THE INFLUENCE OF PENTOXIFYLLINE ON DAMAGE RESPONSES IN TUMOUR CELLS.

By

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature Date

ABSTRACT

Pentoxifylline enhances the toxicity of radiation and has emerged as an effective modulator of the radiation response of tumour cells. The molecular mechanisms involved in the enhancement of radiotoxicity by pentoxifylline have not yet been elucidated. Cell cycle blocks, DNA repair and programmed cell death (apoptosis) are all part of the cellular response to DNA damage and as such must be considered as targets of the drug. In this study, the influence of pentoxifylline on radiosensitisation, G2 block abrogation, DNA repair inhibition and the induction of apoptosis have been investigated in Be11 and MeWo melanoma and 4197 and 4451 squamous cell carcinoma (SCC) cell lines. The influence of pentoxifylline on radiation-induced apoptosis in Jurkat J5 T-lymphocytic leukemia cells has also been assessed. HeLa cervical carcinoma cells were used to investigate the molecular events involved in the abrogation of the G2 block by pentoxifylline. It is shown that pentoxifylline preferentially sensitises the TP53 mutant MeWo and 4451 cell lines and enhances radiotoxicity by factors of up to 14.5. In the MeWo melanoma, but not in the 4451 SCC cell line, radiosensitisation is accompanied by inhibition of DNA repair. No significant enhancement of radiation-induced apoptosis was observed in MeWo melanoma and 4451 SCC cells. However, Jurkat J5 cells showed an increase in apoptosis after irradiation in the presence of the drug. In irradiated HeLa cervical carcinoma cells, pentoxifylline affects the expression of the two components of the mitosis promoting factor (MPF), namely cyclin B1 and $p34^{cdc2}$, and rapidly restores cyclin B1/$p34^{cdc2}$ ratios to control levels. Analysis of cyclin B1 expression in whole cells and isolated

nuclei furthermore reveals an influence of the drug on the subcellular translocation of the MPF.

It is concluded that G2 block abrogation is not the only mechanism involved in the radiosensitisation of tumour cells by pentoxifylline, but that DNA repair inhibition plays a role in certain cell types. Although pentoxifylline induces apoptosis in Jurkat J5 thymocytes, radiation-induced apoptosis plays no role in the radiosensitisation of the two TP53 mutant melanoma and SCC cell lines. Abrogation of the G2 block by pentoxifylline, which sensitises tumour cells to a second irradiation or chemotherapeutic challenge, involves a modulation of the levels of cyclin B1 and $p34^{cdc2}$, and the subcellular location of the MPF. These results are of utmost importance for the clinical potential of pentoxifylline as a dose modifier in cancer therapy.

OPSOMMING

Pentoxifylline verhoog die toksisiteit van bestraling en het dus na vore getree as 'n effektiewe modulator van die sellulêre stralingsrespons in kankerselle. Die molekulêre meganismes betrokke by die verhoging van stralings-toksisiteit deur pentoxifylline is egter nog nie duidelik nie. Blokkering van die selsiklus, die herstel van DNS skade en geprogrammeerde seldood (apoptose) vorm almal deel van die sellulêre respons ná bestraling en word as sulks beskou as potensiële teikens van die middel. In hierdie studie is die invloed van pentoxifylline op stralings-sensitiwiteit, G2 blok verwydering, die vertraging van DNS herstel en die indusering van apoptose ondersoek in die Be11 en MeWo melanoom en 4197 en 4451 plaveisel-sel karsinoom sellyne. Die invloed van pentoxifylline op stralings-geïnduseerde apoptose in Jurkat J5 T-limfosiete is ook bestudeer. HeLa servikale karsinoom selle is gebruik om die molekulêre gebeurtenisse rondom die verwydering van die G2 blok deur pentoxifylline te ondersoek. Dit word aangetoon dat pentoxifylline by voorkeur die radiosensitiwiteit van die TP53 mutante MeWo en 4451 sellyne verhoog, en stralingstoksisiteits verhogingsfaktore van tot 14.5 genereer. Hierdie effek gaan gepaard met die vertraging van DNS herstel in die MeWo melanoom, maar nie in die 4451 plaveisel-sel karsinoom sellyn nie. Die meting van apoptose toon geen noemenswaardige verhoging van stralings-geïnduseerde apoptose in MeWo melanoom óf in 4451 plaveisel-sel karsinoom selle nie. Jurkat J5 T-limfosiete toon egter wel 'n verhoging in apoptose wanneer bestraling in die teenwoordigheid van pentoxifylline gedoen word. In HeLa servikale karsinoom selle affekteer pentoxifylline die uitdrukking van die twee komponente van die mitose promoverings faktor (MPF), naamlik siklien B1 en

$p34^{cdc2}$, en restoreer die siklien B1/$p34^{cdc2}$ verhoudings vinnig na normale vlakke. Ontleding van die siklien B1 uitdrukking in heel selle en in geïsoleerde selkerne toon verder dat die middel ook die sub-sellulêre ligging van die MPF affekteer.

Die gevolgtrekking word gemaak dat G2 blok verwydering nie die enigste meganisme is waardeur pentoxifylline radiosensitiwiteit verhoog nie, maar dat die vertraging van DNS herstel in sommige seltipes 'n rol speel. Alhoewel pentoxifylline apoptose verhoog in T-limfosiete, speel dit nie 'n rol in die verhoogde radiotoksisiteit wat waargeneem is in die TP53 mutante melanoom en plaveisel-sel karsinoom sellyne nie. Verwydering van die G2 blok deur pentoxifylline, wat selle meer sensitief kan maak vir 'n tweede stralings- of chemoterapie aanslag, behels die modulasie van siklien B1 en $p34^{cdc2}$ vlakke en die sub-sellulêre ligging van die MPF. Hierdie resultate is van uiterste belang vir die kliniese aanwending van pentoxifylline as 'n dosis-modifiseerder in kankerterapie.

Opgedra aan my ouers, Kobus en Elma Theron, my suster en twee broers en hulle families - juwele in my lewe wat met emmersvol liefde, geloof en ondersteuning my konstante inspirasie is.

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LIST OF ABBREVIATIONS

TNF	tumour necrosis factor
cdk	cyclin dependent kinases
MPF	mitosis promoting factor
dsb	double strand break
ssb	single strand break
DNA-PK	DNA-dependent protein kinase
PARP	poly- (ADP-ribose) polymerase
SAPK/JNK	stress-activated protein kinase/ Jun kinase
MAPK/ERK	mitogen-activated protein kinase/ extra-cellular signal regulated
SCC	squamous cell carcinoma
FBS	foetal bovine serum
SSD	source-to-sample distance
CFGE	constant field gel electrophoresis
PBS	phosphate-buffered saline
FITC	fluorescein isothiocyanate
PI	propidium iodide
SD/SEM	standard deviation/ standard error margins
REF	radiotoxicity enhancement factor
RIF	repair inhibition factor
AUC	area under the curve

CHAPTER 1

General Background

1.1 Radiotherapy and radiosensitising drugs in cancer treatment

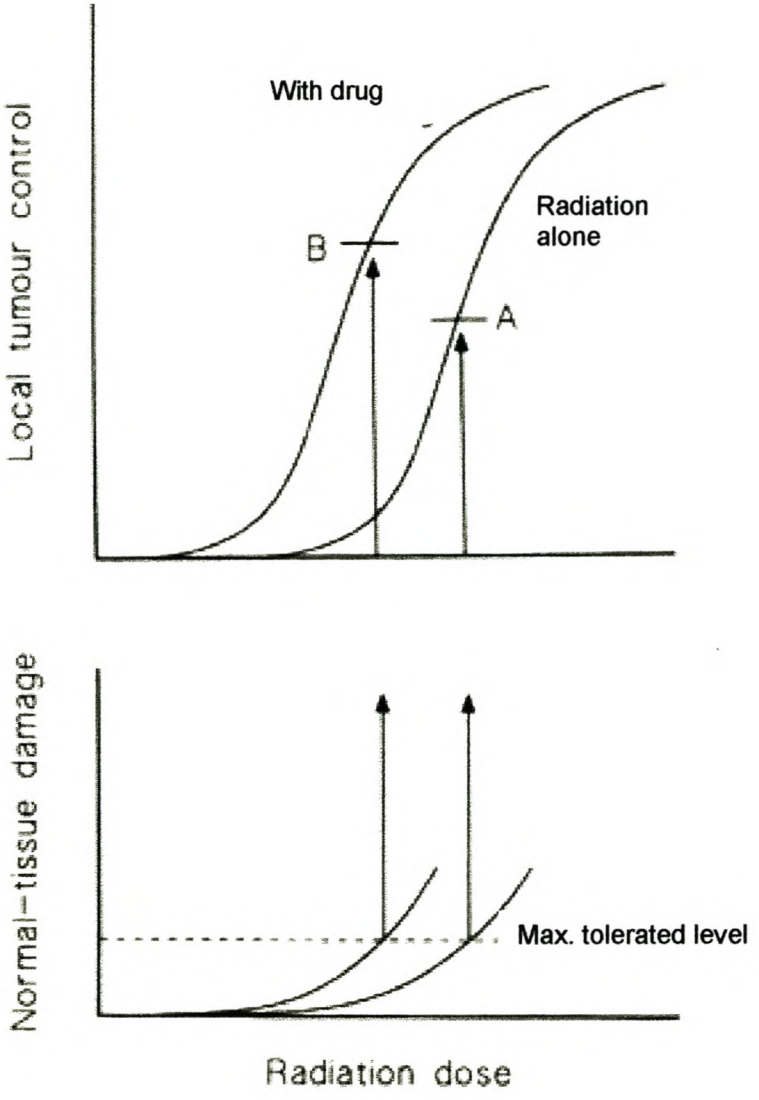
Radiation and surgery are currently the most effective treatment modalities in cancer therapy. Nearly 60% of all tumours receive radiotherapy and radiotherapy in combination with surgery remains the cornerstone for the long-term control of most tumours (Steel, 1993). Radiotherapy often achieves excellent tumour control with good cosmetic results in tumours of the head and neck, cervix, bladder, prostate and skin (Bartelink, 1988). In addition to the curative role, radiation is also used for palliation in a variety of malignancies to reduce pain and increase survival time (Ashbey, 1991). However, radioresistance and late effects remain major problems in the clinic (Steel, 1991; Merkle, 1987; Turesson 1989; Dunne-Daly, 1995). Thus considerable efforts are being devoted to the improvement of radiotherapy.

The possibility of improving tumour control by a change in treatment strategy must always be accompanied by a consideration of the effects on tumour response and damage to the surrounding normal tissue. In curative cancer therapy, the so-called therapeutic index is of critical importance (Steel, 1988). This is defined as the tumour response for a fixed level of normal-tissue damage and carries the notion of a "cost-benefit" analysis (Steel, 1993). A simplified example of how this can be attained by combining a cytotoxic drug

with radiotherapy is given in Figure 1. These drugs are called radiosensitisers, and consist of pharmacological agents which increase radiotoxicity when administered in conjunction with radiation (Adams, 1972). Many radiosensitisers have been discovered over the years, but most offer no practical gain in clinical radiotherapy because the toxicity difference between tumour and normal tissues, also called therapeutic index, is too low. Application of this concept in the clinic is not straightforward, and the clinical evaluation of potential radiosensitisers is difficult. The search for effective radiosensitisers which enhance tumour cell death and spare normal tissue thus continues.

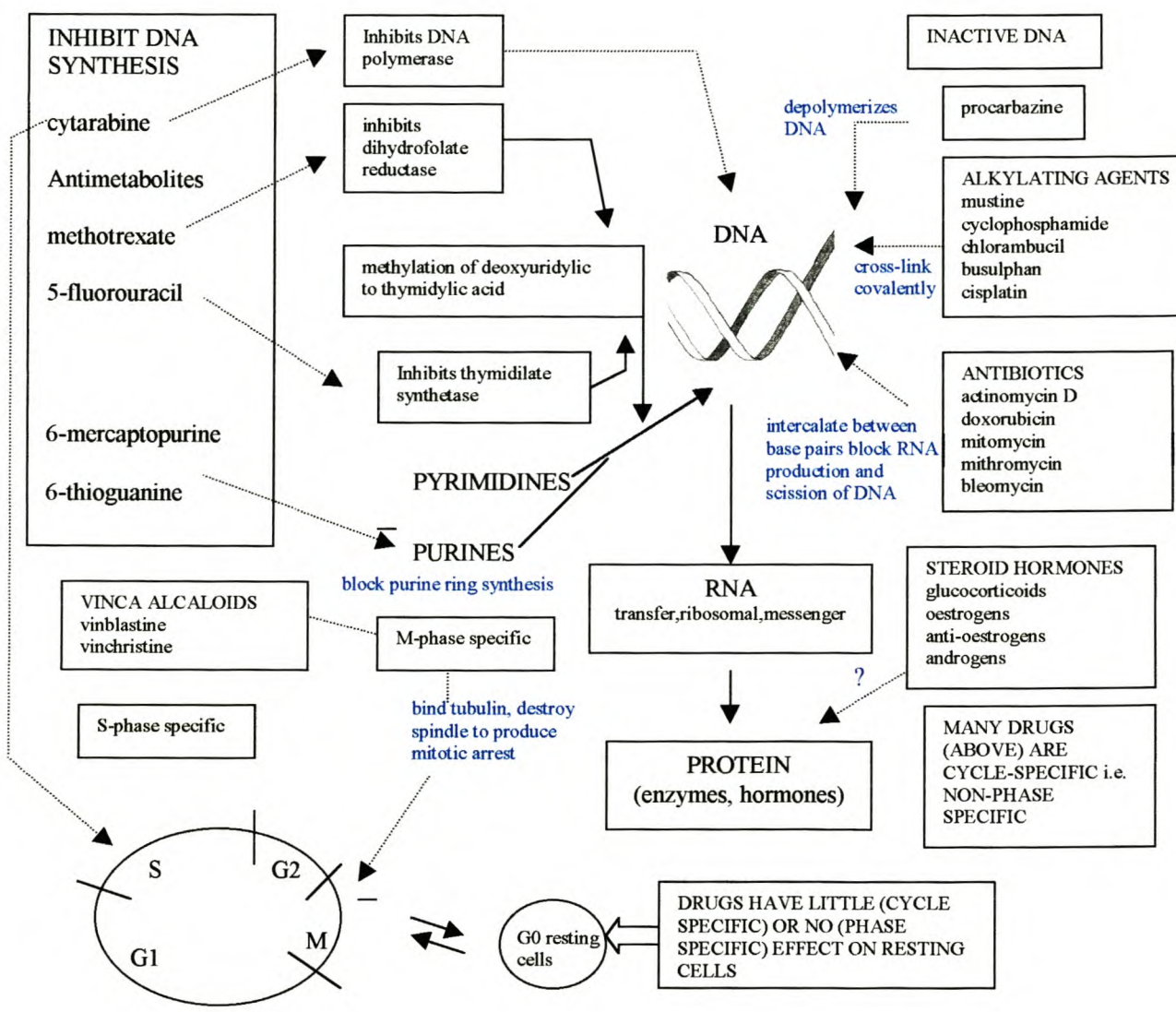
Radiosensitisers currently in use include hypoxic sensitisers such as metronidazole, halogenated pyrimidines such as BrdU and IrdU, DNA synthesis inhibitors e.g. fluorouracil and methotrexate, alkylating agents e.g. cyclophosphamide and antibiotics such as doxorubicin, which intercalate between DNA strands and block RNA production (Nias, 1990). Some of these drugs and their site of action are shown in Figure 2. When the all important criterion of a differential effect between tumour and normal cells are applied, only the halogenated pyrimidines and the hypoxic cell sensitisers have found practical use in clinical radiotherapy on the basis of the faster cycling of tumour cells, and the preferential sensitisation of oxygen deprived tumour cells, respectively (Spiro et al, 1985; Kinsella 1992, McGinn and Kinsella, 1993). Effective dose levels are often associated with high toxicity and the use of these drugs remains problematic (Fowler and Kinsella 1996).

Figure 1



The procedure by which an improvement in therapeutic index might be identified, as a result of combining radiotherapy and a cytotoxic drug. The maximum tolerated level of normal tissue, indicated in the lower diagram, remains the same. However, tumour control with the drug (B) improves as compared to that with irradiation alone (A), as shown in the upper diagram (From Steel. 1993)

Figure 2



Drugs currently used in cancer treatment and their cellular sites of action (Adapted from Nias, 1990).

The rapidly increasing knowledge of cell and molecular biology now offers better insight into how cells respond to radiation and this has lead to new concepts of improving radiotherapy with drugs (Maity et al, 1997). The aim is to achieve a favourable therapeutic index at non-toxic dose levels. Genes and transcription pathways activated by radiation are now being defined. The mechanisms involved in radiation-induced DNA repair and cell cycle delays are also being elucidated. The role of the tumour suppressor gene TP53 in cell cycle delay and apoptosis is a particularly active field of study. The formation and abrogation of radiation-induced cell cycle blocks, and drug-induced modulation of radiation-induced apoptosis and DNA repair offer exciting new avenues for the improvement of cancer therapy. Agents which influence damage response mechanisms of tumour cells are now the focus of oncological research world wide.

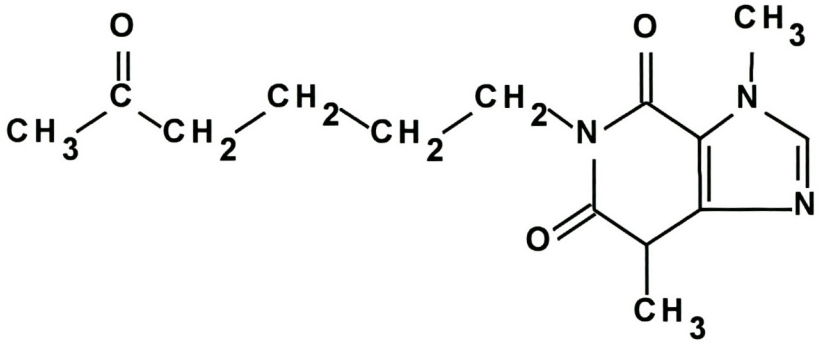
1.2 Pentoxifylline

1.2.1 Chemical structure, toxicity and pharmacology

The chemical structures of pentoxifylline [1-(5-oxohexyl)-3,7-dimethylxanthine] and caffeine [1,3,7-trimethylxanthine] are closely related and are given in Figure 3. Toxicity studies using four representative tumour cell lines, show TD_{50} (toxic dose causing 50% cell death) values of 3.17 - 6.0 mM and 2.24 - 3.92 mM for pentoxifylline and caffeine respectively. This indicates that pentoxifylline is less toxic than caffeine in cell culture by a factor of 0.5, and hence also more suitable for human administration. Throughout this study, pentoxifylline was used at a sub-toxic dose of 2 mM.

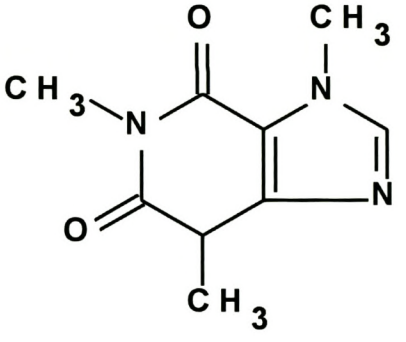
Figure 3

Pentoxifylline



MW 278.31

Caffeine



MW 194.19

Chemical structures and molecular weights of the two closely related methylxanthines, pentoxifylline and caffeine.

In the human body, absorption of pentoxifylline from the gastro-intestinal tract following oral administration is rapid and almost complete (Smith et al, 1986; Bryce et al, 1989). Peak plasma levels are attained within 2-4 hours. Metabolism occurs in both erythrocytes and the liver where pentoxifylline is metabolised to Metabolite I [1-(5-hydroxyhexyl)-3,7-dimethylxanthine] and Metabolite V [1-(3-carboxypropyl-3,7-dimethylxanthine] respectively (Hawkins and Ribeiro, 1987; Ward and Clissold, 1987, Honess et al, 1993). Plasma levels of these metabolites are 5 to 8 times higher than that of pentoxifylline. The plasma half lives of pentoxifylline and its metabolites are 0.4-0.8 h and 1.6-1.8 h respectively. Excretion is primarily renal. The pharmacokinetics of pentoxifylline in cell culture is not known.

1.2.2 Current clinical applications

Pentoxifylline is used clinically for a wide variety of disorders. These include: intermittent claudication (Aviado and Porter, 1989), chronic peripheral heart and cerebrovascular disease (Ward and Clissold, 1987; Windmeier and Gressner, 1997), and the improvement of sperm motility (Shen et al, 1991). The efficacy of pentoxifylline is based on the following: inhibition of phosphodiesterase, increase of cAMP activity, reduction of plasma fibrogen concentrations, and improved erythrocyte flexibility, blood viscosity and blood flow.

Pentoxifylline also inhibits the production of tumour necrosis factor alpha (TNF-α), a naturally occuring cytokine with many functions in the human

immune system (Tracey and Cerami, 1994; Schendene et al, 1992). TNF-α activates CD44, a cell adhesion receptor which facilitates the attachment of white cells to the endothelium in the inflammatory immune response (Tracey and Cerami, 1994; Weiss et al, 1995). Its role in the inhibition of the synthesis and action of several other cytokines like TNF-γ, IL-1, IL-6 and GM-CSF in CD8+ cytotoxic lymphocytes has more recently been demonstrated (Lundblad et al, 1995; Heinkelein et al, 1995). Pentoxifylline reduces the replication of HIV-1 in vitro in acutely infected human T-cells (Fazely et al, 1991). This is also attributed to its reduction of cellular TNF levels. It is therefore not surprising that pentoxifylline is effective in the treatment of inflammatory diseases, septic (endotoxic) shock and infection, thrombosis, ulcers and leprosy (Lundblad et al, 1995). Pentoxifylline is also applied in patients for the treatment of HIV infection, and in organ transplantation as an anti-inflammatory agent (Eugene-Jolchine and Milpied, 1995).

Pentoxifylline clearly has a multi-functional pharmacological profile and operates at various levels. It is therefore not surprising that the full spectrum of pentoxifylline action has not been elucidated and that more effects of the drug are being discovered and investigated. The use of pentoxifylline in cancer therapy as a radio- and chemosensitiser also is a new area of research.

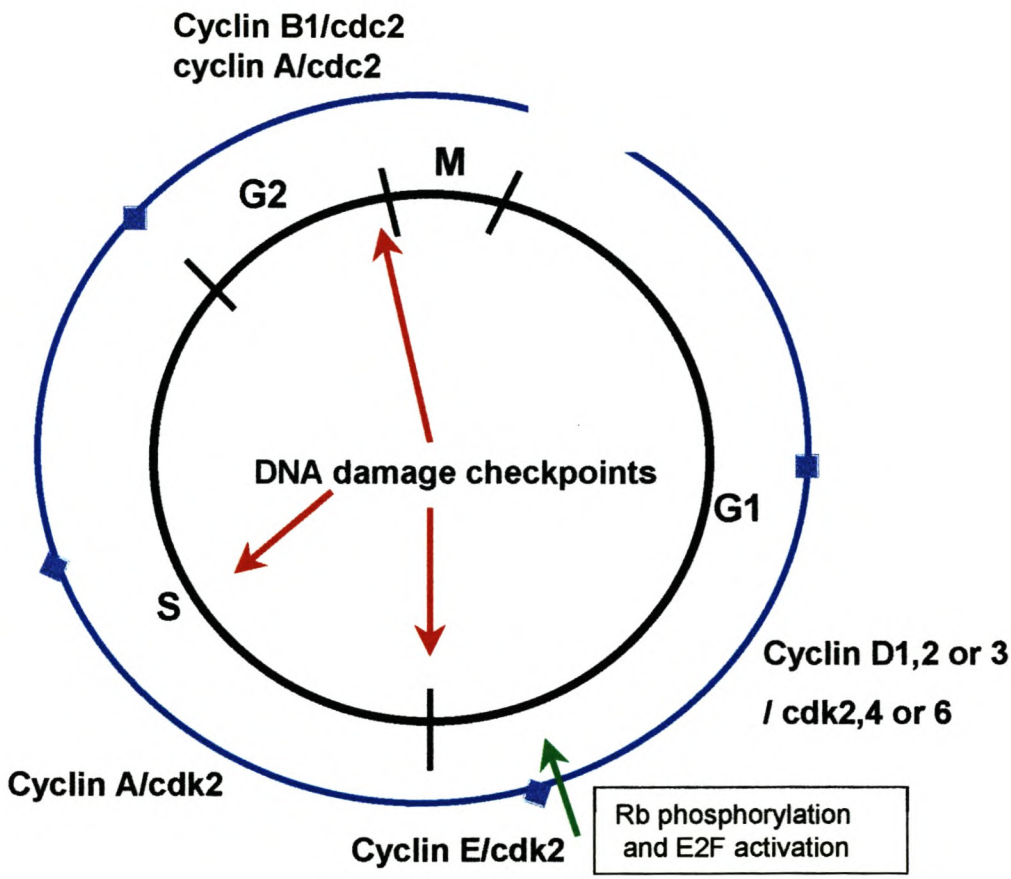
1.3 Potential molecular targets for manipulating the radiation response

1.3.1 Cell cycle checkpoints

Irradiation of cells causes a transient division delay which may include a G1 arrest, an S-phase delay, or a G2 arrest (Figure 4). It is attractive to speculate that cell cycle delays may play an important role in cell survival following DNA damage and there is currently a great interest in understanding the controlling mechanisms. The movement of cells through the cell cycle, and the induction of cell cycle blocks, e.g. by irradiation, are controlled by cyclin proteins and enzymes called cyclin dependent kinases (cdk's) (Maclachlan et al, 1995; Dirks and Rutka, 1997). Specific cyclins act with specific cdk's at different points in the cell cycle as shown in Figure 4. For example, the cyclin B1/p34^{cdc2} and the cyclin E/cdk 2 complexes are crucial for the G2/M and G1/S transitions, respectively (Gong et al, 1994). There is also a third class of proteins involved, namely the cdk inhibitors, which can regulate the cell cycle by binding to and inactivating cdk's. Several cdk inhibitors have been identified, of which p21(*waf/cip*), which is induced via TP53, is a very important example (Harper et al, 1995; Gartel et al, 1996).

While the molecular events resulting in the formation of the G1 block are relatively well established and involve the TP53 pathway (Kastan et al, 1991), very little is known about the signalling initiated by DNA damage which gives rise to a G2 delay. The G2 block occurs as a result of many different types of DNA damage regardless of TP53 status (Hwang and Muschel, 1998). Some

Figure 4

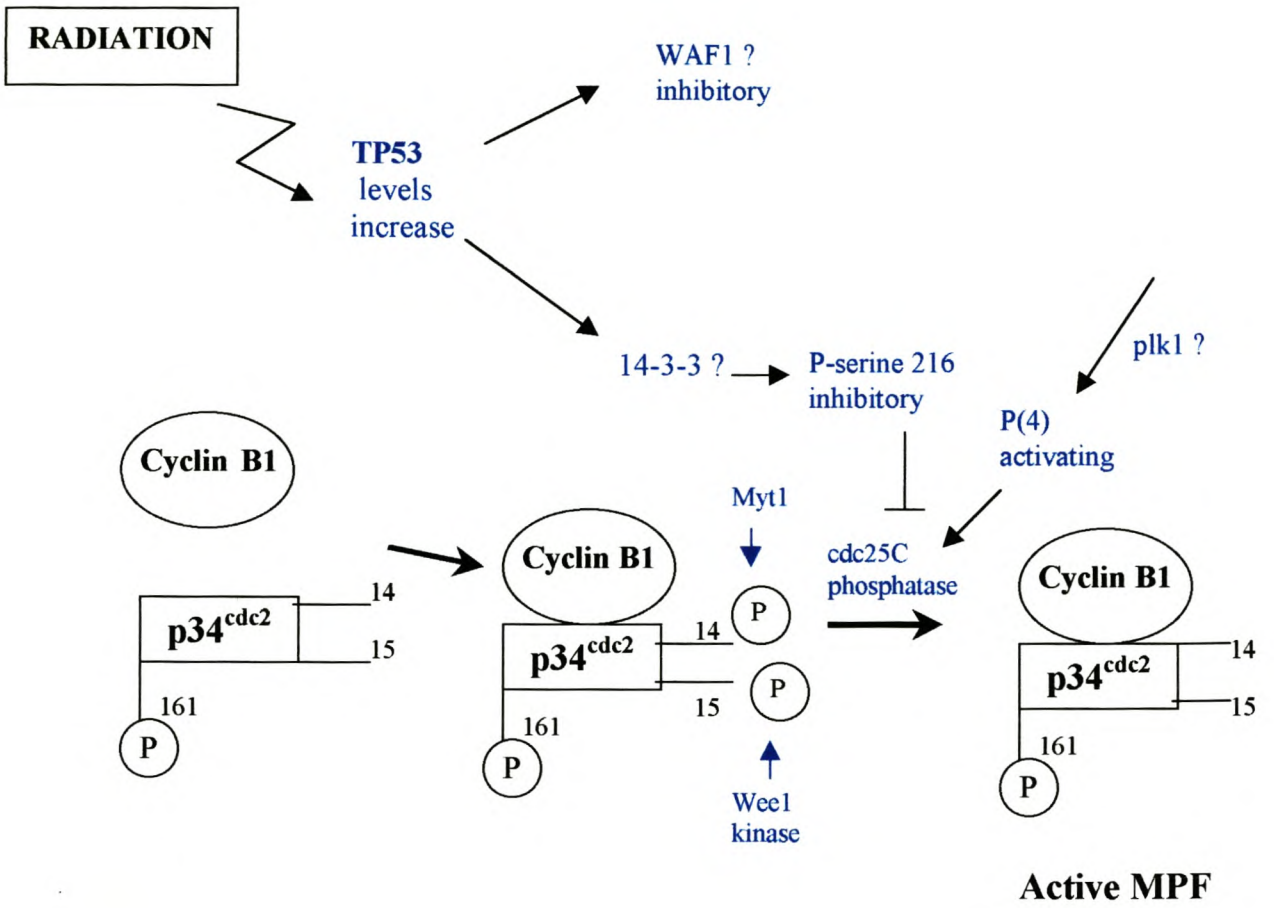


Molecular regulation of the cell cycle. Different cyclins such as A, B1, D1, D2, D3 and E complex with specific cyclin dependent kinases (cdk's) during specific phases of the cell cycle. DNA-damaging agents, including ionising radiation, can arrest cells in the G1, S and G2 phases of the cell cycle (Adapted from Maity et al, 1997).

evidence does exist for a role of TP53-activities in the G2 block (Winters et al, 1998) and it is also possible that other TP53 independent mechanisms are involved. It has now been established that the formation of an active mitosis promoting factor (MPF), consisting of cyclin B1 and its cdk, $p34^{cdc2}$, is essential for the transition of cells from G2 into mitosis (Murray et al, 1989, Broek et al, 1991). The activity of this complex is regulated by two independent mechanisms: firstly by the synthesis of cyclin B1 and secondly by a series of phosphorylations on $p34^{cdc2}$ (also known as cdk1). Formation of a cyclin B1/$p34^{cdc2}$ complex is required for $p34^{cdc2}$ histone H1 activity. For this purpose, there is a ten-fold rise in cyclin B1 expression in the G2 cell cycle phase, and a rapid elimination of cyclin B1 by cell cycle-specific ubiquitination during the final stages of mitosis (Murray 1992). The $p34^{cdc2}$ molecule is phosphorylated at threonine 161. When cyclin B1 binding occurs, phosphorylation at threonine 14 and tyrosine 15, mediated by Myt1 and Wee1 kinases respectively, blocks activity of the complex. Enzymatic activity of the complex is triggered through the activation of cdc25C, a dual-specific phosphatase, which removes both phosphates at Thr14 and Tyr15 (Murray 1992). Activation is further regulated by the sub-cellular location of the MPF complex (Li et al, 1997). The biochemical reactions involved in the activation of the MPF are represented in Figure 5.

There is currently a great deal of interest to increase radiation-induced tumour cell death by manipulating cell cycle checkpoints, especially the G2/M checkpoint. Agents which inhibit the G2 cell cycle delay are caffeine and pentoxifylline, which belong to the family of methylxanthines (Russell et al,

Figure 5



The molecular control of mitotic entry. The expression and binding of cyclin B1 and $p34^{cdc2}$ leads to the formation of an inactive mitosis promoting complex (MPF). This complex is activated by various phosphorylation and dephosphorylation steps as shown. Pi refers to a phosphate group, and the numbers indicate the amino acids on $p34^{cdc2}$ involved in the phosphorylation reaction. Cyclin B1 is rapidly eliminated following mitotic entry (adapted from Hwang and Muschel, 1998).

1996). Since many tumour cells have a mutated TP53 gene and consequently cannot arrest at the G1 checkpoint, a possibility exists to target cancer cells (Russell et al, 1996; Hwang and Muschel, 1998). In normal TP53 wild type cells, the G1 checkpoint would be intact. Irradiation of normal tissues thus would induce a G1 arrest to permit repair and cellular recovery. This could have far-reaching clinical implications as it facilitates the sparing of normal tissue. It has been argued that selective activation of checkpoints may be the underlying reason why some drugs are effective at all in tumour therapy (Waldman et al, 1997). The detailed mechanisms of action of such drugs however remains to be elucidated.

1.3.2 DNA repair

Ionising radiation induces various types of DNA damage, including double strand breaks (dsb), single strand breaks (ssb), base damage and DNA-protein crosslinks (Arrand and Michael, 1992). DNA dsb induction and repair, and the fidelity of DNA repair, seem to be critical factors which determine radiosensitivity in human tumour cell lines (Nunez et al, 1996). Because of this, much effort has been made in trying to understand the molecular mechanisms leading to the repair of radiation-induced DNA damage. This has lead to the identification of the XRCC5 gene, which encodes the 80-kD sub-unit of Ku (Weaver, 1995). Along with another protein called p350, this forms the DNA-dependent protein kinase (DNA-PK) complex thought to be involved in DNA dsb repair (Anderson, 1993). Ku targets the damaged DNA

by attaching to the DNA termini, whereas p350 is the catalytic sub-unit containing kinase activity. Enzymes which are activated by DNA strand breaks, e.g poly(ADP-ribose) Polymerase (PARP) and Mdm-2, have been implicated as possible role-players in the sensing of DNA damage (Burkle et al, 1992; Momand and Zambetti, 1997). PARP is thought to indirectly activate TP53, p21 and DNA-PK (Le Ruhn et al, 1998).

Further work in determining the specific proteins involved in detecting and repairing radiation induced DNA damage may have potential clinical applications. One could target Ku, p350, PARP, or another protein yet to be discovered which acts downstream, thus rendering cells more radiosensitive by preventing the recognition of DNA damage. New drugs which are capable of inactivating these repair proteins and hence inhibit dsb repair are being sought. In this strategy, the preferential targeting of tumour cells over normal tissues remains an ever important problem.

1.3.3 Programmed cell death (Apoptosis)

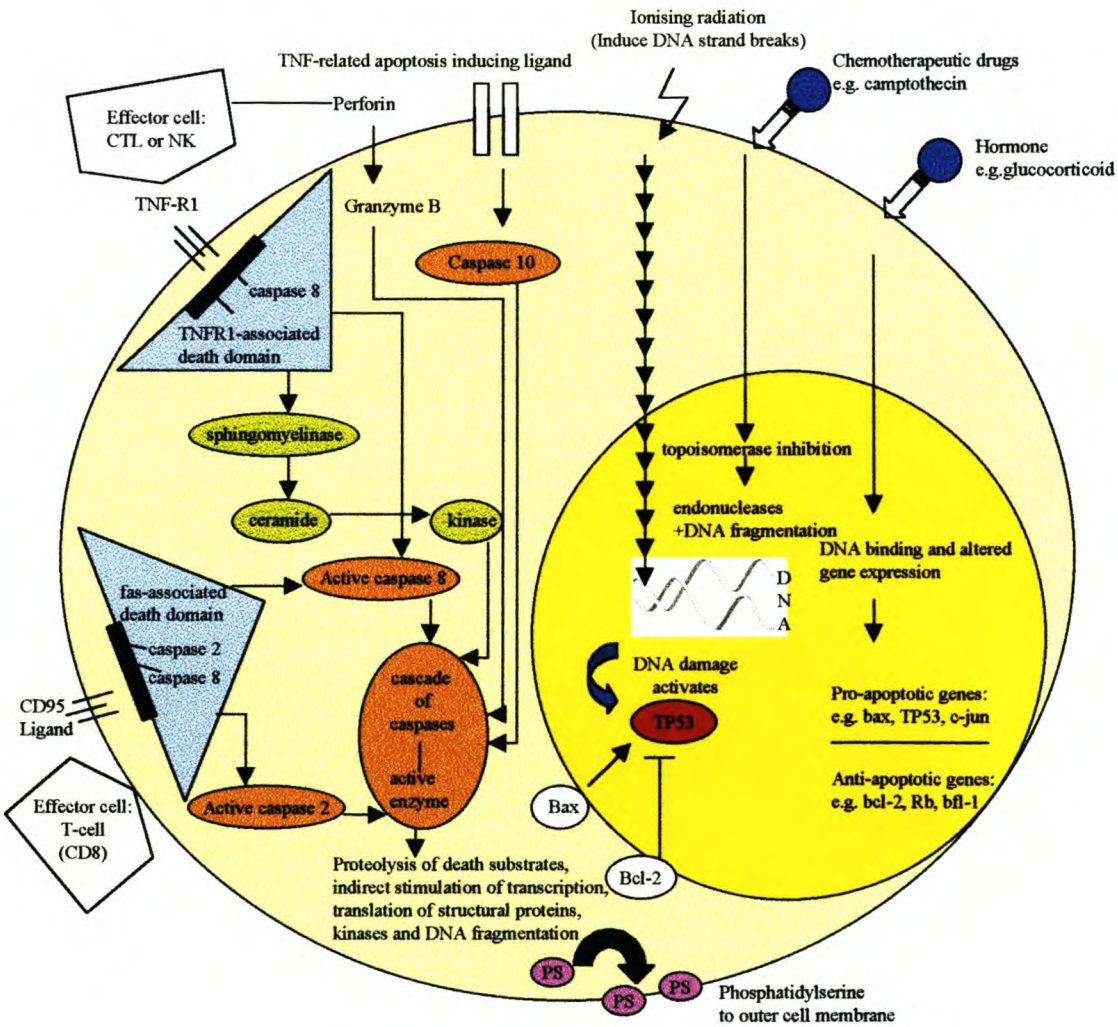
Apoptosis is a distinct form of cell death which is essential for normal development and tissue homeostasis (Wyllie et al, 1980). Apoptosis has several distinctive features which distinguish it from necrosis (Searle et al, 1982). These are chromatin condensation, cell shrinkage, membrane blebbing and DNA degradation at internucleosomal linkages (Wyllie et al, 1981). This results in the characteristic DNA repeat ladder which becomes apparent in DNA electrophoretic gels. Apoptotic cells are usually scattered

throughout tissue rather than occurring in a large block of contiguous cells as found in necrosis (Searle et al, 1982). In contrast to necrosis, inflammation is absent in apoptosis. Unlike necrosis, apoptosis arises from activation of a specific gene program. It has been described as death from the inside out. In the cell, the apoptosis is initiated by a cascade of highly specialised non-random events of protein and DNA-cleavage. Protein cleavage results from activation of a family of cysteine proteases or "caspases", while DNA is cleaved by endonucleases (Peter and Krammer, 1998). The activation of these processes can only start if the cell has sensed damage and signalled this to the appropriate cell machinery. A number of tumour-suppressor genes such as TP53 and oncogenes such as *myc* and *bcl-2* have been implicated in the recognition and signalling process (Konopleva et al, 1999). Intricate pathways leading to apoptotic death have been identified, e.g. the ceramide pathway (Venable et al, 1995; Mizushima et al, 1996), the stress-activated protein/jun kinase (SAPK/JNK) cascade (Kasibhatla et al, 1998), and the mitogen-activated protein kinase/extracellular-signal regulated (MAPK/ERK) pathway (Blagosklonny, 1998). Several other molecules like bcl-2, BAX and other pro- and anti-apoptotic molecules determine the survival "threshold" of a certain cell type (Reed, 1995). A simplified diagram describing the basic apoptotic pathways is given in Figure 6.

There is some evidence that apoptosis may play a role in radiosensitivity (Zhivotovsky et al, 1999). It has been noted that certain cells in normal mammalian tissues which are susceptible to natural apoptosis e.g. haematopoetic tissues, are particularly radiosensitive (West 1995). Since

Figure 6

Apoptotic pathways

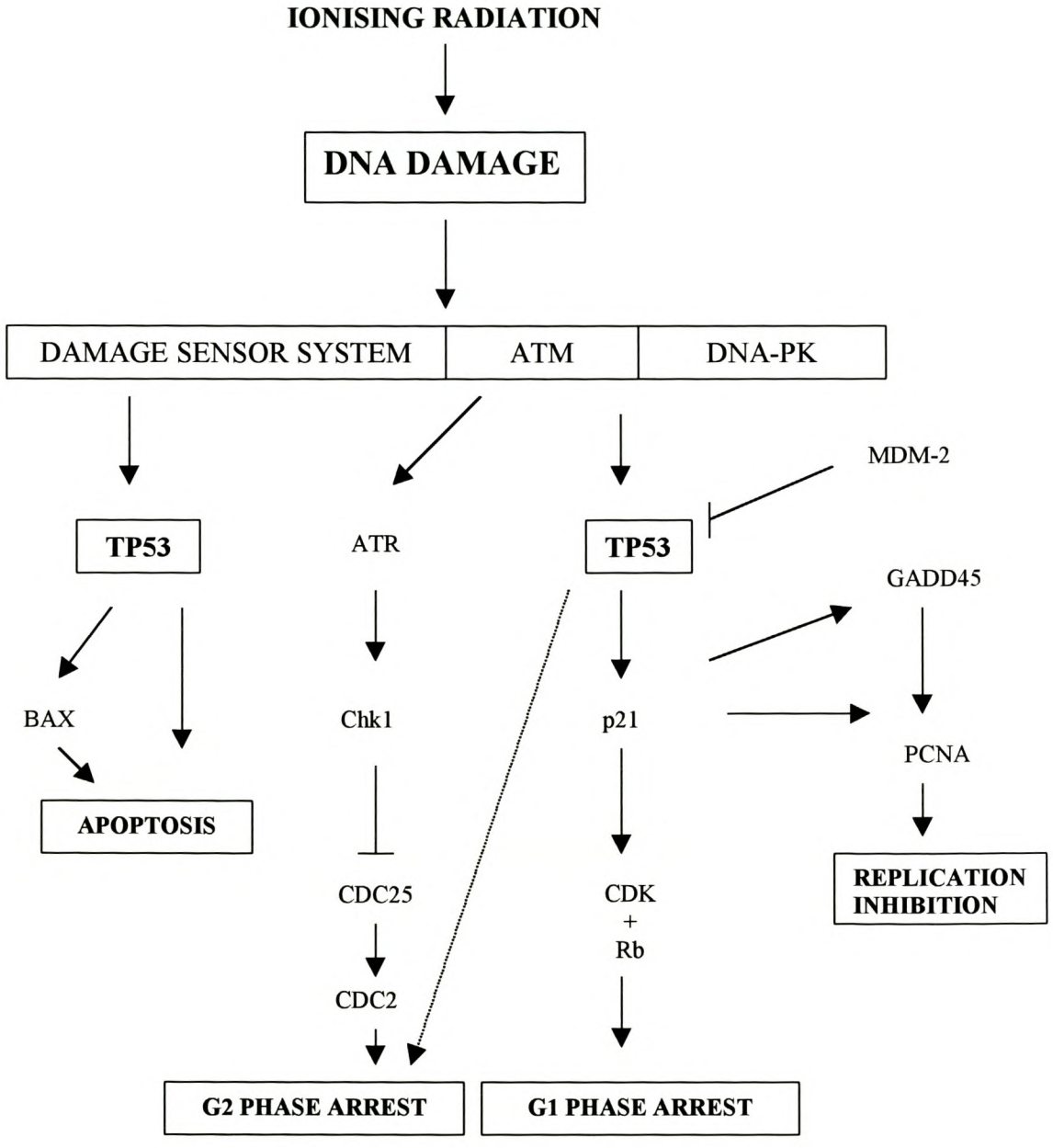


apoptosis appears to be a natural process inherent in cells and is inducible by specific stimuli, understanding of its regulation has considerable application in tumour biology. The hope is to selectively activate apoptotic pathways in tumour cells but not in normal tissue, thereby achieving effective tumour cell death while minimising normal tissue complications. This goal has not yet been accomplished. Various kinase and phosphatase inhibitors as well as growth factors and cytokines have been shown to alter the apoptotic response of cells *in vitro*, and it is possible that the same approach could someday be used clinically. Since TP53 is an important regulator of apoptosis which is mutated in 60% of all tumours (Lee and Bernstein, 1995), restoring wild type function may reverse tumour resistance to apoptosis. However, this is no trivial task. An alternative approach would be to prevent apoptosis in normal tissues and thereby reduce late radiation side-effects. Cytokines which inhibit apoptosis, such as basic fibroblast growth factor (bFGF), have been administered to mice immediately before a lethal dose of irradiation to the whole lung. This prevented the development of radiation pneumonitis (Fuks et al, 1994). Although these results are preliminary in nature, they are extremely interesting and suggest a way in which the apoptotic response may be manipulated for clinical benefit. Another future approach for selective killing of tumour cells lies in the understanding of the reasons for tumour survival despite substantial genomic damage. If the DNA damage and mutations which induce tumours could be harnessed as a signal for the cell to self-destruct (apoptose), the survival threshold for cancer cells would be lower, while normal cells lacking the DNA damage-signal may be spared (Hickman, 1998).

1.4 Role of TP53 in damage responses

DNA damage initiates a signal transduction pathway ultimately resulting in increased levels of TP53 protein. Through sequence-specific DNA binding, TP53 can modulate transcription of a variety of target genes following DNA damage (Enoch and Norbury, 1995). Wild type TP53 plays a role in the induction of a G1 arrest, DNA repair and apoptosis following DNA damage (Lee and Bernstein, 1995). The TP53 mediated G1 arrest results from the transactivation of several cellular molecules, in particular p21 and gadd45 (O'Connor, 1997; Hwang and Muschel, 1998). p21 inhibits CDK/cyclin activity which indirectly suppresses the expression of the transcription factor E2F, required for the expression of S-phase-specific genes. This causes a G1 arrest (Gartel et al, 1996). The high levels of p21 and gadd45 increases repair mediated by proliferating cell nuclear antigen (PCNA) and DNA excision (Prosperi, 1997). The mdm-2 gene is positively regulated by TP53, and inhibits TP53 function (Wetzel and Berberich, 1998). TP53 activation also leads to the synthesis of cyclin G, which is thought to be involved in G2 arrest (Shimitzu et al, 1998). TP53 induces apoptosis through pathways which are not yet fully understood, but is thought to involve activation of the ICE proteases and the BAX gene which acts as a positive regulator (Enoch and Norbury, 1995). Bcl-2 inhibits apoptosis (Hickman, 1998). The rise in TP53 levels also brings about transcription of a number of other genes, summarised in Figure 7.

Figure 7



A diagram of TP-53 signalling in the irradiated mammalian cell (From Szumiel, 1998). TP53 plays a key role in cell cycle blocks, DNA repair and apoptosis following DNA damage.

Since the TP53 protein is involved in all known damage responses and might also play a role in G2 arrest (this is still ill-defined), the cellular TP53 status plays a crucial role in cell survival. The downstream effects of TP53 have lead to contradictory hypotheses regarding the influence of TP53 status on radiosensitivity (Bristow et al, 1996). For example, TP53 wild type could increase apoptosis leading to increased cell death, while it could also induce a G1 or/and G2 arrest and increase DNA repair leading to increased cell survival. Many contradicting reports have been published in this regard (for review see Morgan and Kastan, 1997), which may be explained by the fact that TP53 dependent apoptosis and G1 arrest followed by DNA repair and cellular recovery appears to be controlled by different donwstream pathways and that the activity of these pathways is cell type specific. The fact that a large amount of tumour types exhibit mutated TP53 genes distinguishes them from normal cells. It may be possible to exploit this to preferetially kill tumour cells. One example is the preferential chemo- and radiosensitisation of TP53 mutant tumour cell lines by radiosensitising agents which abrogates the G2 cell cycle block.

1.5 Thesis objective

The search for radiosensitisers which can enhance radiotoxicity at non-toxic dose levels, is currently a focal point in radiobiological research. It is of critical importance that these drugs can distinguish between normal and tumour cells. This would lead to improved local tumour control, while late radiation effects would be reduced through sparing of the surrounding normal tissue. Pentoxifylline, a methylxanthine closely related to caffeine, has been shown to act as a radiosensitiser both *in vitro* and *in vivo* at sub-toxic doses (Lau and Pardee, 1982; Vernimmen et al, 1984; Kim et al, 1993). However, its mechanism of action have not yet been elucidated.

This study addresses the following important questions:

1. Can the enhancement of radiotoxicity by pentoxifylline be solely attributed to the early induction of mitosis through G2 block abrogation?
2. Does pentoxifylline influence the repair of radiation-induced DNA damage?
3. Does the drug enhance radiotoxicity by increasing radiation-induced apoptosis?
4. Which molecular events are involved in the abrogation of radiation-induced G2 cell cycle blocks by pentoxifylline?
5. Does the drug have the potential to discriminate between normal and tumour cells?

This information will contribute greatly to a better understanding of the radiosensitising effects of pentoxifylline. Specific tumour types likely to benefit

from a radiotherapy/pentoxifylline combination may also be identified. This study thus could have far-reaching implications for the improvement of cancer therapy.

Chapter 2

Introduction

The G1/S and G2/M transitions have been recognised as important checkpoints in the response of cells to DNA damage. It is commonly thought that delays in the G1 and G2 phases of the cell cycle, which typically reaches a maximum at 12-24 hours post-irradiation, are protective mechanisms which prevent propagation of defective DNA (for review see Maity et al, 1997). The G1 cell cycle checkpoint is dependent on the activation of wild type TP53 and is absent in TP53 mutant cells (Kastan et al, 1991). Although a recent study on human ovarian cells shows that wild type TP53 can inhibit G2/M transition by decreasing intracellular cyclin B1 levels (Innocente et al, 1999), the G2/M checkpoint is generally activated in response to DNA damage irrespective of TP53 status (Kastan et al, 1991). G2 delays are induced by exposure to a variety of DNA damaging agents including ionising radiation (Weinert *et al*, 1988), DNA alkylators (Konopa *et al*, 1988) and topoisomerase inhibitors (Barlogie *et al*, 1976).

The relationship between G2 arrest and cell survival is not clearly established. Prolongation of the G2 arrest has been correlated with increased radioresistance in HeLa cervical carcinoma and MeWo melanoma cells (Tamamoto et al, 1999). Agents which reduce the G2 delay sensitise cells to ionising irradiation (Bernhard et al, 1996; Kim et al, 1992). It has also been shown that the irradiation-induced G2 block in human lymphocytes is

associated with unrejoined chromosome fragments (Durante et al, 1999). Other authors have failed to demonstrate a correlation between the duration of the G2 block and radiosensitivity, although the frequency of unrejoined chromosome breaks increased with attenuation of the G2 block (Schwartz et al, 1996).

Methylxanthines like caffeine sensitise human tumour cells to ionising irradiation when the TP53 gene is disrupted (Bracey et al, 1997). The clinical use of caffeine is limited, however, because of neurological and cardiac toxicities (Dobmeyer et al, 1983; Curatolo and Robertson, 1983). More recent studies have evaluated other derivatives, which are more potent and less toxic (Russell et al, 1996). Pentoxifylline has been shown to sensitise human and rodent cells to ionising irradiation (Vernimmen et al, 1994; Kim et al, 1993) and chemotherapeutic agents (Fingert et al, 1988; Li et al, 1999). Its toxicity is lower than that of caffeine (Fingert et al, 1986). Pentoxifylline rapidly abrogates the irradiation induced G2 cell cycle checkpoint (Russell et al, 1996).

The increased cell death observed when pentoxifylline is combined with irradiation has been attributed to the ability of the drug to induce early mitosis (Li et al, 1998), which shortens the time for DNA repair (O'Connor, 1997, Durante et al, 1999). However, the evidence for this hypothesis is inconclusive, because DNA repair is completed within 12-16 hours after irradiation and G2 blocks in the absence of pentoxifylline generally last up to 40 hrs (Binder et al, 2000). Therefore, earlier damage response processes

like DNA repair and apoptosis may also play a role in the radiosensitisation effects of pentoxifylline.

The influence of caffeine and pentoxifylline on the induction of DNA repair is still ambiguous. Several authors report caffeine to inhibit DNA repair in human cells (Yasutake et al, 1995; Link et al, 1995; Tempel and Von Zallinger, 1997) while others found no effect (Smeets et al, 1994). Split dose experiments on V79 and HeLa cells showed pentoxifylline to inhibit DNA repair (Vernimmen et al, 1994). Data on chinese hamster ovary (CHO) cells have shown that pentoxifylline inhibits the elimination of cyclobutane pyrimidine dimers (CPD's) induced by UV irradiation (Link et al, 1996). If pentoxifylline indeed is an agent which inhibits the repair process, one may expect the number of DNA dsb remaining after the completion of repair to be greater.

Data on the role of caffeine and pentoxifylline in apoptosis induction are also inconclusive. In irradiated HL-60 cells, caffeine has been found to decrease apoptosis by up to 50% (Ning and Knox, 1999). Studies on human bladder cancer cell lines have shown that the drug has no influence on apoptosis (Ribeiro et al, 1999). This is in contrast with findings that G2 block abrogation by caffeine leads to increased apoptosis when combined with ionising irradiation in human colorectal cells (Bracey et al, 1997), human leukaemic cells (Efferth et al, 1995), murine T-lymphoma cells (Palyoor et al, 1995) and HeLa human cervical carcinoma cells (Bernhard et al, 1996). It had been suggested that the induction of apoptosis by caffeine is cell type specific

(Crompton, 1998) and that the cytocidal effects of caffeine are separate from cell cycle modulation (Takagi et al, 1999). Pentoxifylline has been shown to inhibit spontaneous and chemically induced apoptosis in neutrophils and U937 cells (Belloc et al, 1995). The inhibition of UVB induced apoptosis in HaCaT cells has been attributed to suppression of TNF-alpha release (Schwartz et al, 1997). However, in mouse leukaemic L1210 cells, drug-induced apoptosis has been shown to increase when cells were treated in the presence of pentoxifylline (Rauko et al, 1998).

The molecular events involved in the G2 block abrogation effect of pentoxifylline have not yet been elucidated. The entry of G2 cells into mitosis is dependent on the activation of the maturation promoting factor (MPF) which contains a 34 kDa serine threonine cyclin dependent protein kinase, $p34^{cdc2}$ (Steinman et al, 1991; Murray 1992; Norbury and Nurse, 1992). Kinase activity is controlled by the formation of a complex between $p34^{cdc2}$ and the mitotic cyclin B1 protein (Murray *et al*, 1989; Broek *et al*, 1991). Upon cyclin B1 binding, $p34^{cdc2}$ becomes phosphorylated at threonine 161, which promotes affinity for cyclin B1, and at threonine 14 and tyrosine 15 by the Wee1 kinase, which inhibits kinase activity (McGowan and Russel, 1993). The subsequent dephosphorylation of threonine 14 and tyrosine 15 residues by the cdc25 phosphatases activates $p34^{cdc2}$ kinase (Murray, 1992). The activation is also regulated by the subcellular location of the $p34^{cdc2}$ complex (Li *et al*, 1997, Moore et al, 1999). The cyclin B1/$p34^{cdc2}$ complex, which is localized in the cytoplasm during interphase, is transported into the nucleus at the onset of mitosis (Pines and Hunter, 1991) and then phosphorylates

nuclear substrates e.g. the condensin complex (Murray, 1998). It is clear that the formation and activation of the MPF complex is controlled at multiple levels. It is now generally thought that control of entry into mitosis involve the expression of cyclin B1 (Muschel *et al*, 1991, Maity *et al*, 1996) and p34 ^{cdc2} (Lock and Ross, 1990), the activation of the MPF complex by dephosphorylation (McGowan and Russel, 1993) and subcellular translocation (Li *et al*, 1997, Moore et al, 1999). The possibility therefore exists that pentoxifylline could exert its block abrogating effect at any of these control levels.

In order to identify the molecular events which control the formation of the G2 cell cycle delay, numerous attempts have been made to determine the expression of the cyclin B1 proteins in response to DNA damage. Using synchronised cells, it was shown that irradiation suppresses cyclin B1 expression when cells go into the G2 block (Muschel *et al.* 1991, O'Connor *et al.* 1993, Ling *et al.* 1996, Tsao *et al.* 1992; Kao et al, 1993). A study on the effects of caffeine on HeLa cells has shown that this drug elevates cyclin B1 expression while diminishing the cell cycle block (Bernhard, McKenna and Muschel, 1994 a). Studies on asynchronous cells however failed to demonstrate a significant reduction in cyclin B1 expression after irradiation (Cohen-Johnathan *et al.* 1997, Villa *et al.* 1996), whereas one study reported a dose-dependent increase in cyclin B1 expression concominant with the rise in the G2/M fraction in both synchronised and asynchronous cultures (Smeets, Mooren and Begg, 1994).

In order to resolve these discrepancies, we wondered about the need of using synchronous cells and the complications arising from unscheduled expression of cyclins and other cellular proteins as described by Gong, Traganos and Darzynkiewics (1995). The confusing reports on changes in protein expression in asynchronous cells can be attributed to the sharp rise in the numbers of G2 cells during block formation, which obscures the change in the relative proportions of cyclin B1 to G2 cells. To avoid these pitfalls, we have used asynchronously growing cells, and calculated the relationship between G2 levels and cyclin B1 levels, which has not previously been measured.

$P34^{cdc2}$ expression has previously been measured by flow cytometry in polyploid cells (Baroja *et al*, 1996). To our knowledge this aspect has not been investigated flow cytometrically in response to DNA damage and G2 block abrogation. Some authors suggest that the levels of $p34^{cdc2}$ remains constant throughout the cell cycle and also after irradiation (McGowan and Russel, 1993; Lock and Ross, 1990; Lock, 1992). Our flow cytometric results in HeLa cells allowed us to correlate $p34^{cdc2}$ expression directly with cell cycle stage and showed a higher percentage of $p34^{cdc2}$ expression in the G1 and G2 phases, with an increase in $p34^{cdc2}$ expression in G2 during the radiation induced G2 block. As with the measurement of cyclin B1 expression, the $p34^{cdc2}$ expression was measured here by calculating $p34^{cdc2}$/G2 ratios.

Previous studies on the subcellular location of MPF have shown that the cyclin B1/$p34^{cdc2}$ complex is localised in the cytoplasm during interphase and transported into the nucleus at the onset of mitosis (Tassen *et al*; 1994, Pines

and Hunter, 1991). This was confirmed by using a fusion protein between cyclin B1 and green fluorescent protein to trace the movement of MPF in subcellular components (Hagting *et al*, 1998). Cyclin B1 has been reported to appear in the cytoplasm during late S phase, and then moves to the perinuclear region and during G_2 enters the nucleus, where it is concentrated during mitosis (Kakino *et al*, 1996). A unique cytoplasmic pool of cyclin B1 has also been documented for actively dividing cells, which rapidly increases during S-phase and G_2 phase and is then translocated to the nucleus during early prophase where it forms a complex with the nuclear subset of $p34^{cdc2}$ (David–Pfeuty *et al*, 1996). It is now generally thought that the inactive cyclin B1/p34 complex indeed shuttles between the nucleus and cytoplasm in human cells (Pines, 1999).

In this study we have examined the influence of pentoxifylline on radiosensitivity, G2 checkpoint control, DNA repair and apoptosis in four human cell lines of defined TP53 status, namely, the human melanoma cell lines Be11 (TP53 wild type) and MeWo (TP53 mutant) and the human squamous cell carcinoma (SCC) cell lines 4197 (TP53 wild type) and 4451 (TP53 mutant). To assess the possibility of a cell type specific effect on apoptosis, we have also measured the influence of pentoxifylline on radiation-induced apoptosis in the Jurkat J5 human lymphocytic leukemia cell line. Using asynchronous HeLa cells, which produce a strong G2 block and in which cyclin B1 expression is well characterised (Bernhard et al, 1994a), we determined the cyclin B1/G2 and $p34^{cdc2}$/G2 ratios over a timespan of 25 hours after the induction of a G2 cell cycle block and in response to G2 block

abrogation by pentoxifylline. The influence of pentoxifylline on the subcellular location of the MPF complex was assessed by analysing cyclin B1 expression in isolated nuclei and in whole cells after exposure to ionising irradiation and after G2 block abrogation.

The objective of this study was to clarify the modus operandi of pentoxifylline as a dose-modifying drug and to highlight the role of repair and apoptosis for cell types in which the drug increases radiotoxicity. These results also clarify the molecular events which contribute to the formation of the radiation induced G2 cell cycle delay and the mechanism of action of pentoxifylline as a G2 block abrogator.

Chapter 3

Materials and methods

Cell culture

The human melanoma cell lines, Be11 (TP53 wild type), MeWo (TP53 mutant), and the human SCC cell lines, 4197 (TP53 wild type) and 4451 (TP53 mutant), were kindly provided by F. Zölzer and C. Streffer, University of Essen, Germany. The TP53 mutations in the MeWo and 4451 are identical, namely a G-to-A transition in codon 258 of exon 7, which lead to the substitution of glutamine with lysine (Zölzer et al, 1995). Cells were grown in MEM with 20% Foetal Bovine Serum (FBS), 2ml/L 1-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) in 75 cm^2 culture flasks incubated at 37^0C in 95% O_2/ 5% CO_2. Jurkat J5 human lymphocytic leukemia cells were grown in suspension in RPMI medium with 10% FBS, containing penicillin (100U/ml) and streptomycin (100 μg/ml). Asynchronous populations of HeLa cervical carcinoma cells were plated in McCoy's medium supplemented with Penicillin (100 U/ml), Streptomycin (100 ug/ml) and 10% FCS in 75 cm^2 cell culture flasks and cultured at 37^0C in 95% O_2 / 5% CO_2.

Drug toxicity

The cytotoxicity of pentoxifylline in the Be11, MeWo , 4197 and 4451 cell lines over a dose range of 0.5-4 mM was measured using the crystal violet assay. Pentoxifylline was added to cells seeded at a density ranging from 5000-10,000 cells/well, depending on the growth rate of each cell line, in 24-well multiwell plates, and medium changed after 24 hours. Cells were fixed four days later in buffered formalin (pH 7.2) and stained with 0.01% crystal violet. To determine the drug concentration at which the cell survival was reduced to 50% (TD_{50}), the extracted dye was read spectrophotometrically at 590 nm, after overnight extraction with 10% SDS.

Irradiation procedures

For measuring cell survival, the Be11, MeWo, 4197 and 4451 cell lines were irradiated with ^{60}Co-γ doses ranging from 0-10 Gy. For the induction of a G2 arrest, a single dose of 7 Gy ^{60}Co- γ-rays were applied in all cell lines. For this setup the source-to-sample distance (SSD) was 80 cm, the field size 30x30cm and the dose rate approximately 2 Gy/min. For the CFGE repair experiments, the Be11, MeWo, 4197 and 4451 cell lines were irradiated on ice with ^{60}Co- γ-ray doses ranging from 0-100 Gy at a dose rate of approximately 3.2 Gy/min. The SSD for this setup was approximately 6 cm and the field size was 30x35 cm.

Flow cytometric determination of G2 block expression and abrogation

Exponentially growing cells in 25 cm^2 culture flasks were irradiated with a dose of 7 Gy ^{60}Co γ-irradiation. This was followed by harvesting cells at 2-hourly time intervals for up to 40 hours by trypsinisation, centrifugation and fixation in 70% ethanol at -20°C. The DNA content of these cells was measured to determine the time of maximum G2 block expression. Briefly, cells were stained in PBS containing 10 μg/ml propidium iodide solution (PI, Sigma, St. Louis) and 100μg/ml RNase (Boehringer Mannheim) at 37°C for 30 minutes. Analysis was done on a FACScan (Becton Dickinson, San Diego, USA) flow cytometer at 488 nm. Red fluorescence (PI) was collected as a linear signal and recorded as a measure of the total DNA content. Cell doublets were gated out by processing red fluorescence into area and width. Estimates of cells in the different cell cycle stages, obtained by placing markers on DNA histograms, revealed the time of maximum G2 block expression.

Cell survival

Radiosensitisation induced by pentoxifylline (2 mM) added immediately prior to irradiation, and pentoxifylline added at the time of maximum G2 block expression was assessed by clonogenic assays in the Be11, MeWo, 4197 and 4451 cell lines. Cells were seeded into 25 cm^2 culture flasks to yield approximately 100-200 colonies. After cell attachment and drug treatment,

these flasks were irradiated with 0,2,4,6,8 and 10 Gy of ^{60}Co γ-irradiation. In samples where pentoxifylline was added at maximum G2 block expression, the cells were exposed to a single dose of 7 Gy ^{60}Co γ-irradiation. After an incubation period of two weeks, colonies containing more than 50 cells were scored to measure cell survival and counts were adjusted for plating efficiency (PE). Radiosensitisation by pentoxifylline is expressed as an enhancement factor (REF), which is given by the ratio of surviving fractions:

REF = SF (Control) / SF (+ pentoxifylline).

DNA repair (CFGE) assay

The amount of DNA double strand break damage was determined by constant-field gel electrophoresis (CFGE) as described previously (Wlodek et al, 1991). Confluent cultures were used to avoid S-phase variations between cell lines (Dikomey et al, 1998). Cells were encapsulated in agarose during irradiation and repair. This procedure was optimized according to Kysela et al (1993) in order to minimize non-specific DNA damage. In short, cells were harvested by trypsinisation and resuspended in a 0.5% low melting agarose solution. Aliquots of 30 μl, containing ~0.5 x 10^5 cells, were placed into each well of a disposable plug mold (BioRad), and allowed to solidify at 4°C for 45 minutes. Plugs were irradiated in ice-cold MEM containing 2% HEPES, over a dose range of 0-100 Gy ^{60}Co γ-irradiation on ice. Samples for the determination of initial damage were immediately submitted to subsequent lysing and washing steps. The residual damage was determined either in the

presence or in the absence of pentoxifylline, by incubating plugs at 37^0C in growth medium for periods of 2h and 20h.

For both protocols (initial and residual damage), plugs were submersed in an ice-cold lysing solution containing 50 mM EDTA, 1% N-lauryl-sarcosine and 1 mg/ml Proteinase K. Incubation of 1h at 4^0C was followed by lysing at 37^0C for 20hrs. Agarose plugs were then washed five times and stored in 2ml of 50 mM EDTA solution.

Agarose plugs were loaded into a 20 x 20 cm 0.6% agarose gel and run in 0.5 x TBE buffer for 30h at a constant field strength of 1.2 V/cm. Gels were stained with ethidium bromide (0.5 μg/ml in 0.5xTBE) and subjected to fluorometric analysis with a GeneSnap (VacuTec) image analysis system. The fraction of DNA released from the plug was obtained from the following equation: $F_{rel} = fl_{rel} / (fl_{plug} + fl_{rel})$, where fl_{rel} and fl_{plug} correspond to fluorescence measured in the lane (DNA released) and in the plug respectively. Untreated control samples were used for each sample subset to subtract background fluorescence caused by non-specific DNA degradation.

Dose response curves were obtained by plotting dose (Gy) vs. the fraction of DNA released (F_{rel}) as calculated above, representing initial damage (0h), residual damage (2h), residual damage (2h) in the presence of pentoxifylline, residual damage (20h) and residual damage (20h) in the presence of pentoxifylline. Since data could not be fitted by linear regression, data points were connected and the area under the curve (AUC) was calculated for each

curve in the GraphPad Prism (GraphPad software, San Diego, USA) computer program. Repair inhibition fators were calculated as follows:

AUC ratio $_{\text{without pentoxifylline}}$ / AUC ratio$_{\text{with pentoxifylline}}$.

Apoptosis measurements

Cells exhibiting the characteristic membrane changes associated with apoptosis were identified with Annexin-V-Fluos (Boehringer Mannheim, Germany) (for review see Van Engeland et al, 1998). Adherent cells were harvested by gentle scraping and were combined with cells floating in the growth medium, whereas cells in suspension (Jurkat J5 cells) were used as is. Approximately 10^6 cells were then incubated for 10-15 min. in Annexin-V-Fluos staining solution containing 1μg/ml Annexin-V reagent and 1μg/ml PI in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 5 mM $CaCl_2$). Analysis was done on a FACScan flow cytometer at 488 nm, using a 515 nm bandpass filter for fluorescein detection, and a bandpass filter >560 nm for PI detection. Dot plots in log-scale of FL-1 vs. FL-3 were used to identify the live (Annexin and PI negative) population, as well as early apoptotic (Annexin-V positive, PI negative), late apoptotic (Annexin-V and PI positive), and necrotic (Annexin negative, PI positive) populations. Apoptosis was measured in cells irradiated with 7 Gy of ^{60}Co γ-irradiation at 24 and 48 hour time intervals after irradiation alone, or in the presence of pentoxifylline. Data trends were confirmed by measuring the appearance of a sub-G1 peak in DNA flow histograms obtained by flow cytometry as described above (data not shown).

Determination of the G2 block in HeLa cells

The determination of the time of maximum cell cycle block in G2 was done by irradiating exponentially growing HeLa cells with 7Gy of ^{60}Co γ-irradiation, and sampling cells at 2-hourly intervals for up to 24 hours. The cells were fixed in 70% ethanol and stored overnight at -20^{0}C. For block abrogation studies, samples in exponential phase were irradiated with 7Gy of ^{60}Co γ-irradiation and allowed to grow for ten hours, which is the time needed for maximum G2 block formation in HeLa cells. Pentoxifylline was then added to a final concentration of 2 mM. At different time intervals thereafter, ranging from 2 - 25 hours, the Pentoxifylline treated and control samples were trypsinised, fixed in 70% ethanol and stored at -20 ^{0}C. After washing in PBS, cells were resuspended in PBS containing 10μg of propidium iodide (Sigma) and 0.1% RNase A. Samples were incubated for 20 minutes at 37^{0}C prior to flow cytometric analysis.

Immunocytochemistry

Fixed cells were prepared for multiparameter flow cytometry to simultaneously measure total DNA content and cyclin B1 (Gong *et al*, 1994) or p34^{cdc2} expression (Baroja *et al*, 1996) as previously described. In short, the cells were washed in PBS and treated with 0.25% Triton X-100 for 5 min. on ice. After another wash in 5ml PBS, the cell suspension of 5x10^{5}cells/100 ul was incubated overnight at 4°C in a 1:400 dilution of mouse monoclonal anti-cyclin

B1 antibody (Pharmingen Clone GNS-1, San Diego, CA), or a purified monoclonal anti-$p34^{cdc2}$ antibody (clone HCDC1, ICN Biochemicals) in PBS/1% Bovine Serum Albumine (BSA). The next morning, cells were washed in PBS and incubated for 30 min at room temperature in a 1:40 dilution of fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma) in PBS/1% BSA. The cells were washed again, resuspended in 10 ug/ml of propidium iodide and 0.1% RNase A in PBS, and incubated for 20 min at room temperature prior to analysis. Negative controls were prepared in a similar way, except that an isotype-specific antibody, mouse IgG (Sigma), was used instead of the cyclin B1 or anti-$p34^{cdc2}$ antibody.

Flow cytometry

To determine cyclin B1 or $p34^{cdc2}$ levels, samples were analysed on a Becton-Dickinson (San Jose, CA) FACScan flow cytometer. Fluorescence data from 10 000 events were collected, stored and analysed using Lysis II software. To determine the time of maximum G_2 block, samples were analysed for red (PI) fluorescence which was displayed as a DNA histogram. Markers placed at the G_2 boundaries served to estimate the G_2 content for each time-point.

Cyclin B1 or $p34^{cdc2}$ expression, and DNA data, were displayed in dot plots of red (PI) vs. green (FITC) fluorescence representing total cellular DNA content and cyclin B1 or $p34^{cdc2}$ expression respectively. Cell doublets were gated

out by using the doublet discrimination module. Determination of the G_2 content was as described above. The fraction of cells expressing either cyclin B1 or $p34^{cdc2}$ was determined by gating only cells which displayed a positive green (FITC) fluorescence. The threshold for FITC positive cells was defined using the gate window set on the negative control sample which was prepared with the isotype-specific antibody IgG1, on all the treated samples. All experiments were repeated at least twice and generated identical trends.

Definition of cyclin B1/ G_2, $p34^{cdc2}/G_2$ and cyclin B1/$p34^{cdc2}$ ratios

The cyclin B1/ G_2 and $p34^{cdc2}/G_2$ ratios were calculated by comparing the fraction of cells expressing either cyclin B1 or $p34^{cdc2}$ to the fraction of cells in the G_2 phase of the cell cycle for each post-irradiation time point. Cyclin B1/ $p34^{cdc2}$ ratios were obtained by dividing the $B1/G_2$ ratios over the $p34^{cdc2}/ G_2$ ratios.

Isolation of HeLa nuclei:

Nuclei were obtained as described (Heussen *et al*, 1987). In short, cells from exponentially growing cultures were washed once in "lysis" buffer consisting of 10mM Tris-HCl, 10mM NaCl, 5mM $MgCl_2$, pH 7.4. Cells were then resuspended in ice-cold lysis buffer at $1\text{-}3x10^6$ cells/ml and allowed to swell for about 15-20 minutes on ice. Lysis of cells were completed by the dropwise addition of 10% (v/v) nonidet- P40 (Shell Chemical Co) in lysis buffer to a final

concentration of 0.5% (v/v). During this step, the sample was vortexed vigorously. Released nuclei were sedimented at 300g for 5 minutes in a swing bucket rotor and resuspended gently by stepwise addition of small volumes of phosphate-buffered saline, pH 7.4, and gentle vortexing, essential to prevent clumping. The isolation of nuclei was confirmed by microscopy, and the sample was then subjected to the normal staining procedure for cyclin B1.

Data evaluation

All experiments were repeated at least three times, and the data are given as a mean ± standard deviation (SD) or standard error margin (SEM) for the independent experiments. Statistical analysis and data fitting were performed by means of the GraphPad Prism (GraphPad Software, San Diego, USA) computer program. A two-sided t test was used to compare the means between sample groups.

Chapter 4

Results

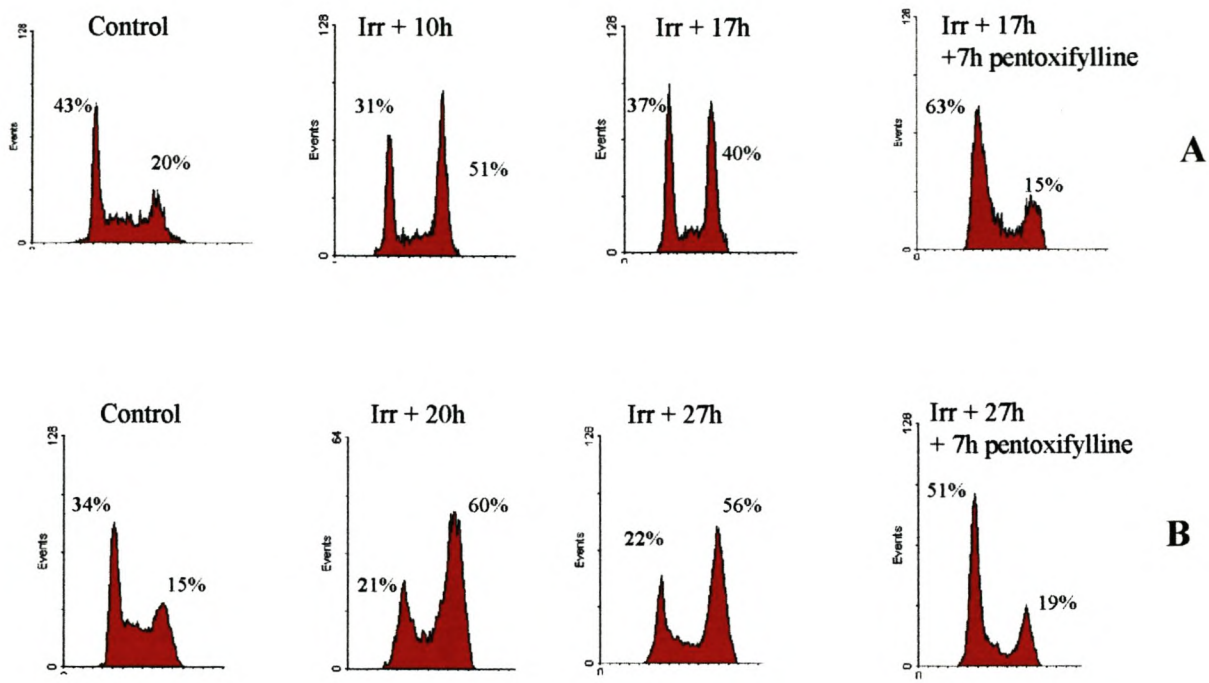
Drug toxicity

The TD_{50} of pentoxifylline (toxic dose causing 50% cell death) was found to be 6.0, 3.2, 5.6 and 4.1 mM for Be11, MeWo, 4197 and 4451 cells respectively (data not shown). A sub-toxic dose of 2mM pentoxifylline was used in all four cell lines in subsequent experiments.

G2 block expression

The G2 block maximum in response to a single dose of 7 Gy was found to be 16, 18, 12 and 24 hours for Be11, MeWo, 4197 and 4451 respectively. Fig. 8 shows examples of G2 block abrogation by pentoxifylline in the 4197 (Fig. 8A) and 4451 (Fig. 8B) SCC cell lines. Similar trends were seen in the Be11 and MeWo melanoma cell lines (data not shown). Both TP53 wild type and mutant cell lines respond to irradiation by an increase in the G2 population. In mutant cells G2 levels reach 60-80% (Fig 8B) and are higher than in TP53 wild type cells (Fig 8A) where G2 levels reach only 40-50%, the remainder of cells being blocked in G1. The time of recovery from the G2 block in irradiated cells in the absence of pentoxifylline is 40-60 hours. In the

Figure 8



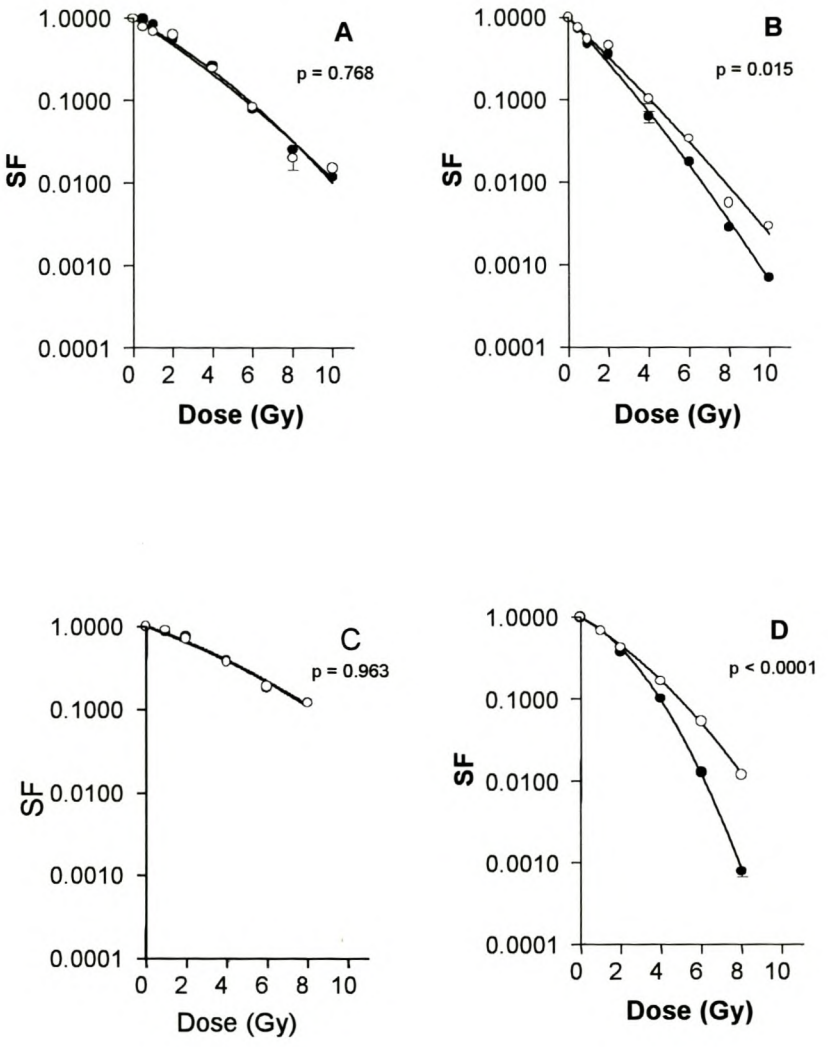
DNA histograms showing the influence of 7 Gy of irradiation, and 7 Gy of irradiation plus pentoxifylline added at maximum G2 block, on the distribution of cells in G1 and G2 cell cycle phases at various post-irradiation times in TP53 wildtype 4197 SCC cells (A) and TP53 mutant 4451 SCC cells (B).

presence of 2 mM pentoxifylline, the G2 population is reduced to control values in approximately 8 hours (Fig. 8).

Cellular radiotoxicity

Cell survival after doses of 0-10 Gy ^{60}Co γ-irradiation was measured for irradiation alone, and for irradiation in the presence of pentoxifylline. Figs. 9 A-D show survival curves obtained from colony assay data in Be11, MeWo, 4197 and 4451 cells respectively. Survival curves were fitted by the linear-quadratic equation, $-\ln S = \alpha D+\beta D^2$. MeWo, 4197 and 4451 cells show similar radiosensitivities with SF_2 values of 0.35, 0.37 and 0.36 respectively. The Be11 cell line emerges as the most radioresistant, showing an SF_2 value of 0.66. There is some variation between our survival data and the original data reported by Zölzer *et al* (1995). This can possibly be attributed to the higher passage numbers used in our laboratory and the experimental design of Zolzer *et al* using X-rays. Pentoxifylline markedly increases cell death in the two TP53 mutant cell lines MeWo ($p = 0.015$) and 4451 ($p < 0.0001$) (Fig.9, B and D), while it has no effect on survival in the two TP53 wild type cell lines Be11 ($p = 0.768$) and 4197 ($p = 0.963$) (Fig.9, A and C). The radiotoxicity enhancement factors (REF's) in the presence of pentoxifylline are given in Table 1. Survival fractions were calculated for doses ranging from 2-10 Gy to ensure comparability with the dose of 7 Gy given to cells under conditions of G2 block abrogation. At 2,4,6 and 8 Gy of ^{60}Co γ-irradiation, pentoxifylline enhances the radiotoxicity by factors of 1.2, 1.64, 4.2 and 14.5

Figure 9



Survival curves of Be11 (A) and MeWo (B) melanoma and 4197 (C) and 4451 (D) SCC cells following irradiation. Pentoxifylline at the subtoxic dose of 2 mM was added to the medium immediately prior to irradiation. Cell survival was measured by the colony assay, and data were fitted to the linear quadratic equation, after irradiation alone (○–○) and irradiation in the presence of pentoxifylline (●–●).

respectively in the 4451 TP53 mutant SCC cell line. In the 4197 TP53 wild type SCC cells, the drug has no influence on radiotoxicity and enhancement factors were found to be close to 1 for the same dose range. In the TP53 mutant MeWo melanoma cell line, pentoxifylline enhances radiotoxicity by factors of 1.29, 1.66, 1.88, 2.0 and 3 at doses of 2,4,6,8, and 10 Gy ^{60}Co γ-irradiation respectively. No enhancement of radiotoxicity by pentoxifylline is seen in the TP53 wild type Be11 cells over the same dose range. When pentoxifylline was added at the G2 maximum after a single dose of 7 Gy, the drug was found to markedly reduce the duration of the G2 block from approximately 20-40 hours to approximately 8 hours (Fig. 8). However, under these conditions there is no influence on radiotoxicity. This is evident from the REF* values (SF_7) which are close to 1 for both the TP53 wild type cell lines Be11 and 4197, and the TP53 mutant cell lines MeWo and 4451 (Table 1).

DNA repair

Dose response curves representing initial DNA damage, and residual DNA dsb damage after 2h and 20h repair times for the dose range 10-100 Gy are shown in Figure 10. Fractions of DNA released (F_{rel}) were plotted against dose, and the area under each curve (AUC) was calculated to compare initial DNA dsb's to the residual unrepaired dsb's after 2h and 20 h of repair in the presence or absence of pentoxifylline. These results are summarised in Table 2. The differences between residual damage after 2h in the presence or absence of pentoxifylline were significant in Be11, MeWo and 4197

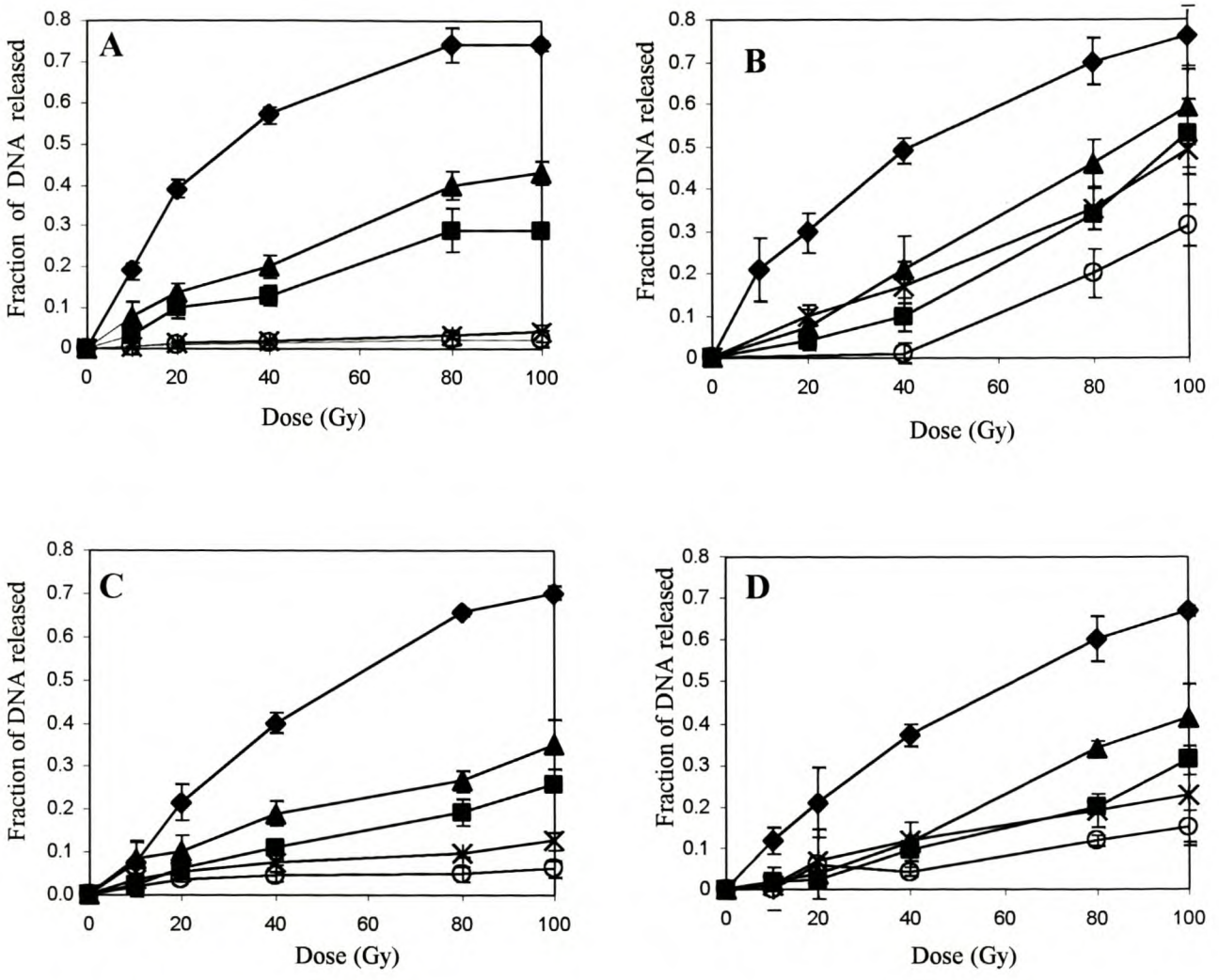
Table 1

Radiotoxicity enhancement factors (REF) for a range of irradiation doses calculated from cell survival fractions. Pentoxifylline was added to the growth medium immediately prior to irradiation.

	Be11	MeWo	4197	4451
SF_2	1.13± 0.07	1.29± 0.03	0.97± 0.02	1.2± 0.03
SF_4	0.93± 0.13	1.66± 0.1	0.97± 0.04	1.64± 0.03
SF_6	1.09± 0.27	1.88± 0.06	1.06± 0.09	4.2± 0.47
SF_7^*	1.01± 0.02	1.1± 0.05	1.01± 0.02	1.1± 0.04
SF_8	0.82± 0.44	2.0± 0.59	1.0± 0.30	14.5± 1.04
SF_{10}	1.2± 0.22	3.0± 0.63	-	-

*REF (SF_7) values were calculated from cell survival fractions by adding the drug at the G2 maximum after a single dose of 7 Gy.

Figure 10



CFGE data showing fractions of DNA released for the dose range of 0-100 Gy in Be11 (A), MeWo (B), 4197 (C) and 4451 (D) cells, as determined by fluorescent densitometry of EtBr stained gels. Area under the curve (AUC) was calculated for each dose response curve and are given in Table 2.

◆–◆ Initial DNA damage measured immediately after irradiation.

■–■ Residual DNA damage measured after 2 hrs. repair incubation at 37°C.

▲–▲ Residual DNA damage measured after 2 hrs. repair incubation at 37°C in the presence of pentoxifylline.

○–○ Residual DNA damage measured after 20 hrs. repair incubation at 37°C.

×–× Residual DNA damage measured after 20 hrs repair incubation at 37°C in presence of pentoxifylline

Table 2

Areas under the dose-response curves (AUC) calculated from fractions of DNA released against irradiation dose (Fig. 4 data) for 0, 2 and 20 hrs. after irradiation over a dose range of 0-100 Gy.

	Be11	MeWo	4197	4451
Initial DNA damage	54.45	49.84	42.63	40.13
2 hrs. repair	17.29	19.30	12.82	12.43
2 hrs. repair +pentoxifylline	25.13	27.40	19.53	18.46
20 hrs repair	1.53	9.5	3.97	7.16
20 hrs. repair +pentoxifylline	2.17	22.5	7.52	13.45

T-test analyses of differences between AUC's after 2h in the presence or absence of pentoxifylline showed p-values of 0.022, 0.049, 0.008 and 0.122 for Be11, MeWo, 4197 and 4451 respectively. Similar analyses of 20h data showed p-values of 0.048, 0.029, 0.021 and 0.049 for Be11, MeWo, 4197 and 4451 respectively.

(p = 0.022, 0.049 and 0.008 respectively). The 4451 did not show a significant difference after 2h repair (p = 0.122). However, after 20h repair incubation, significant differences were seen between pentoxifylline treated and control samples in all four cell lines (p = 0.048, 0.029, 0.021 and 0.049 for Be11, MeWo, 4197 and 4451 respectively).

To assess the quality of dsb repair in untreated cells, ratios of the AUC's representing initial damage vs. residual damage after 2h and after 20h were calculated. These ratios are summarised in Table 3. After 2 h of repair, the Be11, MeWo, 4197 and 4451 cells showed repair ratios of 3.15, 2.58, 3.33 and 3.23 respectively. In the presence of pentoxifylline, these ratios were reduced to 2.17, 1.82, 2.18 and 2.17 in Be11, MeWo, 4197 and 4451 respectively. The 20h control repair ratios were found to be 35.59, 5.25, 10.74 and 5.6 in Be11, MeWo, 4197 and 4451 respectively. In the presence of pentoxifylline these ratios were reduced to 25.09, 2.22, 5.67 and 2.98 in Be11, MeWo, 4197 and 4451 respectively. These repair ratios (Table 3) were used to calculate repair inhibition factors (RIF's) for the two repair incubation periods (Table 4). After 2 h of repair in the presence of pentoxifylline, RIF's were found to be 1.45, 1.42, 1.52 and 1.49 in Be11, MeWo, 4197 and 4451 respectively. The 20h repair data showed RIF's of 1.42, 2.37, 1.89 and 1.88 in Be11, MeWo, 4197 and 4451 respectively (Table 4).

Table 3

Ratios of initial vs. residual DNA damage derived from AUC data (Table 2) for various repair times in the absence or presence of pentoxifylline.

	Be11	MeWo	4197	4451
2 hrs. repair	3.15	2.58	3.33	3.23
2 hrs. repair +pentoxifylline	2.17	1.82	2.18	2.17
20 hrs. repair	35.59	5.25	10.74	5.60
20 hrs. repair +pentoxifylline	25.09	2.22	5.67	2.98

Table 4

Repair inhibition factors (RIF's) as calculated from repair ratios (Table 3) in the absence and presence of pentoxifylline.

	Be11	MeWo	4197	4451
RIF 2 hrs.	1.45	1.42	1.52	1.49
RIF 20 hrs.	1.42	2.37	1.89	1.88

Apoptosis in TP53 mutant melanoma and SCC cell lines:

The influence of pentoxifylline on irradiation-induced apoptosis was assessed in the two TP53 mutant MeWo and 4451 cell lines which show dose modification factors of up to 3 and 14.5 respectively (Table 5). Control levels of apoptotic fractions were 8 ± 1.2% and 3 ± 0.5% in MeWo and 4451 cells respectively. Twenty-four hrs. after a single dose of 7 Gy, these fractions increased to 27 ± 9 % and 34% ± 11 in MeWo and 4451 cells respectively. Addition of pentoxifylline immediately prior to irradiation under the same conditions lead to apoptotic fractions of 27 ± 12% and 35 ± 15% in MeWo and 4451 cells respectively. Identical trends were seen in samples analysed after 48h (Table 5). The rather large error margins are attributed to the fact that the scraping of adherent cells causes membrane damage. However, the Annexin V method was used since it allows for the analysis of a large number of cells and it distinguishes between apoptotic cells and necrotic cells or debris. The data trends were confirmed by analysis of a sub-G1 peak in DNA flow histograms (data not shown).

Apoptosis in Jurkat J5 cells:

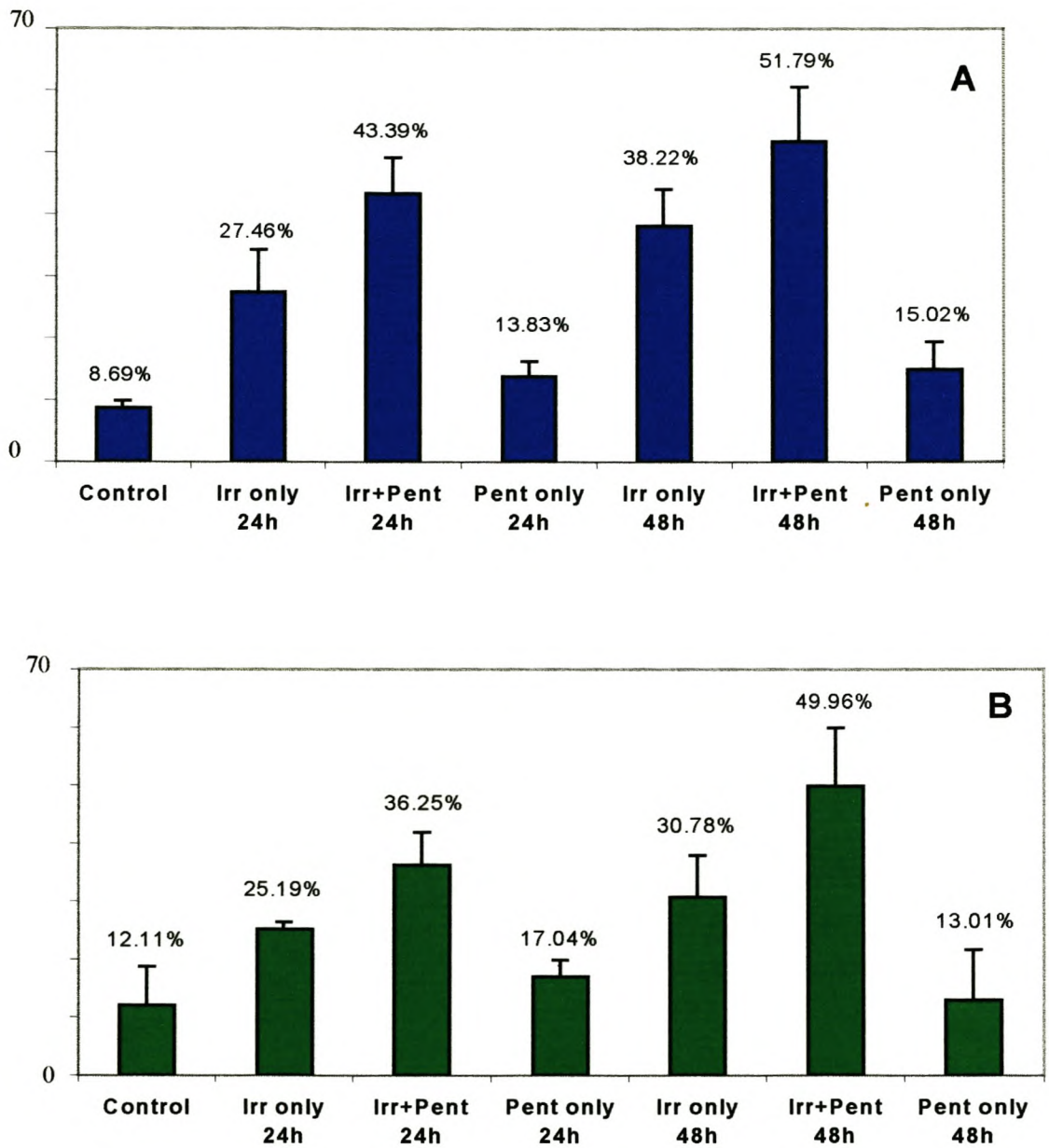
To investigate the possibility of a cell type specific effect, the influence of pentoxifylline on radiation-induced apoptosis was also measured in the Jurkat J5 lymphocytic leukemia cells (Fig.11). Pentoxifylline was added at irradiation and the influence on apoptosis measured by Annexin V (Fig. 11A) and sub-

Table 5

Effect of pentoxifylline on apoptotic fractions (% apoptosis) in irradiated TP53 mutant cells. A single dose of 7 Gy ^{60}Co-γ-irradiation was used.

Cell line	Time	Control	Irradiation only	Irradiation + pentoxifylline
MeWo	24 hrs	8 ± 1.2	27 ± 9	27 ± 12
	48 hrs	8 ± 1.2	31 ± 8	27 ± 9
4451	24 hrs	3 ± 0.5	34 ± 11	35 ± 15
	48 hrs	3 ± 0.5	40 ± 3	40 ± 12

Figure 11



The influence of pentoxifylline on radiation-induced apoptosis as measured by the Annexin V method (A) or by measuring sub-G1 peaks by flow cytometry (B). Cells were irradiated with 7Gy of ^{60}Co-rays in the presence of the drug.

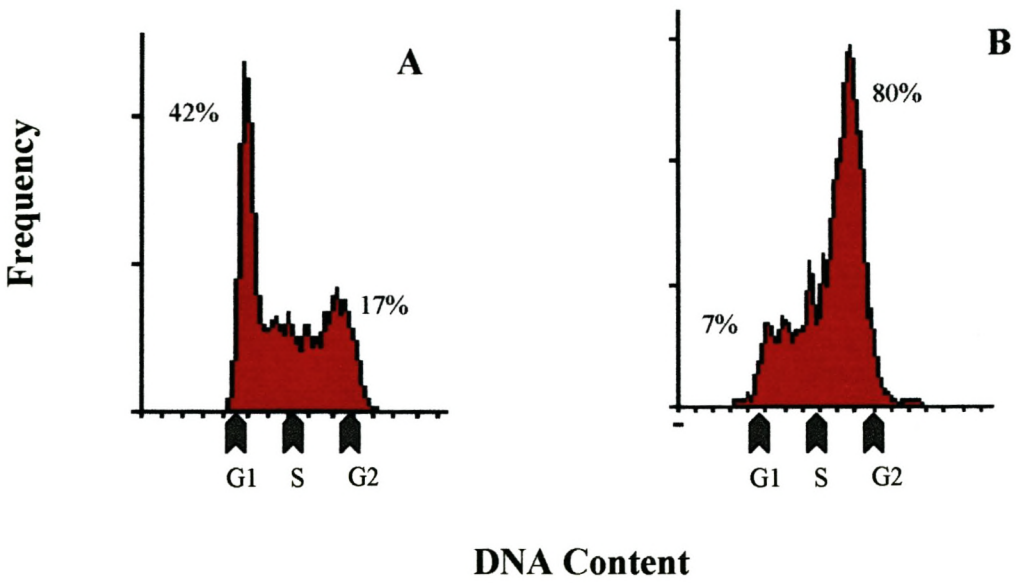
G1 peaks (Fig. 11B) after 24h and 48h. Similar trends are observed for both methods. Pentoxifylline increases irradiation-induced apoptosis in these cells from approximately 26.4 ± 4.5% to 39.8 ± 5% after 24h, and from approximately 34.5 ± 5.5% to 50.8 ± 8.5% after 48h. These values are the means calculated from the two methods, corrected for control apoptosis at 24h and 48h, and show significant differences between apoptosis in the presence of pentoxifylline and with irradiation alone. Pentoxifylline alone had virtually no effect on apoptosis over 48h in this cell line, as indicated by the apoptotic fractions of approximately 15% in the presence of pentoxifylline alone (Fig 11A and B).

Cyclin B1 expression in HeLa cells

In HeLa cells (Fig. 12A), the radiation induced G2 cell cycle block reaches a maximum at 10 hours after a single dose of 7 Gy (Fig. 12B). From the dot-plot of red (PI / total DNA) vs. green (FITC / cyclin B1) fluorescence, it is clear that cyclin B1 is only synthesised in late S-phase and reaches maximum expression in G2 (Fig. 13A). No cyclin B1 is seen in G1-phase because of the rapid degradation of this protein in early mitosis. As the cells accumulate in G2, there is a concominant rise in cyclin B1 content (Fig. 13B).

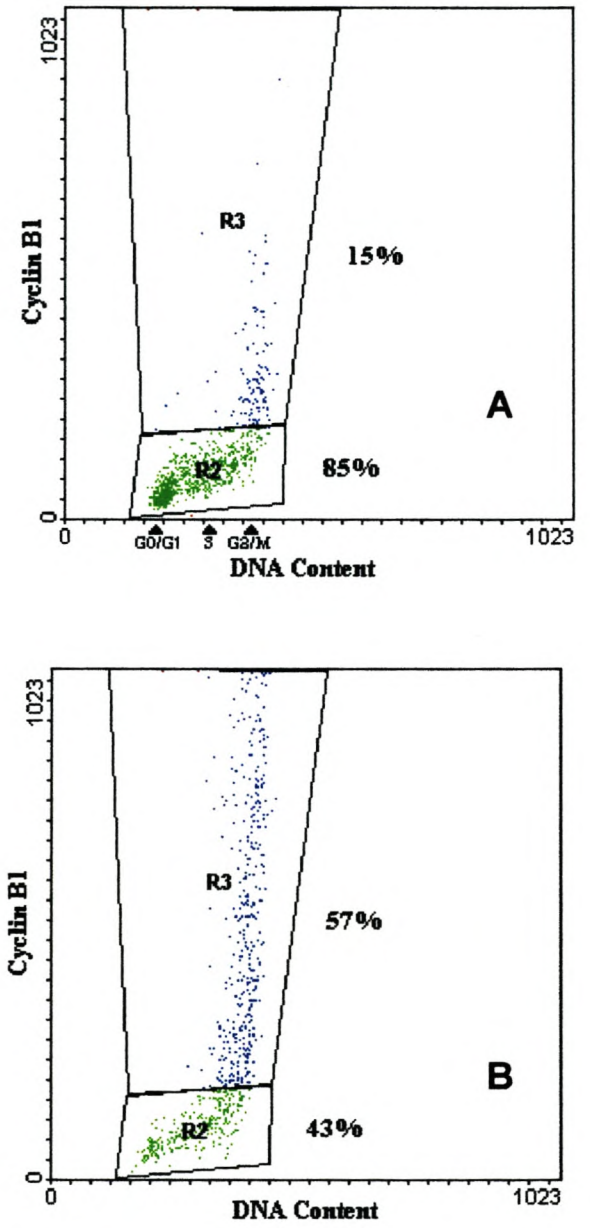
The increase in the G2 fraction of the cell cycle during expression of the block and the decrease of this fraction as the block resolves over 35 hours are shown in Fig. 14 (solid line). Addition of 2 mM pentoxifylline at 10 hours after irradiation, when the G2 delay had reached a maximum, rapidly abrogates the

Figure 12



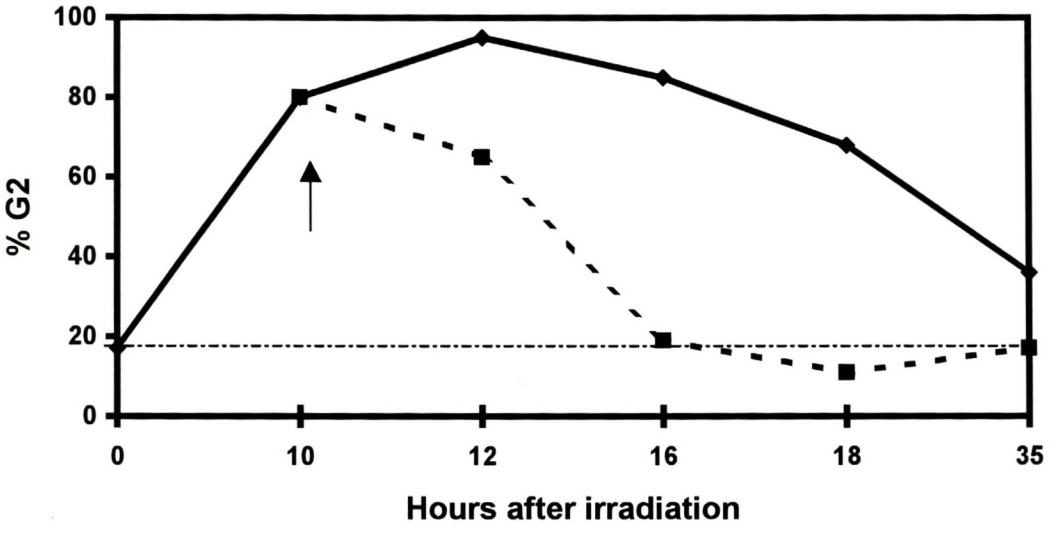
Flow cytometric histogram of the total DNA content of HeLa cells, indicating the normal cell cycle distribution (A) and the maximum formation of the G2 cell cycle delay at 10-12 hours after irradiation (B).

Figure 13



Dot plots of green fluorescence (FITC) on the vertical scale vs. red fluorescence (PI) on the horizontal scale, representing cyclin B1 and total DNA content respectively, indicating the G2-phase specific expression of cyclin B1 in HeLa cells before (A) and 10 hours after irradiation (B).

Figure 14



Increase of the G2 fraction of the cell cycle during expression of the G2 block in HeLa cells (——) and the decrease of this fraction as the block resolves over 36 hours (·····) to return to control levels (- - - - - -). Arrow indicates pentoxifylline addition.

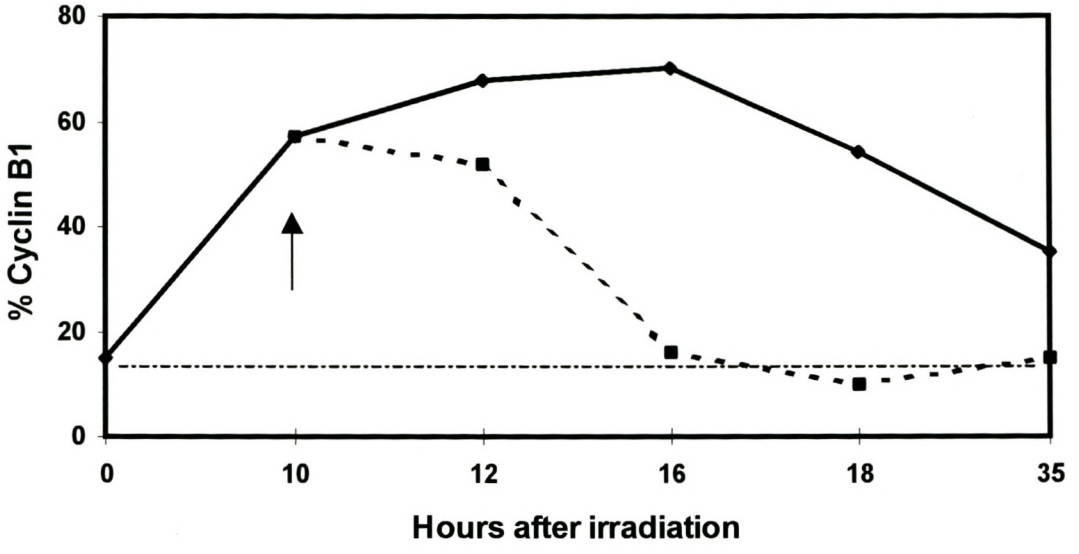
G2 delay and cells return into cycle (Fig. 14, dashed line). Over the same time window, cyclin B1 content rises and parallels the increase of the G2-phase fraction after irradiation (Fig. 15 - solid line), and the decrease after pentoxifylline treatment (Fig. 15 - dashed line).

When the ratio of cyclin B1 fraction vs. G2 fraction is plotted, it is seen that cyclin B1 expression actually decreases at the induction of the G2 block, and stays below control levels for the entire duration of the G2 block (Fig. 16 - solid line). When pentoxifylline is added at 10 hours after irradiation, this ratio is rapidly restored to the level seen in the unirradiated control sample (Fig. 16 - dashed line).

$p34^{cdc2}$ expression in HeLa cells

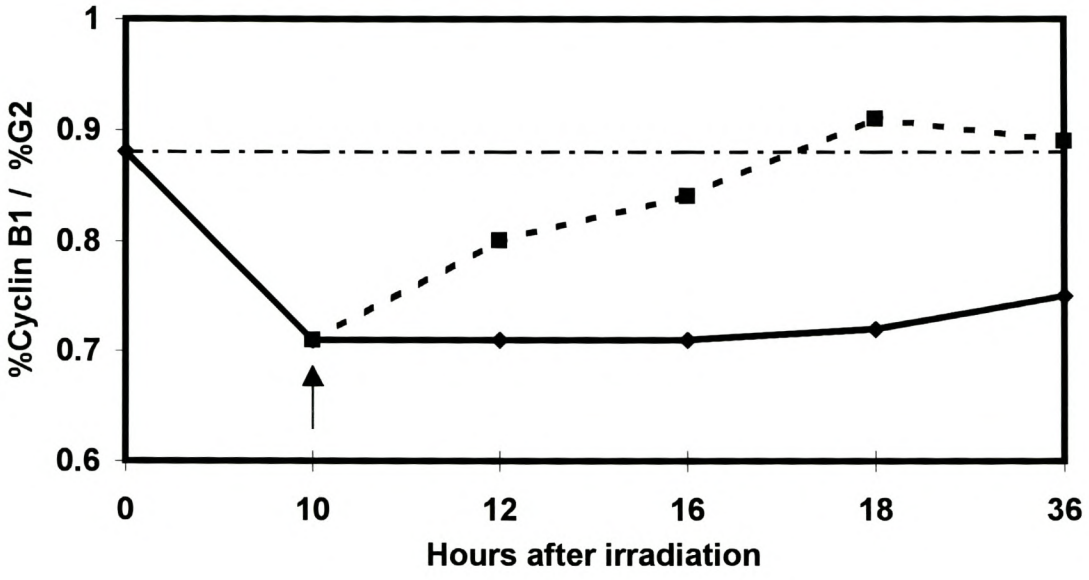
From the dot-plot of red (PI/DNA content) vs. green (FITC/p34) fluorescence it is evident that, unlike cyclin B1, $p34^{cdc2}$ is not uniquely expressed in the G_2 phase of the cell cycle, but also exists in the G_1 phase (Fig. 17A). However, it is predominantly expressed in the G_2 phase during G_2 arrest (Fig. 17B). Similar to cyclin B1, the expression of $p34^{cdc2}$ increases after irradiation, at the formation of the G2 block, and drops after pentoxifylline treatment (Fig 18). When the ratios of $p34^{cdc2}$ to G_2 fraction are plotted over time, an over-expression of $p34^{cdc2}$ during G_2 arrest becomes evident as the ratio increases from 0.1 in controls to approximately 0.48 in irradiated cells. Pentoxifylline treatment reduces the $p34^{cdc2}$ expression to the control level of 0.1 (Fig. 19). Fig. 16 shows the cyclin B1/G_2 ratios for similar dose and time points in HeLa. Since cyclin B1 and $p34^{cdc2}$ act in concert we also plotted the cyclin B1/$p34^{cdc2}$

Figure 15



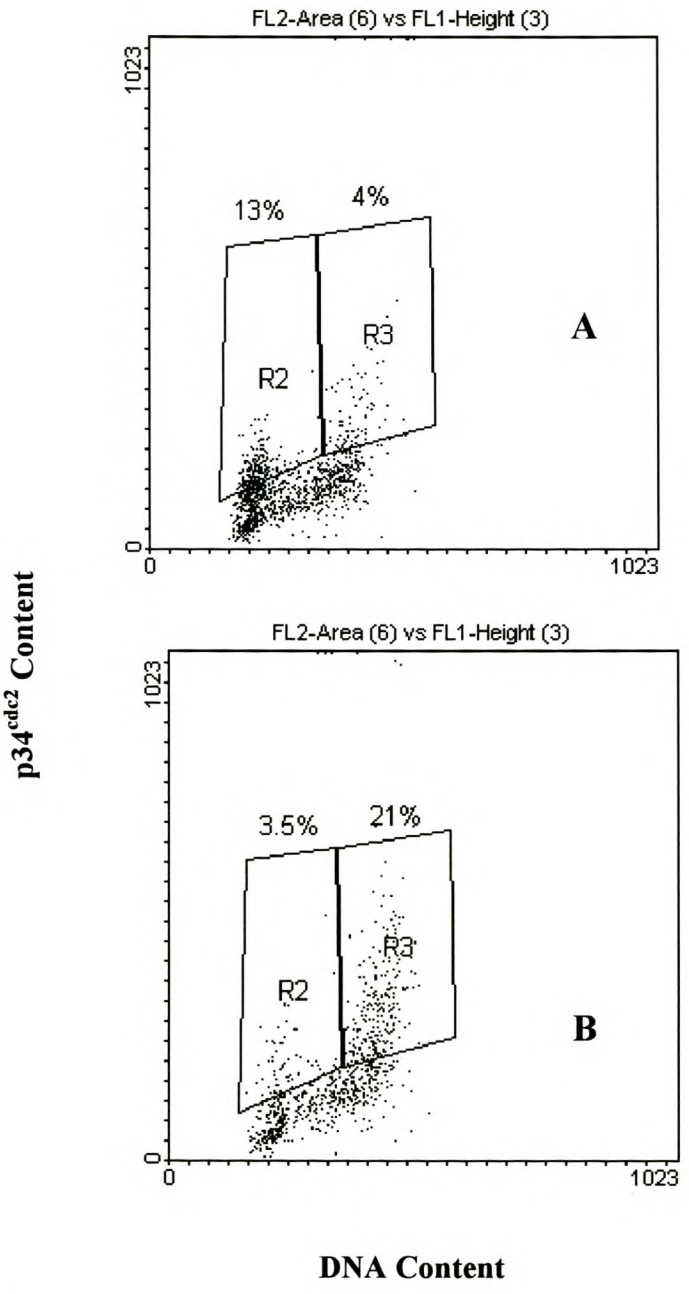
Increase of the cyclin B1 content which parallels the rise of the G2 fraction after irradiation (——), and the abrogation of the block by Pentoxifylline (.....) to restore control levels (------) in HeLa cells . Arrow indicates pentoxifylline addition.

Figure 16



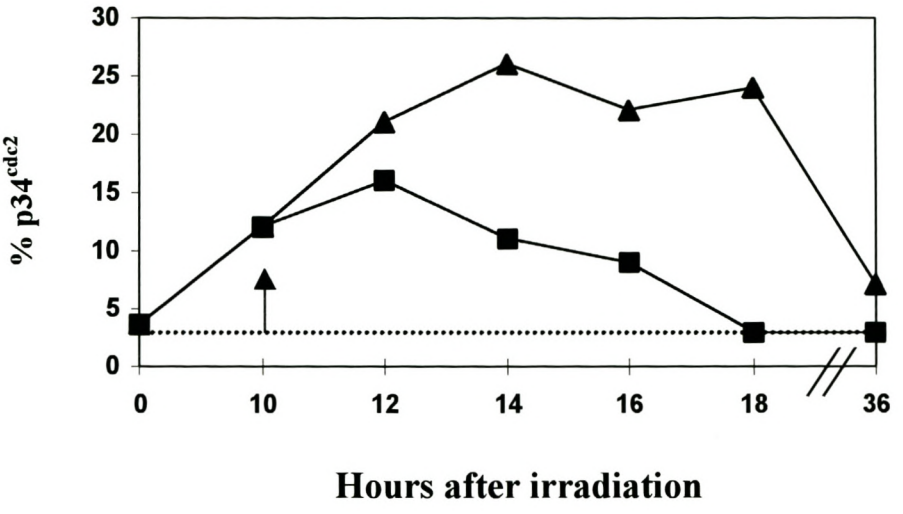
Cyclin B1/ G2 phase ratio plotted over 35 hours after irradiation in HeLa cells. This ratio is suppressed after irradiation at the formation of the G2 block, and recovers slowly as the block resolves (———). After the addition of Pentoxifylline (·····), this ratio rapidly recovers to reach the control level (-·-·-·-) within 6-8 hours.

Figure 17



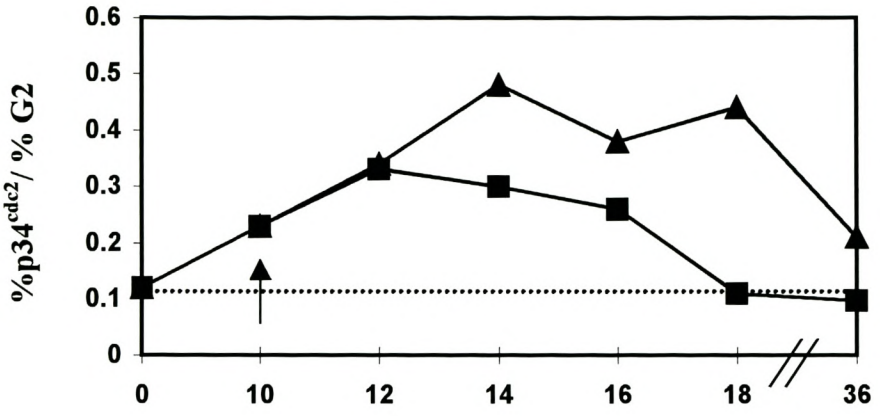
Dot plots of green fluorescence (FITC) on the vertical scale vs. red fluorescence (PI) on the horizontal scale, representing $p34^{cdc2}$ content and total DNA content, respectively. Percentages of cells expressing $p34^{cdc2}$ are indicated for HeLa cells in the G_1 and G_2 phases, before (A) and at maximum G2 block (B).

Figure 18



Increase of the $p34^{cdc2}$ content which parallels the rise of the G2 fraction after irradiation (▲) , and the abrogation of the block by Pentoxifylline (■) over 36h following irradiation in HeLa cells. Arrow indicates the addition of pentoxifylline.

Figure 19



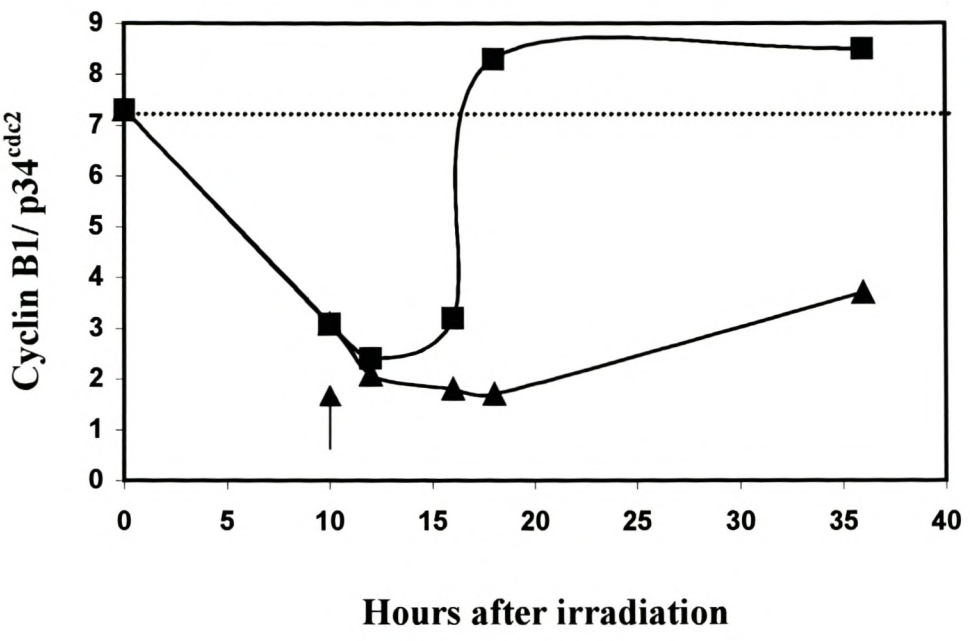
p34^{cdc2}/G2 ratios plotted over a 36h time period after irradiation in HeLa cells. This ratio increases after irradiation (▲), but is reduced to control levels within 8h after the addition of pentoxifylline (■). Arrow indicates the addition of pentoxifylline.

ratios (see definition in material and method section). Fig. 20 shows that the G_2 maximum is associated with a very low cyclin B1/p34^{cdc2} ratio which gradually increases as the cells recover from the G_2 block and enter mitosis after 40 h. Pentoxifylline added at the maximum G_2 block rapidly restores the cyclin B1/p34^{cdc2} ratio over a very narrow time window, which reaches the control value after ~7 h. Abrogation of the G_2 block thus is associated with a rapid restoration of the critical cyclin B1/p34^{cdc2} ratio.

Subcellular location of MPF

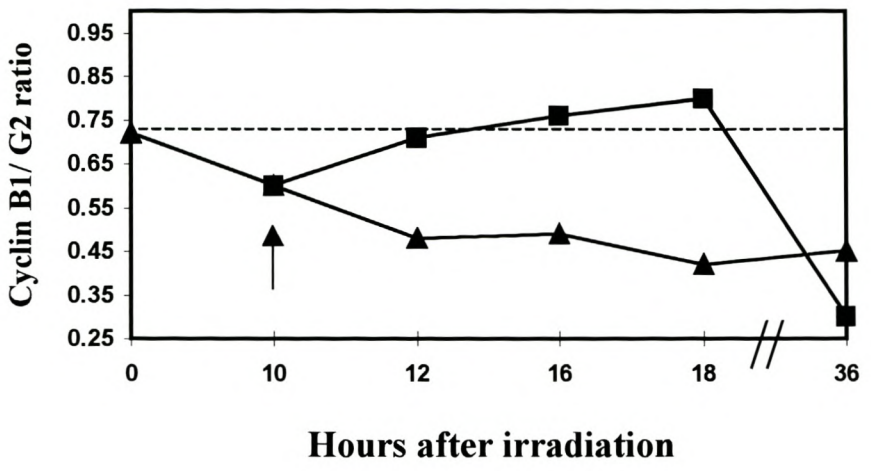
In order to study the influence of pentoxifylline on subcellular translocation of the cyclin/cdk complex, we compared cyclin B1 expression in isolated HeLa nuclei and whole HeLa cells. Addition of pentoxifylline to whole cells at the time of maximum G_2 block results in a rapid restoration of the control cyclin B1/G_2 ratio within ~7 h which remains at control levels for up to 35 h. In the absence of pentoxifylline, these ratios were not fully restored within 35 h, similar to the trends shown in Fig. 16. In nuclei (Fig. 21) the G_2 block abrogation by pentoxifylline induces a sharp drop in cyclin B1 expression after 18h. This suggests that cyclin B1 crosses the nuclear membrane after G_2 block abrogation and reenters the cytoplasm where it is probably degraded by ubiquitin mediated proteolysis (Pines, 1999). Identical data trends were observed in A549 human lung carcinoma cells (not shown).

Figure 20



Ratios of cyclin B1/ $p34^{cdc2}$ plotted over 36h after irradiation in HeLa. During the formation of the radiation-induced G2 block, this ratio is drastically reduced, and recovers slowly during the normal recovery of the cell cycle (▲). After the addition of pentoxifylline at maximum G2 block (arrow), this ratio is rapidly restored to control levels within approximately 6h (■).

Figure 21



Decrease in the cyclin B1/ G2 ratio following irradiation in isolated HeLa nuclei (▲), and rapid recovery to control ratios in 6-8 h in the presence of pentoxifylline (■) as shown before for whole cells (Fig. 16). After G2 block abrogation, cyclin B1 is exported from the nucleus as indicated by the sharp decrease in cyclin B1/G2 ratios between 18h and 36h. Arrow shows pentoxifylline addition.

Chapter 5

Discussion

The enhancement of radiotoxicity by caffeine and pentoxifylline has been attributed to a strong inhibition of the damage-induced G2 cell cycle checkpoint (Russell et al, 1995, Li et al 1998). However, no experimental evidence exists to support this conclusion. Data presented here show that the presence of pentoxifylline at the time of irradiation significantly enhances radiotoxicity in the two TP53 mutant cell lines MeWo and 4451 by factors of up to 14.5 (Fig 9, B and D). No enhancement of radiotoxicity is seen in the TP53 wild type cell lines (Fig. 9, A and C). This is in agreement with previous reports on the methylxanthine-induced radiosensitisation in TP53 mutant and deficient cells (Powell et al, 1995; Russell et al, 1995; Russell et al, 1996; Li et al, 1998; Li et al, 1999; Fan et al, 1995).

When cells were irradiated with a single dose of 7 Gy, and pentoxifylline was added at the time of maximum G2 block expression, no enhancement of radiotoxicity, measured as a reduction of clonogens, was seen in any of the four cell lines. It would thus seem that the early induction of mitosis by pentoxifylline through G2 cell cycle block abrogation cannot be solely responsible for the enhancement of radiotoxicity seen in TP53 mutant cells. That G2 block abrogation indeed induces mitosis has been demonstrated by measurements of histone H3-phosphorylation levels in the Be11, MeWo, 4197

and 4451 cell lines. These levels showed a marked increase immediately after G2 block abrogation, which is an indication that G2 cells enter mitosis (A. Binder, personal communication; Juan et al 1998; Juan et al, 1999).

The possibility that the enhancement of radiotoxicity in the TP53 mutant cell lines MeWo and 4451 is due to increased apoptosis was considered. Our experimental data show no significant differences between apoptotic fractions as measured by Annexin-V binding (Table 5). In the two TP53 mutant cell lines, MeWo and 4451, which exhibit irradiation-induced apoptosis and show a significant increase of radiotoxicity, the apoptotic yield is not influenced by pentoxifylline. This conclusion is in agreement with recent data on caffeine, which demonstrate that the mechanism of action of this drug as a radiosensitiser is not dependent on G2 block abrogation or apoptosis induction in human bladder cancer cells (Ribeiro et al, 1999).

It is well documented that many cell types, especially of haematopoetic origin, mainly die via apoptotic pathways following DNA damage, whereas other cell types are more resistant to apoptosis and die instead by necrosis, mitotic death or terminal cell cycle arrest (Blank et al, 1997). Mitotic death can be defined as a loss of clonogenic survival from the formation of cells that have lost the ability to divide, but remain metabolically active (Bernhard et al, 1996). These cells may undergo apoptosis at a later stage or die by a pathway involving the formation of micronuclei. The possibility that the effect of pentoxifylline on apoptosis may be dependent on cell type was investigated by measuring the influence on radiation-induced apoptosis in Jurkat J5

lymphocytic leukemia cells. This cell line has TP53-null status and shows no G1 arrest following irradiation. A strong G2 block is induced which reaches a maximum approximately 18h post-irradiation (data not shown). The significant increase in apoptosis observed in these cells following radiation exposure in the presence of pentoxifylline as compared to radiation alone (Fig. 11) confirms that the drug can indeed increase apoptosis in certain cell types. Although the influence of pentoxifylline on radiotoxicity in Jurkat cells could not be measured by the clonogenic assay, due to their growth in suspension, it is presumed that the observed increase in apoptosis was accompanied by radiosensitisation, as with other cells lacking TP53 expression. It seems likely that the increase in apoptosis by pentoxifylline may be restricted to cell types such as those from haematopoetic origin, in which the apoptotic pathways are strongly expressed. This conclusion is however at variance with a study showing caffeine enhancement of radiation-induced apoptosis in HeLa cells, which predominantly undergo mitotic death (Bernhard et al, 1996). The reasons for the apparent cell type specificity of apoptosis induction by methylxanthines therefore must be considered as unresolved. The pathways involved in the induction of apoptosis by pentoxifylline is also unknown, but in the HeLa and Jurkat cell lines must be TP53-independent, as neither of these lines express wild type TP53.

DNA repair consists of a fast component (<4h) during which the bulk of DNA is repaired, and a slow component which is completed 16-24 hrs after irradiation (Dikomey et al, 1998). In the four cancer cell lines studied here, the G2 block maximum is reached 12-20 hours after irradiation, and an

additional 20-40 hours are required for the G2 block to resolve in the absence of pentoxifylline. While G2 checkpoint activation and repair may be interrelated, a definite requirement of a G2 block as a repair facilitator is not obvious. It has been suggested that pentoxifylline inhibits early repair events (Fingert et al, 1992). Pentoxifylline has been found to inhibit repair of UV-induced DNA damage in hamster cells (Link et al, 1996). For human cells no data exist on the role of pentoxifylline in repair, although caffeine has been found to inhibit repair-replication after UV-irradiation in human xeroderma pigmentosum cells (Link et al, 1995). Here we demonstrate by constant field gel electrophoresis (CFGE) that pentoxifylline significantly increases the remaining unrepaired irradiation-induced DNA dsb's after a 20h repair period in four human tumour cell lines.

A comparison of residual dsb's after 20h between untreated controls, reveal differences between the four cell lines which correspond with radiosensitivity (Table 3). No significant variation was observed in the initial DNA damage. This is in agreement with other reports showing a correlation between radiosensitivity and repair of dsb's (Schwartz et al, 1988; Whitaker et al, 1995; Dikomey et al, 1998), but contrasts with studies finding no relationship between repair and radiosensitivity (Smeets et al 1993; Olive et al, 1994), and others showing large differences in the induction of dsb (Zaffaroni et al, 1994; Ruiz de Almodovar et al, 1994). The reason for these discrepancies is not known at present, and may require more sensitive repair assays. In this study and others, differences in repair ratios only became evident after high doses (> 30 Gy). The control 20h repair ratios in the two TP53 wild type cell lines

Be11 and 4197 are higher compared to those in the two mutant cell lines, indicating a better repair capacity for the TP53 wild type cell lines.

That DNA repair is also not the major mechanism involved in the radiosensitisation by pentoxifylline is clear from the fact that the drug significantly inhibits repair in all four cell lines, but enhances radiotoxicity only in the two mutant cell lines. In the TP53 mutant MeWo melanoma cell line, repair inhibition at 20h shows an RIF of 2.37. In the TP53 wild type Be11 melanoma cell line, the 20h RIF was found to be 1.42 which is considerably lower. In MeWo cells, the 20h repair data furthermore indicate that repair inhibition by pentoxifylline is statistically signficant ($p = 0.029$), while the corresponding measurements in Be11 cells only show borderline significance ($p = 0.048$). MeWo cells exhibit an REF of 3 indicating a relatively strong radiosensitisation effect, whereas Be11 shows an REF close to 1.0 indicating no radiosensitisation. In the melanoma cell lines, repair inhibition thus strongly correlates with the enhancement of radiotoxicity and also corresponds with TP53 status and radiosensitivity (Table 1). However, this correlation is not apparent in the SCC cell lines, where no simple relationship between radiation-induced dsb's and cell survival exists. This is highlighted by the fact that in the 4451 SCC cell line, which undergoes the largest radiosensitisation, the 2h repair in the presence of pentoxifylline is not significantly different from controls ($p = 0.122$). After 20h of repair, the 4451 cells show borderline significance of repair inhibition ($p = 0.049$). In the 4197 SCC cell line, which does not undergo radiosensitisation by pentoxifylline, repair is significantly influenced by pentoxifylline at 2h and 20h ($p = 0.008$ and

p = 0.021 respectively). A lack of correlation between DNA dsb's and cell survival has previously been reported in 8 SCC and 2 lymphoblastoid cell lines, where G2 block abrogation has been found to lead to a higher frequency of unrejoined chromosome breaks, but there was no correlation between G2 phase duration and radiosensitivity (Schwartz et al, 1996). Another study on two human SCC cell lines revealed a poor correlation between dsb's and cell death, and led to the suggestion that the inherent radiosensitivity in these cells may be influenced by misrepair and damage tolerance (Smeets et al, 1994). More studies on SCC cells are needed to clarify these observations. In the TP53 wild type Be11 and 4197 cells, which show significant DNA repair inhibition by pentoxifylline, the poor radiosensitisation and better survival may possibly be attributed to the fact that these cells show a strong G1 cell cycle block (Fig. 8). DNA repair during the G1 cell cycle arrest is mediated by the GADD45 protein. GADD45 is induced in TP53 wild type cells by doses as low as 0.5 Gy, and has been shown to bind to proliferating cell nuclear antigen (PCNA) and to stimulate DNA excision repair *in vitro* (Maity et al, 1997). Whether the G1 block or this repair pathway plays a role in the survival of these SCC cells however remains to be established.

It is demonstrated here that, in certain cell types, pentoxifylline enhances radiotoxicity by inhibiting DNA dsb repair. The assumption that G2 block abrogation is the main mechanism by which radiosensitisation is accomplished (O'Connor et al, 1996; Husain et al, 1998; Li et al, 1998), could not be corroborated by these data. In the melanoma and SCC cell lines used

here, induction of apoptosis also does not play a role. From these experiments it is clear that radiosensitisation by pentoxifylline should be evaluated separately from G2 block abrogation. In the Be11 and MeWo melanoma cell lines, repair inhibition correlates strongly with radiosensitisation and TP53 status. However, in the 4197 and 4451 SCC cells, the strong radiosensitisation effect seen in the mutant cell line is not reflected by repair inhibition. The possibility that repair inhibition and G2 block abrogation act in concert, or that other mechanisms are involved in the enhancement of radiotoxicity can therefore not be excluded. The different behaviour of melanoma and SCC cell lines indicates that the mechanism by which pentoxifylline sensitises cells to ionising radiation is cell type specific. The mechanisms by which pentoxifylline influences repair also remains to be elucidated. Since TP53 mutant cancer cells generally show enhanced radiosensitisation, sparing of TP53 proficient normal tissue by pentoxifylline treatment is a real possibility.

It has been demonstrated elsewhere that suppression of the G2 checkpoint by pentoxifylline is an effective strategy whereby TP53 defective cells can be targeted for destruction by a second irradiation or chemotherapeutic insult (Wang *et al*, 1996; Binder et al, 2000). The molecular mechanisms involved in G2 block abrogation by pentoxifylline has not been elucidated, and is of critical importance for a better understanding of the potential clinical application of this drug as a radio- or chemo-sensitiser. The possibility of a modulation of the expression levels and sub-cellular location of the two

components of the mitosis promoting factor (MPF), cyclin B1 and $p34^{cdc2}$ has therefore been investigated.

The simultaneous measurement of total DNA content and cyclin B1 expression allows for a quick and relatively easy way of directly correlating cyclin B1 expression to cell cycle phase. Most previous studies have employed synchronised cells and the more laborious western blotting method to analyse for cyclin B1 expression (Muschel *et al* 1991, Bernhard *et al.* 1994 b, Muschel, Zhang and McKenna, 1993) and thus could not correlate cyclin expression and DNA content directly. Other authors have however indicated the value of flow cytometric analysis of asynchronous populations for this type of study (Sherwood *et al.* 1994). Flow cytometric results reported here revealed that cyclin B1 levels in HeLa cells begin rising in late S-phase and reach a maximum in G2 phase. No cyclin B1 expression was seen in other cell cycle phases. This confirms that HeLa cells do not exert unscheduled cyclin expression often seen in other tumour cells (Gong *et al.* 1994 b). The tendency of synchronised cells to show an unscheduled expression of cyclins and other cellular proteins has been demonstrated (Gong *et al.* 1995). For this reason we opted to use asynchronously growing undisturbed cell cultures.

My results demonstrate that the down-regulation of cyclin B1 expression plays a role in the formation of the radiation induced cell cycle delay in the G2-phase. This suppression of cyclin B1 expression probably prevents the optimum formation of the MPF which is essential for cells to cross the threshold from G2 into mitosis. These conclusions are in agreement with

other studies on synchronised cells which have relied on different methods of analysing cyclin expression after exposure to irradiation (Muschel *et al.* 1991, Bernhard *et al.* 1994 a) and after treatment with DNA damaging agents like camptothecin, etoposide and nitrogen mustard (Maity *et al.* 1996). A dose-dependency of cyclin B1 suppression observed by Villa et al (1996) cannot be refuted since we used a constant dose of 7 Gy throughout. My finding that an increase in cyclin B1 expression is concominant with the increase in G2 content is in agreement with data from Smeets *et al* (1994). However, the percentage of cells in G2-phase in asynchronous cells is small (10-15%) and virtually all the cyclin B1 originates from this G2 fraction. When these cells are irradiated, the G2 levels rise to approximately 80% and this explains the massive rise in cyclin B1 (Figure 2). However, when the cyclin B1 content is expressed in relation to the G2 content, it is seen that irradiation in fact reduces the B1/G2 ratio to below control levels. When we followed this ratio at regular intervals over 25 hours, we found that it slowly returns to normal, concominant with the recovery of the cell cycle. The approach used here solves two problems: it eliminates the need for synchronised cells, and it puts the cyclin B1 levels in perspective to the G2 fraction.

These results demonstrate that abrogation of the G2 block by Pentoxifylline restores the cyclin B1/G2 ratio to control levels within 6-8 hours after addition of the drug. This is in agreement with other studies on the influence of Pentoxifylline (O'Connor *et al.* 1993) and Caffeine (Bernhard *et al.* 1994 b) on cyclin B1 expression in nitrogen mustard treated and irradiated cells respectively. The induction of cyclin B1 expression by Pentoxifylline in G2

blocked cells resembles data on a dexamethasone-inducible promotor which abrogates the G2 block (Kao *et al.* 1997). It is therefore concluded that the level of cyclin B1 is indeed a controlling factor in the length of the G2 delay.

Formation of the MPF by the availibility of sufficient quantities of cyclin B1 and $p34^{cdc2}$ is only part of the mechanism by which cells recognise DNA damage and control the cell cycle at the G2 checkpoint. This complex has to be activated by different kinases and phosphatases in order to be functional (reviewed by Murray 1992). The decrease in cyclin B1 expression below control levels after induction of the G2 block is rather small and hence may not be solely responsible for the formation of the G2 delay. The move of cyclin B1 to the cell nucleus may also be an important event (Kakino *et al.* 1996) and has been shown to drop sharply after irradiation (Smeets *et al.* 1994). Various studies on the expression of the $p34^{cdc2}$ protein (Lock 1992, Lock and Ross, 1990), the activity of the MPF as regulated by $P34^{cdc2}$ phosphorylation (Ling *et al.* 1996, Orlandi *et al.* 1996) and the localisation of cyclin B1 (David-Pfeuty and Nouvain-Dooghe 1996, Li, Meyer and Donoghue 1997) and $p34^{cdc2}$ (Cohen-Johnathan *et al.* 1997) have indicated other levels of control at the G2 checkpoint. Answers to the role of cdc25 phosphatase and mik1/wee1 kinase activity which plays a crucial role in the activation of the MPF may further help to unravel the complexities of the G2 delay mechanism.

Expression levels of the $p34^{cdc2}$ cyclin dependent kinase was therefore measured in a similar way. These results show that irradiation induced DNA

damage also has a profound influence on the expression of $p34^{cdc2}$. The rise in $p34^{cdc2}$ expression (Fig. 18B) is in agreement with results from Smeets *et al* (1994) who reported an increase of the hyperphosphorylated inactive form of $p34^{cdc2}$ based on Western blots. Since G_2 block formation, as with the cyclin B1 measurements, is associated with an increase in the number of G_2 cells and in the level of $p34^{cdc2}$ (Fig. 18) we decided to use the $p34/G_2$ ratios to assess changes of $p34^{cdc2}$ expression relative to the number of cells in G_2 (Fig. 19). G_2, $p34^{cdc2}$ and the ratio of $p34^{cdc2}/G_2$ show a maximum at 10-14 hours after irradiation. Addition of pentoxifylline rapidly restores the $p34^{cdc2}$/G2 ratio to the control values within 8 hours (Fig. 19). It therefore can be safely concluded that $p34^{cdc2}$ is upregulated in response to DNA damage. It is possible that this upregulation results in the expression of a hyperphosphorylated, inactive form for the duration of the G_2 block (Smeets *et al*, 1994).

It appears that the existence of the two MPF components enhances the sensitivity of the complex to small changes of the concentration of either constituent. This would result in an "all-or-nothing" reaction resembling a switch. The required changes in substrate concentrations do not follow the normal Michaelis-Menten enzyme kinetics but are characterised by an ultrasensitive response (Ferrel and Machlader, 1998; Koshland, 1998). In ultrasensitive kinetics the maximum velocity is achieved by a 1-4 fold change in substrate concentration, rather than the 80-fold change observed in normal Michaelis-Menten kinetics (Koshland, 1998). The switch-like response has previously been attributed to the translocation efficiency of the cyclin/cdk

complex (Ferrel 1998, TIBS). My data suggest that changes in the concentration of cyclin B1 and $p34^{cdc2}$ indeed act in concert, resembling an ultrasensitive switch (Fig. 20). Translocation of the complex accross the nuclear membrane would further ensure rapid control of mitotic entry. The change of the cyclin B1/$p34^{cdc2}$ ratios closely resembles other ultrasensitive reactions e.g. the cooperativity of O_2 binding by hemoglobin (Koshland, 1998). These experiments only assess the expression of the required amounts of cyclin B1 and $p34^{cdc2}$ and do not address the additional activation expected from MPF phosphorylation. It is suggested here that the restoration of the control ratio of cyclin B1/$p34^{cdc2}$ expression by pentoxifylline forms a crucial part of the mechanism of action of this G_2 block abrogator.

When the influence of block abrogation on the subcellular location of cyclin B1 was assessed, no significant differences were seen between cyclin B1 expression in whole cells and in nuclei, although overall levels seem consistently higher in whole cells. A sharp increase in cyclin B1 expression occurs in nuclei during G_2 block abrogation by pentoxifylline (Fig. 21). Subsequently (after ~8 h) the B1 levels in nuclei falls sharply to even below the levels of irradiated control cells. This is most likely followed by ubiquitin mediated proteolysis of the cyclin B1 required for exit from mitosis (King *et al*, 1996). It thus appears that pentoxifylline prompts mitotic entry and also exit from mitosis.

These data suggest that G_2-block abrogation by pentoxifylline does not result in cell cycle perturbations and the uncoupling of mitosis, but in the resumption

of normal cell cycle progression. The results presented here are consistent with the hypothesis that the cell cycle is regulated in dimensions of both time (the timely synthesis and proteolysis of regulatory proteins) and space (localising these regulators to the correct site) and that methylxanthines like pentoxifylline act at both levels to disrupt the G_2 cell cycle delay. The manipulation of cell cycle checkpoints remain an important topic, not only in the application of methylxanthines like pentoxifylline, but also in general for new strategies in anti-cancer therapy.

Chapter 6

General Conclusions

The human melanoma cell lines Be11 and MeWo, the human SCC cell lines 4197 and 4451, and the human lymphocytic leukemia cell line, Jurkat J5, were used to investigate the role of the G2 cell cycle delay, DNA repair and apoptosis in cellular radiosensitisation by the methylxanthine drug, pentoxifylline. The HeLa human cervical carcinoma cell line was used to study the target molecules and mechanisms involved in the abrogation of the G2 block by pentoxifylline.

The results of this study demonstrate that G2 block abrogation is not the main mechanism involved in the radiosensitising effect of pentoxifylline, which is selective for TP53 mutant cell lines. This observation disqualifies several previous suggestions from the literature that the early enforcement of mitosis by the drug plays a major role in the enhancement of radiotoxicity (O'Connor et al, 1996; Husain et al, 1998; Li et al, 1998).

It is concluded that DNA repair inhibition and the modulation of radiation-induced apoptosis also play roles in the mechanism of action of pentoxifylline as a radiosensitiser, depending on cell type. In the TP53 mutant MeWo melanoma cell line, DNA repair inhibition is larger by a factor of 1.7 compared to that in the TP53 wild type Be11 melanoma cell line. This strongly correlates with the radiosensitisation effects measured in the melanoma cell

pair. Although the two melanoma cell lines studied here are non-syngeneic, these observations suggest an opportunity to attain a favourable therapeutic index with pentoxifylline in this tumour type. This is particularly relevant as mutations in TP53 is the most frequently noted gene alteration in the pathogenesis of malignant melanoma, and currently receives a lot of attention for its potential as prognostic indicator (Weiss et al, 1995). The specific mechanisms involved in DNA repair inhibition remains to be elucidated, but may involve the ataxia telangiectasia mutant (ATM)-related DNA-PK protein which is activated in response to DNA damage and plays an important role in DNA repair (Sarkaria et al, 1999).

That the DNA repair inhibition effect is cell type specific is clearly demonstrated by the fact that no correlation was seen between radiosensitisation and DNA repair inhibition in the 4197 and 4451 SCC cell lines. It is noted that the two SCC cell lines lack the correspondence between radiosensitivity, normal DNA repair capability and TP53 status seen in the melanoma cells, but still show preferential radiosensitisation of the TP53 cell line with pentoxifylline.

Induction of apoptotic pathways plays no role in the radiosenstisation of the two TP53 mutant cell lines MeWo and 4451. An increase in radiation-induced apoptosis is however evident in the Jurkat J5 lymphocytic leukemia cell line, which suggests that the effect of pentoxifylline on apoptosis is also cell type specific. While an enhancement of radiation-induced apoptosis by pentoxifylline may be expected in cells normally dying via apoptotic pathways

following radiation damage, this effect has also been demonstrated for caffeine in HeLa cells which normally die by mitotic death (Bernhard et al, 1996). If the induction of apoptosis by methylxanthines is not dependent on TP53 status or cellular origin, the identification of cell types likely to show increased apoptosis with pentoxifylline requires further investigation. The possibility that DNA repair inhibition or apoptosis induction and G2 block abrogation may act in concert to enhance radiotoxicity, cannot be excluded.

Taking into consideration the rather short plasma half lives of pentoxifylline and its metabolites (Honess et al, 1993), it is not surprising that the early damage responses like repair and apoptosis play a role in radiosensitisation. The formation of the G2 block is a relatively slow response which generally occurs over approximately 24h after irradiation. The mechanism of action of pentoxifylline as a G2 block abrogator is of critical importance in cancer therapy, because tumour cells can be sensitised to a second therapeutic insult by this approach (Wang et al, 1996; Binder et al, 2000).

By calculating cyclin B1/$p34^{cdc2}$ ratios, it is demonstrated that pentoxifylline stimulates cyclin B1 expression during G2 block abrogation. This novel approach may also explain contradictions in literature. A rise in cyclin B1 expression, due to the rise in the G2 population during G2 block formation, identified as increased cyclin B1 expression during G2 arrest (Smeets et al, 1994) may be an error because it neglects the level of G2 cells which also increases. My experiments on $p34^{cdc2}$ suggest an ultrasensitive "switch" which consists of two components (cyclin B1 and $p34^{cdc2}$). The ratio of these

two molecules is rapidly restored to control levels by pentoxifylline. The nuclear export of MPF after G2 block abrogation is also demonstrated. It is not known whether this modulation of cyclin B1 and $p34^{cdc2}$ expression is a direct or an indirect effect involving signal transduction pathways. The possibility exists that pentoxifylline may influence kinases involved in the phosphorylation steps responsible for MPF activity. This is supported by a recent study on caffeine-mediated suppression of the ATM and ATR kinases needed for inhibitory phosphorylation of $p34^{cdc2}$ (Sarkaria et al, 1999) and is also in line with the known function of pentoxifylline as a phosphodiesterase inhibitor.

The results presented here contribute greatly to a better understanding of the mechanism of action of pentoxifylline, both as radiosensitiser and as G2 block abrogator. The neccessity for an independent evaluation of these two effects is a novel concept. These results furthermore highlight that the drug is cell type specific, and the need to identify tumour types likely to benefit from a pentoxifylline/radiotherapy combination and treatment modalities likely to show improved therapy outcome. The selective radiosensitisation of TP53 mutant cell lines by pentoxifylline shows that the clinical application of the drug at a non-toxic dose indeed could lead to increased TP53 mutant tumour cell death, while TP53 wild type surrounding normal tissue could be spared. Since TP53 mutations occur in 60% of all tumour types, this drug and its metabolites emerge as radiosensitisers with the potential of a marked improvement of therapy. One could speculate that the benefit may be even greater in a clinical setup involving tumour hypoxia, because pentoxifylline

has the known effect of improving tumour vasculature, which could lead to an oxygen enhancement ratio (OER) of larger than one.

Publications from this thesis

1. Theron T and Böhm L: Cyclin B1 expression in response to abrogation of the radiation-induced G2/M block in HeLa cells. Cell Proliferation 31, 49-57, 1998.

2. Theron T and Böhm L: Influence of the G2 cell cycle block abrogator pentoxifylline on the expression and subcellular location of cyclin B1 and $p34^{cdc2}$ in HeLa cervical carcinoma cells. Cell Proliferation 33, 39-50, 2000.

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