How does p53 status influence cytotoxicity during regulatory *in vitro* mammalian cell genotoxicity tests?

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1 Abstract

The number of *in vitro* mammalian cell positives that do not correlate with follow-up *in vivo* genotoxicity and carcinogenicity testing is of concern (Kirkland, *et al*, 2005). These misleading *in vitro* positives result in significant animal usage, increased cost or loss of compounds from development. Using p53 competent human cells provides more predictive data for the assessment of human hazard and risk with less misleading *in vitro* positives compared to traditionally used rodent cell lines that lack wild-type p53 function (Fowler, *et al*, 2012a). However, it remains unclear whether the species origin or p53 status of the cells impacts their ability to accurately predict genotoxicity in the *in vitro* mammalian cell tests.

Cells lacking wild-type p53 may underestimate cytotoxicity with analysis of high concentrations genotoxiciy assessment, compared to a p53 functional cell line. Three closely related human lymphoblastoid cell lines that differ in their p53 status were tested; TK6 cells express wild-type p53, NH32 are p53 null and WTK1 overexpress mutant p53, similar to the commonly used rodent cells. Ethyl methanesulfonate (EMS), etoposide and paclitaxel (taxol) were tested according to regulatory guidelines (OECD, 2010) and cytotoxicity determined using relative populating doubling. Relative caspase-3/7 activity was also determined as a measure for apoptosis to aid interpretation of the cytotoxicity data.

NH32 were sensitive to the cytotoxic effects of EMS compared to TK6 and WTK1. In contrast NH32 underestimated cytotoxicity with etoposide compared to TK6 and WTK1. A similar cytotoxic response was observed with all three cell lines with taxol; however cytotoxicity was observed at lower concentrations in TK6. The apoptotic response to each compound in WTK1 was significantly reduced compared to TK6, which demonstrate a typical wild-type p53 response. NH32 demonstrated similar levels of apoptosis to WTK1 following etoposide and taxol treatments but was more similar to TK6 with EMS.

The results showed that p53 deficient cell lines do not consistently underestimate cytotoxicity and that cytotoxicity is drug specific, therefore other factors may be more relevant to the high number of *in vitro* positive in p53 compromised cells. An increase in mutability with loss of wild-type p53 function is discussed which lead to the increased sensitivity observed with the rodent cells lines (Fowler, *et al*, 2012a). Other differences between cells of human and rodent origin are also explored, identifying relevant factors in addition to p53 status.

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5 Acknowledgements

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6 Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of York or any other institution.

7 Introduction

The ability of chemicals to cause changes in the genetic material of both germ cells and somatic cells is of concern for human health; both to the immediate population and future generations (Spalding, 1987; Wassom, 1993). This is evident from the role that gene mutations as well as chromosome and numerical aberrations play in cancer development and inherited genetic disorders (Carere, *et al*, 2002; Popp and Bohlander, 2010). The role of genetic toxicology is to detect potential carcinogens and study the mechanisms of genetically hazardous chemicals and eliminate their impact on the environment and human population. Compounds that are positive in genetic toxicology tests have the potential to be human carcinogens and, or mutagens (Legator and Zimmering, 1975).

Conventional testing for carcinogenicity is lengthy and expensive often involving extensive animal usage. Genotoxicity testing offers a much quicker, relatively inexpensive and earlier screening tool in the development of a compound, as well as providing alternatives to extensive animal usage (Long, 2007). The result of early *in vitro* genotoxicity tests can determine the fate of a compound and it's progression through development. Therefore, it is of concern that the relevance of some genotoxicity tests has been questioned in recent years (Kirkland, *et al*, 2007a).

There are a high number of *in vitro* mammalian cell positives that do not correlate with follow-up *in vivo* genotoxicity and carcinogenicity testing (Kirkland, *et al*, 2005). These misleading (or false) *in vitro* positives result in compounds being unnecessarily removed from development or the requirement for further testing with unnecessary extra cost, time and animal use at an early stage of compound development all aimed at checking the relevance of the *in vitro* positive result.

There are a number of factors that may influence the high rate of misleading *in vitro* positives (Kirkland, *et al*, 2006; 2007a). Fowler, *et al*, (2012a; 2012b) has demonstrated the importance of cell type and the cytotoxicity measures used on the genotoxicity outcome. Recommendations have been made to consider the p53 status of the cell lines used for genotoxicity to improve the relevance of the *in vitro* tests (COM, 2011; Kirkland, 2011; Pfuhler, *et al*, 2011). It has been further demonstrated that the cell species origin (human *versus* rodent) should be considered over p53 status (Hashimoto, *et al*, 2011; Whitwell, *et al*, 2012). However it is still unclear whether p53 status or species origin is

most relevant. In addition, underestimation of cytotoxicity has been shown to increase the incidence of positive results. Using cytotoxicity measures based on cell proliferation has been shown to allow the selection of lower, more pharmacologically relevant concentrations for genotoxicity assessment thus helping to avoid artefacts (Fowler, *et al*, 2012b).

With these considerations in mind, the aim of this study was to investigate the influence of p53 function on the estimation of cytotoxicity within a regulatory *in vitro* genotoxicity assay: the *in vitro* mammalian cell micronucleus test (OECD, 2010).

7.1 Regulatory genetic toxicology (purpose and requirements)

In order for a chemical to gain appropriate licence for use (as a pharmaceutical, industrial chemical, food additive, or cosmetic ingredient), the manufacturer must satisfy regulators, such as the United States Food and Drug Administration (FDA) or the United Kingdom (UK) Medical and Healthcare Products Regulatory Agency (MHRA) by providing evidence that the compound is not a potential hazard for human health at the intended usage concentrations. Currently no single genotoxicity test is capable of detecting all relevant genotoxic endpoints, therefore UK and international regulatory guidelines recommend a defined battery of genotoxicity tests with distinct stages covering *in vitro* and *in vivo* endpoints (Figure 1), depending on its intended use (COM, 2011; ICH, 2012).

Stage one involves a minimal battery of *in vitro* tests capable of detecting the formation of gene mutations and chromosomal changes (large-scale chromosomal damage, recombination and numerical chromosome changes). Compounds are initially screened for genotoxicity in the bacterial gene mutation test (Ames test), known to detect a majority of genotoxic carcinogens (Zeiger, *et al*, 1992). The bacterial test is not considered appropriate to detect all DNA damage relevant to mammalian cells, therefore a mammalian *in vitro* test for clastogenicity (chromosome aberration or micronucleus assay) and aneugenicity (micronucleus assay) is also performed. This provides a highly sensitive battery for detecting the majority of genotoxic compounds (Kirkland, *et al*, 2011). If a chemical is expected to have direct human exposure, a further test to detect gene mutations in mammalian cells, such as the forward mutation mouse lymphoma *tk* assay, is required as part of the minimal test battery (ICH, 2012).

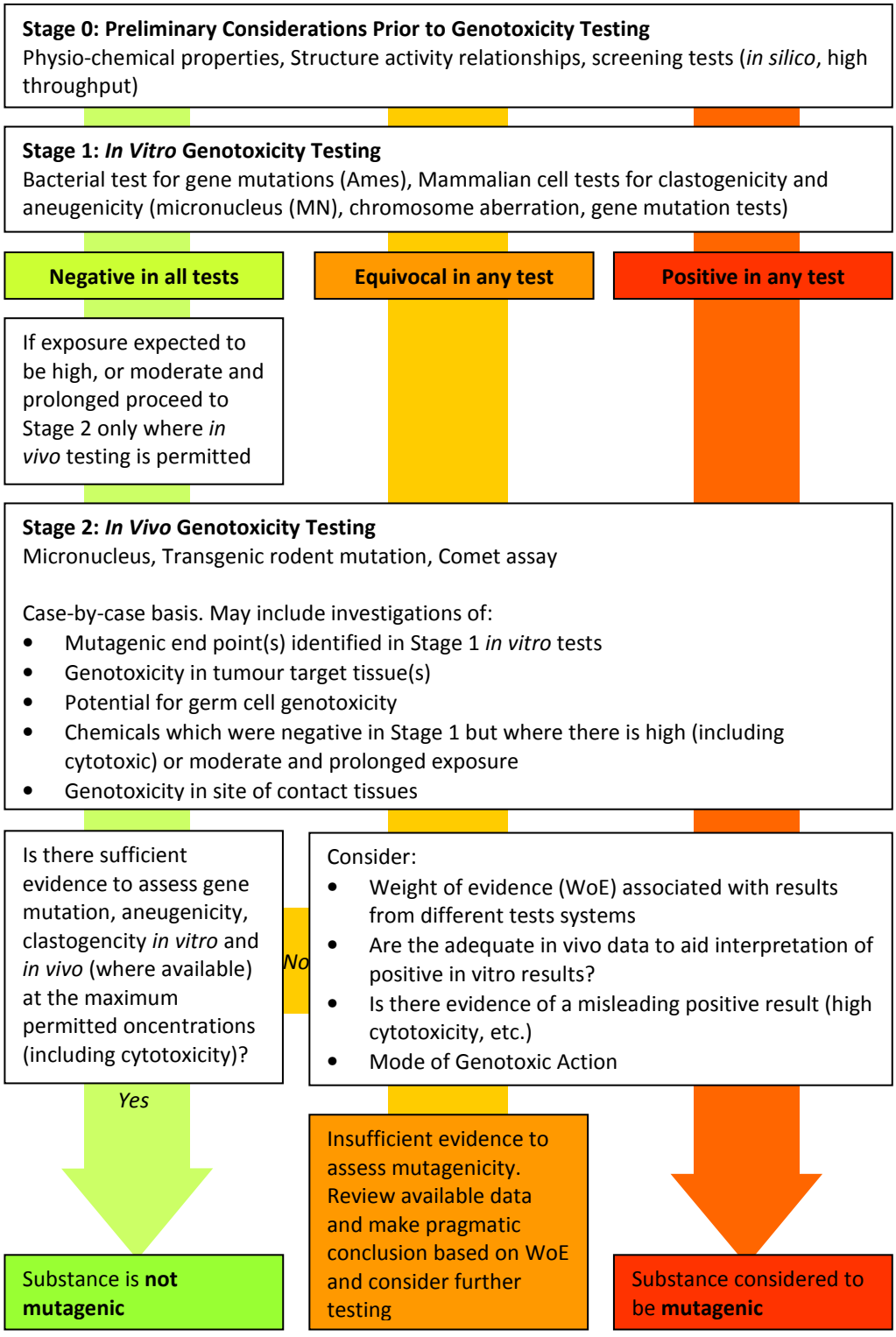


Figure 1: Overview of Strategy for genotoxicity testing.

Figure adapted from COM, 2011.

Depending on the type of chemical and its intended use, a clear negative at stage one of testing is enough to conclude that that a chemical has no mutagenic activity and no further testing is required (COM, 2011). For pharmaceuticals and compounds where human exposure is expected to be high or moderate and sustained, a second stage of testing is required encompassing additional factors relevant to human health (adsorption, distribution, metabolism and excretion). This can be a single additional *in vivo* test for chromosomal damage using rodent haemopoietic cells (ICH, 2012).

A clear positive in any stage one test is sufficient to define a chemical as an *in vitro* mutagen and additional testing is required to assess the relevance and activity in *in vivo* somatic cells, where justified. Further *in vitro* mechanistic studies may also be used to evaluate potential dose-response relationships for genotoxicity and establish possible threshold (no-effect) levels below which DNA damage does not occur. Mechanisms such as inhibition of DNA synthesis, overloading of defence mechanisms, aneuploidy resulting from interaction with microtubules, topoisomerase inhibition and high cytotoxicity can demonstrate a threshold (non-linear) dose-response relationships for genotoxicity and provide 'safe' concentrations for further testing (Henderson, *et al*, 2000, Gollapudi, *et al*, 2013).

In vivo testing is designed to mimic route of exposure, duration of treatment, metabolism and target organ exposure at concentrations relevant to humans. Negative *in vivo* data can aid interpretation and provide supporting evidence for a lack of relevance of an *in vitro* positive result for the intended use of a compound. However, even if the initial *in vivo* test is negative, a second *in vivo* test is still required following an equivocal or positive *in vitro* result (ICH, 2012).

In summary, the genotoxicity test battery is a tool for hazard identification and provides information to assess the potential risk of a novel compound. The current test battery is considered to be sufficiently sensitive to predict the majority of genotoxins. However, sensitivity often comes at the price of the specificity, especially with the mammalian cell tests. With tighter controls, greater awareness of animal use, cost and time in the development and testing of novel compounds this lack of specificity cannot continue.

7.1.1 The *in vitro* micronucleus assay in mammalian cells

Since micronuclei were first studied in cultured human cells exposed to chemicals in the 1970s (Countryman and Heddle, 1976), the mammalian cell micronucleus test has developed into an important tool within regulatory genotoxicity testing. Micronuclei are formed when chromosome fragments or whole chromosomes fail to be incorporated into one of the daughter nuclei during cell division either spontaneous or following chemically induced chromosomal damage. A nuclear envelope forms around the lagging chromosomal material giving the appearance of a small interphase nucleus which is easily identified and analysed by microscopy (Fenech, 2000).

The micronucleus assay offers a more practical endpoint for analysis than the traditional analysis of metaphase chromosomes. As a result, it is used extensively for genotoxicity testing, both as a pre-screening tool in many research laboratories and during the first stage of the regulatory test battery. The assay detects chromosome breakage and chromosome loss events and with the use of centromeric labelling (Schuler, *et al*, 1997), provides information on the mechanisms of chromosome damage and micronucleus formation. Published validation of the *in vitro* micronucleus methodology shows that the assay is reliable, reproducible, transferable and predictive (Corvi, *et al*, 2008). The assay was fully endorsed for application within regulatory genotoxicity testing with the adoption of an internationally accepted OECD guideline (OECD, 2010).

The current OECD guideline (2010) states that the treatment and recovery duration should cover all phases of the cell cycle to allow the test chemical to interact with all potentially relevant cellular components for genotoxicity (Lorge, *et al*, 2006). Asynchronous cell cultures are generally treated with the test compound for a short (pulse) period (3-6 hours) and, following the removal of the test chemical, are allowed to recover for 1.5-2 cell cycles. If negative or equivocal results are obtained from the pulse treatment a further extended treatment is required to confirm absence of genotoxicity. If the mode of action of the test chemical is known to significantly interfere with the cell cycle (e.g. nucleoside analogues), treatment and recovery durations can be adapted to allow a longer treatment and, or recovery period.

7.2 Misleading *in vitro* positives

The validity of a genotoxicity test can be described in terms of sensitivity or specificity. Sensitivity is the proportion of known carcinogens that give a positive result in a genotoxicity test; specificity is the proportion of known non-carcinogens which give a negative result (Cooper, *et al*, 1979). The most predictive genotoxicity tests will have a high sensitivity and high specificity and therefore be relevant to human health.

Current *in vitro* genotoxicity tests have a high sensitivity but suffer from a relatively low specificity with a high number of misleading (false) positives (Thybaud, *et al*, 2007; Pfuhler, *et al*, 2011). These misleading *in vitro* positives are generally negative in the Ames assay and are either non-carcinogenic or are rodent carcinogens but with an assumed non-mutagenic mode of action (Kirkland, *et al*, 2008). The *in vitro* positive often results from analysis of inappropriately high concentrations at high levels of cytotoxicity that would be irrelevant to any therapeutic concentration in humans. Cell lines that have questionable DNA repair mechanisms have been routinely used for mammalian cell tests and have been suggested as a possible cause for the prevalence of *in vitro* positive results (Kirkland, *et a*l, 2007a; 2007b).

The consequence of misleading *in vitro* positives is that heavy reliance has been placed on the *in vivo* genotoxicity and carcinogenicity data to aid the interpretation of the *in vitro* results and to provide weight of evidence to question the relevance of the *in vitro* result allowing a compound to progress through regulatory testing (Kirkland, *et al*, 2007b). A positive *in vitro* result can, therefore, lead to greater and earlier *in vivo* testing. One publication suggested that if 200-400 pharmaceuticals per year gave misleading positive results, the additional animal testing would be estimated at approximately 5,000-10,000 rodents per year (Kirkland, *et al*, 2007a). In many cases compounds are removed from development following positive results *in vitro* due to the significant increase in development time and cost.

There are a high number of reported *in vitro* mammalian cell positives that do not correlate with follow-up *in vivo* genotoxicity and carcinogenicity testing (Kirkland, *et al*, 2005). Improvements to the existing assay design and evaluation criteria (not testing to excessive levels of cytotoxicity, pH or osmolality, for example) have improved the specificity of the *in vitro* mammalian cell tests. A recent report from one pharmaceutical company which

reviewed data generated from their own laboratory demonstrated a reduced rate of positive results from assumed non-carcinogenic pharmaceuticals; 15% compared to the 61% misleading (or false) positive rate demonstrated from the Kirkland, *et al* (2005) review of the published literature from the same endpoint (Fellows, *et al*, 2011). In addition, Fowler, *et al* (2012a) were not able to reproduce positive results for 9 out of 19 previously reported misleading positive results from mammalian cell tests *in vitro* using a modern Protocol design. However, 10 of the misleading positive chemicals still remained positive in at least one of the cell lines tested, even with current testing criteria applied; therefore the impact of misleading *in vitro* positive results still remains a topical issue.

Misleading *in vitro* positives often result in compounds being unnecessarily removed from development or the requirement for further testing with unnecessary extra cost, time and animal use at an early stage of compound development all aimed at checking the relevance of the *in vitro* positive result.

It is clear that that the mammalian cell tests must be further improved and the high incidence of *in vitro* positives can not continue to be supported. Initiatives such as the National Centre of Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (NC3Rs/LASA, 2009) and the European Centre for the Validation of Alternative Methods to Animal Experimentation (ECVAM) (Pfuhler, *et al*, 2009) have placed greater emphasis on improving the quality of *in vitro* data to try and minimise the requirement on *in vivo* testing, so that the number of animals used can be reduced.

7.2.1 Cytotoxicity and selection of upper concentrations

In all *in vitro* genotoxicity tests used for human risk assessment compounds must be tested up to the recommendations maximum top concentration (5000 µg/mL or 10 mM, for example[OECD, 2010]), the limit of solubility in culture, or the highest level permitted by cytotoxicity. In this case, 'cytotoxicity' is used to describe the level of both cell death and growth inhibition (cytostasis) induced by a test chemical for *in vitro* genotoxicity testing (Scott, *et al*, 1998; Fellows and O'Donovan, 2007).

Initially a concentration achieving 70-80 % cytotoxicity was recommended as the maximum top concentration as at higher concentrations there would be insufficient cells available for analysis (Scott, *et al*, 1991); however, a much more complex picture has

evolved with the relationship between cytotoxicity and genotoxicity. Although some genotoxins are only correctly predicted when the concentrations tested induce some degree of cytotoxicity (Kirkland, 1992; Galloway, 2000), the majority of DNA damaging compounds induce an *in vitro* positive response without marked increases in initial levels of cytotoxicity (Greenwood, *et al*, 2004, Kirkland, 2010). Alternatively, misleading *in vitro* positives can occur by secondary mechanisms associated with cytotoxicity and not as a result of direct DNA damage. Double strand breaks and chromosome aberrations are known to be induced by non-mutagenic non-carcinogens only at cytotoxic concentrations, usually above 50% cytotoxicity, that are not relevant to human risk (Storer, *et al*, 1996; Hilliard, *et al*, 1998), which does not occur at lower, more pharmacologically relevant concentrations. Galloway (2000) collected *in vitro* genotoxicity data from 253 chemicals from 27 pharmaceutical and chemical companies and contract laboratories and determined that a an upper limit of 50-60% cytotoxicity would allow detection of the majority of DNA-damaging agents, whilst significantly reducing the proportion of misleading positive results. As a result, current recommend a limit of 50-60% (OECD, 2010) or up to 50% cytotoxicity (ICH, 2012) for the maximum test concentration for the genotoxicity test.

Although the limit of cytotoxicity is relatively well defined in the test guidelines, a method of estimating cytotoxicity is not as clearly prescribed. A number of methods are suggested, with some measurements only considering cell death or cytostasis induced by a test chemical, where as other measures of cytotoxicity will consider both. The relevant sensitivity of the cytotoxicity measure must be taken into account as they can lead to different outcomes and selection of different concentrations for genotoxicity assessment (Fellows and O'Donovan, 2007; Fellows, *et al*, 2008; Lorge, *et al*, 2008).

Fowler, *et al*, (2012b) recently demonstrated the importance of selecting an accurate cytotoxicity measure in the *in vitro* mammalian cell micronucleus assay. Four methods of estimating cytotoxicity were assessed using compounds that have previously reported misleading positives results. Measures of cytotoxicity that take proliferation as well as survival into account, such as relative population doubling (RPD) consistently selected lower concentrations for genotoxicity analysis at the upper cytotoxicity limit (approximately 55% cytotoxicity). For a number of compounds this led to a negative result using RPD. However, using measures that underestimate cytotoxicity resulted in a greater number of misleading positive results due to analysing higher concentrations for potential

genotoxicity. Detection of known genotoxins was still possible using RPD (Kirkland, 2010).

7.2.2 Importance of cell type

International guidelines for the testing of chemicals recommend various cell lines or primary cell cultures, such as primary human peripheral blood lymphocytes, human (TK6, HepG2) and rodent cells lines (CHO, V79, CHL, syrian hamster embryo and mouse L5178Y). Justification for the choice of cell type is based on large historical data sets. Other cell lines can be validated based on acceptability criteria described in the guidelines (OECD, 1997; 2010).

Due to issues with the limited availability and donor-to-door variability (Odagiri, *et al*, 1997) of primary human lymphocytes, immortalised cell lines are commonly used for genetic toxiciology testing (Lorge, *et al*, 2006; Kirsch-Volders, *et al*, 2011). Cell lines derived from malignancies in rodents have been traditionally used over cell lines derived from human origin (Aardema, *et al*, 2006; Wakata, *et al*, 2006; Oliver, *et al*, 2006). p53 mutations are prevalent in the tumours subsequently used by researchers to develop the immortalised cell lines currently in use (Levine, *et al*, 1991; Blakey, *et al*, 2008). At the time of their introduction little or nothing was known about the role of p53 yet these cells have been used to generate many years of historical data and knowledge within genotoxicity testing. Changing established methods and introducing new, more stable cell lines will require further validation, despite the urgent need to improve the tests.

It is important to consider the consequence of cell type on the sensitivity and specificity of the *in vitro* mammalian cell test. Using rodent cell lines (CHO, CHL and V79) with impaired p53 function has been shown to result in a greater number of positive results compared to p53 competent human cells (TK6, human peripheral blood lymphocytes and HepG2) using known misleading *in vitro* positives (Fowler, *et al*, 2012a). These observations are acknowledged in recent guidelines (COM, 2010). However, the question still remains whether the loss of wild-type p53 or the rodent origin of these cell lines plays the greater role in the generation of misleading *in vitro* positives.

7.3 Tumour protein p53

Human p53 (encoded by *TP53*) is a tumour suppressor protein playing a central role in maintaining genomic stability and preventing tumour development (Ryan, *et al*, 2001). The presence of p53 gene mutations in more than 50% all tumours and disrupted p53 signalling in a further 30% of tumours (80% in total) demonstrates the significance of the tumour suppressor role of wild-type p53 (Olivier, *et al*, 2002; Joerger and Fersht, 2011), and why it has been extensively studied since its discovery over 30 years ago (Levin and Oren, 2009).

The p53 protein consists of 393 amino acids and can be divided into five domains (Figure 2): (i) the amino-terminus (region 1-42) containing the highly conserved domain (HCD) I, the acidic transactivation domain and the MDM2 binding site; (ii) second transactivation domain (43-92) and proline rich domain; (iii) the DNA binding domain (101-306) containing HCD II to V, the most commonly mutated region of the p53 protein; (iv) the oligomerisation domain (307-355) consists of a beta-strand, followed by an alpha-helix necessary for dimerisation, as p53 is composed of a dimer of two dimers; (v) the carboxy-terminus of p53 (356-393) contains 3 nuclear localisation signals and a non-specific DNA binding domain that binds to damaged DNA, which is also involved in down regulation of DNA binding of the central domain.

p53 is usually tightly regulated in normal, unstressed cells and maintained at low levels through targeted degradation by MDM2, an E3 ubiquitin ligase (Alarcon-Vargas and Ronai, 2002). Various intrinsic and extrinsic cellular stresses, including DNA damage, hypoxia, oxidative damage, spindle damage, oncogene activation and DNA replication stress, initiate specific signalling pathways that mediate modifications to p53 (Figure 3). These interrupt the p53-MDM2 interactions, which leads to increased levels and activity of the p53 protein (Gu and Zho, 2012). As p53 becomes stable it accumulates and acts as a transcription factor with a number of stress specific effects. Depending on the conditions of cell cycle progression, the type and duration of the stress inflicted on the cell, p53 selectively activates genes that will result in cell-cycle arrest, DNA repair, differentiation, apoptosis, senescence and energy metabolism (Vogelstein, *et al*, 2000; Vousden and Lu, 2002; Feng, *et al*, 2011).

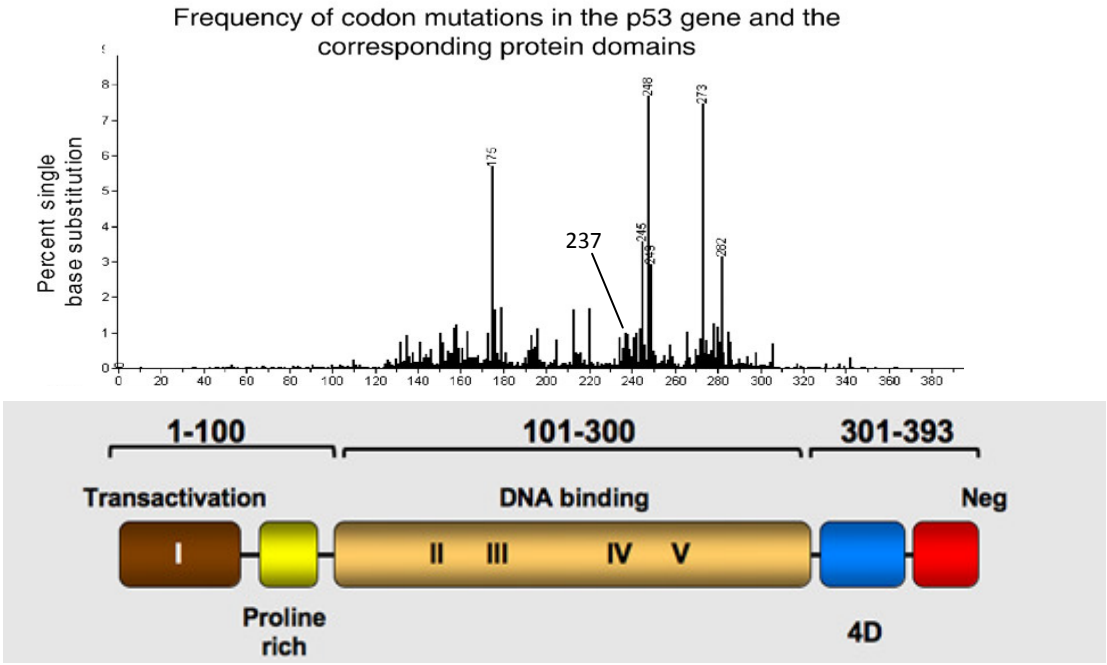


Figure 2: Simplified schematic of human p53.

A schematic of the 393 amino acid long human p53 protein with its major domains highlighted: (i) N-terminus, containing two transactivation domains, (ii) DNA binding domain, (iii) C-terminus. Frequency of mutations along the different codons of the p53 gene with the hotspot mutants (175, 245, 248, 273 and 282) shown in the core DNA binding domain. Location of p53 mutation relevant to the human lymphoblastoid cell line WTK1 (237) is also identified. Reproduced from: http://p53.free.fr/p53_info/p53_Protein.html (accessed 7 January 2013) and Malaguarnera, *et al* (2007).

The central role of p53 in maintaining genomic stability and the cellular response to cytotoxicity (Figure 3) suggests that when p53 is not properly regulated the impact on genotoxicity assessment could be dramatic. p53 plays a major role in DNA repair and the maintenance of genomic stability (Liu, *et al*, 2004), and cells lacking proper p53 regulation have the potential to show genetic drift in culture at high passage numbers (Kirkland, *et al*, 2007a). Activation of p53 causes cell cycle arrest (at the G1/S, G2/M), allowing time for the cell to overcome stress and repair DNA damage (Stewart, *et al*, 1995; Amundson, *et al*., 1998). If DNA repair is unsuccessful p53-mediated apoptosis is triggered (Shu, *et al*, 2007), removing irreparably damaged cells from the population and eliminating developing tumour cells (Yee and Vousden, 2005). p53 will directly influence cytotoxicity in mammalian cell genotoxicity tests and cells with impaired p53 would be expected to have a less sensitive phenotype compared to p53 competent cells.

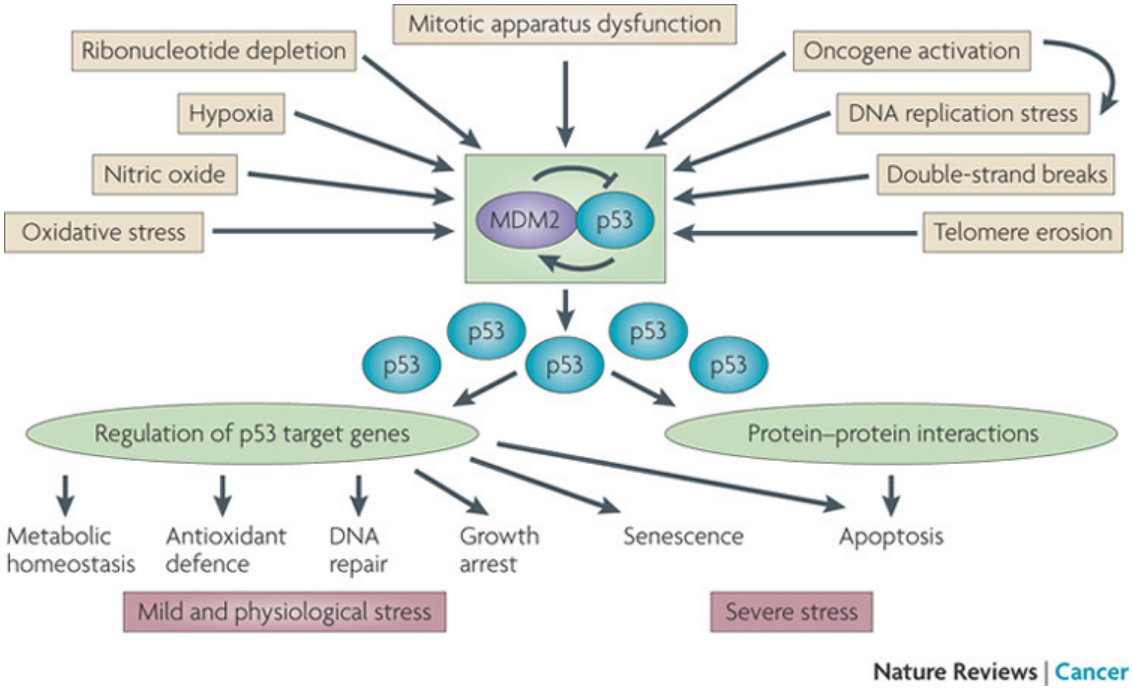


Figure 3: Simplified diagram of the functional interactions of the p53 pathway.
Under normal cellular conditions, p53 is maintained at low levels and activity in a p53-MDM2 feedback loop. Following stress signals (a representative example of these are given above) p53 is released from the feedback loop allowing p53 levels and activity to increase. Diagram taken from Levine and Oren, 2009.

7.4 Cell lines (wild-type p53, mutant p53 and p53 knock-out)

Many of the established rodent cell lines that are used for genotoxicity testing overexpress mutant p53 (CHO-K1, CHO-WBL and V79 cells) and wild-type p53 (CHL cells) protein. p53 is not induced in these cell lines following ionising irradiation (Chuang, *et al*, 1999; Hu, *et al*, 1999). Fowler, *et al*., (2012a) has demonstrated a greater chance of a misleading positive using p53 compromised rodent cell lines than human p53 functional cells. Although differences in the p53 status have been highlighted as an influence for these phenotypes, it must be acknowledged that the p53 mutant cells were all of hamster origin and the p53 functional cells were all human derived cell lines. Therefore, the species origin of the cells may have a greater relevance on their sensitivity to chemical insult. This could be further investigated with isogenic cell lines that differ in their p53 status to assess the influence of p53 in the *in vitro* genotoxicity assays.

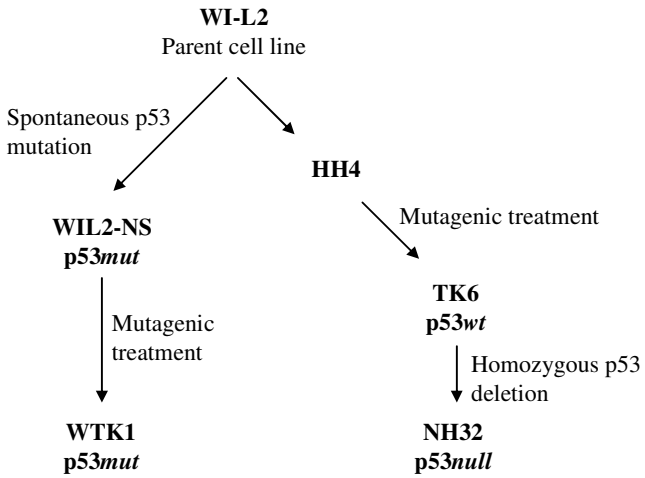
The closely related TK6, NH32 and WTK1 cell lines are derived from the same parent human lymphoblastoid cell line (Little, *at al*, 1995; Xia, *et al*, 1995; Chuang, *et al*, 1999). TK6 cells express functional wild-type p53, NH32 are p53 null derived from TK6 through

targeted p53 knockout and WTK1 overexpress mutant p53 (cells were kindly provided by Professor Howard Liber, Department of Environmental and Radiological Health Sciences, Colorado State University, USA)

TK6 cells are used extensively for genotoxicity analysis due to their well documented wild-type p53 status (Islaih, *et al*., 2005, Hastwell, *et al*., 2006, Nesslany and Marzin, 2010; Shi, *et al*., 2010). The TK6 cell line was first established from the HH4 cell line by repeated treatment with the frameshift mutagen, ICR-191, in order to select a thymidine kinase (*tk*) heterozygote for mutagenic analysis using the *tk* gene mutation assay (Skopek, *et al*., 1978; Liber and Thilly, 1982). HH4 was itself, a subclone of the WI-L2 lymphoblastoid cell line (Figure 4), which was selected for its ability to form colonies in soft agarose without the need for a feeder layer of fibroblasts (Skopek, *et al*., 1978; Gupta, 1980). The WI-L2 cell line was established in 1968 from cell cultures recovered from a 5-year-old male's spleen, which was removed in the treatment of hereditary splenocytosis (Levy, *et al*., 1968).

NH32 is a double *p53* knockout cell line derived directly from the TK6 cell line using promoterless gene targeting of the neomycin phosphotransferase and the histidinol dehydrogenase genes into exon 2 of the *p53* gene, resulting in no constitutive and no induction of p53 protein (Chuang, *et al*., 1999).

WTK1 cells originate from the same parental cell line as TK6 (Figure 4). The WTK1 cell line was established from WI-L2-NS by repeated treatment with the frameshift mutagen ICR-191 in order to select a thymidine kinase (*tk*) heterozygote (Benjamin, *et al*., 1991). WI-L2-NS is a subclone of WI-L2, the same donor cell line as HH4 and TK6. The WTK1 karyotype (47, XY, +13, 14q+, 21p+) is indistinguishable from TK6. WTK1 overexpress mutant p53; direct sequencing of TK6 and WTK1 revealed a single base pair substitution, a transition of ATG to ATA in codon 237 of exon 7 of the *p53* gene of WTK1. This has resulted in a methionine to isoleucine amino acid substitution in the p53 protein at residue 237 (M237I). In contrast the TK6 *p53* gene showed a wild-type sequence (Little, *et al*., 1995; Xia, *et al*., 1995).



Cell line	p53 status	Reason for loss of p53 function
TK6	Wild-type	-
WTK1	Mutant	Homozygous G → A mutation at codon 237 (exon 7) resulting in a methionine to isoleucine
NH32	Null	Targeted homozygous knockout

Figure 4: Cell linage and p53 status of TK6, NH32 and WTK1 cell lines.
Cell linage and p53 status of the human lymphoblastoid cell lines used in this study show the relationship between TK6, NH32 and WTK1. TK6 and WTK1 were selected following treatments with ICR-191 in order to select *tk* heterozygote cells for the *tk* gene mutation assay. NH32 were derived from TK6 following targeted knockout of *p53*. TK6 cells have remained wild-type (*wt*) p53, whereas, a spontaneous point mutation occurred in the linage of WTK1 resulting in a mutated (*mut*) p53.

The M237 amino acid lies within the L3 loop of the DNA binding surface of p53 (Figure 2)**,** the structure of which is stabilised by a zinc ion, termed the zinc binding region (Joerger and Fersht, 2007). The M237I mutation has a typical phenotype of mutations in the zinc binding region, such as R175H (one of the hotspot p53 mutants found in cancers [Olivier, *et al*., 2002]) and C242S, and appears to have major inhibitory effects on the function of p53. The R175H mutation causes a globally denatured state of p53 resulting in complete loss of wild-type p53 binding affinity and DNA-binding activity (Bullock, *et al*., 2000; Dearth, *et al*., 2006). The M237I mutation, found in the WTK1 cell line, is expected to have a similar effect on the function of p53, as the p53 binding affinity of the M237I p53 variant reduced to below 15% (Bullock, *et al*., 2000). Both M237I and R175H mutants do not bind to antibody raised against wild-type p53 (PAb1620), but have been shown to bind to antibody raised against denatured p53 (PAb240) (Ory, *et al*., 1994; Rolley, *et al*., 1995). p53 is not noticeably induced in WTK1 following γ-irradiation and the p21 protein,

known to be regulated by p53, showed no induction following γ-irradiation. TK6, however, showed a robust induction of p53 and consequently p21 following the same level of γ-irradiation (Zhang, *et al*, 2007). The loss of function of the M237I p53 mutant is further demonstrated by the overexpression of the mutant protein in WTK1 cells. Wild-type p53 is maintained at low levels by a regulatory feedback loop with MDM2. Non functional mutant p53 is unable to stimulate transcription of MDM2 in the negative feedback loop, resulting in accumulation and overexpression of the mutant protein (Midgley and Lane, 1997). These results suggest that the DNA binding of the M237I p53 mutant is severely restricted, if not lost completely.

7.5 Cellular impact of chemicals used in this study

Three genotoxins were selected in order to examine the effects of p53 status on assessment of cytotoxicity in the *in vitro* mammalian cell micronucleus assay. They were chosen to highlight the likelihood that p53 compromised cell lines underestimate cytotoxicity in the micronucleus test.

7.5.1 Ethyl methanesulfonate

Ethyl methanesulfonate (EMS) is an ethylating agent that is known to be mutagenic. Ethylation of DNA results in unstable apurinic sites leading to replication fork stalling and breaks in DNA, inducing p53 mediated cell cycle arrest and apoptosis (Zhou, *et al*., 2001; Stopper and Lutz, 2002).

Replication fork stalling and the resulting formation of double strand breaks causes accumulation of protein kinases ATM, ATR and DNA-PKs which increase the stability and activity of p53. Cell cycle arrest can occur through inactivation of specific cyclin/Cdk2 complexes required for cell cycle progression, by p53 associated proteins such as p21 (El-Deiry, *et al*, 19954; Akyűz, *et al*, 2002).

p53 has been shown to play another, potentially more significant role in response to EMS. Base excision repair (BER) is considered to be the main pathway handling damage by alkylating agents (Seo, *et al*, 2002). p53 has been shown to play a significant role in the BER pathway through direct interaction with DNA polymerase *β* (*β*-pol), a mechanism that is absent in p53-null cell lines (Akyűz, *et al*, 2002, Seo, *et al*, 2002).

7.5.2 Etoposide

Etoposide is a topoisomerase II inhibitor. Topoisomerases are responsible for regulating DNA topology (Schoeffler and Berger, 2008). The topoisomerase II enzyme aids the relaxation of over-coiled DNA by making transient double strand breaks allowing DNA to pass through the break before religation. Etoposide stabilises the binding of topoisomerase II to the cleaved DNA generating a reaction product termed 'stable cleavable complex' (Watt and Hickson, 1994). Essentially etoposide treatment converts topoisomerase II into a cell poison, resulting in high levels of double strand breaks regardless of the cell cycle stage (Hande, 2006).

The accumulation of double strand breaks by etoposide is regulated by ATM, ATR and DNA-PKs (Shrivastav, *et al*, 2008). Activation of p53 occurs via phosphorylation, in particular the Ser-15 in p53. This results in the up-regulation of proteins which are involved in cell cycle control and apoptosis. Etoposide is a potent inducer of p53-mediated apoptosis via transcription of pro-apoptotic proteins such as Fas receptors and members of the Bcl-2 family, in particular Bax and eventually inducing apoptosis with the participation of the caspase family of proteins (Karpinich, *et al*, 2002; Brantley-Finley, *et al*., 2003).

7.5.3 Paclitaxel

Paclitaxel (taxol) enhances the polymerisation of tubulin to stabilise microtubules, which blocks cells in the G2/M phase of the cell cycle. Mitotic irregularities causes nuclear accumulation of p53 resulting in p53-dependent cell cycle arrest or apoptosis (Rathinasamy, *et al*., 2010).

In response to mitotic spindle damage, multiple mitotic kinases phosphorylate p53 to activate p53 mediate signalling pathways. The stabilised p53 regulates the expression of mitotic kinases, such as BubR1, which is a potent inducer of apoptosis, in order to prevent the replication of chromosomally abnormal cells (Oikawa, *et al*; Ha, *et al*, 2007).

7.6 Goals

Current regulatory guidelines recommend a number of cell lines for use in the *in vitro* mammalian cell tests, these include rodent cell lines with compromised p53 function, which are more likely to give a misleading (false) positive result in the test compared to

human cell lines with functional p53 (Fowler, *et al*., 2012a). More recent guidelines and proposed changes to guidelines state that the p53 status of the cell must be considered (COM, 2010; ICH, 2012). This ambiguity within the guidelines requires resolution in order for the *in vitro* mammalian cell tests to be improved.

Selecting a human, p53 competent cell line coupled with a measure that does not underestimate cytotoxicity leads to a reduction in the potential for a misleading positive result (Fowler, *et al*., 2012b). It is therefore hypothesised that a cell line deficient in p53 or with impaired p53 function will lead to the underestimation of cytotoxicity, increasing the concentration at which a compound can be analysed for the genotoxicity endpoint and providing a greater chance of a false positive result.

The specific goals of my research were to show that p53 compromised cell lines underestimate cytotoxicity following treatment with known genotoxins that are expected to induce a p53-mediated response.

8 Materials and Methods

8.1 Details of chemicals

Chemical	CAS number	Molecular weight	Purity	Diluent
Ethyl methanesulfonate	62-50-0	124.16	>98%	DMSO
Etoposide	33419-42-0	588.56	>98%	DMSO
Paclitaxel	33069-62-4	853.91	>97%	DMSO

Table 1: Details of chemicals showing manufacturer, CAS number, molecular weight, purity, diluents.

All chemicals were obtained from Sigma-Aldrich, UK and formulated in reagent grade dimethyl sulfoxide (DMSO, Sigma-Aldrich). 100X stock solutions were prepared approximately two hours prior to treatment and were added directly to cultures with mixing.

Information for other chemicals, solutions and reagents used is supplied (where available) following their first appearance in the text. The contents and activity of some commercial products are propriety and cannot be obtained, therefore only the manufacturer's details are given.

8.2 Cell lines and routine culturing

8.2.1 Culture media

Cells were maintained in complete RPMI medium, prepared as detailed below:

Per 500 mL volume: 50 mL heat inactivated foetal calf serum (Gibco®, UK), 5 mL 1000 IU/1000 µg/mL Penicillin/Streptomycin (PAA, UK) made up to 500 mL with Roswell Park Memorial Institute (RPMI) 1640 medium, with GlutaMAX^{TM}.

8.2.2 Cell lines

TK6, WTK1 and NH32 cells were obtained from Dr Howard Liber, Colorado State University, USA. Master stocks were created and held under nitrogen at Covance Laboratories Ltd., Harrogate, UK. Frozen stocks were stored at approximately

1 x 10^6 cells/mL 10% DMSO in complete RPMI. Cells stocks were verified as mycoplasma free.

8.2.3 Culture initiation and maintenance

At least seven days prior to each experiment, cells were resuscitated from frozen stocks by rapidly thawing the vial(s) at 37°C. The resulting suspension was immediately diluted in 50 mL pre-warmed (at 37°C) complete RPMI to give a final concentration of approximately 2 x 10^4 cells/mL in a 75 cm^2 vented tissue culture flask and incubated at 37°C, 5% CO_2, 95% humidity.

Cell cultures were subcultured at least once prior to treatment (every 2-3 days as appropriate) at an initial density between 0.5 and 1 x 10^5 cells/mL and maintained such that the culture density did not exceed approximately 1 x 10^6 cells/mL at the time of passage.

Cells for treatment were subcultured at a density of approximately 1 x 10^5 cells/mL in 4.95 mL culture medium on the day prior to treatment. Cells were maintained is suspension by mixing prior to incubation 37 ± 1°C, 5% (v/v) CO2 in air, 95% humidity for treatment the following day.

8.3 p53 status of cells

8.3.1 Treatment

Exponentially growing cultures of all cell types were treated with either DMSO (vehicle controls) or in the presence of etoposide (0.0325 and 0.0625 µg/mL) for 24 hours.

The final culture volume was 5 mL at the time of treatment. Cells were maintained in suspension by mixing prior to incubation on a slope at 37±1°C, 5% (v/v) CO_2 in air, 95% humidity for the twenty-four hours.

8.3.2 Post-treatment

Twenty-four hours from the start of treatment, cells were sampled (1:200) into Isoton II (Beckman Coulter, UK) prior to counting on a coulter counter to determine the cell concentration of each culture.

Following determination of culture concentrations, an equal number of cells from each culture (1 x 10^6 cells) were sampled into a labelled centrifuge tube to ensure equal protein content in each cell sample, and cell pelleted by centrifugation at 200 *g* for 5 minutes in a Sigma 4-15 centrifuge.

The supernatant was removed and cells resuspended in 2 mL PBS, pH 6.8 (Severn Biotech, UK). Cells were pelleted by centrifugation at 200 *g* for 5 minutes and the wash step was repeated.

The supernatant was removed, being careful to remove all supernatant without disrupting the cell pellet. 100 µL of 2X SDS loading buffer was added to each culture and cell immediately resuspended and heated in a water bath at 90°C for 10 minutes. The samples were immediately chilled on ice prior to mixing by vortex and stored at -20°C until use.

8.3.3 Western blot analysis

8% gel was added to the running chamber with 1X running buffer. 20 µL of each sample (previously corrected to 5 x 10^5 cells/mL, maintaining an equal protein content between samples) was loaded into the gel. 15 µL of a molecular weight marker (Precision Plus Protein™ All Blue, Bio-Rad, UK) was added to one well for a marker control. The gel was run for approximately 1 hour at 150 V.

Filter papers and the nitrocellulose membrane were soaked in 1X transfer buffer. The gel was placed in a 'transfer sandwich' (filter paper-membrane-gel-filter paper) in the transfer chamber containing 1X transfer buffer. The protein was transferred from the gel to the membrane for 1.5 hour at 77 mA at 2-8°C.

The membrane was removed and submerged in 1% bovine serum albumin (BSA) buffer (blocking buffer) and incubated at room temperature for approximately 1 hour with shaking.

The membrane was then stained with anti-p53 primary antibody (1:5,000 with 1% BSA buffer) for 2 hours at room temperature with rocking. The membrane was then washed in 1% BSA buffer to remove excess stain, followed by three washes in 1% BSA buffer for 10 minutes each, at room temperature with rocking.

Horseradish peroxidise (HRP)-labelled secondary antibody (1:10,000 with 1% BSA buffer) was added to the membrane and incubated at room temperature for 1 hour with rocking, followed by three washed in 1% BSA buffer for 10 minutes each with rocking.

The membrane was developed using the EZ-Enhanced Chemiluminescence (EZ-ECL) Detection kit (Biological industries, Israel). The detection solution was prepared by mixing EZ-ECL Solution A and EZ-ECL solution B (1:1), which was allowed to equilibrate for approximately 10 minutes prior to use. The detection solution was added to the membrane for approximately 1 minute at room temperature in the dark. A sheet of film was placed over the blot and exposed for 10 seconds.

Quantification of protein levels was performed using ImageJ 1.47 (Rasband, W.S., ImageJ, U.S. National Institutes of Heath [NIH], Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012). p53 protein intensity was normalised to the concurrent histone control and presented as a fold change in the level of p53 protein in the etoposide treated cultures of TK6, WTK1 and NH32 cells compared to the concurrent vehicle treated controls.

8.4 Cell cycle times (average generation times)

8.4.1 Treatment

Exponentially growing cultures of all cell types were treated for 24 hours in the presence of 10 µM BrdU (Sigma, UK).

The final culture volume was 5 mL at the time of treatment. Cells were maintained in suspension by mixing prior to incubation on a slope at 37±1°C, 5% (v/v) CO_2 in air, 95% humidity for the 24 hours.

8.4.2 Post-treatment

Approximately 2 hours prior to harvest, colchicine was added at a final concentration of 1 µg/mL in order to arrest cells in metaphase for analysis.

Following 24 hours exposure to 10 µM BrdU, cells were collected by centrifugation and treated with a hypotonic solution of 75 mM KCl at 37°C for 15 minutes to swell the cells

to aid microscope analysis. Cells were fixed with several washes by centrifugation with a methanol:acetic acid (3:1) fixative. Tubes were stored in fixative for a minimum of 3 hours at 2-8°C prior to slide preparation and staining.

8.4.3 Slide analysis

Fixed cells were collected by centrifugation and resuspended in a few drops of 45% v/v aqueous acetic acid. Approximately 50 µL of cell suspension was dropped onto each of two slides per replicate and dried on a slide drier.

Slides were stained by immersion in Hoechst 33258 stain for 25 minutes at room temperature, protected from light. Slides were rinsed twice in McIlvaines Buffer, pH 8.0 at room temperature and stored flat, immersed in fresh McIlvaines buffer, pH 8.0 at 40°C. Slides were exposed to UV light from 365 nm bulb for 35 minutes at 40°C in McIlvaines buffer, pH 8.0. Slides were then immersed in 4% v/v Giemsa stain in Gurr's phosphate buffer for 35 minutes, rinsed once in Gurr's phosphate buffer followed by a rinse in ROHP and allowed to air dry. Once dry a cover slip was added with the application of DPX mountant.

100 cells were analysed per culture (six cultures per cell type) by light microscopy and the relative proportion of cells in 1st, 2nd and 3rd division recorded, as previously described (Palma, *et al*, 1993, Corona-Rivera, *et al*, 2005).

8.4.4 Calculation of average generation time

The cell cycle durations for the TK6, WTK1 and NH32 cell types were determined using BrdU incorporation to calculate the average generation time (AGT) (Palma, *et al*, 1993, Corona-Rivera, *et al*, 2005), as shown below:

$$AGT\ (h) = \frac{\text{Time of BrdU incorporation (h) x number of cell scored}}{M1 + (2 \times M2) + (3 \times M3)}$$

Where; M1, M2, M3 are the relative proportion of cells in that division (i.e. 1st, 2nd, 3rd division, respectively).

8.5 Cytotoxicity treatments

8.5.1 Treatment and recovery

Population doublings (PD) were determined for each cell line from the time the culture initiation to the time of treatment (approximately 24 hours). Treatments were only performed if PD were above approximately 1.5 for TK6 and WTK1 and 1.0 for NH32 (ensuring cell cultures were in exponential growth). Cultures of all cell types were treated with the vehicle (six replicates) or test chemicals (in triplicate) (Table 2), under the following conditions:

Cell line	Treatment	S-9	Addition of test chemical (h)	Removal of test chemical (h)	Harvest time (h)
TK6 and WIL2-NS	3+24, -S-9	-	0	3	27
NH32	3+40, -S-9	-	0	3	43
TK6 and WIL2-NS	24+24, -S-9	-	0	24	48
NH32	24+40, -S-9	-	0	24	64

Table 2: Treatment conditions for each experimental occasion and cell type.

The final culture volume was 5 mL at the time of treatment. Cells were maintained in suspension by mixing prior to incubation on a slope at 37±1°C, 5% (v/v) CO_2 in air, 95% humidity for the designated treatment time.

8.5.2 Baseline cell counts

Immediately prior to treatment four cultures were sampled (1:200) into Isoton II prior to counting on a coulter counter for determination cytotoxicity based on population doubling.

8.5.3 Post-treatment and recovery

Following the exposure period (3 h or 24h), test chemicals were removed and cells washed with sterile saline by centrifugation. Cultures were resuspended in a final culture volume of 5 mL in fresh pre-warmed complete RPMI medium (see 8.2.1).

8.5.4 Determination of relative population doubling (cytotoxicity)

From each coulter cells were sampled (1:200) into Isoton II prior to counting on a coulter counter for determination cytotoxicity based on population doubling.

Relative population doubling (RPD), expressed as a percentage relative to a concurrent vehicle control, was used to estimate cell survival and calculated as below:

Population doubling (PD) = $[\log (N \div X_0)] \div \log 2$

Where; N = the post treatment cell count, X_0 = the cell count at the time of treatment (baseline).

RPD (%) = $(PD_{treat} \div PD_{vc}) \times 100$

Where; PD_{treat} = mean treated PD value, PD_{vc} = mean vehicle control PD value.

8.5.5 Determination of caspase-3/7 activity (apoptosis)

The Capase-Glo® 3/7 assay (Promega, UK) was performed according to manufacturer's protocol to assess relative caspase-3 and -7 activity as a measure of apoptosis. 100 µL of cell suspension was aliquoted from each culture (from all vehicle and test chemical treated cultures) at time of harvest and added to corresponding wells of a 96-well Luminometer plate. 100µL of Caspase Reagent was added to each sample in the 96-well plate and mixed on a plate-shaker for at least 30 seconds (300-500 rpm). Following mixing, plates were incubated at room temperature for at least 1 hour (and no more than 3 hours).

Luminescence was analysed using a Spectramax Gemini EM plate reader (Molecular Devices). Relative luminescent units (RLU) of a blank (complete RPMI medium) control were subtracted from each culture. RLU was calculated per 1000 cells and an increase in caspase-3 and -7 activity was presented as a fold increase in test chemical treated cultures relative to the vehicle control, as previously described (Fowler, *et al*, 2012a).

8.5.6 Statistics

The results have been presented as mean ± standard deviation (SD). The mean values of each cell line were compared by one-sided analysis of variance (ANOVA) with pairwise t-

test performed to compare each cell line where ANOVA demonstrated significance. A *p* value of ≤0.05 was considered statistically significant. Where ANOVA was not significant the results of the pairwise t-test was not reported.

9 Results

9.1 Cell line characterisation

9.1.1 Aims

In order to use the cells in the proposed experiments (outlined in section 9.2) the p53 status of each cell line was confirmed. The expression of both constitutive and induced p53 protein was tested in all three cell lines (TK6, NH32 and WTK1) to see whether:

(i) the TK6 cell line expresses normal constitutive levels of p53 that can be induced following etoposide treatment;
(ii) the NH32 cell line does not express any constitutive levels of p53 and that the protein is not induced or present following etoposide treatment;
(iii) the WTK1 cell line overexpresses p53, which is not induced following treatment with etoposide.

In order to relate these data to current in vitro genotoxicity testing, the experiments are required to be conducted in concordance with current international guidelines. The *in vitro* micronucleus assay is a well established and validated genotoxicity test with internationally accepted guidelines (OECD, 2012) and is used extensively for both investigatory and regulatory genotoxicity testing. A critical consideration for the micronucleus assay is ensuring that cells have undergone mitosis during the treatment or the post-treatment recovery period, to reduce the risk of false negative results. Therefore, the cell cycle times for each cell line will be determined to tailor the recovery period for each cell line tested.

9.1.2 Experimental approach

In order to determine the presence or absence of both the constitutive and induced p53 protein cell cultures were treated in the presence of etoposide for 24 hours, as previously described (Section 8.3). The concentrations selected were expected to induce cytotoxicity and induce functional p53. Negative (vehicle) controls were included to demonstrate and confirm constitutive expression of p53, without chemical insult.

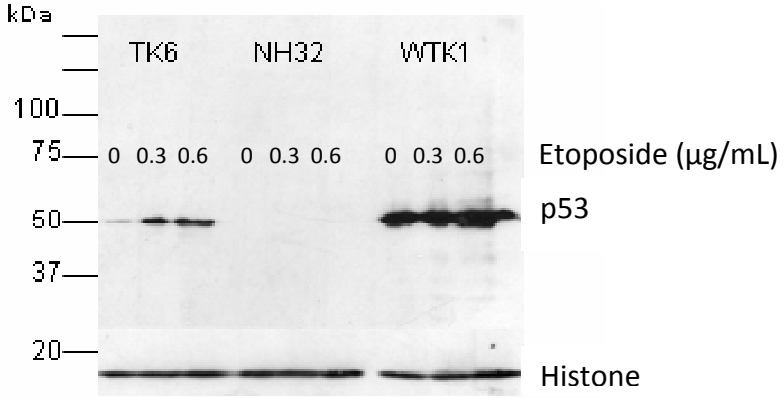
Western blot technique using an anti-p53 mouse monoclonal antibody (clone DO-1) against both wild-type and mutant human p53 was employed to detect p53 protein in negative and etoposide treated cultures of TK6, NH32 and WTK1 cells. This antibody has been used extensively for p53 protein expression in TK6, WTK1 and NH32 (Little, *et al*, 1995; Chuang, *et al*, 1999; Chou and Huang, 2002; Zhang, *et al*, 2007).

The cell cycle times were assessed using BrdU incorporation over a 24 hour period. BrdU is a synthetic nucleoside analogue of thymidine, incorporated as a substitute of thymidine in the DNA of replicating cells during S phase of the cell cycle. BrdU substitution is assessed with Hoechst plus Giemsa differential chromatid staining, also known as the fluorescence plus Giemsa staining technique (Perry and Wolff, 1974; Goto, *et al*, 1975). Slides are exposed to UV light and heat, which results in the photolysis of the BrdU-containing DNA. The addition of Hoechst sensitises and enhances the photolytic effect on BrdU-incorporated DNA (Goto, *et al*, 1978; González-Gil and Navarrete, 1982). Metaphases that have progressed through first, second and third cell divisions can be easily distinguished using light microscopy. Where both chromatids have incorporated BrdU into only a single strand of DNA (first cell cycle) they will stain dark blue with Giemsa. Following a second cell cycle one chromatid will have incorporated BrdU into both strands of DNA and will stain a light blue-grey with Geimsa, in contrast to dark staining of the unifilarly substituted chromatid (Figure 6). Cells that have passed through the third cell cycle are distinguishable as they will have approximately 75% of chromatids that are stained light blue-grey (i.e. bifilarly BrdU-incorporated).

9.1.3 p53 status

Low levels of p53 protein were detected in untreated TK6 cell cultures, demonstrating the expected constitutive levels of a wild-type p53 cell lines (Figure 5). p53 levels were clearly induced in TK6 cells following 24 hour exposure to both 0.3 µg/mL and 0.6 µg/mL etoposide, demonstrating the expected normal wild-type response of p53. Dosimetric analysis of the western blot showed a 9.17 and 10.59 fold induction of p53 protein following 0.3 and 0.6 µg/mL etoposide relative to the vehicle treated culture (Figure 5b). In contrast, NH32 cells harbour no p53, either in untreated cultures or following treatment with etoposide at concentrations that induce p53 in the wild-type TK6 cell line, confirming their previously reported p53 null status (Chuang, et al, 1999; Léger and Drobetsky, 2002; Li, *et al*, 2006).

a)



b)

Cell type	Concentration (µg/mL)	p53 intensity	Histone intensity	Normalised values	Fold induction
TK6	0	382.9	5477.2	0.0699	1.00
	0.3	3770.4	5879.1	0.6413	9.17
	0.6	5015.8	6776.6	0.7402	10.59
NH32	0	0	6688.0	0	-
	0.3	0	6423.2	0	-
	0.6	0	7098.0	0	-
WTK1	0	10409.4	5890.7	1.7671	1.00
	0.3	11169.3	6664.9	1.6758	0.95
	0.6	12583.0	7342.3	1.7138	0.97

Figure 5: p53 protein levels in TK6, NH32 and WTK1 cells.

a) Western blot analysis of p53 protein levels in TK6, NH32 and WTK1 cells, showing results from untreated cells (0 µg/mL), 0.3 µg/mL and 0.6 µg/mL etoposide treated cell cultures. b) Quantification of protein levels was performed using ImageJ 1.47 (NIH, Bethesda, Maryland, USA). p53 protein intensity was normalised to the histone control and presented as a fold change in the level of p53 protein in the etoposide treated cultures relative to the level of p53 in the concurrent vehicle controls for TK6, WTK1 and NH32 cells. Whole cell lysates were prepared from untreated cell cultures and cell cultures treated for 24 hours with etoposide (0.3 and 0.6 µg/mL). Cell number (protein) were corrected to equal concentration following treatment and stored in 4x loading buffer prior to western blotting. Total p53 was probed by immunoblotting using mouse anti-p53 (DO01) monoclonal antibody, which recognises p53 wild-type and mutant forms. The housekeeping protein histone H3 is included as a loading control.

The results show that p53 levels in untreated WTK1 cell cultures are much greater than the normal p53 levels in the untreated TK6 (Figure 5). This is expected and correlates with reports of over expression of mutant p53 in WTK1 cells, with some reporting up to 4-times the constitutive levels observed in WTK1 compared to TK6 (Little, *et al*, 1995; Xia, *et al* 1995). Dosimetric analysis showed that there was no accumulation of p53 protein in WTK1 cell cultures treated with 0.3 µg/mL or 0.6 µg/mL etoposide (Figure 5b). p53 has

previously been shown not to be noticeably induced in WTK1 cells following irradiation with both X- and γ-rays or following chemical treatment (Little, *et al*, 1995; Xia, *et al*, 1995, Zhang, *et al*, 2007).

Levels of the concurrent histone loading control are similar or equal in each sample (Figure 5). Therefore, increases and absence of p53 signals are accepted as a true response and not as a result of underexposure during processing or from significant differences in total protein levels in each sample.

9.1.4 Cell cycle times

In order to determine treatment times for analysis of cytotoxicity and the induction of apoptosis, the average generation times for each cell type were determined by BrdU incorporation. The number of cells in first, second and third division were recorded for each cell type following 24 hours culture in medium containing BrdU (Table 3). Staining was optimised by using various UV light exposure times to enable clear differentiation between bifilarly and unifilarly BrdU-incorporated chromatids (Figure 6).

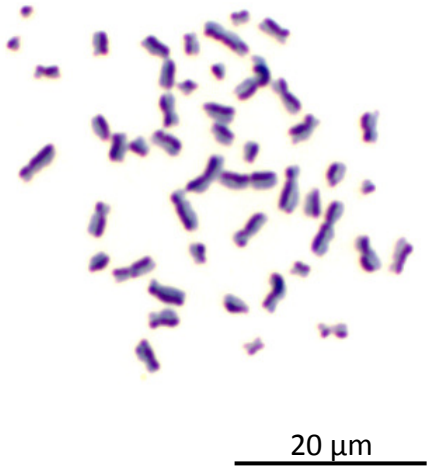


Figure 6: TK6 metaphase in the second cell division.
Representative image of a Hoechst plus Giemsa stained metaphase of TK6 cells that has progressed through a second cell cycle (with 50% of chromatids stained light blue-grey and 50% dark blue) following 24 hours in the presence of BrdU. Bifilarly BrdU-incorporated chromatids stain dark blue, where as unifilarly incorporated chromatids stain light blue-grey. The above images were captured using a Zeiss Axio Imager fluorescent microscope equipped with a CoolCube CCD camera (MetaSystems) and a x63 objective. The image was captured and processed using *in situ* imaging system (ISIS) imaging software (MetaSystems).

BrdU incorporation indicated that the average generation time for TK6 and WTK1 cells cycles were 12.9 and 13.3 hours, respectively, with an average generation time of 23.1 hours for NH32 cell cultures (Table 3) which indicated a longer cell cycle time for NH32 under the same culture conditions for each of the three cell types.

For TK6 and WTK1 cultures a recovery time of 1.5 to 2 cell cycles would be the equivalent to approximately 19 to 26 hours, with a 24 hour recovery selected for experimentation. For NH32 cell an equivalent recovery of would be between approximately 34 to 46 hours, with 40 hour recovery period used in the treatments.

Cell type	Replicate	Cells in each division			Average generation time (SD)
		Fist division (M1)	Second division (M2)	Third division (M3)	
TK6	A	13	85	2	12.7 h
	B	16	83	1	13.0 h
	C	13	87	0	12.8 h
	D	12	88	0	12.8 h
	E	16	84	0	13.0 h
	F	15	85	0	13.0 h
	Total	85	512	3	12.9 (±0.1) h
NH32	A	93	7	0	22.4 h
	B	96	4	0	23.1 h
	C	98	2	0	23.5 h
	D	96	4	0	23.1 h
	E	97	3	0	23.3 h
	F	96	4	0	23.1 h
	Total	576	24	0	23.1 (±0.4) h
WTK1	A	23	77	0	13.6 h
	B	21	79	0	13.4 h
	C	19	81	0	13.3 h
	D	18	82	0	13.2 h
	E	22	78	0	13.5 h
	F	17	83	0	13.1 h
	Total	120	480	0	13.3 (±0.2) h

Table 3: Analysis of cell cycle average generation time, Cell cycle time.
Average generation times of the human lymphoblastoid p53 wild-type TK6, null NH32 and mutant WTK1 was determined using BrdU incorporation for 24 hours. Differentiation between cells in first, second and thirds division was performed using the Hoechst plus Giemsa staining technique. Average generation time (h) = (Total time with BrdU [h] x total number of cells analysed) ÷ (M1 + (2 x M2) + (3 x M3)). SD = standard deviation of six replicates.

9.2 Influence of p53 status on cytotoxicity

9.2.1 Introduction and aims

The data presented in Chapter 9 demonstrates the differences in both intrinsic and induced p53 between the TK6, NH32 and WTK1 and, therefore, that the cell lines were appropriate to investigate the influence of p53 status on cytotoxicity and assess the goals discussed in Section 7.6.

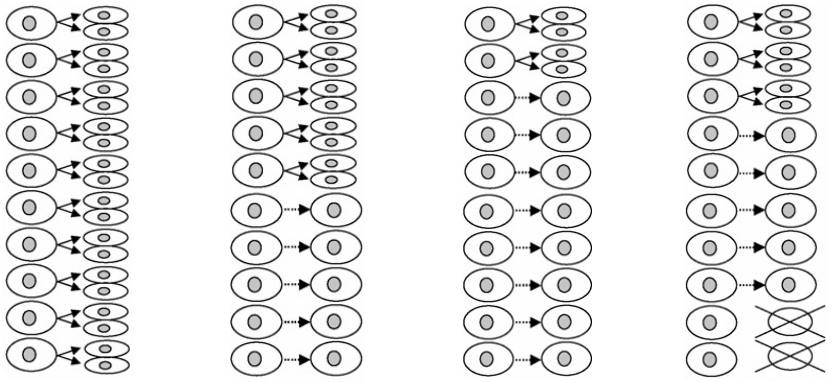
The aim of these experiments was to demonstrate that the loss of p53 wild-type function in NH32 and WTK1 cell lines underestimate cytotoxicity as measured by relative population doubling (RPD), and provide discussion for the impact of testing inappropriately high concentrations for genotoxicity analysis when these cell lines are compared to a p53 wild-type cell line (TK6).

9.2.2 Experimental approach

In order to demonstrate the impact of p53 status on cytotoxicity, three closely related human lymphoblastoid cell lines (TK6, NH32 andWTK1), differing in p53 status were treated with EMS, etoposide and taxol according to international guidelines for the *in vitro* micronucleus (IVMN) assay (OECD, 2010). The three compounds were selected as they have been shown to illicit p53 induction and cytotoxic responses via p53 induction (Section 7.5).

As discussed previously, in order to accurately assess the extent of cytotoxicity both cell death and cytostasis induced by a compound must be considered (Fellows and O'Donovan, 2007; O'Donovan, 2013). For these experiments, relative population doubling (RPD) was used to determine cytotoxicity induced by each of the chemicals tested. RPD was selected as it is an efficient technique that is widely used when assessing the levels of cytotoxicity for *in vitro* cytogenetics assays used for human health risk assessment (Kirkland, 2010; OECD, 2010). Relative population doubling (RPD) has been shown to be a more accurate cytotoxicity measure, where other measures can underestimate cytotoxicity (Greenwood, *et al*, 2004, Fowler, *et al*, 2012b). For determination of RPD a baseline cell count is determined for the start of treatment. The number of population doublings (PDs) from the beginning to the end of treatment is calculated for each culture by determining the cell number at the time of harvest. The PD value is then compared against the concurrent

controls to determine the percentage RPD. RPD therefore takes into account both loss of cells from the population through cell death and cytostasis and is therefore a preferred measure of cytotoxicity (Fellows and O'Donovan, 2007). Figure 7 demonstrates these calculations and provides justification for using RPD. For example, if only half of the population of cells have divided from the start of treatment to the point of harvest (Figure 7b), RCC (based on the harvest counts alone and therefore does not consider growth over the treatment period) would equal 25% cytotoxicity, where as cytotoxicity would be 42% (58% RPD) measured by RPD, a more accurate assessment of the reduction in cell numbers compared to the control.



	a; control culture	b; treated culture 1	c; treated culture 2	d; treated culture 3
Initial cell number (start of treatment)	10 cells	10 cells	10 cells	10 cells
Final cell number (harvest)	20 cells	15 cells	12 cells	11 cells
PD *	1.0	0.6	0.3	0.2
RPD (Cytotoxicity)*	100% (0%)	58% (42%)	26% (74%)	14% (86%)
RCC (Cytotoxicity) **	100% (0%)	75% (25%)	60% (40%)	55% (45%)

Figure 7: Theoretical examples of cytotoxicity using relative population doubling.
a) An example of a vehicle control with division in 100% of cells; and examples of chemically treated cultures; b) with inhibition of division in 50% of cells; c) with inhibition of division in 80% of cells; d) with inhibition of cell division and cell death. PD: population doubling; RPD: relative population doubling; RCC: relative cell counts; * calculated according to formulae in Section 8.5.4; ** RCC (%) = (treated final cell number ÷ control final cell number) x 100, cytotoxicity based on RCC (%) = 100 - RCC. Figure adapted from Lorge, *et al*, 2008.

p53 is known to control induction of apoptosis following cellular stresses (Fridman and Lowe, 2003; Haupt, *et al*, 2003). In order to discuss differences in cytotoxicity, apoptosis was assessed in the three cell lines for each compound using the Caspase-Glo® 3/7 assay

(Promega, UK). Caspase-3 and -7 belong to the caspase family of proteases, which play a central role in initiating and executing apoptosis (Launay, *at al*, 2005). Caspase-3 and -7 are activated by caspases that initiate apoptosis, such as Caspase-2, -8, -9 and -10, either directly or though regulation of other proteins such as Bid which causes cytotochrome c release from the mitochondria. Active caspases-3 and -7 then execute apoptosis via subsequent cleavage of cellular proteins such as poly(ADP-ribose) polymerase, lamin, fodrin, and also Bcl-2, for example (Fan, *et al*, 2005). In the caspase-Glo assay, active caspase-3 and -7 cleaves a specific substrate, resulting in a luminescent signal. Luminescence is proportional to the amount of caspase activity present in the sample. Luminescence in each culture is calculated per cell and compared to the control, giving a fold increase in caspase-3 and -7 activity over the control (baseline) values.

For all experiments described TK6, NH32 and WTK1 treatments were performed and analysed in parallel using the same test chemical formulations. Triplicate replicates were performed for each concentration tested and an average of the triplicate values are displayed in the figures below.

9.2.3 Cytotoxicity

To assess the influence of p53 on the cytotoxicity of EMS, etoposide and taxol, RPD in wild-type TK6, p53 null NH32 and p53 mutant WTK1 was determined. Determination of cytotoxicity following treatments with each of the 3 compounds show two clear points; i) none of the cell lines used consistently underestimated cytotoxicity and ii) none of the cell lines showed greater cytotoxic response, compared to the other cell types (Figure 8). This suggests that induction of cytotoxicity in cell lines with different p53 status is compound-specific.

Following a three hour exposure to EMS and recovery for approximately 1.5 cell cycles in fresh culture medium, TK6 and WTK1 show similar levels of cytotoxicity across the concentration range tested. RPD steadily decreased from 92% to 31% (TK6) and 79% to 41% (WTK1) between 50 and 600 µg/mL EMS. Unexpectedly the p53 null NH32 cells were significantly more sensitive to cytotoxicity induced by EMS at all concentrations tested, with the exception of 50 µg/mL, where WTK1 and NH32 are compared (Figure 8a and Table 4a). With NH32, there was a sharp decrease in RPD from 66% to 42% between

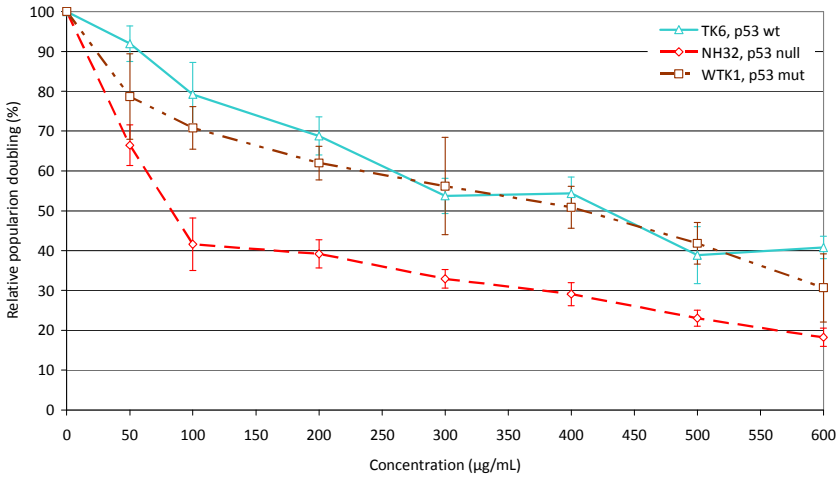
50 µg/mL and 100 µg/mL followed by a steady decline to 16% RPD of the control at 600 µg/mL EMS.

With a three hour exposure to etoposide and recovery for approximately 1.5 cell cycles, cytotoxicity induced in NH32 was significantly less compared to both TK6 and WTK1 at all concentration tested (Figure 8b and Table 4b), in contrast to the response observed with EMS treatment. Cytotoxicity induced in TK6 was also significantly more than that induced by etoposide in WTK1 at all concentrations tested, excluding the lowest and highest two concentrations tested (0.05, 0.5 and 0.7 µg/mL) (Figure 8b and Table 4b). At the highest concentration tested (0.7 µg/mL) NH32 cells demonstrated an RPD of 49% where as TK6 and WTK1 demonstrated RPDs of 0% and 1% (100% and 99% cytotoxicity), respectively. To reach a similar level of cytotoxicity (40-50% RPD) as seen in NH32 cells, etoposide concentrations of only 0.15 µg/mL and 0.2 µg/mL were required in TK6 and WTK1, respectively.

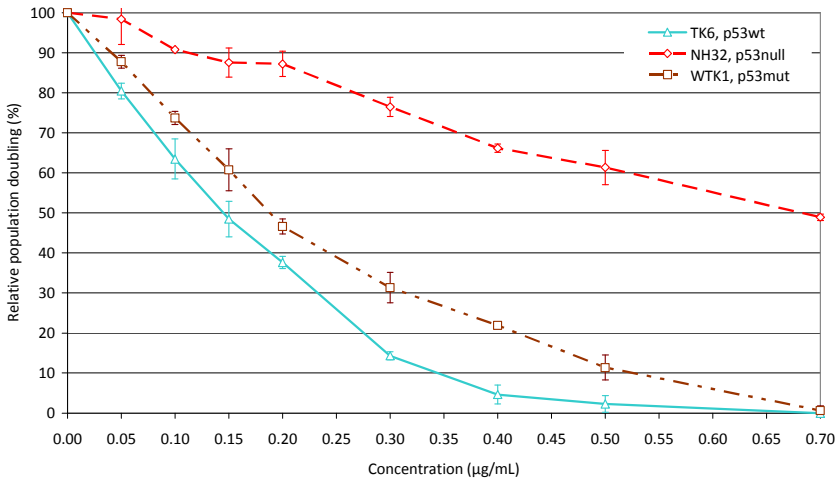
Following a twenty-four hour exposure to taxol and recovery for approximately 1.5 cell cycles, WTK1 and NH32 show similar levels of cytotoxicity across the concentration range tested, however significant differences were observed at 0.003 and 0.006 µg/mL (Figure 8c and Table 4c). RPD decreased from 90% and 80% at 0.003 µg/mL to 66% and 60% of the control at 0.0045 µg/mL, respectively. 0.006 µg/mL taxol was required to achieve a 51% and 46% reduction in RPD in NH32 and WTK1 cells, respectively. The p53 wild-type TK6 cells showed similar levels of cytotoxicity to WTK1 and NH32 up to 0.003 µg/mL (83% RPD). At 0.0045 µg/mL and above TK6 was significantly more sensitive to cytotoxicity induced by taxol (Figure 8c and Table 4c); with TK6 there was a much greater decrease in RPD from 83% to 46% of the control between 0.003 and 0.0045 µg/mL.

Experiments were considered valid as vehicle (diluent) control replicates consistently demonstrated that they had passed through more than approximately 1.5 to 2 cell divisions, as described in section 9.1.4. Therefore, any decreases in the RPD of the test compound treated cultures were due to the effects of treatment and not suboptimal culturing conditions.

a) EMS



b) Etoposide



c) Taxol

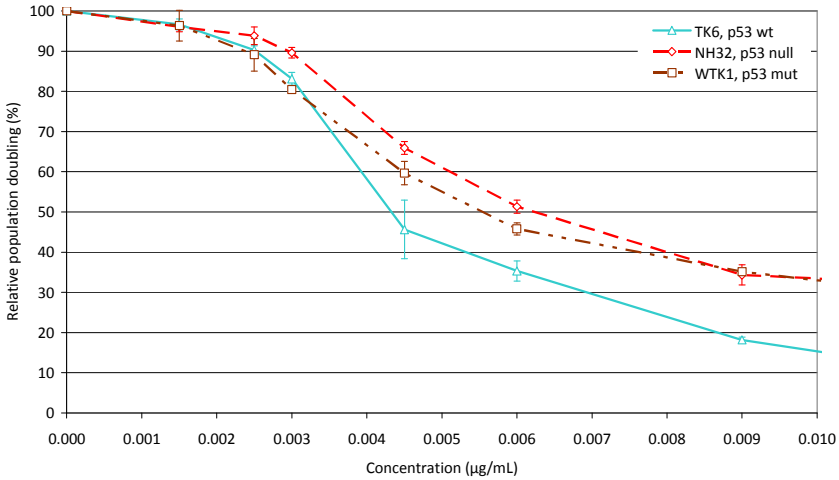


Figure 8: Cytotoxicity in human lymphoblastoid cells differing in p53 status.

Relative population doubling was determined in p53 wild-typeTK6 (△), p53 null NH32 (◇) and p53 mutant WTK1(□) cell cultures following treatment with various concentrations of a) EMS, b) etoposide and c) taxol. Standard deviations were calculated from three concurrent replicate cultures per concentration.

		p value						
a) EMS	**Concentration (µg/mL)**	**50.0**	**100**	**200**	**300**	**400**	**500**	**600**
	ANOVA	0.0165 *	0.0010 **	0.0007 ***	0.0154 *	0.0005 ***	0.0082 **	0.0073 **
	TK6 v NH32	0.0058 **	0.0004 ***	0.0003 ***	0.0155 *	0.0003 ***	0.0088 **	0.0025 **
	TK6 v WTK1	0.0697	0.1906	0.1006	0.5624	0.3120	0.5009	0.0602
	NH32 v WTK1	0.0945	0.0016 **	0.0014 **	0.0075 **	0.0006 ***	0.0040 **	0.0364 *
b) Etop	**Concentration (µg/mL)**	**0.05**	**0.10**	**0.15**	**0.20**	**0.30**	**0.40**	**0.50**	**0.70**
	ANOVA	0.0036 **	$8.2x10^{-5}$ ***	$8.1x10^{-5}$ ***	$4.1x10^{-7}$ ***	$2.6x10^{-7}$ ***	$1.0x10^{-8}$ ***	$1.0x10^{-6}$ ***	$9.5x10^{-10}$ ***
	TK6 v NH32	0.0012 **	$2.8x10^{-5}$ ***	$2.9x10^{-5}$ ***	$1.9x10^{-7}$ ***	$9.9x10^{-9}$ ***	$3.8x10^{-7}$ ***	$5.1x10^{-7}$ ***	$6.8x10^{-10}$ ***
	TK6 v WTK1	0.0566	0.0054 ***	0.0124 *	0.0029 **	0.0002 ***	$6.7x10^{-6}$ ***	0.0140	0.3900
	NH32 v WTK1	0.0149 *	0.0004 ***	0.0003 ***	$6.1x10^{-7}$ ***	$6.6x10^{-7}$ ***	$2.9x10^{-8}$ ***	$1.4x10^{-6}$ ***	$7.4x10^{-10}$ ***
c) Taxol	**Concentration (µg/mL)**	**0.0015**	**0.0025**	**0.0030**	**0.0045**	**0.0060**	**0.0090**	**0.0120**
	ANOVA	0.9820	0.1710	0.0004 ***	0.0037 **	0.0002 ***	$7.5x10^{-6}$ ***	$2.6x10^{-5}$ ***
	TK6 v NH32	-	-	0.0010 **	0.0014 **	$6.8x10^{-5}$ ***	$6.6x10^{-6}$ ***	$1.3x10^{-5}$ ***
	TK6 v WTK1	-	-	0.0550	0.0082 **	0.0007 ***	$4.7x10^{-6}$ ***	$3.1x10^{-5}$ ***
	NH32 v WTK1	-	-	0.0002 ***	0.1380	0.0162 *	0.4100	0.1300

Table 4: Results of ANOVA and pairwise t-tests for cytotoxicity in TK6, WTK1 and NH32.
The mean RPD values of the human lymphoblastoid p53 wild-type TK6, null NH32 and mutant WTK1 cell lines were compared by one-sided ANOVA with pairwise t-test for (a) EMS, (b) etoposide and (c) taxol. *p* values and significance are presented. Where ANOVA did not demonstrate significance, results from the pairwise t-test were not reported. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The results have been presented as mean ± standard deviation (SD). The mean values of each cell line were compared by one-sided analysis of variance (ANOVA) with pairwise t-test performed to compare each cell line where ANOVA was significant. A *p* value of ≤0.05 was considered statistically significant. Where ANOVA was not significant the results of the pairwise t-test was not reported.

9.2.4 Apoptosis

In order to further assess the influence of p53 on the cytotoxicity of the three chemicals tested, caspase-3 and -7 activity relative to concurrent vehicle control cultures in wild-type TK6, p53 null NH32 and p53 mutant WTK1 was determined as a measure of induction of apoptosis.

For all three compounds TK6 showed an expected p53 wild-type function and more readily induced apoptosis following treatment with the three compounds, compared to both NH32 and WTK1 (Figure 9).

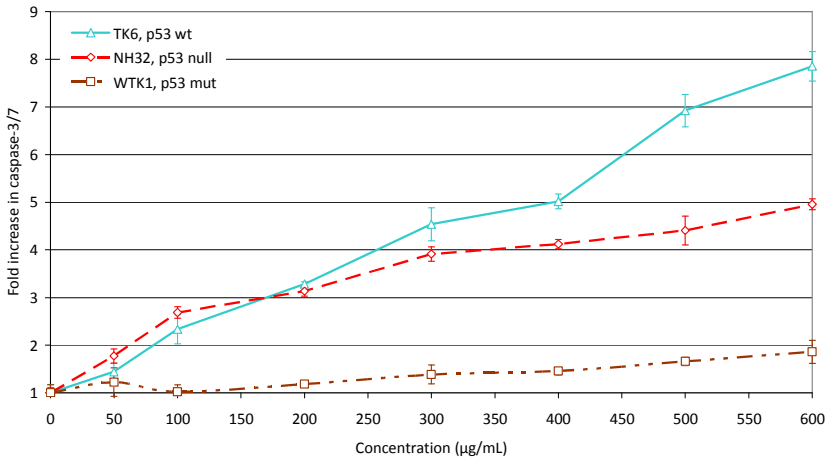
Following a three hour exposure to EMS and recovery for approximately 1.5 cell cycles (Table 3) in fresh culture medium, TK6 and NH32 displayed similar levels of casapase-3 and -7 activity up to 300 µg/mL,(approximately 4- to 5-fold increase over the concurrent vehicle control) at which point the level of caspase activity in NH32 began to plateau between 4- and 5-fold over the vehicle control compared to TK6 where caspase-3 and -7 activity continued to approximately 8 fold over the concurrent vehicle control at 600 µg/mL, significantly more than caspase-3 and -7 activity in NH32. The level of capase-3 and -7 activity in the p53 mutant WTK1 cells was significantly less than both TK6 and NH32 at all concentrations tested and never rose above a 2-fold level of the vehicle control throughout the concentration range (Figure 9a).

Following three hour exposure to etoposide and recovery for approximately 1.5 cell cycles TK6 exhibited a marked increase in caspase-3 and -7 activity rising from approximately 3-fold at 0.2 µg/mL to almost 14-fold over the concurrent control at 0.7 µg/mL. Both NH32 and WTK1 demonstrated significantly less induction of apoptosis with WTK1 rising from approximately 2-fold at 0.2 µg/mL to approximately 4-fold at 0.7 µg/mL. NH32 demonstrated a further significant reduction in apoptotic response to etoposide treatments never reaching more than a 3-fold increase in caspase-3-and -7 activity at 0.7 µg/mL (Figure 9b).

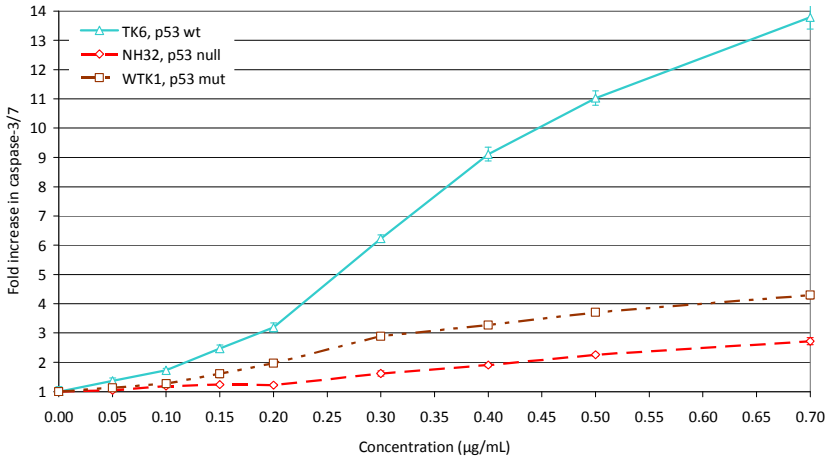
Following a twenty four hour exposure to taxol and recovery for approximately 1.5 cell cycles TK6 again showed a marked increase in apoptosis induction rising sharply between approximately 2-fold and 6.5-fold between 0.003 and 0.0045 µg/mL taxol, respectively, up to nearly 11-fold at 0.009 µg/mL. WTK1 was again similar to NH32, rising from almost

2-fold to approximately 5-fold active caspase-3 and -7 between 0.003 and 0.009 µg/mL, however caspase-3 and -7 activity was significantly more in WTK1 cells at 0.003, 0.006, 0.009 and 0.012 µg/mL compared to NH32. In NH32 cultures caspase activity rose from approximately 1.5 to almost 5-fold at these same concentrations (Figure 9c).

a) EMS



b) Etoposide



c) Taxol

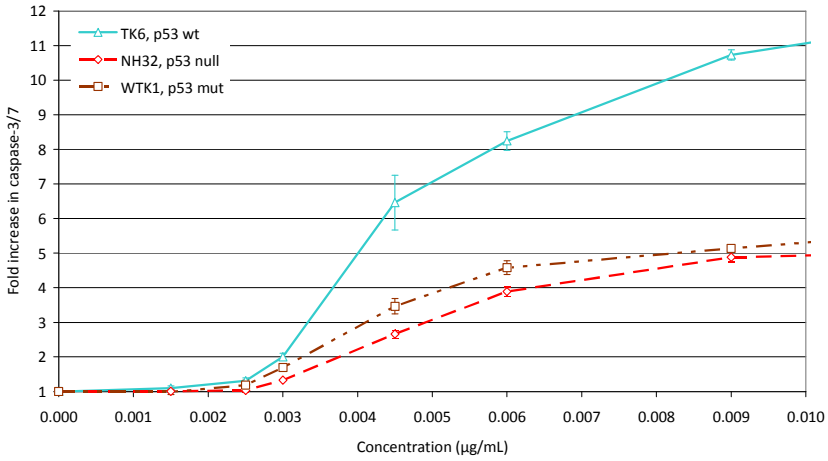


Figure 9: Induction of apoptosis in human lymphoblastoid cells differing in p53 status.
Relative caspase-3/7 activity was determined using the caspase-3/7-Glo® assay (Promega, UK) in p53 wild-type TK6 (△), p53 null NH32 (◇) and p53 mutant WTK1(□) cell cultures following treatment with various concentrations of a) EMS, b) etoposide and c) taxol. Standard deviations were calculated from three concurrent replicates per concentration.

		p value						
a) EMS	**Concentration (µg/mL)**	**50.0**	**100**	**200**	**300**	**400**	**500**	**600**
	ANOVA	0.0552	0.0002 ***	6.1×10^{-8} ***	9.8×10^{-9} ***	6.5×10^{-8} ***	1.1×10^{-6} ***	2.4×10^{-7} ***
	TK6 v NH32	-	0.0866	0.044 *	0.021 *	8.7×10^{-5} ***	2.6×10^{-5} ***	5.5×10^{-6} ***
	TK6 v WTK1	-	0.0003 ***	3.7×10^{-8} ***	4.3×10^{-6} ***	2.6×10^{-8} ***	3.4×10^{-7} ***	7.5×10^{-8} ***
	NH32 v WTK1	-	6.8×10^{-5} ***	5.8×10^{-8} ***	1.6×10^{-5} ***	1.6×10^{-7} ***	1.6×10^{-5} ***	3.8×10^{-6} ***
b) Etop	**Concentration (µg/mL)**	**0.05**	**0.10**	**0.15**	**0.20**	**0.30**	**0.40**	**0.50**	**0.70**
	ANOVA	0.0013 ***	2.8×10^{-5} ***	1.7×10^{-5} ***	8.1×10^{-7} ***	1.4×10^{-8} ***	2.8×10^{-9} ***	1.1×10^{-9} ***	5.3×10^{-9} ***
	TK6 v NH32	0.0005 ***	1.3×10^{-5} ***	6.2×10^{-6} ***	2.7×10^{-7} ***	5.3×10^{-9} ***	1.3×10^{-9} ***	5.2×10^{-10} ***	2.7×10^{-9} ***
	TK6 v WTK1	0.0023 ***	3.6×10^{-5} ***	4.8×10^{-5} ***	4.5×10^{-6} ***	3.6×10^{-8} ***	4.5×10^{-9} ***	1.5×10^{-9} ***	6.7×10^{-9} ***
	NH32 v WTK1	0.1347 ***	0.085	0.0048 **	8.0×10^{-5} ***	1.1×10^{-5} ***	2.4×10^{-5} ***	2.6×10^{-5} ***	0.00025 ***
c) Taxol	**Concentration (µg/mL)**	**0.0015**	**0.0025**	**0.0030**	**0.0045**	**0.0060**	**0.0090**	**0.0120**
	ANOVA	0.1410	0.0404 *	0.0002 ***	0.0002 ***	5.0×10^{-7} ***	2.9×10^{-9} ***	1.1×10^{-7} ***
	TK6 v NH32	-	0.015 *	6.7×10^{-5} ***	7.1×10^{-5} ***	2.4×10^{-7} ***	1.9×10^{-9} ***	6.2×10^{-8} ***
	TK6 v WTK1	-	0.181	0.0041 **	0.0003 ***	6.7×10^{-7} ***	2.5×10^{-9} ***	1.1×10^{-7} ***
	NH32 v WTK1	-	0.111	0.0019 **	0.0867	0.0066 **	0.048 *	0.027 *

Table 5: Results of ANOVA and pairwise t-tests for induction of apoptosis in TK6, WTK1 and NH32.
The mean fold change in caspase-3 and -7 for the human lymphoblastoid p53 wild-type TK6, null NH32 and mutant WTK1 cell lines were compared by one-sided ANOVA with pairwise t-test for (a) EMS, (b) etoposide and (c) taxol. *p* values and significance are presented. Where ANOVA did not demonstrate significance, results from the pairwise t-test were not reported. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

10 Discussion chapter

10.1 p53 deficient cells can underestimate cytotoxicity

Following treatment with etoposide, the p53 wild-type TK6 cells demonstrate an expected reduction in RPD with clear increases in p53-dependent apoptosis, measured as an increase in caspase-3 and -7 activity, at the higher cytotoxic concentrations (Figure 8b and Figure 9b). In comparison to TK6, NH32 demonstrate the expected response of a p53 deficient cell line, with greatly reduced cytotoxicity over the concentration range of etoposide tested. A significantly reduced apoptotic response was also observed when comparing NH32 and TK6 at equal concentrations.

The cytotoxicity and apoptosis profiles observed with TK6 and NH32 cells were expected as etoposide is a potent topoisomerase II inhibitor resulting in cytotoxicity due to formation of double strand breaks (Watt and Hickson, 1994; Hande, 2008). The role of p53 in response to etoposide and double strand breaks has been widely demonstrated (Akyűz , *et al*, 2002; Clifford, *et al*, 2005; Nam, *et al*, 2006; Dai, *et al*, 2011). Double strand breaks results in the stabilisation and increased p53 levels. Increased levels of p53 in the presence of etoposide is known to elicit G_2 cell cycle arrest and facilitate DNA repair via non-homologous end joining (NHEJ) (Akyűz , *et al*, 2002; Jackson, 2002). Where G_2 arrest is prolonged due excessive DNA damage p53 induces apoptosis to remove irreversibly damaged cells (Nam, *et al*, 2006, Roos and Kaina, 2012). Cytotoxicity observed at lower concentrations with TK6 demonstrates the role of p53 in G_2 arrest, resulting in a significant decrease in proliferation during the treatment period and therefore yielding a reduction in both cell number and the percentage RPD at the time of harvest compared to the control. It is interesting to note that when comparing equi-toxic concentrations, similar levels of relative active caspase-3 and -7 are observed in TK6 and NH32 (Table 6) suggesting that the majority of cytotoxicity observed in TK6 cells may be due to p53-induced cell cycle delay, rather than significant cell loss from apoptosis. Smart, *et al* (2008) has previously demonstrated significant reduction in G_2 accumulation in response to other topoisomerase II inhibitors with NH32, compared to TK6, which showed expected G_2 arrest. Therefore, the limited reduction in RPD to etoposide observed with NH32 cells compared to TK6 would be expected. There may also be argument that the method used to determine cell numbers at the time of harvest may underestimate cytotoxicity where significant increases

in caspase-3/7 are observed. RPD was determined from whole cell counts, therefore cells in apoptosis (with active caspase-3 ad -7) may have been included in the analysis. One consideration would be to include a stain for viability or a stain to distinguish apoptotic cells, such as against caspase-3 or annexin V to distinguish and exclude apoptotic cells and measure the impact of this on cytotoxicity determination by RPD.

With regards to genotoxicity testing, RPD was 48% at 0.15 µg/mL in TK6 and 49% of the control at 0.70 µg/mL NH32, respectively. The impact of using the p53 null NH32 cell line with this compound is a significant underestimation of cytotoxicity resulting in almost a 5-fold increase in the concentration selected for assessment of the genotoxic potential. Selection of an inappropriately high concentration due to underestimating cytotoxicity has been shown to adversely impact the outcome of a genotoxicity assay (Hilliard, *et al*, 1999; Fowler, *et al*, 2012b). Although this has been demonstrated with etoposide which is known to induce double strand breaks, the results here highlight the importance of p53 status and cell selection on the cytotoxic response in the *in vitro* assays.

Chemical	Cell line	Concentration at 40-50% RPD (µg/mL)	RPD (%)	Fold caspase-3/7 activity
EMS	TK6	500	39	7.8
	NH32	**100***	**42**	2.7
	WTK1	500	42	1.7
Etoposide	**TK6**	**0.1***	**48**	1.7
	NNH32	0.7	49	2.7
	WTK1	0.2	47	1.6
Taxol	**TK6**	**0.0045***	**46**	6.5
	NH32	0.006	51	3.9
	WTK1	0.006	46	4.6

Table 6: Concentrations that induce 40-50 % RPD

Concentrations of EMS, etoposide and taxol that induce 40-50% RPD (50-60 % cytotoxicity) in the p53 wild-type TK6, p53 null NH32 and p53 mutant WTK1 cell lines. Fold caspase-3/-7 activity (measured using caspase-3/7 Glo® assay, Promega, UK) is also shown. * Highlighted concentrations demonstrate the most sensitive cytotoxic response. RPD = relative population doubling.

10.2 p53 deficient cells can demonstrate enhanced cytotoxicity

With EMS a much greater induction of cytotoxicity was observed with NH32 cells than both p53 wild-type TK6 and p53 mutant WTK1 (Figure 8a). With 100 µg/mL EMS RPD was 42% of the control using NH32 cell however a concentration of 500 µg/mL EMS had to be tested to achieve similar cytotoxicity (39% RPD) with TK6 cells, effectively a five-fold increase in EMS concentration to reach equi-toxic concentrations.

EMS is a DNA alkylating agent, reacting with DNA to produce ethylated nucleotides which predominantly targets the highly nucleophilic centers, such as N^7-guanine and N^3-adenine and to a lesser extent the O-atoms of O^6-guanine and O^2-thymidine (Vidal, *et al*, 1995, Doak, *et al*, 2007; Gocke, *et al*, 2009). This type of damage may explain the increase in the magnitude of cytotoxicity observed in the p53 null NH32 compared to the p53 wild-type TK6. *N*-alkylation of DNA is predominantly repaired by base excision repair (BER) (Pastink, *et al*, 1991; Doak, *et al*, 2007). BER has been found to be directly regulated by p53 (Offer, *et al*, 1999) through complex formation of DNA polymerase-beta (β-poly), a rate limiting step in BER (Zhou, *et al*, 2001; Seo, *et al*, 2002). It has been demonstrated that cells deficient in BER, particularly through loss of p53, are hypersensitive to various alkylating agents (Zurer, *et al*, 2004). As BER is deficient in p53 null cell lines, the highly cytotoxic DNA lesions resulting from EMS are allowed to rapidly accumulate resulting in mitotic catastrophe and p53-independent apoptosis (Offer, *et al*, 1999; Zhou, *et al*, 2001). The accumulation of DNA damage leading to increased cell cycle stress and apoptosis (cytotoxicity) with the absence of p53 ($p53^{-/-}$) has also been demonstrated with other DNA damaging agents, which explain the increased sensitivity observed with NH32 (Neito, *et al*, 2004; Hawkins, *et al*, 1996)

The enhanced sensitivity to EMS observed with NH32 does not fit with the hypothesis that p53 deficient cell lines underestimate cytotoxicity leading to selection of inappropriately high concentration for genotoxicity analysis. However, it is of concern that with the p53 null cell line, a loss of p53-mediated regulation of DNA repair mechanisms, such as BER, may lead to a hypermutable phenotype. Damage induced by a compound such as EMS is allowed to accumulate until the cell is overloaded and unable to progress through cellular division and die as a result. Could this damage be expressed in the genotoxicity tests to a greater extend that with p53 functional cells? Chuang, *et al* (1999) showed X-ray induced mutations are not elevated in NH32 cells compared to TK6 when measured at the

autosomal thymidine kinase locus. Other studies have also demonstrated similar levels of genotoxic response between p53 functional and p53 null cell lines with a number of clastogenic and aneugenic compounds (Hashimoto, *et al*, 2011). However, Hashimoto, *et al* (2012) demonstrated that NH32 were more susceptible to chromosome loss events with aneugens compared to TK6, as they are more prone to escape from the G_1 checkpoint and mitotic slippage leading to increased aneuploidy (Hashimoto, *et al*, 2012).

10.3 Cytotoxicity can be different with p53 mutant and p53 null cells

Although similarities are observed in taxol treatments, this study showed that p53 null and mutant cells can demonstrate a different a cytotoxic response to the same compound. With EMS, the p53 null NH32 cells were cytotoxic at significantly lower concentrations compared to the p53 mutant WTK1, which showed a cytotoxicity to the p53 wild-type TK6 cell line with concentrations tested (Figure 8a). With WTK1 500 µg/mL EMS was required to induce 42% RPD, similar to TK6. However, 100 µg/mL EMS achieved an equal level of cytotoxicity in the NH32 cells (Table 6). The sensitivity of NH32 to the cytotoxicity of EMS has been discussed (Section 10.1).

WTK1 also exhibit cytotoxicity similar to TK6 and not NH32 with etoposide. In contrast to observations with EMS, WTK1 (as with TK6) demonstrate greater sensitivity to cytotoxicity induced by etoposide compared to NH32. RPD of 47% of the controls was achieved with 0.2 µg/mL etoposide in WTK1; however 49% RPD was achieved at a significantly higher concentration of 0.7 µg/mL in NH32 cells (Figure 8b). Although RPD was similar with TK6 and WTK1 following EMS and etoposide, the two cell lines demonstrate a significant difference in caspase-3 and -7 activity with all three compounds. At approximately 40% RPD following etoposide treatments, WTK1 and TK6 express similar levels of active caspase-3 and -7; 2-fold and 2.5-fold over the control, respectively. However, in TK6 the levels of caspase-3 and -7 rose to 13.8-fold over the control at 0.7 µg/mL etoposide where as in WTK1 only 4.3-fold over the control was achieved at 0.7 µg/mL. These values were observed at highly cytotoxic concentrations in TK6 and WTK1 therefore the influence from this difference in caspase-3 and -7 on the genotoxicity endpoint may be limited.

Following irradiation WTK1 have previously demonstrated similar levels of apoptosis when compared to TK6. However, increases in apoptosis in WTK1, measured by both

morphological analysis of apoptotic cells and ELISA, were delayed by at least a day compared to TK6 (Xia, *et al*, 1995). This supports the reduced response observed in WTK1 in this study where both cell lines were sampled 24 hours following the end of treatment. However, the discrepancy in the levels of apoptosis between the TK6 and WTK1 does not explain the similarities in RPD observed with etoposide. As previously discussed p53 facilitates G_2 accumulation following exposure to topoisomerase II inhibitors (Akyűz, *et al*, 2002; Jackson, 2002) which reduces RPD as cells are unable to divide. Interestingly, etoposide has been shown to elicit prolonged G_2 arrest in p53 mutated human non-small cell lung cancer cells as well as delayed induction of apoptotic cell death compared to p53 wild-type function (Chiu, *et al*, 2005). Similar levels of G_2 accumulation in TK6 has also been observed in WTK1 following exposure to other topoisomerase II inhibitors (Smart, *et al*, 2008). As discussed above, Smart, *et al*, (2008) demonstrated that NH32 had a delayed and much reduced G_2 accumulation following exposure to ciprofloxacin, an effect mirrored here with etoposide with greater RPD than TK6 and WTK1. Therefore, the ability of both p53 wild-type and mutant cells to elicit G_2 arrest following exposure to topoisomerase II inhibitors may explain the similarities observed between TK6 and WTK1 in the short-term tests.

10.3.1 Impact of cell origin

The majority of p53 deficient cell lines used for *in vitro* genotoxicity testing are also of from rodent origin. The hamster CHL cell line has shown significantly greater MN frequencies at comparable levels of cytotoxicity compared to TK6 where as MN frequency with the same compounds were comparable between human TK6 and NH32 (Hashimoto, *et al*, 2011). Differences due to the species origin rather than the p53 status of the cell may be more relevant to the high number of positives in the *in vitro* mammalian cell tests, demonstrated by Fowler, *et al*, (2012a) and should be considered in more detail.

Mammalian cells have evolved a number of defence mechanisms for maintaining genomic integrity through cell cycle checkpoint regulation or DNA repair capabilities to prevent permanent genetic damage induced by endogenous and environmental mutagens. However, differences in control and rate of DNA repair and cell transformation have evolved between human and rodent cells. Much work has shown that UV irradiation induced cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidone UV products (6-4PPs) are efficiently repaired in cultured human cells by nucleotide excision repair (NER)

(through the transcription coupled repair (TCR) and global genomic repair (GGR) subpathways) (Hanawalt, 2001; Vink and Roza, 2001). Although rodent cells are proficient in TCR they are deficient in GGR of CPDs leading to a greater frequency of mutations in rodent cells with the same doses of UV. Similar survival characteristics are, however, still observed between rodent and human cells. This suggests that rodent cells are able to survive higher levels of unrepaired damage than human cells giving them a mutable phenotype (reviewed in Hanawalt, 2001). The argument may not be as clear as a human versus rodent response. Much of this work has also utilised immortalised rodent cells with mutant p53 function, such as the hamster CHO and V79 cells. GGR is p53-dependent in primary human cell cultures and directly transactivates proteins associated with the GGR pathway, such as p48 (a component of UV-DDB) and gadd45 in human cells (Hwang, *et al*, 1999, Hanawalt, 2002). GGR deficiency observed in these rodent cells may, therefore, be attributed to the loss of p53 function rather than differences between the species. Interestingly, Tan and Chu (2002) have demonstrated that the mouse *p48* gene does not contain a functional p53 response and that loss of wild-type p53 does not affect the response to UV-irradiation in mice cells. This suggests that fundamental differences in DNA repair have evolved between humans and rodents and these differences are not solely a result of mutated p53. In addition, mice cells have also demonstrated reduced mismatch repair in response to methylating agents with increased tolerance of unrepaired damage following similar levels of DNA methylation compared with equivalent human cells (Humbert, *et al*, 1999).

Differences in regulation of cell immortalisation and tumour development between human and rodent cells may also sensitise rodent cells in the genotoxicity tests. It is known that mice develop malignant tumours with multiple genetic changes within a relatively short time (6-8 months), where similar tumours in humans can take many years to become life-threatening. Rodent cells are also much easier to transform in culture through chemical treatment or oncogene introduction (reviewed in Balmain and Harris, 2000) compared to human cells, even with the same level of mutations (Humbert, *et al*, 1999). Another example is the role of retinoblastoma protein (RB) and p53 in maintaining stable cell proliferation. DNA damage signal pathways induce cellular senescence by activation of either p53 or RB. Suppression of both p53 and RB pathways are required to inactivate cellular senescence in human cells, where as loss of p53 function alone is sufficient for cell immortalisation in mouse cells (Smogorzewska and de Lange, 2002). In addition, rodent

cells showed increases in aneuploidy with microtubule toxins. Human cells show strict checkpoint control and more readily arrest in metaphase, however, rodent cells were more prone to cell cycle progression as a result of a less stringent spindle assembly checkpoint (Haller, *et al*, 2006).

It has been speculated that more stringent mechanism have developed in humans to maintain the genome as they are of greater benefit to the longer life span of humans (who can live 30-50 times longer than a mouse) compared to the shorter life-span of rodents (Haller, *et al*, 2006). It does appear that fundamental differences have evolved that may facilitate accumulation of genomic instability which would adversely impact on the outcome of *in vitro* genotoxicity testing with rodent cells compared to human.

10.4 Relevance to genetic toxicology

This present work has shown that with the three chosen chemicals (EMS, etoposide and taxol), a loss of wild-type p53 function by spontaneous mutation or genetic inactivation of *p53* does not lead to a universal underestimation of cytotoxicity in the *in vitro* micronucleus assay. In addition, cytotoxicity induced in the human WTK1 cell line was more similar to TK6 than NH32 with two out of the three compounds tested. Therefore, it may appear that loss of wild-type p53 may not impact the maximum concentration selection of all compounds for *in vitro* genotoxicity testing as expected. Additional chemicals would show if these effects were compound specific or related to the chemical class.

In addition to the impact of cytotoxicity it is important to consider if a loss or mutant p53 function has been shown to impact different genotoxicity endpoints with these cell lines? WTK1 have showed approximately 30-50 fold increased spontaneously and radiation induced mutations at the autosomal heterozygous thymidine kinase (*tk*) locus compared to TK6 (Honma, *et al*, 1997; Chuang, *et al*, 1999). *tk* mutations in NH32 were similar to TK6 rather than WTK1 (Chuang, *et al*, 1999) therefore loss of p53 function due to homozygous deletion and mutation do not induce the same response to DNA damage.

The results of this present work showed caspase-3 and -7 activity in WTK1 is significantly lower than with TK6 cells where cytotoxicity was similar or reduced following EMS, etoposide and treatments. WTK1 have previously been shown they are able to tolerate

unrepaired DNA damage with a delayed apoptotic response compared to TK6 cells (Xia, *et al*, 1995). WTK1 were able to divide at least once following treatment with X-rays which resulted in an increase in mutations observed at the tk locus. Abrogation of p53 function by human papillomavirus E6, which stimulates proteasomal degradation of p53, resulted in delays in apoptosis with only moderate increases in mutability (Yu, *et al*, 1997). Apoptosis, therefore, may not play a significant role in the increased mutation frequency observed with WTK1.

WTK1 are able to escape the p53 dependent checkpoints and apoptosis as they preferably repair damage through an abnormally higher rate of error prone recombination with increased translocations, a process which is inhibited in TK6 (Honma, 2005). This has lead to the M237I p53 mutant in WTK1 being described as a gain of function mutant with a hypermutable phenotype. Alternatively, NH32 demonstrate a loss of p53 function as they demonstrate some increase in mutability compared to TK6, but not to the extent of that seen in WTK1 (Xia, *et al*, 1995; Honma, *et al*, 1997). Interestingly, comparisons of WTK1 and TK6 did not affect the positive or negative judgement in the micronucleus test, although WTK1 generally demonstrated higher micronucleus frequencies than TK6 (Honma and Hayashi, 2011). Comparisons between NH32 and TK6 showed that NH32 were more sensitive to direct acting genotoxin but again this not affect the genotoxic response in the micronucleus assay, with a number of compounds showing no difference in the magnitude of micronucleus induction between TK6 and NH32 (Hashimoto, *et al*, 2011; 2012). Compared to TK6, NH32 were more susceptible to whole chromosome loss events following aneugen treatment as a result of escape from the G1 checkpoint as described by Hashimoto, *et al* (2012). This may help to explain the results from RPD determination for the aneugen taxol (Figure 8c). The response between the three cell lines are similar following treatment with taxol. Taxol blocks cells in the G2/M phase of the cell cycle (Rathinasamy, *et al*, 2010), therefore the reduction in RPD in each cell line may be a result of this inhibition of proliferation at a similar dose response manner in each cell type. However, the ability of NH32, which lacks wild-type p53, to escape the G1 checkpoint allowing some cells to progress through additional cellular divisions at lower concentrations may explain the significant difference between the p53 wild-type TK6 and the p53 compromised cell lines with taxol.

Differences in p53 has been shown not to effect the outcome of the micronucleus assay with known genotoxins, however, the micronucleus frequency and mutation frequency (at the *tk* locus) is increased in cells that have lost wild-type p53 function. Further work needs to be performed to demonstrate if differences in the sensitivity of these genotoxicity endpoints will affect the outcome of the *in vitro* tests with the misleading positives identified by Kirkland, *et al*, (2008). This will help determine if p53 wild-type cell lines improve the accuracy and therefore relevance of the *in vitro* assays.

10.5 Conclusion

In conclusion, the results of this work show that underestimation of cytotoxicity leading to analysis of irrelevant concentrations through loss of wild-type p53 function (through mutation or genetic inactivation) is not the major cause of misleading positive results in *in vitro* mammalian genotoxicity tests. Since rodent cell lines show a greater level of genotoxicity with less cytotoxicity (Hashimoto, *et al*, 2011; Fowler, *et al*, 2012a) species differences are likely to play a bigger role in the genotoxic response in the *in vitro* micronucleus assay. Therefore, human cell lines should be recommended for use.

However, WTK1 (Chuang, *et al*, 1999), and to a lesser extent NH32 (Honma, 2005), demonstrate a mutable phenotype resulting in increased sensitivity to genotoxins. Therefore, despite no underestimation of cytotoxicity, loss of wild-type p53 function may still be a confounding factor which has lead to the current inaccuracy of *in vitro* mammalian cell tests.

10.6 Further work

In order to improve the current project and aid the interpretation of the results from the RPD and caspase-3 and -7 determination I intend to examine p53 protein in samples from EMS and taxol treated cultures, in addition to that already performed for etoposide treated cultures. p53 phosphorylation on serine 15 induces resistance to MDM2 dependent degradation of p53, therefore additional analysis to show p53 induction would also be improved by detecting levels of phosphorylated p53.

This project was undertaken with the longer term aim of improving existing mammalian cell *in vitro* genetic toxicology assays. Further work needs to be performed to determine if

the p53 status of the cell type used effects the outcome of *in vitro* genotoxicity tests and examine whether p53 wild-type cells reduce the incidence of misleading positive results in order to make further recommendation for testing for human health risk assessment. In order to demonstrate this, cytotoxicity and genotoxicity could be performed with a number of the reported misleading positives identified by Kirkland, *et al*, (2008) with the three closely related cell lines used in this project to directly compare the effects p53 status. In order to justify these follow up experiments, genotoxicity (induction of micronuclei) will be assessed for the three test chemicals used in this project. Although some work has shown that the magnitude of genotoxic response in NH32 cells in significantly higher than TK6 at the same cytotoxic concentrations (Hashimoto, *et al*, 2011), no direct comparison of induction of micronucleus has been performed for these classes of chemical.

11 Appendices

11.1 List of reagents

11.1.1 Western blot analysis

2X sodium dodcyl sulphate (SDS) loading buffer was prepared as follows:

Per 10 mL final volume: 0.34 mL of 2-mercaptoethanol (Sigma, UK) was added to 4.66 mL 4X SDS, immediately prior to use. 4 mL of the resulting solution made up to a total volume of 10 mL with PBS, pH 6.8. Solution used immediately following preparation.

4X SDS loading buffer was prepared as follows:

Per 10 mL final volume: 1.6 g of SDS (Sigma, UK) was dissolved in 6 mL glycerol (Fluka), 0.32 mL water for irrigation (Baxter), 0.68 mL 2-mercaptoethanol. A pinch of bromoethanol blue (Sigma-Aldrich) was added and the resulting solution stored at -20°C until use.

1M Tris, pH 6.8 prepared as follows:

Per 100 mL volume: 12.11 g tris (Merck) made up to 80 mL with purified water. The pH was adjusted to 6.8 with 1M HCl (Merck), and the resulting solution made up to a total volume of 100 mL and stored at room temperature, protected from light, until use.

1X tris-glycine-SDS PAGE running buffer prepared as follows:

Per 1 litre volume: 100 mL of 10X tris-glycine SDS stock, pH 8.8 (Geneflow) made up to 1 litre with purified water.

8% running gel was prepared as follows: 9.09 mL purified water, 5.3 mL 30% acrylamide, 5 mL 1.5 M Tris, pH 8.8, 0.2 mL 10% SDS, 0.2 mL 10% ammonium persulfate (APS), 50 µL Tetramethylethylenediamine (TMED) (Sigma, UK)

4% staking gel was prepared as follows: 2.72 mL purified water, 0.67 mL 30 % acrylamide, 0.5 mL 0.5 M Tris, pH 6.8, 40 µL 10% SDS, 40 µL 10% APS, 12.5 µL TMED

Transfer buffer was prepared as follows:

Per 200 mL volume: 7.2 g tris and 0.44 g 3-[cyclohexamino]-1-propanesulfonic acid (CAPS) were dissolved in 100 mL purified water and mixed. To this solution, 20 mL methanol and 0.4 mL 10% SDS was added and the resulting solution made up to 200 mL with purified water.

1% bovine serum albumin (BSA) buffer prepared as follows:

Per 200 mL volume: 2 g BSA (Sigma) plus 0.2 mL Tween-20 (Sigma) dissolved in 200 mL 1X PBS.

Primary antibody prepared as follows (1:5000 dilution):

Per 10 mL volume: 2 µL anti-p53 (Ab-6) (Pantropic) Mouse (DO-1) monoclonal antibody (Merck Millipore, UK) made up to 10 mL with BSA buffer.

Horseradish peroxidase (HRP)-labelled secondary antibody was prepared as follows (1:10000 dilution):

Per 10 mL volume: 1 µL Goat anti-Mouse IgG, HRP conjugated antibody (Merck Millipore, UK) made up to 10 mL with BSA buffer (1:10000 dilution).

11.1.2 BrdU incorporation (cell average generation times)

75 mM KCl was prepared as follows:

Per 5 litre volume: 28 g of KCl (Fisher Scientific, UK) made up to 5 litres with purified water.

Hoechst 33258 stain (26.7 µg/mL) was prepared as follows:

Per 2.5 litre volume: 66.75 µL of Hoechst 33258 made up to 2.5 litres with McIlvaine's buffer.

McIlvaine's buffer was prepared as follows:

Per 2 litre volume: 55.2 g di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) plus 1.2 g citric acid dissolved in 2 litres of purified water. pH adjusted to 8.0 with citric acid.

4% v/v Geimsa was prepared as follows:

Per 400 mL volume: 15 ml filtered Geimsa made up to 400 mL with Gurr's phosphate buffer, pH 6.8.

12 Abbreviations

AGT	Average generation time
ANOVA	Analysis of variance
ATM	Ataxia-telangiectasia mutated
ATR	ATM- and Rad3-elated
Bax	Bcl-2-associated X protein
BER	Base excision repair
Bcl	B cell lymphoma protein
β-poly	DNA polymerase-beta
BSA	Bovine serum albumin
BrdU	5-bromo-deoxyuridine
CAPS	3-[cyclohexamino]-1-propanesulfonic acid
CC	Cell counts
Cdk1	Cyclin-dependent kinase 1
CHO	Chinese hamster ovary
CHL	Chinese hamster lung
COM	Committee on Mutagenicity
CPD	Cyclobutane pyrimidine dimmer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
ECVAM	European Centre for the Validation of Alternative Methods to Animal Experimentation
EMS	Ethyl methanesulfonate
ELISA	Enzyme-linked immunosorbent assay
Fas	Tumor Necrosis Factor Receptor Superfamily, Member 6
FDA	Food and Drugs Administration
GGR	Global genomic repair (GGR)
HCD	Highly conserved domain
HIFCS	Heat inactivated foetal calf serum
HPBL	Human peripheral blood lymphocytes
HR	Homologous recombination
HRP	Horseradish peroxidase

ICH	International Conference on Harmonisation
IVMN	*In vitro* micronucleus assay
LASA	Laboratory Animal Science Association
MDM2	p53 E3 ubiquitin protein ligase homolog (mouse double minute 2)
MHRA	Medical and Healthcare products Regulatory Agency
MN	Micronucleus
NER	Nucleoside excision repair
NH32	Human lymphoblast NH32
NHEJ	Non-homologous end joining
NIH	U.S. National Institutes of Health
OECD	Organisation for Economic Co-operation and Development
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer solution
PD	Population doubling
6-4PP	6-4 pyrimidine pyrimidone UV product
RCC	Relative cell counts
RPMI	Roswell Park Memorial Institute
RPD	Relative population doubling
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Ser-15	Serine-15
TCR	Transcription coupled repair
TK6	Human lymphoblast TK6
UK	United Kingdom
UKEMS	United Kingdom Environmental Mutagen Society
US	United States
UV	Ultraviolet
UV-DDB	UV-damaged-DNA binding protein
V79	Chinese hamster V79
WTK	Human lymphoblast WTK1

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