The development of a differential assay for the determination of ataxia-telangiectasia heterozygosity in human subjects.

by

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Abstract

Ataxia-telangiectasia (A-T) patients exhibit increased sensitivity to ionising radiation and also demonstrate a higher than normal risk of developing cancer. Heterozygote phenotypes exhibit intermediate levels of radiation sensitivity compared to A-T homozygotes and controls and also have an increased risk of cancer, but are asymptomatic. Radiation used in diagnostic procedures could predispose such individuals to the development of cancer. Classically, clinical diagnosis only discriminates between homozygote and non-homozygote individuals, necessitating the development of a routine approach that facilitates identification of A-T heterozygotes.

Tissue culture patient derived cell lines, when used in combination with western blot analysis revealed it was possible to identify cell lines with ATM mutations compared to controls, but discrimination of each genotype was not possible using this methodology.

Similarly, flow cytometry was used to determine the cell death profiles of the same patient-derived cell lines upon exposure to a variety of cell damaging agents (ionising radiation, chemicals or chemotherapeutic drugs), with the aim of discriminating A-T heterozygotes from homozygotes and controls. This methodology revealed that cell death profiles generated in response to hydrogen peroxide were able to discriminate each of the three genotypes in both lymphoblast and fibroblast cell lineages. In SV40 patient derived lymphoblasts, the increase in late apoptotic cells and the reduction in necrotic cells in the A-T homozygote cells was found to be statistically significant compared to controls. Likewise, the reduction in late apoptotic cells and the increase in necrotic cells in the A-T heterozygote cells was also found to be statistically significant compared to both A-T homozygotes and controls (P <0.05 one-way ANOVA).

In untransformed fibroblasts, using a telomerase transformed fibroblast cell line as the control, the increase in early apoptotic cells and total apoptotic cell death (early and late apoptotic cells combined) in the A-T homozygote cells was found to be statistically significant compared to the control. The increase in early apoptotic cells and total apoptotic cell death in the A-T heterozygote cells was also found to be statistically significant compared to both the A-T homozygotes and the control (P <0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes in two different cell lineages in response to hydrogen peroxide treatment.

It had previously been reported that virus transformed cell lines produced different cell death profiles to primary or untransformed cell lines from A-T patients and carriers compared to controls following exposure to ionising radiation. Data presented here confirms these observations with additional A-T homozygote, A-T heterozygote and control cell lines in SV40 transformed lymphoblasts and untransformed fibroblasts. Data is also presented using the same patient-derived cell lines with the chemicals hydrogen peroxide and staurosphinor, and the anticancer drugs etoposide and bleomycin. The differential response of SV40 transformed cell lines and untransformed cell lines to these treatments calls into question whether virally transformed cell lines should be used to predict in vivo cellular responses.
List of Abbreviations

ALL : Acute lymphocytic leukaemia
A-T : Ataxia-telangiectasia
ATR : Ataxia and rad-related protein kinase
B-CLL : B-cell chronic lymphocytic leukaemia
bp : Base pairs
CAP kinase : Ceramide-activated protein kinase
CDC2 : Cell division cycle 2
CDK : Cyclin-dependent kinase
CDNA : Complementary DNA
Chk1 : Checkpoint kinase-1
Chk2 : Checkpoint kinase-2
CL : Cytoplasmic lysate
DD : Death domain
DED : Death effector domain
DEM : Diethylmaleate
DNA-PK : DNA protein kinase
DNA-PKcs : DNA protein kinase catalytic subunit
dUTP : Deoxyuridine triphosphate
FACS : Fluorescence activated cell sorting
GSH : Reduced glutathione
Het : Heterozygote
IGF-1R : Insulin-like growth factor-1 receptor
IR : Ionising radiation
kb : Kilo bases
kDa : Kilo Daltons
MAP kinase : Mitogen-activated protein kinase
MARCKS : Myristoylated alanine-rich C kinase substrate
MDM2 : Mouse double minute 2
NL : Nuclear lysate
ORF : Open reading frame
PARP-1 : Poly (ADP-ribose) polymerase
PBLs : Peripheral blood lymphocytes
PCNA : Proliferating cell nuclear antigen
PHA : Phytohaemagglutinin
PI : Propidium iodide
PI-3-kinase : Phosphatidylinositol-3-kinase
PKC : Protein kinase C
PKCS : Protein kinase C δ
pRB : Retinoblastoma protein
PS : Phosphatidylerine
RDS : Radio-resistant DNA synthesis
ROS : Reactive oxygen species
RPA : Replication protein A
SAPK : Stress-activated protein kinase
TCR : T-cell receptor
UTR : Untranslated region
VP-16 : Etoposide
WL : Whole cell lysate
γ-H2AX : Phosphorylated H2AX
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XVIII
General Introduction.

1.1 Ataxia-telangiectasia.

Ataxia-telangiectasia (A-T) is a genetic disease that displays an autosomal recessive pattern of inheritance (Taylor et al. 1994), and was first named as a disorder and described in terms of its pathogenesis in 1957 (Boder and Sedgwick, 1957; 1958). These were the first reports to indicate that the condition was hereditary, along with documenting the neurological symptoms of the disease and the incidence of sinopulmonary infection in patients with the condition. A fuller picture of the clinical features evolved over the years, and includes cerebellar ataxia, dilated blood vessels in the eyes and skin (oculocutaneous telangiectasias), underdevelopment of some organ systems, and a 100-fold increase in the risk of some types of cancer such as lymphoma (reviewed by Gatti et al. 1991). Growth retardation, premature ageing (reviewed in Jackson, 1995; Lehmann and Carr, 1995; Zakian et al. 1995), delayed puberty, and less commonly, insulin resistant diabetes (Rotman and Shiloh, 1997) are also clinical features of this syndrome. At the cellular level, immunodeficiency, chromosomal instability, hypersensitivity to ionising radiation (IR) and radiomimetic drugs, (reviewed by Gatti, 1991) and radio-resistant DNA synthesis are common occurrences (Reviewed in Jackson, 1995; Meyn, 1995). A-T is estimated to affect between 1 in 40,000 and 1 in 100,000 live births (Swift et al. 1986). The disease has been recognised in all races throughout the world, and seems to be equally distributed between the sexes (Boder and Sedgwick, 1970).

1.2 A-T heterozygotes.

It has been estimated that between 1-2% of the general population are A-T heterozygotes (Gatti et al. 1991). Unlike A-T homozygotes, A-T heterozygotes are clinically asymptomatic (Swift et al. 1986, 1991; Taylor et al. 1996; Wright et al. 1996) and show intermediate levels of radiosensitivity, between that of A-T homozygotes and normal subjects (Taylor et al. 1985; Gatti et al. 1991; Zakian, 1995). It has been reported that this carrier status confers a 3-5-fold greater risk of cancer (Swift et al. 1991; Easton, 1994; Weeks et al. 1991) including a higher incidence of breast cancer (reviewed in Jackson, 1995; Lehmann and Carr, 1995; Zakian, 1995). Stankovic et al. (1998) reported the identification of a single ATM mutation (7271T→G) that could confer an increased risk of developing breast cancer in both homozygotes and heterozygotes. This mutation was identified in five people within two families, and both families had a history of breast
cancer. In family 46, two out of three sisters of the heterozygote father carrying this mutation developed breast cancer, at the ages of 50 and 55 years. Studies conducted on breast cancer patients, show however, that there are discrepancies in the contribution of A-T heterozygosity to the development of breast cancer. For example, Fitzgerald et al. (1997) concluded that ATM heterozygous germline mutations did not contribute to early onset sporadic breast cancer, a notion reinforced by Chen et al. (1998) who concluded that the contribution of heterozygous ATM mutations to familial breast cancer was minimal, and Bebb et al. (1999) failed to identify any A-T heterozygotes in 47 women with later onset breast cancer. However, a recent study on Dutch breast cancer patients indicated a nine-fold increased risk of heterozygous ATM carriers developing breast cancer (Broeks et al. 2000). The fact that A-T homozygotes are highly sensitive to the effects of IR is well documented, as is the intermediate radiation sensitivity of A-T heterozygote fibroblasts (Paterson et al. 1979; West et al. 1995). Interestingly, all the breast cancer patients tested for ATM mutations in this study had low-dose diagnostic radiation in the early years of life, to screen for tuberculosis. In addition to this, all the patients had received radiation treatment for their first breast tumour. It is therefore possible that the radiation treatment or x-rays may have acted as a stimulant for the development of breast cancer in the A-T heterozygotes identified in the study. Another report suggests that 8.8% of all breast cancer patients could be A-T heterozygotes, and furthermore, they may be responsible for underestimating radiation therapy tolerance doses by as much as 15-20%. In 1965, a boy with A-T died after being treated with only 70% of the tolerance dose (Gotoff et al. 1967). Following this event, fibroblast cell lines were generated from A-T homozygote and heterozygote subjects. The cell lines were used in radiation experiments to determine the dose required to kill 90% of the cells compared to normal cells. The cells from A-T homozygotes were shown to be extremely sensitive to IR such that only 32% of the dose used on normal cells was sufficient to kill the same number of A-T cells. A-T heterozygote cells were also found to be sensitive to IR, but not as sensitive as the A-T homozygote cell lines. The dose required to kill 90% of A-T heterozygote cells was 75% (Nagasawa et al. 1987). If the sub clinical radiosensitivity of A-T carriers is responsible for lowering the threshold for the normal tolerance dose, and 8.8% of breast cancer patients are A-T heterozygotes, this means that >90% of breast cancer patients could be treated with higher doses of radiotherapy. This would have major effects on the efficacy of radiotherapy in the treatment of breast and other types of cancer (Gatti et al. 1991). This factor alone warrants the development of a diagnostic screening procedure for the identification of A-T
heterozygotes within the general population, especially for patients who develop breast cancer. Additionally, if diagnostic radiation was indeed the stimulus for the development of breast cancer in the study carried out on Dutch breast cancer patients, then there are obvious implications for using routine mammograms in screening for breast cancer, the use of X-rays to identify pathological conditions such as lung disease, and the routine diagnostic X-rays employed to identify injuries such as bone fractures and in dentistry to identify dental decay. Considering the bystander effect of radiation, these routine, diagnostic screening procedures may also aid the development of other types of malignancy for those individuals carrying ATM mutations.

The ability to identify patients carrying ATM mutations would benefit these individuals in several ways. Identification of ATM carrier status would allow clinicians to minimise the use of x-rays for diagnosis, and where a malignancy was identified would permit a more appropriate treatment regime to be used, specifically tailored to the degree of sensitivity observed to the treatment, and also, the treatment could be more closely monitored if the clinician was aware of the possible risk to the patient in using radiotherapy and chemotherapeutic drugs. This close monitoring of individual patients would also benefit people without mutations in the ATM gene, as these patients may therefore be able to be treated with a more effective dose of radiation/chemotherapy, which may increase dramatically the survival rates in patients without ATM mutations. Additionally, although ATM carrier status has been linked to an increased risk of developing breast cancer, identification of A-T carriers may also aid in the collection of data to determine the incidence of A-T heterozygotes with other types of cancer. For example, it has been reported that A-T heterozygotes may also have an increased risk for the development of B-cell chronic lymphocytic leukaemia (B-CLL) (Stankovic et al. 1999; Bullrich et al. 1999). A-T heterozygote cell lines have been shown to be just as resistant to early apoptosis as A-T homozygote cell lines (Shigetta et al. 1999), which may contribute to the notion that defective apoptosis may aid survival of B-CLL tumours.

1.3 Clinical diagnosis of A-T.

Clinical diagnosis for the presence of A-T is usually sought after the presentation of ocular telangiectasia and symptoms linked to neurodegeneration. Diagnosis is usually confirmed by demonstrating increased α-fetoprotein levels in these patients. However, some young patients do not show progressive ataxia, others do not develop telangiectasia, and some
patients may present with a mild phenotype. Due to these factors, additional tests are also used to confirm the presence of A-T, and include the absence of ATM protein on immunoblots, the lack of ATM kinase activity, and increase in chromosomal breaks, radioresistant DNA synthesis and reduced cell colony survival after exposure to γ-radiation. None of the currently employed methods for the diagnosis of A-T are 100% specific or 100% sensitive, and for these reasons, laboratory tests always need to be correlated with the clinical presenting features of the patient (Lavin et al. 2007). Diagnosis of A-T would, ideally be identified by detecting the ATM mutations present in A-T patients. This involves detecting mutations in the genetic code by amplifying genomic DNA, which due to the large size of the gene, has to be carried out using multiple reactions. Once the DNA has been amplified, additional procedures are then required to detect the mutations. Methods that have been used for this purpose include DNA sequencing, protein truncation test, restriction endonuclease fingerprinting, single strand conformation polymorphism and heteroduplex analysis. However, the number of mutations detected using these methods was lower than the number of mutations that were expected (Telatar et al. 1996; Stancovic et al. 1998; Sandoval et al. 1999), and so it would seem that these methods do not offer any additional sensitivity or specificity over diagnostic procedures currently used for the clinical diagnosis of A-T. Additionally, the exceptionally large size of the ATM gene, and the fact that there are no mutational hot spots, renders these methods of analysis impractical for routine clinical screening (Lavin et al. 2007). Due to the many tests that are required to confirm a clinical diagnosis of A-T, the process can take weeks to perform. For example, genetic screening, which is only one of the tests required to confirm a diagnosis takes 8-10 weeks to complete. Genetic screening is available for carriers within A-T families where the mutations are already known, and in this instance takes 3 weeks to complete. However, these tests are only usually conducted if there is a family history of A-T. At present, mutation screening costs $4800.00 to sequence the ATM gene for possible mutations and $400.00 where the mutation has already been identified in a family member (A-T children’s project; http://www.communityatcp.org/NETCOMMUNITY/Page.aspx?pid=596&sreid=1200).

Both the time required for testing and the cost of the procedure emphasise why this approach to routine clinical screening has not been adopted to date. This thesis was therefore interested in investigating methods that could be used as a preliminary screening procedure to discriminate A-T heterozygotes from A-T homozygotes and controls.
1.4 Study design.

1.4.1 Western blot for measurement of ATM expression.

Genetic sequence analysis has been considered not to be practical for clinical screening due to the exceptionally large size of the ATM gene (Lavin et al. 2007). Additionally, genetic mutation analysis often requires the use of sophisticated and expensive equipment, precluding the number of research facilities able to carry out this work. Screening of patient derived cell lines has also shown that genetic analysis does not always detect ATM mutations and does not offer increased sensitivity or specificity over methods currently employed to make a diagnosis of A-T (Telatar et al. 1996; Stancovic et al. 1998; Sandoval et al. 1999). However, the absence of ATM protein on immunoblots is one of the tests already used in the differential diagnosis of A-T (Lavin et al. 2007), and relies that the majority of mutations that occur in the ATM protein are predicted to lead to protein truncation (Stankovic et al. 1998). Gilad et al. (1996) also observed this phenomenon in three of five A-T cell lines, and concluded that elements near the carboxyl terminus of the protein were required for protein stability. Therefore, it should be possible to detect A-T homozygotes, who exhibit little or no protein on western blots, as well as A-T heterozygotes, exhibiting a reduced ATM protein expression (40-60%) when compared to normal samples. The obvious advantages to this type of protein assay (western blots) are its ease and efficiency of use when compared to DNA-based assays, the equipment and reagents are relatively inexpensive, and the procedure could be carried out extensively in a wide range of laboratories. Western blotting has previously been used to demonstrate the absence or reduced expression of the protein in A-T cell lines, and the reporting of less than normal protein levels in A-T heterozygote cell lines (Telatar et al. 1996). In a study which examined the correlation between the ATM genotype and phenotype using western blotting, 85% of the cell lines expressed no ATM protein, and 15% produced reduced levels of the protein. Additionally, 90% of A-T heterozygotes also expressed less ATM protein (Becker-Catania et al. 2000). Although the study highlighted difficulties with quantifying the amount of ATM protein detected, the results observed for ATM expression in A-T homozygote and heterozygote cell lines was encouraging for its use in a screening assay.

The experimental design included using a panel of antibodies with specific peptide target sequences to different regions of the ATM protein, to determine if this approach could improve the sensitivity and specificity of the assay in detecting ATM mutations in patient-
derived cell lines. The experimental design also included the use of both SV40 transformed lymphoblastoid cells and untransformed fibroblast cell lines to determine if A-T heterozygotes could be identified in both cell types, or whether the use of a specific cell type would aid discrimination in detecting A-T heterozygotes.

1.4.2 Induction of cell death profiles using γ-irradiation, chemicals and chemotherapeutic agents.

It has previously been suggested that radiation induced apoptosis could form the basis of a screening assay for the identification of A-T heterozygotes (Bebb et al. 2001) as A-T heterozygotes were able to be discriminated from A-T homozygote and control cells using the hypodiploid and TUNEL assays to measure apoptosis in Epstein-Barr virus transformed B lymphocytes. However, IR is only one treatment that is used for the treatment of cancer. Chemotherapeutic drugs whether used alone or in combined therapy are frequently employed as well. A-T heterozygote cells have also been shown to exhibit intermediate sensitivity to cell death following treatment with neocarzinostatin (Shiloh, et al. 1982), streptonigrin and etoposide (Pernin et al. 1999), and these observations suggest that A-T heterozygotes may show intermediate sensitivity to a range of anti-cancer drugs, which would mirror the response of these cells to IR. These drugs may therefore also be useful in a screening assay to discriminate A-T heterozygotes from A-T homozygotes and controls. This thesis was therefore interested in determining the response of A-T homozygote and heterozygote cells in response to a variety of DNA damaging agents including IR, etoposide, hydrogen peroxide (HP), staurosporine, doxorubicin and bleomycin in combination with tissue culture and flow cytometry. In undertaking these approaches it was hoped that the efforts would aid the differential determination of suitable markers of A-T heterozygosity.

1.4.3 Traditional assays used for measuring cell death profiles.

Although the hypodiploid and TUNEL assays had previously demonstrated that both these methods were independently able to discriminate A-T heterozygotes from A-T homozygotes and controls, different levels of cell death were produced using these assays in response to IR (Bebb et al. 2001). Additionally, the hypodiploid and TUNEL methods are used to detect apoptosis by measuring the DNA content or the presence of nicked DNA respectively. The hypodiploid method is based on a method by Nicoletti et al. 1991, and measures decreases in DNA content of the cells to detect apoptosis, as cells with normal
DNA content show G1-M-G2 cell cycle peaks on histograms measured by flow cytometry. However, this method does not work equally well in all cell lines. For example MCF-7 cells are deficient in caspase-3 (Smith, G.C.M. et al. 1999), which is required to cleave ICAD so that CAD can enter the nucleus and degrade the DNA, and so would not be an appropriate cell line to use with the hypodiploid method. The TUNEL assay also measures apoptosis by detecting DNA fragmentation at “nicked” sites. In this instance, an enzyme (terminal deoxynucleotidyl transferase) adds labelled deoxyuridine triphosphate (dUTP’s) to the nicked DNA ends (Negoescu et al. 1996; 1998). Although the original methodology for TUNEL analysis exhibited problems in not being able to discriminate apoptotic from necrotic cells, the improved methodology has been optimised to identify cells in the late stages of apoptosis (Negoescu et al. 1996; 1998). However, this thesis was interested in determining the cell death profiles of A-T homozygotes and heterozygotes in response to a variety of DNA damaging agents, and while the literature frequently reports on the apoptotic response on a wide variety of cell lines in response to various treatments, data showing necrotic cell death profiles is less frequent. This may be due in part to the type of assay used to measure cell death, e.g. TUNEL assay, but the fact that A-T patients can suffer massive tissue necrosis in response to IR necessitated inclusion of the measurement of this form of cell death in addition to apoptosis. For these reasons, cell death profiles were measured using annexin-V and propidium iodide in combination with flow cytometry.

1.4.4 The use of annexin-V and propidium iodide to measure apoptosis and necrosis.
Annexin-V and propidium iodide (PI) in combination with flow cytometry is commonly used to measure apoptotic and necrotic cells. Phosphatidylserine (PS) resides on the inside of the plasma membrane, but is externalised to the outside of the membrane when cells are undergoing apoptosis. This event has been shown to precede the nuclear changes that occur during this process, such as DNA degradation, and has also been shown to occur several hours before membrane integrity is breached, and so this translocation is detected early in the apoptotic process. This event has been demonstrated in various human and mouse tissues, and has been previously detected after treatment with a variety of apoptosis-inducing agents (Martin et al. 1995). The means by which this translocation was visualised was by utilising FITC conjugated annexin-v, which has been shown to bind preferentially to PS (Tait et al. 1989; Andree et al. 1990; Thiagarajan and Tait. 1990). The DNA stain PI was utilised as a marker of necrosis as this stain is only able to enter the cell following a breach in the plasma membrane. This has been demonstrated by its use to show membrane translocation.
integrity to confirm that annexin v binds to PS on the outside and not the inside of the membrane during apoptosis (Martin et al. 1995).

1.4.5 The use of SV40 transformed lymphoblasts and untransformed fibroblasts to induce cell death profiles.
A range of cell types were used in this study because it was previously shown that it was not possible to discriminate A-T heterozygotes from A-T homozygotes or control peripheral blood lymphocytes (PBLs) as these cells responded differently to transformed cells when treated with γ-radiation (Bebb et al. 2001). Cell lines utilised therefore included both SV40 transformed lymphoblastoid cell lines and untransformed fibroblast cell lines. It was hoped that using these two types of cell line, would provide data on whether these cells exhibited similar or different cell death profiles and whether the cell death profiles observed were cell type specific or whether transformation of the cell lines has an effect on cell death profiles.

1.4.6 Selection of cell death-inducing stimuli.
In selecting the DNA damaging agents to be utilised in the study, γ-radiation was selected to determine if the cell death profiles produced by the A-T homozygote and heterozygote cells would also include necrotic as well as apoptotic cells, as research in this area seems to have only reported apoptosis in A-T cell lines. The chemotherapeutic agents etoposide and doxorubicin were chosen for inclusion in the study as these drugs are used to treat a wide variety of different types of cancer, including cancers of lymphoid origin that commonly develop in A-T patients (Fujino et al. 2002; Róžalski et al. 2005). Both these drugs are Topoisomerase II inhibitors, and while staurosporine is not classed as an anti-cancer drug, like etoposide and doxorubicin, staurosporine is also a Topoisomerase II inhibitor. However, this agent is also a protein kinase inhibitor and so has the capacity to inhibit ATM (Tee and Proud, 2001) and so for these reasons was considered to be an interesting agent for comparative studies with etoposide and doxorubicin. Bleomycin is an anticancer drug that exerts its effects by producing reactive oxygen species (ROS) such as hydroxyl radicals, superoxide and HP, that are able to interact with DNA (Mahmutoglu et al. 1987) causing both single and double strand breaks in DNA (Shaham et al. 1983). Ionising radiation and etoposide also produce ROS that induce DNA double strand breaks (Benítez-Bribiesca and Sánchez-Suárez, 1999; Kurtz et al. 2004). However, HP in contrast to these other treatments is one of the ROS produced by the action of the other treatments, and
induces single strand breaks in DNA (Benítez-Bribiesca and Sánchez-Suárez, 1999). A-T cells have been reported as being in a continuous state of oxidative stress (Rotman and Shiloh, 1997a; 1997b), and therefore artificially inducing oxidative stress in these cells utilising HP was also considered to be a valuable agent for inclusion in the study.

It was therefore hoped that this research would aid the discrimination of A-T heterozygotes from A-T homozygotes and controls using an optimised western blotting assay. Additionally, it was hoped that the research would provide valuable information on the cell death profiles in response to a variety of cell death-inducing stimuli, with the specific aim of identifying if the cell death profiles exhibited by the cell lines, could be used as a marker (s) for A-T heterozygosity.

1.2 The ATM gene.

Analysis of A-T patients, suggested that A-T could be divided into five complementation groups (Savitsky, et al. 1995a), based on the introduction of a mutant chromosome that was able to restore normal function to a cell that already had a mutation in the homologous chromosome. As each mutation is different, the two chromosomes together, contain one complete copy of the genetic material. However, a single gene has since been found to be mutated in all complementation groups, and has been identified by positional cloning (Savitsky et al. 1995a). The genetic locus has been demonstrated as chromosome 11q22-23 (Gatti et al. 1998). This gene, designated ATM (Ataxia-Telangiectasia Mutated), spans approximately 150 kilo bases (kb) of genomic DNA (Uziel et al. 1996), which codes for a 13kb transcript (Savitsky et al. 1995b; Uziel et al.1996), consisting of 9168 nucleotides. This transcript codes for a protein that is estimated to contain 3056-amino acids, with an approximate molecular weight of 350 kilo Daltons (kDa) (Savitsky et al. 1995b; Byrd et al. 1996). The ATM gene contains 66 exons, i.e. the translated regions that code for protein (Uziel et al. 1996). The first two exons were found to be alternative leader exons, and were designated 1a and 1b respectively. The first methionine of the open reading frame lies within exon 4. The stop codon is located in the 3' and largest exon, which is 3.8kb. The same exon has a 3' untranslated region (UTR) of approximately 3600 nucleotides. The majority of exons in the ATM gene, with the exception of the 3' exon, range from 43 to 634 base pairs (bp), with a mean of 152bp. There is also considerable variation in the size of the introns in the ATM gene. They range from 100 bp to 11kb, with the mean falling into a range of 1-3kb. The consensus dinucleotides GT and AG were found to be present at the
donor and acceptor splice sites of all introns except one. This variant donor site had a GC dinucleotide which was located in the intron 3' to exon 52 (Uziel et al. 1996). The protein product of the wild type ATM gene (so called because it is mutated in the genetic disease A-T) has been shown to contain sequence homologies with a number of proteins in both mammals and yeast that have been conserved throughout evolution.

ATM has been designated as a member of a family of unusually large (250 to 460kDa) proteins, which function in cell cycle regulation and/or DNA damage recognition and repair (Meyn, 1995; Zakian, 1995). This family of proteins, shares a highly conserved carboxyl terminal region of about 300 amino acids that shows high sequence similarity to the catalytic domain of phosphatidylinositol-3-kinases (PI-3-kinases) (approximately residues 2500-3000) (Savitsky et al. 1995a,b). PI-3 kinases function in signal transduction and phosphorylate inositol lipids that act as intracellular second messengers (Toker and Cantley, 1997). ATM, however, shares most sequence similarity with a subgroup of the PI-3 kinases that are involved in phosphorylation of proteins rather than lipids, and like ATM, are involved in cell cycle regulation, and/or detection and signalling of DNA damage (Zakian, 1995). The family members share 30% identity and 60% similarity in the PI3-like kinase domain and 20% identity and 50% similarity in the RAD3 domain. (Morrow et al. 1995).
Figure 1.1 Schematic diagram showing the areas of sequence homology in the PI-3 kinase and RAD3 domains between several proteins (redrawn from Savitsky et al. 1995a). Additional information is provided on the position of the ATM mutation(s) with respect to areas of sequence homology for the cell lines used in the current research.
Members of this family include the *Saccharomyces cerevisiae* proteins, Tor1p and Tor2p and their mammalian homologues, FRAP and RAFT1, which are all involved in progression of the cell cycle from G1 to S-phase (Brown *et al.* 1994; Sabatini *et al.* 1994). FRAP is 44% identical to Tor1 and 46% identical to Tor2 overall. The area with the greatest sequence homology is the C-terminal 660 amino acids, where FRAP is 57% identical to Tor1 and 59% identical to Tor2 (Brown *et al.* 1994). RAFT also shares identical regions with Tor1 and 2 (39% and 43% respectively) (Sabatini *et al.* 1994). TEL1p and MEC1p in the budding yeast *Saccharomyces cerevisiae* (Morrow *et al.* 1995), and the Mec1p homologues, from *Schizosaccharomyces pombe*, (RAD3) (Bentley *et al.* 1996) and *Drosophila melanogaster*, (mei-41) (Hari *et al.* 1995), are the proteins that are most closely related to ATM. The RAD3 domain contains a leucine zipper (Chen, 1996). As is the case with ATM, defects in or absence of these proteins leads to genomic instability, hypersensitivity to DNA damaging agents, and defects in DNA damage-induced cell cycle checkpoint regulation, which are classical features of A-T. Mutations in TEL1 result in a decrease in telomeric length (Greenwell *et al.* 1995), a feature that is also present in the A-T syndrome (Metcalf, 1996). The same mutation results in an increase in both chromosome loss and mitotic recombination, but does not lead to sensitivity to IR or radiomimetic drugs. Deletion or truncation of TEL1 has no effect on growth or viability, whereas similar mutations in MEC1 resulted in non-viable cells. The same loss of telomeric length was not observed in MEC1, Tor1 or Tor2-carrying mutations; therefore, this property is not a feature of all proteins included in this group (Greenwell *et al.* 1995).

In humans, ATM, ataxia and rad-related protein kinase (ATR) (Cimprich *et al.* 1996) and DNA protein kinase catalytic subunit (DNA-PKcs) (Hartley *et al.* 1995) are also members of this sub-group. DNA-PKcs interacts with the heterodimeric protein Ku, which itself binds to DNA ends (Dvir *et al.* 1992). When this complex binds to DNA double strand breaks, the kinase function is activated (Gottlieb and Jackson, 1993). Cells that lack DNA-PKcs are also hypersensitive to IR (Jackson, 1995). In view of these findings, and sequence homologies, it would be reasonable to suggest that ATM may have functional similarities with these genes.

The N-terminal half of the gene was sequenced by Byrd *et al.* (1996). The sequence contained 1348 amino acids. No homologies between the N-terminal half of ATM and MEI-41, TEL-1 or MEC-1 proteins were found. A leucine zipper motif was identified in
ATM in a similar region to the leucine zipper in DNA protein kinase (DNA-PK), although there appeared to be little sequence homology between them. The N-terminal half of the protein was sequenced for mutations, and like the C-terminal, mutations were spread throughout the length of the protein with no hotspots. There was one region between amino acids 709-880 that contained three mutations, and so the authors suggested that this region could be an important area for correct ATM function. They also observed this cluster of mutations to be outside the region of the leucine zipper.

Beside these sequence homologies, which suggest a similar functional role to family members in cell cycle checkpoints and DNA damage and repair, there are features of the A-T phenotype that offer additional roles for the function of ATM.

Although ATM is almost exclusively a nuclear protein, in some cells, it has been found to be cytoplasmic, and has been shown to bind to the vesicle protein β-adaptin (Lim et al. 1998), which is associated with coated pits formed from plasma membrane-forming coated vesicles. Cytoskeletal alterations and cell membrane defects have also been reported in A-T (Shiloh, 2001).

ATM function in growth factor mediated pathways has also been reported. Epidermal growth factor causes transcriptional down-regulation of the ATM gene. The decrease in ATM expression substantially reduced activation of transcription factor Sp1 (Shiloh, 2001). ATM has also been shown to interact with the insulin-like growth factor-1 receptor (IGF-1R). This is a transmembrane tyrosine kinase receptor that functions in growth regulation, cell differentiation, cell transformation and apoptosis (Baserga et al. 1997). A-T cells express low levels of IGF-1R, and exhibit reduced IGF-1R promoter activity. Transcriptional expression of IGF-1R was found to be ATM-dependent (Peretz et al. 2001).

1.5.1 Mutations in the ATM gene.
A large number of different mutant ATM alleles exist in the general population, as over 300 mutations have been identified in the open reading frame (ORF). The A-T phenotype arises due to ATM null alleles that truncate (frame shift or stop codon mutations), or severely destabilise (mutations that affect mRNA splicing) the protein (Gilad et al. 1996). In one study alone, investigating ATM mutations in 36 unrelated A-T patients and two
control cell lines, the sequence alterations observed included nucleotide substitutions (2), insertions (1) but more frequently, deletions (27) of from 2 to 298 nucleotides (Wright et al. 1996). When the location of these sequence alterations were analysed, taking into account the exon-intron structure of the ATM gene (Uziel et al. 1996), many of the deletions were found to occur due to either one or two exons being incorrectly spliced. Of the 30 sequence differences observed, 25 of these were shown to be different, one occurred three times, and the same alteration in complementary DNA (cDNA) was observed in two pairs of cell lines. Of the 25 different sequences identified, 19 were predicted to lead to protein truncation. Where the same alteration in cDNA was detected, genotyping confirmed that the loss of exons detected in these samples arose due to different mutations. The mutations detected were broadly distributed throughout the gene, which highlights the difficulty in correlating specific genetic aberrations with defects in a specific protein function. Two fibroblast cell lines not derived from A-T patients or their families, GM00637 and LM217, were screened using single strand conformation polymorphism for mutations across the entire ATM cDNA sequence. Both GM00637 and LM217 have been used frequently as controls in radiation biology experiments, and their responses to radiation are well characterised (e.g. Ziv et al. 1995). Despite their apparently normal radiation response, aberrant splicing products were detected in both LM217 and GM00637, which would be predicted to truncate the ATM protein (Wright et al. 1996). As the aberrant splicing products detected in these cell lines do not seem to be linked to IR sensitivity, analysis of mutations at these sites, and analysis of A-T phenotypes in such patients may yield information on the involvement of these genetic loci in ATM function. These results suggest that there may be a large number of different mutant ATM alleles in the general population, but as no one abnormal cDNA sequence was detected more than three times in this panel of 36 A-T patients, this suggests that they do not occur at a high frequency (Wright et al. 1996). Similar observations were made in a study of ATM mutations in A-T families native to the British Isles. In this study, 51 different mutations were found in 60 A-T families, 71% of these, were predicted to lead to protein truncation. None of the cell lines generated from the patients with ATM truncating mutations showed detectable ATM protein (Stankovic et al. 1998). Gilad et al. (1996) also observed this phenomenon in three of five A-T cell lines. They suggest that the reason for this is that elements near the carboxyl terminus are required for its stability and that when these elements are mutated or deleted, the protein becomes unstable and is degraded. This is also the view of Professor A.M.R. Taylor (University of Birmingham) (personal communication). However, patients
who were identified as having either missense mutations or in-frame deletions of the ATM protein expressed detectable full length ATM protein (Stankovic et al. 1998). A further study carried out on German A-T patients identified 46 different mutations on 25 of the 132 alleles, as well as 26 polymorphisms. The truncating mutations occurred throughout the whole coding sequence of the gene, again indicating the absence of hot spots for mutation. Three termination mutations were localised downstream of the kinase domain (Sandoval et al. 1998). In the same study, an A-T cell line with a valine deletion near to the kinase domain only showed a 20% reduction in protein expression, indicating near normal expression in this cell line. Lakin et al. (1996) reported a group of A-T patients that exhibited a less severe phenotype of the disease. Interestingly, they showed residual ATM expression, and furthermore, the ATM was full length. Residual protein function has also been documented in A-T patients with 5762ins137 splice site or a 7271T→G missense mutation. Despite the protein expression in 5762ins137 cells being at low levels, the kinase activity of this protein was no different from control cells. However, although the protein expression of 7271T→G cells was near normal, its kinase activity was only 6% of that obtained with controls. Induction of p53 was intermediate between classical A-T cells and controls with both these mutant cell types. However, phosphorylation of Nbs1 and hMre11, which are the proteins involved in double strand break repair, was only evident in normal and 7271T→G cells. Both these mutations have been linked with the patients having a less severe phenotype than classical A-T patients, and the ATM kinase activity correlates with the severity of neurological disorder of the patients (Stewart et al. 2001). There is obviously a link between the amount of full-length functional protein present, and severity of the syndrome in A-T patients. However, in one family a total homozygous deletion of the ATM gene has been identified. This shows that the ATM gene is not essential for life (Lehmann and Carr, 1995).

The Drosophila melanogaster gene, mei-41, is one of the closest related proteins to ATM, sharing both functional and primary sequence homology with ATM. This gene is also characterised by a large number of different mutant alleles. Like A-T homozygotes, and to a lesser extent, heterozygotes, Drosophila larvae are sensitive to IR (Hari et al. 1995).
1.6 Clinical symptoms of A-T

1.6.1 Cerebellar Ataxia.

Ataxia means an imbalance of muscle control, and the cerebellum is part of the hindbrain that is responsible for motor function, muscle tone and balance. Hence, clinical symptoms of cerebellar ataxia are uncontrolled muscle movements that lead to an unsteady posture, and are a direct result of degeneration of purkinje cells. Cerebellar ataxia is present in all cases of A-T and is always the first clinical symptom to be recognised. It is usually detected between 12-14 months of age, when the child is learning to walk. Young children usually show other signs of cerebellar disease including speech disorders resulting from weakness or incoordination of the speech muscles (dysarthria), low muscular tone (muscular hypotonia), resulting in slow initiation and performance of all voluntary movements, and poor posture. Low facial muscle tone (hypotonic facies) is often accompanied by drooling (Boder and Sedgwick, 1958; Zecevic and Rakic, 1976). The truncal and gait ataxia that is also seen in A-T patients is not as evident in the early years of life due to the child developing better motor skills with age, but the progressive nature of the disease results in patients requiring the use of a wheelchair by the age of 10 or 11 years (Gatti, et al. 1991), and being wheelchair-dependent by the age of 20 (Urhammer et al. 2000). Oculomotor abnormalities are present in nearly all A-T patients (Boder and Sedgwick, 1958; Zecevic and Rakic, 1976). When the head is stationary, voluntary eye movements are slowly initiated and are quite often interrupted, but can be completed given sufficient time. However, when the head is turned quickly, the eyes initially move in the opposite direction before following the direction in which the head was turned (Zecevic and Rakic, 1976).

Most young children with A-T have normal muscle strength. The plantar response is a test used to identify upper neuron lesions. An equivocal or flexor response is usually evident. Children with A-T have intact superficial sensation and normal deep tendon reflexes in their early years, but these usually decrease by the age of 7 or 8 years. Following adolescence, the neurological features of the disease are intensely progressive. There is peripheral neuropathy and loss of both vibratory and position sense. The severity of the neurological features appears to differ between patients (Zecevic and Rakic, 1976). Patients in their twenties and early thirties suffer progressive spinal muscular atrophy, which results in sustained, involuntary muscle spasms (dystonia). These involuntary movements also
cause abnormal postures that additionally affect the hands and feet (Aguillar et al. 1968; Amromin, et al. 1979).

Although there is progressive cerebellar degeneration in these patients, they are usually of normal intelligence (Gatti et al. 1991; Uhrhammer et al. 2000), as most patients have IQ scores that are within the normal range or higher. Some patients in their twenties and thirties experience an unexpected severe loss of short-term memory, but it is thought that this may be more to do with premature ageing (Boder, 1985; Sedgwick and Boder, 1972). A-T patients usually die in their twenties or thirties (Savitsky et al. 1995a).

1.6.2 Telangiectasias.
Telangiectasia means dilated capillary vessels, and in A-T patients, the vessels are permanently dilated (Lehmann and Carr, 1995), and usually present in the eyes first. Other common sites of capillary dilation are the ears, the bridge of the nose and behind the knees. Telangiectasias normally develop between the age of 3 and 5 years, which is usually two to four years after the development of the ataxia syndrome. This aspect of the A-T syndrome is also progressive, and increases in severity with age. Long-term studies into oculocutaneous telangiectasias suggest that the changes are progeric, as they resemble those found in the aged, both in appearance and location. There is also evidence for other progeric changes, including grey hair, and atrophic and sclerodermoid facial skin. The appearance of grey hair occurs even in young children with A-T, and increases until adolescence. However, the rate of progression following adolescence appears to be normal. The changes that occur in the facial skin usually accompany adolescence (Zecevic and Rakic, 1976). It was previously thought that telangiectasias only affected the external surfaces of the body, but recently, three cases of haemorrhage due to bladder telangiectasia have been reported. One child had been treated for refractory steroid-resistant and intravenous immunoglobulin-resistant severe autoimmune thrombocytopenia 3 years before presenting with severe gross hematuria and recurrent bladder tamponade (Suzuki et al. 2008), and two teenagers who presented with haemorrhage due to bladder telangiectasia had previously been treated for lymphoma (Cohen et al. 2008).

1.6.3 Underdevelopment of some organ systems.
Nearly all A-T patients examined at post mortem show an abnormality of the immune system in that the thymus is severely degenerated or totally absent (Rotman and Shiloh,
Primary cerebellar degeneration, often with large areas lacking perinje cells are routinely seen at autopsy. The absence of ovaries (Gatti et al. 1991), and hypoplasia or dysplasia of the gonads have also been documented (Amromin et al. 1979), which probably account for the delayed onset of puberty seen in A-T. Skull x-rays usually show a reduction or absence of adenoidal tissue in the nasopharynx. Growth retardation, and less commonly, insulin resistant diabetes are evident. Pulmonary changes similar to those seen in cystic fibrosis have also been noted, but these changes are associated with recurrent sino-pulmonary infections that are a feature of this syndrome, and which, is the largest single cause of death in these patients (Gatti et al. 1991).

1.6.4 Hypersensitivity to ionising radiation.
When A-T patients are treated with conventional doses of radiotherapy, for example, in treating leukaemia or lymphoma, they suffer massive tissue necrosis (Gotoff et al. 1967; Cunliffe et al. 1975). The major effect that IR has on cellular DNA is the induction of single strand and double strand breaks. The rate at which these are produced depends on the dose, the type of radiation (high or low LET) and the time after irradiation. Immediately following irradiation, single strand breaks predominate. At 3 hours post-irradiation there is a marked increase in double strand breaks, which is the same time that the first indicators of apoptosis are present. At 24 hours after a dose of 10 Gy, the double strand breaks account for nearly 80% of all strand breaks (Benitez-Bribiesca and Sanchez-Suarez, 1999).

A-T homozygotes are extremely sensitive to the effects of IR (Taylor et al. 1975). When normal cells are irradiated with X-rays, the cells inhibit replicon initiation and chain elongation. However, replicon initiation is inhibited to a much lesser degree in A-T cells, and there is no inhibition of chain elongation (Painter and Young, 1980). As A-T cells take longer to repair DNA double strand breaks (Urhammer et al. 2000), the radiosensitivity of A-T cells seems not to be due to their inability to repair DNA damage, but their failure to undergo delays that allow the DNA to be repaired before it is replicated (Painter and Young, 1980). This phenomenon is termed radio-resistant DNA synthesis (RDS). It is a common feature of A-T cells, and arises due to a defect in the S phase cell cycle checkpoint (Zakian, 1995). However, A-T cells are also sensitive to IR under conditions when cell cycle checkpoints are not operating (reviewed in Thacker, 1994). The radio-sensitivity of A-T cells in culture has been reported to be due to inappropriate apoptosis (Meyn, 1995).
The fact that A-T homozygotes and to a lesser extent, A-T heterozygotes, are sensitive to IR-induced DNA damage, has long been known, and is well documented (e.g. Taylor et al. 1975) by studying the type of DNA damage caused by IR, its effect on cell cycle checkpoints, and the clonogenic survival of A-T cells following exposure to radiation compared to normal subjects. However, while it has been reported that A-T cells undergo inappropriate apoptosis in response to such treatments (Meyn, 1995), it has also been reported that A-T patients suffer massive tissue necrosis in response to IR (Gotoff et al. 1967; Cunliffe et al. 1975). These two cell death mechanisms are distinct from each other, and so it is unclear from the literature whether A-T cells, in response to IR and other agents that cause DNA damage, induce an apoptotic or necrotic cell death profile or both. The current research study was therefore interested in investigating the cell death mechanisms activated by A-T cells in response to γ-radiation and a variety of chemicals and anti-cancer drugs to determine how A-T homozygote and heterozygote cell lines responded to these treatments compared to controls.

Most researchers have demonstrated that A-T cells in vitro are not sensitive to the effects of UV damage. This is in contrast to a group of 4 Saudi A-T patients, who showed increased sensitivity to germicidal UV (254nm). The same patients showed less inhibition of [3H] thymidine incorporation after the same dose of UV as the controls, and showed delayed kinetics in the removal of pyrimidine dimers (Hannan et al. 2002). However, as ATR is largely responsible for dealing with damage caused by exposure to UV-irradiation, it may be possible that these cells have mutations in both the ATM and ATR genes.

1.6.5 Increased risk of developing cancer.

It is known that A-T patients have a higher risk of developing cancer than people without A-T. Some suggest that the increased risk is about 100 times higher for developing certain types of cancer, such as lymphoma (reviewed by Gatti et al. 1991), but there are also reports that A-T homozygotes have an increased risk of developing cancer that is between 61 to 184 times higher than normal individuals (Swift et al. 1991). Approximately 85% of the malignancies A-T patients develop are lymphomas or leukaemia and there is a total absence of myeloid tumours (Taylor et al. 1996). One study involving 3 different A-T patients showed chromosomal breaks of 20-30% in lymphocyte cells from these patients (Hecht et al. 1966). This factor along with the abnormal response to the mitogen, phytohaemagglutinin (PHA) led the authors to suggest that the lymphocytes from A-T
patients may be abnormal and that the result of this abnormality may be the development of leukaemia and lymphoma, which are the most common cancers observed in A-T patients. The most common cancer in children with A-T is acute lymphocytic leukaemia (ALL) of T-cell origin, which can sometimes present before ataxia is evident. The ALL that is most common in unaffected children is usually of a pre-B-cell form, so children with A-T differ in this respect. In older A-T patients, the leukaemia is also of T-cell origin, but is much more aggressive, and cell morphology is more similar to a chronic lymphoblastic leukaemia (Taylor et al. 1996). Where lymphomas are present, these are usually non-Hodgkin’s lymphomas, which are extra-nodal and of B-cell origin. The occurrence of T cell tumours are 4-5-fold higher than B cell tumours in these patients (Taylor et al. 1996). Solid tumours that occur at sites distinct from B and T cell origins increase with increasing age of patients (Morrell et al. 1986). Due to the sensitivity of A-T patients to radiation, individuals that develop cancer can only be treated with a very small fraction of the normal therapeutic radiation dose (Gatti, et al. 1999). This factor, in addition to the fact that A-T patients suffer massive tissue necrosis in response to IR (Gotoff et al.1967; Cunliffe et al. 1975) and tumour necrosis is associated with a poor prognosis (Edwards et al. 2003; Langner et al. 2003; Chang et al. 2006; Sang et al. 2006), further highlight the need to determine the cell death mechanisms involved in response to these treatments in A-T patients, and may indicate treatments that may be beneficial for use in treating malignancy in A-T patients.

1.7 Cellular features of A-T.
1.7.1 Immunodeficiency.

The immunodeficiencies seen in A-T patients are very diverse, and are thought to arise due to problems in B and T cell maturation. The most common deficiencies in immunoglobulin classes are IgG^2 (80%) and IgA (60%) (Gatti et al. 1991), and IgE (Rotman and Shiloh, 1997). The most common consequence of these immunodeficiencies is recurrent sinopulmonary infections, which differ in severity between patients (Urhammer et al. 2000). Serum IgM levels are sometimes elevated and can occasionally lead to a high blood viscosity syndrome, with complications such as enlargement of the spleen (splenomegaly), abnormal lymph nodes (lymphadenopathy), decrease in circulating neutrophils and platelets (neutropenia and thrombocytopenia respectively), and congestive heart failure. Nearly half of all A-T patients suffer cellular immunity defects. T-cell deficiencies are
common, and correlate with abnormal responses to skin test antigen and to the mitogen PHA (Paganelli, 1992).

Peripheral lymphocytes from A-T patients show chromosomal translocations and inversions, most commonly involving chromosomes 7 and 14. These rearrangements involve the following sites: inv(7)(p13q35), t(7;7)(p13q35), t(7;14)(p13q11), t(7;14)(q35;q11), t(14;14)(q11;q32), inv(14)(q11;q32) and t(a;14)(q28;q11). All of these breakpoints, except 14q32 and q28, are the regions where the T-cell receptor (TCR) genes are located. In some instances, the breakpoints occur within them. Chromosome rearrangements of this type do occur in people without A-T, but their occurrence is a lot less frequent (Taylor et al. 1996).

The TCR is generated by combination of various genes. These involve variable (V), joining (J) and constant (C) regions that are located on the α gene on chromosome 14 in both man and mouse, and the same segments with an additional diversity (D) region, all from the β gene located on chromosome 7 in man, and on chromosome 6 in mouse. The variability of these genes arises due to the joining of different segments as well as the addition of random nucleotides (N), which are inserted between VJ or VDJ rearrangements. This TCR is known as TCRαβ, because it is made up of α and β genes. Two other TCR genes have also been identified (TCR γ and TCR δ). During development, the γ gene rearrangements appear first, followed by rearrangements of the TCR γ and δ genes to form TCRγδ. This receptor is only present on 1-10% of T cells in man, as most T cells express the TCRαβ moiety. It is the rearrangement of these 4 genes that gives the TCR its diverse repertoire (Strominger, 1989).

B cell and T cell deficiencies seen in A-T patients are thought to result from a defect in the rearrangement of immunoglobulin and TCR genes. T cells recognise antigens via two different types of receptors, those containing αβ chains, which are present on mature T cells, and γδ chains on immature T cells. A study on 10 A-T patients showed that these patients had a relative increase in T cells expressing γδ chains rather than αβ chains compared to normal subjects. However, this relative increase in γδ configured T cells, was found to be due to a decrease in cells bearing αβ chains rather than an actual increase in cells with γδ chains, which may indicate a defect in genetic recombination in these cells.
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The authors also suggest that these observations could also be due to an associated defect in DNA repair (Carbonari et al. 1990).

Hybrid genes also occur with greater frequency in A-T patients compared with normal subjects. Although hybrid genes have been observed in normal subjects, TCRγ/TCRβ genes occur with a frequency 70 times higher in A-T patients, the occurrence of which, has been proposed to arise due to over expression or hyperactivity of a recombinase. These hybrid genes could increase the T cell repertoire, could result in ineffective TCRs, resulting in immunodeficiency, or alternatively, could have little or no effect (Lipkowitz et al. 1990). Kojis et al. (1991) also favour a recombinase to explain the A-T phenotype, and propose that the immunodeficiency seen in A-T patients arises due to generation of ineffective hybrid TCRs. Defects in repair efficiency and fidelity that lead to increased rates of chromosomal damage and telomeric fusions can also be explained within this hypothesis. They also suggest that the activation of an oncogene or growth promoting factor at 14q31.2 could account for the T-cell malignancies, which are a common feature in A-T.

In a study of 70 British patients, 10% had severe immunodeficiencies, while 40% had normal immunologic function (Woods, 1992). These diverse differences in immunologic function could explain why some patients with A-T suffer debilitating bouts of e.g. sinopulmonary infection, while other A-T patients appear relatively unaffected.

1.7.2 Chromosome instability.

A-T cells also show an increase in intrachromosomal recombination rates. In an experiment on four transformants from an A-T cell line, the recombination rates were calculated to be greater than 100 times higher than in the control cell line (Meyn, 1993). Telomere shortening and telomere fusions have also been observed in lymphocytes from A-T patients, (Pandita et al. 1995; Metcalfe et al. 1996). A-T cells show higher rates of telomere fusions at metaphase and in the interphase, when compared to normal cells. Higher rates of chromosome breaks are also observed at metaphase (Pandita et al. 1995). One study involving 3 different A-T patients showed chromosomal breaks of 20-30% in lymphocyte cells from these patients (Hecht et al. 1966). Telomere fusions occur in pre-leukaemic translocation clones, but have not been found in A-T patients with leukaemia. Telomere fusions between a single pair of chromosomes were not observed, but there were more fusions observed for chromosomes 19-22. A-T patients seem to have a predisposition to
shortened telomeres, and this feature increases with age (Metcalfe et al. 1996). A defect in telomerase is unlikely to be responsible for the telomere fusions, as telomerase activity has been found to be normal in A-T cells (Pandita et al. 1995; Metcalfe et al. 1996). Thus, the telomere shortening itself may be a prerequisite for the development of telomere fusions observed in A-T patients.

1.8 Cellular defects in A-T cell lines.
Cells from A-T patients have a reduced lifespan (reviewed in Thacker, 1994), higher requirement for serum growth factors, chromosome instability and defects in the cytoskeleton, when in culture. In addition, they display a phenotype that includes increased sensitivity to killing by IR and radiomimetic chemicals, RDS, and failure of cell cycle checkpoint controls after exposure to IR (Taylor et al. 1975; Young and Painter, 1989; Beamish and Lavin, 1994). A-T cells are defective in the radiation induced G1-S, S and G2 cell cycle checkpoints (reviewed in Thacker, 1994). These checkpoints allow arrest of the cell cycle, so that damaged DNA can be repaired before the DNA is replicated, and before the DNA is passed on to daughter cells, thereby maintaining the integrity and fidelity of the genome (Beamish and Lavin, 1994).

Defective cell cycle checkpoint control is accompanied by defective or severely delayed activation of p53 in A-T cells, compared to activation of this protein in wild type cells (Kastan et al. 1992; Khanna and Lavin, 1993; Lu and lane, 1993), which places ATM upstream of p53 in the damage response pathway. These factors indicate that ATM plays an essential role in the mechanisms controlling cell cycle arrest and DNA repair following exposure to IR and other DNA damaging agents (Lakin et al. 1996). Because A-T-defective cell lines fail to activate the G1, S and G2 cell cycle checkpoints, the defect is likely to occur early along the DNA-damage response pathway, and furthermore, there may be a single pathway that is common to all three checkpoints (Gately et al. 1998).

p21, GADD45 and MDM2 are all involved in signal transduction pathways that are mediated by p53 in response to IR. The activation of these proteins has been demonstrated to be either delayed or reduced in A-T cells, when the cells are exposed to IR (Kastan et al. 1992). Lack of post-irradiation inhibition of cyclin-dependent kinase (CDK) activities in S phase and G2 phase has also been noted in A-T cells which correlated with the lack of significant increase in the amount of CDK associated p21. These results suggest that the
defective p53/p21 mediated response to IR in A-T cells is related to the abnormalities in cell cycle checkpoints (Rotman and Shiloh, 1997). Failure of the S phase cell cycle checkpoint results in A-T cells replicating DNA, even when there is DNA damage present (Zakian, 1995), a feature known as RDS.

When full length ATM cDNA is over expressed in A-T cells, the survival of these cells is enhanced in response to IR. The cells also show reduction in radiation-induced chromosome aberrations, reduction in RDS, and the cell cycle checkpoints were partly corrected (Zhang et al. 1997).

1.9 Treatment for clinical symptoms of A-T.
There is currently no cure for A-T, and no therapeutic regime that is able to prevent its progression. Treatment therefore focuses on treating the clinical symptoms such as the neurological features, immunodeficiency and sinopulmonary infection associated with this condition. These treatments are often problematic due to the nature of the syndrome, but the problems of treating malignancy in these patients is exacerbated by the unusually high sensitivity of A-T patients to radiotherapy (Lavin et al. 2007). A-T patients are at increased risk for developing cancer (Morrell et al. 1986), and strategies to treat these malignancies require a delicate balance between minimising tissue damage while maintaining the effectiveness of the therapy in treating the tumour. This involves reducing the intensity of γ-radiation and lowering the concentration of chemotherapeutic drugs (Lavin et al. 2007). These preventative measures to reduce tissue damage are able to be implemented due to the early diagnosis of this genetic condition.

1.10 ATM localisation/expression.
ATM exists as a dimer or higher order multimer within the cell (Bakkenist and Kastan, 2003). It is predominately a nuclear protein, where it associates with chromatin and the nuclear matrix (Gately et al. 1998), and is located in the nucleus throughout all phases of the cell cycle (Brown et al. 1997). Even though most of the ATM is localised to the nucleus, it only constitutes ≈ 0.005% of total nuclear protein by weight (Smith and Jackson, 1999). ATM is expressed at similar levels in all tissues and cell lines analysed (Lakin et al. 1996). Ionising radiation has no significant effect on the distribution pattern of the protein, and furthermore, the level of protein expression remains constant throughout the cell cycle (Gately et al. 1998), which indicates that ATM is not synthesised in response to DNA.
damage, but is constitutively expressed. ATM has been shown to associate with DNA, with particular affinity for DNA ends (Smith et al. 1999). These findings would be consistent with ATM being a component of the DNA damage detection apparatus rather than being an inducible downstream effector of the DNA damage response.

ATM knockout mice exhibit neurological defects that can be demonstrated using a variety of tests. However, histological examination of the cerebellum shows no abnormality in these animals. It has therefore been proposed that the neurological dysfunction is associated with pre-degenerative lesions. The ATM protein was found to be exclusively cytoplasmic in purkinje cells, as it is in human cerebellum, and was also present in the cytoplasm of a subset of cells in the dorsal root ganglia (Barlow et al. 2000). The existence of ATM in the cytoplasm of neurons may therefore be important, and its absence has been proposed to cause abnormalities in cytoplasmic organelles, seen as an increase in lysosomal numbers (Barlow et al. 2000).

ATM shares sequence homology with the PI-3-kinases (discussed earlier). These proteins on the whole are cytoplasmic in location and function in signal transduction pathways in response to growth factor stimulation. ATM has been found to associate with β-adaptin, a cytoplasmic protein involved in vesicle and protein transport (Lim et al. 1998). ATM is predominantly nuclear in A-T cells in culture and these cells show defects in the cytoskeleton. A small amount of ATM is present in the cytoplasm of mitotically active cells, but the ATM remains predominantly nuclear. ATM is exclusively cytoplasmic in oocytes that are arrested in meiosis I (Barlow et al. 1998). The localisation of ATM in proliferating cells is predominantly nuclear, which would be consistent with its role in cell cycle control, DNA repair and replication. However, in non-proliferating cells such as oocytes that undergo meiosis rather than mitosis and neurons, the ATM seems to be exclusively cytoplasmic. This seems to suggest that during embryogenesis, ATM is predominantly cytoplasmic, or there are two pools of ATM, one cytoplasmic and one nuclear, that have distinct functions. The cytoplasmic pool of ATM seems to help to function in development, and the nuclear pool seems to function in maintaining the integrity of the genome. Whether the cytoplasmic pool of ATM translocates to the nucleus following development, or is degraded, remains to be established. However, this notion would explain how A-T patients with no detectable ATM protein are born with no physical defects, and that the A-T phenotype develops after birth, and is associated with loss of
genome integrity. ATM is one of the key proteins involved in apoptosis (Smith et al. 1999) and amongst other functions, apoptosis is involved in the shaping of digits, in the generation of the T cell repertoire and in the maintenance of organ size, such that the generation of new cells is matched by apoptosis in these organs (Young, 1992). A-T patients are not born with webbing between the digits, so apoptosis must be functional in embryogenesis. There have been no reports of A-T patients suffering from autoimmune diseases, so auto-reactive T cells are presumably removed by apoptosis in A-T patients. Although there is underdevelopment of some organ systems, most notably, the appearance of a small embryonic thymus at autopsy, and underdevelopment of the sex organs, this does not suggest that these organs are underdeveloped at birth. In addition to these observations, there is no documented evidence of babies with A-T being born with any neoplastic transformations, which suggests that the development of cancer is a feature related to the loss of cell proliferation control after birth, not before birth. There is also the possibility that these differing pools of ATM are tissue-specific, but it would be very interesting to identify the ATM pool(s) at different stages of embryogenesis, and to determine if there are indeed two pools of ATM during this process, and at what stage of development, if any, the cytoplasmic pool is degraded, or if there is only one ATM pool that translocates to the nucleus at some stage in development.

1.11 The cell cycle.
The cell cycle is positively controlled by cyclin/cdk complexes, and negatively controlled by CDK inhibitors. It is up-regulation and down-regulation of the expression of these molecules, at different times in the cell cycle that regulates progression or arrest of the cycle.
Key.

- $G_1 = G_1$ phase (growth/resting phase)
- $S = S$ phase (DNA synthesis)
- $G_2 = G_2$ phase (growth/resting phase)

Figure 1.2 Schematic diagram showing the stages of the cell cycle. (Redrawn from Bowen et al., 1998)

The cycle of cdk expression at different phases of the cell cycle is best characterised in the yeast *Saccharomyces cerevisiae*. In this instance, a single kinase, which is the product of the CDC28 gene sequentially interacts with a series of different cyclins that are transiently expressed (Nasmyth 1993). Each cyclin gene, except $CLN3$, is transcribed for only a short period of time within the cell cycle. The messenger RNA is translated, but because the protein is rapidly degraded, each cyclin protein produced is only active in one phase of the cell cycle. However, in mammalian cells, the expression of cyclins and cdks is not quite as simplistic as in yeast.
Figure 1.3 Schematic diagram showing the expression of cdk4 and their associated cyclins during the cell cycle. (Redrawn from Bowen et al., 1998)

Entry into the cell cycle is controlled by growth factors, which stimulate the cells to re-enter the cell cycle from G0, by inducing synthesis of D cyclins that form complexes with CDK4/CDK6. G1 phase, S phase, where DNA synthesis occurs, and G2 phase are together known as interphase. Progression through these phases is controlled by the oscillating expression of A, B, C, and E cyclins, and their associated CDKs at different points in the cycle, with the dimers requiring phosphorylation on Thr-161 by CAK to generate the active form (Lewin 1997). CDK2, which may form a complex with cyclin E or A or both, functions in S phase, and cell division cycle-2 (CDC2) complexed with cyclins A and B is essential for mitosis (Hartwell and Kastan 1994). M phase is where cell division takes place to generate two new daughter cells. Progression through this phase is not only dependent on the phosphorylation reactions by M phase kinase, but also relies upon the deregulation of cyclins A and B, via the “ubiquitin conjugation” pathway, and degradation of a target protein in anaphase. G1 and G2 are gap phases. Progression through these phases only occurs when mitosis or DNA synthesis has been successfully completed, respectively, and constitutes two points in the cycle where cell cycle progression can be arrested (Lewin 1997). Although greater numbers of these molecules are required for progression of the cell...
cycle in mammalian cells, compared to progression of the cell cycle in yeast, functionally, both the CDKs and cyclins are conserved in eukaryotic cells. Most mammalian cyclins and CDKs can replace the function of corresponding yeast proteins. The same can also be said for enzymes that control kinase activity (Hartwell and Kastan 1994). Timing of entry into the cell cycle, and progression through it is very tightly controlled by a number of sequential, inter-related events, which includes transcription of cyclin genes, degradation of cyclin proteins, and phosphorylation reactions that modify the kinase subunits (Nurse 1990). Added to this are both positive and negative feedback loops, the latter playing a crucial part in cell cycle arrest (Hartwell and Kastan 1994). Such cell cycle arrest provides the necessary time for the cell to assess and repair any damage before re-entering the cell cycle, and occurs when integrity of the genome has been compromised, with the aim of preventing the propagation of mutations.

1.12. DNA damage.
Cell cycle arrest is induced when the DNA becomes damaged, which can occur in a variety of ways. DNA damage can arise through normal cellular metabolism and via environmental insult. During meiotic and V(D)J recombination, breaks in DNA are required to enable these processes to occur correctly. It is important that the DNA strand breaks that occur during these processes do not activate the cells normal safety mechanism for removal of cells harbouring compromised genetic material.

Reactive oxygen species may be formed during normal cellular metabolism, or may be formed from breakdown of chemicals in the environment. These ROS are highly reactive with cellular DNA.

DNA replication often induces sequence alterations, and although these may not be detected as strand breaks, they are equally important, as sequence alterations can affect both the fidelity and integrity of the DNA.

The cell responds to DNA damage by inducing expression of tumour suppressor genes such as p53, which, besides being induced by genotoxic damage, is also induced by oxygen deficiency and altered ribonucleotide pools. The most damaging lesion that occurs in DNA is the double strand break. This is where both DNA strands of the α-helix are severed. Only
one unrepaird double strand break is lethal to the cell (Shiloh, 2001). DNA double strand breaks are also the most severe lesions to be induced by IR (Bakkenist and Kastan, 2003).

**Figure 1.4** Schematic diagram showing ATM-dependent activation of p53 following ionising radiation-induced DNA damage. Ionising radiation induces DNA strand breaks which results in ATM-dependent activation of p53. Once activated, p53 can promote cell cycle arrest and apoptosis. ATM positively regulates the pathway by activation of Chk2, but is also able to negatively regulate the pathway by activation of Brca-1.

1.13 ATM and DNA double strand break repair.
Just how cells respond to damage depends on where the cell is in terms of the cell cycle. When cells are irradiated in G₀ or G₁, the damage is repaired by non-homologous end joining, whereas cells irradiated in G₂ repair the damage by homologous recombination between sister chromatids. A-T cells are slower in repairing double strand breaks compared
to normal cells. ATM is involved in the rejoining of double strand breaks by regulating the process through its interaction with other proteins (Paterson et al. 1976).

A-T cells have previously been evaluated for their ability to repair single strand and double strand breaks in DNA after exposure to γ-irradiation. A-T cells were able to repair single strand breaks (i.e. scissions in the sugar phosphate backbone) as efficiently as normal cells, and so it appears that A-T cells have the normal enzymatic apparatus to repair γ-irradiation-induced single strand breaks. However, excision repair was found to be suboptimal and delayed in A-T cells compared to controls. This was due to a defect in the excision of γ-modified base residues. The excision sites, and hence the gaps that needed to be filled were measured by the amount of repair replication occurring in the cells. Repair replication in A-T cells was only half that observed in controls, confirming that A-T cells are defective in excising γ-modified base defects, which is probably the result of these cells lacking a fully functional γ endonuclease to excise damaged bases (Paterson et al. 1976).

In normal cells not undergoing insult, ATM exists as a dimer with its kinase domain bound to another ATM protein containing amino acid 1981. This interaction enables ATM to fold correctly and is also required for its stability. While ATM is dimerised, this prevents ATM interacting with other proteins and substrates. However, following exposure to IR, ATM autophosphorylation occurs such that the kinase domain of ATM phosphorylates its partner in the dimer on serine 1981. These phosphorylations result in separation of the dimer into two ATM monomers that are then available to interact with other target substrates (Bakkenist and Kastan, 2003). Another potential ATM autophosphorylation site has been identified at serine 440 (Kim et al. 1999). The ATM dimer is extremely sensitive to double strand breaks, as a dose of radiation predicted to result in 18 double strand breaks in the genome of a human cell is sufficient to activate over 50% of the ATM contained within the cell within 5 minutes of the radiation treatment. However, these rapid events are not consistent with ATM binding to DNA double strand breaks itself, and so it has been suggested that a signal is generated to induce the active ATM, and the signal could possibly be due to changes in chromatin structure consistent with unwinding of the tightly coiled DNA due to double strand breaks (Bakkenist and Kastan, 2003).
1.14 GI/S phase cell cycle arrest.
One of the main features of the A-T phenotype is RDS, which arises due to failure of cell cycle checkpoints, and hence, replication of DNA in spite of the presence of damage. This phenomenon has been well characterised, mainly due to experiments with irradiated A-T cells. As the lack of cell cycle control in A-T cells is best characterised in response to IR, the following discussion on cell cycle checkpoints will focus on the cells response to IR where possible. However, it is also important to realise that these cell cycle checkpoints have the same functions and responses when stimulated by the other types of DNA damage.

Following exposure to ionising radiation, ATM interacts with a variety of proteins and transcription factors to induce GI/S phase cell cycle arrest. The interaction of ATM with these substrates is discussed.
Figure 1.5 Schematic diagram showing $G_1/S$ phase cell cycle arrest and DNA repair pathways activated by IR-induced DNA damage.
1.14.1 ATM, MRE and histone H2AX.

One of the earliest cellular responses to double strand breaks is the phosphorylation of histone H2AX, a variant of histone H2A, at the sites where damage is present (Modesti and Kanaar, 2001). H2AX is phosphorylated at serine 139 within seconds of double strand breaks occurring in mammalian cells (Rogakou et al., 1998). ATM phosphorylates H2AX following exposure to IR, etoposide and bleomycin (Burma et al. 2001), and H2AX is phosphorylated rapidly in cells induced to undergo oxidative stress by treatment with HP (Zhao et al., 2008). Phosphorylated H2AX (γ-H2AX) forms foci at sites of DNA damage (Rogakou et al., 1999). Phosphorylation of H2AX has been shown to be specific to the sites of DNA damage in experiments using “laser scissors” to introduce breaks into living cells. In these experiments, the foci followed the path of the laser through the cell nuclei (Rogakou et al., 1999; Paull et al., 2000). H2AX phosphorylation has also been shown to occur during endogenous generation of double strand break intermediates including V(D)J recombination in lymphoid cells (Chen et al., 2000) as well as meiotic recombination in the mouse model (Mahadevaiah et al., 2001). Serine 129 of yeast H2A is homologous to serine 139 of H2AX in mammals. Phosphorylation at this site causes chromatin to become decondensed, and it is required for efficient DNA double strand break repair (Mahadevaiah et al., 2001). However, in mammalian cells, γ-H2AX is responsible for recruiting repair and/or damage signalling factors to sites where DNA is damaged (Paull et al., 2000; Rappold et al., 2001). The MRN complex, consisting of Mre11, Rad50 and Nbs1, senses DNA double strand breaks and recruits ATM to the site of damage. (Lee and Paul, 2005; Paull and Lee, 2005). This event is facilitated by at least two protein-protein interactions; one between ATM and Nbs1 and another between ATM and Mre11/Rad50 (Paull and Lee, 2005). ATM dimers used in experiments in vitro with DNA and MRN, produce active ATM monomers that phosphorylate the downstream targets, p53 and Chk2. In the presence of MRN, ATM autophosphorylation was not required to produce active ATM monomers in vitro (Lee and Paul, 2005), however, ATM autophosphorylation on ser 1981 was found to be essential in human cells in vivo (Paull and Lee, 2005).

1.14.2 ATM and p53.

A tumour suppressor is a gene that codes for a product that negatively regulates the cell cycle. In order for the cell to undergo rapid division, this product must be inactivated or mutated. p53 protein is a transcription factor that is the product of such a tumour
suppressor gene. p53 protein levels increase rapidly following various types of cellular stress, including genotoxic radiation (x-rays, γ-rays and uv-rays) and genotoxic drugs, cell stresses such as heat shock, hypoxia and osmotic shock (Fritsche et al., 1993), and inhibitors of DNA replication and transcription (Yamaizumi and Sugano, 1994). Posttranslational modifications to the protein, that include phosphorylation, dephosphorylation and acetylation at various sites on it have been associated with its stability (Agarwal, 1998; Giaccia and Kastan, 1998; Sakaguchi et al., 1998; Waterman et al., 1998; Shieh et al., 1999; Unger et al., 1999). The protein plays a key role in pathways activated via the G1/S phase checkpoint, that lead to cell cycle arrest to allow DNA to be repaired, or programmed cell death (Levine, 1997; Gottlieb and Oren, 1996; Agarwal, 1998) to prevent the cell from replicating damaged DNA.

Transcriptional activation of p53 following DNA damage has long been established (Hollstein et al., 1991), placing p53 as a key player in the cell’s response to DNA damage. Another key player in this pathway is ATM, which acts upstream of p53 by phosphorylating p53 on ser-15 (Rotman and Shiloh, 1997; Siliciano et al., 1997; Canman et al., 1998; Khanna et al., 1998; Banin et al., 1998; Nakagawa et al., 1999). This residue is evolutionary conserved, and corresponds to ser-18 in murine (mouse) p53. Both these residues can be phosphorylated in vitro by several ATM family members including ATR (Rotman and Shiloh, 1997) and DNA-PK (Lees-Miller et al., 1992). However, ATM has been shown to be required for optimal induction of p53 following exposure to IR (Kastan et al., 1992; Dulic et al., 1994; Khanna et al. 1998), and ATM-dependent phosphorylation at ser-15 of p53 correlates with p53 stabilisation following DNA double strand breaks (Nakagawa et al., 1999). ATM induces G1/S phase arrest through the action of several intermediates. One of the most important targets is the phosphorylation of p53 on ser15 (Canman, 1998; Khanna et al., 1998). Cells defective for p53 fail to arrest at the G1/S phase checkpoint (Kuerbitz, et al., 1992).

Radiation-induced phosphorylation of p53 at serine 15 by ATM results in the accumulation of p53 protein, which is then able to activate p21 and MDM2 in vivo. A-T cells are also able to phosphorylate p53 on ser15, although accumulation of p53 in these cells is sub-optimal and delayed (Rotman and Shiloh, 1997; Siliciano et al. 1997; Khanna and Lavin, 1993), as it is in the mouse model (Khanna and Lavin, 1993), and both exhibit defects in G1/S phase, S phase and G2/M phase cell cycle checkpoints (Rotman and Shiloh, 1997).
1.14.3 ATM and MDM2

The mouse double minute 2 (MDM2) gene is activated by p53, but activation of MDM2 results in degradation of p53 forming a negative feedback loop (Haupt et al. 1997). The MDM2 gene has two adjacent p53 binding sites within its first intron (Oren, 2003), which when bound to p53, inhibits the transactivation activity of p53, and targets it for degradation by the 26S proteosome (Oliner et al., 1993; Haupt et al. 1997; Kubbutat et al. 1997; Oren, 2003), preventing p53 from activating its target genes. The destabilization of p53 is dependent on two functional domains of MDM2 binding to p53, involving both the amino- and carboxyl-terminal regions of MDM2 (Haupt et al. 1997; Kubbutat et al. 1997; Bottger et al. 1997; Kubbutat et al. 1999), as well as its nuclear export capability (Roth et al. 1998; Tao and Levine, 1999). MDM2 is an E3 ubiquitin-protein ligase that functions in ubiquitination of p53 leading to its degradation (Honda et al. 1997; Honda and Yasuda, 1999; Honda and Yasuda, 2000; Fang et al. 2000). The E3 ubiquitin ligase activity of MDM2 is dependent on its RING finger domain (Fang et al. 2000). As a direct consequence of these events, the intracellular levels of p53 are maintained at low levels throughout the cell cycle (Gudas et al., 1994).

It has been reported that phosphorylation of p53 at Ser-15, following exposure to genotoxic agents, including UV and γ radiation, causes a structural change in p53 which allows it to evade complex formation with MDM2 (Shieh et al., 1997), thereby promoting accumulation of p53. ATM is the major kinase responsible for phosphorylating p53 on serine 15 (Rotman and Shiloh, 1997; Siliciano et al., 1997; Canman et al., 1998; Khanna et al., 1998; Banin et al., 1998; Nakagawa et al., 1999).

In response to IR and neocarzinostatin, a radiomimetic chemical, MDM2 was found to be phosphorylated rapidly in an ATM-dependent manner. This phosphorylation of MDM2 occurred prior to p53 accumulation, and was not dependent upon p53 serine 15 phosphorylation. ATM was also found to phosphorylate recombinant MDM2 in vitro on at least two sites, suggesting that MDM2 might be a direct in vivo target of ATM after DNA damage (Goldberg et al. 1995). It was subsequently found that ATM phosphorylates MDM2 on serine 395. This phosphorylation event inhibits nuclear export of the p53/MDM2 complex, which is required for p53 degradation (Shiloh, 2001).
Phosphorylation of MDM2 on serine 395 by ATM therefore facilitates indirect regulation and stabilisation of p53 by ATM.

1.14.4. ATM and Chk2.
Checkpoint kinase-1 (Chk1) and checkpoint kinase-2 (Chk2) phosphorylate p53 at serine 20 (Shieh et al., 2000; Chehab et al., 2000) and both proteins are required for p53 stabilisation after exposure to IR. Chk1 and Chk2 are activated by ATM (Chehab et al., 2000; Hirao et al., 2000; Matsuoka et al., 1998), thereby producing an indirect route for p53 stabilisation by ATM. Phosphorylation at this site also interferes with the binding of MDM2 to p53, and Chk2 has been shown to disrupt p53/MDM2 complexes in vitro (Chehab et al. 2000). ATM therefore indirectly participates in the stabilisation of p53 through its interaction with Chk1 and Chk2, and results in G1 cell cycle arrest. Chehab et al. (2000) propose that Chk2 is also involved in S and G2 cell cycle arrest as post-translational modifications of Chk2 in response to IR occurred in all phases of the cell cycle.

1.14.5. ATM and p21.
p21/CIP1/WAF1 is involved in cell cycle control and is transcriptionally activated and directly regulated by p53. This protein suppresses tumour cell growth in culture (El-Deiry et al. 1993), giving an insight into its role in regulating cell growth. The p21 protein is an inhibitor of a variety of cyclin dependent kinases. The most likely targets for controlling the G1/S checkpoint are cyclin dependent kinases containing cyclins A, D, and E, as these are the cyclins expressed early in the cell cycle, and added to this, the knowledge that the activity of cyclins E and A complexed to CDK2 are inhibited by IR in a p53-dependent manner, possibly through the transcription of p21 (Dulic et al. 1994). Expression of p21 also inhibits phosphorylation of pRb by CDKs. pRb is the product of the retinoblastoma gene (Kouzarides, 1995). Hyperphosphorylated pRb ties up the transcription factor E2F. This event prevents progression through G1, by preventing expression of E2F responsive genes, the products of which, are required for progression into S phase. A-T cells fail to undergo cell cycle arrest at the G1-S cell cycle checkpoint, and consistent with this aberrant response, A-T cells do not inhibit cyclin E and cyclin A-associated kinase activity. This lack of inhibition was reflected in accumulation of the hyperphosphorylated (inactive) form of pRb, in these cells. Induction of p21 following exposure to IR has also been shown to be sub-optimal and/or delayed in these cells (Khanna et al. 1998). p21-mediated protection
against IR-induced apoptosis is also thought to result from inhibition of CDKs that are required for the activation of the caspase cascade downstream of mitochondria (Sohn et al. 2006).

p21 also inhibits DNA replication factor proliferating cell nuclear antigen (PCNA). PCNA is a subunit of the DNA polymerase δ enzyme complex. It is thought that this complex is involved in both DNA replication and DNA repair (Rotman and Shiloh 1997).

p21 is also involved in the G2/M phase cell cycle checkpoint, as although 21 is not an absolute requirement for G2/M cell cycle arrest, it is necessary to sustain the arrest (Deng et al. 1995; Brugarolas et al. 1995).

1.14.6 ATM and GADD45.
Phosphorylation of p53 by ATM results in the activation of a nuclear protein called GADD45 (Urhammer et al. 2000; Kastan et al., 1992). The protein product of GADD45 interacts with the CDK inhibitor p21, to arrest the cell cycle so that DNA can be repaired. GADD 45 also interacts with PCNA, an accessory factor in DNA repair and replication (Chen et al., 1995), and so it is thought that this protein could also be involved in DNA replication as well as in DNA repair (Rotman and Shiloh 1997). Transcription of PCNA is regulated by p53. A-T cells do not induce GADD45 as effectively as normal cells in response to IR (Kastan et al. 1992), demonstrating that ATM is a requirement for optimum GADD45 activation.

1.14.7 ATM and c-abl.
ATM constitutively binds c-abl, with binding occurring at the SH3 domain of c-abl with amino acids 1373-1382 of ATM (Shafman et al. 1997). ATM induces G1/S phase arrest through phosphorylation of c-abl on ser 465 in response to IR (Shafman 1997; Baskaran, 1997). Phosphorylation of c-abl results in the phosphorylation of RNA polymerase II (Baskaran, 1997), and activates the p53 homologue p73 and the Stress-Activated Protein Kinase (SAPK) pathway to block progression to S phase (Urhammer et al. 2000).
1.14.8 ATM and BRCA-1.

BRCA is the breast cancer related gene, of which there are two types, BRCA-1 and BRCA-2. BRCA-1 (wild type) suppresses cancer by its involvement in signalling the presence of DNA damage and in its repair (Zhang et al., 1998; Scully and Livingston, 2000). Following exposure to DNA damaging agents, BRCA-1 is hyperphosphorylated (Scully et al., 1997) at several sites. Ser-1387 is specifically phosphorylated after IR, and ATM has been shown to phosphorylate BRCA-1 at this site (Kim et al. 1999). Following IR, BRCA-1 is also phosphorylated by Chk2 (Cortez et al., 1999; Gatei et al., 2000; Lee et al., 2000). Chk2 co-localises with BRCA-1 in discreet nuclear foci, but exposure to IR induces separation of these two proteins. This separation is a direct result of Chk2 phosphorylating BRCA-1 on serine 988, along with autophosphorylation of Chk2 (Lee et al. 2000). ATM also phosphorylates Chk2, providing an indirect route to BRCA-1 for ATM.

ATM also phosphorylates BRCA-1 in response to double strand breaks (Cortez, 1999). BRCA-1 is required for the assembly of the Rad 50/MreII/nibrin repair complex, as well as Rad 51 complexes at double strand break sites. ATM can phosphorylate Mre11 at ser264, and also phosphorylates nibrin (Kim et al. 1999; Shiloh, 2001). Both Rad 50 and Rad 51 are required for DNA recombination and repair. ATM and c-abl-mediated phosphorylation of Rad51 enhances the association between Rad51 and Rad52, and has been shown to be required for post-translational modification of Rad51, which is necessary for assembly of the Rad51 repair complex in response to IR. ATM may therefore act as the initiator of double strand break repair through these pathways.

In response to IR, ATM phosphorylates, hMreII and Nbs1, which results in the generation of foci (Shiloh, 2001; Kim et al. 1999). These foci are not present in hMreII mutant cells, which places ATM and the MRE complex in the same pathway. Mutations in hMreII are similar to those found in A-T as well as NBS. In fact, four patients thought to have A-T in two families have been found not to have mutations in ATM but do have mutations in hMreII, indicating the similarity in the pathology exhibited by mutations in these genes (Stewart et al. 1999).
Figure 1.6 Schematic diagram showing S phase cell cycle arrest activated by IR-induced DNA damage.

1.15 S phase cell cycle arrest.
Phosphorylation of c-abl by ATM also serves to halt cell cycle progression within S-phase by inhibiting Rad 51, a single strand DNA binding protein essential for replication. Cdc25A phosphatase activates cyclin-dependent kinase 2 (CDK2), which is required for DNA synthesis (Galaktionov and Beach 1991; Hoffmann et al., 1994; Jinno et al., 1994). When DNA damage is present replication is stalled or Cdc25A is degraded (Mailand et al., 2000).

Following IR, destruction of Cdc25A is dependent on the activity of ATM and the phosphorylation of Cdc25A by Chk2 on serine 123. The degradation of Cdc25A in response to IR prevents dephosphorylation of CDK2, which then results in a transient DNA replication block (Falck et al., 2001).
Replication protein A (RPA), another protein that is essential for the progression of DNA replication, is inhibited by ATM through phosphorylation of its p34 subunit. Replication protein A is also a single strand DNA-binding complex. This complex, which is essential for DNA replication, DNA excision repair and recombination, contains three subunits, 70kDa, 34kDa and 14kDa (RPA 1, 2 and 3 respectively). The protein complex associates with the kinase domain of ATM during meiotic prophase at sites where homologous regions of DNA interact. The complex also associates with ATM at sites of DNA strand breaks associated with meiotic recombination after synopsis. Phosphorylation of the p34 subunit of RPA is rapidly induced in response to IR (Liu and Weaver, 1993), but A-T cells exhibit a decreased and delayed ability to phosphorylate the p34 subunit of the RPA complex in response to IR (Liu and Weaver, 1993). This defect is also observed in yeast with mutations in MEC1, which is homologous to ATM (Brush et al., 1996), in response to IR.

Gately et al., (1998) demonstrated that immunoprecipitated ATM specifically phosphorylates the p34 subunit of RPA on serine and threonine residues, both in irradiated and non-irradiated cells, and that it does not phosphorylate the p14 and p70 subunits. The phosphorylation of p34 was found to be dependent on the presence of single strand DNA (Wold, 1997; Gately et al. 1998). An additional finding was that only background levels of p34 phosphorylation were detected in the absence of double strand linear DNA. This led to the hypothesis that the requirement for linear double strand DNA may mimic the radiation-induced DNA damage response by ATM. Additionally, it was also hypothesised that as the damaged DNA is repaired, the decrease in its availability might be in itself, a trigger for release from cell cycle arrest and continuance of progression through the cell cycle, thereby controlling the protein kinase activity of ATM (Gately et al., 1998).
Figure 1.7 Schematic diagram showing G2 phase cell cycle arrest. 
IR-induced DNA damage results in activation of ATM. ATM-dependent phosphorylation of Chk1 results in phosphorylation of Cdc25A, which in association with 14-3-3 proteins, inhibits CDK2 leading to G2 cell cycle arrest.

1.16 G2 phase cell cycle arrest.
ATM inhibits cells from entering mitosis after irradiation through the phosphorylation of at least two targets, Chk1 and Chk2. Brown et al. (1999) identified a protein that they designated HuCds1 (Chk2). DNA damage and replication checkpoints block progression to mitosis by inactivating Cdc2. Cdc2 is the cyclin-dependent kinase responsible for initiating mitosis (Enoch and Nurse 1990; Jin et al., 1996). This is partly accomplished by maintaining the phosphorylation of Cdc25 phosphatase. The phosphorylated form of Cdc25A binds 14-3-3 proteins (Zeng et al., 1998; Peng et al., 1997; Kumagai et al., 1999). When Cdc25 is bound to 14-3-3 proteins, it is prevented from activating Cdc2, resulting in G2 arrest (Brown et al., 1999). Both Schizosaccharomyces pombe Cds1 (Zeng et al., 1998; Murakami and Okayama 1995) and Chk1 from Homo sapiens (Hu), and Xenopus (Peng et al., 1997; Walworth et al., 1993; Al-Khodairy et al., 1994; Sanchez et al., 1997; Kumagai
et al., 1999) are involved in regulating the interactions between Cdc25 and 14-3-3 proteins. Brown et al. (1999) showed that phosphorylation of HuCds1/Chk2 in response to IR required functional ATM protein. Furthermore, the HuCds1 phosphorylated human Cdc25C at Ser 216, which is the 14-3-3 binding site, demonstrating conservation of the checkpoint function of Cds1 in yeast and mammals (Peng et al., 1997). Serine 216 is involved in negatively regulating Cdc21c, which results in inhibition of Cdc25c's ability to dephosphorylate and activate Cdc2/Cyclin B complexes, needed for cell cycle progression, resulting in cell cycle arrest (Matsuoka et al. 1998).

The relationship between Chk2 and ATM was determined using radiation treatment of cells. In response to this stimulus, Chk2 was phosphorylated by ATM on threonine 68 (Matsuoka et al. 2000). Phosphorylation at this site results in Chk2 forming nuclear foci. ATM phosphorylates BRCA-1 at Ser-1423 in response to IR (Xu et al., 2001; Gatei et al. 2001), and this event functions specifically in the G2/M checkpoint (Xu et al., 2001).

The subject of G2 arrest in A-T cells is not as well characterised as the other checkpoints, most likely because there are two arrest points, and only one is defective in A-T. Immediately after DNA damage, the defective cell cycle checkpoint can be measured as a failure to diminish the numbers of cells that enter mitosis in the hours that follow irradiation. In contrast, at later times, there is clearly an increase in G2 cells, which is readily detectable by FACS analysis. The late G2 accumulation is due to cells that were in G1 or S at the time of irradiation, which replicated their DNA in spite of the presence of DNA damage. Thus the DNA damage triggered an additional and distinct G2 checkpoint (Urhammer et al. 2000).

1.17 APOPTOSIS.

Apoptosis is an intrinsic suicide programme, the features of which, have been specifically characterised by the biochemical and morphological changes that occur within the cell. These cellular events are distinct from cell death by necrosis. In necrosis, the cell swells and bursts. The contents of the cell are disrupted and released into the extracellular environment. The presence of these nuclear and cytoplasmic cellular contents in the extracellular environment results in the generation of an inflammatory response. In contrast to this, during apoptosis, the cell shrinks, the cytoplasm and nucleoplasm condense, and
there is blebbing of cytoplasmic membranes, which form apoptotic bodies. These apoptotic bodies are utilised by neighbouring cells or are phagocytosed by resident macrophages. The internal contents of the cell are packaged within these apoptotic bodies, thereby preventing their release into the extracellular environment (reviewed in Elmore, 2007).

Malfunction in apoptosis has been suggested to be involved in many forms of human disease, e.g. neurodegenerative disease, AIDS, ischemic stroke and cancer (Luo, 1998).

In humans, approximately 100 thousand cells are produced by mitosis every second, and a similar number are destroyed by apoptosis (Vaux, 1999). This same process is responsible for the evolution of tissues in embryonic development, in the formation of digits (Young, 1992), and in the adult, occurs for example, in the uterus, post partem, in the breast after weaning, in the adrenal cortex or thyroid after hypophysectomy, and in the liver during starvation (Bursch, 1992). It is responsible for elimination of autoreactive T lymphocytes during their maturation in the thymus, and is responsible for controlling the size of organs by matching the rate of cell death with the rate of cell division (Young, 1992). It is also the means by which excessive cells or damaged cells are removed. In fact, during the lifetime of the human, 99.9% of cells undergo this process (Vaux, 1999), highlighting the importance of this cellular function in both health and disease.

The basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells at all times, but the activation of apoptosis is regulated by many different signals that originate from both the intracellular and extracellular environment. Apoptosis does not appear to occur at random in all cells of a tissue. Old, neoplastic, damaged or excessive cells are, or can be destroyed preferentially (Bursch, 1992). It appears that certain cells may be more susceptible to death signals than others. This could be dependent on the nature of the stimulus or on the particular routes to cell death that exist in different cell types. A good example of this is the steroid receptors, which can signal some cells to die and others to survive. The decision for whether a cell will undergo apoptosis is usually determined by the nature of signals received from outside the cell.
Fig 1.8 Schematic diagram showing induction of apoptosis via death receptor signalling. Apoptosis is initiated by the binding of specific ligands to cell membrane receptors. Fas receptor activation results in caspase-mediated apoptosis, whereas activation of TNFR1 can result in either caspase-mediated apoptosis or cell survival.

1.17.1 Induction of apoptosis via binding of death ligands.
Apoptosis is mediated by a family of proteases called caspases, although there are different ways in which these proteins become activated. One way in which apoptosis is induced, is by the binding of specific ligands to their receptors on the cell surface. Two such receptors are Fas/APO-1 and TNFR1. Activation of these receptors occurs by the binding of specific
proteins, Fas L (ligand) in the case of Fas (forming DISC), and TNF in the case of TNFR1. Fas and TNFR1 transmit their death signals to the ICE family members inside the cell, but this process requires the association of other components in the pathway. Fas and TNFR1 both contain an intracellular motif called the death domain (DD), which they use to bind adaptor proteins, which also contain this domain. The adaptor protein that binds to Fas is MORT-1/FADD, and the adaptor that binds to TNFR1 is TRADD (Wallach, 1997). The DD has been conserved throughout much of metazoan evolution. It shares some homology with the protein product of the Drosophila reaper gene, which has been shown to be essential for all of the many cell deaths that occur during normal Drosophila embryogenesis. MORT-1/FADD contains another motif called the “death effector domain” (DED). This is located upstream of the DD. The DED also occurs in duplicate in two of the caspases, caspase 8 (MACH/FLICE), and caspase 10 (Mch-4/FLICE-2). These caspases are able to bind directly to MORT-1/FADD through association of the DEDs. The death domain in TRADD, unlike that in MORT-1/FADD, is required to receive the death signal from the receptor and to pass it on to the next component in the pathway. Further components of this pathway have not been specifically identified, but MORT-1/FADD and FLICE/MACH have been implicated by experiments showing that cell killing by TNF is blocked when these proteins are altered so that they can receive the death signal, but are inhibited from passing it on. MACH/FLICE also contains a sequence of 260 amino acids at its C-terminal end that has been shown to be required for protease activity. It is this interaction and subsequent proteolytic cleavage, which may be self induced, that are thought to be critical steps in the initiation of the killing process (Wallach, 1997).
1.17.2 Induction of apoptosis in T-Lymphocytes.

Cytotoxic T lymphocytes function in the removal of malignant and virus infected cells, and are involved in transplant rejection and auto-immune disease. These cells induce death in target cells by releasing their granule contents, which include perforin and granzyme A and B. Perforin forms a pore in the plasma membrane allowing granzyme B to enter the cell. Once inside the cell, granzyme B activates caspase 3 and caspase 10, which is also able to activate caspase 3. Activation of caspase 3 then feeds into the execution phase of apoptosis (Nicholson and Thornberry, 1997).
Apoptotic stimuli.

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Apoptotic stimuli.
Caspase 8
Pro-apoptotic BCL-2
Release of cyt. C from mitochondrion
DATP/ATP
Cytochrome c binds to Apaf-1
Apaf-1
Apoptosome
Caspase-3
ICAD
CAD
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**Figure 1.10** Schematic diagram showing the execution phase of apoptosis. Activation of caspase-8 induces the execution phase of apoptosis. Cytochrome c released from the mitochondria is involved in the formation of the apoptosome with Apaf1 and pro-caspase 9, which activates caspase 3. Activation of caspase 3 releases CAD from ICAD, which degrades chromosomal DNA. ATM is cleaved by caspase-3, which inactivates the protein preventing inhibition of apoptosis.
1.17.3 Execution phase of apoptosis.

Caspase 8 cleaves the COOH end of BID, a BH3 domain-containing protein known to interact with both Bcl-2 and BAX. BAX is a member of the Bcl-2 family of proteins that functions as a proapoptotic death effector (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Oltvai et al., 1993), and Bcl-2 functions as an antagonist in this pathway. Another protein that has been shown to regulate the function of these two proteins is p53. The cleavage of BID causes translocation of BAX from the cytosol to the mitochondria (Luo 1998). BAX and adenine nucleotide translocator, a mitochondrial protein that is constitutively expressed, cooperate within the permeability transition pore complex to increase permeability of the mitochondrial membrane (Marzo et al., 1998). BAX then triggers the release of cytochrome c (Luo, 1998). Activation of caspase 3 and DNA fragmentation are two of the well-characterised biochemical markers of apoptosis. Caspase 3 is required for DNA laddering as well as the characteristic morphological changes such as membrane blebbing that occur during apoptosis (Jänicke et al. 1998) Cytochrome c binds to Apaf-1 to form a complex with and activate the initiator procaspase 9, which in turn activates downstream effector caspases, unleashing the death cascade (Li, 1998), until caspase 3 cleaves CAD from its chaperone ICAD. This cleavage releases the DNase activity of CAD, so that it can enter the nucleus and degrade the chromosomal DNA (Enari et al., 1998). However, before this DNase activity of CAD occurs, caspase 3 cleaves ATM at approximately 100kDa from the N-terminus. Even after cleavage, the two fragments remain complexed to one another, and both retain DNA binding activity. However, the kinase activity is diminished, noted by its reduced ability to phosphorylate p53 (Smith, G.C.M. et al. 1999). Functional ATM normally acts to inhibit apoptosis, so that it can initiate cell cycle arrest and promote DNA repair. These activities of ATM require a functional kinase domain, as most of the functions of ATM are accomplished by it phosphorylating its target substrates. This inactivation of ATM’s kinase domain by caspase 3 cleavage, excludes ATM from interfering with the apoptosis cascade. As caspase 3 is a key factor in both the death receptor apoptosis cascade and the intrinsic pathway of apoptosis, it can inhibit ATM in both pathways, thereby ensuring that the cascade comes to fruition.

Another negative regulator of this cascade is Bcl-2, which is located on the outer membrane of mitochondria. Bcl-2 prevents activation of caspase 3, and prevents cytochrome c release from the mitochondria (Susin et al., 1996; Kluck et al., 1997).
Soluble growth factors and cytokines can give survival signals by binding to cellular receptors, preventing activation of apoptosis by default. (Vaux, 1999). One mechanism that has been proposed for the way in which IL-3 survival factor induces cell survival centres around the homodimerisation and heterodimerisation of the Bcl-2 family of proteins, which show homology with the *C. elegans* protein Ced-9. The outcome is dependent on whether the protein elements act as inhibitors or promoters of apoptosis, which is largely determined by the way in which these proteins combine.

**Figure 1.11 Schematic diagram showing the role of BAX in apoptosis.** The formation of Bax homodimers increases sensitivity to apoptosis, while heterodimers formed by Bcl-2 or Bcl-XL with Bax favours cell survival by preventing Bax homodimerisation. Bad is also able to dimerise with Bcl-2 or Bcl-XL, which increases Bax homodimers and sensitivity to apoptosis.

For example, when Bax homodimerises, the cell's sensitivity to apoptosis increases. However, if Bcl-2 or Bcl-XL heterodimerise with Bax, this prevents Bax homodimerisation,
which acts to protect the cells from apoptosis. However, BAD appears to sequester Bcl-xl and Bcl-2 away from BAX, which in turn results in an increase in BAX homodimers, and therefore an increase in apoptosis (Bowen et al., 1998).

1.18 Poly (ADP-ribose) Polymerase.

Poly (ADP-ribose) polymerase (PARP-1) is a chromatin associated enzyme that binds to DNA strand breaks and uses NAD⁺ as a substrate to transfer long, branched ADP-ribose polymers (Nicholson et al. 1995). PARP-1 is activated by DNA damage caused by strand breaks, and is able to bind to the damaged DNA. PARP also interacts with many of the cell cycle checkpoint proteins that respond to DNA damage (Pleschke et al., 2000). NAD⁺ acts as a substrate for the automodification of PARP with poly ADP-ribose (Jacobson and Jacobson 1997), and this automodification allows PARP to regulate its interactions with both DNA and proteins (Pleschke et al., 2000). Pleschke had participated in identifying PAR binding to p53 and to myristoylated alanine-rich C kinase substrate (MARCKS) proteins and identified a likely PAR binding domain. SWISSPROT database was used to identify proteins that were likely to bind to this PAR binding domain. PARP-1 bound PAR was demonstrated to bind to p21 at the C terminal region (aa 140-163); DNA-PKcs (aa 2228-2752); Ku 70 (aa 243-264); p52 subunit of NF-κβ (aa 179-199); CAD (aa 148-169) and Telomerase (aa 962-983), as well as other proteins involved in maintaining the integrity of the genome. Through its interaction with these various proteins, PARP can participate in cell cycle checkpoints, DNA damage recognition and repair as well as programmed cell death.

Herceg and Wang (1999) introduced a point mutation into the PARP cleavage site (DEVD) that made it resistant to caspase cleavage. Cells expressing this mutated PARP treated with TNFα accelerated cell death, which was accompanied by depletion of NAD⁺ and ATP. The PARP inhibitor 3-aminobenzamide prevented NAD⁺ depletion in cells expressing wild-type PARP, and inhibited this accelerated cell death, which was shown to be mainly by necrosis, as well as apoptosis. They found that this accelerated death was due to NAD⁺ depletion, following PARP cleavage. This led to the conclusion that PARP cleavage prevents the induction of necrosis and promotes caspase-mediated apoptosis.
Consistent with the view that high levels of ATP allow the cells to undergo apoptosis, whereas low levels of ATP result in necrosis, it was proposed that PARP acts as a molecular switch between apoptosis and necrosis (Los et al. 2002). Supporting this view is the fact that PARP-1 deficient mice are protected from both ATP depletion and necrosis, but are not protected from apoptosis, indicating that this pathway is active in the absence of PARP (Ha and Snyder, 1999). Furthermore, during CD95 induced apoptosis, PARP is cleaved by caspases, and ATP levels are maintained, ensuring execution of apoptosis, whereas TNF induced PARP cleavage results in ATP depletion and necrosis. The difference between these two processes is in the active state of PARP. TNF-induced PARP cleavage activates PARP, whereas PARP cleavage by caspases inactivates the protein (Los et al. 2002).

The principle caspases involved in PARP cleavage are caspase 3 and caspase 7. They cleave the 116kDa PARP-1 at the DEVD site to generate an 85kDa and a 24kDa fragment (Lazebnik et al. 1994; Nicholson et al. 1995). The IR-induced response to DNA damage has been well characterised, but what is missing is the stimulating factor that causes the cell to die by apoptosis or necrosis. In light of the fact that TNF cleavage of PARP induces a necrotic pathway and caspase cleavage of PARP ensures an apoptotic pathway, it does seem that PARP is one of the regulating factors in which pathway is chosen.

1.19 ATM, p53 and Cancer.
The various functions of the ATM protein that have been discussed demonstrate the enormity of the various cellular processes in which ATM is involved. When we consider that the majority of A-T patients have truncating mutations of both ATM alleles, which ultimately result in its destabilisation and/or inactivation, it is not difficult to realise the impact of the absence of this protein in these patients. It is not only involved in surveillance of the DNA for damage, but it is involved in its removal. It is involved in cell cycle checkpoints that halt the cell cycle, providing the necessary time for the damage to be repaired, as well as repairing the damage by indirectly recruiting the proteins required for this process. Once this has been completed it is involved although indirectly, in DNA replication. If the damage cannot be repaired in time, the cell can be removed by apoptosis. This pathway is normally inhibited by ATM while the DNA is being repaired. In response to IR, A-T cells exhibit increased numbers of cells taking this pathway, which would be expected, as without ATM, this abrogates the negative control imposed by this protein.
ATM is also involved with proteins that regulate cell proliferation such as the retinoblastoma protein. Malfunction in these pathways can lead to unrestricted cell cycling and the development of cancer (reviewed in: Classon and Harlow, 2002).

An important cell cycle checkpoint for the development of cancer seems to be the G1/S phase checkpoint. ATM plays a pivotal role in this pathway by phosphorylating p53 on serine 15 (Rotman and Shiloh, 1997; Siliciano et al., 1997; Canman et al., 1997; Canman et al., 1998; Khanna et al., 1998; Banin et al., 1998; Nakagawa et al., 1999). p53 also plays a major role in this pathway but has been shown to be mutated in 60% of human cancers, and loss of cell signalling upstream and downstream of p53 has been implicated in the remaining cancers continuing to express wild type p53 (Bourdon, 2007). No fewer than nine different isoforms of p53 have been identified (Ghosh et al., 2004) that are abnormally expressed in tumour tissues. In normal human tissues, expression of these p53 isoforms is tissue-specific, and would account for the differences observed in different tissue types to the same stress stimulus. For example, IR induces p21 and p53-dependent cell cycle arrest in the liver, while the same dose of radiation induces robust p53-dependent apoptosis in the spleen and thymus (Midgley et al. 1995; Bouvard et al. 2000; Fei et al. 2002). A-T patients, defective for ATM, are sensitive to IR and have a higher incidence of cancer, especially lymphoblastic lymphomas (Hartwell and Kastan, 1994; Peterson et al., 1992). Lymphoblastic lymphomas are also the primary cancers seen in mice with disrupted p53 alleles (Hartwell and Kastan, 1994; Donehower et al., 1992). Considering that the G1/S phase checkpoint is abrogated in cells deficient for ATM and p53, and that deficiencies of both these proteins are associated with higher incidences of cancer, and the same primary cancers, this does suggest that the G1/S phase checkpoint is a critical control point for the development of cancer, with both p53 and ATM being essential for maintaining the integrity of the genome.

The A-T phenotype not only arises due to loss of function in the ATM protein, but also the loss of function of ATM target proteins that may be functionally altered as a direct result of defective or absent ATM protein. Considering the vast array of proteins with which ATM interacts and controls, it is not surprising that clinically A-T has a diverse phenotype. Where a protein with which ATM interacts is mutated or deleted, such patients express part of the A-T phenotype, but no other single loss of protein function exhibits the same phenotype as ATM.
1.20 Aims.
Principally, the aim of this thesis was to investigate methodologies that could be used to discriminate A-T heterozygotes from A-T homozygotes and wild type cells, using either:

1) Western blot analysis to measure ATM protein expression in A-T homozygous, A-T heterozygous and wild type patient-derived cell lines, or

2) Measurement of cell death profiles in the same patient-derived cell lines, to determine sensitivity to a range of cell damaging agents including IR, Etoposide, hydrogen peroxide, staurosporine, doxorubicin and bleomycin in combination with flow cytometry.

This thesis was also interested in the response of virally transformed lymphoblasts and untransformed fibroblasts to these DNA-damaging agents, as it has previously been reported that transformed cell lines show different cell death profiles to primary cell lines in response to IR (Duchaud et al. 1996; Bebb et al. 2001).

In undertaking these approaches, it was hoped that the efforts would aid the determination of suitable markers of A-T heterozygosity, as unlike A-T homozygotes, A-T heterozygotes are asymptomatic, and routine exposure to ionising radiation used for diagnosis of various medical conditions, as well as in screening and treatment for various malignancies could aid the development of neoplastic conditions in these susceptible individuals.
2.1 GENERAL CHEMICALS.

Coomasie brilliant blue R-250, acetic acid-glacial, sodium dodecyl sulphate, bromophenol blue, ammonium persulphate, glycine, TEMED, propidium iodide, Trizma base, EDTA, deoxycholate-sodium salt, DL-lactic acid, tertitol NP-40, sodium orthovanadate, aprotinin solution, phenylmethylsulfonyl fluoride (PMSF), Kodak developer, Kodak fixer, biamax light (light-1) chemiluminescent film, phenazine ethosulfate, dimethylsulfoxide (DMSO), staurosporine, immobilon-p PVDF membrane and bicinechonic acid kit were purchased from Sigma-Aldrich Company Ltd. (Dorset, U.K.).

Acrylamide, N, N'-Methylene-bis-acrylamide and DC protein assay kit were purchased from Bio-Rad Laboratories Ltd (Hemel Hempstead, U.K.).

RPMI 1640 medium without L-glutamine, nutrient medium F12-Ham with L-glutamine, MEM medium, DMEM medium, L-glutamine, penicillin/streptomycin, mark 12 unstained protein standard, WesternBreeze chemiluminescence detection kit (anti-mouse, anti-rabbit, anti-goat), NuPage sample buffer, NuPAGE sample reducing agent, NuPage antioxidant, 7% tris-acetate gels, tris-acetate running buffer, gel loading tips, tris-acetate transfer buffer, versene, trypsin and geneticin were purchased from Invitrogen life technologies (Paisley, U.K.).

Methanol (analytical grade) and hydrogen peroxide were purchased from Fisher Scientific U.K. (Leicestershire, U.K.).

Protogold was purchased from British Biocell International (Cardiff, U.K.).

Australian Foetal calf serum was purchased from PAA Laboratories Ltd. (Somerset, U.K.).

Phosphate buffered saline tablets were purchased from Oxoid (Basingstoke, U.K.).

DNA protein kinase (DNA-PK; V5811) was purchased from Promega (Southampton, U.K.).

Nuclear extraction kit (40010) was purchased from Active Motif (Rixensart, Belgium).
CHAPTER 2: METHODS

2.1.1 Antibodies.
Rabbit anti-ATM polyclonal antibody (Ab-3; PC116) was purchased from Merck Chemicals Ltd. (Nottingham, U.K.). Goat anti-ATM polyclonal antibody (Sc-1212), goat anti-ATM polyclonal antibody (sc-7128) and blocking peptide (sc-7128p) and mouse anti-ATM monoclonal antibody (QED; 3310) were purchased from Autogen Bioclear (Wiltshire, U.K.).

Mouse anti-ATM monoclonal antibody (Ab-78; Genetex); mouse anti-β-actin monoclonal antibody ((AC-15)-ab6276), alkaline phosphatase-conjugated goat anti-rabbit polyclonal antibody (ab6722) and rabbit anti-ATM polyclonal antibody (ab2887) and blocking peptide (ab13769) were purchased from Abcam (Cambridge, U.K.).

FITC-conjugated annexin-v antibody was purchased from BD Biosciences (Oxford, U.K.).

2.1.2 Chemotherapeutic drugs.
Etoposide, bleomycin, and doxorubicin were purchased from Merck Chemicals Ltd. (Nottingham, U.K.).

2.2 EQUIPMENT
2.2.1 Cell Culture.
Haemocytometer chamber, serological pipettes, sterile 2ml cryotubes, 24 well tissue culture plates and vented 25 and 75cm³ tissue culture flasks were purchased from (Fischer Scientific (Leicestershire, U.K.).

2.2.2 SDS-PAGE.

2.2.3 Quantitation of chemiluminescence and Coomassie blue stained protein bands.
Bio-Rad Fluor-S™ Multilamger with Quantity One software.
2.2.4 Flow cytometric analysis.
Beckton Dickinson FACScan™ with class 1 laser product incorporating class 3B laser and Cell Quest software. Results were further analysed using WinMDI 2.8 software.

2.2.5 Radiation source.
Nordion Gammacell 1000. Cesium-137 source with 22 becquerels (Bq) specific activity.

2.3.0 CELL CULTURE MATERIALS AND METHODS.
2.3.1 Cell Lines.
The following SV40 transformed lymphoblastoid cell lines (LCLs) were kindly donated by Professor A.M.R. Taylor, CRC Institute for Cancer Studies, The University of Birmingham, UK.

A-T1 = A-T homozygote. Two truncating mutations. 794 ins 4 and 2839del183 (loss of exon 21)
A-T1a = Same cell line as A-T1 (different batch)
A-T2 = A-T homozygote. Two truncating mutations. 4388 del T and 7928 del 83 (loss of exon 56)
A-T2a = Same cell line as A-T2 (different batch)
Het1 = A-T heterozygote. Mother of A-T1. Truncating mutation.
Het3 = A-T heterozygote. Truncating mutation. 6096 del 103 (loss of exon 44).
Het5 = A-T heterozygote. 822 del T or 7660C>G
PHet = Possible heterozygote. Mutation unknown.
Control cells were generated from the general population and were not genetically tested for A-T.

Untransformed fibroblast cell lines purchased from Coriell Cell Repositories, Coriell Institute for Medical Research (New Jersey, U.S.A.).


**GM03489** A-T heterozygote. Mother of GM03487. Mutation = A>T substitution at nucleotide 8266 in exon 58 resulting in truncation at codon 2756.

**GM08387** A-T heterozygote. Mutation = substitution at nucleotide 5932 (G>T) in exon 42 resulting in a truncation at codon 1978.

**GM08389** A-T heterozygote. Mutation = 4bp deletion at nucleotide 4642 in exon 33 resulting in a frame shift and truncation at codon 1548.

**GM03395** A-T homozygote. Homozygous for a G>A transition at nucleotide 7913 of the ATM gene (7913G>A) resulting in a substitution of a termination codon for tryptophan at codon 2638 [Trp2638Ter (W2638X)]. Increased sensitivity to cell killing by X-irradiation. Deceased sister had A-T.


Control cell lines kindly donated by Dr. Carole Rickards, Department of Medical microbiology, University of Wales College of Medicine (UWCM).

Human promyelocytic leukaemia HL-60 cells.
T lymphocyte Jurkat cells.

A-T homozygote and control cells kindly donated by Dr. Chris Jones, Department of Medical Biochemistry (UWCM).
A-T hTERT (Telomerase-transformed fibroblast A-T cell line)
MRC-5 hTERT (MRC-T; Telomerase-transformed fibroblast control cell line)

2.3.2 Preparation of Supplemented Media.
2.3.2.1 Preparation of culture medium for SV40 transformed lymphoblastoid cell lines, HL-60 and Jurkat cells.

Roswell Park Memorial Institute (RPMI) 1640 culture medium without L-glutamine, was supplemented (v/v) with 15% Australian foetal calf serum (FCS), 1% 2mM L-glutamine, 1% penicillin (50IU/ml)/Streptomycin (100µg/ml). All supplements were stored at –20°C prior to use. RPMI 1640 medium and supplemented medium was stored at 4°C. Supplemented medium was allowed to equilibrate to room temperature prior to use.

2.3.2.2 Preparation of culture medium for untransformed fibroblast cell lines.
Minimum essential medium (MEM) culture medium without L-glutamine, was supplemented (v/v) with 15% un-inactivated Australian foetal calf serum (FCS), 2mM L-glutamine, 1% penicillin (50IU/ml)/Streptomycin (100µg/ml). All supplements were stored at –20°C prior to use. MEM culture medium and supplemented medium was stored at 4°C. Supplemented medium was allowed to equilibrate to room temperature prior to use.
2.3.2.3 Preparation of culture medium for A-T hTERT fibroblast cell line.
Minimum essential medium (MEM) culture medium without L-glutamine, was supplemented (v/v) with 10% FCS, 2mM L-glutamine, 1% penicillin (50IU/ml)/Streptomycin (100μg/ml) and 500μg/ml geneticin (G418). All supplements were stored at -20°c prior to use. MEM culture medium and supplemented medium was stored at 4°c. Supplemented medium was allowed to equilibrate to room temperature prior to use.

2.3.2.4 Preparation of culture medium for MRC-5 hTERT fibroblast cell line.
Dulbecco's minimum essential medium (DME/M) culture medium without L-glutamine, was supplemented (v/v) with 10% FCS, 2mM L-glutamine, and 1% penicillin (50IU/ml)/Streptomycin (100μg/ml). All supplements were stored at -20°c prior to use. MEM culture medium and supplemented medium was stored at 4°c. Supplemented medium was allowed to equilibrate to room temperature prior to use.

2.3.3 Sub-culturing cells.
Supplemented culture medium was pre-equilibrated to 37°c prior to use. Lymphoblast cells were sub-cultured when the cells had grown to confluence. Confluence was determined by visual inspection and defined by the appearance of colonies at the bottom of the wells of the culture plate, accompanied by a colour change of culture medium from orange to yellow. Sub-culturing was performed by the replacement of half the cell suspension with the same volume of supplemented culture medium.

Fibroblasts were sub cultured when the cells had grown to confluence. Confluence was determined by visual inspection and defined by a spindle appearance of the cells, which covered approximately 80% of the bottom of the tissue culture flask. Sub-culturing was performed by removing the culture medium; adding 5ml of versene, and tilting the flask to ensure all cells were covered in versene. The versene was aspirated and 5ml of trypsin was added, and the procedure repeated. After aspirating the trypsin, the flask was returned to the 37°c incubator until the cells dislodged by tapping the flask. Ten ml of supplemented culture medium was added to the flask and the cell suspension was centrifuged at 600 x g for 5 minutes. The supernatant was removed and the cell pellet was resuspended in 30ml supplemented culture medium. Ten ml of cell suspension was added to each of three tissue
culture flasks containing an equal volume of supplemented culture medium. Flasks were then transferred to the 37°C incubator (37°C, 5% CO₂).

2.3.4 Cell counting.
A haemocytometer, a glass slide containing two chambers and a flat cover slip was used to estimate cell number. Each chamber consists of a grid of known volume (0.1mm³ or 0.1μl) etched on glass. Dispersal of cell colonies by gentle aspiration using a serological pipette was required prior to counting cells. Briefly, a 15μl of evenly suspended cells was applied to both chambers. The haemocytometer chamber was placed in a wet box for 5 minutes to allow the cells to settle into one plane before counting. Using a light microscope cells were counted in each grid. A mean cell count was taken and multiplied by 10⁴ to obtain a cell count in million cells/ml. The cell count was performed in duplicate and the mean value calculated.

2.3.5 Estimation of cell viability.
Cell viability was assessed by the exclusion of the dye Trypan blue. Equal volumes of cell suspension and 1% Trypan Blue (0.085g NaCl and 0.1g Trypan blue in 10ml distilled water) were incubated together for 15 minutes at room temperature. The percentage of cells remaining unstained correlated to the percentage of viable cells within the culture. The test was performed prior to sub-culturing cells and prior to all experimental procedures to ensure that a cell viability of 95% or greater was maintained.

2.3.6 Preparation of Freezing Medium.
Culture medium was supplemented with 30% FCS and 10% DMSO.

2.3.7 Long-term storage of cells.
Lymphoblastoid cell lines were suspended at 50 x 10⁶ and fibroblast cell lines were suspended at 10 x 10⁶ in 1ml freezing medium in sterile cryotubes. Cells were initially frozen at a slow rate in an insulated freezing box at -80°C overnight before being transferred to liquid nitrogen for long-term storage.
2.3.8 Freezing of cell pellets for subsequent protein extraction.
Cells were counted using a haemocytometer (2.3.8). The volume of cell culture required to pellet $15 \times 10^6$ lymphoblastoid or $7.5 \times 10^6$ fibroblast cells was centrifuged at 600 $\times$ g for 5 minutes. Pelleted cells were washed twice with sterile, ice-cold phosphate buffered saline (PBS) pH 7.3, and the cell pellet stored at $-70^\circ$C.

2.3.9 Thawing Cells.
Frozen cells were removed from liquid nitrogen and thawed rapidly in a water bath at $37^\circ$C. The cryotube was wiped with methanol and the contents were mixed with 10 ml culture medium. After centrifugation (500 $\times$ g, 10 minutes), lymphoblastoid cells were resuspended in 3 ml supplemented culture medium at room temperature, and 1ml was transferred to each of 3 wells in a 24 well tissue culture plate. Cultures were transferred to a humidified incubator ($37^\circ$C, 5% CO$_2$). Fibroblasts were treated identically, but resuspended in 10ml supplemented culture medium in a 25cm$^3$ vented tissue culture flask before being transferred to a humidified incubator ($37^\circ$C, 5% CO$_2$).

2.4 WESTERN BLOTTING FOR ATM PROTEIN.
2.4.1 Preparation of protein samples.
2.4.1.1 Preparation of whole cell lysate.
Lymphoblastoid ($15 \times 10^6$) or fibroblast ($7.5 \times 10^6$) cells were washed twice in ice-cold PBS and lysed for 30 minutes on ice in 100$\mu$l of Radio Immuno Precipitation Assay (RIPA) buffer (100mMNaCl, 10mM Tris-HCl (pH 7.2), 2mM EDTA, 0.5% (w/v) deoxycholate, 1% (v/v) NP40, 10mM MgCl$_2$, 1mM PMSF and 100$\mu$M sodium orthovandate). Samples were sonicated for 10 seconds on ice to shear DNA and to reduce sample viscosity. Cell lysate samples were centrifuged at 12000 $\times$ g for 5 minutes at 4$^\circ$C and the lysate supernatant fraction was removed into a fresh eppendorf.

2.4.1.2 Preparation of nuclear and cytoplasmic cell lysate.
Nuclear and cytoplasmic cell lysate was prepared using a nuclear extraction kit (40010, Active Motif) according to the manufacturers instructions.

2.4.2 Protein determination of cell extract.
The Bio-Rad DC Protein assay (Bio-Rad Laboratories, Herts, U.K.) determined colorimetrically, the protein concentration of the cell lysate after detergent solubilisation.
The reaction is based on the reaction between protein and copper in an alkaline medium and the subsequent reduction of Folin reagent by the copper-treated protein. Bovine serum albumin (BSA) standards were prepared over a concentration range of 0.25-5.0 mg/ml by dissolving BSA in cell lysis buffer. BSA standards and cell lysates were prepared in duplicate according to the manufacturers’ instructions before the absorbance was read at 750nm. A BSA standard curve was obtained by performing regression analysis using Minitab release version 12, and was used to calculate the protein concentration of samples, using the mean absorbance of samples assayed in duplicate.

2.4.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Note: Limited experiments were carried out using the Mini-Protean III Mini-Cell using pre-cast and hand-cast gels made from acrylamide/bis-acrylamide for evaluation of equipment. Gels were constructed according to the manufacturers instructions.

2.4.3.1 SDS-PAGE for ATM protein.

Samples of lysate supernatants were subjected to electrophoresis to separate the proteins. Denaturing gel electrophoresis was performed using the NuPage™ Tris-Acetate electrophoresis system; NuPage lithium dodecyl sulphate (LDS) sample buffer, NuPage sample reducing agent, NuPage antioxidant and NuPage Tris-Acetate running buffer (formulated according to the manufacturer’s instructions) and 7% Tris-Acetate gels with the XCell SureLock™ Mini-Cell and PowerEase™ power pack. Tris-Acetate pre-cast gels (7%) were assembled according to the manufacturer’s instructions. Running buffer solution was diluted 1:20 in a final volume of 1L. Antioxidant (0.5ml) was added to 200ml of prepared running buffer, which was used to fill the inner buffer chamber of the mini-cell. The remainder of the buffer was used to fill the outer buffer chamber. Sample supernatants were adjusted to 50µg/100µl protein concentration using 10% reducing agent and 12.5% LDS sample buffer, which were added to 50µg total cell protein. The sample was made up to 100µl with cell lysis buffer. Samples were vortexed and heated for 5 minutes at 95°C, to denature proteins. To each well of the gel, 10µl of heated sample supernatant (5µg protein) was loaded and electrophoresis carried out for 21h at 70V and constant current at 4°C.
2.4.3.2 SDS-PAGE for β-actin protein.
The same cell lysate samples used for electrophoresis of the ATM protein were used for electrophoresis of β-actin for use as a measure of protein loading. To each well of the gel, 10μl of heated sample supernatant was loaded and electrophoresis carried out for 55 minutes at 200V and constant current at room temperature (RT).

2.4.4 Electroblootning of proteins from gel to PVDF membrane.
2.4.4.1 Electroblootning of ATM protein.
Electrophoretic transfer of proteins was performed following SDS-PAGE, using the mini-cell blot module. PVDF membranes were pre-wetted in methanol and washed three times with deionised water. Transfer buffer solution was prepared at a 1:20 dilution, using 10% analytical grade methanol for 1 gel and 20% for two gels run at the same time, and making the volume up to 1L with deionised water. Antioxidant (1ml) was then added to the buffer. Electrophoresis gels were removed from the casing and the gel/membrane sandwich assembled according to the manufacturers’ instructions, orientated in such a way as the transfer of proteins from gel to PVDF membrane (0.45μm pore), would occur in the direction of cathode to anode. The blotting module was placed in an XCell Mini-Cell tank, transfer buffer poured into the blotting module and proteins electroblotted for 3h at a constant 60V at 4°C.

2.4.4.2 Electroblootning of β-actin protein.
Electroblootning for β-actin protein was carried out identically as detailed for ATM protein, except that transfer was carried out for 1h at a constant 30V at RT.

2.4.5 Western immunoblot detection of ATM and β-actin proteins.
Western immunoblot detection was carried out using the appropriate WesternBreeze chemiluminescent western blot immunodetection kit (anti-mouse, anti-goat, anti-rabbit) according to the manufacturers’ instructions. Briefly, following electrophoretic transfer, the PVDF membranes were placed immediately into blocking buffer and incubated for 30 minutes on a rotary shaker. The membranes were washed (x 2) for five minutes before being incubated with the primary antibody solution (antibody diluted in primary antibody solution at the required concentration) for 1h. The membranes were washed (x 4) with antibody wash before being incubated with the secondary antibody for 30 minutes. Membranes were washed (x 4) with antibody wash for 5 minutes before being washed (x 3).
with deionised water for 2 minutes. The membranes were then placed on a sheet of transparency plastic and the chemiluminescent substrate was added evenly to the membrane. After 5 minutes, the excess substrate was blotted from the membrane using filter paper and the membrane was covered with a second piece of transparency plastic. The membranes were then exposed to Biomax light chemiluminescence film for the desired length of time. The films were developed with 20% (v/v) developer and fixing agents (Sigma-Aldrich, Dorset, U.K.) before being rinsed in deionised water and air dried on blotting paper. Densitometry was then performed on either the ATM or β-actin protein bands using the Bio-Rad Fluor-S™ Multilimage with Quantity One software.

2.4.6 Western immunoblot detection of ATM protein ± blocking peptides.

Cell lysate samples were subjected to SDS-PAGE (2.4.3) and electroblotted (2.4.4) as detailed previously. Western immunodetection was performed with the appropriate WesternBreeze chemiluminescence kit as detailed in 2.4.5, with the following alterations: following transfer of the proteins to PVDF membranes, the membranes were blocked overnight at 4°C and an additional step using the primary antibodies with blocking peptides was performed. Goat anti-ATM polyclonal antibody (sc-7128; Santa Cruz) and blocking peptide (sc-7128p; Santa Cruz) were purchased from Autogen Bioclear (Wiltshire, U.K.), and rabbit anti-ATM polyclonal antibody (ab2887) and blocking peptide (ab13769) were purchased from Abcam (Cambridge, U.K.). Primary antibodies (2 µg/ml) were mixed with the appropriate blocking peptide (20 µg/ml), and made up to 500 µl in primary antibody dilution buffer (WesternBreeze kit) and incubated at RT for 2 hours or overnight at 4°C. After blocking, membranes were washed (x 4) for 5 minutes in WesternBreeze wash buffer and incubated with primary antibody or the previously mixed primary antibody and immunogenic peptide overnight at 4°C. The WesternBreeze kit was used as detailed in 2.4.5. An additional secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit polyclonal antibody; 1:3000 dilution; Abcam) was also used with the rabbit primary antibody ± blocking peptide, replacing the secondary antibody in the WesternBreeze kit for comparative studies.

2.5 STAINING GELS/MEMBRANES.
2.5.1 Coomassie Blue Staining.

Gels or membranes were gently agitated in staining solution (1% (w/v) Coomassie blue R-250, 10% glacial acetic acid (v/v), 45% methanol (v/v) and 45% deionised water (v/v)) for
10 minutes, then placed in destaining solution (10% glacial acetic acid (v/v), 10% methanol (v/v) and 80% deionised water (v/v)) for 1 hour. Protein bands were recorded using Quantity One® software (Bio-Rad).

2.5.2 Membrane staining with Protogold.
Membranes were stained with protogold according to the manufacturer's instructions (British Biocell International, Cardiff, U.K.). Briefly, following immunodetection of ATM protein, the membrane was washed in PBS (pH 7.4) twice for 5 minutes and blocked in PBS containing 0.3% Tween 20 for 30 minutes at 37°C. Membranes were washed (x 3) in PBS containing 0.3% Tween 20 for 5 minutes, followed by washing in deionised water to remove buffer salts. Membranes were incubated with protogold with agitation until protein staining was at the required level to permit visual observation of protein bands. Membranes were washed (x 2) for 5 minutes prior to drying the membranes.

2.6 TREATMENT OF CELLS WITH γ-Irradiation.
2.6.1 To determine the effect of transporting cells to an off-site radiation facility.
Preparation of cell lines for transportation to an “off-site” facility for irradiation.

All cell lines were seeded at 1 x 10⁶/ml 24 hours before transportation to ensure the cells were in log phase of the cell growth cycle. Prior to use in any experimental procedures, a trypan blue exclusion test (as detailed in 2.3.5) was carried out to determine cell viability. One flask from each of three cell lines (A-T1; Het 1; C5) were processed as follows: Maintained in a 37°C humidified incubator consisting of 95% air and 5% CO₂.

The flasks were sealed with parafilm to preserve the CO₂ concentration within the flask before being transferred to a polystyrene box containing an ice pack. These flasks were not transported to the “off-site” facility.

The flasks were sealed with parafilm to preserve the CO₂ concentration within the flask before being transferred to a polystyrene box containing a heat pack that had been equilibrated to 37°C. These flasks were transported to the “off-site” facility.

All the samples were assayed at 8 hours post transportation for apoptosis (using Annexin-V) and necrosis (using propidium iodide, PI) as described in 2.8.
2.6.2 Transport and γ-irradiation of cell lines.
All cell lines were seeded at 1 x 10⁶/ml 24 hours (Lymphoblastoid cells) or 48h (fibroblast cells) before transportation to ensure the cells were in log phase of the cell growth cycle. Prior to use in any experimental procedures, a trypan blue exclusion test (as detailed in 2.3.5) was carried out to determine cell viability. Flasks were sealed with parafilm to preserve the CO₂ concentration within the flask before being transferred to a polystyrene box containing a heat pack that had been equilibrated to 37°C. The flasks were transported to the off-site facility and irradiated/mock irradiated (treated exactly the same as irradiated cells with the exception that they were not irradiated) at various exposures. All cultures were assayed at 8 hours post irradiation for apoptosis (using Annexin-V) and necrosis (using propidium iodide, PI) as described in 2.8 unless otherwise stated.

2.7 TREATMENT OF CELLS WITH CHEMICALS/CHemoth ERAPEUTIC DRUGS.
2.7.1 Formulation of chemicals/chemotherapeutic drugs.
2.7.1.1 Hydrogen peroxide.
Hydrogen peroxide was used for continual exposure experiments (various times) in a 9.8M/L solution, and 25μM was added to cells 4 times at 30-minute intervals to a final concentration of 100μM. Hydrogen peroxide solution was stored refrigerated until required.

2.7.1.2 Staurosporine
Staurosporine (100μg) was dissolved in 21.4μl sterile DMSO to give a 10mM/L stock solution. The solution was further diluted by mixing 10μl of stock solution with 90μl DMSO to give a working stock solution of 1mM/L. Stock solutions were stored refrigerated and protected from light until required.

2.7.1.3 Etoposide
Etoposide (25mg) was dissolved in 425μl sterile ultra-filtered water to give a stock solution of 100mM/L. A working stock solution of 50mM/L was generated by mixing an equal volume of the 100mM/L stock solution, with an equal volume of sterile ultra-filtered water as required. Stock solutions were stored at -20°C and protected from light until required.
2.7.1.4 Doxorubicin
Doxorubicin (0.019g) was dissolved in sterile ultra-filtered water (1 ml) to provide a stock concentration of 10 mg/ml. A working stock of 500μg/ml was produced as required by mixing equal volumes of the 10mg/ml stock solution with sterile ultra-filtered water. Stock solutions were stored refrigerated and protected from light until required.

2.7.1.5 Bleomycin
Bleomycin (15U) was dissolved in 143μl normal saline (0.9% NaCl in sterile ultra-filtered water) in a glass vial to give a stock solution of 50mM/L. Stock solution was stored at -20°C until required.

2.7.1.6 Propidium Iodide (PI).
PI (10μg) was dissolved in 333μl PBS (pH7.4) to generate a working stock solution of 30μg/ml. The solution was stored refrigerated and protected from light until required.

2.8 ANNEXIN-V AND PROPIDIUM IODIDE APOPTOSIS/NECROSIS ASSAY.
Lymphoblastoid cells were seeded at 1 x 10⁶/ml and cultured for 24h to ensure cells were in log phase at the time of apoptosis induction. Cell viability was determined using trypan blue exclusion (as detailed in 2.3.5) and apoptosis induction was only performed on cultures with >95% viability. Fibroblast cells were seeded at the same concentration and apoptosis was induced in most cases, 48h later. Apoptosis was only induced in these cell lines when the cells had reached 60% confluency. The time at which this occurred was not uniform between the cell lines, as some cell lines required an additional 24h of culture.

Apoptosis was induced using γ-irradiation, chemicals, or chemotherapeutic agents at various exposures/concentrations. Following treatment, fibroblast cells were dislodged from the flask (as detailed in 2.3.3), and lymphoblastoid and fibroblast cells were washed twice in cold PBS (pH7.4). Cells were resuspended in cold binding buffer (0.1M Hepes (pH7.4) 1.4M NaCl, 25mM CaCl) at a concentration of 1 x 10⁶/ml, and 500μl (5 x 10⁵ cells) was transferred to a microfuge tube. FITC-conjugated annexin-v (5μl) was added to each tube, and the contents of the tubes mixed by gentle pipetting. Tubes were incubated at room temperature (18-24°C) in the dark for 15 minutes. Tubes were centrifuged at 600 x g for 5 minutes and the supernatant removed. Cells were resuspended in 500μl cold binding buffer with 10μl PI and placed on ice. Samples were analysed immediately by flow
cytometry. Trypan blue exclusion was performed after Flow cytometric results were then analysed using WinMDI 2.8 software to determine quadrant percentages of viable, early apoptotic, late apoptotic and necrotic cells. The percentage of cells in each quadrant in the untreated control sample was subtracted from values obtained for treated cells to give the percentage increase in apoptosis/necrosis for each cell line.

2.9 Statistical Analysis.
All statistical analysis was performed on Minitab® version 15 (Havertown, Pa). Multiple group comparison involved one-way ANOVA and two group comparisons used the two-sample t-test. Results were deemed significant when p <0.05 throughout.

2.10 Ethical Approval.
Ethical approval was granted by UWIC ethics committee for the use of transformed cell lines in this research. (document in appendix)

2.11 Risk Assessment.
All experimental procedures were carried out in line with COSHH (Control Of Substances Hazardous to Health) regulations.
CHAPTER 3: RESULTS

3.0 ATM protein analysis using western blotting.

3.1 Optimisation of Experimental Conditions for Western Blotting.

3.1.1 Optimising voltage and time parameters for protein resolution.

To optimise the electrophoresis conditions that permitted full resolution of the proteins, samples were electrophoresed for either A) 1 hour (180V) and transferred to PVDF membrane for 1.5h (200v), or B) electrophoresed for 21h (40V) and transferred to PVDF membrane for 3h (30V). It has been reported previously that most ATM mutations identified in A-T patients are expected to completely inactivate the protein due to protein truncation (Savitsky et al. 1995a; Byrd et al. 1996; Gilad et al. 1996; Telatar et al. 1996), and that truncating the protein affects its stability (Gately et al. 1998). Therefore, the majority of A-T homozygotes fail to produce any western blot species in protein recognition experiments. The ATM protein is routinely identified in western blotting by the presence of a band from control cell lysates and the absence of a band in A-T homozygote cell lysate (Gately et al. 1998).

The results indicated that enhanced resolution of the proteins was obtained by running the gel for longer with a lower voltage (Fig. 3.1.1B). However, this better resolution also indicated the ATM band on immunoblot A had resolved into two bands on immunoblot B, (ATM and ATM-A). The results suggested that the band at the top of the blot (Fig. 3.1.1B) was the ATM band, indicated by the absence of a band in lane 4, which corresponded to the cell lysate sample from the A-T homozygote cell line A-T1. The additional bands were therefore either truncated ATM proteins or non-specific signals. The PVDF membrane was washed and counterstained with protogold, a colloidal gold solution that binds to proteins (as detailed in the methods). The results demonstrated that the blot was heavily stained, which suggested a good transfer of the proteins to the PVDF membrane (data not shown). However, staining the gel used for immunoblotting with Coomassie blue following transfer of proteins to PVDF membrane indicated an incomplete transfer of proteins (data not shown), which highlighted the need to optimise these conditions further.
Figure 3.1.1 Optimising voltage and time for protein resolution.

Cells were lysed in RIPA buffer and protein concentration determined (both as detailed in the methods). Conditions for electrophoresis and transfer of proteins to PVDF membranes are detailed below. Membranes were probed for ATM using Rabbit Polyclonal anti-ATM (1µg/ml; PC116, Merck) primary antibody and WesternBreeze anti-rabbit chemiluminescence kit (as detailed in the methods).

A) 50µg whole cell lysate (Lane 1 = control C1, Lane 2 = A-T heterozygote Het 1, Lane 3 = A-T heterozygote Het 2, Lane 4 = A-T homozygote A-T 1) was electrophoresed for 1 hour (180V), transferred to PVDF membrane for 1.5h (200v), and photographic film was exposed for 3 minutes (as detailed in the methods).

B) A duplicate gel was prepared as detailed in (A) and electrophoresis was conducted for 21h (40V), transferred to PVDF membrane for 3h (30V), and photographic film was exposed for 3 minutes (as detailed in the methods).

Resolution positions of ATM, a protein species underneath the ATM band designated ATM-A, and an additional band (Band I) are indicated on immunoblots A and B.
3.1.2 Electrophoretic transfer optimisation.

Previous data established that enhanced resolution of protein bands (electrophoresed for 21h, 40V) was obtained by extending the electrophoresis time. To determine if the resolution of the proteins could be improved by increasing the voltage, electrophoresis was conducted at 70V for 21h. However, previous data also indicated that transfer of proteins to PVDF membrane (3h, 30V) had resulted in incomplete transfer. To determine conditions for optimum transfer of proteins, following electrophoresis of whole cell lysate (100µg, control cell line C1), protein transfer was performed at 45V for 9 hours, with the first two membranes being removed at 3 and 6h respectively. Membranes were probed for ATM and the gel used for transfer was then stained with Coomassie blue to determine transfer efficiency.

Data indicated that better resolution of protein bands was obtained by increasing the voltage (Fig 3.1.2B), and immunoblots indicated that the band intensity was greater at 3h (Fig 3.1.2C) than at 6 or 9h (Fig 3.1.2 D and E respectively). However, staining the gels with Coomassie blue after transfer suggested an incomplete transfer (data not shown), indicating further optimisation of the transfer conditions was required. Additional membrane sections were included to prove complete transfer and immobilisation onto the PVDF membrane (data not shown). Based on these collective results, electrophoresis at 70V for 21h, and transfer of proteins to PVDF membrane for 3h were adopted as standard conditions in subsequent experiments.
Figure 3.1.2 Electrophoretic transfer optimisation.

Control cells (C1) were lysed in RIPA buffer and protein concentration determined (both as detailed in the methods). 100μg whole cell lysate was loaded in each well on a 7% Tris-Acetate gel and Electrophoresis was performed for 21h at 70V (constant voltage). Proteins were transferred at 45V for up to 9 hours. At 3, 6 and 9 hours, membranes were probed for ATM using Rabbit polyclonal anti-ATM primary antibody (1μg/ml; PC116, Merck) and anti-rabbit WesternBreeze kit (as detailed in the methods). Photographic film was exposed for 3 minutes.

Key to Figure:
A = ATM immunoblot (lane 1) from Fig. 3.1.1B (electrophoresis 21h, 40V; transferred to PVDF membrane for 3h, 30V)
B = ATM immunoblot (electrophoresis 21h, 70V; transferred to PVDF membrane for 3h, 45V)
C = ATM immunoblot (electrophoresis 21h, 70V; transferred to PVDF membrane for 3h, 45V)
D = ATM immunoblot (electrophoresis 21h, 70V; transferred to PVDF membrane for 6h, 45V)
E = ATM immunoblot (electrophoresis 21h, 70V; transferred to PVDF membrane for 9h, 45V)
3.1.3 Investigation to determine the identity of the additional western blot protein species detected.

During experiments to isolate the ATM protein as a single protein species on the gel, it was apparent that both the rabbit polyclonal (Fig 3.1.1B) and mouse monoclonal (data not shown) anti-ATM primary antibodies were detecting a number of protein species on the same blot. The results suggested that the band detected at the top of the blot was the ATM band, indicated by the presence of a band in lanes 1-3, which corresponded to control and A-T heterozygote samples, and the absence of a band in lane 4, which corresponded to the cell lysate sample from the A-T homozygote cell line A-T1 (Fig 3.1.1B). The additional bands were therefore either truncated ATM proteins, other A-T family members sharing sequence homology with the ATM protein or non-specific signals. DNA-PK is a member of the same protein family as ATM and shares sequence homology with the ATM protein. To investigate whether the additional bands were likely to be members of the same family of proteins as ATM, commercially available DNA protein kinase (DNA-PK; Promega), was run under reducing conditions (data not shown) and loaded neat onto the gel (Fig 3.1.3A). Additional gels were run with control, A-T heterozygote and A-T homozygote cell lysate (Fig 3.1.3B) and with 10 µl Mark 12 unstained molecular weight standard (Fig 3.1.3C). Data demonstrated that the position of the DNA-PK protein band did not correspond to any of the additional protein bands detected by the primary anti-ATM antibodies (Fig 3.1.3A). Data also demonstrated that DNA-PK (460kDa) exhibited slower migration within the gel compared to ATM (350kDa), which would be consistent with its molecular weight. Additionally, the ATM band (Fig 3.1.3B) resolved above the 200kDa myosin band (Fig 3.1.5C). The data were also useful, as molecular weight markers above 200kDa (myosin) are not commercially available, and so by running DNA-PK (460kDa) on the gel, the migratory resolution point of this protein generated a molecular weight marker with a higher molecular weight than ATM, to which ATM could be compared. Under these electrophoresis conditions, the DNA-PK migrated 1.3cm, myosin migrated 2.5cm and the ATM protein was located 2.1cm from the bottom of the loading well. The position of the ATM protein band (350kDa) was located between the DNA-PK (460kDa) and myosin (200kDa) bands, which helped to affirm the position and size of the ATM protein in all subsequent blots.
Figure 3.1.3 Investigation of additional protein species to the ATM protein detected following electroblotting.

A) 5μl DNA-PK (460kDa) loaded on a 7% Tris-Acetate gel.

B) Control (C1; lane 1), A-T heterozygote (Het1; lane 2) and A-T homozygote (A-T1; lane 3) cells were lysed in RIPA buffer and protein concentration determined (both as detailed in the methods). 50μg whole cell lysate from each sample were loaded on a 7% Tris-Acetate gel.

C) 10μl Mark 12 unstained molecular weight standard.

Electrophoresis was performed for 21h at 70V (constant voltage). Transfer of proteins to PVDF membrane (gel B) was performed at 45V for 3h and the membrane was probed for ATM using rabbit polyclonal anti-ATM primary antibody (1μg/ml; PC116, Merck) and anti-rabbit WesternBreeze kit (as detailed in the methods). Photographic film was exposed for 3 minutes. Gels A and C were stained with Coomassie blue as detailed in the methods.
3.1.4 Evaluation of anti-human ATM mouse monoclonal primary antibody.

Previous data showed the existence of two putative ATM species within western blots (see Fig 3.1.2A) using the rabbit polyclonal anti-ATM primary antibody. Therefore, additional primary antibodies were obtained to determine if the same western blot species would be detected on immuno-labelled western blots, as those generated by the rabbit anti-ATM primary Antibody. The immunogen used for the rabbit anti-ATM primary antibody used in the initial data was a synthetic peptide (CKSLASFIKPFDRGEVESMEDDTNG) corresponding to amino acids 819-844 of human ATM protein. A mouse monoclonal anti-ATM primary antibody (ab78; Abcam) where the immunogen was a fusion protein expressed in E. coli corresponding to amino acids 2577-3056 was chosen for further studies.

Protein samples were prepared from one A-T homozygote (A-T 1), one A-T heterozygote (Het 1), and one control (C1), and results demonstrated that the protein bands produced by the primary monoclonal antibody were superior to those observed using the rabbit polyclonal antibody (Fig 3.1.4). The bands were clear, intense, defined, and there was no background staining. A single band was produced for the A-T heterozygote and control cells and no band was present from the A-T homozygote sample (3 min exposure; Fig 3.1.4A). However, prolonged exposure (10 mins) revealed the presence of >20 additional bands from the A-T heterozygote and control samples (Fig 3.1.4B). The number of smaller bands observed from the A-T homozygote sample (deficient in ATM staining) was considerably less. These results indicate the usefulness of this antibody in ATM detection. Additional data evaluating recognition of the ATM protein with additional polyclonal antibodies (rabbit anti-ATM; ab2887, Abcam and goat anti-ATM; C1-1212, Autogen Bioclear) failed to generate an adequate ATM signal (data not shown).
Figure 3.1.4 Evaluation of anti-human ATM mouse monoclonal primary antibody.
Control Cl (lane 1), A-T heterozygote Het 1 (lane 2) and A-T homozygote A-T 1 (lane 3) were lysed in RIPA buffer and protein concentration determined (both as detailed in the methods). 100μg whole cell lysate from each sample was loaded on a 7% Tris-Acetate gel and Electrophoresis was performed for 21h at 70V (constant voltage). Transfer of proteins to PVDF membrane was performed at 70V for 3h. The membrane was probed for ATM using Mouse monoclonal anti-ATM primary antibody (2μg/ml; ab78, Abcam) and anti-mouse WesternBreeze kit (as detailed in the methods). Photographic film was exposed for 3 minutes (A) or 10 minutes (B).
3.1.5 Evaluation of two anti-human ATM mouse monoclonal antibodies with the same target sequence.

Three anti ATM primary antibodies, each with a different ATM target sequence had been previously used to detect ATM in the current research study. An additional anti-ATM monoclonal primary antibody (3310, QED), with the same target sequence as the Abcam anti-ATM antibody (amino acids 2577-3056) was used to determine (1) similar specificity to the Abcam antibody and (2) if the additional bands that were being detected by the Abcam antibody would also be detected by the QED antibody. Results demonstrate that the sensitivity of the QED anti-ATM antibody (Fig. 3.1.5B) was slightly less than the Abcam antibody (Fig. 3.1.5A), as the band intensity using the QED antibody was not as great as that observed for the Abcam antibody, and that the bands detected by the Abcam antibody were also detected by the QED antibody.
null
Figure 3.1.5 Evaluation of two anti-ATM mouse monoclonal antibodies with the same target sequence.
3.1.6 Sample optimisation.

The basis of a screening assay necessitates the identification of A-T heterozygotes from controls. It had been observed that the whole cell lysate from the A-T heterozygote cell line (Het 1) was generating a band intensity that was similar to, and on some occasions, greater than that generated by the control cell line C1. To determine whether the concentration of ATM in the control sample was sufficiently high enough to interfere with access of the primary antibody to its target sequence, protein samples were prepared at 30, 15, 10 and 5µg for both cell lines, which were run on the same gel under standard conditions. Results indicate the greatest difference in signal intensity observed between control (Fig 3.1.6A) and A-T heterozygote (Fig 3.1.6B) occurred using 5µg whole cell lysate. Therefore, 5µg was adopted as the standard concentration for subsequent experiments.
Figure 3.1.6 Optimisation of signal intensity.

Control C1 (A) and A-T heterozygote Het 1 (B) were lysed in RIPA buffer and protein concentration determined (both as detailed in the methods). Whole cell lysate (30µg lane 1, 15µg lane 2, 10µg lane 3 and 5µg lane 4) were prepared for both cell lines and loaded on a 7% Tris-Acetate gel. Electrophoresis was performed for 21h at 70V (constant voltage) and proteins were transferred to PVDF membrane (3h at 60v). The membrane was probed for ATM using Mouse monoclonal anti-ATM primary antibody (1µg/ml; ab78, Abcam) and anti-mouse WesternBreeze kit (as detailed in the methods). Photographic film was exposed for 3 minutes.
3.1.7 Limits of detection using mouse monoclonal anti-ATM primary antibody.

To determine the limits of detection of the mouse monoclonal anti-ATM primary antibody, doubling dilutions were made from a 10μg/ml whole cell lysate stock solution to give protein concentrations of 10, 5, 2.5 and 1.25μg/ml for each cell line (A-T 1 (Fig 3.1.7A); Het 1 (Fig 3.1.7B), Het 4 (Fig 3.1.7C) and control C1 (Fig 3.1.7D). Results (Fig 3.1.7) indicate that with the control and heterozygote cell lines, a faint band was observed at 2.5μg/ml, but was not of sufficient quality to measure band intensity between the three cell lines. Therefore, the limits of detection for this antibody were determined as 5μg whole cell lysate/lane. The A-T homozygote cell line A-T 1 did not produce an ATM signal.
Figure 3.1.7 Limits of detection using mouse monoclonal anti-ATM primary antibody. Control C1 (A), Het 1 (B), Het 4 (C) and A-T 1 (D) cells were lysed in RIPA buffer and protein concentration determined (both as detailed in the methods). Doubling dilutions of a 10µg protein sample for each cell line were prepared and loaded on a 7% Tris-Acetate gel. Electrophoresis was performed for 21h at 70V (constant voltage) and proteins were transferred to PVDF membrane (3h at 60V). The membrane was probed for ATM using Mouse monoclonal anti-ATM primary antibody (1µg/ml; Abcam) and anti-mouse WesternBreeze kit (as detailed in the methods). Photographic film was exposed for 3 minutes. The 10, 5, 2.5 and 1.25µg whole cell lysate samples from each of the cell lines are shown in lanes 1-4 respectively.
3.1.8 Optimisation of ATM extraction procedure.
To determine the localisation and expression of ATM in different cell lines, whole cell lysate was obtained from each of the cell lines and nuclear and cytoplasmic fractions were obtained using a nuclear extraction kit (Active Motif) according to the manufacturers instructions. Results demonstrate that ATM was predominantly localised to the nucleus, with a small level of ATM being present in the cytosolic fractions. The intensity of the ATM band from the nuclear fractions and the whole cell lysate were similar in all cell lines analysed (Fig 3.1.8). Results indicate that either nuclear or whole cell lysate could be used in the assay to detect the level of ATM present. However, as separation of nuclear and cytoplasmic fractions could be subject to variability, due to the loss of nuclear lysate into the cytosolic fraction, and using nuclear cell lysate would introduce more steps into the assay that could have a cumulative effect on reproducibility of the assay, it was decided to use whole cell lysate in further optimisation procedures. Interestingly, ATM protein was not detected in HL-60 cells.
A

Control C5  A-T heterozygote Het 1  A-T homozygote A-T 1

B

A-T heterozygote Het 2  HL-60  A-T heterozygote Het 5

C

Control C4  A-T heterozygote Het 4  A-T homozygote A-T1a

Figure 3.1.8 ATM localisation and Expression.
3.1.9 To determine specificity of antibody binding.

Identification of the ATM protein on western blots to date has relied on the absence of an ATM band from A-T homozygote cells when compared to A-T heterozygote or wild-type cells (Gately et al. 1998). The aim of this work was to prove the specificity of the ATM antibody using competition with a specific ATM immunogenic peptide, to compete with immobilised ATM protein on PVDF membrane for the anti-ATM primary antibody. This experiment was undertaken due to the unavailability of competing ATM protein. Two ATM peptides were used with rabbit anti-ATM, and goat anti-ATM polyclonal primary antibodies respectively. Results demonstrated a faint ATM band using the goat anti-ATM primary antibody (Fig 3.1.9A), but when the blocking peptide was used (Fig 3.1.9B) the ATM band was not detected indicating the competitive nature of the immunogenic peptide. The rabbit anti-ATM primary antibody failed to produce an ATM band, and using the blocking peptide resulted in heavy background staining, which could not be corrected by using higher concentrations of the antibody, by washing the membranes for longer, or with different washing or blocking buffers (data not shown).
To determine specificity of antibody binding.

Control (C1) lymphoblastoid cells were lysed in RIPA buffer and protein concentration was determined (both as detailed in the methods). 50μg whole cell lysate was loaded on a 7% Tris-Acetate gel. Electrophoresis was performed for 21h at 70V (constant voltage). Transfer of proteins to PVDF membrane was performed at 60V for 3h.

Goat anti ATM primary antibody (2μg/ml; C1-7128, Autogen Bioclear) and blocking peptide (20μg/ml; C1-7128p, Autogen Bioclear) were incubated together overnight at 4°C (as detailed in the methods). Membranes used with the goat anti-ATM primary antibody and/or blocking peptide were blocked overnight at 4°C. Membranes were washed and incubated with Goat anti-ATM primary antibody (A) or goat anti-ATM primary antibody and blocking peptide (B) overnight at 4°C. Goat WesternBreeze kit was used for immunodetection (as detailed in the methods).

An additional membrane (C) was probed for ATM with mouse monoclonal primary antibody (1μg/ml, Abcam) and mouse WesternBreeze kit (as detailed in the methods).

Photographic film was developed for 2 minutes.
3.1.10 Test to determine if it is possible to discriminate A-T heterozygotes from A-T homozygotes and controls.

Having optimised and standardised the variables to be used in the assay, the feasibility of using western blotting as a method to discriminate A-T heterozygotes from A-T homozygotes and controls was tested. A-T homozygote (A-T 1, A-T1a, A-T 2), A-T heterozygote (Het 1; Het 1a; Het 2; Het 2a; Het 3; Het 4; Het 5), control (C1; C1a; C2; C3; C4; C5; C6; C7) and a cell line of unknown genotype (P Het), thought to be a possible A-T heterozygote lymphoblastoid cell lines were assayed under constant conditions (see legend) and the membranes were probed for ATM and β-Actin respectively. The β-Actin membranes were exposed to photographic film for 10 and 20 seconds, and 1 and 5 minutes. The mean β-Actin expression ± standard deviation was calculated for 10 cell lines at each time point and plotted against time. The graph indicated that the chemiluminescent signal for β-Actin was biphasic, with increased chemiluminescence occurring between 0-20 seconds, and a rapid increase occurring from 1-5 minutes. The only time point at which there was no increase in chemiluminescence was between 20 seconds and 1 minute (data not shown). Measuring the chemiluminescent signal at this time point would minimise errors due to manual handling time for removal of the membranes from the dark box, and developing the membranes. The β-Actin densitometry results (minus background) at this time point were therefore used to normalise the ATM densitometry readings (minus background). The mean ± standard deviation of the control cells was calculated (97.9% ± 37.7%, n=7) and the A-T homozygote and heterozygote cells were expressed as the percentage expression of the mean control value (Fig 3.1.10 A). As demonstrated in Figure 3.1.10A, all of the A-T homozygote and heterozygote cells expressed <50% of mean control values. Additionally, using this method, two A-T homozygote cell lines (A-T1a, A-T 2), and one A-T heterozygote cell line (Het 1), did not produce sufficient ATM protein to be measured in the assay, and so would be classed correctly as carrying at least one ATM mutation. The cell line of unknown genotype (but thought to be an A-T heterozygote; P Het) also did not produce enough protein to be measured in the assay. Results therefore suggest that P Het is carrying at least one ATM mutation.
Figure 3.1.10A Graph showing ATM expression in A-T homozygote and heterozygote cell lines normalised to β-Actin and expressed as % of controls (n=7).

Figure 3.1.10B ATM expression in A-T homozygote, A-T heterozygote and control lymphoblastoid cell lines
3.1.11 Testing untransformed A-T homozygote and heterozygote fibroblast cells.

Having demonstrated that it was possible to discriminate A-T homozygote and heterozygote whole cell lysate samples from control samples, the same assay conditions as detailed in 3.1.10 were applied to untransformed fibroblast cells. One A-T homozygote (GM03395), and four A-T heterozygote (GM08387; GM08389; GM03396; GM03397) untransformed cell lines were used with four of the cell lines used in 3.1.10, which were used to generate control values (C2; C3; C4; C5). Cell lines were assayed under constant conditions and the membranes were probed for ATM and β-Actin respectively (see legend).

The β-Actin densitometry results (minus background) were used to normalise the ATM densitometry readings (minus background). The mean ± standard deviation of the control cells was calculated (76.41% ± 13.96%, n=4) and the A-T homozygote and heterozygote cells were expressed as the percentage expression of the mean control value (Fig 3.1.11A). Results demonstrate all of the A-T heterozygote cells expressed <35% of mean control values. Additionally, using this method, the A-T homozygote cell line (GM03395), did not produce sufficient protein to be measured in the assay (Fig. 3.1.11), and so would be classed correctly as carrying at least one ATM mutation.
Figure 3.1.11 Testing untransformed A-T homozygote and heterozygote fibroblast cells.
3.2 Treatment of Human Cell Lines with Gamma Radiation.

Radiotherapy is used to treat cancers associated with lymphoid cells such as lymphomas, myelomas or leukaemias (Fertil and Malaise, 1985), which are the main types of cancer that patients with A-T develop. A-T cells are extremely sensitive to IR; (Stilgenbauer et al. 1997) as it only takes 32% of the normal tolerance dose to kill the same number of A-T cells as normal cells (Nagasawa et al. 1987). A-T heterozygotes show intermediate radiosensitivity between that of A-T homozygotes and normal subjects (Taylor et al. 1985; Gatti et al., 1991; Zakian, 1995) and 75% of the normal tolerance dose of radiation is able to kill the same number of A-T heterozygote cells compared to normal cells (Nagasawa et al. 1987). In patients with A-T, the dose can be adjusted to take into account the patient's medical condition and the possible response of the patient to the treatment, but A-T carriers are asymptomatic, and so would receive the same radiation dose as a person with two normal copies of the gene. These factors necessitate research into the response of A-T carriers to the effects of IR.

3.2.1. Assessment of the effect of cell transportation, to an off site facility, on cell viability.

Due to the lack of an in house radiation source, an off site radiation facility had to be used. This posed the question of whether the transportation of the cells would be detrimental to cell viability. During the transportation, there was the possibility that the cells could suffer from a decrease in both temperature and CO₂ concentration. To minimise these effects, it was decided to transport the cells in a sealed polystyrene box containing a heat pack that was pre-equilibrated to 37°C in an incubator, and to seal the flasks with parafilm to minimise the loss of CO₂. Transport to and from the facility was timed at 20 minutes in total, plus the time it took to irradiate the cells (transportation and irradiation, 60 minutes maximum).

An experiment was therefore devised to determine if possible decreases in temperature and CO₂ would have adverse effects on cell viability. Cells were either kept in a 37°C in house incubator, kept in house within a sealed polystyrene box containing a freezer pack, or transported to the off site radiation facility in the afore mentioned heated box. The transported cells were kept at the facility for 45 minutes before being transported back. This was to ensure that if a high radiation dose, and therefore long radiation exposure time

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was required, the time taken to transport and irradiate the samples would not be detrimental and have an adverse effect on the percentage of cell death recorded for that experiment.

Cell death profile analysis was conducted at 8 hours post-irradiation as previously described (Elward et al. 2005; Elward, K. personal communication) for apoptosis (using annexin-V) and necrosis (using PI) as detailed in the methods section. The results indicated that the conditions used for cellular transportation to an off-site facility reduced cell viability by <10% (Fig. 3.2.1).

To confirm these results, cell viability was also tested at 9 and 24 hours post transportation using the trypan blue exclusion test as detailed in the methods section. All cell lines used in the assay showed >90% viability. This was also confirmed by a colour change in the medium from orange to yellow along with cell aggregation which is a marker of cell proliferation in these cells (Taylor, personal communication).
Figure 3.2.1A.

Figure 3.2.1B.

Figure 3.2.1C
3.2.2 Assessment of the effect of $\gamma$-irradiation (4 and 8Gy) on Jurkat cells (24h post irradiation analysis).

The initial radiation experiments were conducted on Jurkat cells. Due to variability in the level of cell death previously reported with Jurkat cells (Kataoka et al. 1998; Gong and Almasan, 2000) and the fact that 5Gy had been previously shown to induce an apoptotic response in A-T cells (Zhang et al. 2001), it was decided to initially use 4 and 8Gy $\gamma$-irradiation with the Jurkat cells to determine the level of response. The cell death profile of Jurkat cells exposed to either 4Gy or 8Gy $\gamma$-irradiation indicated necrotic cell death (24h post irradiation analysis; Fig 3.2.2). To determine whether the cells were capable of death via a putative apoptotic pathway, cells were exposed to a lower level of radiation and assayed for apoptosis and necrosis (A-V, PI) using an earlier time point.
Figure 3.2.2A
Figure 3.2.2B
3.2.3 Assessment of the effect of \( \gamma \)-irradiation (4Gy) on human cell lines and Jurkat (control) cells (8 hours post irradiation).

The aim of this study was twofold; i) to determine the effect of a lower radiation exposure (4Gy) and earlier time point analysis (8h) on the apoptotic/necrotic profiles of Jurkat cells, and ii) to determine the effect that these altered experimental parameters had on the apoptotic/necrotic profiles of human lymphoblast A-T homozygote and heterozygote, and control cells.

Results indicated that A-T homozygote (A-T 1, A-Tla) and heterozygote (Het 1, Het 2) cells were unaffected by 4Gy \( \gamma \)-irradiation and exhibited no apoptosis or necrosis when compared to control (C5) or Jurkat cell lines (Fig 3.2.3). Control (C5) lymphoblastoid cells exhibited substantial levels of necrosis, compared to the other lymphoblastoid cell lines and Jurkat cells. The cell death profile of the Jurkat cells exposed to the lower dose (4Gy) and assayed at an earlier time point indicated an apoptotic cell death profile. This is in contrast to the previous study (Fig 3.2.2B) using a later analysis time point (24h) in which a necrotic cell death profile was exhibited.
Figure 3.2.3 Assessment of the effect of γ-irradiation (4Gy) on human cell lines and Jurkat (control) cells (8 hours post irradiation).

A-T homozygote (A-T 1, A-T1a), A-T heterozygote (Het 1, Het 2) and control (C5) lymphoblastoid cell lines and Jurkat control cells ($2 \times 10^6$) were irradiated (4Gy) and following an 8 hour incubation in a 5% CO₂ humidified incubator at 37°C, were analysed for Annexin-V binding and PI uptake as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using WinMDI, and the results expressed are the mean (±) SD of three separate experiments.
3.2.4 Assessment of the effect of γ-irradiation (4Gy) on human lymphoblastoid cell lines (2-24 hours post irradiation).

Results indicate that all of the lymphoblastoid cell lines (A-T homozygote, A-T heterozygote and control) exhibit a necrotic cell death profile using these experimental conditions. Results also indicate that cell necrosis in the A-T homozygote cells (A-Tl) exhibit a time-dependent increase in their necrotic profile, (at the latter time points (8, 24h), when compared to the early time points (2, 5h), using the experimental conditions employed. The A-T heterozygote cell line (Het l) however, did not exhibit any increase in cell death profiles over the same time frame. The control cells (C5) also exhibited a time-dependent increase in their necrotic profile, (at the latter time points (8, 24h), when compared to the early time points (2, 5h), using the experimental conditions employed, and exhibited the greatest number of necrotic cells compared to the other lymphoblast cells (A-T l, Het l) utilised.
Figure 3.2.4 Assessment of the effect of γ-irradiation (4Gy) on human lymphoblastoid cell lines (2-24 hours post irradiation).

A-T homozygote (A-T 1), A-T heterozygote (Het 1) and control (C5) lymphoblastoid cell lines (2 x 10⁶) were irradiated (4Gy) and processed for Annexin-V binding and PI uptake (2-24h post irradiation) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.2.5 Assessment of the effect of γ-irradiation (20-60Gy) on Jurkat cells (24 hours post irradiation).

From the previous experiments, it was apparent that the radiation dose was insufficient to elicit an appropriate apoptotic/necrotic effect in the A-T homozygote and heterozygote cell lines presently being used. Therefore in order to establish the response of control (Kataoka et al. 1998; Gong and Almasan, 2000) Jurkat cells, to which other cell lines could be compared, a dose response assay was carried out for these purposes. Results obtained indicate that the level of early apoptotic cells remain fairly constant whereas there is a dose-dependent increase in the numbers of late apoptotic and necrotic cells upon exposure to higher levels of radiation.
Figure 3.2.5 Assessment of the effect of $\gamma$-irradiation (20-60Gy) on Jurkat cells (24 hours post irradiation).

Jurkat cells ($2 \times 10^6$) were irradiated (20-60Gy) and processed for Annexin-V binding and PI uptake (24h post irradiation) as previously described in the methods section. Data indicates the level of cells depicted to be in:
- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean ($\pm$) SD of three separate experiments.
Figure 3.2.6 Assessment of the effect of $\gamma$-irradiation (30Gy) on human lymphoblastoid cell lines (2, 5, 8 and 24 hours post irradiation).

The results indicate that unlike Jurkat cells (Fig 3.2.5) using radiation as a DNA damaging agent, lymphoblastoid cells die by necrosis rather than apoptosis, even at earlier time points after irradiation. The A-T heterozygote cells show more resistance to cell death compared to the A-T homozygote and control cells, at later assay time points. Increasing the radiation dose in these cell lines to 30Gy resulted in a dose dependent increase in necrotic cells.
Figure 3.2.6 Assessment of the effect of γ-irradiation (30Gy) on human lymphoblastoid cell lines (2, 5, 8 and 24 hours post irradiation).

A-T homozygote (A-T 1), A-T heterozygote (Het 1) and control (C5) lymphoblastoid cell lines (2 x 10⁶) were irradiated (30Gy) and processed for Annexin-V binding and PI uptake (2, 5, 8 and 24h post irradiation) as previously described in the methods section. Data indicates the level of cells depicted to be in:
- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.2.7 Assessment of the effect of γ-irradiation (30Gy) on human lymphoblastoid and fibroblast cell lines (24, 48 and 72 hours post irradiation).

To determine if previous results with the lymphoblastoid cell lines were cell line or cell type specific, the same level of radiation and assay time points were used with different cell lines. The A-T homozygote (A-Tla) and control (C2) were both SV40 transformed lymphoblast cell lines, and the A-T heterozygote (GM08389) was an untransformed fibroblast cell line. The A-T homozygote cell line was produced from the affected child of A-T heterozygote (Het 1) used previously. This cell line was utilised here to determine if the A-T homozygote also showed resistance to radiation-induced cell death. In addition to reproducing the previous experimental conditions, the methodology was taken a step further by including 48 and 72 hour assay time points to determine if time after irradiation also had an effect on the cell death profiles of the cells.

The results indicate that at all assay time points after irradiation, all the cell lines exhibited an apoptotic cell death profile (Fig 3.2.7), unlike the previous cells used (Fig 3.2.6). The A-T homozygote was more resistant to radiation-induced cell death compared to its heterozygote mother (Het 1) used previously (Fig 3.2.6) at the 24h assay time point. However, at later post irradiation assay time points (48, 72h) this resistance was overcome, with greater numbers of early apoptotic cells exhibited at 48h and both early and late apoptotic cells at 72h. The A-T heterozygote cell line again showed more resistance to cell death compared to the A-T homozygote and control, but overall cell death (apoptotic and necrotic cells) did exceed 10% albeit after 72 hours.
A-T homozygote A-T1a

A

A-T heterozygote GM08389

B

Control C2

C

Figure 3.2.7 Assessment of the effect of γ-irradiation (30Gy) on human lymphoblastoid and fibroblast cell lines (24, 48 and 72 hours post irradiation).
3.2.8 Assessment of the effect of $\gamma$-irradiation (60Gy) on human lymphoblastoid and fibroblast cell lines (24 and 48 hours post irradiation).

The aim of this experiment was to determine if increasing the dose of radiation caused significant increases in cell death at earlier time points. For this reason, the assay time was shortened to 48h post irradiation, compared to the 72 hours previously analysed.

The results indicate that there is a further increase in cell death at later time points post irradiation, and furthermore, that the lymphoblastoid cell lines progress to a necrotic state. The A-T heterozygote GM08389 remained resistant to this dose of radiation, when compared to the effect produced in the other cell lines. The control (C2) cell line was more sensitive to radiation-induced cell death than the A-T homozygote and heterozygote cell lines, indicated by this cell line producing the greatest number of late apoptotic cells. Using this dose of radiation was insufficient to induce cell death in the HL-60 cell line.
Figure 3.2.8 Assessment of the effect of γ-irradiation (60Gy) on human lymphoblastoid and fibroblast cell lines (24 and 48 hours post irradiation).

A-T homozygote (A-T1a) and control (C2) lymphoblastoid cell lines, A-T heterozygote (GM08389) fibroblast and HL-60 cells (2 x 10⁶) were irradiated (60Gy) and processed for Annexin-V binding and PI uptake (24 and 48h post irradiation) as previously described in the methods section. Data indicates the level of cells depicted to be in:

early apoptosis □ late apoptosis □ and necrosis□

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
Figure 3.2.9 Assessment of the effect of $\gamma$-irradiation (60Gy) on human lymphoblastoid and fibroblast cell lines (24 hours post irradiation).

The aim of this experiment was to use the same dose of radiation (60 Gy) as previously used but to use a variety of A-T homozygote, A-T heterozygote and control cell lines to compare the overall response of the three different genotypes.

The results show that at the higher dose of irradiation (60 Gy), more late apoptotic cells were generated when compared with the previously used lower dose of 30 Gy, in all cell lines tested. No clear distinction could be made between the cell lines and how they responded to the irradiation treatment based on their ATM status. The A-T homozygote cell line A-T2 showed the most sensitivity to the higher dose (60Gy) of radiation by producing the largest percentage of cells undergoing late apoptosis. Under these experimental conditions all of the A-T heterozygote lymphoblastoid cell lines were no longer resistant to the effects of the radiation, and produced a high percentage (>30%) of late apoptotic cells. The A-T heterozygote (GM08389) fibroblast cell line remained resistant to the induction of cell death, producing <10% cells exhibiting apoptosis/necrosis as demonstrated previously (Fig’s 3.2.7, 3.2.8). The controls also showed differing sensitivities to this dose of radiation, with at least two of the four controls showing less cell death than the A-T homozygote and heterozygote lymphoblastoid cells.
Figure 3.2.9 Assessment of the effect of γ-irradiation (60Gy) on human lymphoblastoid and fibroblast cell lines (24 hours post irradiation).

A-T homozygote (A-T1a, A-T2) A-T heterozygote (Het 1, Het 2, Het 3, Het 5) and control (C1, C2, C3, C5) lymphoblastoid cell lines, and A-T heterozygote (GM08389) fibroblast cells (2 x 10^6) were irradiated (60Gy) and processed for Annexin-V binding and PI uptake (24h post irradiation) as previously described in the methods section. Data indicates the level of cells depicted to be in:

early apoptosis □ late apoptosis □ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.3 Chemical treatment of human cell lines with hydrogen peroxide.

Hydrogen peroxide (HP) is one of the intermediates formed by the reduction of molecular oxygen, and is one of the products generated during normal cellular metabolism (Gutteridge, 1994). Hydrogen peroxide can react with iron ions and other redox active transitional metals to produce hydroxyl radicals that have the potential to damage lipids, amino acids in proteins, carbohydrates and cellular DNA, and these molecules have all been shown to be targets for free radical attack (Byung, 1994). Reactive oxygen species (ROS) including HP and the hydroxyl radical are also produced when cells are exposed to IR (Riley, 1994). There are several enzymes that function in detoxifying ROS, but under circumstances where these mechanisms are overwhelmed, the cells enter a state of oxidative stress (Gutteridge, 1994). It has been suggested that the cells of ataxia-telangiectasia patients are in a continuous state of oxidative stress, especially neuronal cells, and this has been proposed as the mechanism responsible for neuronal degeneration in these patients (Rotman and Shiloh, 1997a; 1997b). Increased oxidative stress has been demonstrated in the cerebellum of ATM null mice, which is the main area of the central nervous system affected in A-T patients (Kamsler et al. 2001). It has also been proposed that ATM protein is involved in prevention or repair of oxidative damage to telomeric DNA as following oxidative stress induced by HP (20μM) treatment, the telomeres of A-T homozygote and heterozygotes demonstrate increased shortening compared to controls (Tchirkov and Lansdorp, 2003). Based on this apparent involvement of ATM in the response to oxidative stress, and the fact that HP is produced as a by-product of normal cellular metabolism, and in response to IR, the most effective treatment for the majority of cancers that develop in A-T patients, HP was considered to be a valuable compound for inclusion in the present study.

The ability of HP to induce apoptosis in HL-60 cells has been demonstrated previously. Morphological changes consistent with apoptosis including condensed and/or fragmented nuclei, reduction of procaspase 3 levels coincidental with an increase in activated caspase 3 levels, and DNA fragmentation have been demonstrated in HL-60 cells treated with 50μM HP for 4 hours (Matsura et al. 1999). Although no reference material could be elucidated that specifically related to percentages of apoptotic/necrotic cells induced by treatment with HP, the afore-mentioned publication demonstrated characteristic features of cell death using this cell line with HP, and also provided a stated dose at which a cell death profile
could be expected. Initial experimental procedures therefore aimed to determine if a cell death profile would be observed using this concentration of HP (50μM) as part of a dose and time study using the annexin-V/PI assay utilised in previous work with γ-irradiation.

3.3.1 Assessment of the effect of hydrogen peroxide (10-100μM) on HL-60 cells (2-6 hours after treatment).

Results indicated (Fig 3.3.1) that at lower doses (10-50μM), and the earliest time point (2h), HL-60 cells predominantly die by apoptosis. These results are therefore consistent with previously published results (Matsura et al. 1999). At the later time points (4, 6h), the cells exhibited more necrotic than apoptotic cell death up to a concentration of 50μM. Under the same experimental conditions, Matsura et al. (1999) only reported the presence of apoptotic cells. However, the method used to evaluate apoptosis in that study relied on the morphological appearance of condensed chromatin and fragmented nuclei. In the current study, utilising annexin-V and PI to measure apoptosis by detecting changes that occur in the cell membrane is an earlier indicator of both apoptotic/necrotic cell death, due to the fact that PS has been shown to be translocated to the outside of the membrane up to several hours before the appearance of DNA fragmentation, and any membrane breach is detected early due to the fact that PI is normally impermeable to an intact cell membrane (Martin et al. 1995). Under these circumstances, early detection of necrotic cells may therefore be expected. At the highest HP concentration (100μM) the percentage of necrotic cells remained constant (~30%) through the time course, whereas a dose-dependent increase in late apoptotic cells was exhibited with time. Interestingly, at the four hour time point, there were less cells exhibiting an apoptotic or necrotic profile than at the earlier (2h) or later (6h) time points up to a concentration of 50 μM. This observation was not present when the HP concentration was increased to 100 μM. In previous experiments using IR, we found that a much higher dose was required to elicit a response in our three genotypes under investigation when compared to Jurkat control cells. Looking at the results with HL60 cells treated with HP, at 100μM continuous exposure for four hours, there was approximately 65% total cell death. This time and dose was therefore considered to be a good starting point for the treatment of the A-T homozygote, heterozygote and control lymphoblastoid cell lines.
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Figure 3.3.1
Figure 3.3.2 Assessment of the effect of hydrogen peroxide (100µM) on human lymphoblastoid and HL-60 cells (4 hours post treatment analysis).

Results indicated that a clear distinction could be made between the response of A-T heterozygotes compared to A-T homozygote and control cell lines (Fig 3.3.2). The A-T heterozygote cell lines exhibited the highest percentage of necrotic cells (>80%) with relatively few apoptotic cells (<10%). The A-T homozygote cell lines exhibited a larger percentage of apoptotic cells (58-67%) than necrotic cells (31-35%). The control lymphoblastoid cell lines, like the A-T heterozygote cell lines, also exhibited a higher percentage of necrotic cells (52-56%) than late apoptotic cells (35-42%), but the percentage of necrotic cells were substantially lower, and the percentage of apoptotic cells were substantially higher when compared to the values obtained with the A-T heterozygote cells.

The increase in late apoptotic cells and the reduction in necrotic cells in the A-T homozygote cells was found to be statistically significant compared to controls. Likewise, the reduction in late apoptotic cells and the increase in necrotic cells in the A-T heterozygote cells was also found to be statistically significant compared to both A-T homozygotes and controls (P <0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes.

The HL-60 control cells produced a similar cell death profile to that observed previously under the same experimental conditions (Fig 3.3.1).
Figure 3.3.2 Assessment of the effect of hydrogen peroxide (100µM) on human lymphoblastoid and HL-60 cells (4 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 4; Het 5), and control (C1; C2) lymphoblastoid cell lines and HL-60 cells (2 x 10⁶) were incubated with 100µM HP for 4 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:
- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.

† Indicates a significant increase in late apoptotic cells in A-T homozygote cells compared to control lymphoblast cells (P < 0.05 one-way ANOVA).
‡ Indicates a significant reduction in necrotic cells in A-T homozygote cells compared to control lymphoblast cells (P < 0.05 one-way ANOVA).
* Indicates a significant reduction in late apoptotic cells in A-T heterozygote cells compared to A-T homozygote and control lymphoblast cells (P < 0.05 one-way ANOVA).
†† Indicates a significant increase in necrotic cells in A-T heterozygote cells compared to A-T homozygote and control lymphoblast cells (P < 0.05 one-way ANOVA).
Figure 3.3.3 Assessment of the effect of hydrogen peroxide (100μM) on human lymphoblastoid, fibroblast and HL-60 cells (6 hours post treatment analysis).

The aim of this study was twofold; i) to determine if the necrotic cell death profile exhibited by the A-T heterozygote cell lines demonstrated previously (Fig 3.3.2) was time-dependent, and ii) if these observations were cell line or cell type specific. For these reasons, the drug incubation time was extended to 6 hours and A-T homozygote and heterozygote untransformed fibroblast cell lines were used in addition to the lymphoblast cells used previously (Fig 3.3.2). At this later time point, the overall cell death profile for the A-T heterozygote lymphoblastoid cell lines remained constant in that there were very high numbers of necrotic cells (>85%) and relatively few apoptotic cells (<10%) (Fig 3.3.3). There was less difference in the numbers of apoptotic/necrotic cells exhibited between the other two genotypes under these experimental conditions. The Jurkat control only produced late apoptotic cells (80%). However, the results obtained with the untransformed fibroblast cell lines show a completely different pattern of cell death compared to the lymphoblastoid cell lines in that there were relatively few necrotic cells (<10%) and much larger numbers of both early (15-75%) and late (9-35%) apoptotic cells.

The A-T heterozygote cell lines exhibited the greatest sensitivity to HP treatment, as a higher percentage of these cells were induced to undergo apoptosis following HP treatment compared to cells from the other two genotypes. The telomerase-transformed control exhibited the least cell death, while the A-T homozygote showed intermediate sensitivity to HP between that of A-T heterozygotes and the control. Using these experimental conditions it was possible to distinguish A-T heterozygote fibroblast cells from A-T homozygote and controls, based on the percentage of HP-induced early apoptosis, and total apoptotic cell death (early and late apoptotic cells). Using a telomerase transformed fibroblast cell line as the control, the increase in early apoptotic cells and total apoptotic cell death (early and late apoptotic cells combined) in the A-T homozygote cells was found to be statistically significant compared to the control. The increase in early apoptotic cells and total apoptotic cell death in the A-T heterozygote cells was also found to be statistically significant compared to both the A-T homozygotes and the control (P <0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes.
Figure 3.3.3 Assessment of the effect of hydrogen peroxide (100μM) on human lymphoblastoid, fibroblast, Jurkat and HL-60 cells (6 hours post treatment analysis).
3.4 Treatment of human cell lines with the chemotherapeutic drug, etoposide.

Since its introduction in the treatment of cancer in 1971, etoposide (VP-16) has become one of the most frequently used anticancer drugs (Slevin, 1991; Baldwin and Osheroff, 2005). It has a broad anti-tumour spectrum and has been used to treat a variety of cancers including haematopoietic malignancies, such as leukaemia (Rózalski et al. 2005). Etoposide is used to treat several different types of leukaemia, including acute lymphocytic leukaemia (Fujino et al. 2002), which is the most common cancer found in children with A-T, but which is also common in adult A-T patients (Taylor et al. 1996). These factors demonstrate the relevance of using etoposide in the present research.

Jurkat and HL-60 cells have previously been used in numerous experimental procedures involving the use of etoposide. However, on reviewing the literature, various etoposide concentrations and assay time points had been used with these cell lines. In a study comparing PBLs to Jurkat cells, 1μM etoposide was used for 12 hours. The results demonstrated that apoptosis was considerably higher in Jurkat cells (38%) compared to PBLs (17%) (Robertson et al. 2000). In a separate study, also using 1μM for 12 hours, the same level of apoptotic cells (38%) was demonstrated using annexin-V/PI to measure apoptosis (Ferraro-Peyret et al. 2002). In another study, Jurkat cells treated with 80μM etoposide for 24 hours resulted in 7% early apoptotic cells, 52% late apoptotic cells and 6% necrotic cells measured by annexin V binding and propidium iodide uptake (Wang et al. 2001). Various other studies have also been conducted on etoposide treated Jurkat cells using a variety of different drug concentrations, drug exposure times, along with different methods to enumerate apoptotic cells, which all generated different results (Eilich et al. 1997; Kataoka et al. 1998; Wesselborg et al. 1999).

Due to the fact that more published data had been identified for Jurkat cells treated with etoposide compared to published work using HL-60 cells, an initial undertaking was made to reproduce published etoposide results with Jurkat cells.
3.4.1 Assessment of the effect of etoposide (80µM) on Jurkat cells (24 hours post treatment analysis).

As demonstrated in Fig 3.4.1, similar numbers of apoptotic and necrotic cells to those published by Wang et al. (2001) were produced when the cells were treated with 80µM etoposide for 24 hours. However, in the current study, slightly higher numbers of both early apoptotic (3% increase) and necrotic (4% increase) cells were produced. Having established that it was possible to reproduce these results with the same cell line, Jurkat cells were included in experimental procedures carried out on the three genotypes under investigation so that other drug concentrations and treatment time points could be utilised.
Figure 3.4.1 Assessment of the effect of etoposide (80μM) on Jurkat cells (24 hours post treatment analysis).

Jurkat cells (2 x 10⁶) were incubated with 80μM etoposide for 24 hours and processed for Annexin-V binding and PI uptake (immediately after 24 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
Figure 3.4.2 Assessment of the effect of etoposide (50µM) on human lymphoblastoid and fibroblast cells and control (Jurkat and HL-60) cells (6 hours post treatment analysis).

The aim of this work was to look at the way in which the different cell lines responded to DNA damage, induced by the anti-cancer treatment, etoposide. Having reproduced published work using Jurkat cells, etoposide was then used with the three genotypes under investigation. A-T cells had previously been treated with 17µM etoposide for 24 hours (Pernin et al. 1999), and this treatment was sufficient to induce apoptosis up to 70% in some cell lines, measured by TUNEL. This factor was taken into account when deciding the etoposide concentration to use with the cells under investigation in the present study, although ideally, a shorter exposure time was required due to the large number of cell lines to be analysed. For these reasons, it was decided to shorten the exposure time to 6 hours, and to increase the etoposide concentration to 50µM. The results indicated that these experimental conditions were unable to induce cell death in any of the lymphoblast cells (Fig 3.4.2A) and that the level of cell death induced in the fibroblast cells (10%) was minimal (Fig 3.4.2B). In addition to this, HL-60 cells exhibited no response under these experimental conditions, and the Jurkat cells exhibited <25% apoptosis, indicating that a higher concentration of etoposide was required to induce cell death in the lymphoblastoid, fibroblast and HL-60 cell lines.
Figure 3.4.2 Assessment of the effect of etoposide (50μM) on human lymphoblastoid and fibroblast cells and control (Jurkat and HL-60) cells (6 hours post treatment analysis).
3.4.3 Assessment of the effect of etoposide (50μM) on human fibroblast cells (24-72 hours post treatment analysis).

The results indicate that increasing the time of exposure failed to induce an appropriate increase in cell death profiles (Fig 3.4.3) compared to the 6h exposure time used previously (fig 3.4.2). As a result of this data it was decided to double the drug concentration (100μM) and to observe an earlier time point (6h).
Figure 3.4.3 Assessment of the effect of etoposide (50μM) on Human fibroblast cells (24-72 hours post treatment analysis).

(A) A-T homozygote (GM03487), and A-T heterozygote (GM03489, GM03396, GM08389), fibroblast cells (2 x 10^5) were incubated with 50μM etoposide for 24 hours and B) A-T heterozygote (GM08389) cells were incubated with 50μM etoposide for 24, 48 and 72 hours. Following incubation with etoposide the cells were processed for Annexin-V binding and PI uptake (immediately after stated continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:
- early apoptosis □
- late apoptosis □
- and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (+) SD of three separate experiments.
3.4.4 Assessment of the effect of etoposide (100μM) on human lymphoblast and fibroblast cells and control (Jurkat and HL-60 cells (6 hours post treatment analysis). Results indicated that increasing the concentration of etoposide (100μM) and observing cell death at an earlier time point (6h) caused a substantial improvement in cell death profiles of both the transformed and untransformed cell lines compared to results found in Fig 3.4.2. The data also indicated that the lymphoblast cells (Fig 3.4.3A) were more responsive to etoposide than the fibroblast cells (Fig 3.4.3B). Results with the lymphoblastoid cells indicate that a clear distinction can be made between the A-T heterozygotes compared to the other cell lines, as the A-T heterozygotes exhibited more necrotic cells, compared to apoptotic cells. This cell death profile was not observed in either the A-T homozygote or control lymphoblast cells. Although Het 1 had fewer necrotic cells compared to the other two A-T heterozygote cell lines, in relation to the level of apoptotic cells for that particular cell line, the result still showed some significance.

The results with the fibroblast cell lines show that despite doubling the etoposide concentration, all the cell lines, with the exception of the A-T heterozygote cell line GM08387 were unaffected by the drug. A-T heterozygote cell line GM08387, exhibited a 16% increase in necrotic cells indicating this cell line was more sensitive to the effects of etoposide than any of the other fibroblast cells.
Figure 3.4.4 Assessment of the effect of etoposide (100μM) on human lymphoblast and fibroblast cells and control (Jurkat and HL-60 cells (6 hours post treatment analysis)).
Figure 3.4.5 Assessment of the effect of etoposide (100μM) on human fibroblasts (24 hours post treatment analysis).

To monitor whether the previously demonstrated resistance to etoposide-induced cell death in the fibroblast cell lines (Fig 3.4.4B) was due to the experimental early (6h) time point, a prolonged time point (24h) was chosen to analyse any delay/change in cell death profiles. However, due to the cost of reagents, it was decided to conduct further experiments on less cell lines until an appropriate apoptotic or necrotic response could be demonstrated. For this reason, three of the cell lines utilised in Fig 3.4.4B (A-T homozygote (GM03487) and A-T heterozygote (GM03489; GM08389) were included in this study, along with an additional cell line (GM03396) that had previously only been treated with 50μM etoposide (Fig 3.4.3). The results indicate however, that increasing the incubation time had no effect on increasing the rate of cell death in these cell lines (Fig 3.4.5).
Figure 3.4.5 Assessment of the effect of etoposide (100μM) on human fibroblasts (24 hours post treatment analysis).

A-T homozygote (GM03487) and A-T heterozygote (GM03489, GM03396, GM08389) fibroblast cells (2 x 10⁶) were incubated with 100μM etoposide for 24 hours and processed for Annexin-V binding and PI uptake (immediately after 24 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis  □
- late apoptosis  □
- and necrosis  □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
Figure 3.4.6 Assessment of the effect of etoposide (100-1000µM) on human fibroblasts. Previous work had demonstrated that the A-T heterozygote cell line GM08389 was highly resistant to γ-irradiation induced cell death (60Gy; see Fig 3.2.9). Therefore this cell line was chosen to monitor the response to drug-induced cell death by exposing the cells to a range of etoposide concentrations (100; 200; 300 µM) for 24-72h or to higher concentrations (500; 750; 1000µM) for 24 hours. The results indicate that increasing the etoposide concentration and drug exposure time had no effect on inducing cell death in this cell line (Fig 3.4.6A and B), as etoposide-induced cell death was <2%. 
Figure 3.4.6 Assessment of the effect of etoposide (100-1000μM) on human fibroblasts.

A) A-T heterozygote GM08389 fibroblast cells were incubated with 100-300μM etoposide for 24-72 hours and B) A-T heterozygote GM08389 fibroblast cells were incubated with 500-1000μM etoposide for 24 hours. Following incubation with the drug, cells (2 x 10^6) were processed for Annexin-V binding and PI uptake (immediately after continuous exposure at times indicated) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.5 Combined treatment of human cell lines with etoposide and hydrogen peroxide.
Phosphatidylserine is a phospholipid that normally resides on the inside of the cell membrane. However, when cells are induced to undergo apoptosis, this phospholipid is oxidised, an event that causes it to flip to the cell surface. Etoposide has been reported to act as a lipid antioxidant and in doing so, prevents oxidation of the membrane phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and PS during HP-induced apoptosis (Kagan et al. 2001; Tyurina et al. 2004). Tyurina et al. (2004) demonstrated that when HL-60 cells were treated with a combination of 50 μM etoposide and 100μM HP (4 hours), there was approximately 50% reduction in apoptotic cells compared to HP used alone. In addition to this, no increase in apoptotic cells were seen above background levels with etoposide (50 μM) used in isolation. However, it is also pertinent to note that although the combined etoposide and HP treatment of HL-60 cells reduced the numbers of apoptotic cells by 50% at 4 hours, this amount only equated to 7% total reduction in apoptosis. Although it was stated that etoposide prevented oxidation of phospholipids in this study, it is also possible that etoposide was acting as a free radical scavenger, and rather than having a direct effect on membrane phospholipids, was in fact acting on the HP to reduce its effect on the cells. This would explain why etoposide did not induce considerable numbers of apoptotic cells in the HL-60 cell line. If etoposide acts as a free radical scavenger, this has implications for its use in combined chemotherapy regimes with agents that exert their effects through the generation of ROS, such as IR (Kurtz et al. 2004), doxorubicin (Peng et al. 2005) and bleomycin (Mahmutoglu et al. 1987). It was therefore decided to treat A-T homozygote and heterozygote cells (with appropriate controls) to observe whether these cell lines would show less sensitivity to the combined treatment, due to etoposide reducing the potent cellular effects of HP, or whether the sensitivity of these cells to this combined treatment were the same as HP used in isolation.

In repeating this work, we decided to repeat the previously published work (Tyurina et al. 2004) at the 4-hour time point, but also decided to include a higher concentration of etoposide (100μM).
3.5.1 Assessment of the effect of etoposide (10-100μM) ± 100μM hydrogen peroxide on HL-60 cells (4 hours post treatment analysis).

The results demonstrate that HL-60 cells respond to etoposide similarly within the dose range 10-100μM (see Fig 3.5.1), as similar numbers of apoptotic and necrotic cells have been recorded for all doses used. The observed results were also very similar to the results obtained with 50μM in the previously published work (Tyurina et al. 2004). However, much higher numbers of apoptotic cells were observed in the current study compared to previously published results following treatment of HL-60 cells with HP at 4h (50% compared to 30.5%). Comparing these results with etoposide used in addition to HP, when the highest (100μM) and lowest (10μM) etoposide concentration was used with HP, the etoposide seemed to have an effect in slightly reducing the level of apoptotic cells, but at the intermediate (50μM) etoposide concentration, the effect seems to be additive, as although the numbers of late apoptotic cells were slightly reduced, the numbers of early apoptotic cells showed a significant increase. The results also indicate that apoptosis is primarily influenced by HP concentration, since the cell death profiles of HL-60 cells treated with etoposide alone had a minimal effect on cell death parameters (<10%). These results are therefore partially in agreement with the findings of Tyurina et al. (2004).

However, an important factor that could explain the differences in the results of the previously published work (Tyurina et al. 2004) compared to the current study is the fact that the previous study used a rapid binding method that involved incubating the cells with both annexin-V and PI for 5 minutes, while the method used in the current study had longer incubation times with annexin-V and PI as well as numerous washing steps. Both these factors could account for the increase seen in annexin-V and PI positive cells.
Figure 3.5.1 Assessment of the effect of etoposide (10-100μM) ± 100μM hydrogen peroxide on HL-60 cells (4 hours post treatment analysis).

HL-60 cells, (2 x 10^6) were incubated with 10-100μM etoposide ± 100μM HP for 4 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:
early apoptosis □ late apoptosis □ and necrosis □
The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.5.2 Assessment of the effect of etoposide (10-100μM) ± 100μM hydrogen peroxide on HL-60 cells (6 hours post treatment analysis).

The aim of this study was to determine if HL60 responded differently to etoposide used in conjunction with HP with time. The results indicate that a similar pattern of cell death was observed at a later (6h) time point (Fig 3.5.2) when compared to the earlier (4h) time point (Fig 3.5.1). The contribution of etoposide to the combinational treatment results for cell death again appears to be minimal when compared to HL-60 cells treated with HP alone. Interestingly, comparison of the results of the 4h exposure (Fig 3.5.1) to the later (6h) exposure (Fig 3.5.2) indicate that at the latter time point there is an increase in the level of late apoptotic and necrotic cells, whilst the level of early apoptotic cells has decreased.
Figure 3.5.2 Assessment of the effect of etoposide (10-100\textmu M) ± 100\textmu M hydrogen peroxide on HL-60 cells (6 hours post treatment analysis).

HL-60 cells, (2 x 10^6) were incubated with 10-100\textmu M etoposide ± 100\textmu M HP for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 6 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis ▢ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
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Figure 3.5.3 Assessment of the effect of etoposide (10μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (4 hours post treatment analysis).

Having established the response of HL-60 cells to etoposide and HP the effect of these two agents on the three genotypes was investigated. Initially, the lowest concentration of etoposide (10μM) used in the drug combination study (Tyurina et al. 2004) was used. The three genotypes under investigation had been treated with etoposide (10μM) in isolation, but this concentration of the drug did not elicit a cell death profile in these cells (data not shown) and so the results were not included. The etoposide/HP data however, was presented due to a few interesting results when the data was compared to HP used in isolation (Fig 3.3.2). Interestingly, all the cell lines showed higher sensitivity to this combination of agents than the HL-60 cells, demonstrated by a much higher percentage of the cells displaying apoptotic/necrotic cell death profiles. However, analysis of data produced when these cell lines were treated with HP alone (Fig 3.3.2) demonstrated that the addition of etoposide had little effect in generating these cell death profiles, as the major contribution was made by treatment of the cells with HP. The results indicate a significant reduction (28%) in late apoptotic cells coincidental with an increase in necrotic cells (17%) in the A-T1 cell line (see Fig. 3.5.3), compared to HP used in isolation (see Fig 3.3.2). However, there were no significant differences observed with the other A-T homozygote, A-T heterozygote or control cells using the combined treatment (Fig 3.5.3) compared to HP used alone (Fig 3.3.2). To examine whether or not the effects seen in the A-T homozygote cell line were time dependent, and also to determine if increasing the exposure time would have an effect on the other cell lines, the experimental conditions were extended to the 6-hour period used previously with HL-60 cells.
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Figure 3.5.3 Assessment of the effect of etoposide (10μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (4 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 4; Het 5), control (C1; C2) lymphoblast cell lines and control (HL-60) cells (2 x 10⁵) were incubated with 10μM etoposide and 100μM HP for 4 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □, late apoptosis □ and necrosis □. The results were analysed using Win MDI, and the results expressed are the mean (+) SD of three separate experiments.
3.5.4 Assessment of the effect of etoposide (10μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (6 hours post treatment analysis).

Comparing the combined treatment (Fig 3.5.4) to HP used alone at this time point (see Fig 3.3.3), both the A-T homozygote and heterozygote cell lines showed no significant differences. Although there were subtle changes in the numbers of cells undergoing apoptosis/necrosis, the overall number of cells undergoing cell death (apoptosis and necrosis) remained similar. The only significant change in cell death profiles was observed with the control C1, which exhibited a 10% increase in late apoptotic cells with a simultaneous 8% reduction in necrotic cells. The cell death profile of the HL-60 cells remained unchanged.
Figure 3.5.4 Assessment of the effect of etoposide (10μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (6 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 4; Het 5), control (C1; C2) lymphoblast cell lines and control (HL-60) cells (2 x 10⁶) were incubated with 10μM etoposide and 100μM HP for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 6 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis ▪ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.5.5 Assessment of the effect of etoposide (50µM) and 100µM hydrogen peroxide on lymphoblast and HL-60 cells (6 hours post treatment analysis).

Having established that there was very little difference in the results obtained with the majority of the cell lines when treated with 10µM etoposide and 100µM HP for 4 and 6 hours, it was decided to increase the etoposide concentration to see if this higher concentration would be able to inhibit externalisation of PS and hence annexin-V binding.

The results (Fig 3.5.5) when compared to HP used in isolation (see Fig 3.3.3) show the A-T homozygote (A-T 2) exhibited a 15% increase in late apoptotic cells with a simultaneous 5% decrease in necrotic cells. The A-T heterozygote (Het 5) exhibited a 9% increase in late apoptotic cells with a simultaneous 14% reduction in necrotic cells. The control cell line C1 showed a 17% increase in late apoptosis with a concomitant 33% decrease in necrotic cells.

It would therefore appear that using 50µM etoposide with 100µM HP has an affect on the mechanism of cell death induced in the lymphoblast cells, as this combination of treatments seems either to favour the induction of apoptosis and/or prevent necrosis in the A-T2, Het 5 and C1 cell lines. Jurkat cells were also included in the current study, (as HL-60 cells had already been analysed several times under these experimental conditions, and produced similar results with each treatment performed) and exhibited an 11% reduction in late apoptotic cells and a 6 % increase in necrotic cells compared to HP used alone (Fig 3.3.3).
Figure 3.5.5 Assessment of the effect of etoposide (50µM) and 100µM hydrogen peroxide on lymphoblast and HL-60 cells (6 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 1; Het 5; Het 4), control (C2; C1) lymphoblast cell lines and control (Jurkat) cells ($2 \times 10^6$) were incubated with 50µM etoposide and 100µM HP for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 6 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean ($\pm$) SD of three separate experiments.
3.5.6 Assessment of the effect of etoposide (50μM) and 100μM hydrogen peroxide on fibroblast and HL-60 cells (6 hours post treatment analysis).

Having established the response of HL-60 cells and lymphoblastoid cell lines from each of the three genotypes, the response of untransformed A-T homozygote and heterozygote cell lines was investigated under the same experimental conditions. The results were similar to other treatments using these cell lines in that the cells died by apoptosis rather than necrosis. The A-T heterozygote cell line GM03489 was generated from the parent of an A-T patient (cell line GM03487). The A-T heterozygote demonstrated more sensitivity to the treatment than the homozygote cell line as the heterozygote cell line produced 27% more apoptotic cells than the A-T homozygote. Addition of etoposide had no effect on fibroblast cell death profiles when compared to HP used alone (see Fig 3.3.3). However, the most striking result was generated by A-T heterozygote GM03397, which produced 29% late apoptotic cells and the highest level (43%) of necrotic cells compared to the other fibroblast cell lines. Unfortunately, data was not available for this cell line treated with HP alone, but this cell line only produced 1.5% necrotic cells when treated with 50μM etoposide for 6 hours (Fig 3.4.2), which suggests that the cell death profile observed for this cell line was most likely due to HP rather than etoposide or the combined treatment. The cell death profile of GM03397 was very different from all the other fibroblast cell lines tested in that necrotic cells predominated under these experimental conditions. Interestingly, the response of GM03397 was also different to cell line GM03396, which only produced apoptotic cells, and which carries the same ATM mutation as cell line GM03397. The results with the HL-60 cells, when compared to the previously used 10μM etoposide and 100μM HP showed similar results in both studies.

Further experiments were performed with fibroblast cells using 100μM etoposide and 100μM HP for 4 and 6 hours. No significant alterations in cell death profiles were observed for any of the cell lines when compared to treatments utilising 50μM etoposide and 100μM HP at the same experimental time points (data not shown).
Figure 3.5.6 Assessment of the effect of etoposide (50μM) and 100μM hydrogen peroxide on fibroblast and HL-60 cells (6 hours post treatment analysis).

A-T homozygote (GM03487), A-T heterozygote (GM03489, GM08387, GM08389, GM03396, GM03397), fibroblast cell lines and control HL-60 cells (2 x 10^6) were incubated with 50μM etoposide and 100μM HP for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 6 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (+) SD of three separate experiments.
3.5.7 Assessment of the effect of etoposide (100μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (4 hours post treatment analysis).

Results indicated that both the A-T homozygote cell lines A-T1 and A-T2 exhibited a decrease in late apoptotic cells (Fig 3.5.7 (A-T1, 27%; A-T2, 10%)) when compared to HP used in isolation (Fig 3.3.2). This decrease in late apoptotic cells in A-T homozygote A-T1 was coincidental with a 10% increase in necrotic cells. The A-T heterozygote and control (C2) cell lines exhibited similar cell death profiles with the combined treatment (Fig 3.5.7) to when HP was used alone (Fig 3.3.2). However, a 5% increase in early apoptotic cells, an 8% reduction in late apoptotic cells with a simultaneous 20% decrease in necrotic cells was observed for control C1 cells. HL-60 cells exhibited a 20% increase in early apoptotic cells with a simultaneous 22% reduction in late apoptotic cells and a 26% reduction in necrotic cells with the combined treatment (Fig 3.5.7) compared to HP used in isolation (Fig 3.3.2). These results indicated that inhibition of PS externalisation in HP treated cells was most prominent in A-T homozygote and control cells.
Figure 3.5.7 Assessment of the effect of etoposide (100μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (4 hours post treatment analysis).

A-T homozygote (A-T1; A-T2), A-T heterozygote (Het 4; Het 5), control (C1; C2) lymphoblastoid cell lines and control HL-60 cells (2 x 10⁶) were incubated with 100μM etoposide and 100μM HP for 4 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis ▢ and necrosis □. The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.5.8 Assessment of the effect of etoposide (100µM) and 100µM hydrogen peroxide on lymphoblast and HL-60 cells (6 hours post treatment analysis).

The only significant differences in cell death profiles compared to HP used alone (Fig 3.3.3) were demonstrated in the Jurkat and C2 control cell lines. The lymphoblast control C2 exhibited a 6% increase in late apoptotic cells and a 14% decrease in necrotic cells in response to the combined treatment (Fig 3.5.8) compared to HP used alone (Fig 3.3.3). Jurkat cells exhibited a 29% reduction in late apoptotic cells in response to the combined treatment (Fig 3.5.8) compared to HP used alone (Fig 3.3.3). These results therefore indicate that 100µM etoposide is able to reduce PS externalisation and annexin-V binding in cells treated with 100µM HP (Fig 3.5.8) compared to 100µM HP used alone (Fig 3.3.3), but the effect is only observed in a proportion of control cell lines.
Figure 3.5.8 Assessment of the effect of etoposide (100μM) and 100μM hydrogen peroxide on lymphoblast and HL-60 cells (6 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 1; Het 4; Het 5), control (C1; C2) lymphoblastoid cell lines and control Jurkat cells (2 x 10^6) were incubated with 100μM etoposide and 100μM HP for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using WinMDI, and the results expressed are the mean (±) SD of three separate experiments.
3.6 Treatment of human cell lines with the chemotherapeutic drug, doxorubicin.

Doxorubicin is an anthracycline antibiotic that is one of the most commonly used and one of the most effective anticancer drugs. It prevents DNA replication and RNA transcription, and by binding to DNA and forming a complex with DNA topoisomerase II, it prevents DNA polymerase II from repairing DNA strand breaks. It also interferes with DNA unwinding, strand separation and the activity of the helicase enzyme, it damages DNA by producing free radicals and damages cell membranes by oxidising lipids (Peng et al. 2005). Doxorubicin has a broad anti-tumour spectrum and has been used to treat a variety of cancers including Hodgkin’s disease, sarcomas, breast cancer (Gamen et al. 1997) and haematopoietic malignancies, such as leukaemia (Róžalski et al. 2005). Leukaemias and Lymphomas account for 85% of the cancers that A-T patients develop (Taylor et al. 1996) and A-T heterozygotes have also been reported to be at greater risk for the development of breast cancer (Jackson, 1995; Lehmann and Carr, 1995; Zakian, 1995; Athma et al. 1996; Broeks et al. 2000). Considering the fact that doxorubicin would be used to treat the majority of malignancies that predominantly affect both A-T homozygote and A-T heterozygote patients, this demonstrates the relevance of using doxorubicin in the present research.

Previous work in treating Jurkat cells with doxorubicin have employed a variety of doxorubicin concentrations, incubation times, and methods of analysis, which have produced a variety of results in the numbers of cells exhibiting cell death (Eischen et al. 1997; Kataoka et al. 1998; Wesselborg et al. 1999; Shiratsuchi and Nakanishi, 1999).

Due to the variability in numbers of apoptotic cells reported in the aforementioned publications, a dose response assay was performed to include three of the concentrations (0.2, 0.5, 1.0μg/ml) utilised in these studies. The majority of previously published work had involved treating the Jurkat cells with doxorubicin for 24h, and so this time point was utilised in the current study along with two further drug exposure times (48, 72h).
3.6.1 Assessment of the effect of doxorubicin (0.2-1.0μg/ml) on Jurkat cells (24-72 hours post treatment analysis).

The results indicated (Fig 3.6.1) that Jurkat cells used in the current study appear to be more responsive to the drug than previously published results (Eischen et al. 1997; Kataoka et al. 1998; Wesselborg et al. 1999). In comparison to the Wesselborg et al. study (1999), which reported the cells produced 80% apoptotic cells when treated with 1μg/ml doxorubicin for 24 hours, the numbers of cells exhibiting cell death under the same experimental conditions in the present study were 45% late apoptotic cells and 43% necrotic cells. There was only 8% difference in the numbers of cells exhibiting a cell death profile between the cells used in the current study and the previously published results. In addition to this, it is not clear from the publication whether all annexin-V positive cells were reported, or only those that were positive for annexin-V and PI negative. Unfortunately, it was not possible to discern the assay procedure used by Wesselborg et al. (1999) from the publication, or the manufacturer of the annexin-V antibody, and so the actual differences in any assay procedure or in the way the cell death profile was analysed and reported remain unclear. The possible differences identified could also be due to the fact that the annexin-V used in this study was from BD Pharmingen, and the annexin-V used in the publication was from Boehringer-Mannheim. Despite this, the numbers of cells exhibiting positive staining for annexin-V were very similar to the published results.

The results also demonstrated that at the lowest concentration (0.2 μg/ml) and within the first 24 hours of treatment, the cells were either undergoing necrosis, or damage to the cell membrane was allowing an early influx of the DNA stain PI (see Fig 3.6.1). These results are consistent with published results that demonstrate doxorubicin damages cell membranes by oxidising lipids (Peng et al. 2005). However, the current results also indicate that this is a transient effect on the cells, as the data (fig 3.6.1) indicates that this effect can be overcome with time. This has been demonstrated as at later time points the cells show a decrease in the level of PI staining, concurrent with an increase in annexin-V staining, which is both dose and time dependent. Interestingly, no early apoptotic cells were present regardless of the dose or incubation time utilised.
Figure 3.6.1 Assessment of the effect of doxorubicin (0.2-1.0μg/ml) on Jurkat cells (24-72 hours post treatment analysis).

Jurkat cells (2 x 10⁶) were incubated with 0.2-1.0μg/ml doxorubicin for 24-72 hours and processed for Annexin-V binding and PI uptake (immediately after stated time for continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis □
- late apoptosis □
- and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
Figure 3.6.2 Assessment of the effect of doxorubicin (0.2μg/ml) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

Initially, it was decided to measure the cellular response of a variety of human cell lines using the experimental conditions used to establish a response in Jurkat cells, namely 0.2μg/ml for 24 hours. Cell lines used to date were either SV40 transformed lymphoblast cells or untransformed fibroblast cells. During the course of the current research we had identified that the fibroblast cells often produced very different results to the lymphoblast cell lines. This was thought to be due to, in part, to viral transformation of the cell lines. The new cell line (A-T hTERT) used in the doxorubicin experimental procedures is a telomerase transformed fibroblast A-T cell line, which was a kind gift from Dr. C. Jones (University Hospital of Wales). This cell line was therefore included in the doxorubicin studies to determine if it would provide information on whether all transformed cells responded differently to DNA damaging agents compared to untransformed cells, or whether the difference was simply between different cell types (i.e. lymphoblasts vs. fibroblasts). The results (Fig 3.6.2) demonstrated the A-T hTERT cell line to be the most responsive to doxorubicin-induced cell death compared to the other A-T compromised cell lines, indicated by the level of necrosis (16%) exhibited by this cell line. Experimental data also revealed that all of the A-T compromised cell lines tested, exhibited predominantly a necrotic cell death profile, which was substantially reduced (<20%) compared to the profile of Jurkat cells (70%). The only apoptotic response, albeit minimal (<5%), was demonstrated in the A-T heterozygote (GM03487, GM03489) cell lines. This lack of response necessitated an increase in doxorubicin concentration to observe whether it was possible to initiate an improved cell death response at a higher concentration. The Jurkat control produced similar numbers of necrotic cells to when this dose and assay time point was previously used (see Fig 3.6.1).
Figure 3.6.2 Assessment of the effect of doxorubicin (0.2μg/ml) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

A-T homozygote (A-T hTERT; GM03487), A-T heterozygote (GM08387; GM08389; GM03396; GM03397; GM03489) fibroblast cell lines and control (Jurkat) cells (2 x 10^6) were incubated with 0.2μg/ml doxorubicin for 24 hours and processed for Annexin-V binding and PI uptake (immediately after 24 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.6.3 Assessment of the effect of doxorubicin (0.5μg/ml) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

The results indicated (Fig 3.6.3) that similar numbers of late apoptotic and necrotic cells were produced by the Jurkat cells to those demonstrated previously when exposed to 0.5μg/ml doxorubicin for 24 hours (Fig3.6.1). The results also demonstrated that increasing the concentration of the drug to 0.5μg/ml had a dramatic effect on the numbers of necrotic cells exhibited by all the A-T compromised cell lines. All the A-T heterozygote cell lines exhibited less necrosis than the A-T homozygote cell line (A-T hTERT), which produced >50% necrotic cells. Interestingly, no early or late apoptotic cells were produced in the A-T homozygote and heterozygote cell lines, in contrast to the 35% late apoptotic cells produced by Jurkat cells under the same experimental conditions.
Figure 3.6.3 Assessment of the effect of doxorubicin (0.5μg/ml) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

A-T homozygote (A-T hTERT), A-T heterozygote (GM08387, GM08389, GM03397), fibroblast cell lines and control (Jurkat) cells (2 x 10⁶) were incubated with 0.5μg/ml doxorubicin for 24 hours and processed for Annexin-V binding and PI uptake (immediately after 24 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis □ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.6.4 Assessment of the effect of doxorubicin (1.0µg/ml) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

In previous work with Jurkat cells, a dose and time dependent increase in late apoptotic cells had been demonstrated with a simultaneous dose and time dependent decrease in necrotic cells. As a consequence of this, it was decided to use the highest dose of doxorubicin that had previously been used (1.0 µg/ml) with Jurkat cells to determine whether it was possible to initiate a similar cell death profile in the A-T compromised cell lines. Data indicated that exposure of the cells to this elevated concentration failed to induce any apoptotic pathways in the A-T homozygote and heterozygote cell lines (Fig 3.6.4). Additionally, there was heterogeneity in the response profiles within the genotypes. The heterozygote cell lines produced a maximal (GM08387) and intermediate (GM08389, GM03397) response and the A-T homozygote (A-T hTERT) cell line exhibited >85% necrotic cell death profile. Jurkat cells produced similar results to those previously demonstrated under these experimental conditions (Fig 3.6.1) in that equal numbers of late apoptotic and necrotic cells were produced, but no early apoptotic cells were present.
Figure 3.6.4 Assessment of the effect of doxorubicin (1.0μg/ml) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

A-T homozygote (A-T hTERT), A-T heterozygote (GM08387, GM08389, GM03397), fibroblast cell lines and control (Jurkat) cells (2 x 10⁶) were incubated with 1.0μg/ml doxorubicin for 24 hours and processed for Annexin-V binding and PI uptake (immediately after 24 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis ■ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (+) SD of three separate experiments.
Figure 3.6.5 Assessment of the effect of doxorubicin (0.2-1.0µg/ml) on A-T heterozygote cell line GM08389 (24-72 hours post treatment analysis).

Having treated the fibroblast cells with various concentrations of doxorubicin it appeared that the response of the cells to the drug was both dose and time dependent. To determine this, the A-T heterozygote cell line GM08389, which had shown sensitivity to doxorubicin, but had shown resistance to gamma radiation and etoposide previously, was utilised for this purpose. In addition to this, in the previous work, the A-T heterozygote GM08387 exhibited lower numbers of necrotic cells than the A-T homozygote cell line A-T hTERT when treated with 0.5µg/ml doxorubicin, but produced the same level of necrotic cells as A-T hTERT when the doxorubicin concentration was doubled to 1.0µg/ml. This could be due to the GM08387 being less sensitive to the drug at lower concentrations. However, it was decided to undertake the current study over a longer time period to determine if a longer exposure time would induce more necrosis in this cell line (GM08389) or whether a different cell death profile would be observed over time. The results demonstrated that there was a linear relationship in the level of necrotic cells produced with both the drug concentration and time, but even at the highest doxorubicin concentration (1.0µg/ml) over 72 hours, GM08389 still produced 20% less necrotic cells compared to A-T hTERT and GM08387 cell lines, indicating GM08389 was more resistant to doxorubicin-induced cell death.
Figure 3.6.5 Assessment of the effect of doxorubicin (0.2-1.0µg/ml) on A-T heterozygote cell line GM08389 (24-72 hours post treatment analysis).

A-T heterozygote GM08389 cells (2 x 10^6) were incubated with 0.2-1.0µg/ml doxorubicin for 24-72 hours and processed for Annexin-V binding and PI uptake (immediately after stated continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis ▣ and necrosis □. The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.7 Chemical treatment of human cell lines with staurosporine.

Originally, staurosporine was thought to be a specific inhibitor of protein kinase C (PKC), although it is now commonly used as a general protein kinase inhibitor, and is a potent inducer of apoptosis (Tee and Proud, 2001). ATM has protein kinase activity (Kastan and Lim, 2000), and may therefore be a target for the inhibitory action of staurosporine. It has also been demonstrated that staurosporine is a potent inhibitor of Topoisomerase II. The mechanism of action has been shown to be the inhibition of ATP-independent transfer of phosphodiester bonds from DNA to the active site tyrosine residues of the enzyme. As such, it is the first topoisomerase II inhibitor shown to directly interfere with the transfer of phosphodiester bonds from DNA to the enzyme (Lassota et al. 1996). Although technically not classed as an anticancer drug, like etoposide and doxorubicin, it is able to interfere with the activity of Topoisomerase II. Based on the ability of this compound to interfere with the activity of Topoisomerase II, in addition to its inhibitory effects on protein kinases, in which ATM may be a target, staurosporine was considered to be an interesting compound for inclusion in the current study as it has the potential to a) target and inactivate ATM, and b) induce apoptosis (which may be due to the inhibitory effects on ATM and/or other protein kinases involved in apoptosis). In addition to these factors, staurosporine was also included, so that the cell death profiles of cells treated with staurosporine could be compared to the other two Topoisomerase poisons (etoposide and doxorubicin) utilised in the current study.

Previous research had shown that Jurkat cells treated with 1μM staurosporine for 4 hours, produced 90% apoptosis using Sytox Green and Hoechst dye 33258 to quantitate apoptosis by observing cells with condensed chromatin and fragmented nuclei (Feng and Kaplowitz, 2002). In another study, 1μM was used to treat Jurkat cells for 12 hours, but far fewer (30%) apoptotic cells were reported, using annexin-V and propidium iodide staining (Ferraro-Peyret et al. 2002). A different study used half the concentration (0.5μM), and double the exposure time (24h) to that used by Ferraro-Peyret et al. (2002), and produced 58% apoptotic cells using PI to measure the hypodiploid peak using a method by Nicoletti et al. (1991) (Dirsch et al. 2003). In an attempt to reproduce these results, the Jurkat cells were initially treated with 1 and 2μM for 4, and 12h.
3.7.1 Assessment of the effect of staurosporine (1 and 2μM) on Jurkat cells (4 and 12 hours post treatment analysis).

The results (Fig 3.7.1) indicated Jurkat cells treated with 1μM staurosporine for 12 hours produced 25% apoptotic cells and 8% necrotic cells compared to the 30% apoptotic cells reported by Ferraro-Peyret et al. (2002), who used the same method of quantifying apoptotic cells as used in the current study. A possible reason for this small variation in results was that the annexin-V used in the Ferraro-Peyret et al. study (2002), was purchased from Bender MedSystems (Vienna, Austria) and the annexin-V used in the current study was purchased from BD Pharmingen (Oxford, U.K.). The results also demonstrated a further increase in apoptosis (30%) and necrosis (10%) using 2μM staurosporine with the same exposure time (12h). Initially it was decided to use the higher concentration of staurosporine with the three genotypes under investigation, but with the earlier time point (4h), due to the fact that there was only 10% difference in the numbers of apoptotic cells between these two assay time points.
Figure 3.7.1 Assessment of the effect of staurosporine (1 and 2μM) on Jurkat cells (4 and 12 hours post treatment analysis).

Jurkat cells (2 x 10⁶) were incubated with 1 and 2μM staurosporine for 4 and 12 hours and processed for Annexin-V binding and PI uptake (immediately after stated continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:
early apoptosis □ late apoptosis ■ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
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3.7.2 Assessment of the effect of staurosporine (2μM) on human lymphoblast and Jurkat cells (4 hours post treatment analysis).

Results indicated (Fig 3.7.2) that all the A-T homozygote and A-T heterozygote cell lines exposed to staurosporine for 4 hours predominantly died by apoptosis rather than necrosis mediated pathways. The two A-T homozygote cell lines (A-T 1; A-T 2) exhibited greater sensitivity to staurosporine compared to the A-T heterozygote cell lines (Het 4; Het 5), indicated by the significantly higher numbers of both early apoptotic (12.5-20.5% compared to 4.5-8%) and late apoptotic (32-35.5% compared to 14-22%) cells exhibited in these cell lines. However, there appears to be no genotypical trend since the control cell lines exhibit sensitive (C1) and less sensitive (C2) cell death profiles. Interestingly, all the A-T homozygote and heterozygote cell lines showed more sensitivity to staurosporine than the Jurkat controls, which produced a similar cell death profile to that reported previously under these experimental conditions (Fig 3.7.1). Additionally, all the lymphoblastoid cells exhibited early as well as late apoptotic cells. This is in contrast to the other DNA-damaging agents utilised, which, when apoptotic cells were demonstrated, were predominantly late apoptotic. Under these experimental conditions, the A-T heterozygote cell lines could not be distinguished from the control cells.
Figure 3.7.2 Assessment of the effect of staurosorine (2μM) on human lymphoblast and Jurkat cells (4 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 4; Het 5) and control (C1; C2) lymphoblastoid cell lines and Jurkat cells (2 x 10⁶) were incubated with 2μM staurosorine for 4 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis ■ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.7.3 Assessment of the effect of staurosporine (2µM) on human lymphoblast cells (6 hours post treatment analysis).

The results indicate that increasing the time from 4 to 6 hours permitted discrimination between the three genotypes on the basis that A-T homozygote cells exhibited 47-48% apoptotic and 2-4.5% necrotic cells, A-T heterozygote cells exhibited 21-23% apoptotic and 7-13% necrotic cells, and control cells exhibited 30-34% apoptotic and 3.5-5.0% necrotic cells.
Cell Lines

Figure 3.7.3 Assessment of the effect of staurosporine (2μM) on human lymphoblast cells (6 hours post treatment analysis).

A-T homozygote (A-T 1; A-T 2), A-T heterozygote (Het 4; Het 5) and control (C1, C2) lymphoblastoid cell lines (2 x 10⁶) were incubated with 2μM staurosporine for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 4 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:
- early apoptosis ■
- late apoptosis □
- and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
Figure 3.7.4 Assessment of the effect of staurosporine (5μM) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

Fibroblast A-T homozygote and heterozygote cell lines had been treated under the same experimental conditions as the lymphoblastoid cells (Fig 3.7.2), but with the exception of GM08387 and Jurkat cells, the cell death profiles were <5% (data not shown), which necessitated the use of a higher staurosporine concentration. Despite increasing the staurosporine concentration to 5μM, at 4h the cell death profiles, again with the exception of GM08387 and Jurkat cells were unchanged (Data not shown). The cells were therefore treated with the same concentration of staurosporine as was used previously (5μM), but over a longer incubation time (24h) to determine if generating cell death profiles in these cell lines was time dependent. However, results indicated that apart from cell line GM08387, which exhibited a further increase in necrotic cells (60%) at this later (24h) time point compared to previous results (43% at 4h, data not shown), all the other fibroblast cells remained resistant to staurosporine-induced cell death.
Figure 3.7.4 Assessment of the effect of staurosporine (5μM) on human fibroblast and Jurkat cells (24 hours post treatment analysis).

A-T homozygote (GM03487) and A-T heterozygote (GM03489; GM08387; GM08389; GM03396; GM03397) fibroblast cell lines and control (Jurkat) cells (2 x 10⁶) were incubated with 5μM staurosporine for 24 hours and processed for Annexin-V binding and PI uptake (immediately after 24 hour continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
Figure 3.7.5  Assessment of the effect of staurosporine (10-150µM) on human fibroblast cell line GM08389 (24-72 hours post treatment analysis).

Due to the fact that most of the fibroblast cell lines had shown considerable resistance to staurosporine-induced cell death when compared to the results obtained with lymphoblast and Jurkat control cell lines, it was decided to select one cell line for further analysis, with the specific aim of determining a staurosporine concentration and exposure time that was able to induce cell death, to which the other fibroblast cell lines could be compared. The A-T heterozygote GM08389 was chosen for this study. This cell line had previously shown resistance to gamma radiation (up to 60 Gy over 48 hours) and etoposide (up to 1000µM). However, it had also been established that cell death pathways were inducible in this cell line, as 41% early apoptotic and 28% late apoptotic cells were produced in response to treatment with 100µM HP for 6 hours. Doxorubicin also induced a response in this cell line, which exhibited 11% necrotic cells when treated with 0.2µg/ml doxorubicin for 24 hours, 32% necrotic cells with 0.5µg/ml doxorubicin and 44% necrotic cells with 1.0µg/ml doxorubicin over the same time period. The doxorubicin results with this cell line not only demonstrated that cell death pathways were inducible, but also that it was possible to increase the level of response by increasing the concentration of the drug. The GM08389 cell line was treated initially with 10-50µM staurosporine for up to 72 hours, to determine if an increased cell death response would be produced with an increased drug dose and extended exposure time to staurosporine (Fig 3.7.5). However, the cell line remained resistant to staurosporine under these experimental conditions as the total cell death was <10% (Fig 3.7.5A). The cell line was then exposed to 75-150µM staurosporine for 24h to determine if much higher concentrations of the drug would induce more cell death in these cells. Despite increasing the staurosporine concentration 15-fold, these experimental conditions had no effect on increasing the number of cells exhibiting cell death profiles with this cell line (Fig 3.7.5B). The GM08389 remained resistant to staurosporine-induced cell death.
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Figure 3.7.5 Assessment of the effect of staurosporine (10-150μM) on human fibroblast cell line GM08389 (24-72 hours post treatment analysis).
3.8 Treatment of human cell lines with the chemotherapeutic drug, bleomycin.

Bleomycin is an anticancer drug that exerts its effects by producing reactive oxygen species such as hydroxyl radicals, superoxide and HP, that are able to interact with DNA causing DNA strand breaks (Mahmutoglu et al. 1987). Bleomycin causes both single and double strand breaks (Shaham et al. 1983), and it has been demonstrated that the type of strand break induced is largely concentration dependent. (Benítez-Bribiesca and Sánchez-Suárez, 1999).

Numerous studies have previously been carried out on the effect bleomycin has on A-T cells. Several independent researchers have reported a significant increase in chromosome damage to bleomycin treated A-T cells compared to controls (Taylor et al. 1979; Cohen et al. 1981; Shaham et al. 1983). A-T cells also exhibit lower survival rates compared to control cells following bleomycin treatment (Taylor et al. 1979; Cohen et al. 1981), while the survival rate of an A-T heterozygote cell line was shown to be intermediate between that of A-T and control cells. However, chromosome damage in A-T heterozygote cells showed no significant alterations from control levels following exposure to bleomycin (Cohen et al. 1981). Previous work in this area seems to have been centred around the type of damage caused to A-T cells and the survival rates of these cells compared to controls following exposure to bleomycin, while the mechanism involved in this decreased cell survival seems to have been overlooked. It was therefore hoped that this research would provide this information, and add to published work in this area.

In deciding the treatment dose range for bleomycin with the three genotypes being utilised in the study, we aimed to treat the A-T cells with doses that had previously been used to determine cell survival rates in A-T cells, to determine whether apoptosis/necrosis was induced at the previously used concentrations. However, we also intended to use Jurkat cells as additional control cells in this work, and aimed to repeat previously published work with bleomycin treated Jurkat cells to ensure consistency of results. Wang et al. (2001) had successfully used 300μM bleomycin for 24 hours to induce apoptosis in Jurkat cells, and recorded 7% early apoptotic cells, 27% late apoptotic cells and 4% necrotic cells. This concentration is within the concentration range (214-428μM) used to demonstrate that this concentration predominantly induced double strand breaks and apoptosis in PBLs (Benítez-Bribiesca and Sánchez-Suárez, 1999). It was therefore decided to use this concentration (300μM) and exposure time (24h) with the Jurkat cells.
3.8.1 Assessment of the effect of bleomycin (300μM) on Jurkat cells (24 hours post treatment analysis).

The results (Fig 3.8.1) indicated that at the 24-hour assay time point, the level of cell death was very similar to results published by Wang et al. (2001), who previously reported 34% apoptotic cells and 4% necrotic cells under the same experimental conditions. Although slightly less (24%) apoptotic cells and slightly higher (15%) numbers of necrotic cells were exhibited in the present study, the level of cell death was similar in the two assays. One possible reason for this small difference in the numbers of apoptotic and necrotic cells between the two studies could lie in the fact that different suppliers were used to purchase both the annexin-V and propidium iodide. In addition to the 24-hour time point, the current assay additionally studied the effects of bleomycin at 48 and 72h. At 48h, the Jurkat cells exhibited increased numbers of late apoptotic (42%) and necrotic (34%) cells. However, at the 72h time point, a further increase in late apoptotic cells (66%) was noted, along with a dramatic reduction in the numbers of necrotic cells (14%). These results therefore agree with the published results of Wang et al. (2001), and also show a drug exposure time-dependent increase in apoptosis in Jurkat cells.

In deciding the drug concentration to use with the A-T homozygote and heterozygote cell lines, Taylor et al. (1979) and Cohen et al. (1981) had previously used 5, 10 and 25μg/ml for 6 hours, and both reported increased damage and reduced cell survival in A-T cells compared to controls at 10μg/ml (7.13μM) following a 6 hour drug exposure time. It was therefore decided to use 7.13μM for 6 hours to treat the A-T homozygote and heterozygote cell lines.
Figure 3.8.1 Assessment of the effect of bleomycin (300µM) on Jurkat cells (24 hours post treatment analysis).

Jurkat cells (2 x 10^6) were incubated with 300µM bleomycin and processed for Annexin-V binding and PI uptake (immediately after 24 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:
- early apoptosis
- late apoptosis
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.8.2 Assessment of the effect of bleomycin (7.13µM) on human lymphoblast and fibroblast cells and Jurkat cells (6 hours post treatment analysis).

Initial experimental procedures were demonstrated using a limited number of cell lines in order to gauge the cell death response of the three genotypes under investigation to these experimental conditions, before testing additional cell lines. A-T homozygote (A-Tla) and two A-T heterozygote (Het 1; GM03397) cell lines in addition to Jurkat control cells used previously (Fig 3.8.1) were used for this purpose. One lymphoblastoid and one fibroblast A-T heterozygote cell line were used in order to gauge differences in the response of the different cell types to the drug. Initial results (Fig 3.8.2) showed that this concentration of bleomycin was ineffective in inducing apoptosis/necrosis, in any of the cell lines, as cell death in these cell lines, including the Jurkat cells did not reach 10%. The same cell lines were then exposed to 21.43µM bleomycin for 6 hours, but cell death did not exceed 10% in any of the cell lines (data not shown). The data therefore suggested that a higher concentration of bleomycin would be required to induce cell death in these cell lines, and for this reason, the concentration was increased to 42.86µM.
Figure 3.8.2 Assessment of the effect of bleomycin (7.13μM) on Human Lymphoblast and Fibroblast cells and Jurkat cells (6 hours post treatment analysis).

A-T homozygote (A-T1) and A-T heterozygote (Het 1196) lymphoblast cells, A-T heterozygote (GM03397) fibroblast cells and Jurkat cells (2 x 10^6) were incubated with 7.13μM bleomycin for 6 hours and processed for Annexin-V binding and PI uptake (immediately after 6 hours continuous exposure) as previously described in the methods section. Data indicates the level of cells depicted to be in:

- early apoptosis 
- late apoptosis 
- and necrosis

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.8.3 Assessment of the effect of bleomycin (42.86µM) on human lymphoblast and fibroblast cells and Jurkat cells (6 hours post treatment analysis).

In the previous work with bleomycin only a limited number of cell lines had been utilised, and the bleomycin concentrations and exposure times had failed to induce cell death in those cell lines. In order to determine if those results were due to the experimental conditions employed previously, or whether those specific cell lines were resistant to staurosporine-induced cell death, additional lymphoblastoid and fibroblast cell lines were utilised in the current study. The results indicated that two of the A-T heterozygote cell lines in the current study that had previously been used with lower concentrations of the drug (Het 1; GM03397) remained resistant to bleomycin-induced cell death even at this elevated concentration (42.86µM; Fig 3.8.3A and B respectively). The data also indicated that all of the fibroblast cells exhibited resistance to staurosporine-induced cell death (Fig 3.8.3B), but the same experimental conditions were able to induce cell death profiles in the majority of the lymphoblast cells. However, no heterogeneity was observed for the A-T homozygote or control genotypes, as they exhibited very different cell death profiles, which differed not only in the type of cells exhibited (apoptotic v necrotic) but also in the numbers of cells exhibited within the same genotype. However, homogeneity was exhibited in the A-T heterozygote cell lines in terms of the fact that they all produced larger numbers of necrotic cells compared to apoptotic cells.
Figure 3.8.3 Assessment of the effect of bleomycin (42.86μM) on human lymphoblast and fibroblast cells and Jurkat cells (6 hours post treatment analysis).
3.8.4 Assessment of the effect of bleomycin (420μM) on human fibroblast cells (24 hours post treatment analysis) and Jurkat cells (24-72 hours post treatment analysis). Having elicited a response in the lymphoblast cells it was decided to try and optimise experimental conditions for the fibroblast cells to determine if similar results could be obtained with these cell lines. Due to the apparent resistant nature of these cell lines to bleomycin treatment, it was decided to elevate the concentration used previously (Fig 3.8.3) 10 fold, and to extend the drug exposure time to 24 hours. Jurkat cells were also included so that in the event that the fibroblast cells did not respond to the treatment, a response in the Jurkat cells would demonstrate that such an observed effect was not due to aberrant experimental conditions. In addition to the 24-hour time point used for the fibroblast cells, 48 and 72-hour assay time points were included for the Jurkat cells. The results (Fig 3.8.4) demonstrated that despite the 10 fold increase in bleomycin concentration (420μM), all the fibroblast cells remained resistant to the effects of bleomycin, indicated by the fact that there was <10% total cell death (apoptotic and necrotic cells) exhibited. The Jurkat cells did however respond to the treatment, indicating that the response of the fibroblast cells was due to resistance to the drug rather than experimental conditions. Increasing the concentration of the drug produced a significant response in the Jurkat cells. Within the first 24 hours, ~25% of the cells were apoptotic and similar numbers of necrotic cells were produced. By 48 hours these numbers had nearly doubled to ~40%. However, at the 72-hour time point there was a dramatic change in the cell death profile. Although the actual numbers of cells undergoing cell death did not change from 48-72 hours, the mechanism of cell death did. There was a sharp increase in late apoptotic cells to ~70%, with a decrease in necrotic cells to ~13%. These results therefore show a dose response in the generation of late apoptotic cells with time at this concentration and the results also indicate a peak for the generation of necrotic cells at 48 hours, which decreases after this time point.
Figure 3.8.4 Assessment of the effect of bleomycin (420µM) on human fibroblast cells (24 hours post treatment analysis) and Jurkat cells (24-72 hours post treatment analysis).

A) A-T homozygote (GM03487) and A-T heterozygote (GM03396; GM03397; GM08389) fibroblast cells were incubated with 420µM bleomycin for 24 hours and B) Jurkat cells were incubated with 420µM bleomycin for 24-72 hours. Following incubation with the drug, the cells (2 x 10⁶) were processed for Annexin-V binding and PI uptake (immediately after continuous exposure at stated time) as previously described in the methods section. Data indicates the level of cells depicted to be in: early apoptosis □ late apoptosis □ and necrosis □

The results were analysed using Win MDI, and the results expressed are the mean (±) SD of three separate experiments.
3.8.5 Assessment of the effect of bleomycin (600μM) on human lymphoblast cells and HL-60 cells (4 and 6 hours post treatment analysis).

Finally, previously published results had demonstrated that a bleomycin concentration of 714μM used to treat PBLs had produced a reduction in both single and double strand breaks which was associated with a reduction in cell viability (49%), a smeared electrophoresis pattern with no ladder formation and evidence of morphological necrotic cell changes (Benítez-Bribiesca and Sánchez-Suárez. 1999). This raised the question of whether a much higher concentration of bleomycin could alter the cell death profiles of the lymphoblast cells, which, with the exception of the A-T heterozygote cells exhibited a predominantly apoptotic cell death profile (Fig 3.8.3). However, the aim was not to totally kill the cells, but rather to determine if it was possible to induce necrosis using a higher concentration to that used previously. For this reason, a bleomycin concentration of 600μM was chosen to treat the cells for both 4 and 6 hours, so that any changes in cell death between these two time points could also be observed. All of the cell lines used in the previous study (Fig 3.8.3) were used in the current study with the exception of A-T heterozygote (Het 1). The results at the four-hour time point (Fig 3.8.5A) showed a similar cell death profile for most of the cell lines, with slight increases in apoptotic (<5%) and necrotic (<5%) cell numbers with two exceptions. One control (C1) exhibited a 9% increase in apoptotic cells and an additional control (C2) demonstrated a 17% increase in apoptotic cells. Interestingly, these increases were reduced by approximately the same levels at the later (6h) time point (Fig 3.8.5B), such that at this time point, the results were practically identical to the results previously obtained with 42.86μM bleomycin over the same exposure time. Therefore, despite this grossly elevated bleomycin concentration, no further increases in the cell death profiles of the lymphoblastoid cell lines were observed under these experimental conditions.
Figure 3.8.5 Assessment of the effect of bleomycin (600μM) on human lymphoblast cells and HL-60 cells (4 and 6 hours post treatment analysis).
4.0 Discussion.

It has long been recognised that ataxia-telangiectasia patients exhibit a variety of deficiencies in normal bodily function that can range from the simple predisposition to a variety of infectious agents to the more severe cases of progressive cerebral neurodegeneration (reviewed in Taylor and Byrd, 2005). Patients who are confirmed for the presence of the disease are at increased risk and sensitivity to ionising radiation and are also at a higher than normal risk of developing cancer, principally tumours of the lymphatic system (Taylor et al. 1975; Taylor et al. 1996; reviewed by Gatti et al. 1991). The classical clinical diagnosis for A-T involves evidential progressive cerebral ataxia associated with oculocutaneous telangiectasia (Boder and Sedgwick, 1958; Zecevic and Rakic, 1976). This can occur through the loss of function of both alleles either through compound heterozygosity or less frequently through homozygous deletion resulting in the production of an unstable or ineffective ATM protein. Ideally, the successful molecular diagnosis of A-T would require the identification of the sequence mutation associated with each allele. However, given the size of the ATM gene, which spans 150,000 bases, the commercial and routine development of a suitable screening exercise facilitating this requirement can be considered as an extremely onerous task. Therefore, given the problematic nature of this approach to screening, it was decided to investigate methodologies that could be used to discriminate A-T heterozygotes from A-T homozygotes and wild type cells, either, using (1) western blot analysis or (2) sensitivity to a range of cell damaging agents including IR, etoposide, hydrogen peroxide, staurosporine, doxorubicin and bleomycin in combination with tissue culture and flow cytometry. In undertaking these approaches it was hoped that the efforts would aid the differential determination of suitable markers of A-T heterozygosity, as A-T carriers are thought to be at increased risk for cancer susceptibility, in particular, breast cancer in women (Athma et al. 1996; Fitzgerald et al. 1997; Chen et al. 1998; Broeks et al. 2000). Additionally, A-T heterozygotes, unlike A-T homozygotes are asymptomatic, and routine exposure to IR used for diagnosis of various medical conditions, as well as in screening and treatment for various malignancies could aid the development of neoplastic conditions in these susceptible individuals.

Methods that have been used to detect ATM mutations have largely focused on amplification of genomic DNA, which has to be done in multiple reactions due to length. Additional approaches have involved DNA sequencing, protein truncation testing, restriction endonuclease fingerprinting, single strand conformation polymorphism and
heteroduplex analysis. However, the number of mutations detected using these methods was lower than the number of mutations that were expected (Telatar et al. 1996; Stancovic et al. 1998; Sandoval et al. 1999). Other researchers have attempted to screen for A-T heterozygotes using a variety of procedures. Leonard et al. (2004) noticed A-T heterozygote cells exhibit levels, of chromosome exchanges that are intermediate between A-T homozygote and control levels after treatment with camptothecin, whereas Nagasawa et al. (1987) proposed the cumulative labelling indices assay as a method for the identification of A-T heterozygotes within known A-T families. A high-density oligonucleotide array assay has also been utilised to detect ATM mutations (Hacia et al. 1998), however, the aforementioned assays are time consuming, often require specialised equipment, and are expensive to perform due to the large size of the ATM gene.

In comparison to the gene-based assays, western blotting involves analysis at the protein level, where proteins are separated in a gel, immobilised on a membrane support, and specific antibodies generated against peptide sequences within the protein of interest are used to detect the protein on the membrane. The obvious advantages to this type of protein assay are that it is quicker and easier to perform than DNA-based assays, the equipment and reagents are relatively inexpensive, and the procedure could be carried out extensively in a wide range of laboratories. Western blotting has been widely used to demonstrate the absence or reduced expression of the ATM protein in A-T cell lines, and the reporting of less than normal protein levels in A-T heterozygote cell lines (Telatar et al. 1996). In a study examining the correlation between the ATM genotype and phenotype, ATM expression was measured by western blotting in 123 A-T patient cell lines; 85% of the cell lines expressed no ATM protein and the remaining 15% produced reduced levels of the protein. Additionally, 90% of A-T heterozygotes also expressed less ATM protein (Becker-Catania et al. 2000). Western blotting is routinely used to measure ATM protein levels in patients with suspected ataxia-telangiectasia, as part of the laboratory investigation to confirm a diagnosis of A-T (Lavin, et al. 2007). The aim of this research was to determine if the same methodology could be used to reliably discriminate A-T heterozygotes from homozygotes and controls, and to investigate the feasibility of using this methodology in a rapid screening assay for identifying A-T heterozygotes in the general population.
4.1.1 Investigation of non-specific bands detected by western blotting.

During experimental procedures to optimise the western blot assay conditions, the rabbit anti-ATM polyclonal antibody in addition to the ATM band, detected additional bands, the identity of which, were unknown. The possibility existed that the additional bands were non-specific signals, degraded, or truncated ATM proteins or members of the same family of proteins to which ATM belongs, and which share sequence homology to ATM. Of particular interest was a protein species with a lower molecular weight than ATM, which resolved close (within 0.2cm) to the ATM protein on the gel (Fig 3.1.1B) and was termed ATM-A. To ensure that the presence of this additionally detected band did not interfere with the immuno-signal generated by the ATM protein, electrophoresis conditions were optimised to further separate these proteins (Fig. 3.1.2B), such that the distance between the bands was increased to 0.5cm.

DNA-PK is a member of the same protein family as ATM, and so this protein was run under reducing conditions and applied neat to the gel to determine if DNA-PK resolved in the same place as one of the additional bands on the gel. However, addition of the reducing agent caused the DNA-PK to precipitate out of solution such that the amount of protein on the gel was insufficient to be stained with Coomassie blue (data not shown). The neat DNA-PK (460kDa; Fig 3.1.3A) resolved above the ATM protein (350kDa; Fig 3.1.3B), and the ATM protein resolved above a 200kDa myosin molecular weight marker (Fig 3.1.3C), demonstrating that all three proteins had been resolved according to their respective molecular weights. Although the neat DNA-PK did not correspond to protein species ATM-A, or any of the other additional bands detected by the rabbit polyclonal antibody (PC116, Merck), the DNA-PK run under these conditions did provide a high molecular weight marker to which ATM could be compared, which was useful as such markers are commercially unavailable. This helped affirm the position and size of the ATM protein.

To determine if the additional bands detected by the rabbit anti-ATM polyclonal antibody were truncated ATM protein products or non-specific signals being detected in other proteins that shared sequence homology to ATM, additional antibodies with different ATM target sequences were used. A mouse monoclonal anti-ATM primary antibody (ab78, Abcam) with a target sequence corresponding to amino acids 2577-3056 in the carboxyl terminal was used, which was in comparison to the rabbit polyclonal anti-ATM antibody
whose target sequence was amino acids 819-844 in the amino terminal domain. Development of the photographic film for 3 minutes (Fig 3.1.4A) produced a prominent single ATM band in the A-T heterozygote and control cell lines, that was absent in the A-T homozygote cell line. Prolonged exposure of the film (10 minutes) resulted in a very strong ATM band being generated in both the A-T heterozygote and control cell lines, that was absent in the A-T homozygote cell line (Fig 3.1.4B) and revealed 15-20 additional bands in the normal and A-T heterozygote cell lines, most of which, were not present in the A-T homozygote cell line. Interestingly, the mouse monoclonal antibody detected the protein species previously detected by the rabbit primary antibody, ATM-A. The detection of this band by both antibodies, one recognising a sequence within the amino terminal and the other recognising a sequence at the carboxyl terminal did imply that this band was being specifically recognised by the two antibodies. The mouse monoclonal antibody also detected an additional lower molecular weight protein species underneath the ATM band in the normal and A-T heterozygote cell lines (Fig. 3.1.4B). This additional protein species was not the previously detected ATM-A protein, detected using the rabbit polyclonal antibody, as electrophoretic conditions had been optimised to separate this protein from the ATM band (as discussed previously), and this band had also been detected by the mouse monoclonal antibody. The additional band detected by the mouse monoclonal antibody was 0.3cm below the ATM band and between ATM and the previously detected additional band ATM-A, and was only present in normal and A-T heterozygote cell lysate samples. Gately et al. (1998) have reported a similar additional protein species, which was detected in whole cell lysate from HeLa cells, and it was proposed that this species might be a different protein other than ATM containing a PI3-like kinase domain. Additionally, a faint band of lower molecular weight than the ATM protein (350kDa) was produced in A-T cell line GM03487C from the Coriell Cell repository. However, Coriell report GM03487 to be the same cell line as AT4Be, and not AT3Be as reported, and do not list GM03487C in the catalogue, so it is unclear from the supplier or from the publication whether the “C” denotes a transformation process was used with this cell line. Interestingly, Jung et al. (1997) reported detecting a truncated ATM protein (180kDa) in AT29RM cells. They also report consistent detection of an ATM double band in MRC5CVI cells. Like the lymphoblast cells used in the current study, in which an additional band was detected in 3/5 A-T heterozygote and 3/6 control cell lines (Fig 3.1.5), the MRC5CVI fibroblasts were SV40 transformed. Jung et al. (1998) proposed that more than one ATM gene product might be present in the MRC5CVI cells due to alternative splicing. In order to further
address this issue, an additional mouse monoclonal anti-ATM antibody with the same target sequence (aa2577-3056) from a different supplier (QED) was compared with the mouse monoclonal antibody used previously (Abcam). The results demonstrated that ATM was detected in A-T heterozygote and control cell lines but not in A-T homozygote cell lines by both antibodies (Fig 3.1.5A and B) and that the additional bands detected by the Abcam antibody (Fig 3.1.5A), were also detected by the QED antibody (Fig 3.1.5B) at identical points on the film. It would have been advantageous to recover these protein bands and sequence them to determine if the deduced amino acid sequence showed sequence homology with the ATM protein, but at the time this research was conducted, access to a peptide sequencer was not available. However, a BLAST sequence search (see BLAST http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi) of the immunogen used to generate the rabbit polyclonal antibody (CKSLASFIKKPFDRGEVESMEDDTNG), as well as identifying the sequence in the ATM protein (Identities = 26/26 (100%), Positives = 26/26 (100%), Gaps = 0/26 (0%)), also detected similar sequences in other proteins. Other than the ATM protein, the protein sharing the most sequence homology with this peptide was the cell division cycle associated 8 gene product, CDCAS (Identities = 11/15 (73%), Positives = 12/15 (80%), Gaps = 2/15 (13%)). Therefore it is conceivably possible that the additional bands detected on the western blots are proteins sharing sequence homology with the ATM protein.

The specificity of antibody binding was investigated using ATM immunogenic peptides to compete with ATM protein immobilised on PVDF membrane, for the primary antibody. This work was undertaken due to the unavailability of full length ATM protein. Two immunogenic peptides were utilised in this work, one was used to raise a rabbit polyclonal antibody, and the other used to generate a goat polyclonal antibody. Results demonstrated the rabbit antibody did not produce a detectable ATM band and using the immunogenic peptide resulted in dense background staining, which could not be corrected (data not shown). However, using the goat polyclonal antibody (sc-7128, Autogen Bioclear), a faint ATM band was detected (Fig 1.1.9A), and using the immunogenic peptide (sc-7128p, Autogen Bioclear, 18 amino acid sequence corresponding to an internal region of the ATM protein) in conjunction with the primary antibody (Fig 3.1.9B), the ATM band was not detected, indicating that the immunogen had bound to the primary antibody, which was then not able to bind to the ATM protein immobilised on PVDF membrane. An additional gel, run under the same conditions was used to probe for ATM protein (Fig 3.1.9C) using a
mouse monoclonal anti-ATM antibody (ab78, Abcam, target sequence corresponding to amino acids 2577-3056 of ATM). ATM protein detected by immunoblotting with the mouse monoclonal and goat polyclonal antibodies detected the ATM protein in the same position on the blots.

4.1.2 Protein sample optimisation.
During experimental procedures to optimise assay conditions, the A-T heterozygote cell line Het 1 had consistently produced an ATM band that was either equal to or greater than that generated by the control cell line C1. To determine if the concentration of ATM protein in the control was sufficiently high enough to prevent binding of the antibody to the target sequence, protein samples from A-T heterozygote and control cell lysate were prepared over the concentration range 5-30µg. Results demonstrated that the signal intensity was greater in the control than the A-T heterozygote cell line only at the lowest (5µg) concentration (Fig. 3.1.6), which indicated that if western blotting was to be used to discriminate A-T heterozygotes from controls, a lower cell lysate concentration was favoured. Additional work on signal optimisation indicated that the limits of detection for the mouse monoclonal anti-ATM primary antibody, was 5µg/ml for the control C1 (Fig. 3.1.7A) and both A-T heterozygotes Het 1 (Fig 3.1.7B) and Het 4 (Fig 3.1.7C) cell lines using a primary antibody concentration of 1µg/ml. Greater sensitivity in the limits of detection using western blotting to measure ATM protein had been reported previously (Becker Catania et al. 2000), which could be explained by the specificity of the different antibodies used in the previously published results and the current research. In the Becker-Catania et al. (2000) study, although the limits of detection were determined as1µg total cell protein using 1µg/ml primary antibody, the experimental procedures were conducted mainly on samples containing 25µg whole cell lysate. In the current research, a cell lysate concentration of 5µg was able to generate a good signal intensity, and was favoured due to greater discrimination between normal and A-T heterozygote samples being observed at lower cell lysate concentrations (Fig 3.1.7).

4.1.3 Comparison of ATM expression in nuclear and whole cell lysate.
As ATM is predominantly a nuclear protein, with some being present in the cytoplasm, these cellular compartments were isolated and fractions obtained to determine if better discrimination of A-T heterozygote samples from A-T homozygote and control samples could be obtained by using nuclear lysate instead of whole cell lysate. The results
demonstrated that the signal intensity generated from the nuclear lysate and whole cell lysate were similar in the A-T heterozygote and control samples (Fig 3.1.8A-C). Additionally, the β-actin band intensity was generally seen to be greater in cytoplasmic extracts than nuclear extracts, which would be consistent with its function as a cytoskeletal protein, but may have affected its use in normalising ATM expression levels. The results therefore demonstrated that no advantage would be obtained by using nuclear cell lysate compared to whole cell lysate, and furthermore, using nuclear lysate could be detrimental to the success of the screening assay as it would introduce additional steps into the assay which could be subject to variability. Interestingly, no ATM protein was detected in HL-60 cells (Fig 3.1.8B), which has also been observed by other researchers (Gately et al. 1998). However, activation of the ATM protein has been demonstrated in HL-60 cells treated with topotecan or mitoxantrone by using an antibody specific for ATM phosphorylated on serine 1981 (Kurose et al. 2005). It is therefore possible that this antibody shows greater sensitivity for the phosphorylated ATM than the monoclonal antibody used to detect unphosphorylated ATM in the present study.

4.1.4 Testing A-T homozygote and heterozygote SV40 transformed lymphoblastoid and untransformed fibroblast cell lines with the optimised western blotting assay.

Having optimised and standardised as many of the variables in the assay as possible, A-T homozygote, A-T heterozygote and control lymphoblastoid cell lines were evaluated for ATM and β-actin expression. The β-actin membranes were exposed to photographic films for 10 seconds to 5 minutes in order to determine the point at which there was the least change in signal intensity. The results showed that the increase in optical density occurred with slower kinetics within the first minute of film exposure, than that observed between one and five minutes, and that between 20 seconds and 1 minute, there was little change in the optical density (data not shown). Using optical density measurements at 20 seconds would therefore minimise the variation in optical density readings that could arise due to the time taken to remove the films from the dark box and manually develop the films, and for this reason, the 20-second film exposure time was used to generate the optical density measurements for β-actin. The optical density recorded for the ATM band in each cell line was normalised to the corresponding optical density value for the beta actin band from that cell line by dividing the ATM optical density values by the beta actin density value. This figure was expressed as a percentage by multiplying the figure obtained by 100. The mean ± standard deviation of the control samples on the gels was calculated (97.9% ± 37.7%, n =
7), and each A-T homozygote and heterozygote value was expressed as the percentage expression of the mean control value. Results indicate all the A-T homozygote and heterozygote lymphoblastoid cell lines expressed <50% of mean control values (Fig 3.1.10). In addition to the results in Fig 3.1.10, using this method of analysis, two A-T homozygote cell lines (A-T1a; A-T2), one A-T heterozygote cell line (Het 1), and one cell line of unknown genotype (but thought to be an A-T heterozygote; P Het) did not produce sufficient ATM protein to be measured in the assay. These A-T homozygote and heterozygote cell lines would therefore be correctly identified as carrying at least one ATM mutation. In the case of cell line P Het, the results would suggest that this cell line is indeed an A-T heterozygote.

The same method of analysis was applied to A-T homozygote and heterozygote untransformed fibroblast cell lines, using four of the control cell lines (C2; C3; C4; C5) used to generate the results with the lymphoblastoid cell lines in Fig 3.1.10. The cell lines were normalised to β-actin (as detailed previously), and the mean ± standard deviation was calculated for the control cell lines (76.41% ± 13.96%, n = 4). The ATM band for each A-T homozygote and heterozygote cell line was expressed as the percentage expression of the mean control value (Fig 3.1.11). The results demonstrate that all of the A-T heterozygote cells expressed <35% of mean control ATM values. In addition to the results in Fig 3.1.11, an A-T homozygote cell line (GM03395) did not produce sufficient protein to be measured in the assay and so would be classed correctly as carrying at least one ATM mutation.

The procedure to normalise the intensity of the ATM band to the β-actin band has been used previously (Becker-Catania et al. 2000). In the same study, the intensity of the ATM band was expressed as a percentage of a single ATM band generated by a control cell line on the same gel. However, the authors were of the opinion that this method was not reproducible, but do not give reasons why they came to this conclusion. It is difficult to speculate on why they came to this conclusion, but a possible reason for this would be that the band intensity generated by the control itself was not reproducible. In the current research, the mean band intensity of several control cell lines was used to calculate the percentage expression of the mean of the controls. This method probably gives a more accurate measurement of the range of ATM protein expression within the control cell lines, and using this method it was possible to detect all of the A-T homozygote and heterozygote cell lines in both SV40 transformed lymphoblastoid cell lines and untransformed fibroblast
cell lines. Using this method it was not possible to distinguish between A-T homozygote and heterozygote cell lines, but the test could still be used to discriminate individuals who were heterozygous for ATM, as homozygote patients are usually diagnosed with the condition at an early age due to phenotypic markers being dominant. The Becker-Catania et al. (2000) study replaced the use of a control to measure ATM expression with a scoring system of 0-4+, related to the intensity of the ATM band observed. However, they do not describe what criteria were used for assigning these scores. If each of these categories had been assigned a range of optical density units for inclusion in the category it might have been possible to interpret the results presented in the current research with those of the published study more accurately. In the current research, three A-T homozygote cell lines (A-T1 and A-T1a were different batches of the same cell line), seven A-T heterozygote cell lines (Het 1 and Het 1a were different batches of the same cell line), and seven control cell lines were used to generate the results. The current research identified that the A-T homozygote cell lines did not produce any ATM protein, consistent with the fact that each of these cell lines had two truncating mutations, and all of the A-T heterozygote cell lines produced less ATM protein than controls. All of the A-T heterozygote cells had truncating mutations, except for Het 1, which had one of two possible mutations; either a truncation mutation or a change in amino acid sequence from C>G. Different batches of the same cell line were used for one of the A-T homozygote and one of the A-T heterozygote cell lines, indicating that the transformation process did not alter the characteristics of the cell line between batches. The Becker-Catania et al. study (2000) used 123 patient cell lines and 10 A-T heterozygote samples. They found that 85% of the A-T cell lines did not express ATM protein and that the level of protein in the remaining 15% was considerably reduced compared to normal expression. They also found that 90% of the A-T heterozygote cell lines expressed reduced amounts of ATM protein. These slight differences between the current research and published results could therefore be due to the different mutations being present in the cell lines used in each study as well as the number of cell lines utilised.

There have also been reports of apparently full length ATM protein being produced in both A-T homozygote and heterozygote cell lines (Stancovic et al. 1998). These cell lines have a founder effect mutation (7271T>G) and the A-T patients from whom the cell lines were generated have an observed milder clinical phenotype. This type of mutation was not represented in the current study, and so it was not possible to report on the accuracy of the current assay representing these cell lines, but may be a consideration for future work.
Interestingly, the three anti-ATM antibodies utilised in the study detected a number of protein species on the gel in addition to the ATM protein. In a publication where one of these antibodies has been used, even though the region of the blot containing the ATM protein has only been published, it was still possible to see the presence of one protein species in addition to the ATM protein (Kozlov et al. 2006 (ab78)). The number of additional bands detected was greater using the monoclonal antibodies compared to the polyclonal antibody, and this was thought to be due to increased sensitivity of the monoclonal antibodies, as the band intensity of the ATM protein was also much greater using the monoclonal antibodies than that observed with the rabbit polyclonal antibody. Attempts were made to source ATM protein so that it could be a) used as a reference standard to more accurately quantify ATM expression, and b) to run alongside the cell lysate samples under reducing conditions to determine if the additional bands were degraded ATM peptide products. However, ATM protein was not commercially available and research groups that had used recombinant protein were unable to provide the peptide to help clarify these issues here. An additional method that could have been used to determine the identity of these additional bands would have been to recover them from the gel and sequence the proteins recovered. The amino acid sequence could then have been compared to the ATM amino acid sequence to determine if the peptides shared sequence homology with the ATM protein. However, at the time this research was conducted, a peptide sequencer was not available. Interestingly, Becker-Catania et al. (2000) did not report the presence of additional bands in their work, and reported that there were no truncated ATM products in A-T homozygote samples. The limits of detection in the current study were 2.5µg/ml whole cell lysate, but 5µg/ml whole cell lysate was used in order to generate an adequate ATM signal that could be measured by densitometry. The antibody used in the Becker-Catania et al. (2000) study showed greater sensitivity, with lower limits of detection (1µg/ml), but despite this, they routinely used 25µg/ml whole cell lysate. It was therefore surprising that they had no reported the presence of additional protein species being detected on the gel in addition to the ATM protein, unless in addition to the increased sensitivity of their antibody, it also showed greater specificity for the ATM protein. Two of the authors in the Becker-Catania et al. (2000) study had previously published work with what appeared to be the same antibody (Chen and Lee, 1996), and in control cell lysate, a similar pattern of additional protein species as those presented here was evident. The importance of determining whether or not these additional protein species
were truncated ATM proteins is exemplified in a study by Butch et al. (2004). This publication reported the development of an immunoassay, which could detect and quantify ATM protein in peripheral blood mononuclear cells (PBMCs) and lymphoblast cell lines (LCLs) using recombinant ATM protein as a reference standard. In nuclear lysates from 22 control LCLs, the ATM concentrations were determined as 49–610 μg/L, and in control PBMCs was 48–943 μg/L. ATM was undetectable (<20μg/L) in 18 of 21 A-T patient cell lines. The remaining three A-T cell lines produced low amounts of ATM protein ranging from 24–61μg/L. ATM was also measured in control whole cell lysate from LCLs (64–463 μg/L) and PBMCs (42–444 μg/L). ATM measured in 8 lymphoblastoid A-T heterozygote cell lines was found to be between 52 and 98μg/L. Based on these figures, at least one of the A-T homozygote cell lines expressed ATM within the concentration range generated by the controls using nuclear lysate, and at least one of the A-T heterozygote cell lines expressed ATM within the concentration range generated by the controls using whole cell lysate. The study evaluated the merits of the immunoassay against western blotting, and found that the immunoassay was 2-fold more sensitive than the western blotting method. The concentration of nuclear lysate used for western blotting with the lymphoblast cells was 20μg, and if the immunoassay was found to be 2-fold as sensitive, this implies that detection of ATM protein using the immunoassay was able to be performed on 10μg nuclear lysate. In the current research, western blotting using 5μg whole cell lysate was able to generate an adequate ATM band that permitted discrimination of A-T homozygote and heterozygote cell lines from controls. Interestingly, in the Butch et al. (2004) study, one of the two antibodies immobilised on the plate to capture the ATM protein was the same mouse monoclonal primary antibody (Genetex antibody from Abcam) as used in the current research. Two mouse monoclonal antibodies with the same target sequence from different suppliers had been used in the current research, and as mentioned previously, both these antibodies detected ATM protein, but also detected 10-15 additional bands. The study by Butch et al. (2004) used a rabbit primary antibody to detect the ATM protein by western blotting, and the current research demonstrated that polyclonal antibodies were not as sensitive as monoclonal antibodies in detecting the ATM protein. This could have implications for their assay, as the additional bands detected in the current research when probing for ATM with the monoclonal antibodies may not have been present on the western blots conducted by Butch et al. (2004) using the rabbit antibody, and so it is possible that the immunoassay was detecting the ATM protein as well as the additional bands demonstrated in the current research. Additionally, the sensitivity of the western
blotting may have been increased by using the same monoclonal antibody to detect ATM in western blots that they had used as the capture antibody in their immunoassay instead of the polyclonal antibody, as research here has demonstrated monoclonal antibodies to be more sensitive.

The western blotting assay investigated in the current research demonstrated problems with reproducibility of the assay. However, this was mainly due to rapid signal generation by the β-actin used to normalise the ATM bands. This assay would have benefited greatly from using ATM reference standards, as development of the ATM signal generated on the films was a much slower process than that observed for β-actin, which would have decreased dramatically the variation in signal intensities observed between experiments.

4.2 Fluorescence Activated Cell Sorting (FACS) cytometric analysis of apoptotic/necrotic cell death profiles of human cell lines in response to DNA-damaging agents.

It has previously been suggested that radiation induced apoptosis could form the basis of a screening assay for the identification of A-T heterozygotes (Bebb et al. 2001) as A-T heterozygotes were able to be discriminated from A-T homozygote and control cells using the hypo diploid and TUNEL assays to measure apoptosis in Epstein-Barr virus transformed B lymphocytes. A range of cell types were used in this study because it was previously shown that it was not possible to discriminate A-T heterozygotes from A-T homozygotes or control PBLs as these cells responded differently to transformed cells when treated with γ-radiation (Bebb et al. 2001).

Additionally, this thesis recounts data generated from SV40 transformed and untransformed patient derived A-T homozygote and heterozygote cells derived from both lymphoblastoid and fibroblastoid lineages, to also determine whether the transformation process might either affect or contribute to the cell death profiles observed in these cells when exposed to a variety of treatment regimes. Furthermore, it was hoped to use the regimen induced specific cell death profiles to gain some insight into the mechanism of ATM function based on the knowledge of ATM mutations within the specific cell lines used.
Ionising radiation has the capacity to induce single and double-strand DNA breaks due to the production of ROS. These molecules can arise due to the ionisation of water molecules within the cell, or can also occur due to the peroxidation of lipids (Kurtz et al. 2004). Double strand breaks are the most severe lesion to be induced by IR (Bakkenist and Kastan, 2003).

Figure 4.1 Schematic diagram showing radiation/chemical-induced DNA damage and Apoptosis.

ROS-induced DNA damage can trigger G1/S cell cycle arrest, facilitating the cell to correct the DNA lesions before the DNA is replicated or can also induce apoptosis (Lee et al. 2000). A-T cells are reported to have a defect in the p53-dependent arrest in G1/S damage-
sensitive checkpoint as well as other cell cycle checkpoints (Canman et al. 1994; Thacker, 1994; Takagi et al. 1998; Painter and Young, 1980), resulting in continuous progression of the cell cycle into the S-phase. The DNA is replicated in spite of the damaged DNA. This phenomenon is known RDS, which is a common feature in A-T cells (Gatti et al. 1991), and arises due to a defect in the S phase cell cycle checkpoint (Zakian, 1995).

Figure 4.2 Schematic diagram of the cell cycle, showing ATM involvement in cell cycle checkpoints (Adapted from nobelprize.org/.../laureates/2001/press.html).

Natural sources of radiation can be found in rocks, soil, building construction materials, food and drink and from solar energy. Man made sources include nuclear energy production, through extraction of oil and natural gas, from nuclear bomb tests conducted in
CHAPTER 4: DISCUSSION

the 1950s and 60s (low residual levels), and from medical applications relating to diagnosis and treatment of cancer (Department for Environment, Food and Rural Affairs). High-energy γ and X-rays are routinely used in the treatment of neoplastic lesions due to their ability to penetrate body tissue and damage DNA (Cancer Research U.K.). It has previously been demonstrated that A-T patients are extremely sensitive to IR and A-T heterozygotes show intermediate sensitivity as it only takes 75% of the normal tolerance dose of radiation to kill the same number of A-T heterozygote cells compared to normal cells (Nagasawa et al. 1987). Despite this however, and the fact that A-T heterozygotes have also been shown to have cell cycle defects (Shiloh, 2001) asymptomatic A-T carriers would be treated as normal individuals so would receive the same dose of radiation as cancer patients with two normal copies of the ATM gene. This could predispose A-T heterozygotes to the development of further malignancies, especially considering a bystander effect of ionising radiation on normal adjacent tissue (Benítez-Bribiesca and Sánchez-Suárez 1999). To address this observation it was decided to expose both patient and carrier derived cell lines to γ-radiation.

4.2.1 The effect of low-dose γ-irradiation on human lymphoblastoid cells.
The cellular response of human lymphoblastoid cells to low dose (4Gy) γ-irradiation enabled A-T homozygote (A-T1; A-T1a) and heterozygote (Het 1; Het 2) cells to be discriminated from normal lymphoblastoid cells (Fig 3.2.3) when cell death profiles were analysed 8 hours post-irradiation. This finding was based on the fact that this level of radiation failed to induce cell death in the A-T homozygote and heterozygote cell lines and only produced cell death profiles in control cells. Interestingly, the control lymphoblastoid cell line (C5) exhibited a necrotic cell death profile, while the same experimental conditions produced an apoptotic cell death profile in Jurkat cells. The apoptotic cell death profile exhibited by the Jurkat cells was consistent with previously published data demonstrating this mechanism of cell death with this cell line (Gong and Almason, 2000; Bebb et al. 2001) and demonstrated the ability of annexin-V to bind its externalised PS target. Further investigations revealed that by extending the assay time post-irradiation to 24 hours, discrimination could be made between the three genotypes (Fig 3.2.4). The results observed for the A-T homozygote (A-T1) and control (C5) cells demonstrate that a response was only exhibited at later time points (8, 24h), and that the level of necrosis in the normal cells (18%) was twice that observed in the A-T homozygote cells (9%) at 24h. The A-T heterozygote cell line (Het 1) did not show any increase in cell death over the
same time period, and it was apparent that this cell line exhibited resistance to radiation-induced cell death under these experimental conditions. The A-T heterozygote cell line (Het 1) was produced from the mother of an affected child, from which the A-T homozygote cell line (A-T1) was generated. Interestingly, the A-T homozygote cell line did not show the same level of radiation-induced resistance to apoptosis exhibited by the A-T heterozygote cell line. These results were in disagreement with the literature. For example, it has previously been demonstrated that A-T heterozygote cells exhibit numbers of apoptotic cells of intermediate value between that of A-T homozygote and wild-type cells when treated with X-IR (Shigeta et al. 1999) and γ-IR (Bebb et al. 2001). This raised concern whether the resistance to IR-induced cell death observed in the A-T heterozygote cell line (Het 1), and the necrotic cell death displayed by the A-T homozygote and control cell lines was cell line specific. In order to address some of these issues, higher radiation doses were employed, and the response of the A-T heterozygote cell line was compared to other A-T heterozygote cell lines under the same experimental conditions.

4.2.2 The effect of medium to high-dose γ-irradiation on human lymphoblastoid cells.

Further investigations with elevated radiation doses (30Gy) revealed that the same lymphoblastoid cells continued to exhibit a necrotic cell death profile in response to radiation exposure, and that the appearance of necrotic cells was only exhibited from 8 hours post-irradiation (Fig 3.2.6). At this time point, the level of necrosis was higher in the control (C5) cell line, but at a later time point (24h), the A-T homozygote (A-T1) cell line exhibited more necrotic cells than the control. Although the cell death profile demonstrated by the A-T homozygote (A-T1) does not agree with previously published data, the fact that A-T cells often show a delayed and/or sub optimal response to irradiation, (Siliciano et al. 1997; Saito et al. 2003) has been demonstrated with this cell line. The A-T heterozygote Het 1 however, remained resistant to irradiation-induced cell death under these experimental conditions.

Researchers at Johns Hopkins Kimmel Cancer Centre treated colon and prostate cancer cells with low dose and high dose radiation exposures. They found that ATM activation was reduced by 40-50% in cells treated with the low dose radiation, and this “suppression” of DNA-damage induced activation of ATM resulted in more cells being killed than was observed in cells receiving high dose radiation. They postulate that small amounts of DNA damage are able to go unrecognised by ATM and that at higher doses of radiation, the
damage is so severe that ATM is activated to preserve as many of the cells as possible by repairing damaged DNA, thereby aiding the survival of cancer cells. The absence of ATM in the A-T homozygotes and reduced ATM in the A-T heterozygotes coupled with the low-dose radiation utilised may explain why an alternative cell death profile was exhibited in these cells compared to Jurkat cells, which express ATM protein (Gately et al. 1998). However, this does not explain why control lymphoblastoid cells exhibited a necrotic rather than apoptotic cell death profile. The lymphoblastoid cells were transformed using SV40 virus. The large T antigen of SV40 binds to and inactivates p53 (Dobbelstein and Roth, 1998), so in this cell type, the control cells were lacking the activity of the major substrate of ATM required for induction of apoptosis. Jurkat cells were derived from a patient with T cell leukaemia, and so have not been subjected to this transformation process, which may further explain why Jurkat cells were able to undergo apoptosis whereas the lymphoblast cells were only able to undergo necrosis in response to radiation exposure at these doses. Additionally, gamma radiation has been shown to induce cell cycle arrest in G1 and up regulate the death receptor Fas in MCF-7 breast carcinoma cells expressing wild-type p53. Cell cycle arrest and up regulation of Fas was abrogated in cells with mutated p53 or in cells not expressing p53 (Sheard et al. 1998). This highlights the importance of activation of p53 in the induction of apoptosis, and would also explain why apoptosis was only seen in control Jurkat cells, as this was the only cell line with both functional ATM and p53. However, a p53-independent pathway has been reported involving the p53 related protein p73, the checkpoint kinases Chk1 and Chk2 and the transcription factor E2F1 in response to cytotoxic drugs (Urist et al. 2004). Interestingly analysing cell death profiles at a later time point post irradiation (24h) and increasing the level of radiation exposure (60Gy), which is far in excess of therapeutic doses (Professor M.A Hannan, personal communication) an apoptotic cell death profile was induced in all the cell lines whether they were A-T homozygotes, A-T heterozygotes or control SV40 transformed cells. The demonstration of an apoptotic cell death profile under these experimental conditions may be due to the activation of other proteins that participate in this pathway, or may be due to induction of the p53-independent apoptotic pathway discussed previously.

The results also indicated there was no homogeneity within the A-T homozygote or control genotypes. Based on these results utilising two A-T homozygote, five A-T heterozygote and five control SV40 transformed lymphoblastoid cell lines, γ-irradiation was not considered to be an appropriate apoptosis-stimulating agent for use in a screening assay to
discriminate A-T heterozygote cell lines from A-T homozygote and control lymphoblastoid cell lines.

4.2.3 The effect of γ-irradiation on untransformed fibroblast cells compared to SV40 transformed lymphoblastoid cells.

An A-T heterozygote cell line (GM08389) had been included to represent the response of untransformed fibroblast cells to γ-irradiation, but even at 60Gy, this cell line was highly resistant to radiation-induced apoptosis (Fig 3.2.9). In fact, a number of A-T homozygote (GM03487) and A-T heterozygote (GM03489, GM08387, GM08389, GM03396, GM03397) untransformed fibroblast cell lines had been exposed to 10-30Gy IR for 0-48h, but even the highest radiation dose failed to elicit a response in these cell lines, and cell death remained under 5% regardless of the level of radiation exposure (data not shown).

In response to DNA double strand breaks, gamma-H2AX (γ-H2AX) which is the phosphorylated form of histone H2AX, functions in retaining DNA repair complexes at sites of DNA double strand breaks (Friesner et al. 2005). This focus formation is therefore used to measure the numbers of DNA strand breaks produced in response to IR. A study conducted on the same untransformed fibroblast cells as used in the current research as well as Epstein-Barr virus transformed cells from the same donors used a single acute high dose rate of 1 Gy or a continuous low dose rate exposure (accumulated dose = 2.4Gy) of radiation and monitored focus formation (Kato et al. 2006). Following 24h of low dose rate γ-irradiation, the number of foci/cell was highest in the A-T homozygote fibroblast cells and A-T heterozygote cells showed intermediate numbers of foci/cell compared with A-T homozygote and control cells. The same level of discrimination between the three genotypes was exhibited by the EBV transformed B-lymphocyte cell lines. However, when the numbers of foci were compared in the untransformed fibroblasts to EBV transformed B lymphocytes from the same donors, the numbers of foci formed in the lymphoblast cells were considerably lower to those observed in the fibroblast cells, demonstrating different cellular responses in these cell types. In comparing the numbers of foci that were still present at 24h following the single dose of radiation, which was the sampling time of cells irradiated with low dose rate γ-irradiation, the pooled data for the A-T homozygotes, A-T heterozygotes and controls showed that less foci were present following the single high dose when compared to the continuous low dose radiation. This may therefore indicate that the administration of a single acute dose of radiation has less effect on inducing double
strand breaks than a prolonged low dose of radiation, and may account for the lack of large numbers of cells exhibiting cell death profiles in the current research, which were exposed to single doses of radiation over a short time. However, it should be noted, the authors did recognise that the experimental procedures were different for the fibroblast and lymphocyte cell lines used, but in addition to this, the single dose of radiation administered was 1 Gy, while the cumulative dose of the low dose rate radiation was 2.4 Gy. The difference in radiation doses administered could therefore also account for the differences in foci formation between the high and low dose rate responses. It has also been reported that the amount of \( \gamma \)-H2AX foci formed in irradiated patient blood samples was different despite the samples receiving the same dose of irradiation, and also that the number of foci formed did not correlate with the numbers of DNA strand breaks measured by the comet assay, which suggested that different numbers of foci were produced in response to DNA strand breaks (Ismail et al. 2007). Under these circumstances, whether the numbers of foci and therefore DNA double strand breaks are sufficient to induce cell death pathways is unclear, but would be an interesting project for further research.

Clonogenic survival assays using fibroblast cells from the same A-T families that were used to generate the results in the current research, have previously been demonstrated (Kinsella et al. 1982) by measuring \( D_0 \) values (the dose of radiation required to reduce survival to 37%). This was the method of evaluation originally proposed by Puck and Marcus (1956), and seems to have been adopted as a standard measurement on observing clonogenic survival data in the public domain. Interestingly, Kinsella et al. (1982) did not find that the A-T heterozygote cell lines were more sensitive to cell killing when compared to control cells. However, the data did demonstrate that A-T homozygote cell lines were more sensitive to radiation than A-T heterozygote and control cells, as the \( D_0 \) value for these cell lines was approximately 50% of the dose required to reduce survival rates to 37% in the A-T heterozygote and normal cells. The fact that the clonogenic survival rates demonstrated in the A-T heterozygotes were similar to those observed for controls (Kinsella et al. 1982), suggests that the ability to repair DNA strand breaks in these cell lines may have been similar, and furthermore, that the lack of radiation induced cell death in the fibroblast cells may be due to cell type rather than genotype-specific effects.

Interestingly, a study carried out by Duchaud et al. (1996) used the same cell lines as used in the Kinsella et al. (1982) study, and consistent with the clonogenic survival data,
demonstrated that the EBV transformed A-T homozygote cells exhibited less apoptosis than the control, but the A-T heterozygote EBV transformed cell lines produced similar numbers of apoptotic cells to the control cells. The cell lines used in both these studies were generated from the same donors that generated the untransformed fibroblast cells in the current research, and which demonstrated a strong resistance to γ-radiation induced apoptosis. Probably the most important result of the Duchaud et al. (1996) study was their use of the same donor generated A-T homozygote untransformed fibroblast cells and EBV transformed B lymphocyte cells, as were used in the current research thesis. In agreement with the current research, Duchaud et al. (1996) found that A-T homozygote (GM03487, also known as AT4Be) did not undergo apoptosis in response to γ-irradiation, whereas SV40 transformed fibroblasts (AT5BI) did undergo apoptosis. An additional study carried out by Bebb et al. (2001) used Epstein-Barr Virus (EBV) transformed B lymphocytes that were generated from the same donors that generated the untransformed A-T homozygote and heterozygote fibroblast cell lines used in the current research. An increase in apoptosis was generated in response to 1.6-12.8 Gy γ-irradiation in the A-T homozygote; A-T heterozygote and control EBV transformed cells, measured using the hypodiploid method and TUNEL analysis. The hypodiploid and TUNEL methods are used to detect apoptosis by measuring the DNA content or the presence of nicked DNA respectively. The hypodiploid method is based on a method by Nicoletti et al. 1991, and measures decreases in DNA content of the cells to detect apoptosis, as cells with normal DNA content show G1-M-G2 cell cycle peaks on histograms measured by flow cytometry. However, this method does not work equally well in all cell lines. For example MCF-7 cells are deficient in caspase-3 (Smith et al. 1999), which is required to cleave ICAD so that CAD can enter the nucleus and degrade the DNA, and so would not be an appropriate cell line to use with the hypodiploid method. The TUNEL assay also measures apoptosis by detecting DNA fragmentation at “nicked” sites. In this instance, an enzyme (terminal deoxynucleotidyl transferase) adds labelled dUTPs to the nicked DNA ends (Negoescu et al. 1996; 1998). In the current research, the assay used to measure apoptosis relies on the externalisation of PS, which is a much earlier event in the apoptosis pathway compared to DNA degradation. The lack of response demonstrated by the untransformed fibroblast cells in the current study using annexin-v and PI could therefore be due to measuring apoptosis using an earlier event than DNA fragmentation. Additionally, the lack of response could also be due to the untransformed nature of the cells. Bebb et al. (2001) also treated primary PBLs under the same experimental conditions as the EBV-transformed cell lines. The level of apoptosis in
PBLs did not differ significantly between the three genotypes. The authors concluded that EBV transformed cells would be useful for discriminating A-T heterozygote cells from A-T homozygotes and controls, but this was not able to be demonstrated in PBLs. Although the cell death profiles demonstrated for the SV40 lymphoblastoid cells in this study (necrosis) was different to that exhibited by the EBV-transformed cell lines (Bebb et al. 2001) using similar radiation doses, this thesis was also able to induce an apoptotic cell death profile in SV40 transformed cell lines using higher radiation doses (60Gy; Fig 3.3.9), and in this regard, the mechanism of cell death, is in agreement with previously published work (Bebb et al. 2001). This thesis was not able to reliably discriminate all the A-T heterozygote cell lines from A-T homozygote and control cells, but the current study used considerably more A-T heterozygote cell lines than were used in the work carried out by Bebb et al. (2001). However, this inability to discriminate all of the A-T heterozygote cell lines was not based on the lack of homogeneity in the A-T heterozygote cell lines, but rather the lack of homogeneity within the A-T homozygote and control genotypes.

It is interesting to note that the cell lines which permitted discrimination of A-T heterozygote cells in both the current study and that carried out by Bebb et al. (2001) were only transformed cell lines, and that primary or untransformed cell lines were not able to exhibit similar results. There is no question that the ATM status of the cell has a contribution to make in the discrimination of A-T heterozygote from A-T homozygote and control cells, but it seems as though transformation of the cells also has a contribution to this process, as without the transformation process, the three genotypes exhibit a similar response to IR (Bebb et al. 2001) or do not respond at all to IR, which was demonstrated in the current study. The Duchaud et al. (1996) study not only demonstrated the same results as those presented in the current research with the same cell line, but also serves to highlight the contribution the transformation process has on the mechanism of cell death in these cell lines. Other researchers have also reported that untransformed A-T and control cell lines do not show an increase in apoptotic cells following exposure to IR (Foray et al. 1997; Enns et al. 1998).

The transcription factor NF-κB is a member of the Rel protein family. In its inactive form it is located in the cytoplasm bound by IκBs, a family of inhibitory proteins, which mask the nuclear localisation signal. A variety of stimuli cause degradation of IκB, which then frees NF-κB, allowing it to translocate to the nucleus, where it regulates gene transcription.
(Karin and Ben-Neriah, 2000). NF-κB can act as an apoptosis inhibitor or promoter in different cell types to a diverse range of stimuli (Barkett and Gilmore, 1999). NF-κB has been shown to induce Fas ligand in response to DNA damaging agents in T lymphocytes (Kasibhatla et al. 1998). NF-κB and p53 can prevent normal expression of each other through binding with the CBP/p300 pathway (Ravi et al. 1998b; Wadgaonkar et al. 1999; Webster and Perkins, 1999), and RelA (part of the NF-κB complex) can block apoptosis induced by 53BP2, a protein that binds p53 (Yang et al. 1999). The inhibitory protein IκBα has been shown to be a target for caspase-3, and has been shown to be cleaved in the amino terminal in A-T cells exposed to γ-radiation (Jung et al. 1998) and in a myeloid cell line (32D) treated with TNFα or by IL-3 deprivation (Reuther and Baldwin, 1999). IκBα cleavage by caspase 3 has also been demonstrated in ν-Rel-transformed cells induced to undergo apoptosis (Barkett et al., 1997; White and Gilmore, 1996; White et al. 1995). NF-κB can promote the development of tumours by activating anti-apoptotic genes such as BclXL and Bfl-1/A1 thereby preventing cell death (Chen et al. 2000). NF-κB is activated by a plethora of diverse stimuli including γ-radiation (Brach et al. 1991a), by oxidative stress caused by HP (Svreck et al. 1991), and by the chemotherapeutic agents doxorubicin (Das and White, 1997) and etoposide (Bessho et al. 1994), as well as viruses used to transform cell lines such as SV40 (Kanno et al. 1989) and EBV (Sugano et al. 1997). NF-κB, which has been shown to protect cells from irradiation-induced apoptosis (Wang et al. 1996), was found to be expressed at constitutively high levels in an SV40 transformed A-T fibroblast cell line, and that the same cell line expressed high levels of IκBa, a gene that is controlled by NF-κB (Jung et al. 1995) In a separate study, basal levels of NF-κB were found to be significantly higher in an SV40 transformed cell line compared to the untransformed cell line from which the transformed cell line was generated (Ashburner et al. 1999). It would therefore appear that the SV40 transformation process has an effect on the expression level of NF-κB in both these studies, and may offer a further explanation for the difference in cell death profiles observed in SV40 transformed and untransformed cell lines.

The sphingomyelin pathway can be activated by many agents including radiation, etoposide and doxorubicin (reviewed in Modrak et al. 2006). Activation of this pathway involves the hydrolysis of sphingomyelin to produce ceramide and phosphocholine. Elevated ceramide results in the activation of various intracellular signalling molecules including ceramide-activated protein (CAP) kinase (Mathias et al. 1991; Zhang and
Kolesnicky (1995), mitogen-activated protein (MAP) kinase (Raines et al. 1993), Raf-1 (Belka et al. 1995), stress-activated/Jun protein kinase (Sanchez et al. 1994), the inhibitor of κB (Henkel et al. 1993), and c-myc (Hermeking and Eick, 1994). Translocation of NF-κB from the cytoplasm to the nucleus has also been observed (Schutze et al. 1992; Yang et al. 1993). Ionising radiation has been shown to induce hydrolysis of sphingomyelin to generate ceramide in bovine aortic endothelial cells, initiating apoptosis. These experimental results were generated from experiments on cell extracts that did not contain nuclei, and so demonstrate that IR can initiate apoptosis in the membranes of these cells independent of DNA damage (Haimovitz-Friedman et al 1994).
Figure 4.3 Schematic diagram showing various apoptotic/necrotic and cell survival pathways.

The sphingomyelin pathway activation signal has not been elucidated for IR, but it has been suggested that ROS produced by IR may play a role in this activation signal (Haimovitz-Friedman et al. 1994). The generation of ceramide within the cell has been
proposed to induce a necrotic cell death profile when activation of caspase-8 is not sufficient to activate caspase-3 during apoptosis, and that the late accumulation of ceramide under these circumstances induces necrosis in such cells that have not already been induced to undergo apoptosis (Hetz, et al. 2002). It has been suggested that the absence of ATM protein in A-T patients renders the cells in a continuous state of oxidative stress (Rotman and Shiloh, 1997a; 1997b). If this is indeed the case, and ROS are able to activate the sphingomyelin pathway, this may explain the necrotic cell death profiles exhibited by the SV40 transformed cell lines at low radiation doses, as the level of ROS generated at these radiation levels may not be sufficient to induce the activation of caspase-3.

An additional factor, which may explain the necrotic cell death profile of SV40 transformed cell lines in response to low dose γ-irradiation, is the mechanism of viral transformation of the cell lines. The large T antigen of SV40 binds to and inactivates p53 (Dobbelstein and Roth, 1998). p53 is a key tumour suppressor protein in the apoptosis cascade in response to IR. A study conducted by Yu and Little (1998) on cells with wild-type p53 (TK6 cells), mutated p53 (WTK1 cells) or abrogated p53 (TK6) exposed to 4Gy γ-irradiation, reported that wild type cells exhibited rapid apoptosis, whereas the apoptotic response was delayed and reduced in the cells with mutated or abrogated p53 protein. The level of apoptosis in these cell lines correlated with caspase-3 activation. An early and late phase of caspase-3 activation was exhibited in wild type cells, whereas caspase-3 activation was delayed in cells with mutated or abrogated p53. Inactivation of p53 by SV40 virus may also lead to late activation of caspase-3, and in accordance with the results of Hetz, et al. (2002) may lead to a necrotic rather than apoptotic cell death profile as demonstrated in the data for the transformed cells.

The lack of radiation-induced cell death in the fibroblasts may also be considered in terms of the relevance of the inducing stimulus in treating malignancies that affect white blood cells and skin cells. Cancers of the lymphoid cells such as lymphomas, myelomas or leukaemias usually respond to radiation therapy, whereas squamous cell carcinoma which is a form of skin cancer is not as responsive to this type of treatment, and melanoma, another type of skin cancer has been shown to be quite resistant to this therapy (Fertil and Malaise, 1985). The reduced/resistant response of skin cancer cells to radiation therapy could therefore explain why γ-irradiation was successful in inducing cell death profiles in lymphoblasts but unsuccessful in inducing cell death profiles in fibroblasts.
It is difficult to interpret whether the SV40 transformed lymphoblast cells or the untransformed fibroblast cells more closely resemble the cellular response to IR in vivo, but the observation that A-T patients, when treated with conventional doses of radiotherapy suffer massive tissue necrosis (Gotoff et al. 1967; Cunliffe et al. 1975), suggests that the results obtained with the transformed cells most closely resemble the response of A-T patients to IR. These results therefore have important implications for both A-T homozygote and A-T heterozygote patients, as tumours linked with a necrotic profile rather than an apoptotic profile are usually more aggressive and generally have a poor prognosis (Edwards et al. 2003; Langner et al. 2003; Chang et al. 2006; Sang et al. 2006). For example, in retinoblastoma patients, extensive ocular tissue and tumour necrosis is associated with high-risk prognosis for development of both metastasis and mortality (Chang et al. 2006). Tumour necrosis was identified in 42.2% of patients with upper urinary tract transitional cell carcinoma. The 5-year metastasis free survival rates were 24% for extensive tumour necrosis, 45% for focal tumour necrosis, and almost double (78%) in patients who had tumours without necrosis. Tumour necrosis was also associated with high tumour stage and grade and poor prognosis (Langner et al. 2003). Macroscopic tumour necrosis was identified in 27% of renal cell carcinoma patients. These patients were found more likely to have a larger tumour, a higher local stage and higher tumour grade and metastatic disease. Pathological features of microvascular invasion and sarcomatoid differentiation were also observed (Sang et al. 2006). Additionally, data derived from murine models suggests the suppression of apoptotic cell death is associated with tumour progression (Naik et al. 1996; Cory et al. 1999). The ability to reliably detect the mechanism of cell death in response to radiation therapy in these patients at an early time after treatment may provide valuable information for treatment options, may serve as an early diagnostic and prognostic indicator, and also may be a valuable asset in monitoring patient treatment regimes.

4.3 Chemical treatment of human cell lines using hydrogen peroxide.

Hydrogen peroxide (HP) is one of the intermediates formed by the reduction of molecular oxygen by the addition of electrons from a variety of biological sources, and is one of the products generated during normal cellular metabolism (Gutteridge, 1994). HP can react with iron ions and other redox active transitional metals to produce hydroxyl radicals that have the potential to damage lipids, amino acids in proteins, carbohydrates and DNA (Byung, 1994). It has been reported that the predominant effect of HP on DNA is the
generation of single strand breaks (Benítez-Bribiesca and Sánchez-Suárez, 1999). In a study conducted on PBLs treated with 10-500μM HP for 5 minutes, the increase in single strand breaks was found to be dose dependent, and at the highest HP concentration (500μM) there was a 96% increase in single strand breaks, and a 19% increase in double strand breaks (Benítez-Bribiesca and Sánchez-Suárez, 1999). However, A-T cells have been shown to be capable of repairing single (Taylor et al. 1975; Vincent et al. 1975; Paterson et al. 1976) and double DNA strand breaks induced by radiation and bleomycin as effectively as wild type cells (Lehmann and Stevens, 1979). Both of these agents can also produce ROS including HP that interact with DNA causing single and double strand breaks (Riley, 1994; Mahmutoglu et al. 1987).

Figure 4.4 Overview of ROS metabolism. (from Nindl, 2004)
Reactive oxygen species (ROS) (yellow) are continuously formed as a by-product of normal cellular metabolism, and are also produced to act as cellular regulators (blue). ROS are removed by anti-oxidants, which protect against accumulation of ROS in the body (red). The effect of ROS on the body can be both beneficial and unbeneificial (green).
There are several enzymes that function in detoxifying ROS, but under circumstances where these mechanisms are overwhelmed, the cells enter a state of oxidative stress (Gutteridge, 1994). It has been suggested that the absence of ATM protein in A-T patients renders the cells in a continuous state of oxidative stress, especially neuronal cells, and this has been proposed as the mechanism responsible for neuronal degeneration in these patients (Rotman and Shiloh, 1997a; 1997b; Kamsler et al. 2001). Increased oxidative stress has been demonstrated in the cerebellum of ATM null mice, which is the main area of the central nervous system affected in A-T patients (Kamsler et al. 2001). Based on these significant observations HP was considered to be a valuable compound to artificially induce oxidative stress in A-T cells and its use was used to monitor the effect of ROS on the cell death profiles.

4.3.1 The effect of hydrogen peroxide (100μM) on human lymphoblastoid cell lines and HL-60 cells.

HL-60 cells are a human promyelocytic leukaemia cell line that are used in studying apoptosis (datasheets from: Abcam, 2007), and were used to generate data in this section of the thesis for comparative studies with the lymphoblastoid cell lines. HL-60 cells exhibited a similar cell death profile (apoptotic and necrotic cells) to the control lymphoblastoid cell lines (Fig 3.3.2), but these results are in disagreement with previously published results demonstrating that 14.5-30.8% apoptotic cells were generated in response to 100μM HP treatment (2-6h), with an absence of necrotic cells (Tyurina et al. 2004). The increased numbers of apoptotic and necrotic cells demonstrated in this study (Fig 3.3.2) in comparison to published data (Tyurina et al. 2004) could be due to differences in (1) annexin-V antibody used in the studies or (2) could be due to the fact that Tyurina et al. (2004) used a rapid binding method for the annexin-v (5 minutes) with only 1 washing step, whereas the present study used longer incubation times with annexin-v and PI and used numerous washing steps, which may have facilitated increased specific binding of annexin-v and PI uptake respectively.

Data generated demonstrated that the A-T heterozygote cell lines could clearly be distinguished from A-T homozygote and control cell lines, based on the cellular response to HP (100μM, 4h treatment; Fig 3.3.2). The A-T heterozygote cell lines exhibited the
highest percentage of necrotic cells (>80%), with relatively few apoptotic cells (<10%). The A-T homozygote cell lines exhibited a larger percentage of apoptotic cells (58-67%) than necrotic cells (31-35%). The control lymphoblastoid cell lines, like the A-T heterozygote cell lines, also exhibited a higher percentage of necrotic cells (52-56%) compared to late apoptotic cells (35-42%), but the number of apoptotic cells in the controls were considerably higher, and the number of necrotic cells were considerably lower than the numbers observed in the A-T heterozygote cell lines. Statistical analysis performed on the results of SV40 transformed cell lines treated with 100μM HP for 4 hours (Fig 3.3.2) demonstrated a significant increase in late apoptotic cells in the A-T homozygote cells compared to controls (P <0.05 one-way ANOVA), and a significant reduction in necrotic cells in the A-T homozygote cells compared to controls (P <0.05 one-way ANOVA). Statistical analysis also demonstrated that the reduction in late apoptotic cells in A-T heterozygote cells compared to both A-T homozygote and control cells was significant (P <0.05 one-way ANOVA), as was the increase in necrotic cells in the A-T heterozygote cells compared to both the A-T homozygote and control cells (P <0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes.

Having established that it was possible to reliably discriminate between the three genotypes when the cells were treated with HP for four hours, this raised two questions. Firstly, was this phenomenon time dependent at this concentration, and secondly, were these observations cell line or cell type specific. To address these issues, the same lymphoblastoid cell lines as used in Fig 3.3.2 were treated with the same dose of HP (100μM), but were treated for an extended time period (6 hours). To address the issue of whether the observations were cell line or cell type specific, additional untransformed A-T homozygote (GM03487) and A-T heterozygote (GM03489, GM08387, GM08389, GM03396) fibroblast cell lines were also included here. Additionally, a telomerase transformed control (MRC-T) fibroblast cell line was also utilised to determine if the transformation process would induce a similar cell death profile to virus (SV40) transformed cells or whether the cell line would produce a similar cell death profile to untransformed fibroblast cells. The untransformed fibroblast cell lines would ideally have included at least one control of the same cell type, however, the inability of these cells to grow impacted on this scenario. Rather than excluding potential data that could be generated by the A-T homozygote and heterozygote cell lines due to the absence of a similar control, it was decided to include these cell lines to evaluate the response of
untransformed A-T homozygote and heterozygote cell lines compared to their SV40 transformed lymphoblastoid counterparts. On this basis it was decided that inclusion of the fibroblast cell lines in the study might still provide valuable information.

Extending the study by two hours and inclusion of additional cell lines (Fig 3.3.3) confirmed the initial findings with the lymphoblastoid cells and helped firmly establish the observation that A-T heterozygote cells could be discriminated, on the basis of their apoptotic and necrotic profiles when compared to A-T homozygotes or controls. However, data confirmed that it was not possible to differentiate between A-T homozygotes and controls under these experimental conditions, as both the A-T heterozygote cell lines exhibited similar cell death profiles to those observed for the control cell lines. Jurkat cells, which are a human T cell lymphoblast-like cell line that have been previously used in studying apoptosis (datasheets from: Abcam, 2007) exhibited a different cell death profile to the SV40 transformed lymphoblast cell lines in that they only produced late apoptotic cells in response to the same experimental conditions (Fig 3.3.3). However, these results show similarities with previously reported data in that HP-induced apoptosis (25%) was demonstrated in Jurkat cells using 50μM HP for 6 hours (Hampton and Orrenius, 1997).

In the current study, 100μM HP induced 80% late apoptotic cells in the Jurkat cell line. The difference in numbers of apoptotic cells recorded between the two studies would be expected due to twice the HP concentration being used in the current research, but the results of the Hampton and Orrenius study (1997) serve to highlight that the same cell death profile was generated in both studies.

4.3.2 The effect of hydrogen peroxide (100μM) on human fibroblast cell lines.

In stark contrast to the lymphoblastoid cell lines, the fibroblasts exhibited an apoptotic cell death profile (Fig. 3.3.3). This profile predominantly consisted of early apoptotic cells, while lymphoblastoid cell lines produced late apoptotic and necrotic cells under the same experimental conditions. The fibroblast cells produced relatively few necrotic cells (<10%) and much larger numbers of both early (15-75%) and late (9-35%) apoptotic cells. Although the mechanism of cell death for the A-T homozygote and heterozygote untransformed fibroblast cell lines and the telomerase transformed control was the same, it was possible to discriminate each of the three genotypes based on the percentage of cells undergoing apoptosis. Apoptosis was determined to be highest in A-T heterozygotes, intermediate in an A-T homozygote cell line and lowest in the telomerase-transformed
control. Statistical analysis demonstrated the level of early apoptotic cells in the A-T homozygote cell line was significantly higher than the control (P < 0.05 one-way ANOVA), as was total apoptotic cell death (early and late apoptotic cells: P < 0.05 one-way ANOVA). The level of early apoptotic cells was also demonstrated to be significantly increased in A-T heterozygote cell lines compared to both the A-T homozygote and control cell lines (P < 0.05 one-way ANOVA), as was the total apoptotic cell death using these cell lines (P < 0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes.

The most striking observation with the untransformed fibroblast cells was the amount of apoptotic cell death exhibited by the A-T heterozygote cell lines (GM08387, GM08389, GM03396). These cell lines produced significant numbers (≥68%) of both early and late apoptotic cells. Previously, these cell lines had been exposed to gamma radiation doses as high as 30Gy, and when assayed over 48 hours showed considerable resistance to γ-irradiation-induced cell death (data not shown). Based on these combined results, it is clear that the fibroblasts were capable of initiating an apoptotic response, but that the inducing stimulus was an important factor in the type of response shown. Interestingly, ionising radiation can induce both single and double strand breaks (Benítez-Bribiesca and Sánchez-Suárez, 1999; Pernin et al. 1999; Ismail et al. 2005). The fibroblasts proved resistant to this form of DNA damage. It has been demonstrated that HP predominantly induces single strand breaks (Benítez-Bribiesca, and Sánchez-Suárez, 1999; Ismail et al. 2005) and the fibroblasts responded to this type of DNA damage. Based on a study conducted by Ismail et al. (2005), 10Gy γ-radiation generated 200-500 double strand breaks/cell. The maximum radiation dose used with the fibroblasts (60Gy) in the current research may therefore generate as many as 3000 double strand breaks/cell. However, in the same study, no double strand breaks were detected for hydrogen peroxide treatment up to 3mM, and from their figures, the HP concentration used with the fibroblast cells (100μM) in the current study would be predicted to induce 13,333 single strand breaks/cell. The Ismail et al. (2005) study also demonstrated that ATM was only activated in response to double strand breaks, and that the amount of ATM activation correlated with the amount of double strand breaks generated. Additionally, the ATM substrates Chk2, H2AX and SMC1 were activated in response to IR but not HP treatment. They also demonstrated that PARP was activated in response to HP treatment indicating that the single strand breaks were detected in the cells. PARP is cleaved by caspase 3 during apoptosis, which at least suggests that the apoptosis
detected in the fibroblast cells in response to HP treatment, might be driven by caspase activation. Since ATM and its substrates were activated specifically in response to double strand breaks induced by IR (Ismail et al. 2005), the lack of response to IR in the fibroblast cells may be due to the cells carrying at least one ATM mutation, and so ATM-dependent cell death was not initiated, and the difference compared to the SV40 transformed cells can be explained due to viral transformation of the cells, as previously demonstrated by Duchaud et al. (1996) and Bebb et al. (2001), as discussed previously. The demonstration that ATM and ATM-dependent activation of Chk-1, H2AX and SMC1 does not occur in response to HP treatment (Ismail et al. 2005), and that HP induced an apoptotic cell death profile in A-T homozygote, A-T heterozygote and telomerase transformed control fibroblast cell lines in the current research, therefore suggests that an ATM-independent pathway was activated in these cell lines.

It has previously been demonstrated that HP induces the binding of protein kinase Cδ (PKCδ) and c-Abl, and that PKCδ is phosphorylated by c-Abl in HP treated cells (Sun et al. 2000; Kumar et al. 2001). However, PKCδ can also phosphorylate c-Abl, indicating a possible feedback loop (Sun et al. 2000). HP-induced activation of PKCδ resulted in translocation of PKCδ from the cytoplasm into the mitochondria. This translocation was associated with the loss of mitochondrial transmembrane potential, the release of cytochrome c, and apoptosis (Majumder et al. 2001). HP treatment of cells has also been demonstrated to cause c-Abl to translocate from the cytoplasm to the mitochondria in addition to PKCδ, and the translocation was found to be mediated by PKCδ. This event was again associated with the loss of mitochondrial transmembrane potential, but in this instance ATP was depleted, which suggested a necrotic cell death profile (Kumar et al. 2001). However, the demonstration of apoptosis in MCF-7 cells (1mM HP; Majumder et al. 2001) and necrosis in mouse embryo fibroblasts (MEF; 1mM HP; Kumar et al. 2001), suggests that the cell death profile induced may be dependent on cell type. In response to IR, ATM has been reported to phosphorylate c-Abl (a proto-oncogene) on serine 465, activating its tyrosine kinase activity (Baskaran et al. 1997). However, the involvement of ATM in response to HP treatment is unlikely as cytoplasmic pools of both c-Abl and PKCδ were translocated to the mitochondria (Majumder et al. 2001) and c-Abl was found to phosphorylate PKCδ on Tyr 512 (Sun et al. 2000). In the same study, PKCδ was also shown to be phosphorylated at Tyr 523, by a yet unknown tyrosine kinase other than c-Abl, and as ATM is a serine/threonine protein kinase, it is therefore unlikely that ATM
participates in this process. Further evidence to support this hypothesis was reported by Ismail et al. (2005) who demonstrated that ATM was activated in response to IR, but not by HP treatment. These events would therefore offer an explanation as to how an apoptotic cell death profile was induced in fibroblasts with ATM mutations, and would suggest an interesting area for further research.

Figure 4.5 Schematic diagram showing the proposed model of HP-induced apoptosis.

Excessive oxidation or control of ROS is, in part, balanced by the enzyme glutathione peroxidase, which functions in the detoxification of HP, and in doing so actively protects the cell against oxidative stress induced by HP (Meredith and Dodson, 1987). In mice with
homozygous mutated glutathione peroxidise-1, increased levels of lipid peroxides were found in the liver, and the liver mitochondria were found to release substantially more HP in these animals (Esposito et al. 2000). The glutathione depleting agent diethylmaleate (DEM; Castillo et al. 2002) was used to treat A-T homozygote, A-T heterozygote and control fibroblast and lymphoblast cells, and the re-synthesis rates of GSH (reduced glutathione) were measured (Meredith and Dodson, 1987). GSH was repleted in normal cells by 6h, in A-T heterozygote cells at 18h, but at 24h the levels of GSH in A-T homozygote cells had only reached 30%. The sub-optimal and delayed re-synthesis and replenishment of GSH in A-T homozygote cells was found to be due to a defect in cystein transport (Meredith and Dodson, 1987). Interestingly, one A-T homozygote (GM03487) and one A-T heterozygote (GM03489) used in the Meredith and Dodson (1987) study were also used to generate the HP data here. The heterozygote cell line (GM03489) was generated from the parent of an A-T patient (cell line GM03487). As the repletion of GSH in the A-T heterozygote was more efficient than in the A-T homozygote cell line (Meredith and Dodson, 1987), it would be reasonable to expect that more HP-induced DNA damage would be induced in the A-T homozygote cell line. However, in the current study, HP treatment of these cells induced 42% apoptotic cells in the A-T homozygote cell line and considerably more (75%) apoptotic cells in the A-T heterozygote cell line. A-T cells often show cell responses that are sub-optimal and/or delayed, as demonstrated in the rate at which intracellular GSH was re-instated (Meredith and Dodson, 1987), and the slower rate at which these events seem to occur in A-T cells may also effect the way in which the cells process physiological and non-physiological molecules, which may directly effect the rate at which damage is induced, detected, and repaired, and also in the response to DNA-damage induced apoptosis in these cells. The slower kinetics of these events would generate a model, which encompassed the results generated in response to HP. It has been demonstrated that extrusion of GSH from the cell takes place during apoptotic signalling (Ghibelli et al. 1995; Van den Dobbelsteen et al. 1996), and furthermore, GSH extrusion has been shown to be necessary in order for downstream apoptotic events to be triggered (Ghibelli et al. 1998). The grossly delayed replacement of GSH following treatment with DEM in A-T homozygote cells may therefore result in GSH extrusion occurring at a later time than that observed in the A-T heterozygote cells, and as this event has been demonstrated as a pre-requisite for downstream apoptotic events to be triggered (Ghibelli et al. 1998), would also explain why far fewer apoptotic cells were observed in the A-T homozygote cultures compared to A-T heterozygote cells (Fig 3.4.2).
The control MRC-T fibroblast cell line was also treated with HP. MRC-T was generated by the introduction of hTERT (human telomerase reverse transcriptase) into MRC-5 fibroblasts. The MRC-5 cells are a normal human diploid fibroblast cell line that is well characterised as they have been used in research as a standard for over 30 years (McSharry et al. 2001). These cells are only able to perform up to 46 population doublings before the onset of replicative cellular senescence. In fibroblasts, this is caused by the erosion of telomeres. Introduction of hTERT therefore allows the cells to avoid senescence and replicate indefinitely (Bodnar et al. 1998), while not conferring changes associated with malignancy (McSharry et al. 2001). The cells remain karyotypically normal, retain cell cycle checkpoints (Jiang et al. 1999; Morales et al. 1999) and retain the morphology of younger cells (McSharry et al. 2001). The telomerase transformed cell line was included to ascertain if the transformation process would be a dominant factor in the cellular response to HP treatment and produce a similar cell death profile to SV40 transformed cells, or whether this cell line would exhibit a similar cell death profile to untransformed fibroblast cells. The data indicated that telomerase transformation did not induce a similar cell death profile to SV40 transformed lymphoblast cells, as the cell death profile was demonstrated to be the same in the MRC-T cells as the other fibroblasts used in the study, although the MRC-T cell line exhibited less apoptotic cells than the other cell lines. The data therefore suggest that telomerase transformation did not affect the way in which the cells responded to HP treatment, which is in contrast to the way in which EBV or SV40 viral transformation produces very different cell death profiles to primary lymphocytes (discussed earlier). The different cell death profiles exhibited by the fibroblasts and SV40 transformed lymphoblast cells were more likely to be due to cell type specific events in this instance. The results for the MRC-T cell line therefore suggest that telomerase transformation of cell lines may be more useful in studying cellular responses to genotoxic agents, as the telomerase transformation seems to more closely resemble the response of untransformed cells than viral transformation of cell lines, and could prove to be a valuable diagnostic tool in future research.

Results using HL-60 cells (Fig 3.3.1) indicated that at lower doses (10-50μM), and the earliest time point (2h), HL-60 cells predominantly die by apoptosis. These results are therefore consistent with previously published results by Matsura et al. (1999), who
demonstrated apoptotic cellular morphology in this cell line under similar experimental conditions.

Collectively, the results obtained helped demonstrate that HP would be a valuable cell death inducing stimulus to be included in a panel of DNA damaging agents for use in detecting A-T heterozygote cells, and for the useful purpose of discriminating them from A-T homozygote and normal cells in both fibroblast and lymphoblast cell lineages. The optimum conditions for SV40 transformed lymphoblast cells were determined as 100μM for 4 hours, and the same concentration at 6 hours was determined as the optimum conditions for the identification and discrimination of A-T heterozygote cells of untransformed and telomerase transformed fibroblast origin.

Additionally, the research highlighted the differences in the cellular response to HP between the SV40 transformed lymphoblast cells and untransformed fibroblast cells, which has been previously reported in an SV40 transformed cell line and an untransformed cell line from the same donor (Duchaud et al. 1996), as well as in Epstein-Barr virus transformed cell lines compared to primary lymphocytes (Bebb et al. 2001). This research therefore not only confirms the results of Duchaud et al. (1996) in response to ionising radiation, but also demonstrates the same effect in these cell lines using HP. Data also highlight the importance of the transformation procedure in elucidating the effects of HP since the process of immortalisation is different depending on the mechanism employed.

4.4 Treatment of human cell lines with the anti-cancer drug etoposide.
Etoposide (VP-16) is one of the most frequently used anticancer drugs, and has been in clinical use for over 20 years (Baldwin and Osheroff, 2005). It has a broad anti-tumour spectrum, but has predominantly been used to treat a variety of haematopoietic malignancies, such as leukaemia (Różalski et al. 2005). As many as one-third of A-T patients are capable of developing a malignancy (Morrell, et al. 1986), approximately 85% of which, are lymphomas or leukaemia (Taylor et al. 1996). Etoposide has been used to treat several different types of leukaemia, including acute lymphocytic leukaemia (Fujino et al. 2002), equally common in children and adult A-T patients (Taylor et al. 1996). Etoposide increases the level of topoisomerase II-mediated DNA breaks, and acts by preventing topoisomerase II from rejoining cleaved DNA molecules (Różalski, et al. 2005),
4.4.1 The effect of etoposide (100µM) on human lymphoblast cell lines.

Data generated demonstrated that concentrations of etoposide <100µM failed to induce an appreciable cell death profile in either the SV40 transformed lymphoblast or untransformed fibroblast cells. However, increasing the concentration to 100µM evoked cell death profiles in both cell types (Fig 3.4.4), although the results observed were more pronounced for the lymphoblasts (Fig 3.4.4A) compared to fibroblasts (Fig 3.4.4B). The data generated by the lymphoblasts demonstrate that etoposide induced predominantly necrotic cells in A-T heterozygote cells compared to apoptotic cells in A-T homozygote and control cells, and in this regard, the cell death profiles of the A-T heterozygote cell lines was distinct from the other two genotypes. This phenomenon was also demonstrated using HP (Fig 3.3.2) with A-T heterozygote cell lines. The A-T heterozygote cell line Het 1 was generated from the mother of A-T patient A-T1 [794 ins 4; 2839 del 183], and so has one of two possible mutations. The A-T homozygote (A-T1) exhibited an apoptotic cell death profile, which was also demonstrated in A-T homozygote cell line A-T2 [4388 del T; 7928 del 83]. The necrotic cell death profile of Het 1 was also shared by the other two A-T heterozygote cell lines, Het 4 [2249 ins 9] and Het 5 [822 del T or 7660 c>G]. The necrotic cell death profiles of the A-T heterozygotes cannot therefore be explained in terms of either the position of the mutation within the gene, or the type of mutation present (mutation information from Prof. A.M.R. Taylor, Birmingham University, UK. Personal Communication). The only common factor for the A-T heterozygote cell lines was that each of them had one truncating mutation. A study using EBV transformed cell lines treated with 17µM etoposide for 96h demonstrated the level of apoptosis to be higher in A-T homozygote cells compared to controls and that A-T heterozygotes exhibited intermediate sensitivity to the drug (Pernin et al. 1999). The A-T heterozygote and control cells displayed similar levels of apoptosis within their respective genotypes, however, the level of apoptosis in the A-T homozygote cell lines showed considerable variation. One A-T homozygote cell line produced 20.1 ± 1.9% apoptotic cells, which was within the normal range for control cells, while a different A-T homozygote cell line produced 69.4 ± 1.25% apoptotic cells. These results are not dissimilar to those observed in the current research using SV40 transformed lymphoblastoid cell lines (Fig. 3.4.4A), with a higher etoposide (100µM) concentration over a shorter exposure time (6h). In the current research, One A-T homozygote cell line (A-T1) produced levels of apoptosis that were similar to three of four control cell lines, while another A-T homozygote (A-T2) cell line produced similar numbers of apoptotic cells to an additional control cell line (C2). However, the level of
Apoptosis was demonstrated to be much higher using the EBV transformed cell lines (Pernin et al. 1999), than that demonstrated in the current research using SV40 transformed cells, which further serves to highlight the heterogeneous response between lymphocyte cells using different methods of viral transformation. Pernin et al. (1999) concluded that it was not possible to discriminate A-T heterozygotes using their experimental procedures, because several of the A-T heterozygote cells showed apoptosis values within those generated for the controls. However, in the current research, A-T heterozygote cells could be discriminated from A-T homozygote and control cells, but A-T homozygote and control cells produced similar cell death profiles. Interestingly, Pernin et al. (1999) also treated the same cell lines with 17µM etoposide for 24h, and under these experimental conditions the level of apoptosis was 10-16%, 12-21% and 14-24% in control, A-T heterozygote and A-T homozygote cells respectively, showing considerable overlap in the level of apoptosis detected in the three genotypes, indicating that assay time after treatment is an important factor in assay design. In a recent study (Gurley and Kemp, 2007), A-T homozygous, A-T heterozygous and control mice were irradiated and various tissues were screened for apoptosis at 2, 4 and 10h. In thymus cells, the level of apoptosis was highest in control cells, lowest in A-T homozygote cells and intermediate in A-T heterozygote cells over the 10h assay time period. However, in the small intestine, apoptosis was higher in control cells at 2h, but between 4 and 10h, the level of apoptosis was the same in A-T homozygous and control cells. Although the authors state that this effect was mainly observed in young mice, these results do parallel the results obtained by treating lymphoblastoid cells with 100µM etoposide for 6h, and demonstrate this effect in another cell type. The Gurley and Kemp (2007) study highlights that different tissues from the same animal respond differently to the apoptosis-inducing stimulus, despite them having the same ATM mutation, and that assay time after administration of the apoptosis-inducing stimulus can affect levels of apoptosis, presumably through the involvement of other signalling molecules involved in the apoptosis pathway.

4.4.2 The effect of etoposide (100µM) on human fibroblast cell lines.

The results with the fibroblast cell lines (Fig 3.4.4B) demonstrate that all the cell lines with the exception of the A-T heterozygote cell line GM08387 remained unaffected by the drug indicating that this cell line was more sensitive to the effects of etoposide than any of the other fibroblast cells tested. These results could not be explained in terms of the position of the ATM mutation in these cell lines (Mutation information from Coriell Institute, U.S.A.
Personal Communication). The A-T homozygote GM03487 [1141 ins 4; 8266 A>T] has one mutation in the amino terminal and the other mutation, which it shares with A-T heterozygote GM03489 [8266 A>T] is located in the carboxyl terminal of the protein. The position of the ATM mutation in A-T heterozygote GM03397 [7913 G>A] is also located in the carboxyl terminal of the protein. All the aforementioned cell lines failed to respond to etoposide, and if it was not for the fact that >15% necrotic cells were produced in the A-T heterozygote cell line GM08387 [5932 G>T], the results would suggest that untransformed fibroblast cells were resistant to etoposide-induced apoptosis. GM08387 carries a mutation in the ATM gene within the region that shares sequence homology with RAD3. Interestingly, GM08389 [4642 del 4] also carries a mutation in the same region of the ATM protein, but was resistant to etoposide-induced cell death (Fig 3.3.4b). It is therefore very likely that other factors besides the position of the mutation, such as the type of mutation, and the effect of the mutation on protein stability, protein folding etc. that plays a more prominent role in the sensitivity and/or resistance to etoposide induced cell death.

To monitor whether the previously demonstrated resistance to etoposide-induced cell death in the fibroblast cell lines (Fig 3.4.4B) was time dependent, a prolonged time point (24h) was additionally chosen to analyse any delay/change in cell death profiles. However, increasing the incubation time had no effect on increasing the rate of cell death in these cell lines (Fig 3.4.5). Previous data with the A-T homozygote cell line GM03487 treated with Amsacrine, which, like etoposide is a topoisomerase II inhibitor, reported the cell line was able to rejoin DNA strand breaks following removal of the drug (Kaufmann et al. 1991). The fact that DNA was able to be repaired in this cell line may therefore account for the lack of etoposide-induced cell death in this cell line, but more importantly may reflect a difference in the efficiency with which DNA damage is repaired between untransformed and SV40 transformed cells. Interestingly, two of the A-T heterozygote fibroblast cell lines (GM03489, GM03396) used in this study were from the same donors as EBV immortalised B-lymphocyte (GM03188, GM03334 respectively) cells treated with 17μM etoposide for 24 and 96h (Pernin et al. 1999) and the transformed cell lines exhibited 15 and 12% apoptotic cells respectively. These results therefore demonstrate another instance where transformed lymphocytes respond to DNA-damaging agents (Pernin et al. 1999) whereas fibroblast cells from the same donors do not respond, even to higher concentrations of the
drug (Fig 3.4.5), a phenomenon that has previously been demonstrated by Duchaud et al (1996).

Previous results (Fig 3.4.4) with etoposide had shown that the cell line that was most resistant to etoposide was the A-T heterozygote fibroblast cell line GM08389. This cell line was also highly resistant to γ-irradiation induced cell death (60Gy; see Fig 3.2.9). Therefore this cell line was chosen to monitor the response to drug-induced cell death by incorporating a range of concentrations (100-1000μM) and a variety of time points (24-72h), to observe any consistencies or discrepancies between the two DNA damaging stimuli in cell death profiles. The results demonstrated that this cell line was highly resistant to etoposide-induced cell death, as etoposide failed to induce apoptosis or necrosis in this cell line (Fig 3.4.6). These results were similar to those obtained when this cell line was irradiated with up to 60 Gy (Fig 3.2.9). In fact, all the fibroblast cells tested demonstrated resistance to both radiation and etoposide-induced cell death with the exception of GM08387, which responded to etoposide. Interestingly, both ionising radiation and etoposide induce double strand breaks (Benítez-Bribiesca and Sánchez-Suárez, 1999; Pernin et al. 1999; Ismail et al. 2005) and etoposide has been characterised as mimicking the effect of ionising radiation (Pernin et al. 1999). Etoposide does not totally inhibit the activity of topoisomerase II, but selectively uses the catalytic activity of the enzyme to increase the frequency and duration of DNA strand breaks, resulting in permanent double-stranded breaks that are lethal to the cell (Froelich-Ammon and Osheroff, 1995). Since ATM has been shown to be specifically activated in response to double strand breaks, as demonstrated during exposure of the cells to IR (Ismail et al. 2005), the lack of response in the fibroblast cells might be explained by the fact that each of the cell lines had at least one ATM mutation, and so ATM-dependent cell death was not initiated, and that at least one of these cell lines has shown normal DNA repair capability (Kaufmann et al. 1991). The difference compared to the SV40 transformed cells may be explained due to viral transformation of the cells, as previously demonstrated by Duchaud et al. (1996) and Bebb et al. (2001). Further evidence to support this hypothesis was reported by Smith et al. (1986), who demonstrated that SV40 transformation increased the sensitivity of both normal and A-T fibroblast cells to etoposide (20μM) compared to the parental cell lines from which the cells were transformed. The up regulation of p53 protein has also been demonstrated to be similar in A-T and control cells following etoposide (17μM) treatment in virus (EBV) transformed lymphoblastoid cells (Canman et al. 1994).
These results may therefore suggest that viral transformation may alter the expression of a number of genes, including those involved in the apoptosis pathway, and furthermore, that the altered expression of these genes may be different in cells of different cell lineages. It has been reported that ATM is cleaved by caspase 3 during etoposide-induced apoptosis (68μM; 5h), and that cleavage of ATM reduces its ability to phosphorylate p53 in HL-60 cells (Smith et al. 1999). Since ATM contributes to the up regulation of p53 in apoptosis, and the up regulation of this protein has been shown to be similar in cells lacking ATM and controls during apoptosis (Canman et al. 1994), this may suggest that viral transformation may affect the expression of other genes that are able to activate p53 expression. If this is indeed the case, this might explain why SV40 transformed cells were able to initiate etoposide-induced apoptotic pathways, despite the presence of ATM mutations, whereas untransformed cell lines were unresponsive to the same treatment (Fig. 3.4.4).

The fact that ATM is cleaved by caspase 3 in response to etoposide, and that cleavage of ATM reduces its ability to phosphorylate p53 may explain why HL-60 cells appeared to be resistant to etoposide-induced apoptosis (Fig 3.4.4). However, immunoblotting of whole cell lysate from untreated HL-60 cells did not detect ATM protein in this cell line (Fig 3.1.15B), which has also been reported previously (Gately et al. 1998).

Considering that anti-cancer drugs are used to treat cancers for which they show specificity, and that etoposide is used to treat haematopoietic malignancies, such as lymphoblastic or myeloid leukaemia (Rózalski et al. 2005), the demonstration of lymphoblastoid sensitivity to etoposide could have been expected. Etoposide is not commonly used to treat skin cancers, and so the lack of response in the fibroblast cells could also have been expected, however, this does not explain why HL-60 cells did not respond to etoposide as this cell line is of promyelocytic lineage. However, as shown in Figure 4.7, endogenous enzymes also protect the cell from the harmful effect of ROS, which may be produced by etoposide, and the level of these enzymes within the different cell types could also be a factor in the cells response to etoposide. Both these scenarios would have important implications for the treatment of A-T patients, as the neuronal degeneration in these patients has been postulated to be the result of these cells being in a continuous state of oxidative stress (Rotman and Shiloh, 1997a; 1997b).
Etoposide was found to be useful in discriminating A-T lymphoblastoid heterozygote cells from A-T homozygotes and controls, but did not allow discrimination between A-T homozygote and control lymphoblastoid cells. Additionally, most of the untransformed fibroblast cells were resistant to etoposide-induced cell death. Based on these collective results, etoposide was not considered to be a useful drug for use in a screening assay for the identification of A-T heterozygote samples.

4.5 Treatment of human cell lines with the anti-cancer drug etoposide and hydrogen peroxide.

A previous study had shown that, HL-60 cells treated with 50μM etoposide and 100μM HP (4 hours) resulted in a 50% reduction in apoptotic cells compared to HP used alone. No increase in apoptotic cells was observed above background levels with etoposide (50 μM) used in isolation. The lack of macrophage clearance of etoposide treated cells and the reduced clearance of combined etoposide and HP treated cells demonstrated that etoposide prevented the oxidation and externalisation of PS; Tyurina et al. (2004). In lieu of this data it was decided to repeat these experimental conditions with the A-T homozygote and heterozygote cells and to compare the response to control cells to determine if the antioxidant properties reported for etoposide would affect the cell death profiles of the cells in response to oxidative stress artificially induced by HP.

This thesis has previously presented data that agrees with the results published by Tyurina et al. (2004), in that no increase in annexin-v binding was detected in HL-60 cells treated with etoposide up to 50μM. The current research also demonstrated this effect at double the etoposide concentration (100μM; Fig 3.4.4B). However, ATM, one of the key proteins involved in apoptosis was not detected on immunoblots from this cell line (Fig 3.1.10B), which has also been reported previously (Gately et al. 1998), which could account for the lack of apoptosis in this cell line. This thesis has also presented data demonstrating the effect of etoposide on SV40 transformed lymphoblast (Fig 3.4.4A) and untransformed fibroblast (Fig 3.4.4B) cell death profiles. Likewise, the response of the same cell lines to HP treatment has also been presented (SV40 transformed lymphoblastoid cells (Fig 3.3.2) and untransformed fibroblast cell lines (Fig 3.3.3)). Interestingly, HL-60 cells exhibited a strong apoptotic response to HP treatment, which may reflect the ability of this cell line to respond to single rather than double strand breaks induced by etoposide, or γ-irradiation (Fig 3.2.8), to which this cell line showed no response.
4.5.1 The effect of etoposide and hydrogen peroxide on HL-60 cells.

In repeating the work by Tyurina et al. 2004 with HL-60 cells, the results demonstrated that using Etopoide ($\leq$50μM) in addition to HP (100μM for 4h) produced more annexin-v binding than HP used alone (Fig 3.5.1). Although using 100μM etoposide with 100μM HP produced similar numbers of apoptotic cells to HP used alone, the results demonstrated that etoposide was not able to reduce PS externalisation in HP treated HL-60 cells and therefore disagree with previously published results (Tyurina et al. 2004). Similar observations were demonstrated at 6h over the same concentration range (Fig 3.5.2).

4.5.2 The effect of etoposide and hydrogen peroxide on human lymphoblastoid cell lines.

In comparing the combined treatment (10μM etoposide and 100μM HP, 4h treatment; Fig 3.5.3) to HP (100μM HP, 4h treatment; Fig 3.3.2) used alone, the only significant result was obtained with the A-T homozygote A-T1, which exhibited a 28% reduction in late apoptotic cells with a simultaneous 17% increase in necrotic cells.

Extending the assay time to 6h, similar results were obtained with all the cell lines using the combined treatment at 6h (Fig 3.5.4) to the 4h treatment time used previously (Fig 3.5.3) with the exception of control cell line C1, which exhibited a 10% increase in late apoptotic cells concomitant with an 8% reduction in necrotic cells. However, in comparing the data from the combined treatment (Fig 3.5.4) to HP used alone (Fig 3.3.3A), the increase in necrotic cells seen in the control C1 at this later time point was also evident with HP used alone indicating that the increase in necrosis was due to extended exposure to HP in this instance.

When the concentration of etoposide used with 100μM HP was increased to 50μM (Fig 3.5.5; 6h treatment) the cell lines A-T1, Het 5 and C1 exhibited a 15%, 9% and 17% increase in late apoptotic cells with a simultaneous 5%, 14% and 33% reduction in necrotic cells respectively, compared to HP used alone (Fig 3.3.3A). It would therefore appear that using 50μM etoposide in combination with 100μM HP has an affect on the mechanism of cell death induced in the lymphoblast cells, as this combination of treatments seems to favour the induction of apoptotic pathways and/or inhibition of necrosis in the A-T2, Het 5
and C1 cell lines. Jurkat cells exhibited an 11% decrease in late apoptotic cells with a 6% increase in necrotic cells.

When the concentration of etoposide used with 100µM HP was increased to 100µM and the cells were treated for 4h, the A-T homozygote A-T1 showed a 27% reduction in late apoptotic cells with a 10% increase in necrotic cells using the combined treatment (Fig 3.5.7) compared to HP used in isolation (Fig 3.3.2). A decrease in late apoptotic cells was also observed for A-T1 (10%) and C1 (8%), although the C1 control also exhibited a reduction in necrotic cells (20%) as well as a 5% increase in early apoptotic cells. Interestingly, the previously used 50µM etoposide in combination with 100µM HP over a longer exposure time (Fig 3.5.7, 6h) had a more dramatic effect on cell death profiles in the C1 control cell line than the higher (100µM) concentration of etoposide in combination with 100µM HP over a shorter (4h) exposure time (Fig 3.5.7). Using the same combination of etoposide and HP (100µM etoposide and 100µM HP) and treating the cells for 6h (Fig 3.5.8), the only change in cell death profiles, were observed for control C2 and Jurkat cells. The control C2 produced a 6% increase in late apoptotic cells with a 14% reduction in necrotic cells, and the Jurkat cells exhibited a 29% reduction in late apoptotic cells.

The results demonstrate that etoposide was able to reduce the amount of externalised PS in these cell lines, demonstrated by the decrease in annexin-V binding, and therefore reduced numbers of apoptotic cells. This observation was based on the fact that late apoptotic cells are stained with annexin-V and PI and necrotic cells stain more heavily with PI. Peroxidation of lipids results not only in the externalisation of PS, but also damage to the cell membrane. The fact that fewer cells were recorded as necrotic with control cell line C1 using 10µM etoposide with HP (Fig 3.5.4, 6h), 50µM etoposide with HP (Fig 3.5.5, 6h) and 100µM etoposide with HP (fig 3.5.7, 4h); and a reduction in necrotic cells was also observed with the cell lines A-T2 and Het 5 using 50µM etoposide with HP (Fig 3.5.5, 6h), and also with control C2 using 100µM etoposide with HP (Fig 3.5.8) compared to HP used alone (Fig 3.3.2 and Fig 3.3.3) therefore suggests that less damage to the cell membranes had occurred due to the addition of etoposide, evidenced by decreased PI uptake in these cells.

Interestingly, the 5%, 14% and 33% decrease in necrotic cells observed in A-T2, Het 5 and C1 cell lines treated with 50µM etoposide and 100µM HP for 6h (Fig 3.5.5) was
accompanied by a 15%, 9% and 17% increase in late apoptotic cells in these cell lines respectively. In this instance, it not only appears that addition of etoposide has a protective effect on the cell membrane, indicated by reduced PI uptake in these cells, but addition of etoposide when artificially inducing oxidative stress using HP, seems to promote the induction of apoptosis in these cells lines, evidenced by the increase in late apoptotic cells using the combined treatment (Fig 3.5.5) compared to HP used alone (Fig 3.3.3A). These results may therefore be important for the treatment of cancer, as tissue necrosis following radiation and chemotherapy treatment has been linked with a poor prognosis (Edwards et al. 2003; Langner et al. 2003; Chang et al. 2006; Sang et al. 2006). Any agent that is able to alter a necrotic cell death profile to favour the induction of apoptosis would be a valuable tool in the treatment of cancer. On this basis, the antioxidant properties of etoposide warrant further study, as its use in combined cancer therapies with radiation and other chemotherapeutic drugs such as doxorubicin and bleomycin which exert their effects in part through the generation of ROS may elucidate more effective treatment regimes, especially as the reduction in necrotic cell death and promotion of apoptosis was found to be concentration dependent using these cell lines.

Based on these results, it would appear that etoposide does have the capacity to act as a lipid antioxidant and is able to prevent the oxidisation of membrane phospholipids. These results are therefore in agreement with previously published results (Tyurina et al. 2004), and additionally demonstrate this effect in A-T homozygote, A-T heterozygote and control SV40 transformed lymphoblastoid cell lines.

4.5.3 The effect of etoposide and hydrogen peroxide on human fibroblast cells.

Data indicated that apart from A-T heterozygote GM08389, which exhibited a 12% reduction in late apoptotic cells and a 3% increase in necrotic cells when 50μM etoposide was used with HP (Fig 3.5.6), compared to HP used alone (Fig 3.3.3B), and which was not observed when 100μM etoposide was used with HP (data not shown), etoposide had no effect in reducing PS externalisation indicated by annexin-v positive cells, as the values obtained for a combination of etoposide with HP were similar to those obtained with HP alone (Fig 3.3.3B).

Interestingly, the A-T heterozygote cell lines GM03396 and GM03397 both carry the same mutation, as the cell lines were generated from the parents of an affected child who is
homozygous for a G>A substitution at nucleotide 7913, and yet these cell lines produced very different cell death profiles. GM03396, like the other A-T homozygote and heterozygote fibroblast cell lines exhibited mostly early apoptotic cells and lower numbers of late apoptotic cells when treated with 50µM etoposide and 100µM HP for 6h, while GM03397 exhibited only late apoptotic and necrotic cells, with more necrotic than late apoptotic cells. Data was unavailable for the A-T heterozygote cell line GM03397 treated with HP alone, however, this cell line was shown to be resistant to etoposide-induced cell death (100µM), and under these circumstances, this would suggest that the late apoptotic/necrotic cell death profile produced in response to etoposide and HP treatment was due to the effect of HP. As GM03396 produced a similar cell death profile to the other fibroblast cells, this suggests that GM03397 may have at least one additional mutation, which contributed to the demonstrated results. The results also suggest that this cell line is either more sensitive to this combination of DNA damaging agents as it has progressed more quickly to a necrotic state, or that a different cell death pathway may have been utilised.

4.6 The effect of doxorubicin on human cell lines.
Doxorubicin is an anthracycline antibiotic that is one of the most commonly used and one of the most effective anticancer drugs. It has been used to treat leukaemia (Różalski et al. 2005), the most frequently observed of the cancers that develop in A-T patients (Taylor et al. 1996), as well as breast cancer (Gamen et al. 1997), which A-T heterozygotes may be more at risk of developing (Jackson, 1995; Lehmann and Carr, 1995; Zakian, 1995; Athma et al. 1996; Broeks et al. 2000).

Doxorubicin has recently been shown to induce ATM-dependent phosphorylation of a variety of proteins involved in the apoptosis cascade. One of the most important of these being phosphorylation of p53 at serine 15 (Kurtz et al. 2004). This residue is important in the stabilisation of p53 following DNA double strand breaks. Probably the most important phosphorylation to be induced by the drug is phosphorylation of ATM itself at serine 1981 (Kurtz et al. 2004). In normal cells not undergoing DNA insult, the inactive ATM kinase can exists as a dimer. Activation of the ATM protein results in autophosphorylation at amino acid 1981, followed by separation of the dimer into active monomers, which are then free to interact with a variety of substrates (Kim et al. 1999).
The implications of these findings are very important in the fact that doxorubicin seems to elicit its action through apoptosis by directly activating the ATM protein. Therefore giving this drug as a treatment to ATM compromised patients may be predisposing them to DNA damage that is unable to be repaired due to lack of functional ATM, and such damage may not be able to be cleared by removal of the affected cell via apoptosis, as direct activation of ATM may not occur in A-T homozygotes, and may be sub optimal in A-T heterozygotes.

Considering the fact that doxorubicin would be used to treat the majority of malignancies that predominantly affect both A-T homozygote and A-T heterozygote patients, this demonstrates the relevance of using doxorubicin in the present research.

4.6.1 The Effect of doxorubicin on human fibroblast cell lines.

Data indicated that all of the cell lines exhibited a necrotic cell death profile (Fig 3.6.2), and only two fibroblast cell lines produced cell death profiles that exceeded 10% when treated with 0.2μg/ml doxorubicin. A-T hTERT produced 17% necrotic cells and A-T heterozygote (GM08389) produced 11% necrotic cells, which was in comparison to >70% necrotic cells produced by Jurkat cells under the same experimental conditions. Doxorubicin-induced necrotic cell death in Jurkat cells has been reported previously using low-dose (40nM) doxorubicin treatment (Sugimoto et al. 2002). Although the doxorubicin concentration used by Sugimoto et al. (2002) was lower than that used in the current study (0.2μg/ml = 0.37μM), this does demonstrate that necrotic pathways were inducible in this cell line in response to doxorubicin treatment. The fact that doxorubicin has been shown to mediate its effects in an ATM-dependent manner (Kurtz et al. 2004), and the fact that Jurkat cells were the only cell line used that did not have mutated ATM protein, may explain the gross difference in cell death profiles between Jurkat and A-T homozygote and heterozygote cell lines. The results necessitated an increase in the doxorubicin concentration to determine if a more appropriate response could be generated in the fibroblast cells, as well as to clarify if the necrotic cell death profiles exhibited were caused by DNA damage induced cell death or caused by damage of the cell membranes due to lipid peroxidation, as it has been reported that doxorubicin induces lipid peroxidation and membrane damage due to the generation of ROS (Reviewed in: Minotti et al. 2004).
Increasing the doxorubicin concentration (0.5\(\mu\)g/ml; Fig 3.6.3) produced a dose-dependent increase in necrotic cells in all of the fibroblast cell lines, with A-T hTERT showing greater sensitivity to the drug than the A-T heterozygote cell lines. Interestingly, the higher doxorubicin concentration induced both late apoptotic (35%) and necrotic (52%) cells in the Jurkat cell line (Fig 3.6.3). Considering that the Jurkat cells were much more sensitive to the drug than any of the fibroblast cells, the cells could have sustained more membrane damage than the fibroblast cells, in which case, annexin-v could be binding to PS on the inside of the membrane rather than the apoptosis-induced externalised moiety.

![Figure 4.6 Schematic diagram showing annexin-V binding to externalised PS during apoptosis compared to annexin-V binding on the inside of the membrane during necrosis.](image)

To clarify these issues, the doxorubicin concentration was increased further (1.0\(\mu\)g/ml) in order to try and match the same level of damage that induced 87% cell death in the Jurkat cells (Fig 3.6.3) with the fibroblast cells, to determine if a threshold of damage was required in order for apoptotic cells to be produced. To ascertain if the annexin-v was
binding to PS on the inside of the membrane or to externalised PS, as shown in Fig 4.6, the cells were subjected to the trypan blue exclusion test (as described in the methods) after doxorubicin treatment.

Using 1.0μg/ml doxorubicin all the fibroblast cells continued to exhibit a necrotic cell death profile (Fig 3.6.4), in contrast to the 41% late apoptotic and 42% necrotic cells exhibited by the Jurkat cells under the same experimental conditions. Similar numbers of Jurkat cells exhibiting cell death have been previously demonstrated (80%; Wesselborg et al. 1999) using the same experimental conditions, although the cell death profile was shown to be distinctly apoptotic. The results in Fig 3.6.4 demonstrate that equal numbers of cells exhibited apoptosis and necrosis (41% and 42% respectively). Wesselborg et al. (1999) used a different anti-annexin-v antibody and propidium iodide (PI) to that used in the current study, and the assay procedure was not apparent from the publication and was not accessible from the manufacturer. Several annexin-v assays use a rapid binding method, previously used by Tyurina et al. (2004). The data generated did not use this technique and could account for the difference in cell death profiles observed between the two studies. At this elevated doxorubicin concentration, A-T hTERT and A-T heterozygote GM08387 fibroblast cells exceeded the level of cell death observed in Jurkat cells, while the other two A-T heterozygote cell lines (GM08389, GM03397) produced considerably less (44-45%) necrotic cells. Interestingly, although the amount of cell death in two of the fibroblast cell lines was higher than observed for Jurkat cells under these experimental conditions, the fibroblast cells continued to exhibit a necrotic cell death profile. Phosphatidylserine normally resides on the inside of the plasma membrane, but is externalised to the outside of the membrane when cells are undergoing apoptosis, and has been shown to occur several hours before membrane integrity is breached. This event has been demonstrated in various human and mouse tissues, and was detected after treatment with a variety of apoptosis-inducing agents (Martin et al. 1995). The means by which this translocation was visualised was by utilising FITC conjugated annexin-v, which has been shown to bind preferentially to phosphatidylserine (PS) (Tait et al. 1989; Andree et al. 1990; Thiagarajan and Tait, 1990). The DNA stain PI was utilised as a marker of necrosis as this stain is only able to enter the cell following a breach in the plasma membrane. This has been demonstrated by its use to show membrane integrity to confirm that annexin v binds to PS on the outside and not the inside of the membrane during apoptosis (Martin et al. 1995).
Although PI has been used to discriminate between apoptotic and necrotic cells previously (Martin et al. 1995), to confirm that the annexin-v was binding to externalised PS using Jurkat cells, and not intracellular PS due to extensive membrane damage, all the cells were additionally subjected to trypan blue exclusion (as detailed in the methods). Both the Jurkat and fibroblast cells exhibited trypan blue exclusion rates >90%, confirming that <10% of the cells had disrupted membranes, in which case, the annexin-v positive cells observed in the Jurkat cells were due to the induction of apoptosis, which in turn confirmed that doxorubicin induced a necrotic cell death profile in fibroblast cells, and furthermore, that the necrotic profile exhibited by these cell lines was both dose and time dependent (Fig 3.6.5). The fact that Jurkat cells were able to initiate an apoptotic cell death profile may be related to the lack of ATM mutations in this cell line compared to the fibroblast cells which all had at least one ATM mutation.

In a study conducted on human prostate cancer cells, doxorubicin was found to increase intracellular HP in a dose-dependent manner up to 1μM, and at this concentration, HP concentration had increased from steady state levels of 13 ± 4pM to 51 ± 13pM (Wagner et al. 2005). The concentration of doxorubicin used with the fibroblast cells at 0.2μg/ml (Fig 3.5.2) and 0.5μg/ml (Fig 3.5.3) were within this dose range. At these concentrations, and at the higher concentration of 1.0μg/ml (Fig 3.6.4), only necrotic cells were produced in the fibroblast cells. Glutathione functions in the detoxification of ROS, in particular HP, and has been proposed to regulate ceramide production (Meredith and Dodson, 1987). Ceramide has been proposed to induce a necrotic cell death pathway in cells that have not been induced to undergo apoptosis (Hetz et al. 2002). Considering that all the fibroblast cells had at least one ATM mutation, this may explain why a necrotic cell death pathway was induced in the fibroblast cells and the expression of wild type ATM in Jurkat cells would also explain how the Jurkat cells were able to induce an apoptotic cell death pathway in response to the same cell death stimulus.

Etoposide and doxorubicin are both Topoisomerase II inhibitors (Różalski, et al. 2005; Peng, et al. 2005), and both these drugs produce ROS (Kurosu et al. 2003; Peng et al. 2005). The ROS HP can also undergo further intracellular modification to produce the hydroxyl radical, which is another type of ROS (Byung et al. 1994). Each of these treatments was used with the fibroblast cells and each produced a very different response in these cells. The majority of fibroblasts were highly resistant to etoposide-induced cell death (Fig 3.4.2), while the same cell lines produced a strong apoptotic cell death profile in
response to HP (Fig 3.3.3B). Doxorubicin induced a necrotic cell death profile in these cells (Fig's 3.6.2-4). While additional mutations or differential expression of cellular proteins cannot be ruled out as factors facilitating the results, it is possible that the intracellular concentration of ROS such as HP or the hydroxyl radical are not induced to the same level following treatment with these two anti-cancer drugs and HP treatment, or that generation of ROS occurs with different kinetics in response to these agents. In such a model, the intracellular concentration of ROS may determine the mechanism of cell death. Further evidence for this lies in the dose response and time course study on doxorubicin treated Jurkat cells (Fig 3.6.1). At the lowest concentration of doxorubicin (0.2μg/ml) and within the first 24h, only necrotic cells were produced. However, using the same concentration of doxorubicin, a time-dependent increase in apoptosis was demonstrated with a simultaneous decrease in necrosis. Increasing the drug concentration resulted in the generation of apoptosis within the first 24h, and the increase in apoptotic cells and reduction of necrotic cells was found to be both dose and time dependent.

There are many other proteins involved in the induction of cell death pathways, such as the pro-apoptotic proteins p53 and caspases and anti-apoptotic proteins such as Bcl-2 and BclxL and the involvement of such proteins in doxorubicin-induced cell death cannot be ruled out. Additionally, differential expression of such proteins in different cell types could also be important in the type of cell death pathway induced to a variety of different anti-cancer drugs. The same anti-cancer drug is not used to treat all forms of cancer, and each drug shows specificity for a particular tissue type, which highlights the complex nature of cell death pathways in different cell types. However, based on the response of fibroblast cells to the anti-cancer drugs etoposide and doxorubicin and the response of these cells to HP added extracellularly, and the fact that the A-T phenotype has been linked to oxidative stress (Rotman and Shiloh, 1997a; 1997b), further work to elucidate the biochemical pathways in response to different types of oxidative stress in different cell types using a range of drugs that generate ROS, may be useful in determining the prognosis of various types of cancer with a range of different anti-cancer drugs.

This thesis was also interested in the response of SV40 transformed lymphoblastoid cells to doxorubicin, however, this work could not be completed. This therefore identifies an area of research for further study; especially considering that this thesis demonstrated that untransformed fibroblast cells respond differently to a variety of DNA damaging agents when compared to SV40 transformed lymphoblast cells. Due to reduced numbers of cell
lines utilised using doxorubicin, compared to the other treatments, it was felt that a fuller analysis was required before deciding whether doxorubicin was able to reliably discriminate A-T heterozygotes from A-T homozygotes and controls.

4.7 The effect of staurosporine on human cell lines.
Originally, staurosporine was thought to be a specific inhibitor of protein kinase C (PKC), although it is now commonly used as a general protein kinase inhibitor, and is a potent inducer of apoptosis (Tee and Proud, 2001). ATM has protein kinase activity (Kastan and Lim, 2000), and may therefore be a target for the inhibitory action of staurosporine. Although technically not classed as an anticancer drug, like etoposide and doxorubicin, it is able to interfere with the activity of Topoisomerase II. Staurosporine was considered to be an interesting compound for inclusion in the current study as it has the potential to a) target and inactivate ATM, and b) induce apoptosis (which may be due to the inhibitory effects on ATM and/or other protein kinases involved in apoptosis). In addition to these factors, staurosporine was also included, so that the cell death profiles of cells treated with staurosporine could be compared to the other two Topoisomerase poisons (etoposide and doxorubicin) utilised in the current study.

4.7.1 The effect of staurosporine on human lymphoblastoid cell lines.
The research findings of this thesis demonstrated A-T homozygote, A-T heterozygote and control SV40 transformed lymphoblast cells exhibited an apoptotic cell death profile when treated with 2µM staurosporine for 4h (Fig 3.7.2) and 6h (Fig 3.7.3). Interestingly, the cell death profiles exhibited by all these cell lines consisted of both early and late apoptotic cells, and these cells predominated the cell death profiles, with necrotic cells only making a small contribution to the numbers of cells undergoing cell death. This cell death profile was distinct from the cell death profiles generated using the other DNA-damaging agents as staurosporine induced high numbers of early apoptotic cells. This agent was the only agent to be used that specifically inhibited protein kinases. At the later (6h; Fig 3.7.3) time point, the A-T homozygote cell lines exhibited the greatest sensitivity, producing the highest numbers of apoptotic cells. The cell death profiles of A-T heterozygotes, exhibited less apoptotic and more necrotic cells than the control cells. These results permitted discrimination of all three genotypes on this basis.
4.7.2 The Effect of staurosporine on human fibroblast cell lines.

Data generated for the fibroblast cells demonstrated that both the A-T homozygote and heterozygote cell lines, with the exception of A-T heterozygote GM08387 were resistant to staurosporine-induced cell death. Despite increasing the staurosporine concentration to 5μM, and exposing the cells to the drug for 4h (data not shown) or 24h (Fig 3.7.4) the results indicated that apart from cell line GM08387, which exhibited a further increase in necrotic cells (60%) at this later (24h) time point compared to previous results (43% at 4h, data not shown), all the other fibroblast cells remained resistant to staurosporine-induced cell death.

Due to the fact that most of the fibroblast cell lines had shown considerable resistance to staurosporine-induced cell death when compared to the results obtained with lymphoblast and Jurkat control cell lines, it was decided to select one cell line for further analysis, with the specific aim of determining a staurosporine concentration and exposure time that was able to induce cell death, to which the other fibroblast cell lines could be compared. The A-T heterozygote GM08389 was chosen for this study, based on the fact that this cell line had shown resistance to gamma radiation (up to 60 Gy over 48 hours; Fig 3.2.8) and etoposide (up to 1000μM; Fig 3.4.6). However, it had also been established that cell death pathways were inducible in this cell line, but that the response was stimulant dependent. For example, this cell line exhibited 41% early apoptotic and 28% late apoptotic cells when treated with 100μM hydrogen peroxide for 6 hours (Fig 3.3.3), and showed 11% necrotic cells when treated with 0.2μg/ml doxorubicin for 24 hours, 32% necrotic cells with 0.5μg/ml doxorubicin and 44% necrotic cells with 1.0μg/ml doxorubicin over the same time period (Fig 3.6.5). The doxorubicin results with this cell line not only demonstrated that cell death pathways were inducible, but also that it was possible to increase the level of response by increasing the concentration of the drug. The GM08389 cell line was treated initially with 10-50μM staurosporine for up to 72 hours, to determine if an increased cell death response would be produced with an increased drug dose and extended exposure time to staurosporine (Fig 3.7.5). However, the cell line did not respond to these experimental conditions, and produced, <5% apoptotic/necrotic cells (Fig 3.7.5A). The cell line was then exposed to 75-150μM staurosporine for 24h to determine if much higher concentrations of the drug would induce more cell death in these cells. Despite increasing the staurosporine concentration 15-fold, these experimental conditions had no effect on increasing the
number of cells exhibiting cell death profiles with this cell line (Fig 3.7.5B). The GM08389 cell line remained resistant to staurosporine-induced cell death.

Based on these results, staurosporine was considered to be an ineffective stimulant to induce cell death in fibroblast cell lines, but also highlighted the difference in cellular responses to the three-topoisomerase II inhibitors (etoposide, doxorubicin and staurosporine) used to generate the data in this thesis. Fibroblast cells were resistant to both etoposide and staurosporine-induced cell death, but produced a significant necrotic cell death profile in response to doxorubicin. This reinforces the requirement for further study in this area to elucidate why the response to doxorubicin was so different to that observed for etoposide and staurosporine.

Staurosporine was able to discriminate A-T heterozygotes from A-T homozygotes and control cells using SV40 transformed cell lines, and in this respect would be useful for inclusion in a screening assay to identify A-T heterozygote samples, but due to the resistance demonstrated by the fibroblast cells to staurosporine-induced cell death, would not permit its use in a screening assay to detect A-T heterozygotes in untransformed cells.

4.8 The effect of bleomycin on human cell lines.

Bleomycin is an anticancer drug that exerts its effects by producing ROS such as hydroxyl radicals, superoxide and hydrogen peroxide, that are able to interact with DNA (Mahmutoglu et al. 1987) causing both single and double strand breaks in DNA (Shaham et al. 1983). Bleomycin is used to treat Hodgkin’s and Non-Hodgkin’s lymphoma’s, cancers of the head and neck, as well as other types of cancer (Cancer Research U.K.). In a retrospective study of patients with immunodeficiency disorders who had developed solid tumours, 15 of 22 patients (68%) analysed were diagnosed with ataxia-telangiectasia. Of these 15 patients, 9 people developed Non-Hodgkin’s lymphoma, 5 had Hodgkin’s lymphoma, and 1 patient was diagnosed with brain stem glioma (Varan et al. 2004). In a separate retrospective study, the incidence of developing lymphoma was determined to be 250 times higher for white A-T patients, and 750 times higher for African-American A-T patients when compared to the general population (Morrell et al. 1986). Based on these figures, it is not surprising that numerous studies have previously been carried out on the effect bleomycin has on A-T cells. Several independent researchers have reported a significant increase in chromosome damage to bleomycin treated A-T cells compared to
controls (Taylor et al. 1979; Cohen et al. 1981; Shaham et al. 1983). A-T cells have also been found to exhibit lower survival rates compared to control cells following bleomycin treatment (Taylor et al. 1979; Cohen et al. 1981), and while the survival rate of an A-T heterozygote cell line was shown to be intermediate between that of A-T and control cells, chromosome damage in A-T heterozygote cells showed no significant alterations from control levels following exposure to bleomycin (Cohen et al. 1981).

Previous work in this area seems to have been centred around the type of damage caused to A-T cells and the survival rates of these cells compared to controls following exposure to bleomycin, and while it is well known that survival of A-T cells following this treatment is reduced, the mechanism involved in this decreased cell survival seems to have been overlooked. It was therefore hoped that this research would provide this information, and add to published work in this area.

4.8.1 The effect of bleomycin on human lymphoblastoid cell lines.

Initial experimental analysis was aimed at determining the cell death profiles at bleomycin concentrations that had previously been used to observe chromatid damage and a reduction in cell survival. Taylor et al. (1979) and Cohen et al. (1981) had previously reported increased chromatid aberrations and reduced cell survival in A-T cells compared to controls at 10µg/ml (7.13µM) following a 6 hour drug exposure time. Data generated however, demonstrated that concentrations of bleomycin <42.86µM were not able to induce cell death profiles in the SV40 transformed lymphoblastoid cell lines. However, in both the aforementioned studies, up to 48 hours was allowed for cell recovery. Since the study was concerned with cell death profiles rather than cell survival rates, the bleomycin concentration was increased to elicit a response. Increasing the bleomycin concentration to 42.86µM had a positive effect in inducing cell death profiles in the lymphoblastoid cell lines (Fig 3.8.3A). Homogeneity in the response of A-T heterozygote cells was indicated by the generation of higher numbers of necrotic cells compared to apoptotic cells, which has been a prominent feature of these cell lines in response to a range of DNA damaging agents. This factor permitted discrimination of the A-T heterozygote cell lines from A-T homozygote and control cells. However, the lack of homogeneity displayed in both the A-T homozygote and control cells did not permit discrimination between these two genotypes. In a study to characterise the type of DNA strand breaks that occur in response to a variety of bleomycin concentrations, the lowest concentration of bleomycin that was used was
53.6μM. This concentration produced 29% single strand breaks and 7.5% double strand breaks in PBLs (Benítez-Bribiesca et al. 1999), which may explain why cell death profiles were not observed at the previous (lower) concentrations utilised.

4.8.2 The Effect of bleomycin on human fibroblast cell lines.
The A-T homozygote and heterozygote fibroblast cells were treated under the same experimental conditions as the lymphoblastoid cell lines (42.86μM bleomycin treatment for 6h; Fig 3.8.3B), but the fibroblast cells exhibited resistance to bleomycin-induced cell death, which indicated a substantial increase in drug concentration and/or an increased exposure time would be required to induce cell death profiles in these cell lines. The cell lines were therefore exposed to a 10-fold increase in the bleomycin concentration, and the exposure time was increased to 24h. However, even at this elevated bleomycin concentration (420μM), and longer exposure time (24h), the cell lines remained resistant to bleomycin-induced cell death, with <10% of any of the cell lines exhibiting cell death profiles (Fig 3.8.4A). The Jurkat cells did however respond to the treatment, with the production of both apoptotic and necrotic cells further highlighting that the response of the fibroblast cells was due to resistance to the drug.

4.8.3 The effect of increasing the bleomycin concentration on human lymphoblastoid cell lines.
Finally, previous work had demonstrated that a bleomycin concentration of 714μM used to treat PBLs had produced a reduction in both single and double strand breaks which was associated with a reduction in cell viability (49%), and evidence of morphological necrotic cell changes (Benítez-Bribiesca et al. 1999). This raised the question of whether a much higher concentration of bleomycin could alter the cell death profiles of the lymphoblast cells, which, with the exception of the A-T heterozygote cells exhibited a predominantly apoptotic cell death profile (Fig 3.8.3). However, the aim was not to totally kill the cells, but rather to determine if it was possible to induce necrosis using a higher concentration to that used previously. For this reason, a bleomycin concentration of 600μM was chosen to treat the cells for both 4 and 6 hours, so that any changes in cell death between these two time points could also be observed. The results at the four-hour time point (Fig 3.8.5A) demonstrated a 17% increase in apoptotic cells for control cell line C1, but apart from this observation, no further changes in cell death profiles were evident at 4h (Fig 3.8.5A) or at the later (6h) time point (Fig 3.8.5B). Therefore, despite this grossly elevated bleomycin
concentration, no further increases in the cell death profiles of the lymphoblastoid cell lines were observed under these experimental conditions. With regards to bleomycin being incorporated into a screening assay for the identification of A-T heterozygotes, the results indicated that it was possible to distinguish A-T heterozygotes from the other two genotypes being investigated, again due to the high numbers of necrotic cells in relation to apoptotic cells, which was the case over the bleomycin concentration range 42.86-600 μM. However, the lack of homogeneity in the A-T homozygote and control genotypes meant that bleomycin was not able to discriminate between each of the three genotypes, and on this basis would not be useful in a screening assay.

4.9 Summary of results and areas for further study.

4.9.1 Western blot analysis of ATM protein expression.

The research presented was constructed to determine a number of specific aims. The first of these was to address if it was possible to reliably discriminate A-T heterozygote cell lines from A-T homozygote and control genotypes. The experimental approach involved utilising an optimised western blotting assay to measure ATM expression in A-T homozygote and A-T heterozygote cell lines with different types of ATM mutations in two different cell lineages compared to controls. Data presented indicated that all of the A-T homozygote and heterozygote SV40 transformed lymphoblastoid and untransformed fibroblast cell lines were able to be identified as carrying at least one ATM mutation based on the ATM expression in these cell lines compared to the mean control values. Although it was not possible to discriminate A-T heterozygote cell lines from A-T homozygote cell lines, the use of such a screening approach would only be used to identify samples that warranted further genetic analysis, and in this regard, the assay would be useful for high throughput screening, especially considering that A-T homozygote patients are usually diagnosed with the condition at an early age (Boder and Sedgwick, 1958; Zecevic and Rakic, 1976). However, the reproducibility of the assay was affected by the rapid kinetics of the β-actin chemiluminescent signal used to normalise the ATM expression levels, and in this regard, the assay would have benefited greatly from using ATM reference standards to which ATM test samples could have been directly compared, as previously used by Butch et al. (2004). However, the unavailability of such standards from sources that had previously used the standards, and the lack of commercial availability of such standards did not permit their use in the current research. Although the optimised assay procedure was
able to identify samples with at least one ATM mutation, over 300 different ATM mutations have been identified to date, and so it is not known what proportion of these mutations would be able to be detected using this assay, especially considering that ATM mutations exist that permit apparently full length ATM protein to be expressed. This mutation has been identified as a founder effect mutation (7271T>G), and has been associated with a milder A-T clinical phenotype (Stankovic et al. 1998). This type of mutation was not represented in the current study, and so this research was not able to report whether such a mutation would be identified using the optimised western blotting procedure. The research presented may therefore be considered to warrant further study, especially if ATM reference standards become commercially available.

4.9.2 Cell death profiles measured using annexin-V and propidium iodide.
Next, the thesis was interested in determining whether the cellular response of A-T heterozygote cell lines to a range of DNA-damaging agents could be used to discriminate A-T heterozygote cell lines from A-T homozygote and control cell lines. The treatments utilised for this purpose included: γ-irradiation, chemicals (HP and staurosporine) and anticancer drugs (etoposide, doxorubicin and bleomycin).

4.9.2.1 Cell death profiles induced by γ-irradiation.
The research presented using γ-irradiation demonstrated that A-T heterozygote cells could be discriminated from A-T homozygote and control lymphoblastoid cell lines based on the fact that these cell lines produced higher numbers of necrotic cells compared to apoptotic cells. However, due to the lack of homogeneity in the response of A-T homozygote and control lymphoblastoid cell lines, it was not possible to reliably discriminate each of the three genotypes. Untransformed A-T homozygote and heterozygote cell lines demonstrated resistance to γ-irradiation-induced cell death. These collective results indicated the use of γ-irradiation as a useful DNA-damaging agent to discriminate A-T heterozygote cells from A-T homozygotes and controls had considerable limitations for its use in a screening assay.

4.9.2.2 Cell death profiles induced by hydrogen peroxide.
Hydrogen peroxide was the only treatment used that could reliably discriminate each of the A-T homozygote, A-T heterozygote and control genotypes in SV40 transformed lymphoblastoid cells in addition to each of the A-T homozygote and A-T heterozygote genotypes in untransformed fibroblast cells. Unfortunately, data was not available for
control untransformed fibroblast cell lines. However, a telomerase transformed fibroblast control cell line could also be discriminated from A-T homozygote and heterozygote untransformed fibroblasts based on the numbers of apoptotic cells produced by this cell line.

In SV40 patient derived lymphoblasts, the increase in late apoptotic cells and the reduction in necrotic cells in the A-T homozygote cells was found to be statistically significant compared to controls. Likewise, the reduction in late apoptotic cells and the increase in necrotic cells in the A-T heterozygote cells was also found to be statistically significant compared to both A-T homozygotes and controls (P<0.05 one-way ANOVA).

In untransformed fibroblasts, using a telomerase transformed fibroblast cell line as the control, the increase in early apoptotic cells and total apoptotic cell death (early and late apoptotic cells combined) in the A-T homozygote cells was found to be statistically significant compared to the control. The increase in early apoptotic cells and total apoptotic cell death in the A-T heterozygote cells was also found to be statistically significant compared to both the A-T homozygotes and the control (P<0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes in two different cell lineages in response to hydrogen peroxide treatment.

Based on the data generated in this thesis, HP was demonstrated to be the most useful DNA-damaging agent in discriminating A-T heterozygotes from A-T homozygotes and controls, and the response of cell lines to oxidative stress may therefore be a reliable marker for A-T heterozygosity. The fact that neurodegeneration in A-T patients has been linked to oxidative stress, and that this thesis has demonstrated the possibility of using oxidative damage as a means of discriminating A-T heterozygotes from homozygotes and controls necessitates further research in this area. Of particular importance would be the type of and amount of ROS produced in A-T homozygote and heterozygote cells, compared to normal cells in response to radiation and chemotherapeutic drugs. This information may have important implications for cancer treatment, and may help in increasing the efficacy of future cancer treatments. Additionally, correlating the type of ROS produced by these treatments with the mechanism of cell death, may elucidate mechanisms to suspend the neurodegeneration, which is a common feature in A-T.
4.9.2.3 Cell death profiles induced by etoposide.
The ability to discriminate A-T heterozygote lymphoblastoid cell lines from A-T homozygote and control cell lines was also demonstrated using etoposide. However, using this treatment it was not possible to discriminate A-T homozygotes from controls. All the A-T homozygote and A-T heterozygote fibroblast cells showed strong resistance to etoposide-induced cell death with the exception of one A-T heterozygote cell line (GM08387), which exhibited a necrotic cell death profile. The effect of the mutation in this cell line may therefore indicate an unusually high sensitivity to etoposide-induced cell death, and may therefore warrant further study. Based on these results etoposide also demonstrated limitations to its use in a screening assay for discriminating A-T heterozygote cell lines from A-T homozygote and control cells.

4.9.2.4 Investigating the lipid antioxidant properties of etoposide in hydrogen peroxide treated cells.
In investigating the report by Tyurina et al. 2004, that etoposide acts as a lipid antioxidant and is able to reduce externalisation of PS in HP treated HL-60 cells, this observation was only observed in two A-T homozygote (A-T1, A-T2) and one control (C1) SV40 transformed lymphoblastoid cell line, one A-T heterozygote untransformed fibroblast cell line (GM08389) and Jurkat control cells. While this effect was only demonstrated on a small number of cell lines, the results demonstrated add to previously published results in demonstrating similar results using SV40 transformed A-T homozygote and control cells, A-T heterozygote untransformed fibroblast cells and Jurkat control cells. Additionally, the data also indicated that 50μM etoposide used in addition to 100μM HP for 6h (Fig 3.5.5) increased late apoptotic cell death and reduced necrotic cell death in 3 three cell lines (A-T1, Het 5 and C1) compared to the same cells treated with HP alone (Fig 3.3.2). These results therefore suggest that this combination of treatments may promote apoptosis and/or inhibit necrotic cell death at these concentrations. These results warrant further investigation into the antioxidant properties of etoposide as well as other antioxidants that may be able to prevent necrosis and preferentially induce apoptosis, as tissue necrosis following treatment with IR and chemotherapeutic drugs is linked with a poor prognosis.

4.9.2.5 Cell death profiles induced by doxorubicin.
Although preliminary results with doxorubicin (0.5μg/ml for 24h) demonstrated it was possible to discriminate A-T heterozygote fibroblast cells from A-T homozygote cells,
based on the number of necrotic cells produced, it was felt that the small number of cell lines utilised in this section of the research was not sufficient to report on the usefulness of this drug in a screening assay for the identification of A-T heterozygote samples. However, this chemotherapeutic agent induced a robust necrotic cell death profile in all the ATM-compromised fibroblasts, whereas Jurkat cells with intact ATM produced late apoptotic and necrotic cells. The cell death profiles produced by the fibroblast cells using this drug were distinct from the cell death profiles using any of the other treatments, and may be an indication of ATM compromised patients showing gross sensitivity to this drug. In view of the fact that chemotherapy and radiation-induced necrosis is usually associated with a poor prognosis, the results presented here may indicate that this specific drug may have a more adverse effect in A-T patients and carriers.

4.9.2.6 Cell death profiles induced by staurosporine.
Using staurosporine (2μM for 6 hours) it was possible to identify each of the three genotypes in the SV40 transformed lymphoblastoid cell lines. All the fibroblast cell lines showed complete resistance to staurosporine-induced cell death except A-T heterozygote GM08387, which produced a necrotic cell death profile. Interestingly, this cell line produced a similar cell death profile when treated with etoposide and doxorubicin, which like staurosporine are topoisomerase II inhibitors. As was the case with staurosporine, all the other fibroblast cells showed resistance to etoposide induced cell death. The mutation in the GM08387 cell line may therefore be important in studying the mechanism of cell death induced by topoisomerase II poisons, and may identify patients who may be clinically over sensitive to such treatments.

4.9.2.7 Cell death profiles induced by bleomycin.
Using bleomycin at 42.86-600μM for 6 hours, A-T heterozygote lymphoblastoid cells could be discriminated from the other two genotypes. However, the A-T homozygote and control cells could not be distinguished under these experimental conditions. All the untransformed fibroblast cells were resistant to bleomycin-induced cell death, and on this basis, bleomycin was not considered to be a useful agent for use in a screening assay for the discrimination of A-T heterozygotes.

4.9.2.8 Investigating reported differences in cell death profiles produced by transformed vs. untransformed cells.
Interestingly, the research also highlighted that cells derived from lymphoblast and fibroblast cell lineages responded differently to $\gamma$-radiation. This observation had previously been reported by Bebb et al. (2001) using EBV transformed lymphocytes compared to PBLs and also by Duchaud et al. (1996), who reported a similar observation using an untransformed fibroblast and EBV transformed lymphocyte cell line from the same donor, following exposure to $\gamma$-radiation. The current research therefore extends this observation by demonstrating similar results with SV40 transformed A-T homozygote; A-T heterozygote and control cell lines compared to untransformed A-T homozygote and heterozygote fibroblast cell lines in response to $\gamma$-radiation. Additionally, data is now also presented demonstrating similar results with SV40 transformed A-T homozygote, A-T heterozygote and control cell lines compared to untransformed A-T homozygote and heterozygote fibroblast cell lines in response to HP, etoposide, staurosporine and bleomycin treatment of the same cell lines.

Also of interest, was the response of the untransformed fibroblast cells to the three topoisomerase II poisons utilised in the study. While the majority of the A-T homozygote and heterozygote untransformed fibroblast cells showed considerable resistance to etoposide and staurosporine-induced cell death, the same cell lines all produced a necrotic cell death profile when treated with doxorubicin. Interestingly, both etoposide and doxorubicin produce ROS (Kurosu et al. 2003; Peng et al. 2005), and the same cell lines artificially placed under oxidative stress by treatment with HP, exhibited apoptotic cell death profiles. The reactive oxygen species HP can also undergo further modification intracellularly to produce the hydroxyl radical (Byung et al. 1994). Collectively, these results suggest that the type and amount of specific ROS generated in response to a variety of DNA-damaging agents might govern the cellular response to such agents, and would therefore be an interesting area for further study.

**4.9.2.9 Investigation to determine if mutational position within the ATM protein affected cell death profiles.**

Next, it had been intended to determine if the mode of cell death was different for cells carrying ATM mutations in different areas of the ATM gene (Amino-terminal domain, carboxyl-terminal domain, or the central part of the protein with sequence homology to RAD3). However using the SV40 transformed lymphoblastoid cell lines, cells within the same genotype tended to show similar cell death profiles, albeit with slight variations in the
numbers of apoptotic vs. necrotic cells. Untransformed fibroblasts also invariably produced similar cell death profiles within the same genotype in response to the same treatments. However, the majority of untransformed cell lines showed considerable resistance to γ-radiation, etoposide and staurosporine-induced cell death, but one A-T heterozygote cell line was able to respond to etoposide and staurosporine treatment. A-T heterozygote GM08387 produced significant numbers of necrotic cells in response to both etoposide and staurosporine treatment, and has a substitution [5932 (G>T)] in exon 42 resulting in truncation at codon 1978. This was the only fibroblast cell line to be affected by these DNA-damaging agents, and may therefore represent a mutation which confers hypersensitivity to clinical etoposide treatment. On this basis, this specific mutation may be an interesting area for further study.

4.10 Conclusions.
In conclusion, the data presented demonstrate that an optimised western blotting procedure could be used to confirm the presence of at least one ATM mutation in A-T homozygote and heterozygote cell lines compared to control cells. However, the assay would have benefited greatly from using ATM reference standards to quantify ATM expression, which are currently commercially unavailable.

Data presented also demonstrate it was possible to discriminate A-T heterozygotes from A-T homozygotes and controls in SV40 transformed lymphoblastoid cell lines using hydrogen peroxide. Additionally, A-T heterozygote untransformed fibroblast cells could be discriminated from A-T homozygote untransformed fibroblasts and a telomerase-transformed fibroblast control cell line.

Statistical analysis performed on the results of SV40 transformed cell lines treated with 100μM HP for 4 hours (Fig 3.3.2) demonstrated a significant increase in late apoptotic cells in the A-T homozygote cells compared to controls, and a significant reduction in necrotic cells in the A-T homozygote cells compared to controls (P <0.05 one-way ANOVA). Statistical analysis also demonstrated a significant reduction in late apoptotic cells in A-T heterozygote cells compared to both A-T homozygote and control cells, and a significant increase in necrotic cells in the A-T heterozygote cells compared to both the A-T homozygote and control cells (P <0.05 one-way ANOVA).
Statistical analysis performed on the results of untransformed A-T homozygote and heterozygote fibroblasts using a telomerase transformed fibroblast cell line as the control, treated with 100µM HP for 6 hours (Fig 3.3.3B), demonstrated the increase in early apoptotic cells in the A-T homozygote cells compared to the control was significant, as was the increase in apoptosis in the A-T homozygote cells compared to the control (P <0.05 one-way ANOVA). Statistical analysis also demonstrated a significant increase in early apoptotic cells in A-T heterozygote cells compared to both A-T homozygote and control cells, and a significant increase in apoptosis in the A-T heterozygote cells compared to both the A-T homozygote and control cells (P <0.05 one-way ANOVA). It was on this basis that a clear distinction could be made between the three genotypes in two different cell lineages in response to HP treatment.

Based on the data generated in this thesis, HP was demonstrated to be the most useful DNA-damaging agent in discriminating A-T heterozygotes from A-T homozygotes and controls, and the response of cell lines to oxidative stress may therefore be a reliable marker for A-T heterozygosity.

Data presented also demonstrate that addition of etoposide to cells artificially induced to undergo oxidative stress with hydrogen peroxide reduced the amount of externalised PS, indicated by the decrease in annexin-v binding. This is in agreement with the work of Tyurina et al. (2004). However, this effect was only demonstrated to significant amounts in two A-T homozygote and control SV40 transformed lymphoblastoid cell line, one A-T heterozygote untransformed heterozygote cell line and Jurkat control cells. The work by Tyurina was conducted on HL-60 cells, but data is now presented with A-T homozygote, A-T heterozygote and control SV40 transformed lymphoblastoid cell lines in addition to A-T homozygote and heterozygote untransformed fibroblast cell lines.

Using SV40 transformed lymphoblast cells; A-T heterozygote cell lines exhibited substantially more necrotic cell death than A-T homozygote and control cell lines when treated with hydrogen peroxide, etoposide and bleomycin. A-T heterozygotes may therefore show increased sensitivity over A-T homozygotes and controls to these treatments. This has important implications for cancer treatment as tissue necrosis following exposure to IR/chemotherapy is linked with a poor prognosis. Also of importance was the fact that necrotic cells were produced by both SV40 transformed
lymphoblasts and untransformed fibroblasts using several of these treatments. This data therefore highlights the importance of using an assay procedure that is able to measure necrosis as well as apoptosis in studying cell death profiles.

Finally, data is also presented in agreement with the work by Bebb et al. (2001) who reported that Epstein-Barr virus transformed lymphoblast cells respond differently to primary cells in response to ionising radiation. The current research therefore extends this observation by demonstrating similar results with SV40 transformed A-T homozygote; A-T heterozygote and control cell lines compared to untransformed A-T homozygote and heterozygote fibroblast cell lines in response to γ-radiation. Additionally, data is now also presented demonstrating similar results using the same cell lines in response to HP, etoposide, staurosporine and bleomycin treatment. The differential response of SV40 transformed cell lines and untransformed cell lines to these treatments calls into question whether virally transformed cell lines should be used to predict \textit{in vivo} cellular responses.
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Dear Applicant

Re: Application for Ethical Approval

Abberant tumour suppressor gene expression and the genetic predisposition to radiation-induced malignant disease.

Your research project proposal, as shown above, was amongst those considered at the meeting of the Applied Sciences Ethics Panel on 08/05/03.

I am pleased to inform you that your application for ethical approval was APPROVED subject to the conditions listed below – please read carefully.

Conditions of approval

That any changes in connection to the proposal as approved, are referred to the Panel.

That any untoward incident which occurs in connection with this proposal should be reported back to the Panel without delay.

Yours sincerely

Prof Ken Jones
Chair of Department of Applied Life Sciences Ethics Panel
Cardiff School of Health Sciences
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