DDP showed increased activity in the absence of p53. The calculated IC₅₀ values were in fact 30-40 μ M for parental A2780 cells and 15 μ M for A2780/E6 cells. It is noteworthy that in the

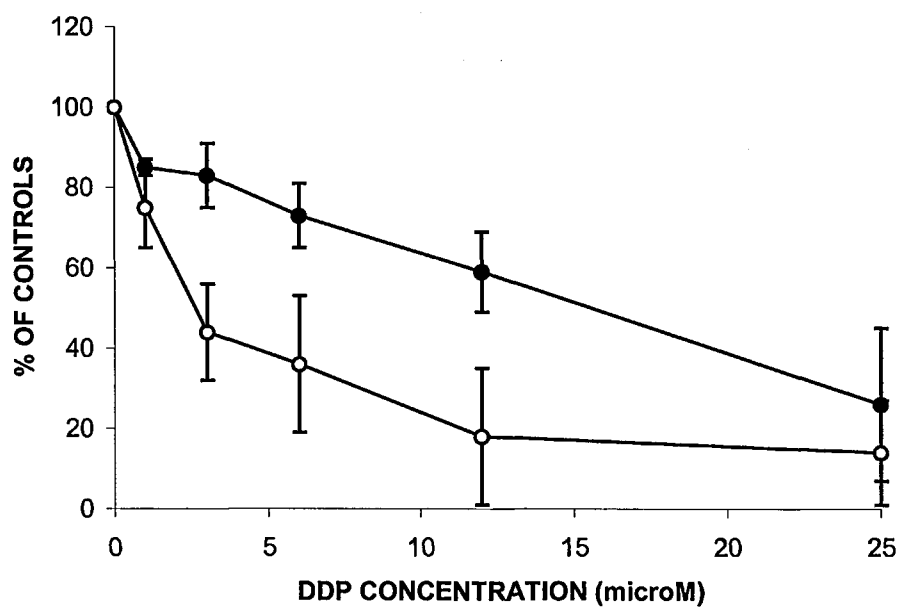


Figure 4.4

In vitro cytotoxicity of DDP in HCT116 parental (●) and in HCT116 p21 -/- cells (○). Cells were treated with different concentrations of DDP for 1 hour. At the end of treatment the drug was removed and the cells incubated in drug free medium for 14 days. The percentage of colonies in treated cells was calculated relative to untreated cells. The results are the mean +/- SD of two experiments each consisting of three replicates.

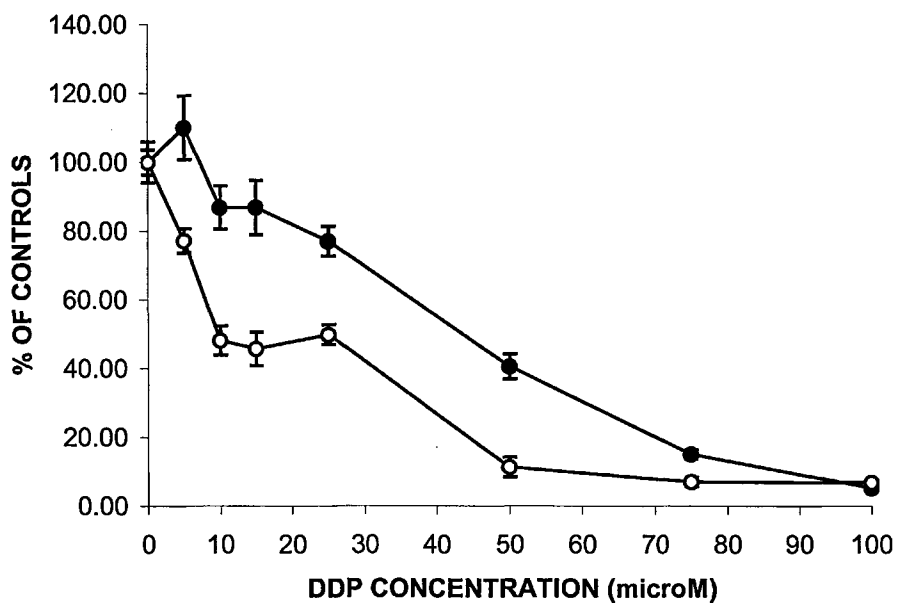


Figure 4.5

In vitro cytotoxicity of DDP in A2780 parental (●) and in A2780/E6 cells (○). Cells were treated with different concentrations of DDP for 1 hour. At the end of treatment the drug was removed and the cells incubated in drug free medium for 72 hours. The percentage of 540nm abs in treated cells was calculated relative to untreated cells. The results are the mean +/- SD of two experiments each consisting of six replicates.

A2780-derived system, the absolute concentration of drug able to inhibit the growth by 50% was slightly higher than in the HCT116 cells. This difference could be due either to the different cellular background or to the different system used to evaluate the cytotoxic activity, which was the MTT test for A2780 cells and the colony assay for HCT116 cells. Nevertheless, the ratio between p53 inactivated and parental cells was roughly the same in the two systems.

4.1.3 p53 and taxol

The results described in the above section dealt with the use of an anticancer agent which induces DNA damage. Here the hypothesis was tested whether another compound, not interacting directly with DNA, would give the same results. Taxol was selected for its outstanding activity in experimental systems and in the clinic. From the data reported in the literature, including those obtained at the National Cancer Institute (USA), taxol was found consistently more active in cells lacking functional p53 (Wahl et al. 1996; O'Connor et al. 1997). The activity of this compound was tested in our system and the results are shown in figure 4.6. In the ovarian cancer-derived system, taxol showed marked differential activity between parental and A2780/E6 cells. The IC₅₀ in the latter (3.9 nM) was 10 times lower than that found in parental cells (41 nM). Again this effect was mostly related to the cell cycle effects induced by p53.

It was previously reported that in A2780 cells taxol induces a "classical"

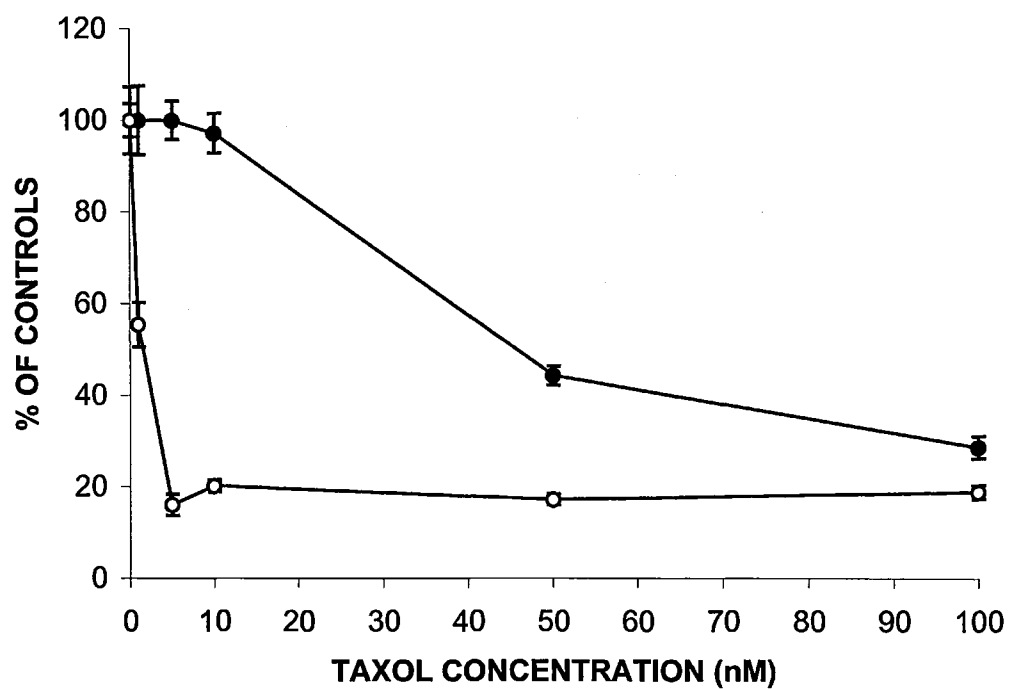
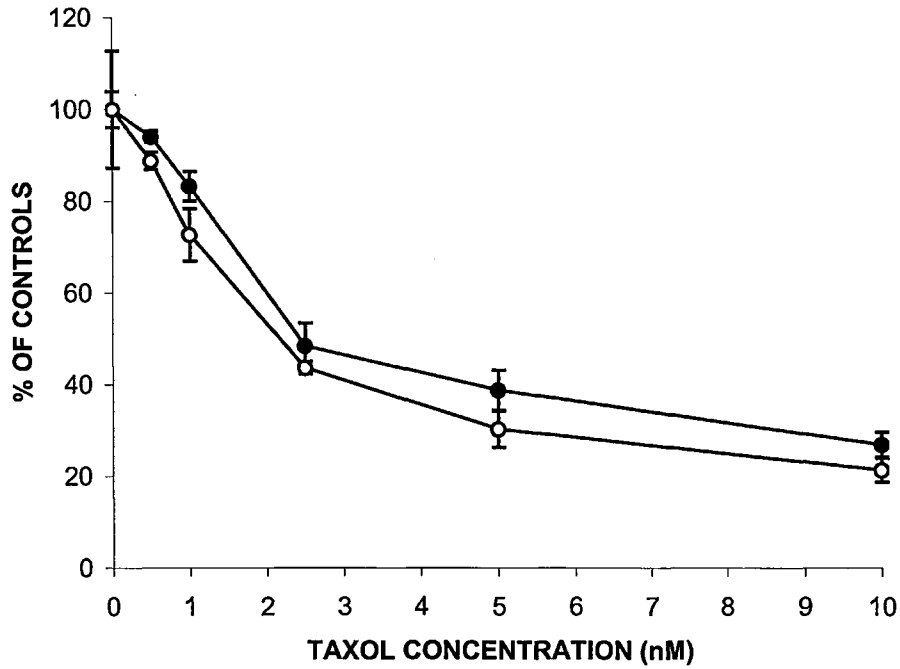
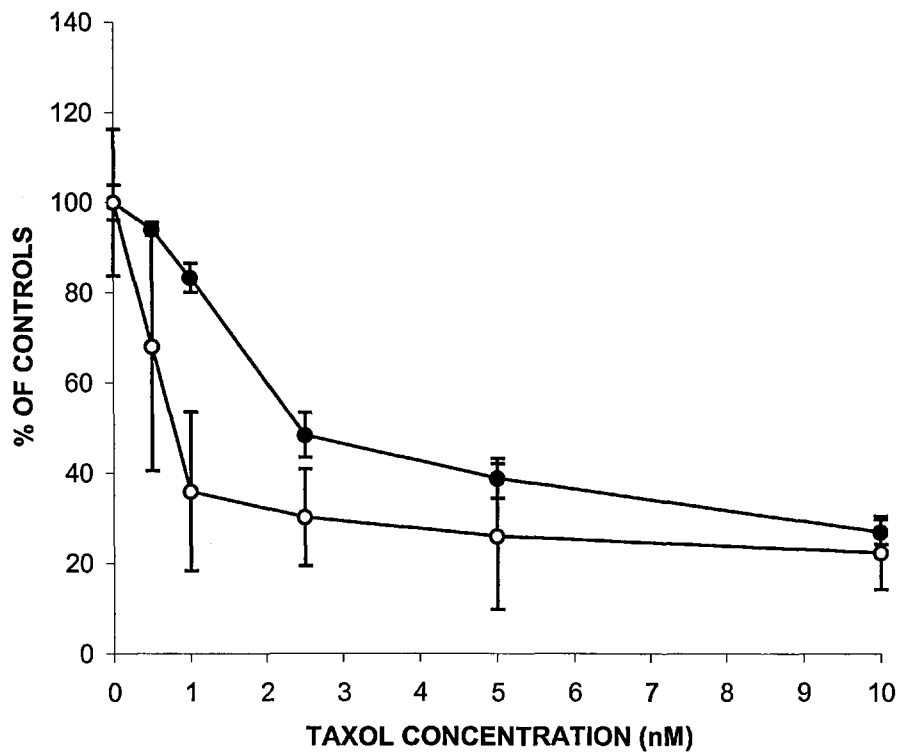


Figure 4.6

In vitro cytotoxicity of taxol in A2780 (filled circle) and in A2780/E6 (open circle) cells. Cells were treated with different concentrations of taxol for 1 hour. At the end of treatment the drug was removed and the cells incubated in drug free medium for 72 hours. The percentage of 540nM abs in treated cells was calculated relative to untreated cells. The results are the mean +/- SD of two experiments each consisting of six replicates.

A**B****Figure 4.7**

Panel A: In vitro cytotoxicity of taxol in HCT116 (●), and in HCT116 p53^{-/-} (○) cells.

Panel B: In vitro cytotoxicity of taxol in HCT116 (●), and in HCT116 p21^{-/-} (○) cells.

Cells were treated with different concentrations of DDP for 1 hour. At the end of treatment the drug was removed and the cells incubated in drug free medium for 12 days.

The percentage of colonies in treated cells was calculated relative to untreated cells.

The results are the mean +/- SD of three replicates.

p53-mediated G1 arrest, while in E6-transfected cells it induces an accumulation of cells in the G2-M phases of the cell cycle (Vikhanskaya et al. 1998). The abrogation of p53-induced G1 arrest following taxol treatment was also previously found to be associated with an increased apoptotic rate (Vikhanskaya et al. 1998). Similar results were obtained in the HCT116-derived system (figure 4.7 panel A), even if the difference in activity of this drug between p53-inactivated and parental cells was much lower than that found in A2780 cells. As was done for DDP, the activity of taxol was also tested in p53-functional but p21-inactivated HCT116 cells (figure 4.7 panel B). Removal of p21 was associated with a marked increase in sensitivity to taxol compared to parental HCT116 cells.

4.1.4 Post-translational p53 modifications

To verify whether the two drugs used were able to induce post-translational modifications of p53, and eventually different pattern of phosphorylation, p53 N-terminal modifications were studied, and specifically phosphorylations at serine 15 and serine 20, in cells treated with DDP or taxol. Both serine residues have been shown to be phosphorylated after UV and IR damage. Figure 4.8 shows a representative Western blot obtained after DDP and taxol treatment in HCT116 cells. Cells were treated for 24 hours with drug concentrations close to their IC50. At the end of treatment, medium containing the drug was removed, and the cells were incubated in drug-free medium. Total

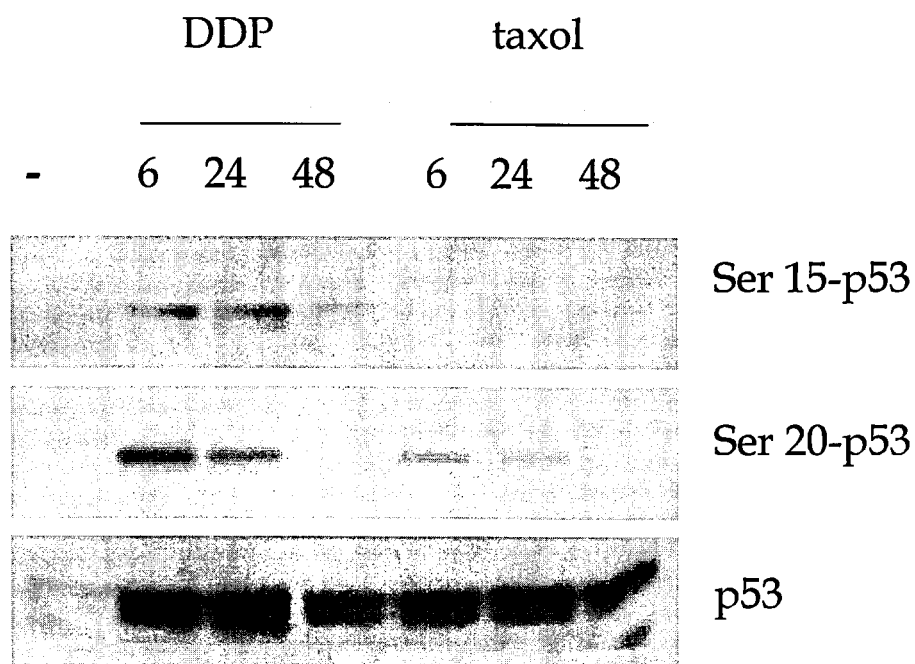


Figure 4.8

Phosphorylation of p53 at serine 15 and serine 20 in HCT116 cells following treatment with 25 μ M DDP or 50 nM taxol.

Cells were treated for 24 hours with the drugs and proteins extracted after 6, 24 and 48 hours incubation in drug-free medium. Proteins were separated on SDS-PAGE and blotted on nitrocellulose filters.

Blots were probed with anti ser15 and ser20 p53 specific antibodies. The same membrane was then blotted with anti p53 antibodies.

cellular extracts were prepared 6, 24 and 48 hours after treatment and the levels of total p53 and of serine 15 and serine 20 modified-p53 were compared to those of untreated cells. Both drugs were able to induce, as expected, an increase in the levels of p53, and both drugs, although with some quantitative differences, were able to induce phosphorylation at serine 20. Differences were found when phosphorylation of serine 15 was analyzed. In this case only DDP treatment was able to induce a modification at this residue, and it was unaffected by taxol. As a further control of p53 activation, the blot was hybridized with anti p21 antibodies, and after DDP or taxol an increase in the levels of this p53 downstream gene was found.

Similar experiments were performed in A2780 cells, which were treated with DDP or taxol at concentrations close to their IC₅₀ (15 μ M and 50 nM, respectively) for 1 hour. Again, both drugs did induce an increase in the levels of p53 and of p21 (figure 4.9), and both drugs were able to induce phosphorylation of p53 at serine 20, but only DDP did induce phosphorylation of serine 15 of p53.

Thus it was established that DDP but not taxol, was inducing post-translational modification of p53 at serine 15. As discussed in the introduction (see chapter 1, paragraph 1.3.1.2) the upstream kinases possibly involved in this post translational modification are ATR, ATM or DNA-PK. Therefore it was investigated which of these kinases was directly activated by DDP and responsible for serine 15 phosphorylation

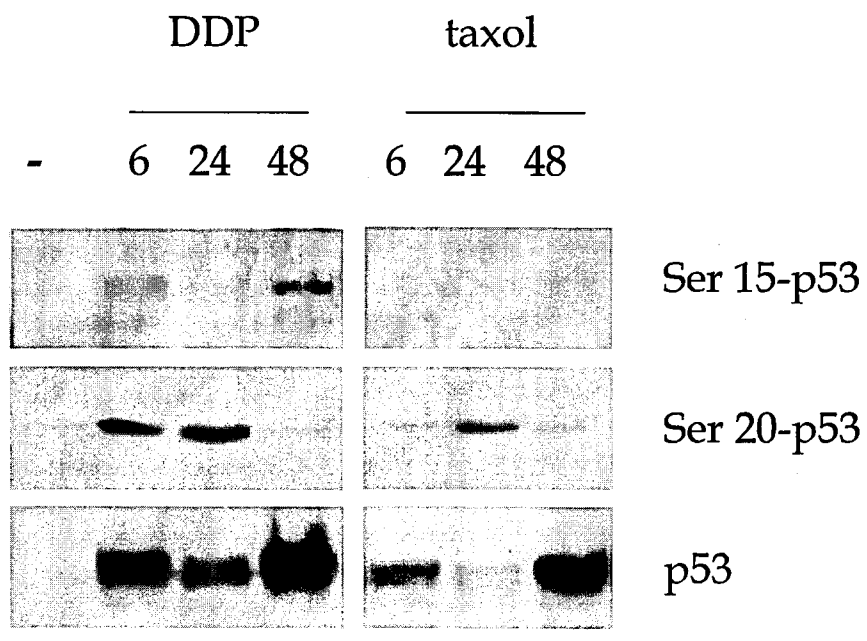


Figure 4.9

Phosphorylation of p53 at serine 15 and serine 20 induced by 25 μ M DDP or 50 nM taxol in human ovarian carcinoma cells A2780.

Cells were treated for 24 hours with the drugs and proteins extracted after 6, 24 and 48 hours incubation in drug-free medium. Proteins were separated on SDS-PAGE and blotted on nitrocellulose.

Blots were probed with anti ser15 and ser20 p53 specific antibodies. The same membrane was then blotted with anti p53 antibodies.

of p53. To address this issue different approaches were used. The rather unspecific inhibitor of PI3 kinases, wortmannin, which has been described to be more specific for DNA-PK than for ATM or ATR was used. Pretreating the cells with this inhibitor, no significant decrease in the degree of p53 phosphorylation induced by DDP either at serine 15 or 20 (figure 4.10, panel A) was observed. The concentration of wortmannin used in these experiments (35.5 μ M) was able to reduce DNA-PK activity by more than 90% (figure 4.10, panel B).

The possible involvement of ATM in DDP-induced phosphorylation of p53 was assessed using lymphoblastoid cell lines, obtained by transformation with the SV40 virus, which are defective in ATM. Two cell lines derived from patients suffering from AT and one from a normal patient were treated with DDP, and the amount of total p53 and of serine 15-phosphorylated p53 was assessed by Western blotting. As can be seen in figure 4.11, panel A, DDP was able to induce phosphorylation at serine 15 of p53 in all three cell lines irrespective of the presence or absence of ATM.

The possible DDP-dependent activation of ATR was assessed by measuring protein levels and kinase activity. We found that DDP did not modify the protein expression of ATR (figure 4.11 panel B) on the contrary, the ATR kinase activity determined after immunoprecipitation from cells untreated and treated with DDP (figure 4.11 panel C) was significantly increased by DDP treatment. Altogether these results indicate

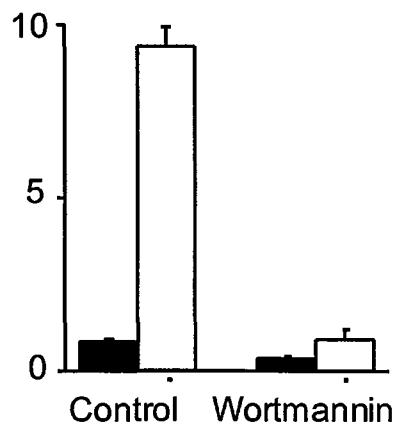
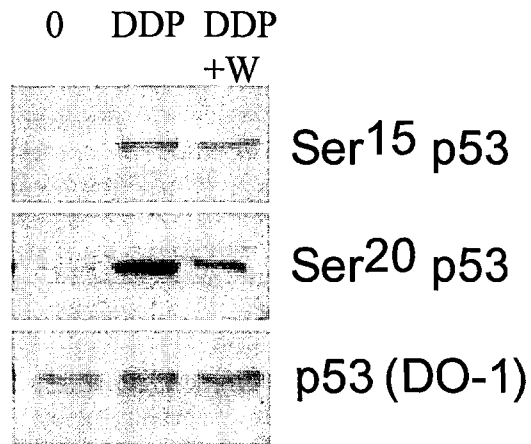


Figure 4.10

Effect of the PI3 kinase inhibitor wortmannin on DDP-induced phosphorylation of p53.

Panel A. Cells were treated for 24 hours with DDP in the absence or presence of 35.5 micromolar wortmannin (W) and proteins extracted after 24 hours incubation in DDP-free medium (containing or not wortmannin).

Blots were probed with anti ser15 and ser20 p53 specific antibodies. The same membrane was then blotted with anti p53 antibodies (monoclonal DO-1).

Panel B. DNA-PK activity in cells treated or untreated with wortmannin (same conditions as in panel A). Values are the mean +/- SD of pmolATP incorporated per minute per mg of protein in the biotinylated substrate in the absence (black bars) or presence (white bars) of double strand DNA

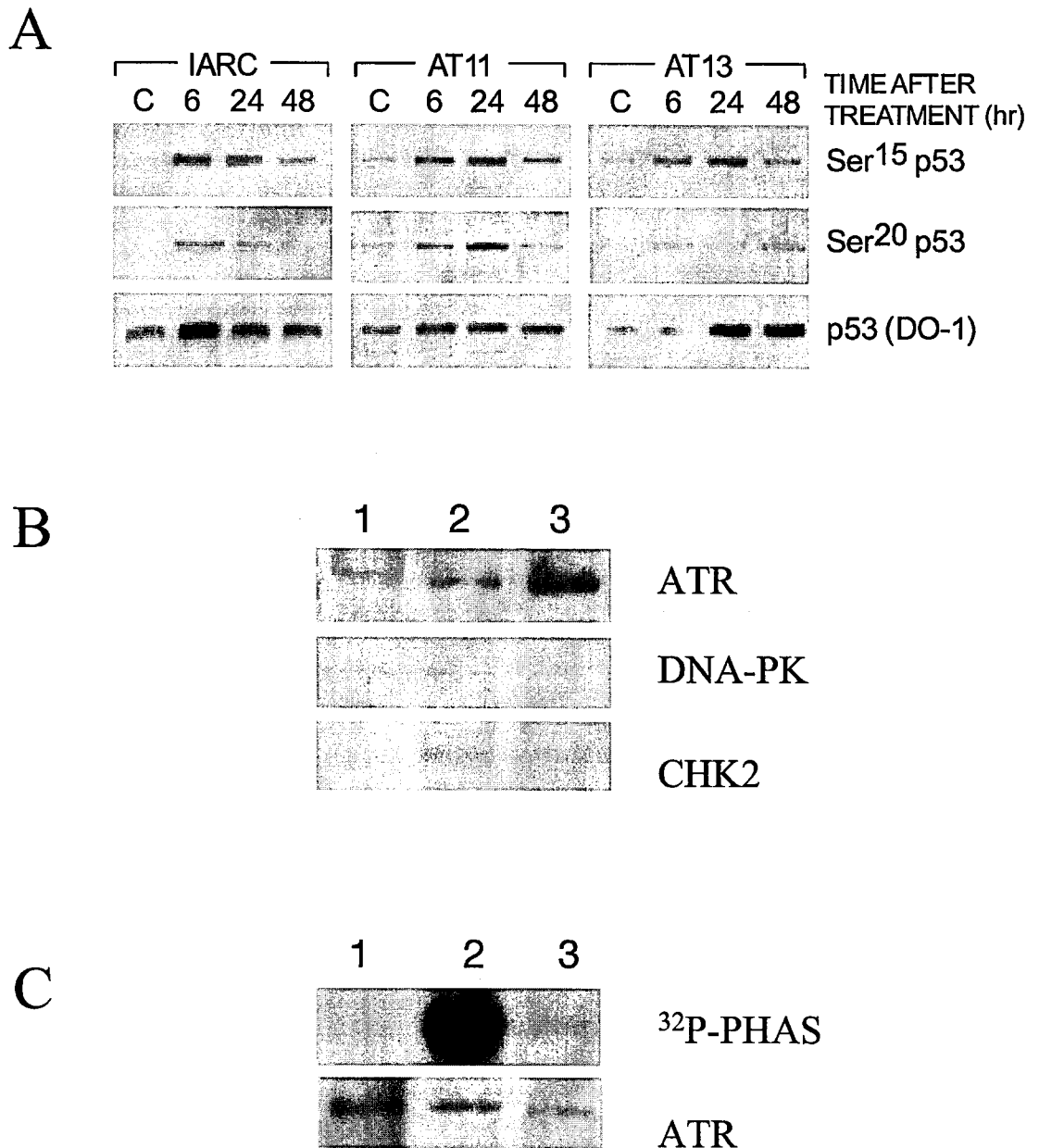


Figure 4.11

Role of ATM and ATR kinases on DDP-induced phosphorylation of p53.

Panel A. WT (IARC1663) and AT (AT11 and AT13) cells were treated with DDP and proteins extracted after 6, 24 and 48 hours incubation in DDP-free medium. Blots were probed with anti ser15 and ser20 p53 specific antibodies. The same membrane was then blotted with anti p53 antibodies (monoclonal DO-1).

Panel B. ATR, DNA-PK and CHK2 protein levels in HCT-116 cells untreated (lane 1) or treated with DDP (lane 2) or taxol (lane 3).

Panel C. ATR kinase activity in HCT-116 cells untreated (lane 1) or treated with DDP (lane 2) or taxol (lane 3). ATR was immunoprecipitated with anti ATR antibodies and kinase activity determined using PHAS as substrate as reported in methods section. The amount of ATR protein in each reaction was determined by western blot and reported in the lower part of the panel.

that DDP, differently from taxol, activates the ATR kinase thus enabling it to phosphorylate p53 at serine 15.

4.1.5 p53 and “new” drugs

The results reported in sections 4.1.1-4.1.3 deal with two clinically used anticancer agents. These drugs were originally selected in large screening programs aimed at identifying new potentially active molecules irrespective of their mechanism of action. As discussed in chapter 1.1, the increased knowledge of the molecular pathways important for normal cellular functions, has helped in identifying potential targets for the development of new anticancer agents. Even if these drugs have been selected to hit a specific target, often not related to DNA, their action is likely to involve the activation of checkpoint proteins and signal transduction pathways. We therefore examined the role of p53 in determining the cytotoxic activity of two “new” compounds which have shown promising activity either because of the target they have been selected for, or for the particular impressive preclinical activity shown. These compounds are the cdk inhibitor roscovitine and the histone deacetylase inhibitor trichostatin A (TSA).

Their activity has been compared in the pair of HCT116-derived cell lines with differential p53 expression. Their cytotoxic activity was tested using the same experimental procedures used for DDP and taxol and the results are shown in figure 4.12. The cell cycle inhibitor roscovitine and the

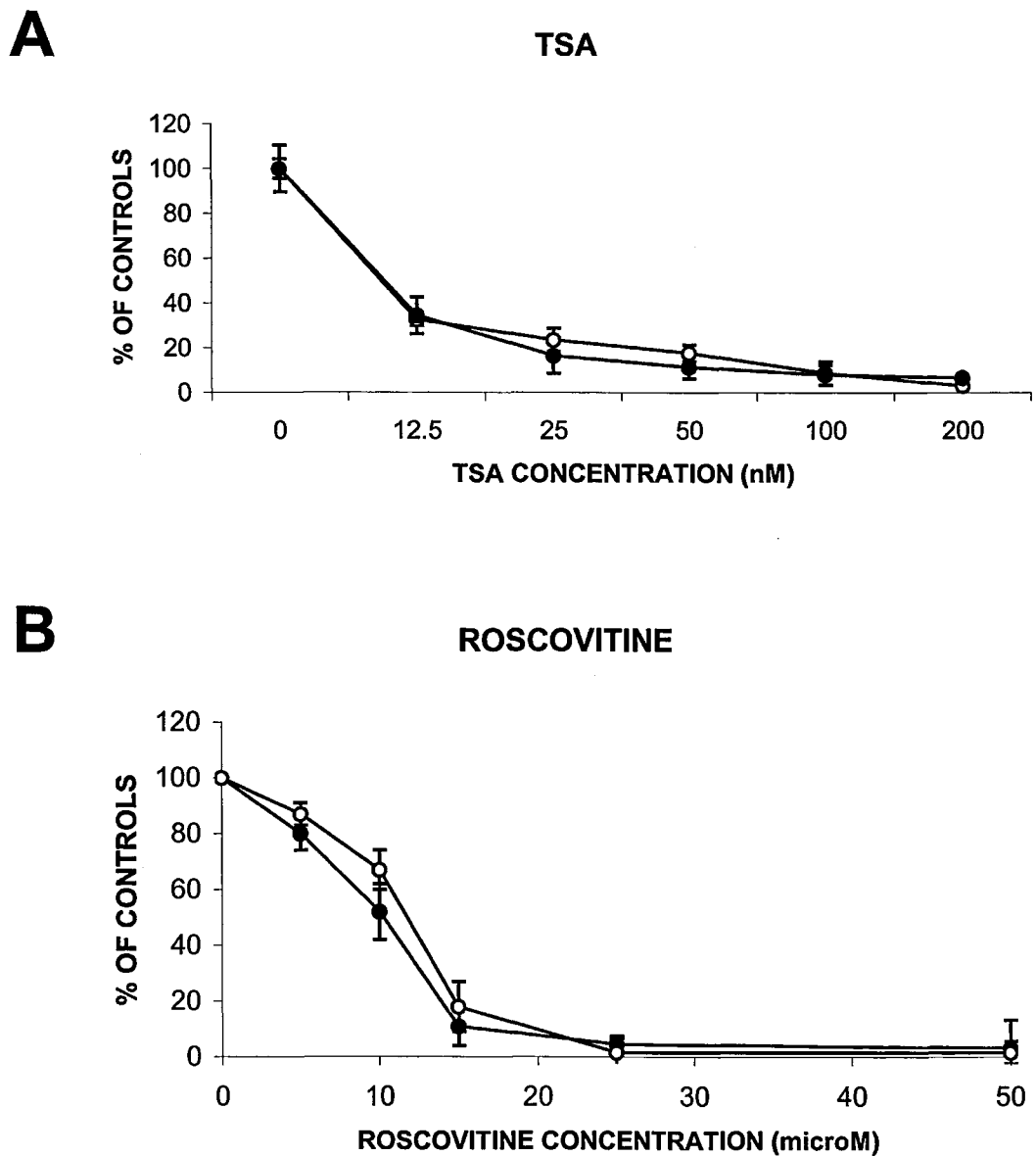


Figure 4.12

In vitro cytotoxicity of TSA and roscovitine in HCT116 p53+/+ (closed circles) and in HCT116 p53-/- (open circles) cells.

Cells were treated with different concentrations of the drugs for 24 hours. At the end of treatment the drugs were removed and the cells incubated in drug free medium for 2 weeks. The percentage of colonies in treated cells was calculated relative to untreated cells. The results are the mean +/- SD of three experiments each consisting of three replicates.

histone deacetylase inhibitor TSA were equally active in p53 expressing or in p53 $-/-$ cells, figure 4.12 panels A, and B.

4.1.6 Discussion

The results reported in this section clearly indicate that the DNA damaging agents DDP and taxol are more active in cells not expressing wild type p53. The results have been obtained in isogenic cell systems which differ only for the status of p53, thus allowing unequivocal evaluation of its role.

Previous experiments obtained in our laboratory, in which p53 status and DDP and taxol sensitivities were compared in different ovarian cancer cell lines with different p53 status, did not reveal any relationship between p53 and response to either drug, most likely because other factors dictated the overall response to treatment in vitro (Debernardis et al. 1997; De Feudis et al. 1997).

It is interesting to note that these cell lines are epithelial in origin with a quite low propensity to activate apoptotic processes. In these cells, the activation of p53 mainly induces the cell cycle arrest pathway, thus potentially allowing, whenever this is possible, the repair of the lesions induced. The removal of p53, and hence of its ability to induce cell cycle arrest, would probably facilitate the induction of apoptosis. This contention is in line with previous observations (in our laboratory) showing that in one of this cell lines (A2780) if p53 is forced to activate the

proapoptotic gene bax rather than the cell cycle inhibitor p21, massive p53-dependent apoptosis and strong increase in cellular sensitivity to taxol occur (De Feudis et al. 2000). These findings indicate that these cells have the potential to activate the apoptotic processes but that their commitment to do this is somehow blocked. The results obtained with DDP and taxol in these epithelial cancer cells are also in agreement with what has been previously found by other laboratories in other cellular systems, such as breast cancer cells (Fan et al. 1995; Wahl et al. 1996). In other cells, particularly leukemic cells, the activation of p53 is instead associated with a marked induction of apoptosis, and hence its presence may contribute to the desired activity of anticancer agents (Chen et al. 1996; Bates and Vousden 1996; Waldman et al. 1997b).

The difference in ability to induce aminoterminal phosphorylation in p53 observed between DDP and taxol as shown above, is consistent with the different mechanisms of action of these drugs, but it does not explain the lack of induction of apoptosis. In fact the ATR-dependent serine 15 phosphorylation observed after DDP but not after taxol, is in line with the strong DNA damage induced by DDP, which is an event sufficient to activate ATR. It is plausible that, at least in these cells, neither taxol nor DDP induces the phosphorylation of p53 at other specific sites, which would lead to the possible preferred activation of p53-dependent proapoptotic genes.

The fact that other drugs such as the cdk inhibitor roscovitine and the HDAC inhibitor trichostatin A did not show differences in activity between p53-expressing or p53-deficient cells, would suggest that these compounds do not activate the p53 pathway, or if they did activate it, the result of this activation would not interfere directly with the mechanism of action and/or repair of the lesions induced by the drugs.

4.2 p73 and its effect on cellular response to anticancer agents

4.2.1 Introduction

The p53 gene is one of the primary response genes in cells following stress induction. Most of its activities are linked to its ability to bind to DNA and to activate the transcription of downstream genes. The p73 gene shares relatively high sequence homology with p53 in the central DNA binding domain, thus enabling it to bind and activate the same downstream genes which are activated by p53 (Kaghad et al. 1997; Kaelin 1999a). This similarity between p73 and p53 would predict that p73 shares common roles in activating downstream pathways following stress induction, eventually leading to cell cycle arrest or apoptosis. Even if marked differences between p53 and p73 have been outlined (see introduction, chapter 1.3.2), relatively few data are available on the possible role of p73 in determining the cellular response to treatment with

anticancer agents. This fact is mostly due to the lack of suitable models specifically addressing this question. For this reason, experiments were designed with the aim of understanding which role p73 could have in determining cellular sensitivity to anticancer agents. Initially, as discussed in the following sections, the focus was on the characterization of the isogenic cell system differing in the expression of p73 previously generated in our laboratory (Vikhanskaya et al. 2000).

4.2.2 Characterization of stable p73 overexpressing clones

In an attempt to understand the role, if any, of p73 as a determinant of cellular sensitivity to anticancer agents, it was important to obtain cellular systems as close as possible to the clinical situation. As discussed in the introduction (section 1.3.2),

p73 is rarely mutated or deleted in human cancer, but rather is frequently overexpressed in its wild-type form. Therefore two clones were selected from the human ovarian cancer cell line A2780, which were established by transfecting A2780 cells with the human p73 alpha cDNA subcloned in the pCDNA3 expression vector. These two clones, named A2780/p73.4 and A2780/p73.5, were compared with a clone obtained by transfecting parental cells with the empty vector used to subclone the human p73 gene (pCDNA3), these cells were named A2780/pCDNA3. Cells were tested for expression of exogenous p73 RNA and protein, and they were found to stably overexpress p73, either at the level of mRNA or

protein (Vikhanskaya et al. 2000). The *in vitro* growth rates of the two p73 overexpressing clones and of the A2780/pCDNA3 clone were compared. The number of cells measured at different days after seeding was plotted against time, and the three different sublines grew at similar rates with doubling times of approximately 24, 25 and 26 hours for A2780/pCDNA3, A2780/p73.4 and A2780/p73.5 cells, respectively (figure 4.13). Having established that the overexpression of p73 does not induce differences in growth rate, the initial approach which was chosen was to characterize the effects of p73 overexpression on overall cell behaviour. To that end the gene expression profile of cells was analysed using microarray methodology. Cells in exponential growth were lysed and total RNA extracted as described in section 3.5.1. Equal amounts of RNA were retrotranscribed to cDNA in the presence of ³²P-labeled dATP and hybridized onto filters containing 588 cDNAs corresponding to human genes with known function.

Replicates of the filters obtained from A2780/pCDNA3, A2780/p73.4 and A2780/p73.5 cells were analyzed by phosphoimager, and the image was acquired using an appropriate software supplied by the manufacturer. On the basis of these sets of data, the expression profiles of the two p73 overexpressing clones were compared with that of vector-transfected cells. Figure 4.14 shows a pseudocolor image resulting from the comparative analysis of expression between each p73 overexpressing clone and the vector-transfected clone.

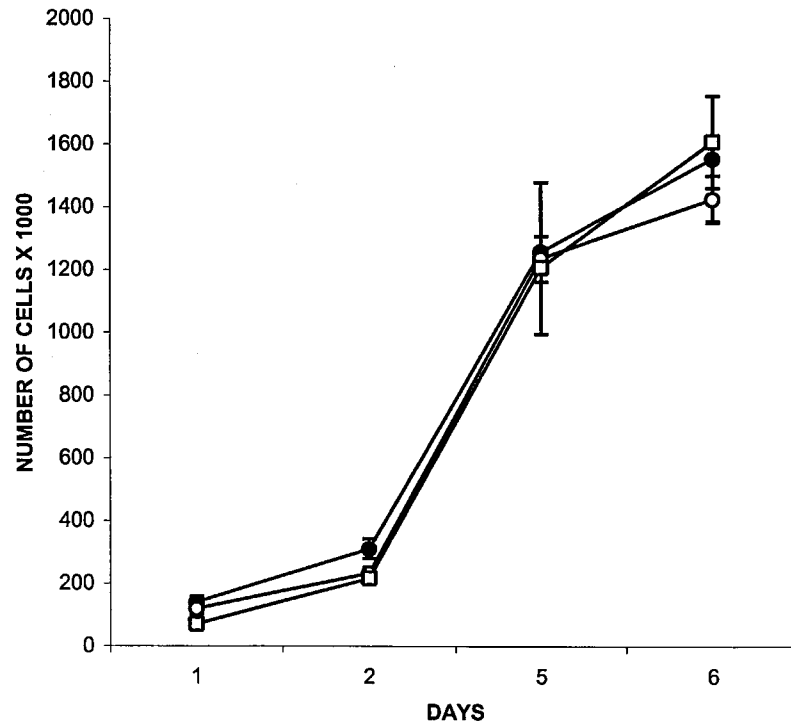


Figure 4.13

In vitro growth of A2780/pCDNA3 cells (○) and of the two p73-overexpressing clones A2780/p73.4 (◻) and A2780/p73.5 (●). Cells were seeded at 100.000 cell/ml and counted at different intervals as indicated. Results are the mean +/- SD of four replicates.

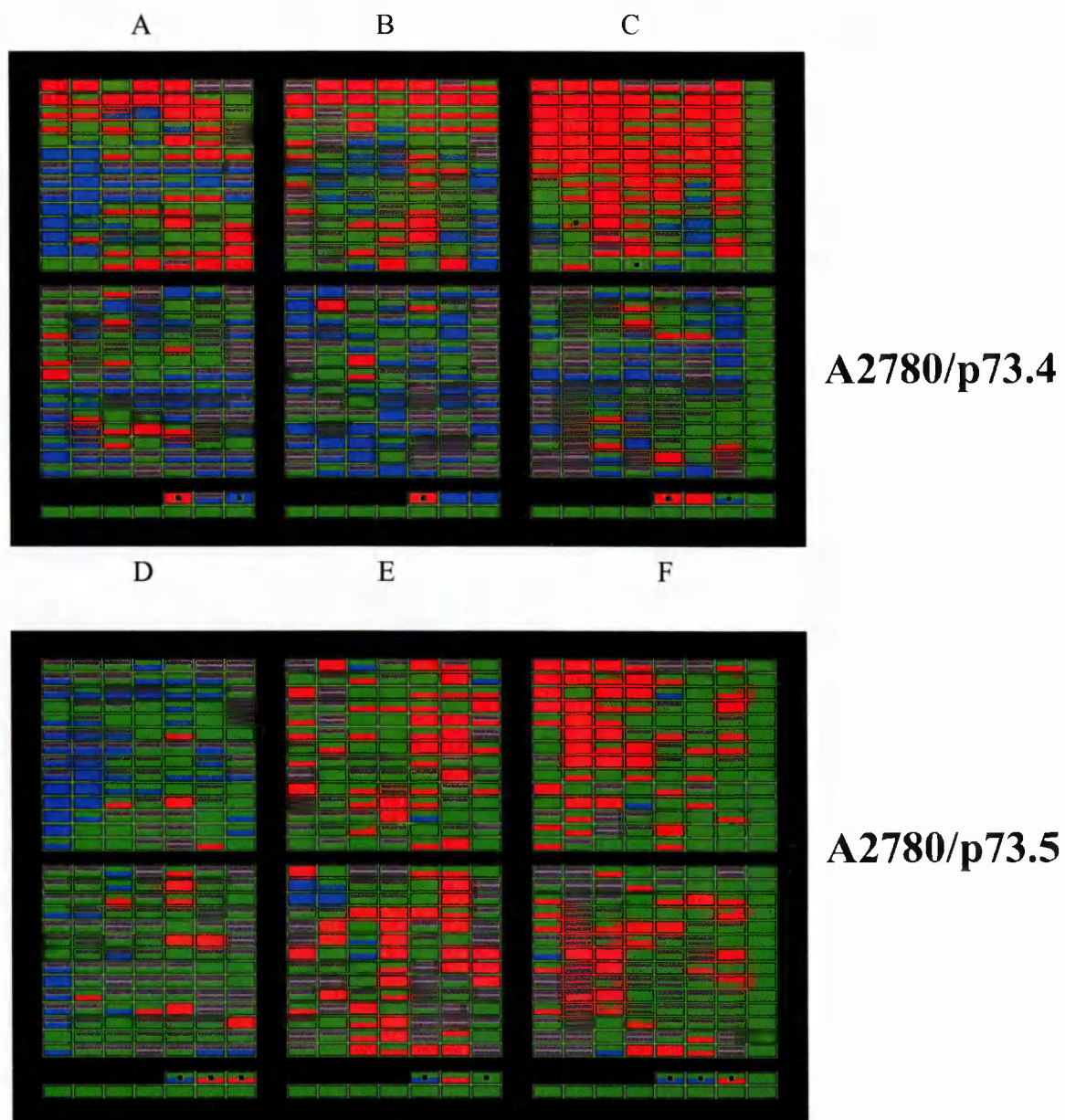


Figure 4.14

Pseudocolor representation of the gene expression profiles obtained from A2780/p73.4 (upper panel) and A2780/p73.5 (lower panel) cells. For each clone the expression level has been compared with A2780/pCDNA3 cells. Red and blue colors indicate those genes upregulated or downregulated, respectively, in the clones compared to parental cells. In green are reported those genes whose levels do not change. The six different panels present on each filter (A,B,C,D,E and F see section 4.2.2) are shown in the upper panel.

Red coloration indicates genes having an expression level greater in p73 overexpressing cells than in parental cells, in blue those with lower expression and in green those whose expression did not change significantly between the two clones. Both clones showed a relatively high number of genes with an expression pattern different from parental cells. It was estimated that approximately 20% of the genes in the two p73 overexpressing clones displayed a more than two fold change in expression compared to parental cells. The filters we used to analyse gene expression were organized in six panels each spotted with cDNAs of genes with common function. The six panels (Fig. 4.14), from left to right, include genes known to participate in cell cycle control (panel A), stress response (panel B), DNA repair and response to damage (panel C), angiogenesis and cell structure (panel D), signal transduction (panel E) and miscellaneous function (panel F).

Differences in the pattern of expression between the two clones could be detected. Of particular interest, in the two lower right panels (panel E and F), there are a significant number of genes with increased expression in A2780/p73.5 clone compared to parental cells, and decreased expression in A2780/p73.4 clone. The expression of many other genes was similarly altered in both clones.

Attention was focussed on the upper left panel of each filter, in which a high number of genes were found to be upregulated at significant levels for both clones. This panel contained cDNAs corresponding to



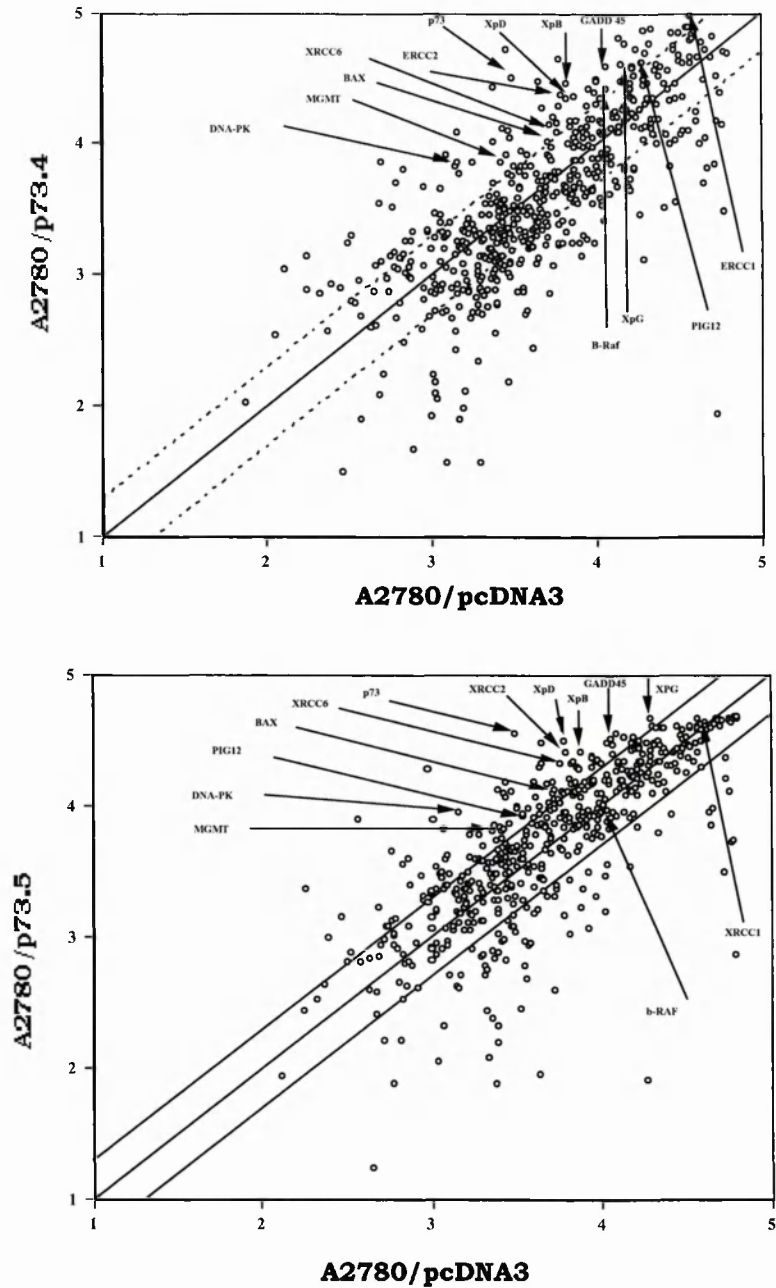


Fig. 4.15

Graphic representation of the relative RNA expression levels. Gene expression data obtained by microarrays in A2780/p73.4 and A2780/p73.5 clones were plotted against those obtained in A2780/pCDNA3 cells. Genes whose expression was two fold (either in positive or in negative) different from parental cells are outside the curves. Some genes relevant for the study are arrowed.

genes belonging to cellular response pathways, including DNA repair and DNA damage response genes, particularly relevant in the context of this thesis. Figure 4.15 shows a graphical view of the results, in which the level of expression of each gene in parental cells (abscissa, data expressed as logarithms) was plotted against the expression in A2780/p73.4 or A2780/p73.5 cells (ordinate, data expressed as logarithms). In these figures, the bisector is reported as a solid line, which helps in identifying those genes with a comparable expression in the two clones, together with the dotted lines representing a 2 fold variation (either in positive or in negative direction) in gene expression level between p73- and vector-transfected clones. This value is an arbitrary value, but it is considered sufficient for this technique to indicate a change in gene expression that, with more than 95% of probability, can be confirmed by other methods. The genes relating to DNA repair and DNA damage response pathways which showed a significant variation in expression are indicated, and they are all in the part of the graph containing those genes with an expression level in p73 overexpressing clone more than two fold greater than in parental cells. Interestingly, both clones were found to overexpress p73 at similar levels, which were 10-11 times higher than in parental cells, thus confirming our initial results. Some of the genes found to be upregulated are listed in table 4.1. The observed changes in gene expression were confirmed using the Northern blot. Figure 4.16 shows the results obtained in a representative experiment, in which both clones overexpressed two

GENE	A2780/p73.4	A2780/p73.5
p73	10	11
DNA-PK	5	6
ATM	3.2	2.3
XRCC6	2.2	3.5
XPB	3.9	4.4
XPD	2.3	2.1
XPB	4.5	3.3
XRCC1	2.5	1
MGMT	2.7	2.6
hMLH1	1.7	2
BAX	2.1	2.3
GADD45	3.5	2.8
PIG3	1.4	2.6
PIG12	2.3	1.8

Table 4.1

Partial list of genes upregulated in A2780/p73.4 and A2780/p73.5 cells.

Data were generated from microarray experiments (figure 4.14) and represent the ratio between the RNA expression in the two clones and in parental A2780/pCDNA3 cells.

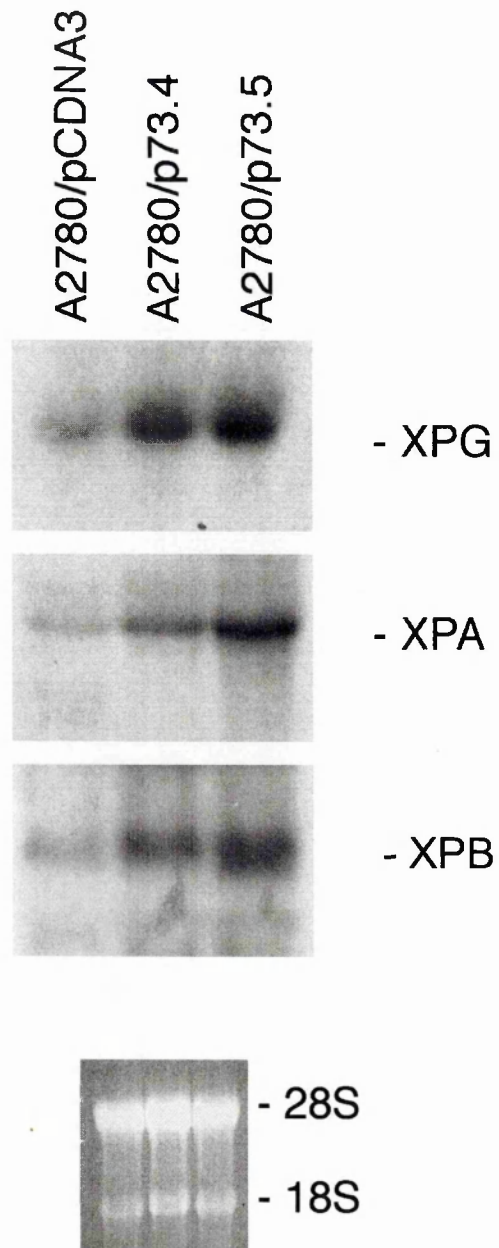


Figure 4.16

RNA levels of the NER belonging genes XPA, XPB and XPD in A2780/pCDNA3, A2780/p73.4 and A2780/p73.5 cells. Total RNA was extracted from exponentially growing cells, electrophoresed and transferred to nylon membrane; The filter was then hybridized with ^{32}P -labeled probes. The lower panel reports the ethidium bromide staining of the gel with the two major ribosomal RNAs as an indication of the loading.

genes belonging to the nucleotide excision repair family, XPB and XPD which were found overexpressed using microarrays. The figure shows another NER gene, XPA, which was not present in the sets of filters used in microarray experiments, which was also found overexpressed in both p73-overexpressing clones. These results suggest a possible concerted activation of all the genes belonging to this particular DNA repair family, and hence the possibility that these clones could have a more efficient repair of the lesions recognized by NER.

It is noteworthy that the lesions induced by DDP are recognized and repaired by NER (Damia et al. 1996; Lindahl and Wood 1999), and, as a consequence one would expect increased resistance against this particular drug in the two overexpressing clones. The experimental data which were obtained confirmed this hypothesis.

4.2.3 DNA repair capacity of the p73 overexpressing clones

The host cell reactivation assay is a validated method to measure the DNA repair capacity of cell lines (Poll et al. 1984; Damia et al. 1998). In this assay a plasmid coding for a reporter gene is damaged in vitro with DDP or UV, and after extensive washing it is transfected into cells. At different time points, the activity of the reporter plasmid (in this case the luciferase gene) is determined in cell extracts. The data are expressed as a percentage of luciferase activity in cells transfected with damaged plasmid calculated against luciferase activity of cells transfected with undamaged plasmid.

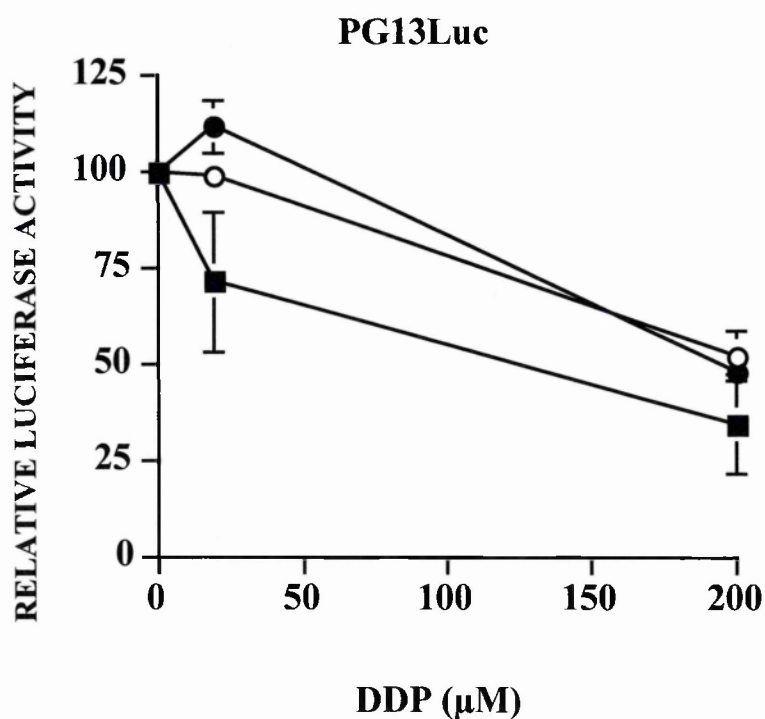
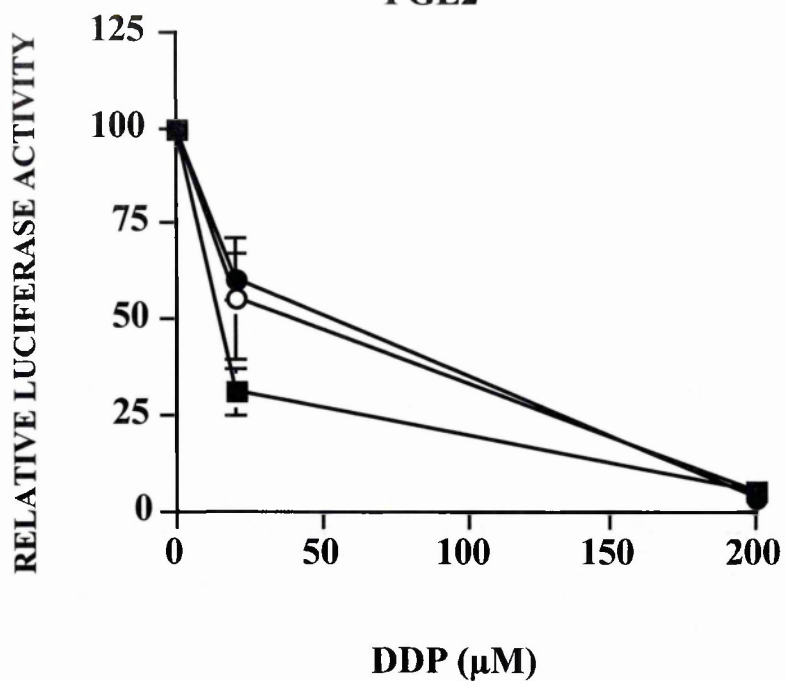


Figure 4.17

DNA repair activity of parental A2780/pCDNA3 (■), A2780/p73.4 (○) and A2780/p73.5 (●) cells. Repair activity was assessed using the host cell reactivation assay (see section 3). Two different DDP-damaged plasmids (pGL2, upper panel and PG13Luc, lower panel) were used. The plasmids were damaged in vitro with 20 or 200 μM DDP for 2 hours as described in chapter 3.4, precipitated and then transfected in the different cell lines. Luciferase activity was determined 24 hours later.

Using this assay, both p73-overexpressing clones were found to repair DNA more efficiently than parental cells when a plasmid damaged in vitro with DDP was used as substrate (figure 4.17). The results were obtained using two different constructs, both containing the luciferase reporter gene but each under the control of a different promoter sequence.

The two promoter sequences were derived from a SV40 promoter region and from a p53 responsive element repeated 13 times, and they are shown in upper and lower panels of figure 4.17, respectively.

The percentages of repair in A2780/pCDNA3, were 30 and 70%, when the SV40 or p53 responsive elements, respectively, were analyzed. In the two p73 overexpressing clones the percentage of repair was higher, being 60 % in A2780/p73.4 and 65 % in A2780/p73.5 with the SV40 promoter, and 100% in both A2780/p73.4 and A2780/p73.5 cells with the p53 responsive element.

These data were obtained using as a substrate to a plasmid damaged with 20 μ M DDP. Increasing the concentration of DDP to 200 μ M (a supra-pharmacological concentration) the repair ability of all three cell lines was much reduced, and the differences between the p73 overexpressing clones and parental cells were less evident, although still present.

4.2.4 Sensitivity of p73 overexpressing clones to several anticancer treatments

Once verified that the two p73 overexpressing clones are characterized by augmented NER-dependent repair ability, the hypothesis was tested that they were resistant against treatment with drugs which produce DNA lesions that are known to be substrates of this repair pathway, but not against drugs inducing lesions which are not repaired by this pathway. In order to test this hypothesis, the MTT test was used (see chapter 3.1.4), which allows detection of growth inhibition induced by a drug in cells growing in culture. P73-overexpressing clones and parental cells were treated with different concentrations of DDP, and with different doses of UV-C, all treatments known to induce lesions recognized by NER (Cleaver et al. 1995; Damia et al. 1996; Lindahl and Wood 1999).

As controls, the topoisomerase I inhibitor topotecan and the microtubule depolymerization inhibitor taxol, the activity of which is independent of the NER status, were selected. The concentration versus growth inhibition curves for these four different treatments are shown in figure 4.18. The two p73-overexpressing clones were more resistant to "NER-sensitive" treatments (DDP and UV, figure 4.18 panels A and B, respectively) than the vector transfected clone, while topotecan and taxol, whose lesions are not recognized by the NER system, showed comparable activity in the three sublines (figure 4.18 panels C and D). The degree of resistance ranged from 3 to 4 for DDP and 2 to 3 for UV. Other drugs

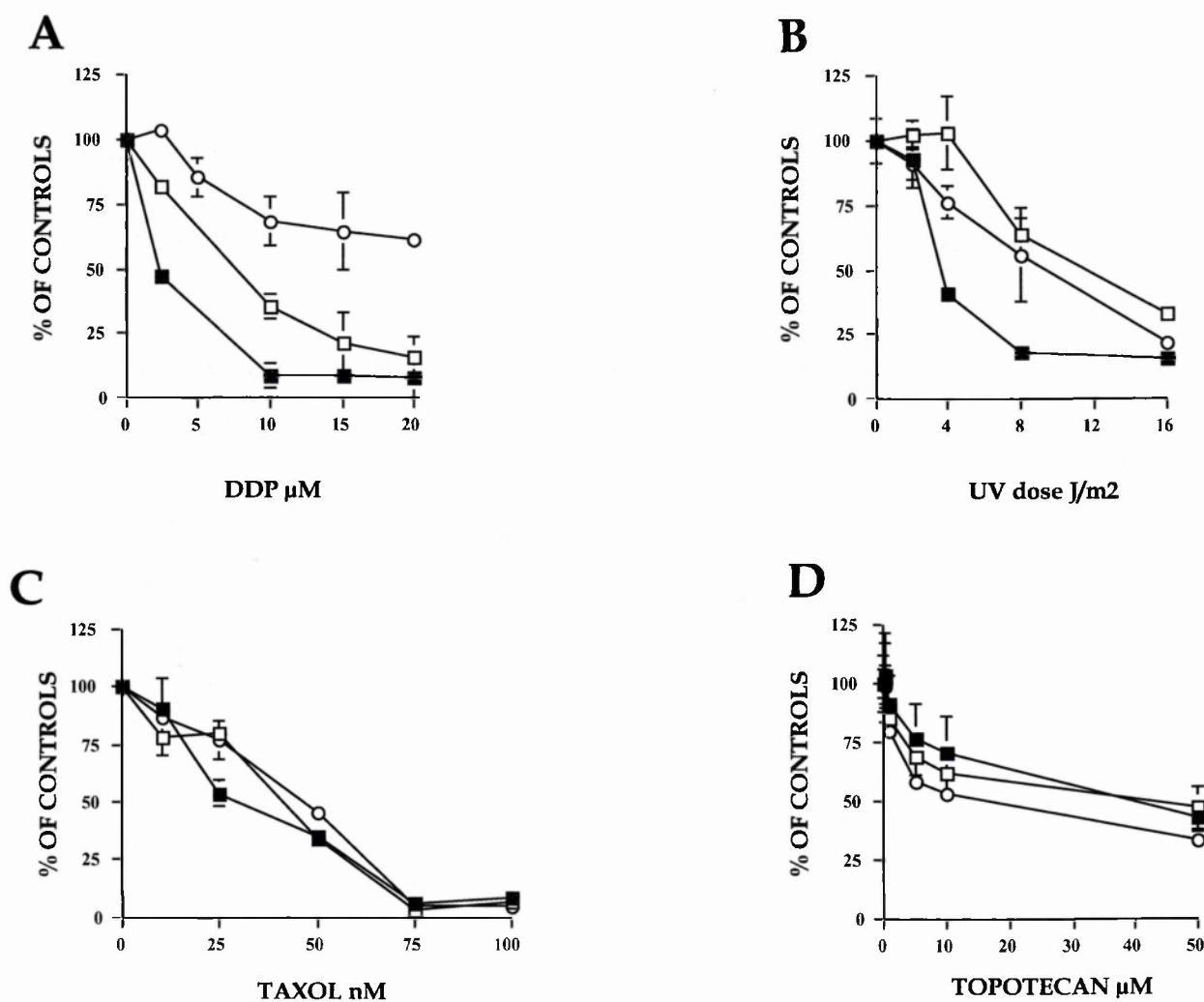


Figure. 4.18

In vitro cytotoxicity of DDP, UV, taxol and topotecan in A2780/pCDNA3 (\blacksquare), A2780/p73.4 (\square) and A2780/p73.5 (\circ) cells.

Cells were treated with different concentrations of the drugs for 1 hour (DDP) or 24 hours (taxol and topotecan). At the end of treatment, the drugs were removed and the cells incubated in drug-free medium for further 72 hours.

The percentage of 540nm abs in treated cells was calculated relative to untreated cells using the MTT test. The results are the mean \pm SD of six samples.

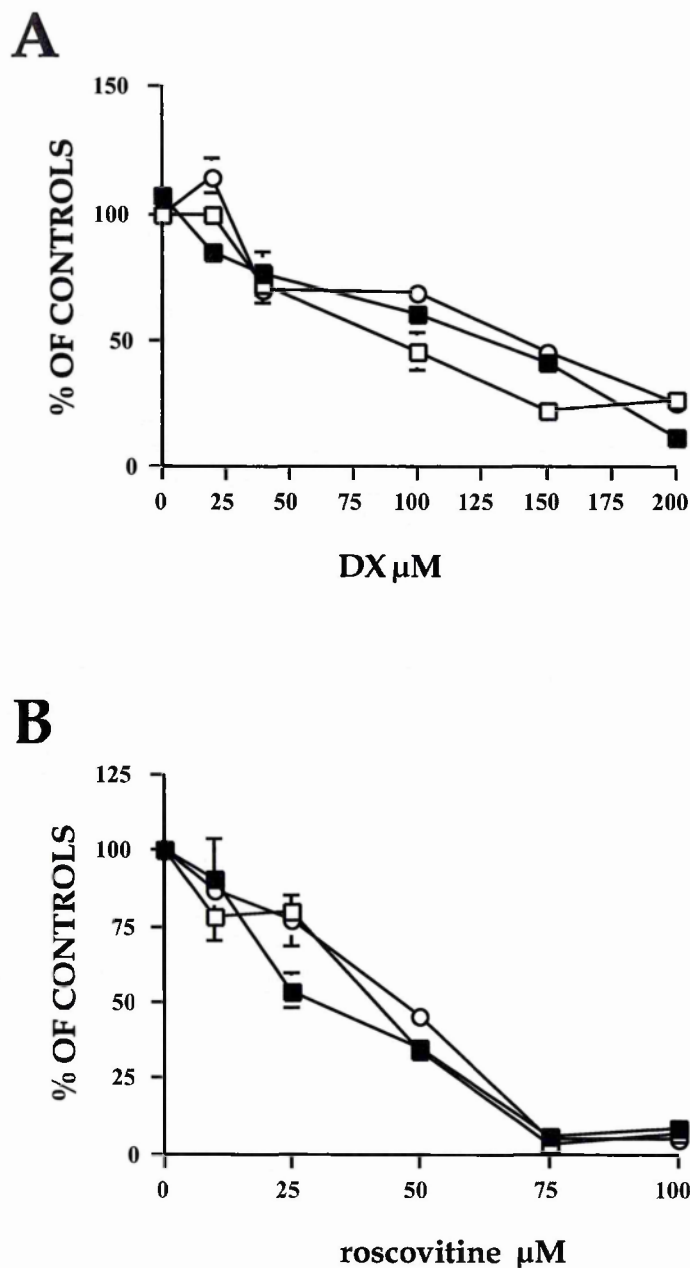


Figure 4.19

In vitro cytotoxicity of DX and roscovitine in A2780/pCDNA3 (●), A2780/p73.4 (□) and A2780/p73.5 (○) cells.

Cells were treated with different concentrations of the drugs for 24 hours. At the end of treatment, the drugs were removed and the cells incubated in drug-free medium for further 72 hours.

The percentage of 540nM abs in treated cells was calculated relative to untreated cells using the MTT test. The results are the mean \pm SD of six replicates.

tested for their activities in differentially p73 overexpressing cells, such as the cdk inhibitor roscovitine and the anthracycline antibiotic doxorubicin, exerted comparable in vitro cytotoxicity in the three sublines, independent of level of expression of p73 (see figure 4.19).

4.2.5 Discussion

Taken together these results indicate that, at least in the experimental system used here, the two p73 overexpressing clones are specifically resistant against “NER-sensitive drugs” and that this resistance is due to increased repair ability of the two p73 overexpressing clones, which in turns is the likely consequence of the increased expression of different NER genes. To my knowledge, these clones are so far the only ones available stably overexpressing p73. Therefore it is difficult to judge the general applicability of the consequences of these findings and to compare results with data available in the literature. It is important that the observed increased resistance against DNA-damaging agents whose lesions are recognized by NER was found in both p73 overexpressing clones, which supports the robustness of the data.

Moreover, these clones were not resistant to all the drugs, rendering unlikely the possibility that they are the result of a selection of particularly resistant clones independently of the overexpression of p73. The relatively recent discovery that a particular form of p73, called DN, which lacks the transactivation domain, could exert effects opposite to those seen with

wild-type p73 (also called TA p73), argues against the possible relevance of the results obtained by overexpressing TA p73 (Melino et al. 2002; Stiewe and Putzer 2002; Ishimoto et al. 2002; Stiewe et al. 2002b).

It has been postulated that it is the overexpression of this “oncogenic” form of p73 which accounts for the high overall p73 levels found in human tumors. However, simultaneous measurement of the levels of the two p73 forms in different cancer cell lines and human tumors, showed that the DN form is overexpressed compared to normal cells, but levels of TA p73 are anyway higher than those of the DN form (Zaika et al. 1999; Grob et al. 2001). Another relevant observation is the finding that the DN form is transcribed through an alternative promoter, different from that of TA p73 (Yang and McKeon 2000; Moll et al. 2001). Intriguingly, the DN promoter contains a p53 responsive element and is activatable by p53 (Vossio et al. 2002; Kartasheva et al. 2002).

It is not yet clear why p53 would activate an “oncogenic” form of p73, and what impact this activation might have for tumor development. Intuition dictates that in tumors in which p53 is inactivated, i.e. the majority, the levels of DN p73 should be reduced compared to tumors or normal tissues expressing wild-type p53. In A2780 parental cells, levels of DN p73 are lower than those of TA p73 (Marabese M. et al, personal communication). This further confirms that the observation in the two A2780 clones is likely to constitute a genuine corollary of overexpression of the TA form of p73.

4.3 CHK1 and its effect on cellular response to anticancer agents

4.3.1 Introduction

CHK1 is an important protein participating in and controlling the G2 checkpoint. In normal cells the function of CHK1 is to prevent the entry of damaged cells into mitosis by phosphorylation the CDC25C phosphatase (Sanchez et al. 1997; Kaneko et al. 1999). CHK1 protein needs to be activated by upstream proteins acting as sensors of damage, such as ATR and ATM, mainly through phosphorylation (Abraham 2001; Tian et al. 2002). As a pivotal protein in controlling G2 checkpoint, it was considered likely that the presence or absence of CHK1 can modulate the response to treatment with anticancer agents.

As discussed in the Introduction, CHK1 seems to play an essential role in the life cycle, at least during development. The absence of CHK1 is in fact incompatible with life, and knock out mouse embryos do not survive to birth (Takai et al. 2000; Liu et al. 2000). Only very recently the generation of a B-lymphoma cell line with CHK1 gene disruption has been achieved (Zachos et al. 2003). Therefore to investigate the role of CHK1 in determining the response to treatment with anticancer agent, different strategies had to be used trying to overcome the lack of availability of cells

without CHK1 gene. Instead, dominant negative mutants and inhibitors of the kinase activity of CHK1 were employed.

4.3.2 The effect of overexpression of mutant CHK1 in human osteosarcoma cells

Initially a U20S-derived cellular systems was used, which has been generated at the European Institute of Oncology, Milan, Italy in the laboratory directed by Dr. G. Draetta. These cells overexpress the wild-type form of human CHK1 or a mutated form of CHK1. The latter is a construct (named D130A), in which the human CHK1 cDNA contains a single mutation leading to a single aminoacid change in the protein in its catalytic domain, the aspartic acid at position 130 has been replaced via site directed mutagenesis by an alanine. Position 130 is inside the kinase domain of the human CHK1 protein and, in vitro, this mutation abolishes almost completely the kinase activity of the protein.

Overexpression of this mutant form could result in a dominant negative function, with a non functioning protein sequestering all the factors otherwise used by the endogenous, wild-type protein. In these experiments the availability of this couple of sublines which overexpress the wild-type or mutated form of CHK1 was exploited. The cells originated from the human osteosarcoma line U20S. In the U20S-derived system, the activity of DDP was tested using the MTT test. Table 4.2 shows the results of four independent experiments.

DDP IC50 (μM)			
Exp.	U2OS/pCDNA3	U2OS/CHK1	U2OS/D130
1	74.0		167.0
2	62.2	79.5	144
3	66.0	135.0	167.0
4	104.0	171.0	235.0
MEAN	76.5	128.5	184.0
SD	16.0	27.0	17.0

Table 4.2

Activity of DDP in U2OS-derived clones. U2OS/pCDNA3, U2OS/CHK1 and U2OS/D130 cells were treated with different concentrations of DDP for 2 hours. 72 hours after, the cells were analyzed with the MTT test. For each experiment, the IC50 of DDP was determined. Values reported in the table are IC50 of single experiments each consisting of six replicates.

The data are presented as IC₅₀, i.e. concentrations able to inhibit the growth by 50% and are derived from the concentrations versus % of control curves. In vector transfected U20S cells, the IC₅₀ of DDP was 77 +/- 16 μ M. The wt CHK1 overexpressing clone was more resistant with a value of 129 +/- 27 μ M. In D130-CHK1 overexpressing cells the IC₅₀ for DDP was 184 +/- 17 μ M.

The differential sensitivity observed with DDP was not seen with other compounds. Figure 4.20 shows the data obtained with the anthracycline antibiotic doxorubicin (DX) and with the methylating agent MNNG. The concentration vs % of controls curves for DX for parental cells, wt CHK1 overexpressing cells and mutated CHK1 overexpressing cells are superimposable.

The calculated IC₅₀ values for DX in the three cell lines were 12.3, 12.3 and 11.9 μ M, respectively. MNNG showed a slightly different pattern of potency. This compound had similar potency in vector transfected and in wt CHK1 overexpressing cells with IC₅₀s of 203 and 210 μ M, respectively, but showed a relatively reduced activity in the clone overexpressing the mutated form of CHK1 (IC₅₀ 353 μ M).

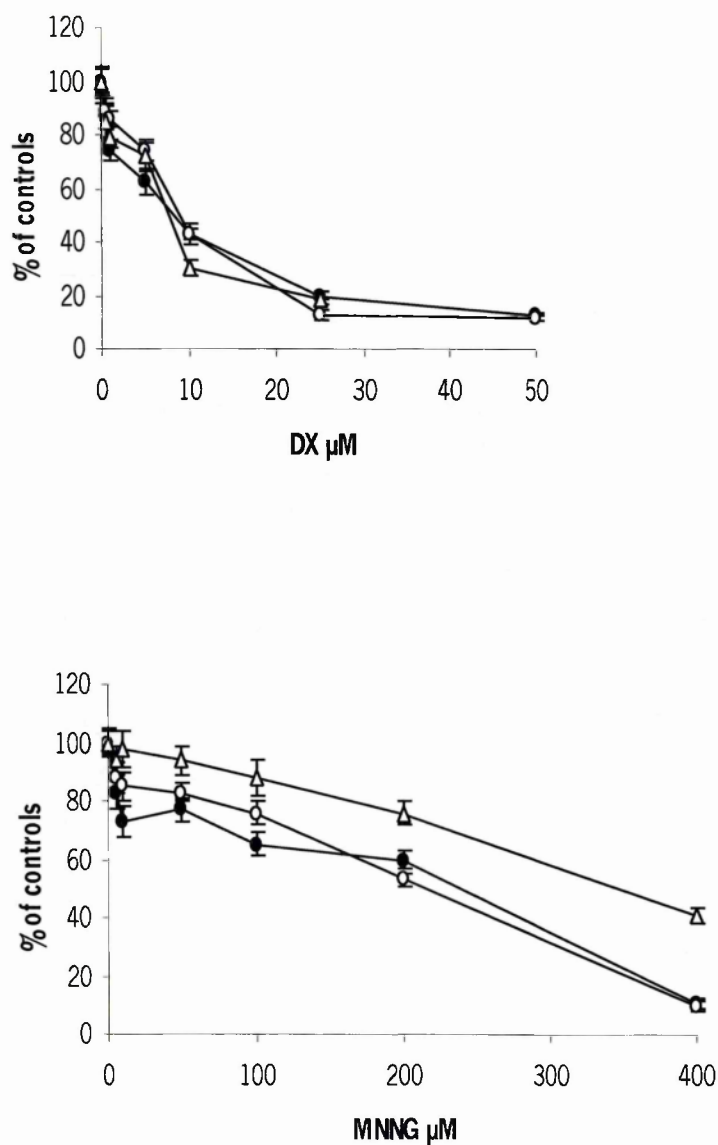


Figure 4.20

In vitro cytotoxicity of DX (upper panel) and MNNG (lower panel) in U2OS/pCDNA3 (\bullet), U2OS/CHK1 (\circ) and U2OS/D130 (Δ) cells.

Cells were treated with different concentrations of the drugs for 24 hours. At the end of treatment, the drugs were removed and the cells incubated in drug-free medium for further 72 hours.

The percentage of 540nM abs in treated cells was calculated relative to untreated cells using the MTT test. The results are the mean \pm SD of six replicates.

4.3.3 The effect of overexpression of mutant CHK1 in human colon cancer cells

Clones analogous to those available in U2OS cells, were generated with the HCT116 background. Parental HCT116 colon cancer cells were transfected with wt CHK1 cDNA or with D130A mutant CHK1 cDNA, both subcloned in the pCDNA3 expression plasmid. After transfection, the cells were seeded at very low density in medium containing the selection antibiotic (in this case G418). Different colonies growing in this medium containing antibiotic were selected and collected from the plates and transferred into 24 wells plates. Once the well was confluent, cells were detached and seeded in duplicate in T25 flasks. One flask was used to freeze the clone while the other was used to allow extraction of total cellular proteins. Proteins were separated on polyacrylamide gel and transferred to nitrocellulose filters. These filters were used to detect by western blotting the presence of the protein coded for by the exogenous, transfected cDNA, using specific anti CHK1 antibodies. The two constructs contained a tag epitope HA at 5' end, which facilitates the detection of the transfected protein and helps in distinguishing between exogenous and endogenous protein. Figure 4.21 shows a Western blot analysis, in which clones selected in the antibiotic-containing medium (G418), were tested for presence of HA-tagged proteins. The two clones selected from the initial screening clearly express the transfected CHK1, visible in the blot as a slightly lower, compared to the endogenous CHK1,

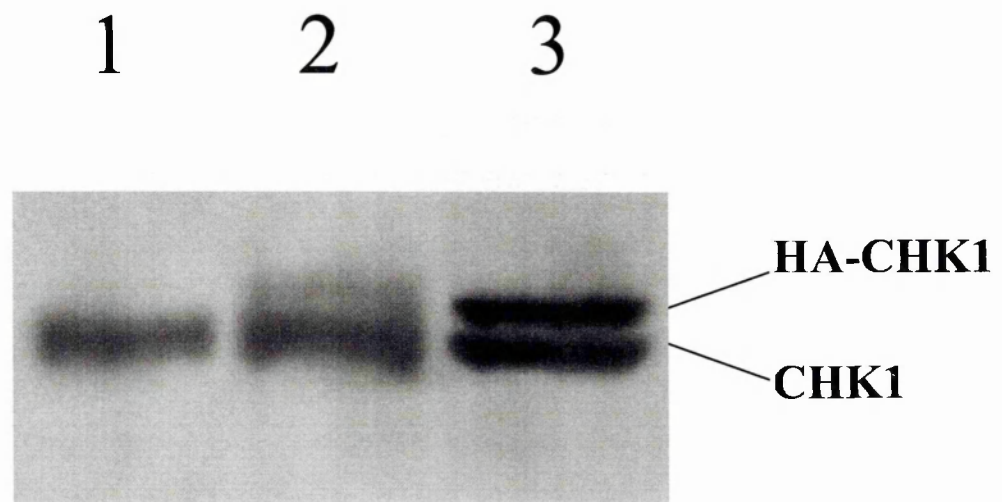


Figure 4.21

Western blot showing the expression of endogenous CHK1 in HCT116 cells.

Total proteins extracted from HCT116/pCDNA3 (lane 1), HCT116/CHK1 (lane 2) and HCT116/D130 (lane 3) were separated on SDS-PAGE, transferred to nitrocellulose and the blot hybridized with anti CHK1 antibodies which detect both endogenous (lower band) and exogenous (upper band) proteins as shown.

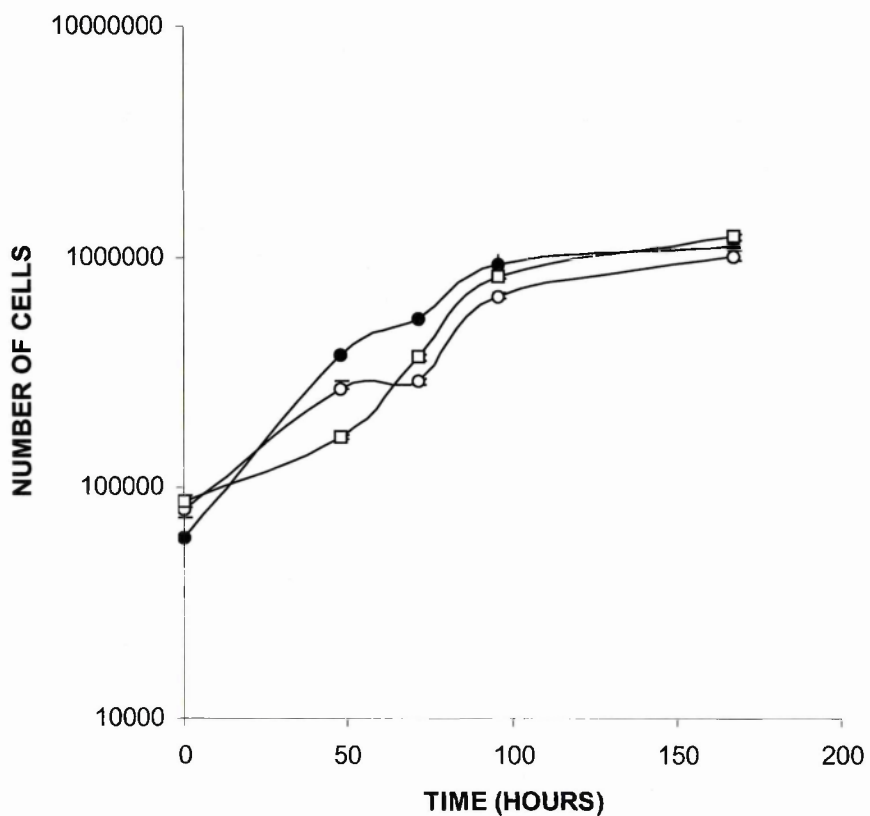


Figure 4.22

In vitro growth of clones derived from HCT116 cells by transfection with empty vector (pCDNA3, ●) with wild-type CHK1 (○) or with D130A mutant CHK1 (□).

Cells were seeded in 6 well plates and cell growth evaluated at the indicated times counting the number of cells per well. Values are expressed as mean + SD of three replicates.

migrating band in the gel. The blot was reprobed with anti CHK1 antibody to confirm the presence of CHK1. The clones were tested for their ability to grow in vitro. They grew at similar rates (figure 4.22) and they were used for further characterisation. A HCT116-derived clone, transfected with the pCDNA3 empty vector, was used as control.

The three clones were investigated for their sensitivity towards DDP. In contrast to the results obtained in U20S-derived system, overexpression of either the wild-type form or of the mutant form of CHK1 did not significantly alter the cellular response to DDP. In fact, when number of colonies was plotted against the concentration of DDP, the curves obtained in parental HCT116 cells and in the two clones overexpressing CHK1 cDNA were superimposable and resulted in comparable IC₅₀ values (Table 4.3).

4.3.4 The effect of CHK1 inhibition on cellular response to DDP

An alternative approach to the study of the effect of CHK1 on anticancer drug sensitivity entailed the use of inhibitors of CHK1 kinase activity in combination with DDP. For these studies the compound UCN-01 was selected, as it has been reported to be effective in inhibiting CHK1 kinase activity, although lacking specificity for this kinase (Busby et al. 2000; Graves et al. 2000). These experiments were performed in the U20S parental cells, which were treated with DDP in the absence or presence of UCN-01. Treatment with UCN-01 was maintained also in post-treatment-

DDP IC50 (μM)			
Exp.	HCT116/pCDNA3	HCT116/CHK1	HCT116/D130
1	17.2	13.8	
2	15.2	16.6	
3	19.7	19.4	
4	16.9		14.8
5	14.7		15.9
6	21.1		15.0
MEAN	17.5	16.6	15.2
SD	2.5	2.8	0.6

Table 4.3

Activity of DDP in HCT116-derived clones. HCT116/pCDNA3, HCT116/CHK1 and HCT116/D130 cells were treated with different concentrations of DDP for 2 hours. 10-14 days after colonies were stained and the analyzed with image analyzer. For each experiment, the IC50 of DDP was determined. Values reported in the table are IC50 of single experiments each consisting of three replicates.

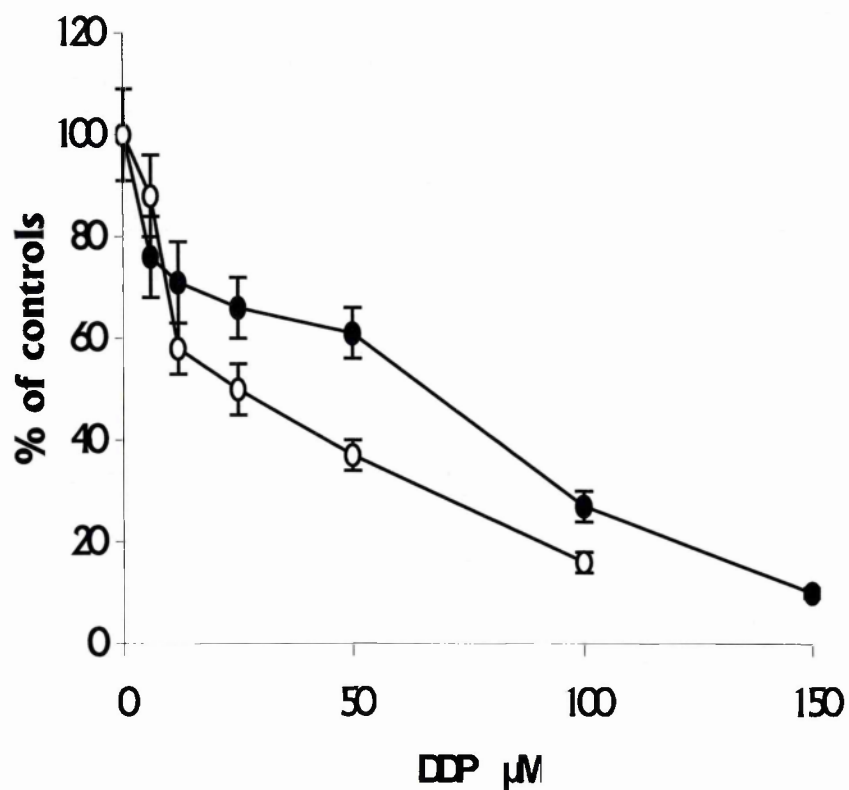


Figure 4.23

In vitro cytotoxicity of DDP alone or in combination with UCN-01 in U2OS cells. Cells were treated with different concentrations of DDP for 2 hours in the absence (○) or in the presence (●) of 0.1 μM UCN-01. At the end of treatment, the drugs were removed and the cells incubated in drug-free medium for further 72 hours. In the DDP+ UCN-01 group, UCN-01 was also added in the post-incubation time. The percentage of 540nm abs in treated cells was calculated relative to untreated cells using the MTT test. The results are the mean \pm SD of six replicates.

time after washing and removal of DDP, in order to keep CHK1 activity low for a long time. The concentration of UCN-01 used in these experiments, slightly reduced the growth of these cells (approximately 25%). When DDP activity was compared between cells untreated or pretreated with UCN-01, this slight effect of UCN-01 was considered and the values normalized.

The results obtained are reported in figure 4.23. DDP was, as expected, cytotoxic in these cells, and the combination of DDP with UCN-01 was more cytotoxic than DDP on its own.

These results are in agreement with previously reported studies, which showed that UCN-01 at concentrations similar to those used here increased the cytotoxicity of the anticancer agents mitomycin C, bis chloroethyl-nitrosourea and DDP (Akinaga et al. 1993; Pollack et al. 1996; Bunch and Eastman 1996; Sugiyama et al. 2000; Monks et al. 2000).

4.3.5 Discussion

The results obtained with CHK1 dominant negative mutants did not allow clear conclusions to be drawn concerning the role of CHK1 in determining cellular sensitivity to anticancer agents. In particular, the overexpression of mutant CHK1 had different effects in the two cellular systems utilised. It is possible that the observed effects in the U20S cell line were not caused directly by the CHK1 dominant negative mutation. In fact, in the U20S cell line the overexpression of wild-type CHK1 per se was

sufficient to alter the cellular response to anticancer agents. The conclusion expected from these experiments was that abrogation of CHK1, i.e. of an important G2 checkpoint participant, would sensitize cells to DNA damaging agent treatment. This was indeed observed when the CHK1 inhibitor UCN-01 was used in combination with DDP, which is in agreement with other data in the literature (Bunch and Eastman 1996; Husain et al. 1997).

The dominant negative effect exerted *in vitro* using CHK1 mutant cells was not reproducible in the various transfected cell types examined. One possible explanation for this unexpected result is that the levels of mutant protein were not sufficiently elevated above the levels of endogenous CHK1. Alternatively it might be difficult to saturate the transcription/translation machinery used by the CHK1 gene in cells in which the endogenous kinase activity is left intact. On the other hand, the result obtained with UCN-01, an inhibitor of CHK1 kinase activity, has been recently confirmed by experiments performed using "small interference RNAs" (siRNA), small RNA molecules able to shut down the expression of the gene of interest (Paddison and Hannon 2002; McManus and Sharp 2002; Scherr et al. 2003).

In a very recent publication, the use of siRNA specific for CHK1 was able to reduce the expression of CHK1 protein and to increase the cellular sensitivity to gamma ray irradiation (Zhao et al. 2002; Xiao et al. 2003).

4.4 CHK2 and its effect on cellular response to anticancer agents

4.4.1 Introduction

As outlined in the Introduction (Chapter 1), CHK2 and CHK1 share some common functional features although they have been reported to participate in distinct checkpoints (Walworth 2000; Rhind and Russell 2000b). CHK2 seems to be mostly involved in the S phase checkpoint, although some activity in the G2 and M checkpoints has also been documented (Rhind and Russell 2000b; Bartek et al. 2001).

Here two questions were addressed. Firstly it was surmised that, by analogy to the hypothesis tested with CHK1, abrogation of CHK2 function affects the response of cells towards treatment with DNA-damaging agents, such as DDP. Secondly the question was studied whether introduction of a dominant negative CHK2 gene into a cellular subline harboring a dominant negative CHK1 elicits the acquisition of additional sensitivity.

4.4.2 Generation and properties of cells overexpressing dominant negative CHK2

U20S pCDNA3 cells and U20S-CHK1 D130A cells were transfected with either the wt form or the mutated form of human CHK2 cDNA. By

analogy to the procedure used for CHK1, the CHK2 mutant was selected in the region containing the kinase domain. The mutated CHK2 cDNA contains a mutation at DNA level generating a protein with a lysine (K) instead of an arginine (R) at position 249. This mutant was previously found *in vitro* to have lost kinase activity (Matsuoka et al. 1998). Both the wild-type and K249R mutant CHK2 cDNAs were inserted in the pCDNA3 vector and co-transfected with a plasmid encoding a hygromycin resistance gene. Using the same method utilized for the generation of clones overexpressing CHK1, different clones were selected from parental and CHK1 D130A cells by double antibiotic selection.

Positive clones growing both in G418 and hygromycin containing media were analyzed for the overexpression of CHK2 by Northern blot analysis, using as a probe the entire CHK2 cDNA (figure 4.24). Five clones were selected, three derived from U20S/pCDNA3 cells, "Up5.10" overexpressing the wt form of CHK2, "Up6.1" and "Up6.21", overexpressing the K249R form of CHK2, and two derived from U20S-CHK1D130A cells, "D130 6.2" and "D130 6.6", both overexpressing the K249R form of CHK2. The *in vitro* growth characteristics of the selected clones was studied and compared with that of the parental cells from which they were derived.

Figure 4.25 shows growth curves for the U20S/pCDNA3-derived clones (panel A) and the U20S-CHK1D130A-derived clones (panel B). The different clones were able to grow at rates similar to their parental cells or

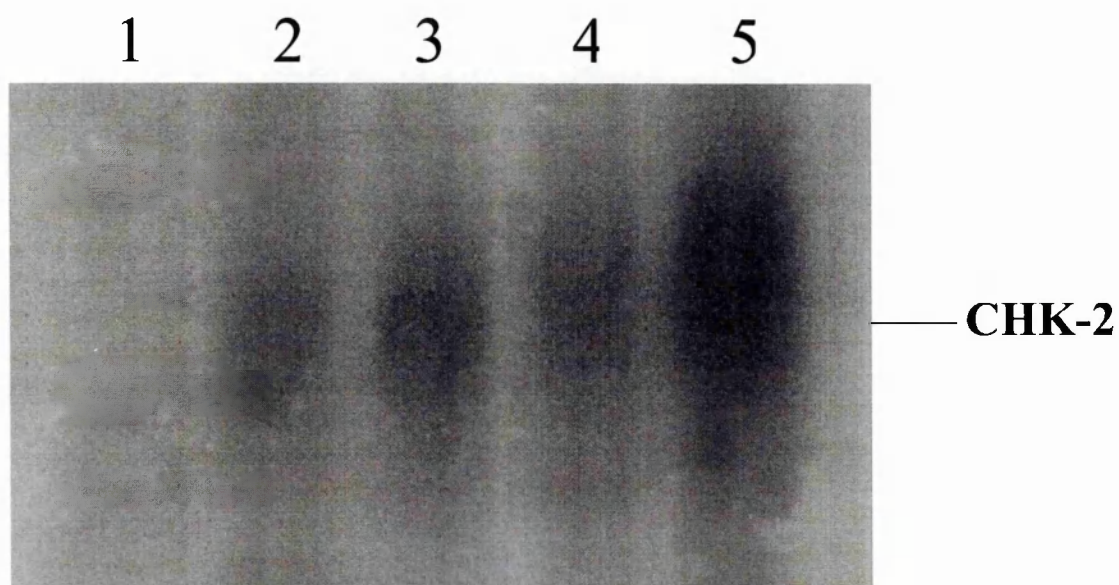


Figure 4.24

Representative Northern blot showing the expression of CHK2 in clones derived from U2OS and U2OS/D130 cells.

Total RNA extracted from U2Os/pCDNA3 (lane 1), U2OS/Up5.10 (lane 2) U2OS/Up6.1 (lane 3) U2OS/D130 6.2 (lane 4) and U2OS/D130 6.6 (lane 5) was separated on agarose gel, transferred to a nylon filter and the blot hybridized with a CHK2 cDNA probe.

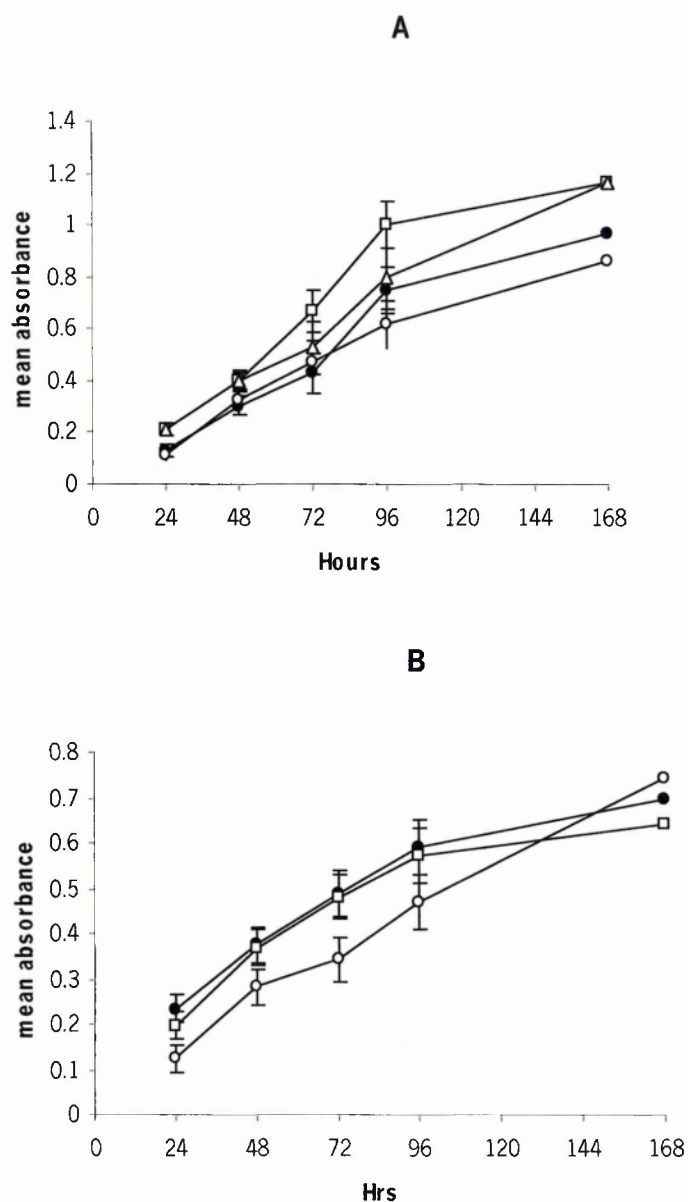


Figure 4.25

In vitro growth of clones derived from U2OS cells and overexpressing CHK2.

Panel A: Cell growth of U2OS parental cells (●) and of clones derived from them Up5 (○), Up6.1 (±) and Up6.21 (Δ). Cells were seeded in 96 well plates and cell growth evaluated at the indicated times using MTT. Values are expressed as mean 540 nm absorbance.

Panel B: Cell growth of D130 cells (●) and of clones derived from them D130 6.2 (○) and D130 6.6 (±). Cells were seeded in 96 well plates and cell growth evaluated at the indicated times using MTT. Values are expressed as mean 540 nm absorbance.

clones. These clones were tested for their ability to respond to DDP treatment. Cells were seeded from exponentially growing cultures and treated with appropriate concentrations of the drug 48 hours after seeding. DDP treatment was for 1 hour, after which the cells were kept in drug-free medium for a further 72 hours, before the MTT solution was added.

The OD obtained for control cells and treated cells were compared and concentration versus % of controls curves were depicted. The overexpression of the dominant form of CHK2 did not substantially alter the cellular response to DDP treatment, reflected by similarity of the curves describing parental cells and the different clones (Fig 4.26).

4.4.3 Discussion

As observed for CHK1, the results obtained with the dominant negative mutant of CHK2 do not allow clear conclusions to be drawn with respect to the relative role of CHK2 in determining cellular sensitivity.

The kinase-dead mutants, while being unable to exert activity in vitro, may well not be able to inactivate completely the endogenous kinase. Again, it is noteworthy that these mutants were effectively reported to act as dominant negative stimuli in cell free systems in which they were able to block the wild-type form of both CHK1 and CHK2 (Sanchez et al. 1997; Matsuoka et al. 1998).

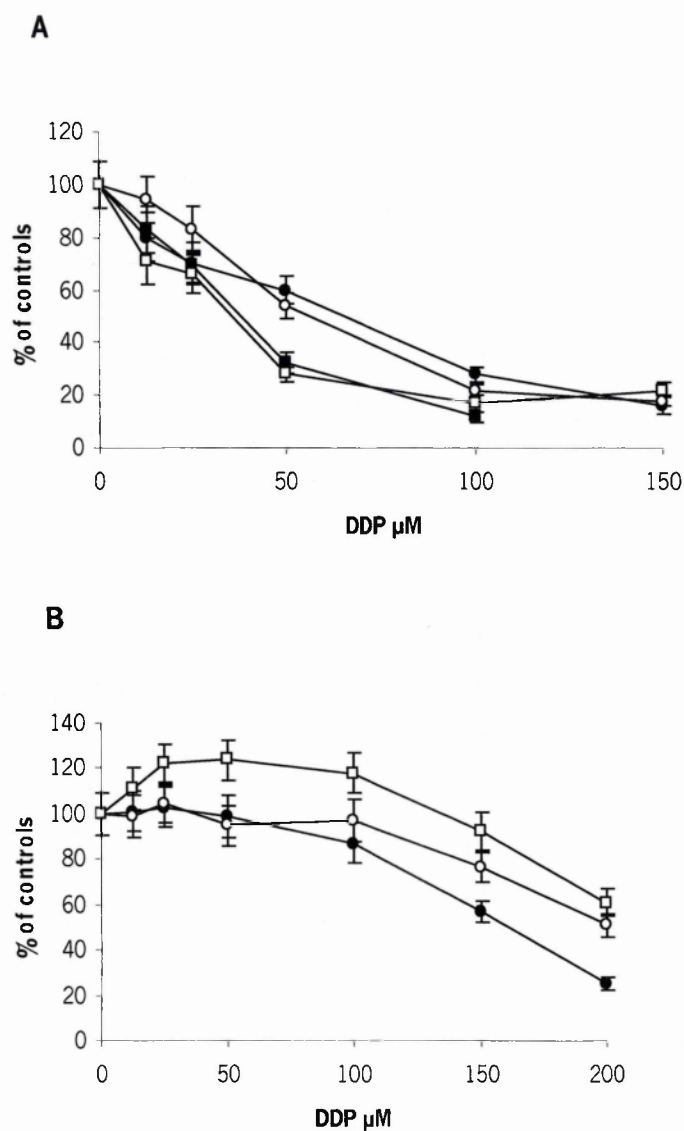


Figure 4.26

In vitro cytotoxicity of DDP in U2OS-derived clones (panel A) and in D130-derived clones (panel B).

Panel A: U2OS parental cells (●), and clones Up 5.10 (■), Up 6.1 (○) and Up 6.10 (□), were treated with different concentrations of DDP for 2 hours. At the end of treatment, the drug was removed and the cells incubated in drug-free medium for further 72 hours.

Panel B: U2OS/D130 cells (●), clone D130 6.2 (○) and clone D130 6.6 (□) were treated with different concentrations of DDP for 2 hours. At the end of treatment, the drug was removed and the cells incubated in drug-free medium for further 72 hours.

The percentage of 540nm abs in treated cells was calculated relative to untreated cells using the MTT test. The results are the mean \pm SD of six replicates.

4.5 Cross-talk between p53 and CHK1

4.5.1 Introduction

The interconnection between different checkpoints may be an important determinant of the cellular response to a given stress such as that exerted by cytotoxicants. This notion is particularly pertinent with respect to a cellular response elicited by an external stress which needs to be abrogated once the damage has been removed, and when constant checkpoint activation would be detrimental for cell life. The removal of checkpoint activation is therefore a mechanism which could involve the reciprocal control between proteins directly participating in checkpoints. The next set of experiments described in this chapter were aimed at evaluating the connections, if any, between p53 and CHK1.

4.5.2 p53 status and CHK1 protein levels

In experiments performed to evaluate the effect of DNA damage on levels of CHK1 in HCT116 cells, it was noticed that at relatively long time periods after treatment with DDP there was a decline in the levels of CHK1. By comparing this effect in the clone HCT116/E6, which does not express p53, the decrease in the levels of CHK1 was unexpectedly found to be much smaller, if not absent, compared to that observable in parental, wild-type p53 expressing cells. This phenomenon was particularly evident

24 and 48 hours after DDP treatment (figure 4.27). The same blot was probed with anti p53 antibodies, to verify the inactivation of p53, and with antibodies against actin, to normalize for potential differences in loading. The experiments were repeated several times and the intensity of the CHK1 band relative to the internal standard actin was measured by densitometry and the degree of inhibition is shown in figure 4.28. Forty-eight hours after treatment with DDP in HCT116 wild-type p53 cells the levels of CHK1 were only 5% of control values, while in the p53-inactivated clone these levels were 65% of controls. As expected, p53 was present only in parental cells after treatment with DDP, while it was almost undetectable in the E6-transfected clone.

To verify whether the differential decline in CHK1 levels observed in parental and E6-transfected cells was really due to the presence of p53, we repeated the experiments in another HCT116-derived subline in which p53 has been inactivated by targeted homologous recombination. In this clone the p53 gene has been directly inactivated and there was no production of RNA and protein. The treatment conditions and experimental procedure were identical to those utilized for the experiments in the HCT116-E6 clone. As reported in figure 4.29, a time dependent decrease in CHK1 protein levels in parental HCT116 cells treated with DDP was again observed, which was not present in HCT116 p53^{-/-} cells. Again, the blot was probed with anti actin antibody to normalize for loading differences and with anti p53 antibody to further

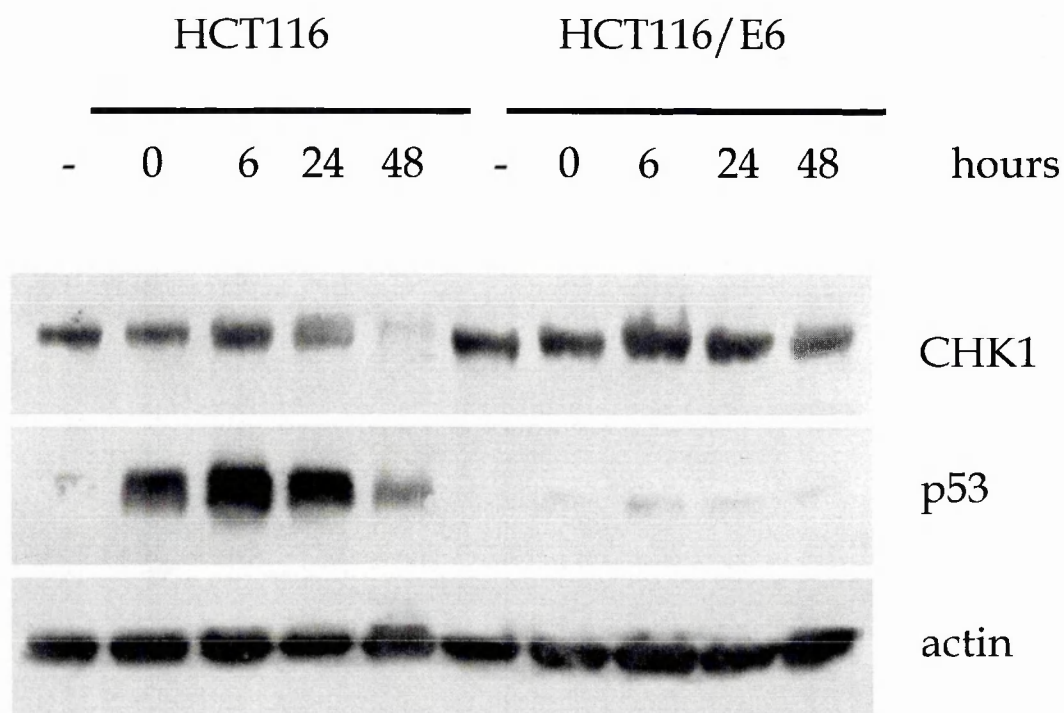


Figure 4.27

Representative western blot showing the effect of DDP treatment on CHK1 protein levels in HCT116 parental and HCT116/E6 cells.

Cells were treated with 25 μ M DDP for 24 hours and cellular extract taken before treatment and after 0, 6, 24 and 48 hours incubation in drug-free medium.

The blot was hybridized with anti CHK1 and anti p53 antibodies. Actin was used to normalize for differences in loading.

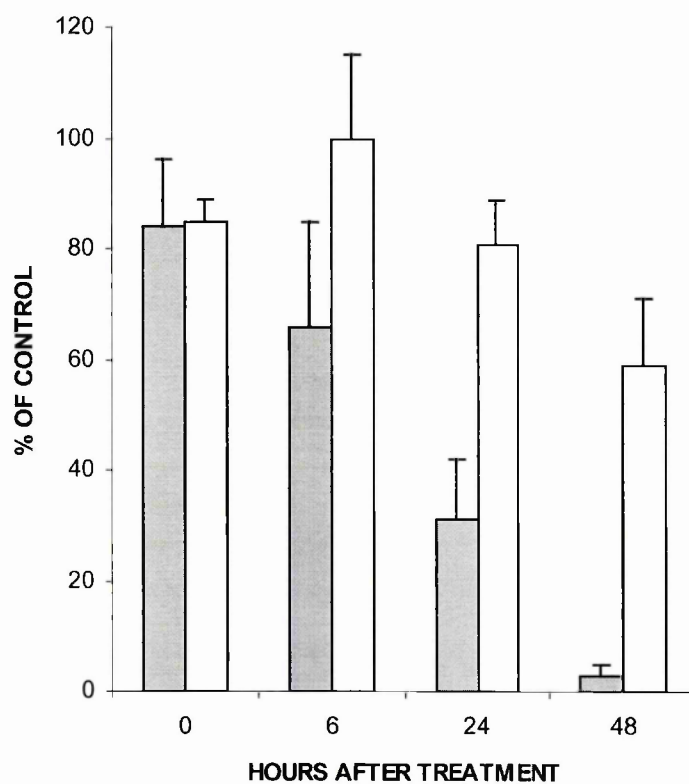


Figure 4.28

Quantitative analysis of the inhibition of CHK1 levels induced by DDP in HCT116 (grey bars) and HCT116/E6 cells (white bars).

Blots of three independent experiments were analyzed with the image analyser and the mean \pm SD of the intensities, normalized for the actin levels calculated.

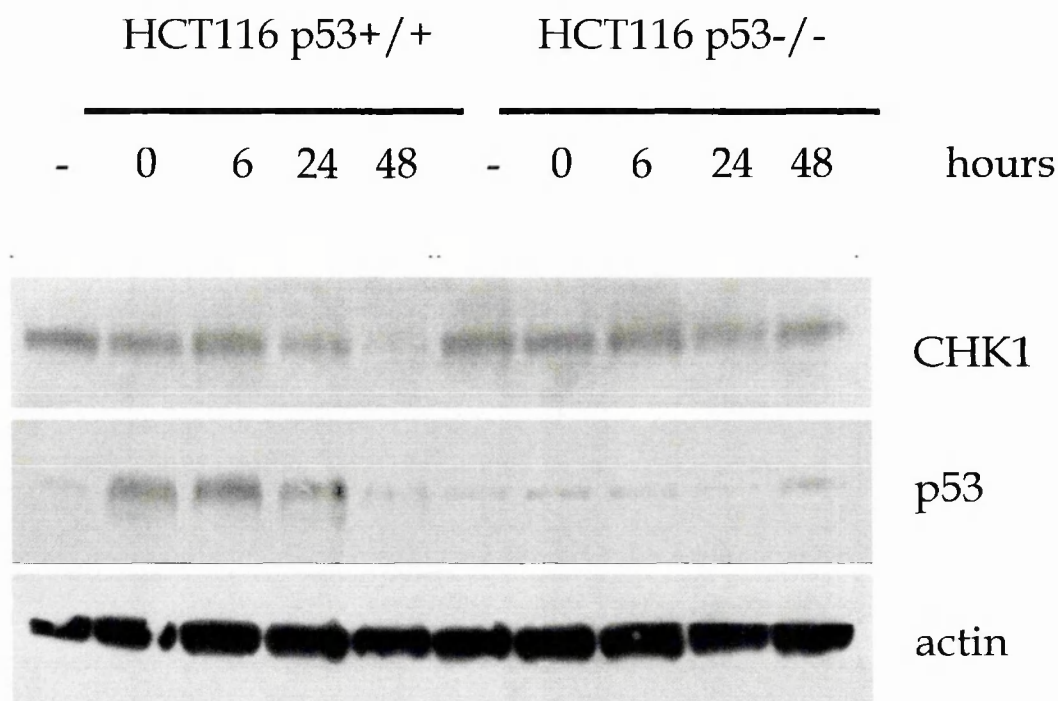


Figure 4.29

Representative western blot showing the effect of DDP treatment on CHK1 protein levels in HCT116 p53+/+ and HCT116 p53-/- cells.

Cells were treated with 25 μ M DDP for 24 hours and cellular extract taken before treatment and after 0, 6, 24 and 48 hours incubation in drug-free medium.

The blot was hybridized with anti CHK1 and anti p53 antibodies. Actin was used to normalize for differences in loading.

check the presence of p53 in parental cells and the absence in p53^{-/-} cells.

The HCT116 p53^{-/-} cells lacked expression of p53, either before or after treatment with DDP. In contrast in parental cells the levels of p53, which was almost undetectable under basal conditions, were rapidly and consistently increased after treatment with DDP.

4.5.3 Cell cycle analysis and CHK1 levels following treatment with DDP

Next the question was addressed whether the decrease in CHK1 levels induced by DDP in a p53-dependent way was due to a different cell cycle effect induced by the drug in cells with or without p53. To do this, HCT116 parental and HCT116/E6 cells were treated with 25 μ M DDP for 24 hours under the same conditions used for the Western blot analysis. At the end of treatment and after 6, 24 and 48 hours incubation in drug-free medium, untreated or DDP-treated cells were removed, washed in PBS, fixed, stained with propidium iodide as described in material and methods section, and analyzed by flow cytometry (figure 4.30, panel A). In parental HCT116 cells, DDP induced initially an arrest of cells both in the G1 and G2-M phases of the cell cycle, and at longer times (24 and 48 hours incubation in drug-free medium) a prevalent G2-M arrest was found. In HCT116/E6 cells, the initial G1 arrest was not observable, while the G2-M arrest at 48 hours incubation in drug-free medium was similar to that achievable in parental HCT116 cells. At this time point, the ratio of

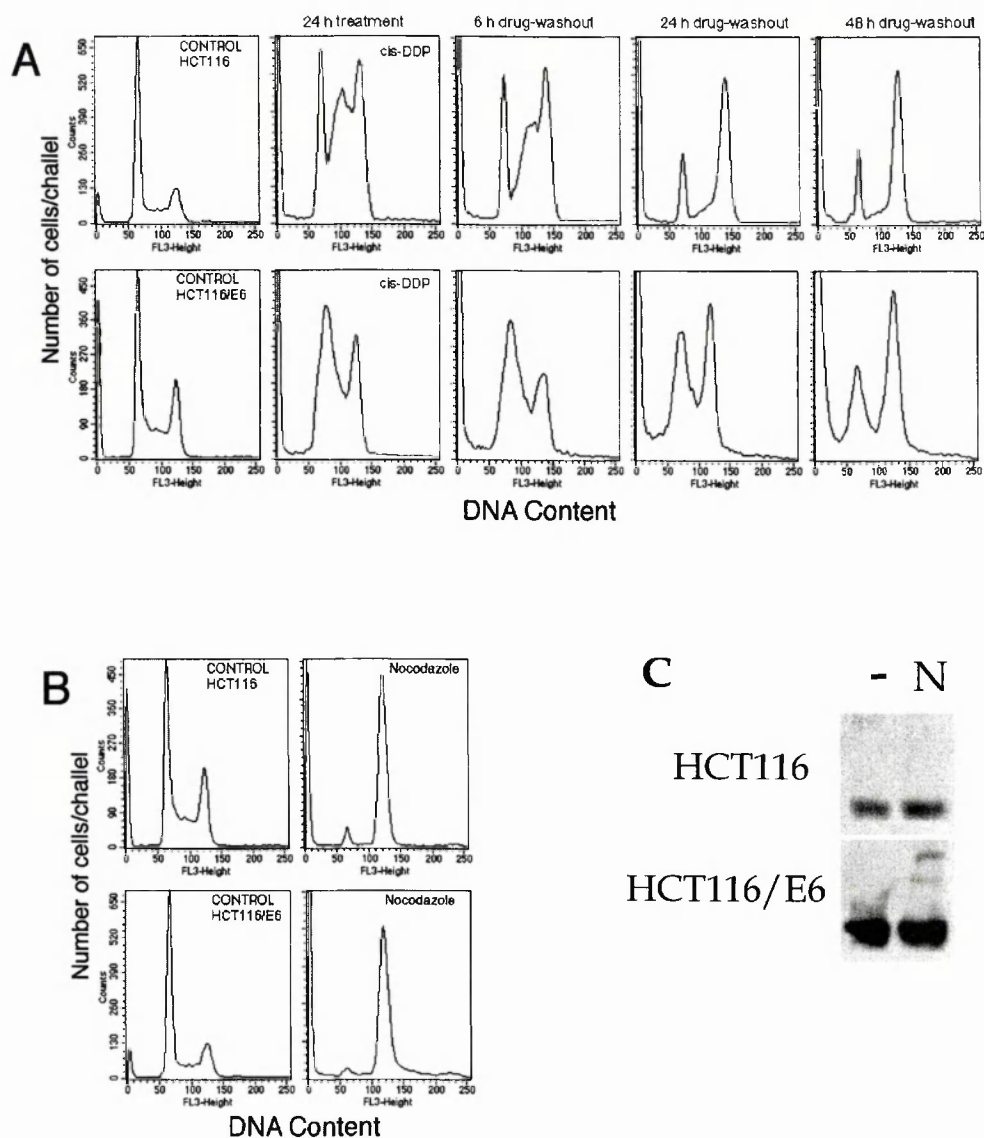


Figure 4.30

Panel A. Cell cycle phases distribution in HCT116 and HCT116/E6 cells treated with 25 μ M DDP for 24 hours. Cell cycle analysis was performed at the end of treatment and after 6, 24 and 48 hours incubation in drug-free medium.

Panel B. Cell cycle perturbation induced by nocodazole in HCT116 and HCT116/E6 cells.

Panel C. CHK1 protein levels in HCT116 and HCT116/E6 cells treated with nocodazole.

percentages of cells in G2-M and G1 phases was 3.1 for HCT116 and and 2.7 for HCT116/E6 cells.

Furthermore, the compound nocodazole, which induces a G2-M arrest interfering with tubulin polymerization, was used. Both HCT116 and HCT116/E6 cells were treated with 400 ng/ml of nocodazole for 24 hours, which caused a clear G2-M accumulation of both cell types (figure 4.30, panel B). However this G2-M arrest was not associated with a decrease in CHK1 protein levels, as shown in figure 4.30 panel C. These results indicate that the observed DDP-induced decrease in CHK1 levels is not merely a consequence of accumulation of cells in the G2-M phase of the cell cycle, but rather is the consequence of the activation of G2 damage checkpoint.

4.5.4 Mechanism of p53-induced downregulation of CHK1 levels

The subsequent step was to try to clarify the reason for the p53 dependency in CHK1 downregulation induced by DDP. First it was investigated whether the observed decline in CHK1 protein levels was also observable at the mRNA level. By using the same treatment conditions utilised for the Western blot and flow cytometry experiments, we isolated total RNA from untreated cells or from cells treated with DDP for 24 hours and incubated in drug-free medium for 0, 6 24 and 48 hours. The RNA was purified and treated as described in Chapter 3.5 and, after transfer to a nylon membrane, hybridized with a radioactive probe

derived from the human CHK1 cDNA. The autoradiograph of a representative experiment is shown in figure 4.31 panel A, in which two bands, corresponding to two different transcript isoforms of CHK1 are clearly visible. In parental HCT116 cells a clear reduction in CHK1 RNA levels following DDP treatment was observed, which was much less evident in E6-transfected cells. By densitometric analysis, the percentage of decrease of CHK1 levels was calculated in both cell lines. After 24 hours incubation in drug-free medium, CHK1 RNA levels were decreased by approximately 80% in parental cells, while no effect was found in HCT116/E6 cells. At 48 hours the inhibition was more than 90% in parental cells and only 35% in p53-inactivated cells (figure 4.31 panel B).

The decrease in CHK1 RNA levels following DDP treatment preceeded the decrease in protein levels, indicating that the primary effect was indeed at the RNA level and that the decrease in protein levels was the consequence of a decreased abundance of the template used for translation.

A different system was used to analyse whether the DDP-induced downregulation of CHK1 mRNA was caused by a transcriptional or posttranscriptional effect. The availability of a construct was taken advantage of in which the human CHK1 cDNA was subcloned in an expression vector in frame with an HA epitope tag consisting of 36 nucleotides, leading to a 12 amino acid peptide located directly at 5' to the CHK1 gene. This construct was stably transfected in U20S cells, and the

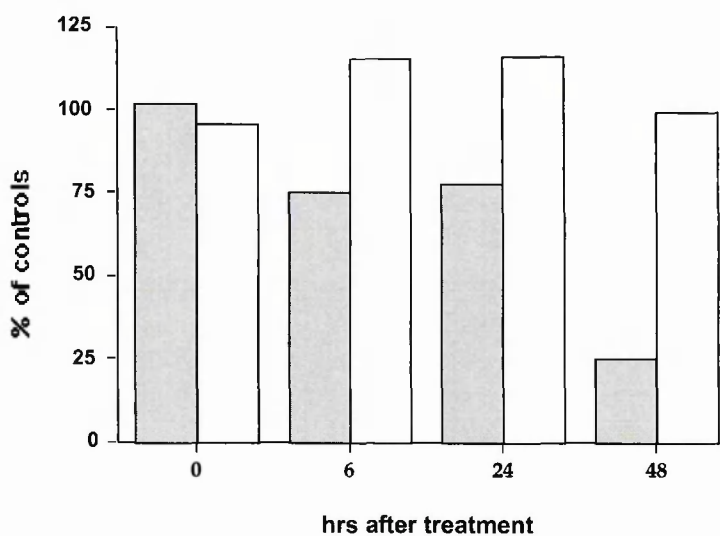
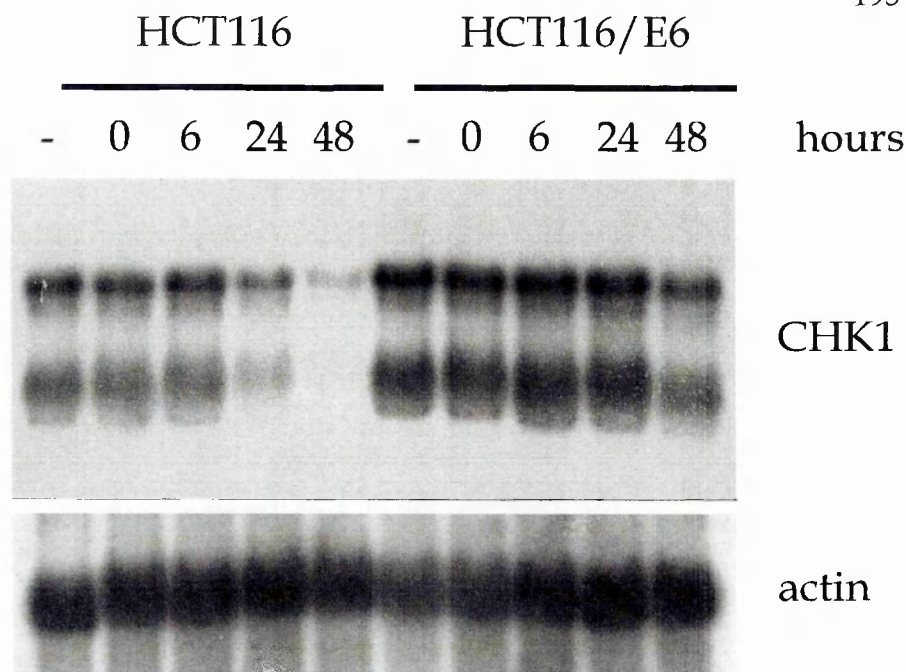


Figure 4.31

Upper panel. Northern blot showing the effect of DDP treatment on CHK1 mRNA levels in HCT116 parental and HCT116/E6 cells.

Cells were treated with 25 μ M DDP for 24 hours and cellular extract taken before treatment and after 0, 6, 24 and 48 hours incubation in drug-free medium.

The blot was hybridized with a cDNA CHK1 probe. Actin was used to normalize for differences in loading.

Lower panel. Quantitative analysis of the inhibition of CHK1 RNA levels induced by DDP in HCT116 (grey bars) and HCT116/E6 cells (white bars).

Blots of three independent experiments were analyzed with the image analyser and the mean \pm SD of the intensities, normalized for the actin levels, calculated. The bigger RNA form of CHK1 was considered.

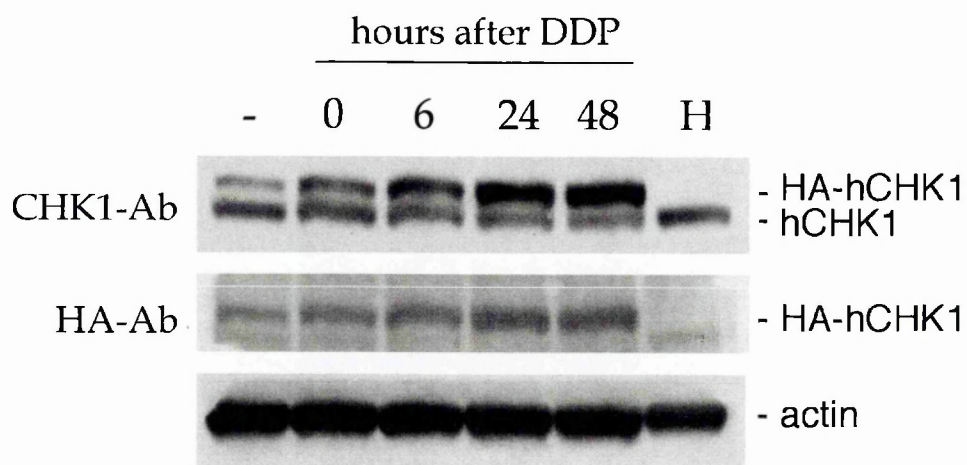


Figure 4.32

Effect of DDP on the levels of endogenous (CHK1) and exogenous (HA-CHK1) CHK1 levels in U2OS cells treated with DDP. Extract were taken at the end of DDP treatment and after 6, 24 and 48 hours incubation in drug free medium. Lane - corresponds to untreated cells and lane H corresponds to extracts obtained from untreated HCT116 cells. The blot was probed with anti CHK1 antibody and subsequently with anti actin antibody. Actin has been used as internal control for normalization.

selected clone, expressing both endogenous CHK1 and exogenous HA-CHK1, was treated with DDP. Figure 4.32 shows the results obtained. The ratio between the exogenous HA-CHK1 and the endogenous CHK1 changed with time in favour of exogenous CHK1, indicating that the observed decrease in CHK1 was likely to be due to a transcriptional effect, not observed when the RNA of the exogenous gene is under the control of a viral promoter.

It was reasoned that if the transcription of the gene was important for the observed decrease in CHK1 RNA and protein in a p53 dependent manner, then it should be possible to see the same effect when the genomic sequences responsible for CHK1 transcription in the human genome are isolated and placed 5' to an exogenous gene normally not present in mammalian cells. By using this construct it should be possible to render observable the modulation of endogenous gene expression mediated by the human CHK1 promoter.

4.5.5 Isolation of CHK1 genomic sequences

To isolate the genomic DNA regions possibly involved in the regulation of CHK1 gene, a human genomic library spotted in duplicate in seven different filters, obtained through the UK Human Genome Mapping Project Resource Centre (UK-HGMP-RC) was used. These filters contain genomic DNA clones and can be hybridized with different probes derived from the human CHK1 cDNA to isolate the genomic sequence of interest.

For the purpose of this study, a probe from the 5' region of the human CHK1 cDNA was selected in order to allow isolation the 5' genomic region which is likely to contain the promoter region of the gene. Figure 4.33 shows an example of an autoradiograph indicating the presence of two positive spots. On the whole set of seven filters, the use of this cDNA probe led to the identification of two positive clones which could be univocally identified through the coordinates of the filters and requested to the UK-HGMP-RC. The two positive clones (53P13 and 253) thus obtained were grown in LB media and the DNA was isolated from the overnight culture using procedures described in the materials and methods section (chapter 3.2.5). Since each of this clone contained approximately 100 Kb of DNA, it was necessary to pinpoint smaller DNA fragments containing the CHK1 gene for further characterization. To do this, DNA was digested with different appropriate restriction enzymes and half of it loaded on 0.8% agarose gels to separate the fragments of different length. At the end of the run the gel was stained with ethidium bromide and the bands visualized under UV light. Figure 4.34, shows fragments of the two clones digested with the restriction enzymes BamHI , EcoRI and HindIII (panel A). The gel was blotted overnight to nylon filter and hybridized with a small cDNA CHK1 sequence to identify the fragments inside the genomic sequence present in the clone which contains the CHK1 gene. The results of the hybridization are shown in the panel B of figure 4.34, from which one can deduce that few digested

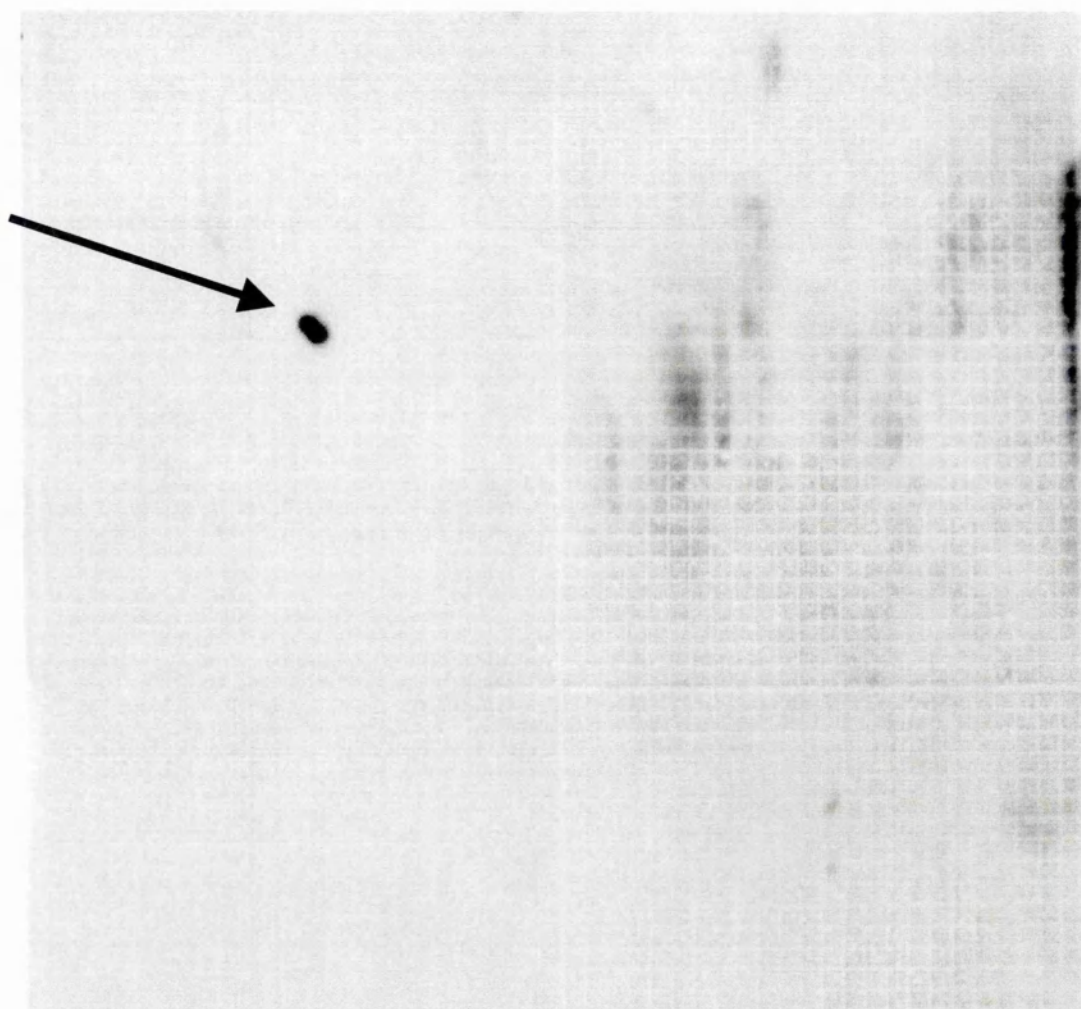


Figure 4.33

Screening of a genomic library.

The figure shows a representative autoradiography obtained from a filter containing genomic DNA that was probed with a ^{32}P -labeled CHK1 cDNA fragment.

Each clone is spotted in double on the filter. The arrow points to a CHK1 positive clone which has been selected from this screening.

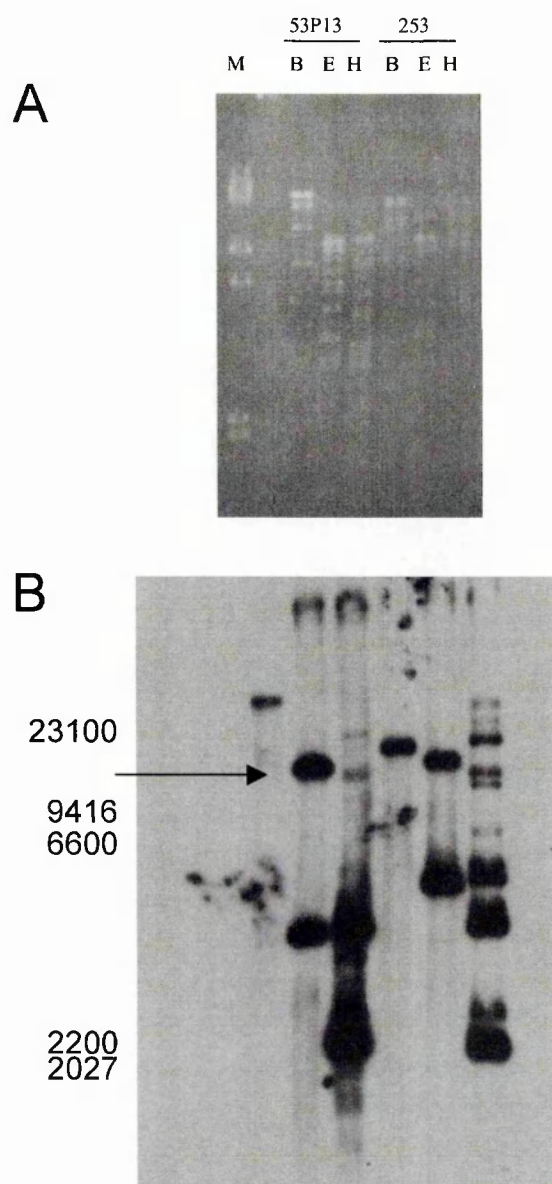


Figure 4.34

Southern blot from a genomic clone isolated from the screening of a genomic library. DNA isolated from the clone was digested with different restriction enzymes and separated on agarose gel.

Panel A. Ethidium bromide staining of the gel. The two clones (53P13 and 253) have been digested with BamHI (B), EcoRI (E) or Hind III (H). M is the molecular weight marker.

Panel B. Autoradiography of the gel transferred to a nylon membrane and hybridized with a ³²P-labelled CHK1 probe. Numbers on the left are derived from molecular weight markers and are bp of DNA. The arrow points to the 10 Kb fragment of the 53P13 clone digested with EcoRI isolated for further studies.

fragments gave positive hybridization and hence contained the sequence of CHK1 used to hybridize. From these experiments a DNA fragment of approximately 10 Kb, obtained from clone 53P13 after digestion with Eco RI was isolated for further characterization.

This DNA fragment was subsequently subcloned in the Eco RI site contained in the multiple cloning site of the pBluescript plasmid. This recombinant plasmid was used to sequence the DNA inserted. Initially the first sequences were obtained using as primers the sequences contained in the plasmid. From these DNA sequences, other oligonucleotides primers were synthesized and used to further sequence the inserted clone. After two steps of sequencing, the obtained sequence was aligned and matched with the human genomic sequences present in the NCBI gene bank database. A complete overlap with a genomic sequence was found, and this sequence was located near to CHK1 cDNA sequence. Once it had been verified that the 10 Kb cloned sequence was indeed a sequence corresponding to the genomic region around the CHK1 cDNA, two oligonucleotide primers were designed and used in PCR reactions to amplify a smaller DNA fragment containing the initial non coding part of the CHK1 gene and a genomic region immediately 5' to this non coding part. The amplified DNA was subcloned in the multiple cloning site of the promoter-less pGL2 vector. This vector contains the luciferase cDNA and a multiple cloning site 5' of the cDNA without promoter and is generally used to determine the efficiency of putative genomic promoter regions to

drive the transcription of the luciferase gene. Using the method reported in sections 3.23-3.25 recombinant clones were selected and the DNA partially sequenced to further verify the presence of the right insert and to determine the orientation. The sequence of the insert isolated and used in subsequent experiments is reported in figure 4.35. This fragment of the genomic CHK1 gene contained a small sequence of the 5' cDNA and a genomic region of 867 bp. Two different colonies, presenting the same sequence subcloned in opposite direction were selected. The colony containing the genomic CHK1 region subcloned in antisense orientation with respect to the luciferase cDNA was used as negative control because it should not be able to drive its transcription.

The ability of p53 to induce downregulation of CHK1 promoter was tested by co-transfecting an expression vector containing human p53 cDNA with the construct containing the luciferase gene under the control of the human CHK1 promoter fragment of 867 bp isolated as described above. From figure 4.36 it appears that p53 reduces the activity of the 867 bp-CHK1 genomic construct. The same construct inserted in antisense orientation, i.e. used as negative control was not affected by p53. The transcriptional repression exerted by p53 on CHK1 promoter fragment was more evident if compared with the transcriptional activation induced on another promoter fragment isolated from the human p21 gene and known to be activated by p53, which was obtained from Dr Carol Prives, Columbia University, NY.

5'
 ATTCTCCTGCCCTCGCCTTCCCAAAGTGCTGGAATTACAGGCAGGA
 GCCACTGGCCCGGCCAGTGCTGGAGAATGTAATGGAGATGATCCT
 GCCTATCCCTGAGTTCCAGTCTAGTTTACTGTCAGAGAATACTTATT
 TCCATTTTTTCTATTTTTGTTCTTGAGTCTCTTTCCTCAGAATTTACTTT
 TCCACTTAAATGACGTACGCAGCTTTTAAATTTGCGTTGTAAGATTT
 ATTTTGGCTCTCCCCGCCTGTTCTTTGCACATTA AAAAATGAAAAAGT
 TTGTAGAACTAAGCTAAGCAGATGGTCTTCCTGCAAAAAGACCGG
 GCTGAAGTAAAGCATTGTTTTGGAGCTGGTTCACAGAAAAAAGGC
 AAAACTGGTTATCCTGACTTCAAGCTCCAACATAAACTGCTCGCTT
 TCTCCGGGAAACTTGCCCCGCCACATACTT**GACTGCGTGGCCA**
GTTCTTTCGAAGCCTCTCGCTCCCAACACGGAGTTCCTCCCATTT
CTTCACAGTCGGCTCTCAGCAGCTGCTGCTGGTTTCTCGGCTCC
AGCACCACGAGTACCGCACTCTGAGGTTTACAAAGCACTCTGCT
TCACCGACTGTGATCCTCACAGTCCTGTCCGGTGGCCTCACGCA
GGTGGCGGTGCAGCCTTTCAGGCCCCAGAGCGGCCAGGAGCGA
AGCCCGCAGCCCCGCCTGGAAGCGCAGCGCGGTTCGGTTCGCGCG
CCCCTGAGGCTTGGAGGCCTGGGCTTCCCCCAGCAGCGCTCGA
GCACCGCCCAGTCGAGCCTCACACCGGATGCCACTTCATATTTG
GGCCCAGAGCTCAATTCGCGCCGATGCGGTCCGCCGTCCTTAA
ATCTCTTCAGCC 3'

Figure 4.35

Sequence of the genomic DNA fragment isolated from the genomic library and used for the determination of CHK1 promoter activity. The sequence is oriented 5'-3'. In bold are sequences of the 5' untranslated region of the gene.

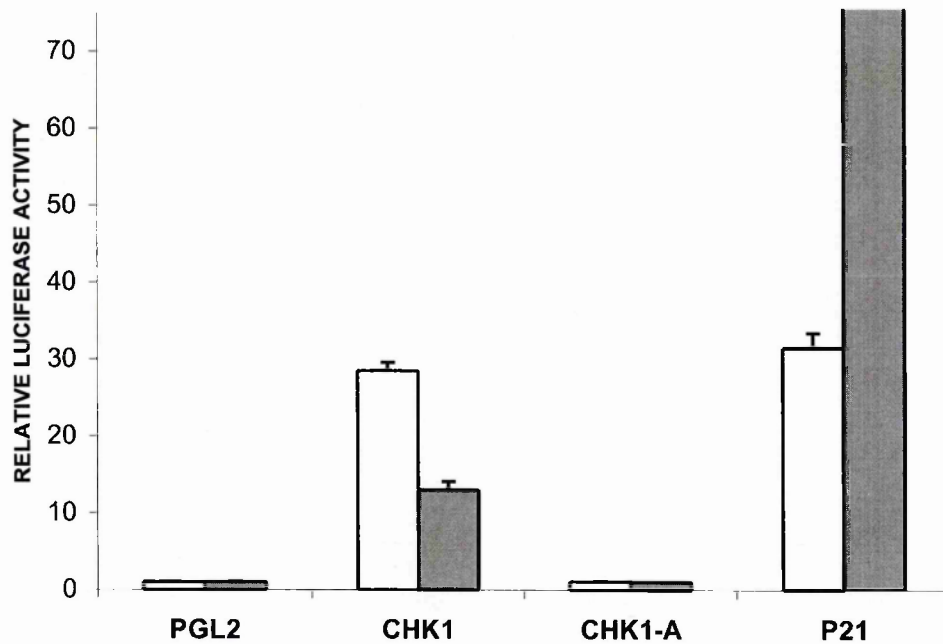


Figure 4.36

Luciferase activity in cells transfected with the 864bp CHK1 construct in sense (CHK1) or antisense (CHK1-A) orientation. As controls, empty vector (pGL2) and the p53-responsive element present in p21 promoter (p21).

Skov-3 cells were co-transfected with the indicated plasmids together with human p53 expressing plasmid (grey bars) or control vector (white bars). Luciferase activity was assessed 48 hours after transfection.

4.5.6 Discussion

The results presented in this section clearly suggest that there is a strict relationship between p53 and CHK1. On one side, CHK1 is activated by phosphorylation following damage (Abraham 2001; Tian et al. 2002), with ATR being one of the kinases responsible for this event. Once activated, CHK1 is able to phosphorylate different substrates, among which CDC25 is probably the most important and best studied (Sanchez et al. 1997; Kaneko et al. 1999). Additionally CHK1 is able to phosphorylate p53 at the amino terminus, at position serine 15 (Shieh et al. 2000). This phosphorylation is thought to be an important event in the activation of p53 thus indicating that the two checkpoint proteins are interrelated. The results presented here indicate that in a wild-type p53 background following DNA damage, and hence implying a p53 dependent mechanism, CHK1 is downregulated, mainly transcriptionally. This notion intimates that there is a cross talk between the two proteins, which in concert could co-operate in the onset of damage-activated checkpoint, through cell cycle arrest, which could then be removed through repression of CHK1. Repression of CHK1 is likely to occur in cells with moderate damage, in which repair of the lesion has occurred and hence the cell cycle needs to be restarted after the transient block. In the case of severe and persistent damage, which renders the repair of the lesions impossible and which entails activation of apoptotic processes, this phenomenon, i.e. repression of CHK1 to resume the cell cycle, might not be relevant.

Moreover, these findings are consistent with the observation that in cancer cells lacking p53 there is a more prolonged and sustained arrest in the G2 phase of the cell cycle than in cells expressing a functional wild-type p53 (Pollack et al. 1996; Sugiyama et al. 2000). In fact, according to the model proposed here, cells lacking functional p53, or expressing mutated p53, would not be able to trigger the signal for downregulation of CHK1, levels of which would thus remain relatively high for prolonged times.

As a consequence, inactivation of both p53 and the CHK1 checkpoint is likely to result in an increased sensitivity to anticancer agents. Such an increase could be a particular therapeutic advantage in the case of cancer cells lacking p53 expression, in which the p53-dependent checkpoint is already compromised, and where therefore the inhibition of the CHK1-dependent checkpoint could result in a more pronounced increase in ability of agents to damage DNA compared to normal cells in which abrogation of the CHK1-dependent checkpoint could leave the p53-dependent checkpoint unaltered.

In addition, the results of this chapter, suggest that different pathways of cell signalling, following damage induction, have to be regarded as a complex cascade of events likely to be interlinked, rather than as a single event dependent on one protein only.

5. GENERAL DISCUSSION

This thesis is concerned with experiments which are aimed at investigating the role of different proteins in the mediation of cellular responses to cytotoxic stress. The proteins considered were p53 and its analogue p73 and the two cell cycle checkpoint proteins CHK1 and CHK2. The underlying rationale of the work was to delineate the role of these proteins in the antitumor activity of certain anticancer agents. It needs to be stressed that in all the experiments performed, particular attention has been paid to the selection and use of appropriate cellular systems.

The underlying idea was to reduce, as much as possible, any interference by other genetic alterations likely to be present when two cells of different origin are used. For this reason, isogenic cell systems were characterized when already available, or generated in the case that cells with the desired genetic alteration were not available. In the case of p53, starting from wild-type p53 expressing cells, two different inactivation methods and two different cell lines were used. The results outlined in section 4.1 suggest that in all cases the presence of p53 was associated with resistance to treatment with the anticancer agents DDP and taxol.

P53 has been reported by many laboratories to play an important role in determining cellular sensitivity to treatment with anticancer agents. There is no doubt that wild-type p53 plays an essential role in controlling the genomic integrity of the cells. The importance of this notion is underlined by the evidence that the majority of human tumors harbor inactivated protein, inactivated either *via* gene mutation, cytoplasmic

sequestration, viral inactivation or other mechanisms (Harris 1996; Hollstein et al. 1997; Vogelstein et al. 2000).

A potential therapeutic application arising from these findings is reflected by attempts to restore p53 function in cancer cells. In cancer cells growing in vitro, the re-introduction of a functional p53 is associated with growth arrest and cell death (Vogelstein and Kinzler 1992; Levine 1997). However, the possibility to use such gene therapy-based approaches in humans, needs additional improvements to be made concerning the currently available delivery systems. Nevertheless clinical trials exploring adenovirus-mediated transfer of wild-type p53 in human cancer have been activated (Merritt et al. 2001; Buller et al. 2002a; Buller et al. 2002b).

Moreover, the finding that tumors often present defects in p53 could offer an advantage for those therapeutic regimens which are characterised by enhanced activity in the absence of p53. Probably the best example is the modified adenovirus which is inactivated in cells expressing wild-type p53, but not in those without p53, in which the virus can replicate, lyse the cells and diffuse to neighboring cells (Heise et al. 1997). This "smart" virus has so far shown promising results, although experience dictates to limit one's enthusiasm concerning such an approach (Nemunaitis et al. 2001; Hecht et al. 2003). In addition, some reports suggest that the virus could also affect cells harboring wild-type p53 (Dix et al. 2001; Georger et al. 2002).

Theoretically, any drug treatment able to preferentially kill cancer cells with inactivated p53 should have a favourable therapeutic index, since its cytotoxic activity against normal and wild-type p53 expressing cells should be reduced. However, there are examples of drugs, the activity of which is increased or decreased by the presence of p53, depending on the cellular context.

One might argue that this fact is not surprising, since the activation of p53 can lead either to activation of apoptosis or to activation of cell cycle arrest. If the latter predominates, it is to be expected that a drug causing DNA damage will have more chances to be active in cells without p53, as the lack of cell cycle arrest reduces the possibilities to repair the lesion. The data reported in sections 4.1.2-4.1.5, which were obtained in epithelial-derived cancer cells, are in agreement with this hypothesis.

In the case of the p53 homologue p73 the situation is somewhat different, in that its role as a tumor suppressor and as a checkpoint control protein is still shrouded in mystery (Grob et al. 2002; Melino et al. 2002; Stiewe and Putzer 2002). The observation that in different tumors the wild type form of p73 is overexpressed (Novak et al. 2001; Nozaki et al. 2001; Moll et al. 2001) mitigates against its function as tumor suppressor. It is not easy to study the effect of overexpression of p73 in isogenic systems, since the introduction of a wt p73 form in cancer cells growing in culture leads to growth arrest and apoptosis (Kaghad et al. 1997).

How human tumors can grow with relatively high levels of p73, compared to normal adjacent tissue, is not yet clear. The evidence reported here (sections 4.2.2 and 4.2.3) using two clones overexpressing p73, in which increased expression of DNA repair genes (and particularly of the NER system) was found, would suggest that tumors with high levels of p73 have a better DNA repair capacity, hence a lower chance to respond to chemotherapy involving drugs inducing lesions that are recognized by the NER system. This notion is only speculative, as the evidence has been observed in only one experimental system, due to the difficulty in obtaining clones overexpressing p73. It seems particularly important to verify whether these results can be generalised, because, if confirmed, they could have a strong impact on the selection of the therapeutic regimens for those tumors overexpressing p73.

DDP, one of the most active and widely used anticancer agent available, is a drug characterised by "sensitivity" to the presence of high DNA repair capability. Therefore the impact of the presence of high p73 levels, in relation to the adjacent normal tissue, would be particularly important concerning the activity of DDP.

Nevertheless, the evidence presented here with respect to p53 or p73 implies that new molecules selected to inhibit targets, such as the cyclin dependent kinases, more specifically than the traditional cytotoxicants, might show antitumor activity independently of the status of p53 or p73. Such new drugs would offer an interesting alternative with

respect to treatment of tumors for which the currently available therapies possess only low efficacy.

The observation that the truncated DN form of p73 could have effects opposite to those of wild-type p73 (Furnari et al. 1999; Moll et al. 2001), raises the possibility to test the impact of overexpression of DNp73 on the cellular response to anticancer agents. In order to test this possibility clones with high levels of DN p73 will have to be isolated, and this is one of the experiments planned on the basis of the results of the studies presented here.

The data obtained with the two checkpoint proteins CHK1 and CHK2 (sections 4.3.2, 4.3.3, 4.4.2) cast doubt on the prudence to use of dominant negative mutants indiscriminately. At least in the HCT116 colon cancer system, they have been hardly useful. Nevertheless the data reported in chapter 4.3.4 supports the idea that abrogation of the G2 checkpoint could result in increased responsiveness of cancer cells towards treatment with anticancer agents.

CHK1 and CHK2 are considered potential targets for the development of new anticancer agents based on the interesting results obtained so far with UCN-01 (Bunch and Eastman 1996; Husain et al. 1997; Monks et al. 2000). The action of UCN-01, however, is not restricted to inhibition of CHK1 and CHK2, and molecules with high specificity for CHK1 or CHK2 would be desirable to allow testing of the relative contribution of these kinases to the overall G2 checkpoint.

The possibility of selectively abrogating the G2 checkpoint in cancer cells is even more attractive considering that in cells lacking p53 (i.e. one of the key proteins governing this checkpoint) the effect of G2 abrogation should be more detrimental than in normal cells, in which the G1 checkpoint is functional and can work as a defense mechanism.

A logical consequence of this hypothesis would be an increased therapeutic index when conventional anticancer agents are combined with G2 abrogators. Another interesting result derived from the experiments reported in chapter 4.5, is the evidence that there is a clear link between proteins participating in different checkpoints. The observation that CHK1 and p53 are mutually regulated is a new finding. It is indicative of a concerted action of these proteins in normal cells throughout the entire cell cycle to ensure the maximal efficiency of control of genomic integrity. Again, the lack of p53 in the majority of human tumors deregulates this connection, which in turn might confer increased vulnerability on to cells towards inhibition of CHK1.

Increasing the knowledge on the links between different checkpoints is an important and suitable topic of study in the future with the aim to allow the selection of better targets for the testing of new drugs with improved specificity to kill cancer cells. The availability of new techniques such as microarray and proteomics offers tremendous increases in the knowledge not only of single gene alterations, but also of cellular pathways which are compromised in a defined pathology.

The increased knowledge of the biology of the tumors is certainly one of the "smartest" ways to identify potential new targets for the development of new anticancer agents. The exploitation of the emerging knowledge of the molecular biology of tumors and of the response of cells defective in a specific gene or pathway, combined with the development of more target-oriented drugs, might render the idea of a "patient-targeted" therapy for individual patients possible. Such an approach might ultimately result in high efficacy and better tolerability of treatments, and hence in achieving the "optimal way" to combat cancer.

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7. APPENDICES

7.1 List of abbreviations

APC	anaphase promoting complex
ATM	ataxia-telangectasia mutated gene
ATP	adenosine 5' triphosphate
ATR	ATM-related gene
bp	base pairs
BRCA 1	breast cancer-associated gene 1
BSA	bovine serum albumin
CAK	cdk activating kinase
cDDP	cis-dichloro-diamine-platinum
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CKII	casein kinase II
CMV	cytomegalo virus
dCTP	deoxy-cytidine-5'-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DTT	dithiothreitol
DX	doxorubicin
ERCC	excision repair cross complementing
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
FHA	forkhead-associated domain
GTC	guanidine thiocyanate
HAT	Histone acetyl transferase

IC ₅₀	concentration inhibiting the growth by 50%
Kb	kilobase pairs
KDa	kilodaltons
MDR	multi drug resistance
MGMT	O ⁶ -methylguanine-DNA methyltransferases
MOPS	3-(N-morpholino) propanesulfonic acid
MMR	mismatch repair
MNNG	N-methyl-N'-nitro-N-nitrosoguanidin
mRNA	messenger ribonucleic acid
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NaAc	sodium acetate
NER	nucleotide excision repair
OD	optical density
PAC	P1 artificial chromosome
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHAS	phosphorylated, heat and acid stable protein
PMSF	phenylmethylsulphonyl fluoride
Rb	Retinoblastoma
RNA	ribonucleic acid
RT	reverse transcription
SAM domain	sterile alpha-motif domain
SD	standard deviation
SDS	sodium dodecylsulphate
TBS	tris buffered saline
TEMED	N',N',N',N'-tetramethylethylenediamine
TSA	Trichostatin A
UV	ultra violet

7.2 List of publications

Publications of the candidate not strictly related to the work presented here:

Broggini M, Marchini SV, Galliera E, Borsotti P, Taraboletti G, Erba E, Sironi M, Jimeno J, Faircloth GT, Giavazzi R, D'Incalci M.

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