ASSESSMENT OF DRUG INDUCED GENOTOXICITY IN MAMMALIAN CELLS AND THE CONTRIBUTION OF TOPOISOMERASE II INHIBITION

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

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This research was carried out in the Department of Drug Safety and Metabolism, AstraZeneca Pharmaceuticals and in the Department of Pharmacology and Therapeutics, The University of Liverpool

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Abstract

There is a common belief that mammalian cell gene mutation assays are prone to false positives, thus questioning the relevance of these tests in regulatory screening paradigms and the mechanisms responsible for these uninterruptable results. False positives can lead to unnecessary animal testing and delays in the development of efficacious new medicines. The initial aim of this thesis was to put into perspective the rate of positives and firstly to consider the extent off target aneugenicity (chromosome loss or gain) may contribute to this rate. Secondly the contribution of topoisomerase II poisoning and its relationship to genotoxicity was considered. Topoisomerase II maintains DNA topology by inducing transient breaks in one strand so a second strand can pass. Chemicals that interact with the enzyme (topoisomerase II poisons; e.g. the antibiotic gemifloxacin and the chemotherapeutin etoposide), yield topoisomerase II bound DNA cleavage complexes, making breaks permanent, leading to mutation or cell death. Structurally, topoisomerase II poisons are diverse, so their genotoxicity is difficult to predict.

To estimate the incidence of positives seen in pharmaceutical research, a retrospective review of data from 10 years of mouse lymphoma assays (MLA) conducted at AstraZeneca was undertaken. This showed that the rate of unexplainable positives was only 5%, vindicating the use of the test in screening paradigms. Consideration was then made of what mechanisms might contribute to this 5%. Aneugenicity was considered but it was shown that the MLA was a poor screen, failing to identify 7 known anuegens.

To gain a better understanding of the relationship between topoisomerase II and genotoxicity, assays to assess enzyme poisoning were examined, including the ability of the cell free decatenation assay to predict the results of the *in vitro* micronucleus test. However, even when combined with an estimate of cellular uptake the predictivity was low.

Assays to investigate topoisomerase II poison / DNA cleavage complexes *in vivo* were then investigated. The TARDIS and ICE assays both use antibodies to target topoisomerase II bound in the complex. TARDIS was found to be insensitive, failing to identify cleavage complex formation with gemifloxacin. Using the ICE assay, cleavage complex formation was seen for etoposide (0.1 µmol/L; FITC intensity 3.53 ± 0.79) and gemifloxacin (100 µmol/L; FITC intensity 3.37 ± 0.86), but not at equivalent concentrations to those inducing micronuclei (MN) (0.03 µmol/L etoposide; MN/1000 6 ± 2.6 and 10 µmol/L gemifloxacin; MN/1000 5 ± 3.4), thus questioning assay sensitivity, or suggesting a role for other mechanisms of genotoxicity e.g. reactive oxygen species (ROS).

The hOGG Comet assay showed that neither etoposide nor gemifloxacin induced ROS related genotoxicity.

Improvements to the sensitivity of the cleavage complex assays were made by preparation of mouse specific antibodies, but TARDIS was still unable to identify gemifloxacin. This work also suggested that when developing antibodies for DNA bound topoisomerase II, the n-terminus of the enzyme should be targeted.

Over the last 4 years, emerging data linked the topoisomerase IIβ isoform to genotoxicity. As research within this thesis had investigated the activity of topoisomerase IIα, this may have explained the difficulty encountered equating topoisomerase II poisoning to genotoxicity. Following siRNA knockdown of topoisomerase IIα, the genotoxicity of etoposide and gemifloxacin was investigated. It was shown that for 0.3 µmol/L etoposide, topoisomerase IIα knockdown of 42% (± 2%) was associated with a reduction in micronuclei of 49% (± 9.7%). For 30 µmol/L gemifloxacin, topoisomerase IIα knockdown of 37% (± 9.5%) was associated with a reduction in micronuclei of 48% (± 0.2%). This was the first time such direct relationships had been demonstrated between the alpha isoform and genotoxicity.

In conclusion, the predictivity of the MLA was confirmed but it was clear the assay is not a suitable screen for aneugenicity. The relative sensitivity of assays to measure topoisomerase II poisoning was shown and linked to genotoxicity. Whilst it was not possible to demonstrate cleavage complex formation at concentrations below which genotoxicity was seen, this was likely due to the insensitivity of the assays used rather than topoisomerase II poisons having other genotoxic mechanisms. For the first time the link between topoisomerase IIα and genotoxicity was confirmed and use of knockdown cells holds real promise as a tool for investigating off target topoisomerase II poisoning.

Publications

Papers relevant for this research

Fellows MD, Jenkins J, Warwicker J, Houghton S, Williams D. The *in vitro* mammalian cell genotoxicity of topoisomerase II poisons etoposide and gemifloxacin is driven by their activity against the alpha isoform of the enzyme. *Manuscript in preparation.*

Fellows MD, Kimzey A, Clare KR, Doherty A, Basarab G. 2014. The relationship between inhibition of DNA decatenation, in vitro genotoxic potency and in vivo genotoxicity for a series of antibiotics targeting bacterial topoisomerase II. *Manuscript prepared, awaiting permission to publish from AstraZeneca R&D.*

Fellows MD, McDermott A, Clare KR, Doherty AT and Aardema MJ. 2014. The Spectral Karyotype of L5178Y $TK^{+/-}$ Mouse Lymphoma Cells Clone 3.7.2C and Factors Affecting Mutant Frequency at the Thymidine Kinase (*tk*) Locus in the Microtitre Mouse Lymphoma Assay. Environ Mol Mutagen. 55(1):35-42.

Fellows M, Luker T, Cooper A, O'Donovan MR. 2012. Unusual structure–genotoxicity relationship in mouse lymphoma cells observed with a series of kinase inhibitors. Mutat Res. 746(1):21-8.

Doherty AT, Hayes J, **Fellows M**, Kirk S, O'Donovan M. 2011. A rapid, semi-automated method for scoring micronuclei in mononucleated mouse lymphoma cells. Mutat Res. 726(1):36-41.

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Fellows MD, Boyer S, O'Donovan MR. 2011 The incidence of positive results in the mouse lymphoma TK assay (MLA) in pharmaceutical screening and their prediction by MultiCase MC4PC. Mutagenesis. 26(4):529-32.

Meeting poster abstracts

Fellows M. The in vitro genotoxicity of topoisomerase II poisons is driven by their activity against the alpha isoform of the enzyme. Presented at the European Environmental Mutagen Society Annual Meeting, Lancaster University July 2014

Fellows M, Basarab G, DohertyA, O'Donovan MR. The relationship between inhibition of DNA decatenation, in vitro genotoxic potency and in vivo genotoxicity for a series of antibiotics targeting bacterial topoisomerase II. Presented at AstraZeneca Scince retreat, Alderley Park, April 2013

Clare K, McDermot A, **Fellows M**. Factors Affecting the Spontaneous Mutant Frequency in the Microtitre L5178Y Mouse Lymphoma Assay. Presented at Industrial Genotoxicity Group Annual Meeting, University of London 2012.

Fellows M. O'Donovan MR. What is the real incidence of positive results in the mouse lymphoma TK assay (MLA) in pharmaceutical screening? Presented at The United Kingdom Environmental Society Meeting, University of Derby, Buxton, July 2010.

Abbreviations

4-NQO	4-nitroquinoline oxide
5Y-CAP	E.coli catabolite acitivator protein
8-oxoGua	8-oxo-7,8-dihydroguanine
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte (German Federal Institute for Drugs and Medical Devices)
BMD	Benchmark dose
$BMDL_{10}$	Estimated benchmark dose producing a 10% increase over background
BSA	Bovine serum albumin
Ca	Circa (approximately)
CAD	Caspase-activated deoxyribonuclease
CCD	Charge coupled device
CE	Cloning efficiency
CHL	Chinese hamster lung cell chromosome aberration test
CHO	Chinese hamster ovary cell chromosome aberration test
ClogP	Concentration partition coefficient neutral molecule in octanol/water
Comet(vit)	In vitro Comet assay
Comet(viv)	In vivo Comet assay
CO_2	Carbon dioxide
Conc	Concentration
CPA	Cyclophosphamide
CsCl	Caesium chloride
C-terminal	Carboxyl-terminus
DAPI	4 6 diamidino-2-phenylindole anti-fade solution
del	Deletion (chromosome)
DHS	Donor horse serum
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dp	Duplication (chromosome)
DPX	Distrene, plasticiser, xylene mounting medium
DTT	Dithiothreitol
ECL	Enzyme-linked chemiluminescence
EMA	European Medicines Agency
EDTA	Ethylenediaminetetraacetic acid
Etop	Etoposide
FITC	Fluorescein isothiocyanate
g	Gravitational acceleration (centrifugation)
G2 phase	Gap phase 2 (cell cycle)
GEF	Global evaluation factor
GHKL	Gyrase, hsp90, bacterial histidine kinase and mutL
G-segment	Gate Segment
GyrA	Gryase subunit A
GyrB	Gryase subunit B

Abbreviations

HCl	Hydrochloric acid
HEPES	Hydroxyethyl-piperazine ethanesulafonic acid buffer
HESI	Health and Environmental Science Institute
his	Histidine
hOGG1	8-hydroxyguanine DNA-glycosylase
HPLC	High pressure liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
hU	Human units (enzyme)
huly	Human lymphocyte chromosome aberration test
Human 2a	Human topoisomerase II alpha
Human 2b	Human topoisomerase II beta
IC_{50}	Concentration inhibiting 50% of control
ICE	In vivo complexes of enzyme
ICH	International Conference on Harmonization
ICRF	Imperial Cancer Research Fund
ILSI	International Life Sciences Institute
IR	Infrared
IVC	In vitro chromosome aberration assay
K^+	Potassium ion
k Da	Kilo daltons
KCl	Potassium chloride
kDNA	Kinetoplast DNA
L	Litre
LOCCEL	Lowest observable cleavable complex effect level
LOGEL	Lowest observable genotoxic effect level
LMA	Low melting point agar
µ	Micro
µMol/L	Micro mole per litre
M	Molar
ma	Miliamps
MCASE	Multicase structural alert analysis programme
MF	Mutant frequency (expressed as mutants per 10^6 viable cells)
mg	Miligram
MLA	Mouse lymphoma assay
MLL	Mixed lineage leukaemia
mMol/L	Milimole per litre
MN	Micronuclei
MN(vit)	In vitro micronucleus assay
MOA	Mode of action
Mol Wt	Molecular weight
MOPS	3-(N-morpholino)propanesulfonic acid
Mouse 2a	Mouse topoisomerase II alpha
Mouse 2b	Mouse topoisomerase II beta
M phase	Mitotic phase (cell cycle)
MRC	Medical Research Council
mRNA	Memory ribonucleic acid
Na^+	Sodium ion
NaCl	Sodium chloride
Na_2EDTA	Sodium ethylenediaminetetraacetic acid

Abbreviations

NaOH	Sodium hydroxide
ND	No data
N-gate	Topoisomerase II hinged dimer region
nMol/L	Nano mole per litre
NOGEL	No observable genotoxic effect level
NT	Not tested
N-terminus	Amino-terminus
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
P	Pluronic (surfactant)
ParC	Topoisomerase IV subunit C
ParE	Topoisomerase IV subunit E
pAQ1	Mutagenesis-enhancing plasmid
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with tween 20
PDP	Protein data Bank
PI	Propidium iodide
pKM101	Mutagenesis-enhancing plasmid
PMSF	Phenylmethylsulfonyl fluoride
POD	Point of departure
Pos Cnt	Positive control
R&D	Research and development
rb	Robertsonian translocation (chromosome)
RBM	Rat bone marrow micronucleus assay
Redox	Reduction and oxidation
rfa	Replication factor-A DNA damage recognition protein
RIPA	Radioimmunoprecipitation assay buffer
RISC	RNA / protein silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPD	Relative population doubling
rpm	Revolutions per minute
RPMI	Roswell park Memorial Institute medium
RPMI10%+P	RPMI medium containing 10% donor horse serum and pluronic
RSG	Relative suspension growth
RTG	Relative total growth
S9	Rat liver enzymes prepared by homogenisation and centrifugation at 9000 x g
SAR	Structural alert relationship
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SG	Propriety supplement product code for Lonza UK electroporation kit
sh-RNA	Short hairpin ribonucleic acid
si-RNA	Silencing ribonucleic acid
SMILES	Simplified molecular-input line-entry system
S phase	DNA synthesis phase (cell cycle)
T	Translocation (chromosome)
TARDIS	Trapped in agarose DNA immunostaining
TBST	Tris(hydroxymethyl)aminomethane) buffered saline with tween 20

Abbreviations

TFT	Trifluorothymidine
tk	Thymidine kinase
to	Toxicity
Topo II	Topoisomerase II
Tris	Tris(hydroxymethyl)aminomethane) buffer
T-segment	Transport segment
trp	Tryptophan
Tyr	Tyrosine
UDS	Unscheduled DNA synthesis
uvrA	Ultraviolet recognition nucleotide excision repair protein
uvrB	Ultraviolet recognition nucleotide excision repair protein
V	Volts
V79	V79 cell chromosome aberration test
V/cm	Volts per centimetre
v/v	Volume to volume
w/v	Weight to volume

CHAPTER 1

General introduction

CONTENTS

Assessment of drug induced genotoxicity in mammalian cells and the contribution of Topoisomerase II inhibition

1 General Introduction

The following introduction to my thesis on drug induced genotoxicity in mammalian cells and the contribution of topoisomerase II inhibition is based on literature available at the start of my research i.e. up to 2010, with two exceptions Wu *et al.,* 2011 which was used to illustrate a recently resolved crystalline structure and Gilroy and Austin, 2011 which was considered important to include as it illustrated a new insight into topoisomerase II isoform activity.

1.1 Background to the research

Genetic toxicology (or genotoxicology) is the study of agents that cause damage to DNA, with the potential for deleterious effects on human health, specifically cancer and possible heritable disease. Since the early seventies, academics, industries and regulatory authorities have instigated a variety of *in vitro* and *in vivo* testing strategies to try to measure the genotoxicity of novel chemicals, hence ensuring appropriate risk assessment for man. However, the perception of the 'over sensitivity' of mammalian cell *in vitro* genotoxicity tests (a core test in nearly all internationally accepted testing strategies) has raised concerns for over three decades. This perception is based on the view that these tests are highly prone to generating 'false positive' results (Kirkland *et al.,* 2005; Kirkland *et al.,* 2007; Kirkland and Speit 2008; Matthews *et al.,* 2006a; Matthews *et al.,* 2006b). Pharmaceutical regulatory authorities make decisions on the safety of new drugs not only based on whether they are positive genotoxicants, but also based on their genotoxic mode of action. Accordingly the generation of 'false' positive results will inevitably lead to additional time and expense during pharmaceutical development as scientists attempt to elucidate the relevant mechanism. This may delay the launch of important new medicines or even, if the genotoxic mechanisms cannot be identified, prevent their use in man. The author has over 25 years of industrial experience working with *in vitro* mammalian cell genotoxicity assays. This experience suggests that the published incidence of false positives greatly exaggerate the actual observed incidence seen during routine pharmaceutical screening. As regulatory testing paradigms and decisions are made based on the

published literature, it is important to gain a more balanced understanding of the predictivity of mammalian cell *in vitro* genotoxicity tests.

Whatever the real rate of unique positives seen during mammalian cell screening, there are some genotoxic positives that cannot be readily explained by their inherent DNA reactivity e.g. by any normal structural alert relationship for mutagenicity. These compounds are unlikely to directly bind to DNA, hence it is far more likely that any observed genotoxicity is induced by indirect DNA targets such as mitotic spindle (aneugencity), reactive oxygen species (ROS) generation or topoisomerase II inhibition. For pharmaceutical research, topoisomerase II inhibition is a particularly interesting genotoxic mechanism, as the enzyme is used as a target for both oncology and anti-infective drugs. Furthermore, some topoisomerase II poisons, be they either cancer drugs or antibiotics, are known to be very potent *in vitro* mammalian cell mutagens (Boos and Stopper, 2000; Smart, 2008a). Whilst there has been a great deal of research into topoisomerase II inhibition, it is surprising that the direct relationship between enzyme poisoning and genotoxicity has not been firmly established. For example, with anti-infectives, there is a known correlation between compounds that target bacterial gyrase and positive responses in mammalian cell genotoxicity assays, but attempts to quantify topoisomerase II inhibition and related genotoxicity have shown that the methods to measure topoisomerase II are either insensitive or (possibly) other genotoxic mechanisms are in play (Lynch *et al.,* 2003). To help with the discovery of safe and efficacious new medicines to tackle the unmet need in such serious conditions as tuberculosis and hospital acquired infections it would clearly be useful to gain a better understanding of the relationship between off-target mammalian cell topoisomerase II inhibition and genotoxicity. Accordingly, a robust method for determining topoisomerase II inhibition in mammalian cells would be a valuable adjunct to available tools and may also help to elucidate the mechanism of genotoxicity for at present unexplainable positive agents.

However, if after using the most sensitive tools available to measure the topoisomerase II poisoning by model genotoxicants (e.g. the oncology drug etoposide and the anti-infective gemifloxacin), the direct relationship between topoisomerase II inhibition and genotoxicity still cannot be established, it is important to determine whether these model compounds are also genotoxic by other mechanisms, for example, induction of ROS. If compounds that are currently 'assumed' to be genotoxic via topoisomerase II inhibition also have other modes of action, this could significantly alter their risk assessment.

To gain a better understanding of the real rate of unexplainable *in vitro* genotoxicity positives, this project will carry out an historical review of *in vitro* mammalian cell

genotoxicity data (specifically the mouse lymphoma assay) generated in an experienced laboratory using modern testing protocols. Initial consideration will be given to whether aneugenicity could contribute to the rate of unexplainable positives. Following on from this the relationship between mammalian cell topoisomerase II inhibition and *in vitro* genotoxicity will be investigated, along with the potential of topoisomerase II poisons to induce mammalian cell genotoxicity by ROS generation. The overall aim will be to gain a clearer understanding of the contribution of topoisomerase II inhibition to mammalian cell *in vitro* genotoxicity.

1.2 Genetic Toxicity Testing For Pharmaceuticals

The science of genotoxicity testing is now some 4 decades old, with Bruce Ames' seminal work on bacterial mutagens in the early 1970's (Ames *et al.,* 1973a) paving the way for the derivation of testing paradigms to be used to ensure new pharmaceuticals, food additives, pesticides and a whole range of other consumer products are not inherently genotoxic (MacGregor *et al.,* 2000). Much has changed over this period. Following the discovery of non-mutagenic and epigenetic carcinogens (Holliday, 1987; Ashby and Purchase, 1988a), Ames' original view that 'mutagens are carcinogens' has long been understood to be a major over simplification. However, the assessment of the genotoxic potential of new chemical entities is still a mainstay of pharmaceutical safety assessment and a regulatory requirement in all Organisation for Economic Cooperation and Development (OECD) territories (OECD 1997; ICHS(R1), 2011).

In practice, the genotoxicological assessment of new pharmaceuticals is very clearly defined in internationally accepted guidelines. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was organised in 1990 to ensure that the safety assessment of new medicines brought to a global market was conducted in a unified way. To this day ICH is continuing to monitor new research and issue and update new guidance documents. The first ICH guidelines for genotoxicity assessment were published in the late 1990's. These were; ICH Topic S2A Genotoxicity: Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1996) and ICH Topic S2B Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals (1997), which detailed specific guidance on tests and the battery of tests to be performed, respectively. The recommended testing in these documents was based on 20 plus years of genotoxicology research. Research that had identified the range of mutations that can lead to heritable change, from gene mutations (both base pair substitution and frame shift mutation), chromosomal aberrations (including mitotic recombination and large DNA deletions) up

to and including loss of whole chromosomes (non-disjunction and aneuploidy). Over this time it was clear that no single test could identify all of the possible chemical induced events involved in mutagenesis. The bacterial reverse mutation test developed by Bruce Ames was effective at identifying DNA reactive compounds that induce base-pair substitution and frame-shift mutations (Ames *et al.,* 1973b), but prokaryotic DNA is limited in its ability to detect damage at the chromosomal level. Mammalian cell gene mutation assays such as the NA^+ K^+ dependent ATPase enzyme assay will not identify large DNA change or deletions because of the small size of the target gene and the essential functionality of surrounding genes (Muriel *et al.,* 1987). The mammalian cell chromosome aberration and micronuclei tests will only identify damage at the chromosome level. Furthermore, no *in vitro* system can mimic the complexities of *in vivo* metabolism and exposure. Accordingly, the concept developed that a battery of short term tests was the best way to identify the widest spectrum of genotoxic mechanisms. It would be incorrect to believe that the current recommended battery is based entirely on good science. Rather, the battery was developed from consensus opinion following a variety of expert group meetings. Hence the current guidelines with their origins in the early 1990's may not be perfect, but they are tried and tested and both their strengths and weaknesses are well understood (ICHS22A, 1996; ICHS22B, 1997). This does not mean that regulatory genotoxicity testing is not open to innovation. Recently the guidelines have been reviewed and revised (Zieger, 2010; ICHS2(R1), 2011). However, the recommended battery of *in vitro* and *in vivo* tests remained broadly the same.

1.2.1 The ICH S2 Recommended Tests

The ICH recommended test battery is comprised of the following assays.

1.2.1.1 The Bacterial Reverse Mutation (Ames) Test

The bacterial strains used in the Ames Test are mutant in their ability to synthesize essential amino acids. The basic study design is presented in Figure 1.1. Mutagens are detected by reverse mutation of histidine-requiring mutants of *Salmonella typhimurium* strains and tryptophan-requiring mutants of *Escherichia coli* strains. Strictly speaking, the ubiquitously used term 'Ames' test only refers to the assay using salmonella strains, the *E.coli* strains were a later addition to Bruce Ames' original protocol (Green *et al.,* 1976; Venitt *et al.,* 1984). When the tester strains are grown on minimal media agar plates containing just a trace amount of histidine (*S.typhimurium* strains) or tryptophan (*E.coli* strains), only those bacteria that have reverted back to the

wild type ability for histidine (his^+) or tryptophan (trp^+) synthesis are able to form discernable colonies.

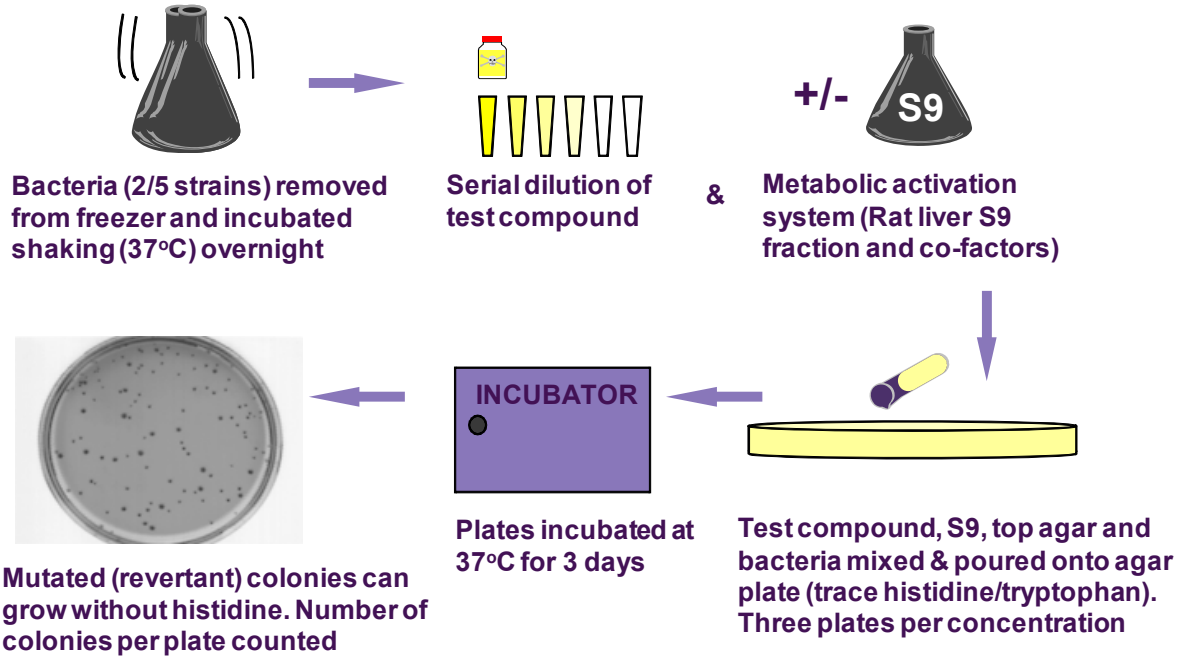


Figure 1.1: The Bacterial Reverse Mutation Test
Schematic of testing regime for a two or 5 strain Ames test

A variety of bacterial strains are used in the test, these differ both in the type of mutation required to cause reverse mutation to histidine/tryptophan independence and with respect to their sensitivity in detecting different mutagens (Table 1.1). The ability of tester strains to detect mutagens is enhanced by mutation, other than *his* or *trp*, and by plasmid insertion. Most contain a *uvrB* mutation, decreasing their DNA excision repair capability and the *rfa* mutation that increases cell wall permeability thus allowing uptake of larger molecules. Some strains (e.g. TA102 and E.coli *uvrA*) do not have the *uvrB* mutation, thus they are DNA excision repair proficient and are capable of detecting mutations by inefficient repair. Some strains also contain the plasmids pKM101 and pAQ1, which encode the SOS-repair genes and enhance error prone repair (Maron and Ames, 1983).

Bacteria	Strain	Plasmid	Reversion event
Salmonella typhimurium	TA1535	None	Base-pair substitution
Salmonella typhimurium	TA100	pKM101	Base-pair substitution
Salmonella typhimurium	TA98	pKM101	Frameshifts
Salmonella typhimurium	TA1537	None	Frameshifts
Escherichia coli	WP2*uvrA*	pKM101	Excision repair proficient, can detect cross-linking agents and small deletions
Salmonella typhimurium	TA102	pKM101, pAQ1	Excision repair proficient, can detect cross-linking agents and small deletions. Capable of identifying gyrase inhibitors
Salmonella typhimurium	TA104	None	Excision repair proficient, can detect cross-linking agents and small deletions. Capable of identifying oxidative stress induced mutation

Table 1.1 The Reverse Bacterial Mutation Assay: Examples of Common Tester Strains

1.2.1.2 The *in vitro* Chromosome Aberration Test (IVC)

The principal of the IVC is to visualize structural chromosomal aberrations using light microscopy (Figure 1.2). After treatment, cells are blocked in metaphase (usually by the addition of colchicine or colcemid). Metaphase slide preparations are stained with a DNA stain (e.g. giemsa) and scored by skilled cytogeneticists who are able to identify chromosomal gaps, breaks and rearrangements (Evans and O'Riordan, 1975; Scott *et al.,* 1990). Structural aberrations may be of two types, chromosome or chromatid (single chromosome arm); the majority of chemical mutagens induce predominantly chromatid aberrations i.e. breaks in a single chromosome arm. An increase in polyploidy can be used as a surrogate for a chemicals potential to induce changes in chromosome number (aneuploidy). Several immortalized cell lines may be utilised for the IVC. However, it also has the advantage that phtyohaemagglutinin stimulated dividing primary human lymphocytes can be used. The disadvantage of the test lies in the difficulty and time consuming task of scoring multiple slides.

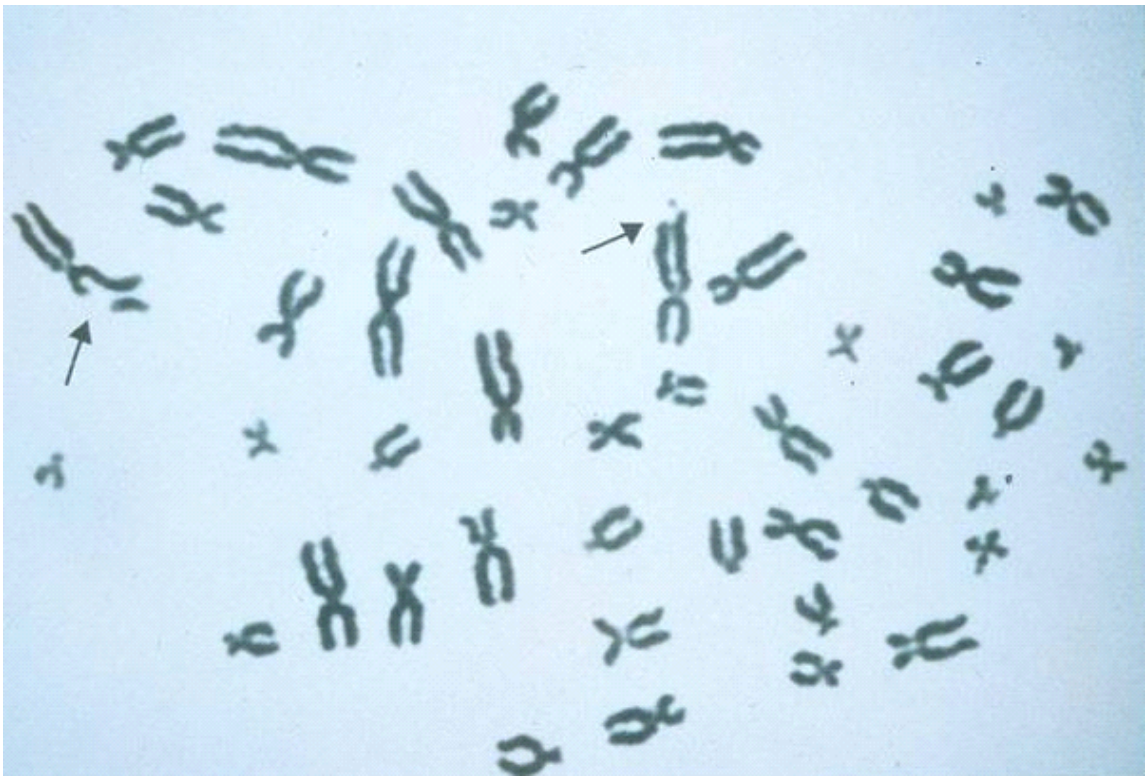


Figure 1.2: Metaphase Preparation of Human Lymphocytes for analysis of Chromosome Aberrations.
The arrows indicate chromosome breaks

1.2.1.3 The Mouse Lymphoma Assay (MLA)

The MLA can be seen to have an advantage over the IVC, and the later to be discussed *in vitro* micronucleus test, as it is capable of detecting point mutations **and** large chromosome deletions (Moore *et al.,* 1985). The assay was initially designed and validated in the 1970's by Don Clive and his co-workers (Clive *et al.,* 1972; Clive *et al.,* 1979). Jane Cole later modified the original agar plate protocol and developed a microtitre technique (Cole *et al.,* 1983, Cole *et al.,* 1986). See Figure 1.3 for the basic microtitre version test design. The MLA uses the mouse lymphoma cell line L5178Y $tk^{+/-}$ clone 3.7.2C, which is heterozygous at the thymidine kinase (*tk*) locus on chromosome 11. Thymidine kinase is a nucleoside salvage enzyme. Loss of functional tk^{+} expression on chromosome 11b, i.e. mutation to $tk^{-/-}$, renders cells resistant to the toxic thymidine analogue trifluorothymidine (TFT). $Tk^{-/-}$ cells are viable as they retain *de novo* thymidine and DNA synthesis. Hence TFT is used to select $tk^{-/-}$ mutant clones in a background of $tk^{+/-}$ cells (Clive *et al et al.,* 1987). Theoretically, a wide variety of mutagenic events can lead to phenotypic TFT resistance, including; small mutations within the tk^{+} gene (genetic mutations), larger chromosomal events within and beyond the tk^{+} gene altering chromosome 11b structure (clastogenicity), mitotic recombinations

and, it has been proposed, loss of whole chromosome 11b via chromosomal non-disjunction or aneuploidy (Clements, 2000). However, there is still much debate regarding whether the MLA can reliably detect *in vitro* aneugens (O'Donovan *et al.,* 1999). As the above events can be induced by various classes of agents, the assay is capable of detecting a wide variety of genotoxic chemicals. Furthermore, $tk^{-/-}$ mutant clones can have slow or wild-type growth rate. The difference in mutant clone growth has been attributed to different mechanisms of DNA damage: i.e. chromosomal mutations extending beyond the *tk* gene produce small slow growing mutant clones, whilst intragenic mutations produce large wild-type growing clones (Combes *et al., 1*995). However, as it has been shown that small mutants can also result from other mechanisms (Blazak *et al.,* 1986 and Blazak *et al.,*1989), mutant colony size should be used only as an indicator, not as a definitive measure of a chemicals mode of mutagenic action.

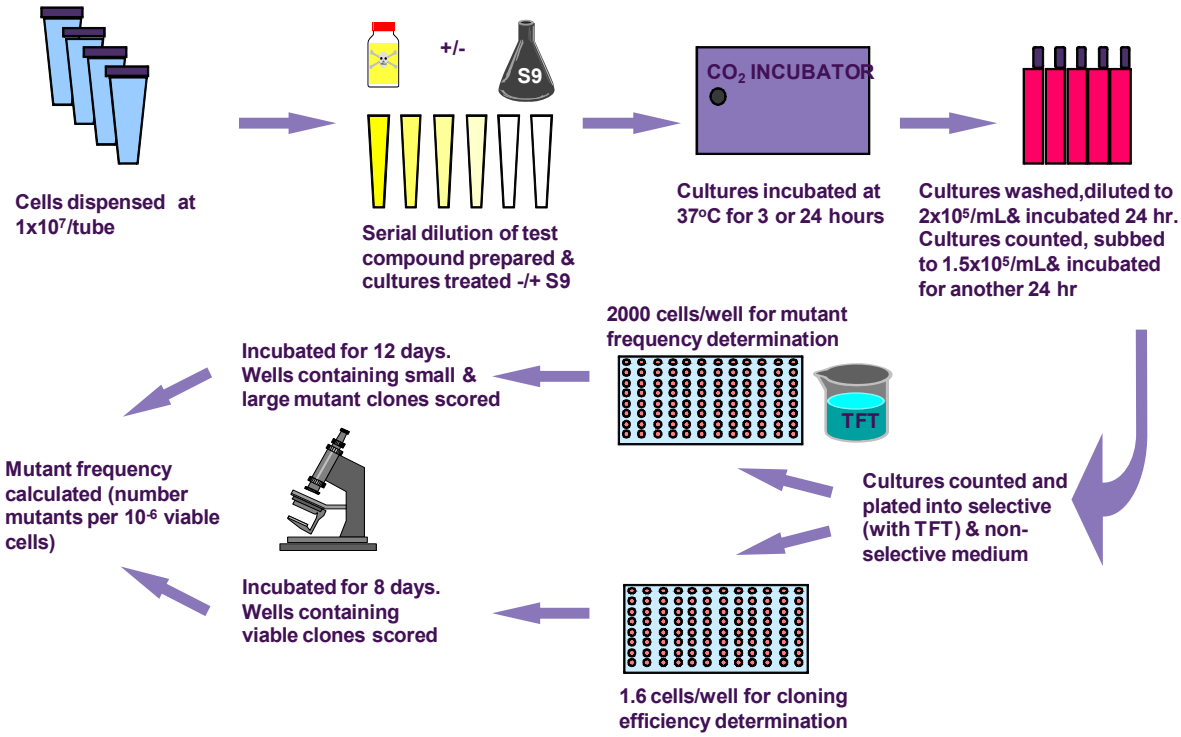


Figure 1.3: The Microtitre Mouse Lymphoma Assay
3 hour incubation use as standard, 24 hour incubation used to identify division dependent mutagens, cells cultured for 2 days post-treatment then plated in selective (TFT containing) or non-selective medium
TFT = trifluorothymidine, selectively kills non-mutant (heterozygous $tk^{+/-}$ cells)

1.2.1.4 The *in vitro* Micronuclei Test (MN(vit))

Analysis of micronuclei formation was originally proposed back in the 1970's as an alternative to *in vivo* analysis of chromosome aberrations (Heddle, 1973; Schmid,

1975). *In vitro* versions of the assay were later developed (see Fenech, 2000 for an example of a common Mn(vit) test design). Micronuclei are fragments of DNA that fail to be integrated into nuclear material following segregation. They may be formed from chromosome/chromatid breaks (clastogenicity) or loss of a whole chromosome (aneugenicity). Accordingly, the MN(vit) is capable of detecting both types of damage and centromere staining of micronuclei can be used to identify the mechanism of their generation (Figure 1.4). Similar to the IVC, the MN(vit) can be performed with a variety of cell lines. The cytochalasin B cytokinesis block MN(vit) has also been developed to facilitate the use of phtyohaemaglutinin stimulated primary human lymphocytes. In this version of the test only binucleated cells are scored, thus ensuring micronuclei are counted in dividing cells (Fenech, 2000). The basic study design of the MN(vit) is presented in Figure 1.5.

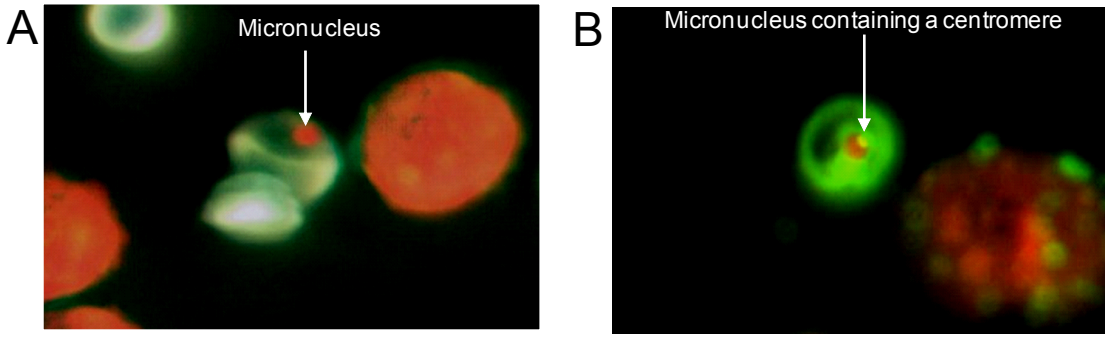


Figure 1.4: L5178Y cell micronuclei stained for presence of an intact centromere
A: micronucleus without a centromere indicating a clastogenic event
B: micronucleus with a centromere indicating an aneugenic event

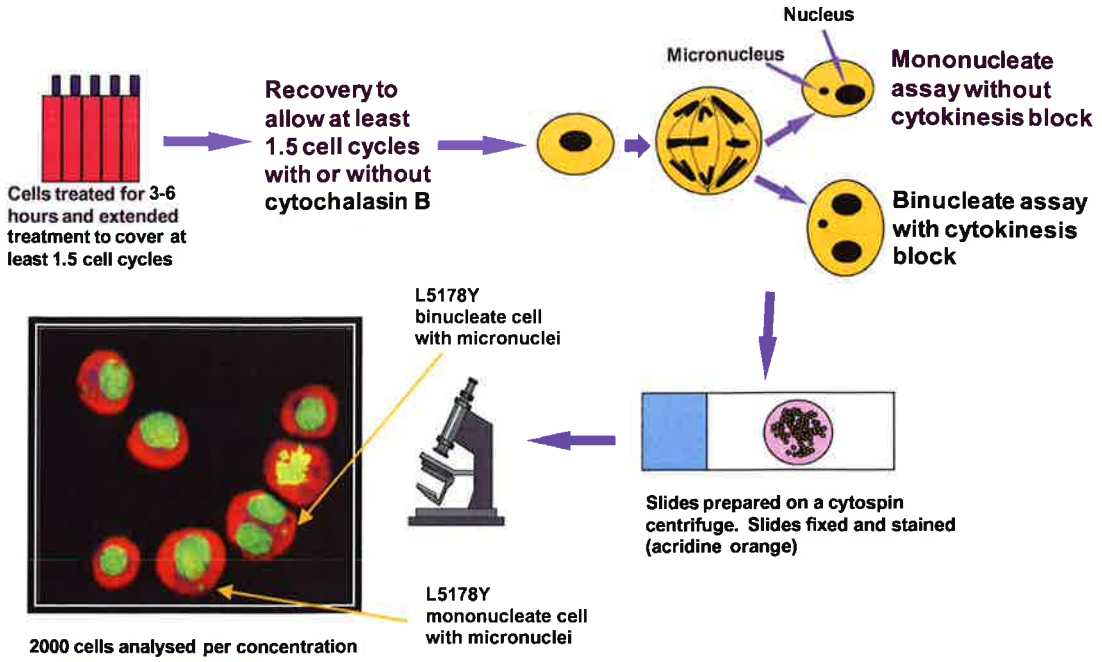


Figure 1.5: The *in vitro* Micronucleus Assay using L5178Y cells
Cells incubated for 1 day after treatment then prepared on microscope slides prior to be fixed and DNA attained with acridine orange for micronuclei determination

Over the last decade, there has been a great deal of work on validation of the *in vitro* micronucleus assay, with several published papers in support of draft OECD Test Guideline 487 (including Fellows *et al.*, 2008 and Fellows and O'Donovan, 2010). The guideline was finally signed off in 2010 (OECD, 2010). Whilst the mouse lymphoma assay can theoretically detect a wider spectrum of mutation than the MN(vit), the MN(vit) has the advantage that it uses less compound (approximately 10 mg compared to 200 mg for a single MN(vit) or MLA experiment, respectively), is quicker to perform (one week compared to three weeks) and has the potential to use automated scoring (Schunck *et al.*, 2004; Fellows, 2008).

1.2.1.5 The *in vivo* Rodent Chromosome Aberration Test and Bone Marrow Micronuclei Test

Following rodent dosing with the agent under investigation, the *in vivo* chromosome aberration test looks for chromosome aberrations in dividing haematopoietic cells after metaphase block and the *in vivo* micronuclei test investigates micronuclei in immature erythrocytes i.e. those formed during chemical exposure (Richold *et al.*,1990). Given the relative ease of analysis, the *in vivo* micronucleus test is generally the assay of choice for chemical safety evaluation. Erythroblasts expel their nuclei a few hours after the last mitotic division, but any micronuclei remain in the cytoplasm of the immature

erythrocyte. By using stains that can differentially image RNA and DNA (e.g. acridine orange; which under fluorescence RNA is red and DNA yellow/green), immature erythrocytes can be distinguished from mature erythrocytes and the frequency of micronucleated immature erythrocytes can be established. Sampling the bone marrow at both 24 hours and 48 hours after treatment allows the detection of compounds with short term and delayed effects (Schmid, 1975). The basic design of the *in vivo* micronucleus test is presented in Figure 1.6. Similarly to the MN(vit), the *in vivo* micronuclei assay has the advantage of being able to detect both clastogenic and aneugenic events.

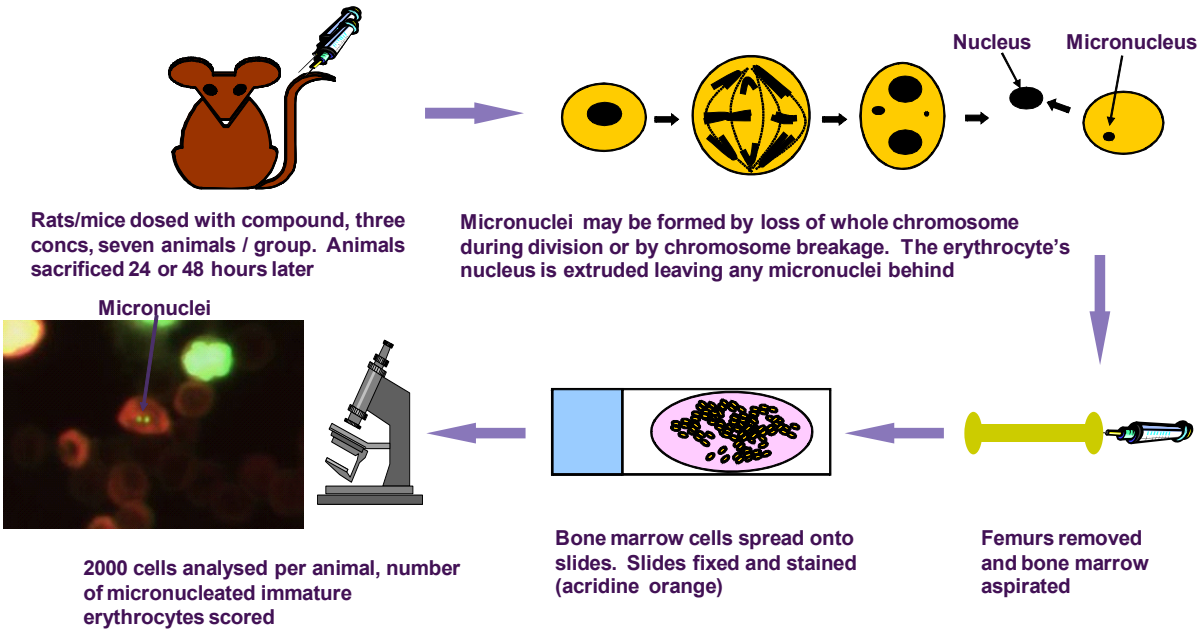


Figure 1.6: The Rodent Haemopoietic Cell *in vivo* Micronucleus Test
Rodents dosed daily twice then sacrifice 24 hours after final dose, bone marrow aspirated from femurs, resultant cells prepared on microscope slides, fixed and DNA stained with acridine orange prior to micronuclei determination

1.2.1.6 The Rodent *in vivo* Comet Assay

The single cell gel electrophoresis or comet assay was first introduced as an *in vitro* genotoxicity screen by Singh *et al* in the late 1980's (Singh *et al.,* 1988). It was later realised that the assay also had potential as an *in vivo* test (Anderson *et al.,* 1998). The major advantage of the *in vivo* comet assay being that DNA damage could be measured in cells from any tissue from which a single cell preparation could be made. This includes the ability to measure DNA damage in hepatocytes, the cells primarily involved in xenobiotic metabolism. Rodents are dosed and sacrificed 3 hours later. Single cell preparations are then made from the relevant tissue samples and set in an agar matrix on a microscope slide (see Figure 1.7 for the basic assay design). The

principal of the assay relies on migration of unwound DNA from the nucleus during electrophoresis. The more DNA breaks there are, the greater the amount of DNA there will be in the comet tail and this can be measured by propidium iodide (PI) staining and image analysis. The comet assay is recommended in ICH S2(R1) as a second/follow on *in vivo* genotoxicity test or as a single assay combined with an *in vivo* micronucleus end-point.

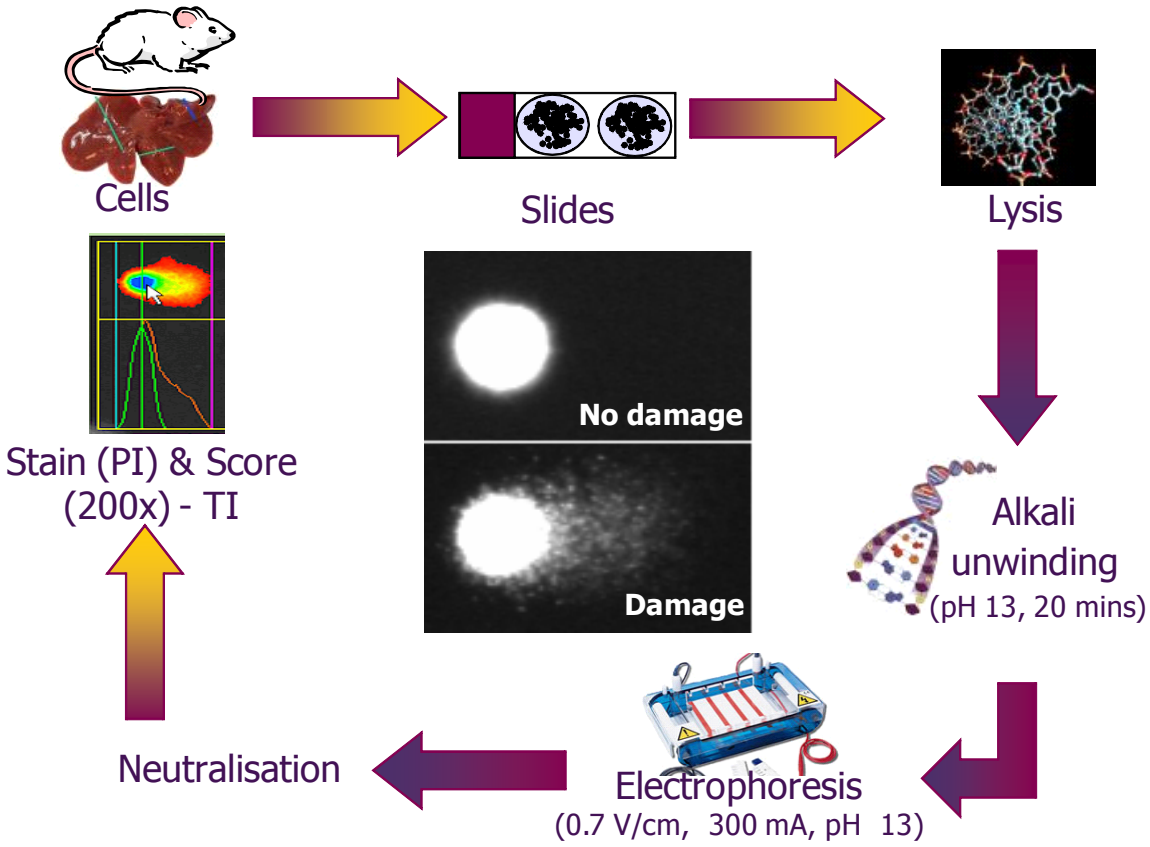


Figure 1.7: The *in vivo* rodent comet assay
Rodents dosed 24 and 3 hours hours prior to sacrifice, single cell suspensions prepared from relevant tissue, cell embedded in agar, lysed, then electrophoresed and staoned with propidium iodide (PI) prior to analysis of amount of DNA in comet tail

1.2.1.7 The history of genotoxicity testing and guidance

The following table (Table 1.2) shows a brief history of the milestones in mutation research and the development of the assays currently recommended in ICH guidance.

Year	Milestone in mutation research and guidance
Late 18th century	Nasal cancer identified in snuff users. Soot wart' reported in Chimney sweeps
1900-1920	Mutation and carcinogen experiments in bacteria, drosophila and *in vivo* (coal tar on rabbit ears)
1920-1940	Single chemical, co-chemical and radiation treatment shown to be carcinogenic in experimental animals
1940-1960	DNA identified as heritable material and structure elucidated, cancer initiators and promoters identified, metabolites shown to be mutagens, concept developed that pharmaceuticals and foodstuffs may be carcinogenic
1960-1970	DNA reactive mutagens shown to be electrophilic species. *In vitro* metabolism of pro-mutagens identified
1971-1975	Development of many of the commonly used genotoxicity screens, including: • Bruce Ames' bacterial reverse mutation (Ames) test • Don Clive's mammalian cell gene mutation assay using L5178Y $tk^{+/-}$ mouse lymphoma cells (MLA) • The *in vitro* chromosome aberration test using human lymphocytes • In vivo tests for chromosome damage and micronuclei formation in rodent haemopoietic material
1975-1980	Concert of non-DNA reactive carcinogens developed e.g. epigenetic, hormonal
1980-1985	Development of *in vitro* and *in vivo* Comet assays
1985-1995	OECD publish first guidance on safety testing of chemical
1996-2000	New international guidelines for genotoxicity testing published including: • OECD specific guidelines for chromosome aberration testes, bacterial and mammalian cell gene mutation assays and *in vivo* tests • ICH guidance on genotoxicity testing of pharmaceuticals including appropriate battery to use
2010	OECD guideline on the *in vitro* micronucleus test published
2010-onwards	Ongoing discussions include: • Updating ICH guidance for genotoxicity testing • Review of OECD guidlines; including development of a guideline for the Comet assay • Institute of Life Sciences looking into use of genotoxicity benchmark dosing for risk assessment

Table 1.2 A brief history of the development of genotoxicity testing
OECD = Organisation for Economic Cooperation and Development
ICH = International Conference on Harmonization

1.2.2 The predictivity of the genotoxicity test battery

Since the drafting of the original ICH guidance in the late 1990's, there has been a great deal of debate over the relative sensitivity and specificity of the assays from the recommended genotoxicity test battery. However, although it is clear that the objective

of genotoxicity assessment is to prevent harm to man, the only database available to judge the predictivity of genotoxicity assays is rodent carcinogenicity data. Whilst such comparisons are inevitably flawed, they are all that is currently available and are hence routinely used by expert groups for design of new guidance. The assessment of the predictivity of genotoxicity assays for rodent carcinogenicity has been extensively reviewed, with five key papers published in the latter half of the last decade (Kirkland *et al.,* 2005; Kirkland *et al.,* 2007; Kirkland and Speit 2008; Matthews *et al.,* 2006a; Matthews *et al.,* 2006b). These researchers made use of data from the National Toxicology Program (NTP) evaluations of the late 1980's and early 1990's. The NTP evaluations compared genotoxicity end-points with the available rodent carcinogenicity data. Initially, 73 chemicals were reviewed. Later over 400 chemicals were added, with evaluation of the predictivity of the Ames, MLA and the rodent bone marrow micronucleus (RBM) test (Zeiger *et al.,* 1990; Zeiger, 1998). Kirkland's and Matthew's reviews of these databases generally supported the value of the Ames test for identification of DNA adduct forming mutagens, and whilst there were some question regarding the sensitivity of the RBM (i.e. the correct prediction of a positive rodent bioassay result), it was considered to be an acceptable *in vivo* genotoxicity screen. However, the re-analyses of the NTP data did cast doubt on the value of other elements of the ICH test battery. In particular, the specificity (the correct prediction of a negative rodent bioassay result) of the *in vitro* mammalian tests. For example, the specificity of the MLA was as low as 39%, indicating that over 60% of non-carcinogenic chemicals are positive in one of the core tests from the ICH battery. The specificity of the IVC and MN(vit) was not markedly better (Kirkland *et al.,* 2005). The NTP data was based on a variety of chemicals from different industries including; industrial chemicals, foodstuffs, agrochemicals and a few pharmaceuticals. As pharmaceuticals were generally under-represented, it is possible that the over sensitivity seen for the mammalian cell tests would not be seen in pharmaceutical screening. However, the reported over sensitivity of these tests was to some extent reflected in data from pharmaceutical submissions in the 1990's, where 20% to 30% of pharmaceutical entities were shown to have a positive result in at least one mammalian cell genotoxicity assay (Muller and Kasper, 2000; Snyder, 2009). Furthermore, from a review by Peter Kasper, the current head of the German pharmaceutical regulatory authority (BfArM), all of the routinely run versions of the ICH recommended mammalian cell tests gave a similar percentage of positive results (Figure 1.8). It should also be noted that most of the pharmaceuticals from these submissions that gave positive responses in mammalian cell genotoxicity tests were considered to be safe to be given to man, hence the majority were not considered likely to be human carcinogens.

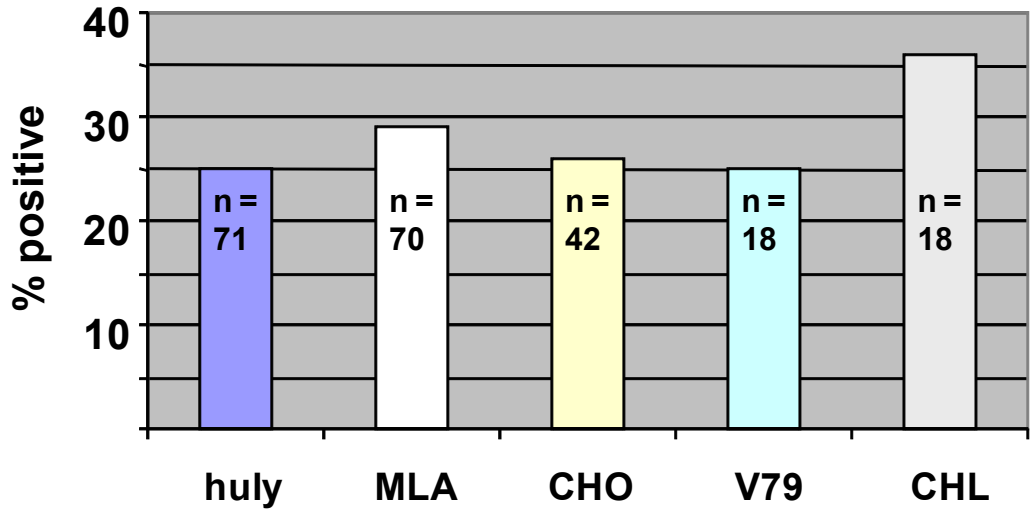


Figure 1.8: Incidence of positive results seen in mammalian cell genotoxicity tests from 104 compounds submitted to the German regulatory authority (BfArM) during the 1990's
huly = human lymphocyte chromosome aberration test, MLA = mouse lymphoma assay, CHO = Chinese hamster ovary cell chromosome aberration test, V79 = V79 cell human lymphocyte chromosome aberration test, CHL = Chinese hamster lung human lymphocyte chromosome aberration test
Image courtesy of Peter Kasper BfArM

These reviews all indicated that the ICH recommended *in vitro* mammalian cell genotoxity tests (including the MLA, IVC and MN(vit)) have a very high positive rate. Hence two fundamental questions need to be addressed:

1. Do pharmaceuticals tested in mammalian cell genotoxicity tests really generate the number of irrelevant positive results that has been indicated by retrospective reviews?
2. What are the likely mechanisms for the positive responses seen?

With reference to point 2, several mechanisms can be responsible for such unexplainable positives; these include induction of reactive oxygen species (ROS), aneugenicity, novel intercalation and non-drug target related topoisomerase II inhibition. Ronald Snyder formerly at Abbott Laboratories, Illinois, has published extensively on the contribution of topoisomerase II inhibitors to unexpected positives in *in vitro* genotoxicity assays (Snyder, 2000; Snyder and Gillies, 2002; Snyder and Hendry, 2005; Snyder *et al.,* 2005, Snyder *et al.,* 2006, Snyder, 2007; Snyder, 2009). Much of Snyder research used agents that block topoisomerase II DNA interactions to modulate the effect of potential novel topoisomerase inhibitors in the *in vitro* micronucleus assay. However, this work could not directly establish induction of stabilised topoisomerase II/DNA cleavage complexes and relate their presence to

induction of genotoxicity. It is perhaps surprising that this direct relationship is yet to be firmly established.

1.2.3 DNA covalently binding and non-covalently binding mutagens and the concept of a biological threshold for mutation

In terms of concentration and effect, a threshold may be considered to be the concentration below which no effect is seen. The traditional standpoint for genotoxic carcinogens has been that there is no safe dose. This dogma reflects the work in radiation research, mainly based on follow-up studies on survivors after the 1945 atomic bomb attacks on Japan, where there has been demonstrated to be a direct correlation with dose and the incidence of solid tumours, with no perceivable low dose threshold. Studies as recently as the last decade confirmed that this relationship was still holding true (Preston *et al.,* 2003). Whether or not this relationship is really the same for chemical genotoxic carcinogens is a matter of much debate. The concept of 'one hit' carcinogenicity has proven to be experimentally very difficult to prove or disprove. Until recently the sensitivity of DNA adduct measurement was not sufficient to delineate true low dose effects. However, the introduction of techniques such as accelerated mass spectrometry have meant that it is now practical to measure very low adduct levels. This has far from ended the debate. There are still advocates of the traditional view, they can experimentally demonstrate DNA adduct formation after treatment with extremely low concentrations of genotoxic carcinogens, and believe that this demonstrates that cellular repair mechanisms alone cannot remove the potential risk from these adducts (Zito, 2001). However, adducts do not directly cause mutation, there has to be some form of error prone repair. More recently several groups have experimentally demonstrated a biological threshold for the genotoxic carcinogen ethyl methane sulphonate, in both *in vitro* and *in vivo* tests (Doak *et al.,* 2007, Gocke and Muller, 2009, Johnson *et al.,* 2009). This threshold is based on the idea of a pragmatic threshold, a threshold below which any effect is considered biologically unimportant (Lutz, 1998) i.e. a concentration below which any induced adducts can be effectively repaired by normal cell mechanisms. This research into thresholds for genotoxic carcinogens is still in its infancy, and whether the same pragmatic threshold will be generally accepted, or whether each genotoxin will require an individual research programme to define the pragmatic threshold, remains to be seen.

Whilst researchers struggle with trying to demonstrate pragmatic thresholds for DNA adduct inducing mutagens, the concept of a threshold for mutagens that do not directly bind to DNA is now accepted for several mechanisms of genotoxic action. For

example, the theory that chromosome loss induced by aneugens has a threshold concentration-response curve has been accepted since the late 1990's (Elhajouji *et al.,* 1997). Although aneugens may induce chromosome number change by a variety of mechanisms, the term aneugen can be considered to describe any agent that can interact and effect cell division apparatus such as microtubules, centrosomes, and centromere associated proteins. Hence pure aneugens do not directly bind to DNA. Accordingly, low concentrations of aneugenic agents may not induce chromosome number changes because deactivation of multiple targets (e.g. microtubules, kinetochores, centrioles) are required to induce effect. For example, spindle poisons such as colchicine or vinblastine inhibit tubulin polymerisation. The spindle consists of many tubulin monomers. Therefore multiple targets have to be damaged before a significant adverse effect occurs (Elhajouji *et al.,* 1997; Aardema *et al.,* 1998). Thresholds have also been demonstrated for other indirect non-DNA adduct forming mechanisms of genotoxicity including oxidative stress, where it has been shown that the levels of ROS induced by low concentration of oxidising agents can be effectively dealt with by normal cellular free radical scavengers. It is only at concentrations above which these mechanisms are saturated that genotoxicity can be seen (Platel *et al.,* 2009). Similarly, thresholds of genotoxicity have been experimentally determined for topoisomerase II poisons (Lynch *et al.,* 2003). However, there is a growing concern amongst pharmaceutical regulatory authorities that topoisomerase II targeting anti-infectives that are shown to be very potent mammalian genotoxic agents are not safe. In 2009 the European Medicines Agency (EMA) withdrew the marketing authorisation application for the fluoroquinolone topoisomerase II targeting antibiotic Factive (gemifloxacin), quoting:

'The EMA was concerned that Factive may be more genotoxic (harmful to the DNA, the genetic material in cells) and that it may therefore cause more damage to the DNA than other fluoroquinolones.'

and

'Therefore, at the time of the withdrawal, the EMA was of the opinion that the benefits of Factive in the treatment of community-acquired pneumonia and acute exacerbation of chronic bronchitis caused by bacterial infection did not outweigh its risks.' (EMA, 2009).

These divergent views demonstrate the importance of gaining a better understanding of the mechanisms of topoisomerase II induced genotoxicity. The role that topoisomerases have in the maintenance of cellular homeostasis, and the reasons why

pharmaceuticals designed to target topoisomerase may have deleterious side-effects is reviewed in the following sections.

1.3 Topoisomerases

Topoisomerases are a range of enzymes that relieve the torsion in DNA by creating temporary strand breaks, thus allowing one DNA strand to pass through another. In simplistic terms, topoisomerases are characterised into two groups. Topoisomerase I enzymes cleave a single strand of DNA, thus allowing the complimentary strand to pass through the nick. Topoisomerase II enzymes cleave both DNA strands, thus allowing duplex DNA to pass through the double strand break. The overall effect of this action is to change the DNA linking number (i.e. the number of times that DNA molecules wind around each other) by -/+ 1 (topoisomerase I enzymes) or -/+ 2 (topoisomerase II enzymes) thus allowing the removal of torsional stress, knots and/or catanes (Bates and Maxwell, 2005). The inherent cytotoxicity of topoisomerase inhibitors has lead to their development as cancer chemotherapeutic agents. Furthermore, anti-infectives targeting bacterial topoisomerases (gyrase and topoisomerase IV) are important medicines in clinical use for the treatment of a range of infectious diseases, with new medicines being developed for the treatment of the unmet needs of hospital acquired infections and tuberculosis. However, the mechanism of cytotoxic action of therapeutic topoisomerase inhibitors can cause permanent DNA strand breaks. Additionally, the homology between bacterial and mammalian topoisomerases (Berger and Wang, 1996) means that many of the compounds that target the DNA binding site of bacterial topoisomerase enzymes also target mammalian cell topoisomerases, albeit to a lesser extent. If inefficiently repaired, the DNA strand breaks formed following topoisomerase inhibition in mammalian cells, can lead to cell death or DNA mutation.

There is a wealth of published data on the genotoxicity of topoisomerase II inhibitors (Anderson and Berger, 1994; Curry *et al.,* 1996; Boos and Stopper, 2000). Their genotoxic potency ranges from the highly potent cancer chemotherapeutic agents such as etoposide (Boos and Stopper, 2000), to the much weaker active fluoroquinolone antibiotics such as ciprofloxacin (Curry *et al.,* 1996). However, it has not proven to be easy to predict the genotoxicity of novel topoisomerase inhibitors based on their activity against the topoisomerase enzyme. A better understanding of these relationships would help with risk assessment for known topoisomerase inhibitors, plus may help

with the elucidation of hitherto unexplained genotoxic mechanisms, e,g. pharmaceuticals that may be genotoxic via off-target topoisomerase inhibition.

1.3.1 The role of topoisomerase in maintaining cell homeostasis

To fully appreciate why and how topoisomerase inhibitors make efficacious pharmaceuticals, and why and how they often have the undesirable side effect of associated genotoxicity, it is important to understand the mechanism of action of these enzymes.

As a group, topoisomerases are very well conserved enzymes. No organism has yet been found that can function without a minimum of one form of both a topoisomerase I and II enzyme (Wang, 2002). The main function of all topoisomerase enzymes is to maintain DNA topology. During transcription, replication and packaging, the DNA double helix is prone to underwinding, overwinding and subsequent catenation and knotting. If left unchecked these changes will lead to problems with transcription or replication and can induce chromosome breaks and subsequent cell death during packaging (Thanbichler *et al.,* 2005). Topoisomerase enzymes relieve these tensions.

Several topoisomerase families have been discovered. Table 1.3 details those that are most relevant for DNA topology maintenance in the majority of bacterial and mammalian cells. This table is not exhaustive, there are other forms of specialised topoisomerase enzymes that have evolved for specific reasons (e.g. reverse gyrases in thermophiles), but these are not relevant in standard pharmaceutical research or for safety assessment in man.

Enzyme	Type	Mechanisms
Bacterial topoisomerase I	IA	Cleaves single DNA strands, help to relax negative supercoils. Cannot decatenate unless one DNA strand already nicked
Eukaryotic topoisomerase I	IB	As bacterial, but found in eukaryotes
Bacterial Topoisomerase III	IA	Potent decatanator if one DNA strand already nicked
Bacterial DNA gyrase	IIA	Cleaves double stranded DNA, can introduce negative supercoils into DNA
Eukaryotic topoisomerase II	IIA	Cleaves double stranded DNA, can relax but not supercoil DNA
Bacterial Topoisomerase IV	IIA	Cleaves double stranded DNA, can introduce negative supercoils into DNA, potent decatanator even without requirement for nicks in one DNA strand
Plant Topoisomerase VI	IIB	Similar to topoisomerase IIA, found in some single cell organisms and plants

Table 1.3: DNA Topoisomerases
Table reproduced and amended from Bates and Maxwell, 2005

There are several anti-cancer therapeutic agents that target topoisomerase I, perhaps the best known example being camptothecin and the more recently discovered Irinotecan. Many topoisomerase I inhibitors are also known to be genotoxic (Cunha *et al.,* 2002, Kontek *et al.,* 2010) and the potential of targeting topoisomerase I for oncology research has been investigated for many years (Ferguson and Baguley, 1996). However, given the number of topoisomerase II inhibitors that are used as both anti-infectives and anti-cancer therapies, topoisomerase II is still a more common drug target and hence investigating the genotoxic activity of topoisomerase II inhibitors will be the main focus of the current research. Accordingly, the mode of action of topoisomerase II and the potential genotoxicity of its inhibitors will be considered in greater detail.

1.3.2 The structure of topoisomerase II enzymes

As detailed in Table 1.3, there are two subfamilies of topoisomerase II enzymes. However, in nature, topoisomerase IIB is fairly uncommon and is not found in bacteria or mammalian cells, hence only the structure and function of topoisomerase IIA will be considered and from here on when topoisomerase II is mentioned the reference will be to topoisomerase IIA. These two subfamilies of topoisomerase II should not be confused with the two isoforms of mammalian topoisomerase IIA, these being alpha and beta. Further detail of the structure and function of the two mammalian isoforms of the enzyme will be given later.

Prokaryotic and eukaryotic topoisomerase II enzymes differ in their basic structure.

1.3.2.1 Prokaryotic topoisomerase II

There are two major forms of bacterial topoisomerase II, based on their structure and function; gyrase and topoisomerase IV. Gyrases are unique in that they can induce negative supercoils. The modes of action of these enzymes will be discussed later. Bacterial forms of the topoisomerase II enzyme are heterotetramers (A_2B_2). Gyrases are made up of two GyrA subunits and two GyrB subunits, whereas topoisomerase IVs are made up of two ParE subunits and two ParC subunits which are analogous to GyrB and GyrA, respectively, see Figure 1.9. The sequence homologogy between the two forms of prokaryotic topoisomerase II is similar, with the exception of a 170 amino acid insertion towards the C terminal domain of the GyrB subunit and the C terminal domains of the 'A' subunits where sequence homology is generally only conserved between very closely related species (Champoux, 2001). The basic structure of the enzyme is such that two B subunits form the 'top' of the enzyme and these are linked to two A subunits which form the 'bottom' of the enzyme.

1.3.2.2 Eukaryotic topoisomerase II

Eukaryotic topoisomerase II enzymes are homodimers. The N terminal half of the enzyme correspond with bacterial GyrB and ParE, whilst the C terminal half corresponds with GyrA and ParC (Figure 1.9). The 'middle' of the eukaryotic topoisomerase enzyme which contains the DNA binding and cleavage site is often designated as $B^{/}$ (N-terminal end) and $A^{/}$ (C-terminal end) in consequence of the homology of this region with the C-terminal end of the prokaryotic B subunit and the N-terminal end of the prokaryotic A subunit. As previously mentioned, most mammals, including humans, have two topoisomerase II isoforms, α and β. These were originally discovered due to their size difference, topoisomerase IIα is approximately 170 kDa

and topoisomerase IIβ is approximately 180 kDa (Drake *et al.,* 1989). It was later discovered that these two different isoforms were encoded by genes on different chromosomes; in human the α encoding gene is on chromosome 17 and the β gene on chromosome 3 (Tan *et al.,* 1992). In mouse the α encoding gene is on chromosome 11 and the β gene on chromosome 14. At the amino acid level, the two human isoforms share approximately 68% homology, with the majority of the variability seen in the C terminal domain, where homology is only 34% (Willmore *et al.,* 1998).

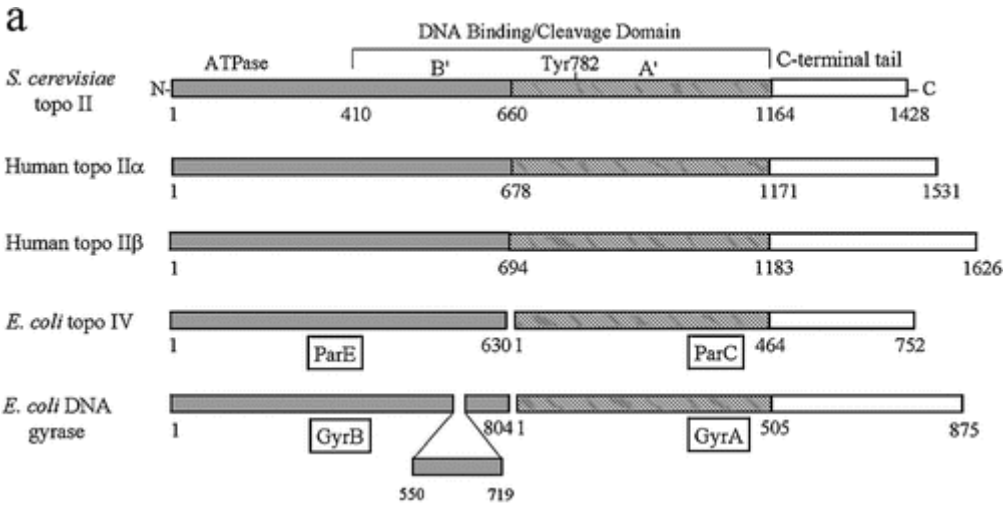


Figure 1.9: Sequence comparisons among type II topoisomerases.
The three-domain structure of the type IIA subfamily of topoisomerases is shown based on amino acid sequence homologies with E. coli DNA gyrase. In each case, the region or subunit that is homologous to the GyrB subunit (excluding the insertion from 550–719) is shown with sequence coordinates and a grey shaded box. The region homologous to the highly conserved first 505 amino acids of GyrA is depicted by a box with diagonal striping. For the *S.cerevisiae* enzyme, the C-terminal half of the GyrB-like region (residues 410–660) is referred to as B′ and the region homologous to GyrA as A′ (residues 661–1164); the combined B′-A′ regions constitute the DNA binding/cleavage domain containing the active site tyrosine site (Tyr782). The N-terminal half of GyrB and the corresponding regions in the other type IIA enzymes contain the ATPase domain (and the DNA capture domain, not indicated). The C-terminal tail domains of the enzymes are depicted as open boxes. The ParC and ParE subunits of *E.coli* topoisomerase IV are also referred to as the A and B subunits, respectively, to denote their relationship to the gyrase subunits. Reproduced from Champoux, 2001

As detailed in Figure 1.9, both eukaryotic and prokaryotic topoisomerase II share a core set of a conserved ATPase domain and a DNA binding domain which contains the active tyrosine site required for DNA cleavage (Champoux, 2001; Schoeffler and Berger, 2005). Most of the early work on eukaryotic topoisomerases used yeast (*S.Cerevisiae*). However, comparisons of topoisomerase structures amongst eukaryotes show that whilst they do not always have completely conserved amino acid sequences they do retain similar overall 3D structural shape. This can be seen for the crystalline structures in Figure 1.10, where the N-terminal half of *S.Cerevisiae*

topoisomerase II is compared against human topoisomerase IIα. The amino acid sequence is not identical but the overall structure and function is conserved (PDP Protein Data Bank, 2011).

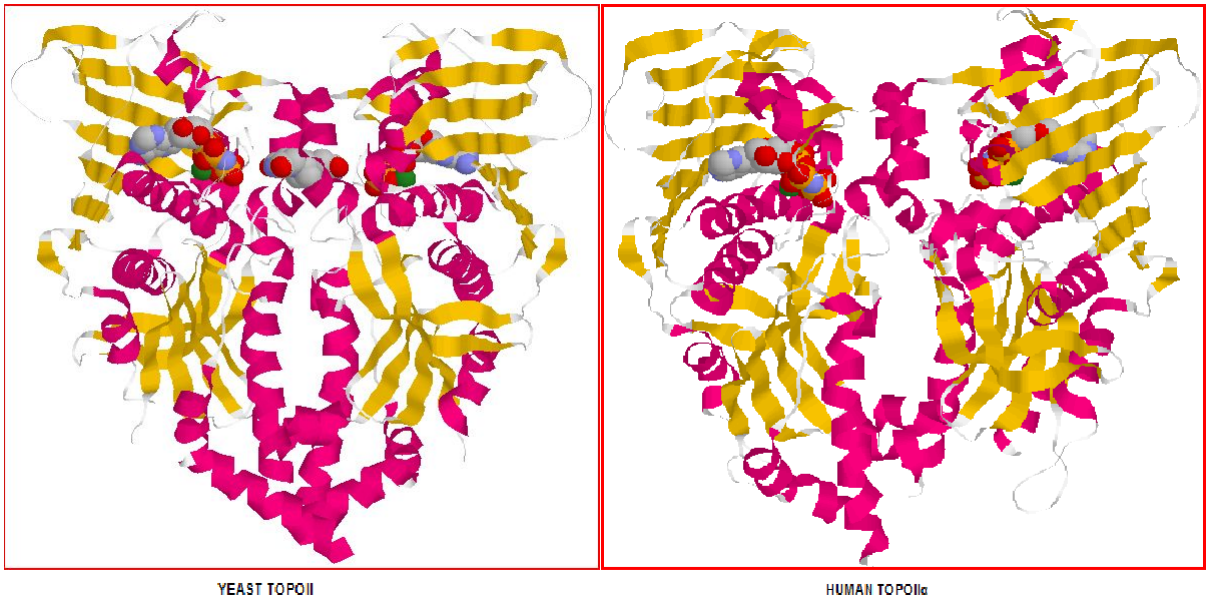


Figure 1.10: ATPase region of Yeast topoisomerase II and human topoisomerase IIα demonstrating similarity in overall structure
Regions are displayed in backbone and ribbon mode. The ligands are shown in spacefill mode. Image from PDP Protein Data Bank, 2011

The structure of both the homodimic eukaryotic enzyme and the heterotetrameric prokaryotic enzyme is exquisitely designed to fulfill the same function. Both the homodimer and the heterotetramer structure have a series of hinged regions between the two or four molecules whose altered conformity facilitates DNA capture, cleavage, reannealing and strand passage through the molecule.

1.3.3 The mechanism of action of topoisomerase II enzymes

The main function of topoisomerase enzymes was solved by the beginning of the last decade and was summarised in the reviews of James Champoux 2001 and Kevin Corbett and James Berger 2004 (Champoux, 2001; Corbett and Berger, 2004).
As previously mentioned, the function of topoisomerase II enzymes is to alleviate DNA torsion stresses and untangle and unknot DNA prior to transcription or pre- and post-replication modifications. The mechanism is via the creation of a transient break in one DNA double strand, known as the gate segment (G-segment) thus allowing a second strand, known as the transport segment (T-segment), to pass through (see Figure

1.11b). Hence changing the DNA linking by -/+ 2. The core activity of the molecule is associated with the hinged dimer interfaces around the ATPase domain and DNA cleavage domain (see Figure 1.11a).

1.3.3.1 Structure and activity of the ATPase domain

The ATPase domain lies within approximately 400 amino acids from the N-terminal of the eukaryotic dimer or prokaryotic ParE or GyrB. Within the ATPase domain there is the N-terminal positioned **GHKL** domain, so called because of its ubiquity in several ATPase containing enzymes i.e. **G**ryase, **H**sp90, bacterial histidine **K**inases and **M**ut**L**. ATP binds to the GHKL domain and facilitates DNA capture by inducing dimerisation of the molecule. The region of the ATPase domain towards the C-terminal contains the transducer domain, in which there is a lysine residue, which projects into the active GHKL domain and hydrogen bonds with bound ATP inducing hydrolysis. ATP hydrolysis is not essential for DNA strand passage as studies with the non-hydrolysable ATP analogue 5'-adenylyl-beta, gamma-imidodiphosphate (ADPNP) have shown. However, hydrolysis of ATP greatly increases the speed of enzymic activity (Champoux, 2001; Corbett and Berger, 2004).

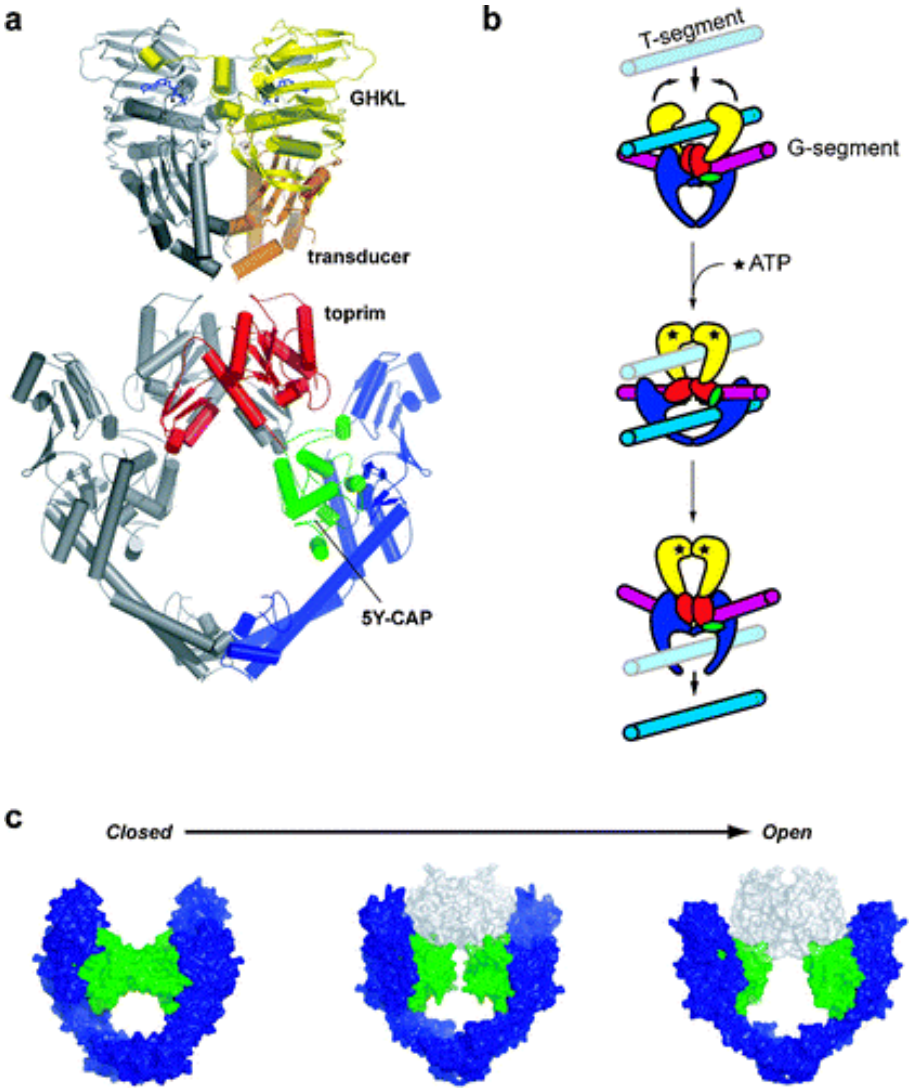


Figure 1.11: Topoisomerase II structure and mechanism
(a) Structure of *S.cerevisiae* topoisomerase II. GHKL domain yellow, transducer domain brown, toprim domain red, 5Y-CAP domain green, accessory domains blue. For clarity, one monomer is shown in grey. (b) Cartoon showing the sequence of events leading to strand passage. Domains are coloured as in (a), with the G-segment in magenta and the T-segment in cyan. (c) Three conformations of the type IIA topo DNA cleavage region. From left to right, a closed DNA gyrase, a partially open and open *S.cerevisiae* topoisomerase II. The *S.cerevisiae* topoisomerase II structures contain the toprim domains light grey, 5Y-CAP green and accessory scaffolding elements blue. Reproduced from Corbett 2004.

1.3.3.2 Structure and activity of the DNA cleavage domain

The DNA cleavage domain lies within the B′ A′ region of the eukaryotic dimer and at the interface of the C-terminal of GyrB and ParE and the N-terminal of ParC and GyrA (see Figure 1.9). The domain consists of two regions, the B′ region located **5Y-CAP**, CAP being a structure similar to the E.coli **C**atabolite **A**ctivator **P**rotein and which contains the active tyrosine residue site that binds to the **5**′ end of DNA following nucleophilic attack. The second region is the A′ located **Toprim** domain, which is conserved between both **Top**oisomerase enzymes and bacterial **prim**ases (enzymes that catalyse short RNA synthesis). The domain contains conserved acidic residues that form a binding site for Mg^{2+} ions that are required for catalytic function. The relative positions of 5Y-CAP and toprim in the topoisomerase II molecule provide the basis for interaction between these two active sites and allow the enzyme to open, hold and orientate the G-segment DNA before and after cleavage (Champoux, 2001; Corbett and Berger, 2004).

1.3.3.3 DNA strand passage

Opening and closing of the dimer hinged ATPase region (N-gate) is facilitated by ATP binding and release. Dimer linkage of this region, along with further dimer linkage around 5Y-CAP and a final dimer linkage at the A′ C-terminal domain gives topoisomerase II enzymes two hollow areas within the enzyme which temporarily hold the DNA T-segment during transport. In its inactive state the N-terminal gate is open. During this open conformation G-segment DNA enters the N-gate and binds to the 5Y-CAP domains. Binding of two ATP molecules within the GHKL region facilitates dimerisation around this site, capture of T-segment DNA and closing of the hinged N-gate. Hydrolysis of one of the ATPs induces a conformational change that helps T-segment transport. The change also forces movement between the dimers around the DNA cleavage domain thus eliciting a 4 base pair staggered break in the G-segment around the active tyrosine bound sites within the 5Y-CAP and toprim domains. Cleavage is achieved by the nucleophilic activity of the active tyrosines on phosphates of each held DNA strand backbone (Champoux, 2001; Corbett and Berger, 2004). The free 4'-phenol of the active tyrosine binds to the phosphate and initiates a transesterification reaction thus cleaving the strand leaving a free 3′ hydroxyl (Meresse *et al.,* 2004), as detailed in the following Figure 1.12.

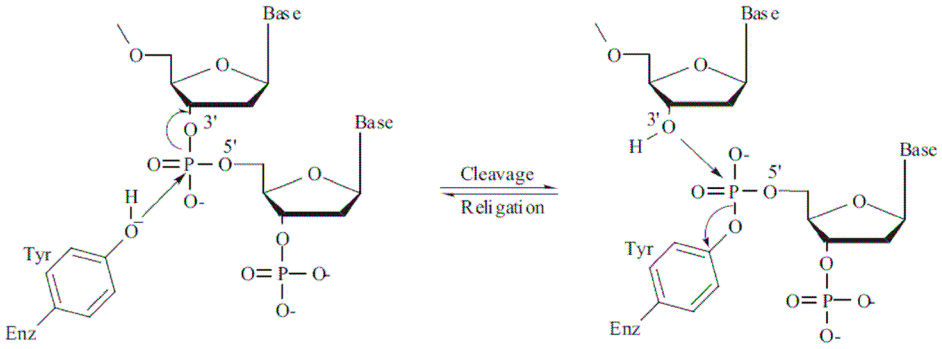


Figure 1.12: Transesterification of phosphate and DNA cleavage by active tyrosine
Reproduced from Meresse *et al.,* 2004

The active tyrosine sites both cleave and hold each single DNA strand by forming a covalent bond with the 5/ terminal phosphates, this helps to conserve DNA phosphate backbone bond energy thus facilitating later religation (Deweese *et al.,* 2009). This G-segment tyrosine/Mg^{2+} held strand break is known as the cleavage complex, and it is xenobiotic stabilisation of this complex that is one of the main mechanisms of topoisomerase II mediated cytotoxicity and genotoxicity. Following formation of the cleavage complex, the T-strand is then released and passes through the G-strand and out of the A/ C-terminal of the dimer (C-gate). Further ATP hydrolysis 'relaxes' the DNA cleavage domain allowing for G-strand reannealing and release from the enzyme. The dimer is thus destabilised and re-set ready for further DNA strand capture (Figure 1.11 b and c) (Champoux, 2001; Corbett and Berger, 2004).

1.3.3.4 Gyrase ability to negatively supercoil

All topoisomerase II enzymes are capable of relaxing supercoils (i.e. changing linking number by +2). However, only gyrases have the capacity to generate negative supercoils (i.e. changing the linking number by -2 (from +1 to -1)). This maintains genomic DNA in a slightly underwound state, which is helpful for bacterial DNA compaction and also replicative and transcriptive processes (Schoeffler and Berger, 2005). Gyrases are able to negatively supercoil due to a specialised DNA binding site within the DNA cleavage C-terminal domain, which facilitates a positive handed wrapping of 140 Kda of a DNA strand around the gyrase enzyme (Bates and Maxwell, 2007). This aids preferential capture and transport of a T-segment from the same DNA molecule as the wrapped G-segment (Corbett and Berger, 2004). Efficient gyrase supercoiling requires significant energy (Ca 114 kJ/mol), which is approximately equivalent to the energy release from hydrolysis of two ATP molecules (Bates and Maxwell, 2007).

1.3.3.5 Specificity of function of human topoisomerase II α and β

The two human topoisomerase II isoforms are not just genetically different, they are also functionally different. Over the last two decades, researchers have begun to understand their diverse roles. It was initially shown that levels of topoisomerase IIα are markedly increased during cell cycle G2 and M phases. Topoisomerase IIα was also found to be located within the nucleus during S-phase, all indicating that it has a role in chromosome untangling and mitotic segregation during and following replication. Furthermore, the α isoform is predominantly expressed in proliferating cells and is found to be closely associated with chromosomes during metaphase, highlighting its crucial function in cell division (Wilmore *et al.,* 1998; Christensen *et al.,* 2002; Linka *et al.,* 2007). Topoisomerase IIβ is maintained at similar levels throughout the cell cycle, is not chromosomally associated during metaphase and is the major isoform found in some end-differentiated cells. In the late 1990's studies using topoisomerase II knock-down yeast demonstrated that topoisomerase IIα and IIβ appeared to be functionally interchangeable, but at this time the specific function of topoisomerase IIβ remained a mystery (Austin *et al.,* 1995; Austin and Marsh, 1998). It was later shown that cells can function with only the α isoform but cannot survive with only the β (Christensen *et al.,* 2002). With the help of more sophisticated techniques the differences in the specific activities of the isoforms were further elucidated. It was found that topoisomerase IIα preferentially relaxes positively supercoiled DNA ahead of the replication fork, whereas topoisomerase IIβ relaxes both positive and negative supercoils with similar efficiency (Pommier *et al.,* 2010). Other work has shown that the β isoform plays a role in facilitating transcriptional activities (Ju *et al.,* 2006) and in cellular maintenance within the central nervous system, with a function in the maintenance of cell differentiation and migration for cells committed to neuronal progression (Heng and Lee, 2010) and for general neuronal growth and brain development (Linka *et al.,* 2007). It has also been experimentally demonstrated that the α and β isoforms have different roles with respect to DNA damage progression and repair in neuroblastoma and astrocytoma cells, with topoisomerase IIα being shown to accelerate DNA damage and β having a role in promotion of DNA repair (Mandraju *et al.,* 2008). Whether β has a similar function in other end-differentiated cells is not clear, but it has been suggested that poisoning of topoisomerase IIβ in non-proliferating cells with a subsequent reduction in the cells ability to repair DNA damage (specifically knockdown of non homologous end-joining) may be partly responsible for the secondary malignancies seen in cancer patients on topoisomerase II poison containing treatment regimes (Nitiss, 2009). The diverse functions and exactly why two similar yet different forms of the topoisomerase II

enzyme have evolved remains to be fully elucidated. With respect to the measurement of topoisomerase II inhibition and *in vitro* genotoxicity in mammalian cells, which are invariable rapidly dividing populations, it would seem probable that the α isoform is likely to be the main target.

1.3.4 Recent developments in the understanding of topoisomerase II mechanism

1.3.4.1 Function of the C-terminal domain

Recent studies on the mechanism of action of human topoisomerase II have highlighted the importance of the C-terminal domain, both on the activity of the enzyme and on the enzymes ability to interact with DNA. As previously mentioned, the C-terminal domain of the eukaryotic homodimer and of prokaryotic A subunits are the least conserved regions of the enzymes. It is also known that phosphorylation sites within the C-terminal domains play a role in topoisomerase activity during mitosis and chromosome condensation (Heck *et al.,* 1989; Escargueil *et al.,* 2000; Ishida *et al.,* 2001). Furthermore, the C-terminal was known to have a role in the cellular localisation of topoisomerase II (Adachi *et al.,* 1997). More recent studies using chimeric forms of the human enzyme (C-terminal swaps between topoisomerase IIα and IIβ) and constructs of the enzyme without C-terminal domains have demonstrated how the C-terminal plays an as yet not fully understood role in both strand passage activity and cell viability. There is mounting evidence to suggest that the C-terminal domain can be thought of as interacting with DNA strands like a magnet, with the traversed DNA as a metal thread, hence orientating movement and localization of the enzyme. Furthermore, the function of the topoisomerase II C-terminal domain differs between the two isoforms of the enzyme, with topoisomerase IIα C-terminal domain being essential for cell growth, but not topoisomerase IIβ, and with the topoisomerase IIβ C-terminal domain acting as a negative regulator for stand passage (Meczes *et al.,* 2008, Gilroy and Austin, 2011).

1.3.4.2 Cleavage and ligation of topoisomerase II mediated DNA breaks

The continuing crystallisation of various forms of topoisomerase II molecules has lead to several breakthroughs in understanding the relationship between structure and function. Crystallisation has shown how topoisomerase II molecules not only preferentially bind to DNA areas with significant curvature, but that the molecule itself can bend DNA by up to 150^{o}, thus facilitating the correct orientation of tyrosine cleavage sites and acidic residue Mg^{2+} binding sites within the toprim domain (Dong

and Berger, 2007). The requirement for divalent Mg^{2+} as a cofactor for DNA cleavage and tyrosine bonding to the 5′ terminal of each cleaved DNA strand has been known for over two decades. However, the role that Mg^{2+} plays in enzymic function has not been fully elucidated. The majority of recent studies have confirmed the enzymic requirement for two divalent cations, but studies have also shown that topoisomerase II uses these ions in an unusual way when compared to other cation DNA and RNA interactions. Topoisomerase II activity requires the divalent cations to stabilise the active groups around cleavage sites. It has been postulated that one Mg^{2+} promotes cleavage and interacts with the bridging 5′-oxygen of the cleaved bond, stabilises a leaving oxygen from the 3′-oxygen whilst also promoting ligation of the ribose 3′-hydroxyl moiety. The second ion would appear to bond in such a way as to hold and stabilise each DNA strand, but the exact mechanism is still unclear. This model is unusual in that only one ion is required for cleavage. The role of the second ion would appear to be primarily to prevent cleavage leading to permanent DNA double strand breaks (Deweese *et al.,* 2008; Deweese *et al.,* 2009; Schmidt *et al.,* 2010). The activity of these divalent cations and the stabilisation of the functional double strand break has direct relevance to both the activity of topoisomerase poisons, which will often interact with these processes, and the potential genotoxicity of chemicals that interfere with metal ion mediated cleavage and ligation. However, there is still much to learn with respect to the cleavage and ligation activity of topoisomerase II enzymes. A recent study suggested that at least for prokaryotic topoisomerase IV, the requirement for two divalent cations to catalyse cleavage activity and/or to stabilise cleaved DNA activity was at best inconclusive. The researchers found no evidence even for a weakly bound second divalent ion, and hence concluded that the enzyme could operate with single 'dynamic' ion coordination (Laponogov *et al.,* 2010). The process of religation has also not been fully elucidated. However, the proposed model is for acidic attack of the 3′-hydroxyl moiety leading to hydrogen removal and the formation of an oxyanion. This in turn initiates breakdown of the phosphotyrosine bond with subsequent re-ligation of the 5′ and 3′ ends (Deweese *et al.,* 2009).

1.3.5 Genotoxicity associated with drug induced topoisomerase II inhibition or poisoning

Whilst the consequence of inhibition of topoisomerase II has been previously alluded to, it is important to have a clear understanding of what is meant by topoisomerase inhibition and how topoisomerase inhibitors may elicit genotoxicity. There are often considered to be two categories of agents that inhibit topoisomerase II. These are:

1. **Topoisomerase poisons**: being agents that either stabilise the DNA/topoisomerase II cleavage complex (see Figures 1.13 and 1.15) or enhance generation of cleavage complexes. Both of these mechanisms increase the number of transient DNA strand breaks, hence turning the topoisomerase enzyme itself into a 'poison'. Furthermore, the increase in the number of bound enzyme DNA complexes results in a 'road block' on the double helix. DNA replicative machinery, such as helicases, stall at this block initiating various DNA repair mechanisms and causing subsequent permanent DNA breaks. Several anticancer agents such as etoposide and teniposide targeting eukaryotic topoisomerase II and the quinolone/fluoroquinolone antibiotics targeting prokaryotic topoisomerase II are topoisomerase II poisons. As chemically stabilised cleavage complexes lead to inefficiently repaired DNA breaks, topoisomerase poisons are genotoxic agents (Deweese *et al.,* 2009).
2. **Catalytic inhibitors**: being agents that prevent the activity of topoisomerase by interaction with the enzymes ATPase region. Catalytic inhibition may be activated by several mechanisms, including:
    a. **Competitive inhibition of the topoisomerase II ATP binding sites.** The aminocoumarin antibiotics (e.g. novobiocin) act in this way. As ATP competitive inhibitors effectively block the activity of the topoisomerase II enzyme before DNA cleavage, they are cytotoxic but generally not genotoxic (Gocke, 1991).
    b. **DNA intercalation.** Intercalation can be considered a form of catalytic inhibition in so far as intercalation and the resultant change in DNA structure can prevent topoisomerase II enzymes from binding to DNA, e.g. doxorubicin (Pommier *et al.,* 2010). At different concentrations, some intercalators may act as topoisomerase poisons by intercalation within cleavage complexes, which can directly induce frame-shift mutations (Ferguson *et al.,* 2007).
    c. **Inhibition of ATPase activity preventing ATP hydrolysis after strand cleavage.** Prevention of ATP hydrolysis can fix the closed clamp form of the enzyme and hence stabilise the topoisomerase II DNA complex. The cancer therapeutic bisdoxopiperazines (ICRF compounds 154, 159, 187 and 193) act in this way. Although these compounds are considered to be catalytic inhibitors, the closed clamp stabilised lesion is similar to that produced by topoisomerase poisons and can lead to DNA road blocks, double strand breaks and consequent genotoxicity (Austin

et al., 1995; Andoh and Ishida 1998; Cortés *et al.,* 2003; Pommier *et al.,* 2010).

Details of the modes of action of non-directly DNA intercalating genotoxic topoisomerase II inhibitors will be considered with reference to putative examples of different classes of drugs with varying targets; i.e. the cancer therapeutic agent etoposide (a mammalian topoisomerase II poison), the fluoroquinolone antibiotics (gyrase/topoisomerase IV poisons with some cross over to activity against eukaryotic topoisomerase II) and the ICRF cancer therapeutic agents (catalytic inhibitors).

Figure 1.13 schematically represents the various targets for topoisomerase inhibition and at which point during the activity of the enzyme drugs may act. Quinolone/fluoroquinolone antibiotics bind with the DNA cleavage domain, interacting close to the ligated strands and enhancing cleavage complex formation. Etoposide also binds within the DNA cleavage domain, but rather than enhancing cleavage complex formation, etoposide blocks religation hence increasing the number of stabilized cleavage complexes. ICRF187 blocks ATP hydrolysis and inhibits gate opening hence trapping DNA (Pommier *et al.,* 2010).

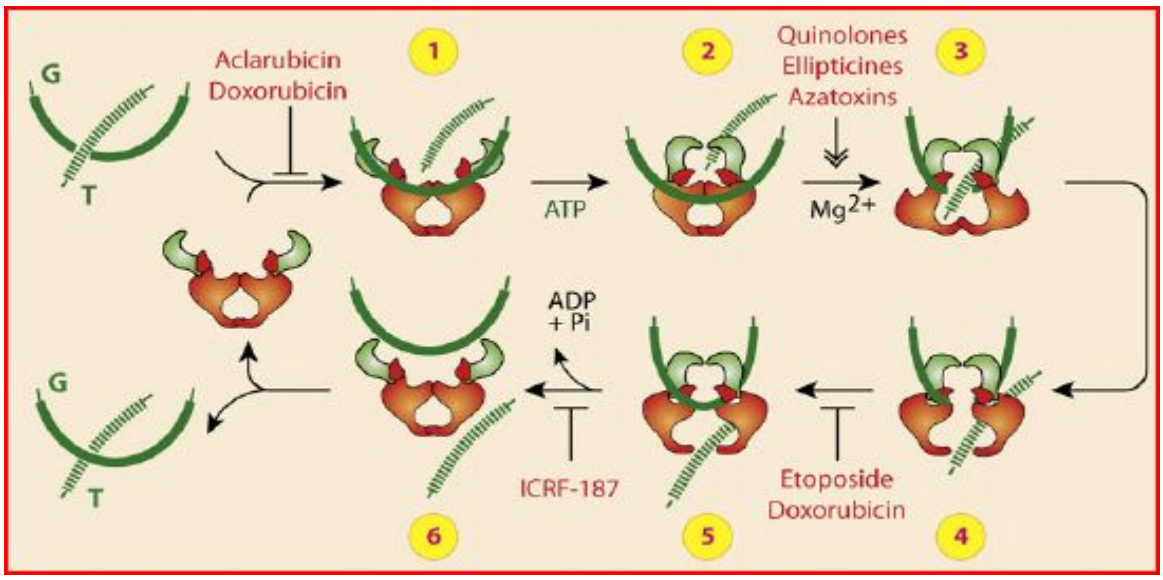


Figure 1.13: Topoisomerase II catalytic cycle and targets of activity of topoisomerase II inhibitors. Complexes of topoisomerase II, DNA and agents such as etoposide which bind within the nicked strand (as in 3 and 4) are stabilised, thus preventing release of the enzyme from DNA. These arrangements are known as Stabilise Cleavage Complexes and are the initial lesions responsible for subsequent permanent strand breaks. Reproduced from Pommier *et al.,* 2010

1.3.5.1 Etoposide: activity and genotoxicity

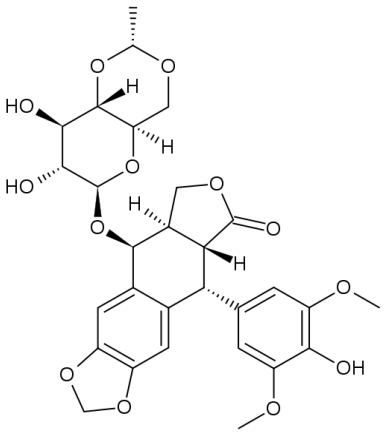


Figure 1.14: Anti-cancer agent etoposide molecule
Molecular Mass 588.557 g/mol

Etoposide (Figure 1.14) is a derivative of podophylotoxin, which is an extract from the mandrake plant that has been used in herbal remedies for millennium. In the latter half of the 20th century podophylotoxin was found to have anti-cancer properties. In the 1960s the more potent etoposide was developed and purified, which following clinical testing received approval for the treatment of several cancers in the 1970's and 1980's. Etoposide and its analogue teniposide have become two of the most widely prescribed anti-cancer drugs. Surprisingly, most of the early clinical trials and marketing was done before the exact mechanism of the anti-tumour activity of etoposide was discovered. It wasn't until the late 1970's and early 1980's that etoposide was shown to be a potent topoisomerase II poison (Hande, 1998).

Within the DNA cleavage domain, two etoposide molecules bind to base pairs flanking either side of the cleaved DNA phosphates. The effect is to displace the 3$^{/}$ hydroxyl such that religation is not possible. The topoisomerase/DNA cleavage complex is thus stabilised. Etoposide is a weak DNA intercalator and binds preferentially to the pre-existing topoisomerase II induced ligated DNA (Wu, 2011). Figure 1.15 shows how the molecules interact.

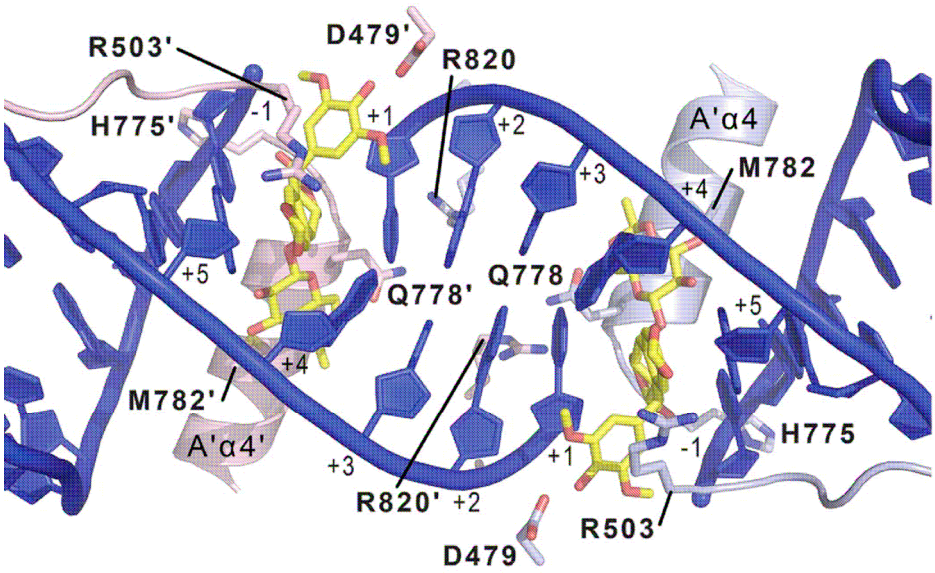


Figure 1.15: Interaction of etoposide with a DNA bound topoisomerase II molecule DNA is blue, helical topoisomerase II monomers in grey and purple, etoposide stick molecule yellow and red. Reproduced from Wu, 2011

Given its ability to stabilise cleaved eukaryotic DNA and road block helicase activity, etoposide has unsurprisingly been reported to be a potent mammalian mutagen in a number of *in vitro* and *in vivo* assays. See Tables 1.4 and 1.5 for data on the genotoxicity of etoposide in the ICH standard battery of tests and the *in vitro* Comet assay.

1.3.5.2 Bisdoxopiperazines: activity and genotoxicity

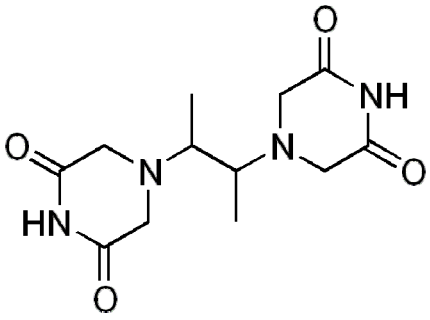


Figure 1.16: Anti-cancer bisdoxopiperazine ICRF193
Molecular mass 282.30 g/mol

Bisdoxopiperazines were initially synthesised as potential pharmaceuticals by the Geigy Corporation in the late 1950's. In the 1970's, following indications of antitumor

activity, the structure was taken up by the Imperial Cancer Research Fund, after which the main compounds from the group were named. ICRF154 was the first compound to be discovered, the more lipophilic and active derivative ICRF159 and the equally potent (+) enantiomer of ICRF159, ICRF187, followed. ICRF193 (Figure 1.16) is a dimethyl derivative with even higher lipophilicity, but also associated excessive toxicity. All of the ICRF compounds are potent ion chealating agents and this was initially thought to be the mechanism of their anti-tumour activity. However, in the early 1990's they were also shown to be potent topoisomerase II inhibitors (Andoh and Ishida, 1998).

Unlike etoposide, the bisdoxopiperazines compounds bind within the ATPase domain not the DNA cleavage domain (See Figure 1.9). Accordingly, bisdoxopiperazines do not act like classic topoisomerase II poisons. A single molecule binds and forms a bridge across the topoisomerase II dimers (see Figure 1.17 for binding of ICRF187). This does not involve direct competitive binding to the ATP site, but the bridging and induced confirmatory change within the ATPase domain prevents hydrolysis of the second ATP molecule and blocks molecular gate opening (Classen *et al.,* 2003, Nitiss, 2009). Hence, although DNA strand passage has occurred, DNA is trapped within the enzyme and a stabilised complex is formed that is similar to that seen with topoisomerase II poisons, accordingly the bisdoxopiperazines are positive in most standard genotoxicity assays (Tables 1.4 and 1.5)

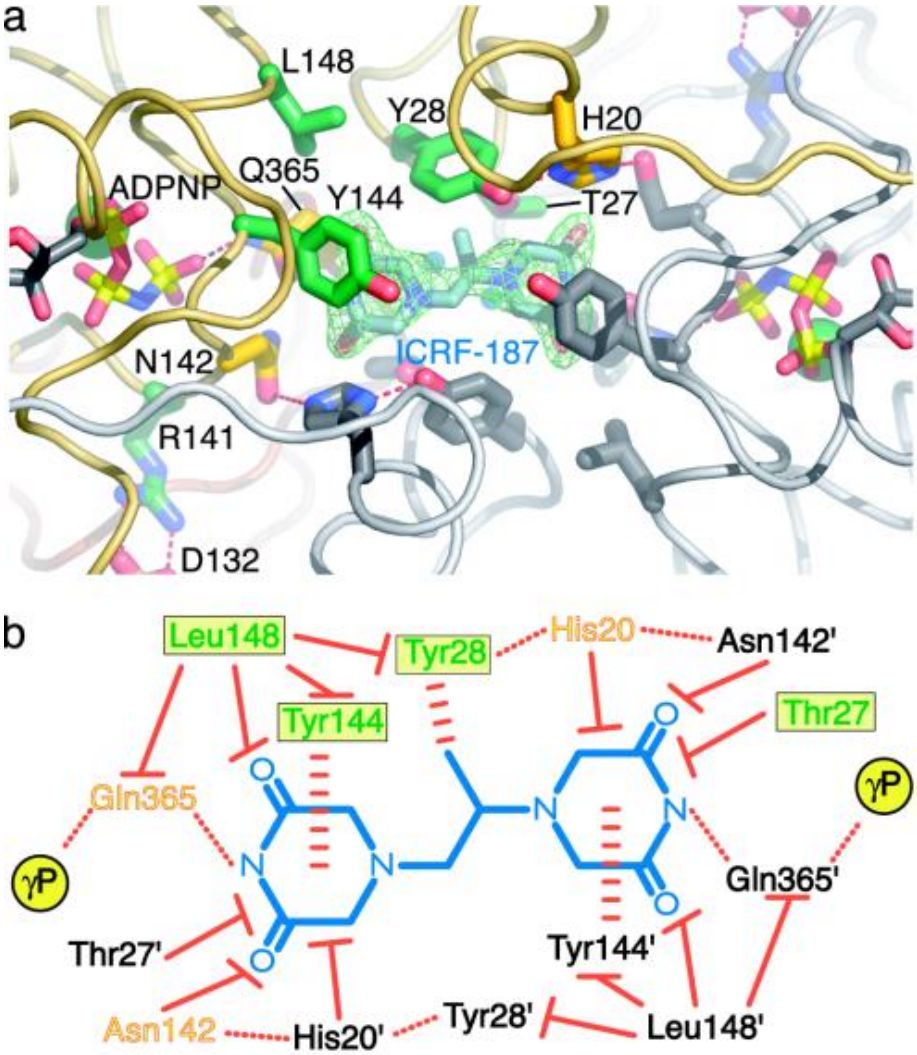


Figure 1.17: ICRF187-binding pocket and protein/drug interactions.
(a) ICRF187-binding pocket seen from the top of the dimer. An Fobs–Fcalc simulated-anneal omit electron density map shown in green is contoured at 1.5σ around ICRF187. ADPNP, ICRF187, and residues within 5 Å of the drug are shown in stick representation. (b) Schematic diagram of protein/drug interactions. ICRF187 is blue. Residues contacting the drug from each of the two protomers are indicated by collared or black text. Hydrogen bonds are indicated by dotted red lines, stacking interactions are indicated by horizontally dashed red lines, and van der Waals interactions are indicated by solid red lines with a flat end. The γ-phosphates of bound ADPNPs are indicated by yellow circles. Reproduced from Classen *et al.,* 2003

1.3.5.3 Quinolone/fluoroquinolone: activity and genotoxicity

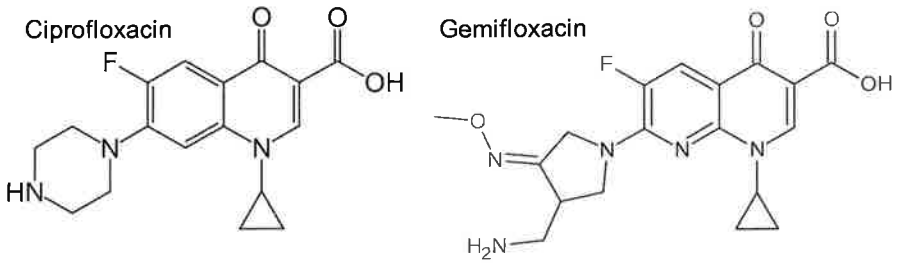


Figure 1.18: Fluoroquinolone antibiotics
Ciprofloxacin (molar mass 331.346 g/mol) Gemifloxacin (molar mass 389.381 g/mol)

The first quinolone antibiotic (nalidixic acid) was discovered in the late 1950s as a by-product from chloroquine manufacturing. As the early quinolones had poor tissue absorption they were initially only used to treat urinary infections (Higgins *et al.,* 2003). However, in the 1970s it was discovered that addition of a fluorine to the quinolone improved both absorption and the activity of the drugs (Koga *et al.,* 1980). These were the first generation of fluoroquinolone antibiotics and included the still prescribed ciprofloxacin (Figure 1.18). Since first introduction, fluoroquinolones have become a potent weapon against infectious disease, with several new generations being developed each with a broader spectrum of activity, including gemifloxacin which was shown to have improved efficacy against hospital acquired gram-negative respiratory pathogens (Yoo *et al.,* 2004). However, with the increase of widespread drug resistance there is still an urgent and unmet need to introduce new and ever more potent antibiotics.

All quinolone antibiotics target bacterial gyrase and/or topoisomerase IV. As an example of fluoroquinolone interaction with prokaryotic topoisomerase II, Figure 1.19 illustrates moxyfloxacin's binding pocket within the DNA cleavage domain. Similarly to etoposide, two fluoroquinolone molecules intercalate at the two sites of DNA cleavage, hence each fluoroquinolone molecule is four bases apart from the other. The exact mechanism of binding is still being investigated, but from recent crystalographical studies, at least for moxyfloxacin, a contribution from both classic hydrogen bonding and Van der Waals forces between the quinolone and DNA bases plus $Mg2^{+}$ associated topoisomerase protein / quinolone hydrogen binding seems to be probable. However, it is likely that the specifics of binding sites will differ for different fluoroquinolone antibiotics, all of which are yet to be fully elucidated (Laponogov *et al.,* 2010; Wohlkonig *et al.,* 2010). Such intercalation is not thought to occur in non-cleaved DNA. This may be because fluoroquinolones are not planar, hence they may

require the more open structure of the cleaved strand to facilitate intercalation within the quinolone binding pocket (Piton *et al.,* 2010; Wohlkonig *et al.,* 2010). The bound enzyme prevents DNA ligation and also increases the rate of cleavage complex formation hence increasing the number of cleavage complexes and DNA road-blocks (Pommier *et al.,* 2010).

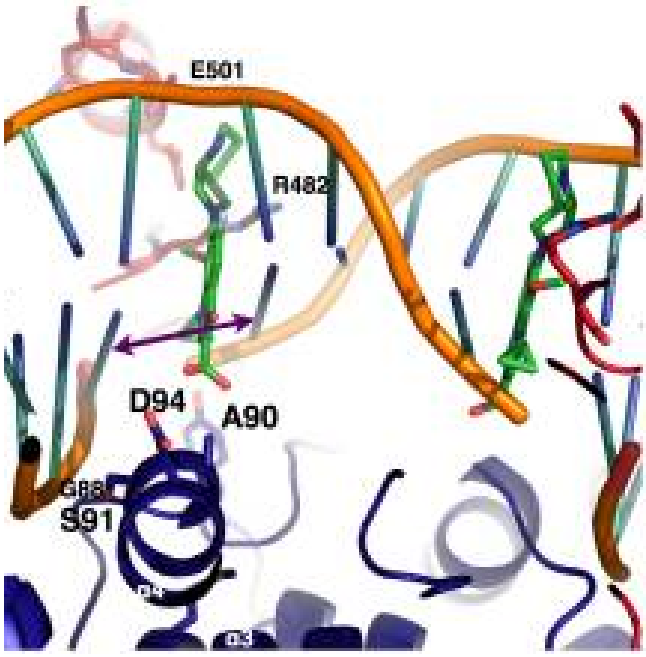


Figure 1.19: Moxyfloxacin binding of *M.tuberculosis* gyrase.
DNA is orange, helical topoisomerase II monomers in blue, moxyfloxacin stick molecule green. Image showing intercalation of the two fluoroquinolone molecule between nucleotides of cleaved DNA. The purple arrow highlights the rise of the intercalated base step that constitutes the DNA walls of the quinolone binding pocket. Reproduced from Piton *et al.,* 2010

The majority of the currently marketed fluoroquinolone antibiotics have a much higher affinity (approximately 100 to 1000-fold) for prokaryotic topoisomerase II than eukaryotic (Heisig, 2009). However, at least some of these drugs do form stabilised cleavage complexes in mammalian cells and hence are capable of inducing mammalian cell genotoxicity (see Tables 1.4 and 1.5 for genotoxicity associated with ciprofloxacin and gemifloxacin and the earlier reference to the EU market withdrawal of gemifloxacin (Section 1.2.3)). It is not known whether the mechanism of fluoroquinolone binding to eukaryotic topoisomerase II / DNA complexes is similar to that as described above for prokaryotic DNA. The challenge for development of new, safe and efficacious topoisomerase II targeting antibiotics is to maintain or preferably improve levels of potency against the prokaryotic target without effects on eukaryotic

DNA. Accordingly, a better understanding of the mechanism of action of these drugs in mammalian cells would be of great benefit for safe anti-infective development.

1.3.5.4 The genotoxicity of etoposide, bisdoxopiperazines, ciprofloxacin and gemifloxacin

The following tables show the genotoxicity and, where available, the lowest observable genotoxic effect levels (LOGEL) for reference compounds taken from published studies:

	***In vitro* Assays LOGEL**			
Compound	**Ames (µM/plate)**	**MLA (µmol/L)**	**MN(vit) (µmol/L)**	**Comet(vit) (µmol/L)**
Etoposide	**+ve 160 µM[1]**	**+ve 0.15 µM[3]**	**+ve 0.15 µM[4]**	**+ve 0.026 µM[6]**
Bisdoxopiperazine	**ICRF159 -ve[2]**	**ICRF193+ve 0.066 µM[6] ICRF187+ve 1 µM[7]**	**ICRF193+ve 0.017 µM[6]**	**ICRF193+ve 4 µM[6]**
Ciprofloxacin	**+ve*0.0003 µM[5]**	**ND**	**+ve 150 µM[4]**	**+ ve 400 µM[8]**
Gemifloxacin	**+ve* LOGEL not available[9]**	**+ve LOGEL not available[9]**	**ND**	**ND**

Table 1.4: *In vitro* genotoxicity of four topoisomerase II inhibitors
LOGEL = Lowest observable genotoxic effect level
MLA = mouse lymphoma assay, MN(vit) = *in vitro* micronucleus test, Comet(vit) = *in vitro* comet test, ICRF = Imperial Cancer Research Fund compounds 159 or 193
[1] Anderson and Berger, 1994; [2] Albanese and Watkins, 1985; [3] Ashby *et al.,* 1994; [4] Lynch *et al.,* 2003; [5] Gocke, 1991; [6] Boos and Stopper 2000; [7] Wang and Eastmond, 2002; [8] Itoh *et al.,* 2006; [9] Rothfuss *et al.,* 2010
* Repair proficient strains only
ND No data

	In vivo Assays LOGEL		
Compound	**MN(viv)**	**Comet(viv)**	**Carcinogenicity**
Etoposide	**+ve 0.1 mg/kg**[1]	**+ve 5 mg/kg**[5]	**Human carcinogen**[6]
Bisdoxopiperazine	**+ve 200 mg/kg**[2]	**ND**	**Rodent +ve**[7]
Ciprofloxacin	**-ve**[3]	**ND (-ve UDS)**[3]	**Rodent -ve***
Gemifloxacin	**+ve 1200 mg/kg**[4]	**+ve 600 mg/kg**[4]	**Rodent -ve***

Table 1.5: *In vivo* genotoxicity of four topoisomerase II inhibitors
LOGEL = Lowest observable genotoxic effect level
MN(viv) = *in vivo* micronucleus test, Comet(viv) = *in vivo* comet test,
[1] Turner *et al.,* 2001; [2] Albanese and Watkins, 1985;
[3] Herbold, 2001, UDS = *in vivo* unscheduled DNA synthesis assay
[4] Positive acute dosing only (Rothfuss *et al.,* 2010)
[5] Godard *et al.,*1999; [6] Anderson and Berger, 1994;
[7] National Toxicology Program, 1978
* No data for ciprofloxacin or gemifloxacin, but no reports of carcinogenicity for any other fluoroquinolones (Iatropoulos *et al.,* 2001)
ND No data

These topoisomerase II inhibitors gave a wide spectrum of results in standard genotoxicity assays. With the exception of the bisdoxopiperazines, they are positive in the Ames test. However, they are generally negative in bacterial strains that identify frameshift mutagens, indicating that direct DNA intercalations is not a likely mechanism for bacterial mutagenicity. The majority of the published data show that they are only positive in excision repair proficient bacterial strains, signifying that incompetent repair is responsible for the mutagenic lesion (Gocke, 1991). Similarly for mammalian cells, it has been proposed that it is inefficient non-homologous end joining repair of topoisomerase II induced DNA breaks that causes DNA mutation (Heisig, 2009; de Campos-Nebel *et al.,* 2010). This explains why potent topoisomerase inhibitors such as etoposide are positive in the mammalian cell HPRT gene mutation assay. The end point of the HPRT gene mutation assays is point mutation or small intragenic deletion rather than large chromosome deletions (Anderson and Berger 1994); hence it is probable that the etoposide induced positive response in this assay is due to inefficient repair rather than direct chromosome breakage. Why mechanistically bisdoxopiperazines are not bacterial mutagens is unclear, but is likely to be due to the lack of complete homology between topoisomerase II ATPase sites in the prokaryotic heterotetramer compared to the eukaryotic dimer. Binding heterology also probably explains why the gyrase/topoisomerase IV targeting fluoroquinolones are far more

potent bacterial mutagens than the mammalian topoisomerase II targeting etoposide, with the reverse seen with respect to mammalian cell mutagenicity. As Ames assay concentrations are expressed as µM/plate and mammalian cell concentrations expressed as µmol/L, it is not possible to quote exact comparisons of relative genotoxic potency between the two assay types. However, it is clear that ciprofloxacin is at least 1000 x more active in bacterial cells than mammalian cells, which is in agreement with its relative prokaryotic / eukaryotic target enzyme potencies (Heisig, 2009). It has been proposed that induction of hydroxy radicals via the Fenton reaction is at least partly responsible for the cytotoxicity of gyrase inhibitors in *E coli* (Dwyer *et al.,* 2007). It is also known that etoposide toxicity may be partly mediated by oxidation (Meresse *et al.,* 2004). Oxidative stress is also a well known mechanism for *in vitro* genotoxicity. It has yet to be elucidated as to whether this mechanism is of relevance to the cytotoxicity and genotoxicity of topoisomerase II poisons in mammalian cells, but it is intriguing to speculate that chromosome breaks as a consequence of replicative stalling at stabilised cleavage complexes may only partly contribute to the observed mammalian cell genotoxic potency. This concept will be investigated during the current programme of research.

The relative potencies of the bisdoxopiperazines ICRF193 and ICRF187, can probably be explained by their respective lipophilicity, where the more highly lipophilic ICRF193 is more readily taken up by mammalian cells and hence is the more potent mammalian cell mutagen (Andoh and Ishida, 1998). It is less clear why these compounds have low mutagenic potency in the comet assay. Boos speculated that the weak comet effect was due to the compounds mechanism of action, i.e. bisdoxopiperazines do not directly stabilise cleaved DNA by interactions within the DNA cleavage domain (Boos and Stopper, 2001), but why these compounds should induce micronuclei (i.e. fragments of broken chromosome) but not DNA breaks as identified by the comet assay is yet to be confirmed.

The relative *in vitro* genotoxic potencies of ciprofloxacin and gemifloxacin (with gemifloxacin being significantly more potent in both bacterial and mammalian cells) may be due to the relative activity of the compounds against both prokaryotic and eukaryotic topoisomerase II. This relative potency is also reflected in their *in vivo* activity, with gemifloxacin being one of the few fluoroquinolones reported to be positive in the rodent bone marrow micronucleus test and the *in vivo* comet assay (Rothfuss *et al.,* 2010). Although few fluoroquinolones have actually been tested in cancer bioassays, ciprofloxacin was negative in a short term liver cancer initiation study. In the same study norfloxacin did induce liver foci (Itoh *et al.,* 2006). However, in a follow on initiation study norfloaxin did not induce liver tumours (Itoh *et al.,* 2007). The

original quinolone antibiotic nalidixic acid has been tested in rat and mouse 2 year cancer bioassays. Whilst positives results were seen with an increase in preputial gland tumours in male rats and clitoral tumours in female rats, these are likely due to hormonal effects. No significant cancer incidence was seen in dosed mice (Morrissey *et al.,* 1991). Accordingly, there is little evidence to suggest that antibiotics targeting bacterial topoisomerase II are carcinogenic. Both etoposide and ICRF159 are rodent carcinogens. Etoposide is also associated with human cancer. Whilst it is not clear whether etoposide can induce human cancers if administered on its own (Anderson and Berger, 1994), the etoposide induced chromosome translocation in the *MLL* (mixed lineage leukaemia) gene has been identified as being involved in the development of secondary acute myeloid leukaemias, seen in 2% to 3% of patients on etoposide combination therapies (Deweese *et al.,* 2009).

Although the results presented in Tables 1.4 and 1.5 were from a very small data set, there is still an interesting relationship between the potency in the *in vitro* mammalian cell tests and *in vivo* effects. Ciprofloxacin which is only weakly active in mammalian cells test is also negative in *in vivo* assays. The more potent fluoroquinolone gemifloxacin is positive in at least two *in vivo* genotoxicity assays. Finally the extremely potent mammalian cell mutagens bisdoxopiperazines and etoposide are not only positive in *in vivo* genotoxicity tests, but are also carcinogenic. If such a relationship was to hold true for all topoisomerase II inhibitors it would prove the value of screening new drugs in both mammalian cell genotoxicity screens and tests to evaluate topoisomerase II inhibition.

1.3.6 Techniques to measure topoisomerase II inhibition

1.3.6.1 Cell free assays to measure topoisomerase II inhibition

The activity of topoisomerase II inhibitors can be measured using kinetoplast linked or linear DNA. These assays incubate DNA with topoisomerase II and ATP along with various concentrations of a suspected topoisomerase II inhibitor. Activity against the enzyme is measured by a reduction in its ability to decatenate kinetoplast or by the promotion of double strand breaks in linear DNA (Fisher and Pan, 2008). A comparison of the available data for etoposide cell free topoisomerase II inhibition and etoposide LOGEL demonstrates the low sensitivity of the end-point. Etoposide was shown to induce weak DNA cleavage in the DNA cleavage assay at 15 µmol/L (Austin *et al.,* 1995), which was at least two orders of magnitude greater than the LOGEL in *in vitro* mammalian cell genotoxicity assays (Table 1.4). There can of course be good reasons why a cell free assay may not predict a cellular effect on a direct dose for dose

comparative level. For example, the use of exogenous ATP and enzymes may be far less effective at DNA interaction than intracellular proteins. Accordingly, whilst cell free assays may be flawed for dose estimation of topoisomerase II inhibition, they may still be useful to screen and rank new pharmaceuticals for their on or off target topoisomerase II inhibitory effect. Whether such ranking can be used to predict the potential for *in vitro* mammalian cell genotoxicity of novel pharmaceuticals remains to be proven. However, whilst the relative insensitivity of cell free assays may prove not to be an issue for target screening, it may not provide sufficient information for elucidation and confirmation of off-target genotoxic mechanisms, hence are cell based assays better?

1.3.6.2 Cell assays to measure topoisomerase II inhibition

Most of the cell assays that have been designed to determine the activity of topoisomerase II poisons measure the relative increase in the formation of stabilised cleavage complexes following treatment i.e. in the presence of a topoisomerase II poison at x time after treatment there will be more topoisomerase II bound to DNA than normal background levels. These assays usually use antibodies to the topoisomerase II enzyme. The most widely used cell assays are the **T**rapped in **A**ga**r**ose **D**NA **i**mmuno**s**taining (TARDIS) assay developed at Newcastle University (Willmore *et al.,* 1998) and the **I**n vivo **C**omplexes of **E**nzyme (ICE) bioassay (Subramanian *et al.,* 2001).

The TARDIS assay sets treated cells in an agar matrix on a microscope slide prior to cell lysis and subsequent topoisomerase II antibody labeling. Stabilised cleavage complexes are measured by the use of a primary antibody to topoisomerase II and a fluorescent secondary antibody that can be scored by image analysis (Willmore *et al.,* 1998).

The ICE assay analyses cleavage complexes in DNA isolated by caesium chloride density gradient centrifugation. Following isolation, DNA is loaded onto a nitrocellulose membrane prior to antibody incubation. In this technique radiolabelled or fluorescent secondary antibodies may be used (Subramanian *et al.,* 2001).

Published data from both the TARDIS assay (Wilmore *et al.,* 1998) and ICE assay (Hawtin *et al.,* 2010) indicate etoposide induces a significant increase in topoisomerase IIα stabilised cleavage complexes at 1 μM. Interestingly, Wilmore *et al* 1998 showed that an even higher concentration of etoposide (10 μM) was required before a significant increase in the level of topoisomerase IIβ cleavage complexes could be seen. Furthermore, in the TARDIS assay, etoposide induced topoisomerase IIα stabilised cleavage complexes dissociated significantly slower than etoposide induced

topoisomerase IIβ stabilised cleavage complexes (Errington *et al.,* 2004). Along with the previously mentioned prevalence of topoisomerase IIα in rapidly dividing cells, these observations further suggests that inhibition of topoisomerase IIα is more likely to be responsible for the *in vitro* genotoxicity seen following short term topoisomerase II poison treatment.

From the available published data, concentrations of etoposide required to induce a measurable increase in stabilised cleavage complexes in the TARDIS and ICE cell bioassays were approximately 10-fold higher than the observed *in vitro* mammalian cell LOGEL (Table 1.4). However, these data should be viewed with some caution. Wilmore *et al* 1998 and Hawtin *et al* 2010 did not specifically perform their studies with the intention of calculating lowest or no effect levels for etoposide in TARDIS and ICE, respectively. Furthermore, the cell lines used for assessment of stabilised cleavage complexes were not the same as the cell lines used for assessment of LOGEL. It is widely accepted that different cell lines will give different responses in a variety of genotoxicity assays. Lynch *et al* 2003 did perform direct comparison of lowest effect levels in TARDIS and MN(vit) for etoposide and ciprofloxacin using L5178Y mouse lymphoma cells. This group also found a large (approximately 10-fold) difference between TARDIS effect level and LOGEL (Lynch *et al.,* 2003). Interestingly, although this group detected formation of stabilised cleavage complexes with ciprofloxacin in the TARDIS assay, this response could not be repeated when ciprofloxacin was tested on human leukemic cells by the Newcastle University group who had originally worked-up the TARDIS assay (Rance 2011). This may again indicate a difference in response between different cell lines.

Given that the primary mechanism for topoisomerase II inhibitor induced DNA strand breakage is likely to be formation of stabilised cleavage complexes and their interaction with the cells replicative machinery, it would seem implausible that genotoxic concentrations would be lower than concentrations required to induce stabilised complexes. Hence, if it could be further established that within the same cell line the LOGEL is several fold lower than concentrations of topoisomerase II inhibitors required to induce measurable stabilised cleavage complexes important questions are raised.

1. Is it simply because the sensitivity of ICE and TARDIS is insufficient to detect low levels of stabilised cleavage complexes, levels that are sufficient to induce strand breakage and genotoxicity? Lynch *et al* speculated that the background fluorescence seen with the TARDIS assay limited its sensitivity. They also considered that the rapid reversibility of stabilised cleavage complexes could contribute to the noted insensitivity (Lynch *et al.,* 2003).

2. Do other mechanisms of DNA damage other than a direct 1:1 stoichiometric relationship between stabilised cleavage complex and strand breakage play a part in the genotoxicity of topoisomerase inhibitors? For example, whilst it is established that stabilisation of cleavage complexes is key to the bacterial toxicity of quinolones, the exact mechanism is less clear. It has been postulated that a cascade of DNA breakage and subsequent induction of ROS may be partly or equally involved (Drlica *et al.,* 2009). Furthermore, etoposide has been reported to undergo redox cycling (Smart *et al.,* 2008). Could the additional ROS load explain the discrepancy between apparent measurable stabilised cleavage complex formation and genotoxicity? The exact contribution of etoposide ROS induction to measured genotoxicity has not been established. Accordingly, additional testing is required to confirm whether ROS induction contributes to the observed *in vitro* mammalian cell genotoxicity of topoisomerase II poisons.

For the development of safe and efficacious new pharmaceuticals, it would clearly be of value to gain a better understanding of the mechanisms of topoisomerase II inhibitor induced mammalian cell genotoxicity. This may also help to elucidate the mode of mutagenic action for some of the drugs that are currently considered to give 'false' positive results in mammalian cell in vitro genotoxicity tests.

1.4 Summary of Aims

The preliminary aim of this project will be put into perspective the real rate of positive responses in *in vitro* mammalian cell genotoxicity test seen during routine pharmaceutical screening. To this end Chapter 3 will include a comprehensive review of data generated at AstraZeneca UK, relating the incidence of positive results in the mouse lymphoma assay to the drugs primary therapeutic target. It was expected that there would be a number of compounds that gave positive results unattributable to the compounds primary pharmacology. If it can be proven that mechanisms other than covalent DNA binding are responsible for the positive response, the drugs may be considered safe for human use. Genotoxicants that can be shown to be aneugens or topoisomerase II poisons are considered as having such a genotoxic threshold. Accordingly, the work detailed in Chapter 3 will also attempt to answer whether aneugenicity could be a contributory factor to the number of unexplained mouse lymphoma assay positive responses.

Given that topoisomerase poisons are potent mutagens but which are considered to have a threshold below which therapeutic doses may be given safely to man, it is surprising that the exact relationship between topoisomerase II inhibition and *in vitro* mammalian cell genotoxicity has not been fully elucidated.

Accordingly the further aims of this project will be:

Chapter 4: to investigate the predictivity of the cell free decatenation assay for mammalian cell *in vitro* genotoxicity test results for AstraZeneca compounds that are thought to be mutagenic by inhibition of topoisomerase II. Can such assays be used to help screen for the mammalian cell mutagenic potential of such drugs and can such assays be used to elucidate unknown genotoxic mechanisms?

Chapter 5: to investigate the topoisomerase II inhibitory effect levels in cell assays (e.g. TARDIS and ICE) for model compounds and relate this to their genotoxic effect levels in *in vitro* mammalian cell tests. Are cell assays for topoisomerase II inhibition more predictive of *in vitro* genotoxicity than cell free assays and similarly can they be better used to evaluate the potential for topoisomerase II induced genotoxicity of novel compounds?

Chapter 6: to investigate whether the reference topoisomerase II poisons used are genotoxic by mechanisms other than inducing DNA breaks directly by initiation of topoisomerase II/DNA stabilised cleavage complexes. The modified Comet assay will be used to investigate possible ROS contribution to genotoxicity.

Chapter 7: following the preparation of a mouse specific antibody to topoisomerase II can the sensitivity of cleavage complex assays be improved?

Chapter 8: the majority of the work in early chapters investigated the relationship between genotoxicity and the activity of topoisomerase II inhibitors against the alpha isoform of the enzyme. In view of recent publications linking the genotoxic activity of topoisomerase II poisons to the beta isoform, can it be demonstrated that at least for the cell line used in the current investigations (L5178Y mouse lymphoma cells) topoisomerase II poison genotoxicity is driven by the alpha isoform?

Chapter 9: will summarise the outcomes of this research and consider the future prospects for elucidating the relationship between topoisomerase II inhibition and mammalian genotoxicity.

Details of the standard methodologies used are detailed in Chapter 2, other specific methodologies are detailed at the beginning of each individual chapter. Throughout this project the topoisomerase II poison reference agents used were etoposide and gemifloxacin. Etoposide was selected due to its high potency against mammalian cell topoisomerase II and gemifloxacin as it is one of the most potent mammalian cell genotoxic fluoroquinolone anti-infectives.

CHAPTER 2

The provenance of the cells used and general material and methods

CONTENTS

2 The provenance of the cells used and general material and methods

Etoposide was purchased from Sigma Aldrich, UK and gemifloxacin was purchased from YES Pharma Ltd, Israel. Both were dissolved in dimethyl sulphoxide (DMSO) before use. All other chemicals and reagents were purchased from Sigma Aldrich, UK or for proprietary compounds were synthesised by AstraZeneca R&D, unless otherwise stated.

2.1 L5178Y mouse lymphoma *tk*$^{+/-}$ clone 3.7.2C cells

Mouse lymphoma L5178Y *tk*$^{+/-}$ cells, clone 3.7.2c, were obtained from Dr Jane Cole, (Medical Research Council, Cell Mutation Unit, Brighton, UK) to whom they had been supplied by Dr Donald Clive (then at Burroughs Welcome, Research Triangle Park, USA) in 1978. The provenance of the cells is such that it can be demonstrated that the cryopreserved master stock cultures in this laboratory have spent no more than 3 weeks in continuous culture since they were supplied by Dr Clive and working cell cultures were cleansed of pre-existing trifluorothymidine (TFT) resistant mutants by growth in medium containing methotrexate on only one occasion (Figure 2.1) (Fellows *et al.,* 2011). The average doubling time of the L5178Y cells in stationary culture was approximately 9 to 10 hours.

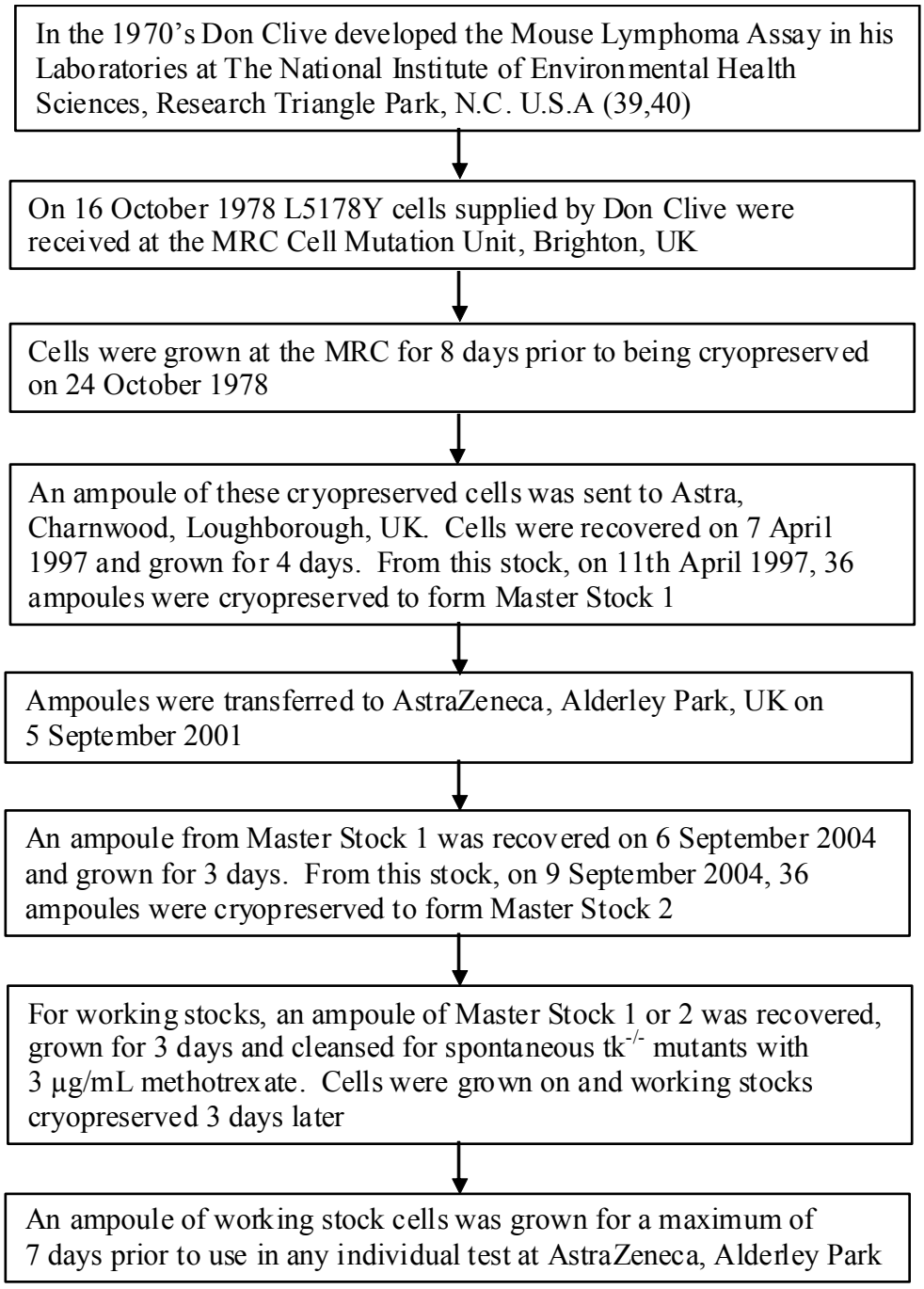


Figure 2.1: The provenance of the L5178Y mouse lymphoma *tk*$^{+/-}$ clone 3.7.2C cells used for this research (Reproduced from Fellows *et al.*, 2011)

2.1.1 Metaphase preparation for modal chromosome number and spectral karyotype analysis

To confirm the provenance of the cells, metaphase preparations were made and slides sent for analysis of spectral karyotype by The BioReliance Corporation, 14920 Broschart Road, Rockville, MD 20850. Dr Ann Doherty (AstraZeneca) assisted with preparation of the samples and arrangement of the spectral karyotype.

Two hours prior to harvest, 0.125 µg/mL final concentration Colcemid™ was added to exponentially growing cultures to arrest the dividing cells in metaphase. Cultures were centrifuged at 300 x *g* for 5 minutes and suspended in 0.075 mol/L potassium chloride hypotonic solution at 37°C for 12 minutes and then fixed in freshly prepared methanol/glacial acetic acid (3:1, v/v) at room temperature. Cultures were re-centrifuged at 300 x *g* for 7 minutes, re-suspended in approximately 10 mL fresh fixative and vortexed. This step was repeated and the cells were then re-suspended in a third 10 mL volume of fixative, spun down and the majority of the supernatant removed leaving approximately 0.5 mL. Metaphase preparations were made by dropping concentrated cell suspensions on to slides and then allowed to air dry.

2.1.2 Solid stain karyology

Slides for the analysis of modal chromosome number were stained in filtered 6% v/v solution Giemsa at pH 6.8 for 5 minutes, then air dried. Slides were then cover-slipped using a permanent Distrene, Plasticiser, Xylene mounting medium, (DPX) (BDH Ltd). Nuclei metaphases were observed microscopically to ensure absence of gross chromosomal aberrations and also to confirm that the modal chromosome number was in agreement with previously reported observations. At least 100 metaphases were scored to assess chromosome number (ploidy) for cultures grown for 3, 14 and 29 days and 3 and 6 months.

2.1.3 Spectral karyotyping

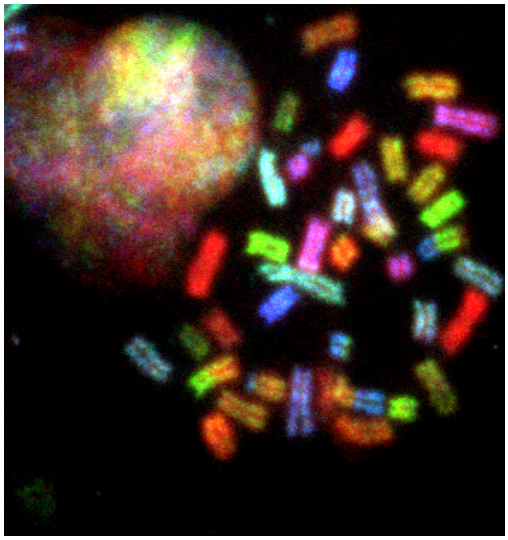
Slides were sent to BioReliance for spectral karyotyping.

The DNA in preparations of metaphases on slides was denatured by heating in 70% formamide at 72ºC prior to being quenched in ice-cold 70% ethanol. Mouse genome specific probes for spectral karyotyping (Oxford Biosystems Cadama, UK) were denatured by heating at 80°C and then incubated at 37°C for 1 hour before application to slides containing denatured metaphases. Each slide was coverslipped and

hybridisation of the mouse probes was allowed to proceed within a humidified chamber equilibrated at 37ºC ± 1°C for approximately 40 hours. After hybridisation, the slides were washed prior to the application of concentrated secondary antibodies (Oxford Biosystems Cadama, UK) each conjugated with specific fluorescent stains. Upon completion of the incubation with the secondary antibodies, test slides were washed and finally counterstained with 4 6 diamidino-2-phenylindole (DAPI) anti-fade solution to facilitate visualisation of chromosome morphology.

Image acquisition was performed using a Charge Coupled Device (CCD) camera containing an interferogram cube mounted on an Olympus BX61 microscope. Metaphase cells stained with Caspase-Activated Deoxyribonuclease (CAD) antibodies were visualised using an optical filter that allows for simultaneous excitation of all dyes and concomitant measurement of their emission spectra. Images of fluorescent stained metaphase cells were captured and examined for modality of chromosome number. Representative spectral karyotypes of metaphase cells were arranged in accordance with the International System for Cytogenetic Nomenclature. Results of analysis are shown in Figure 2.2.

A



B

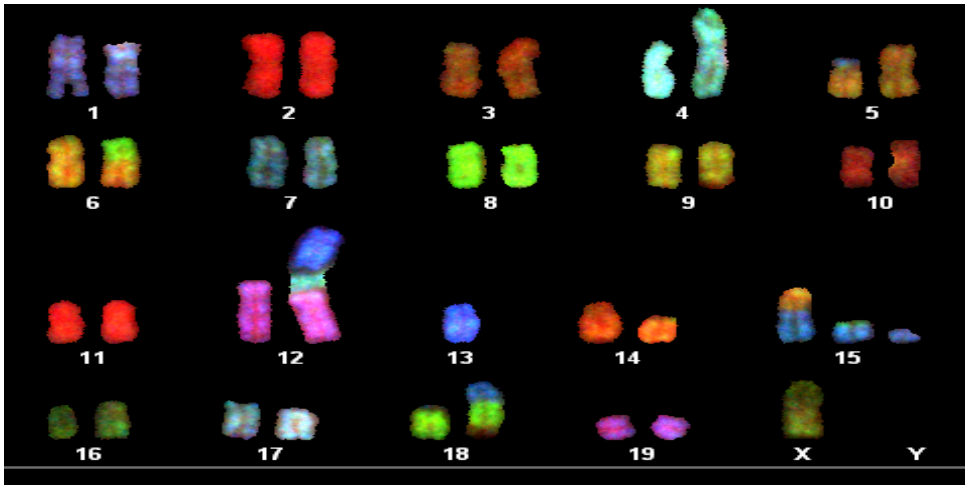


Figure 2.2: AstraZeneca master stock L5178Y mouse lymphoma tk+/- clone 3.7.2C karyotype
A: Fluorescently Stained Metaphase.
B: Arranged karyotype demonstrating pseudocolour resolution of spectrally labelled chromosomes. Arranged karyotype demonstrating cytogenetic aberrations at chromosome 4; Dp(4), chromosome 5; T(15:5), chromosome 6; T(18:6), chromosome 9; T(9:6), chromosome 14; del T(14:6), chromosome 15; T(5:15), and a derivative chromosome demonstrating a portion of chromosome 15 at the proximal end, chromosome 18 at the intermediate section and chromosome 14 at the distal end; T(15:18:14) and Rb(Dp12:13)

As expected a modal chromosome number of 40 was seen and there was no markedly increased variability in chromosome number following continual culturing from 3 days to six months.

Spectral karyology confirmed a composite karyotype 40 with the aberrations detailed in Fig. 2.2. This result is essentially the same as the published karyotype by SKY®-FISH [Sawyer et al., 2006]. The three observed differences were considered to be due to improved resolution in karyology. Specifically:

1. The previous spectral karyotype indicated as T(18;6) is now identified as T(6;18)

2. Previously identified as chromosome 6 in origin 6 (T6;14) is now identified as 14 (T14;6)

3. Previously identified as chromosome 15 (T15;18;14) is now identified as chromosome derivative 18 (t15;18;14).

We consider the karyotype presented here to be the most up to date karyology of L5178Y TK+/- clone 3.7.2C cells.

This work has been published by the author (Appendix 1)

2.1.3 Preparation of cells for each test

Between approximately one to two weeks before each test was performed a single vial of working stock cells was removed from liquid nitrogen, quickly defrosted, centrifuged to remove the dimethyl sulphoxide (DMSO) used in the cryopreservation medium and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Paisley, UK) containing 10% heat inactivated donor horse serum (DHS), 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, 1% Pluronic F68, 200 IU/mL penicillin, 200 µg/mL streptomycin (RPMI10%+P). Cells were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 in air, at concentrations of approximately 1 x 10^5 cells per mL and 1 x 10^6 cells/mL before being used in each individual test.

2.2 Treatment of L5178Y mouse lymphoma cells with test chemicals

The standard treatment regime used was as follows. Variations required for individual assay types will be detailed in the relevant section:

Treatment exposure was for 3 or 24 hours in the absence of an exogenous metabolising system. Three hours exposure has previously been demonstrated to be adequate to assess the genotoxicity of topoisomerase II inhibitors in the author's

laboratory (data not shown) and 24 hours exposure is suitable for detecting division dependent damage such as aneugenicity. Between 5 x 10^6 and 1 x 10^7 L5178Y cells were suspended in between 5 or 20 mL RPMI medium containing 2.5% DHS (3 hours exposure) or 10% DHS (24 hours exposure). Prior to treatment the relevant test compound or positive control was dissolved in DMSO and further dilution made with DMSO as required for each test. Test compound, solvent or, where used, positive control solution was added to the cell cultures at 1% v/v. Treatment cultures were either single culture or in duplicate or triplicate depending on test requirements.

Following treatment, the cells were centrifuged at 200 x *g*, washed once with RPMI10%+P, re-centrifuged and re-suspended in fresh RPMI10%+P at a final cell concentration after washing of 2 x 10^5/mL. When further post-treatment incubation was required, cultures were incubated for the appropriate time at 37^{o}C in a humidified atmosphere of 5% CO_2 in air and then counted for assessment of toxicity one day after treatment (relative suspension growth or relative population doubling) and when required microscope slides prepared for micronucleus assessment or cells lysed or prepared for additional investigative tests as detailed in the later section.

2.3 Metabolic activation

For bacterial reverse mutation and mouse lymphoma tests performed at AstraZeneca between 2001 and 2010 which were reviewed for analysis of the rate of positive responses, treatments were performed in the absence and presence of an exogenous metabolising system. For treatments in the presence of exogenous metabolism, S9 from the livers of Aroclor 1254 treated rats was purchased from Molecular Toxicology Inc. (Boone, NC, USA) and stored frozen at a temperature of 70°C or below until use. On the day of use, S9 mix was prepared by the addition of culture medium containing cofactors for NADPH generation to the S9 fraction. For mouse lymphoma tests a final S9 concentration of 4% v/v was used for tests from 2001 to 2007, and 2% v/v subsequently. For bacterial reverse mutation tests a final S9 concentration of 10% was used.

2.4 Bacterial reverse mutation tests (Ames)

For the Ames tests performed at AstraZeneca between 2001 and 2010 the basic assay study design was as detailed in Introduction Figure 1.1. The majority of tests included *Salmonella typhimurium* strains TA1535, TA100, TA98 and TA1537, plus *Escherichia coli* WP2 uvrA/pKM101. Compounds from AstraZeneca's infection portfolio were

tested using the *Salmonella typhimurium* strain TA102 rather than the *E.coli* strain. An increase in the number of revertant colonies was considered to be significant if it exceeded 2-fold the concurrent solvent control level for each strain.

2.5 Microtitre method mouse lymphoma assay to determine mutant frequency at the tk locus (MLA)

The MLA was performed essentially as described by Clements, 2000. For basic assay design see Introduction Figure 1.3. Brief method was as follows.

2.5.1 Compound exposure

See Section 2.2 Treatment of L5178Y mouse lymphoma cells with test chemicals. Duplicate or triplicate cultures were tested.

2.5.2 Toxicity measurement and mutant selection

Following treatment, cultures were incubated at 37ºC for 1 day then cells counted using a Coulter Counter and sub-cultured in RPMI10%+P medium to 1.5 x 10^5 cell per mL. Cultures were then incubated at 37ºC for a further day, cells were counted and diluted to 1 x 10^4 cells per mL (for triflurothymidine (TFT) resistance selection) or 8 cells per mL for assessment of viability using RPMI medium containing 20% DHS. TFT was added to the appropriate cultures at a final concentration of 3 µg/mL. To determine viability, 200 µL of cultures at 8 cells per mL were plated into two 96-well plates giving approximately 1.6 cells per well. To determine TFT resistance, 200 µL of cultures at 1 x 10^4 cells per mL containing 3 µg/mL TFT were plated into two 96-well plates at approximately 2000 cells per well. Plates were incubated for up to 14 days, prior to scoring. For TFT resistant colonies, mutant clones were designated as large (covering approximately one quarter or more of the area of the well) or small (covering less than one quarter of the area of the well) see Figure 2.3. Cloning efficiency (mutant or viable cells), relative total growth (used to estimate toxicity) and mutant frequency were calculated using standard methods (Clements, 2000) as follows:

Cloning efficiency (CE) in either selective or non-selective medium was based on the zero term of the Poisson distribution. P(0) was calculated from the proportion of wells in which a colony has <u>not</u> grown, P(0):

$$CE = \frac{-\ln P(0)}{\text{Number of cells per well}}$$

Relative total growth (RTG) was used as the definitive measure of toxicity and was the product of relative suspension growth (RSG) and viability at the time of selection for TFT-resistant mutants. The viability for each culture was expressed as the cloning efficiency in non-selective medium where the plates were seeded with 1.6 cells per well.

Relative cloning efficiency (RCE) is:

$$\%RCE = \frac{CE}{\text{Mean control CE}} \times 100$$

Relative total growth (RTG) is:

$$\%RTG = \frac{\%RSG \times \%RCE}{100}$$

Mutant frequency (MF) for each culture was calculated as the cloning efficiency in selective (TFT) medium, where the plates were seeded with 2000 cells per well. It was corrected for viability in non-selective medium from the same culture and expressed as mutants per 10^6 viable cells, i.e.

MF (per 10^6 cells) = [CE (mutant) / CE (viable)] x 10^6

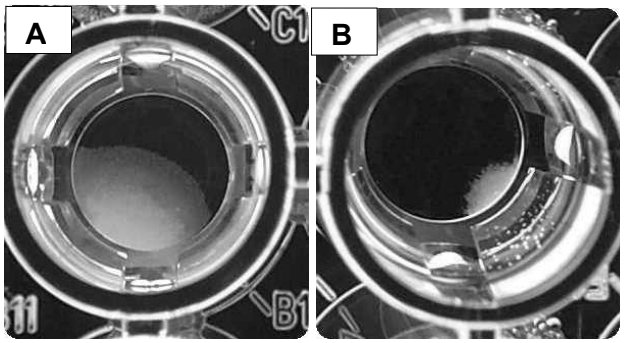


Fig 2.3 L5178Y mouse lymphoma cell mutant clones after 12 days growth A: Large colony. B: Small colony

2.5.3 Evaluation criteria for the MLA

A positive result was determined using the Global Evaluation Factor (GEF), i.e. for any concentration an increase in mutant frequency above concurrent solvent control of greater or equal to 126 x 10^{-6} was considered significant. Toxicity was measured by

RTG, this is the recommended method for toxicity assessment in the MLA (OECD, 1997).

2.5.4 MultiCase (MCASE) MC4PC Analysis

For predictivity of MLA results the mulitticase structural alert analysis programme (MCASE) module of MC4PC (MCASE, Inc.) was used. MCASE is an analysis programme which is cable of learning from the addition of new structures and their results in genotoxicity assays to the internal database (Matthews and Contrera, 1998). The standard SMILES format was used as the entry and the 'expert call' produced by MCASE was used as the activity output. SMILES formats being a standard way of defining chemical structures. MC4PC does provide several qualifications for the activity calls and these 'summary expert calls' were all 'equivocal' so the simple 'expert call' was used. No MLA result generated at AstraZeneca had previously been added to the MCASE learning set. MCASE entry was performed by Scott Boyer, AstraZeneca, Computational Toxicology, and results provided to the author.

2.6 The standard *in vitro* micronucleus test (MN(vit))

Methodology was essentially as described in Fellows *et al.,* 2008. For basic assay design see Introduction Figure 1.5. Brief method was as follows.

2.6.1 Compound exposure

See Section 2.2. Unless otherwise stated, triplicate cultures were tested.

2.6.2 Preparation and scoring of slides for micronuclei determination

Following treatment, cultures were incubated at 37ºC for 1 day then cells counted using a Coulter Counter. Microscope slides were prepared by centrifuging 1 x 10^5 cells in a Cytospin 3 (Shandon) centrifuge (100 x *g* for 8 minutes). Slides were allowed to air dry and cells fixed with 100% methanol (10 minutes). Prior to staining cells were re-hydrated in phosphate buffered saline. For tests where semi-automated scoring was used, slides were stained with DAPI (4', 6-diamido-2-phenylindole). For tests where manual scoring by microscope was used, slides were stained with acridine orange.

2.6.3 Semi-automated scoring

Slides were initially scanned to find micronucleated cells using MetaSystems' Metafer 4, comprising of a Zeiss Axioplan Imager Z1. Where possible, at least 2000 cells per culture were scored. All identified micronuclei were confirmed by eye to be separate and within the cytoplasm, to have intact cytoplasmic membrane and to be less than one third of the diameter of the main nucleus (See Figure 2.4).

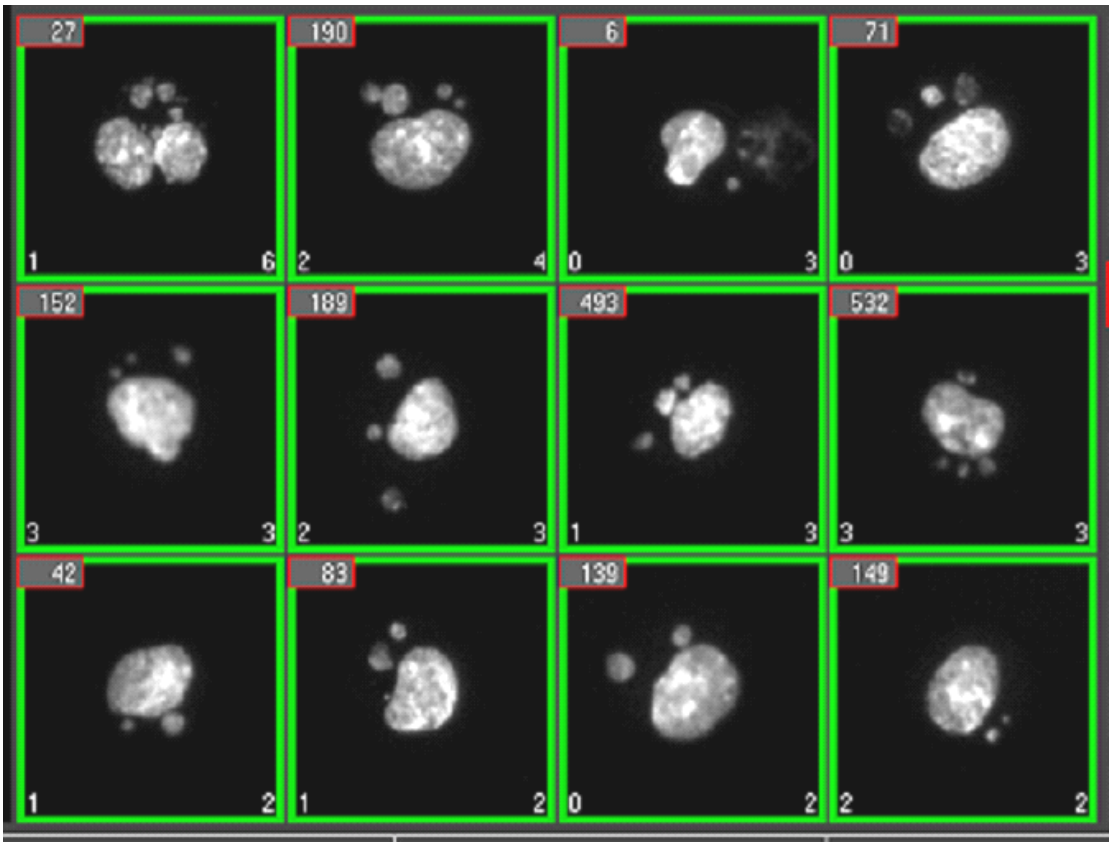


Figure 2.4: Micronucleated mononuclear L5178Y cells following 3 hour treatment with the potent mutagen 4-nitroquinolene oxide followed by 24 hour recovery. Identified and sorted by Metafer 4, where possible, at least 2000 cells per culture were scored and then confirmed by eye Large amount of micronuclei indicate how effectively 4-nitroquinolene oxide breaks chromosomes

2.6.4 Manual scoring

Using the same criteria for identification of micronuclei, slides were scored manually at x 200 magnification using a Zeiss Axioplan microscope. Where possible, at least 1000 cells per culture were scored for the presence of micronucleaed mononuclear cells.

2.6.5 Mn(vit) determination of cytotoxicity

For each treatment, cytotoxicity was determined by calculation of a reduction in relative population doubling (RPD) (Lorge *et al.,* 2008). RPD is one of the OECD recommended methods of cytotoxicity assessment in the MN(vit) (OECD, 2010):

RPD was determined as:

$$\frac{\text{Number of Population doublings in treated cultures}}{\text{Number of Population doublings in control cultures}} \times 100$$

where

Population Doubling = [log (Post-treatment cell number/Initial cell number)] / log 2

2.6.6 Mn(vit) evaluation criteria

The significance of the comparison between treated cultures and negative controls was determined on pooled cultures (where appropriate) using a Chi-square test with Yates' like correction, as recommended by Karen Oldman, AstraZeneca Alderley Park, Safety Assessment UK Statistics. As only increases in micronucleated mononuclear cells above control were of interest, a one-sided test was used. Increases in micronuclei were reported as statistically significant if the P-value was less than 0.05 (at the 5% level). Levels of significance were also assessed at the 1% and 0.1% level.

2.7 The MN(vit) test with chloroquine or novobiocin block

For MN(vit) tests where topoisomerase II DNA interactions were blocked with chloroquine (a DNA intercalater) or novobiocin (a topoisomerase II catalytic inhibitor); prior to test compound treatment cells were pre-incubated for 1 hour with 40 µg/mL chloroquine or 240 µg/mL novobiocin. Test compound dilutions were then added to the pre-treated cultures and the cultures incubated for a further 3 hours. The assay was then performed as for a standard MN(vit). All of these tests were scored manually. Duplicate cultures were used.

2.7.1 Blocked Mn(vit) evaluation criteria

The significance of the difference in response between concentrations tested in the absence and presence of the topoisomerase II block was determined on pooled cultures using a 2-sided Continuity-Adjusted Chi-Square Test, as recommended by Karen Oldman, AstraZeneca Alderley Park, Safety Assessment UK Statistics. Accordingly both significant increases and decreases in micronuclei formation in the presence and absence of the topoisomerase II block were assessed. Effects were

reported as statistically significant if the P-value was less than 0.05 (at the 5% level). Levels of significance were also assessed at the 1% and 0.1% level.

2.8 The *in vitro* alkali Comet Assay

Methodology essentially as described in Smith *et al.,* 2006. The basic assay design of the *in vitro* comet assay was as illustrated for the *in vivo* comet assay in Introduction Figure 1.7. However, rather than using single cell preparation from dosed animal tissue, the *in vitro* comet assay used chemically exposed L5178Y mouse lymphoma cells.

2.8.1 Buffers and other reagents

2.8.1.1 Preparation of agarose pre-coated slides

0.5% w/v normal melting agarose in phosphate buffered saline (PBS) was melted in a microwave and kept molten at approximately 37ºC. Microscope slides were dipped into agarose for approximately 1 second, the back of slides was wiped and slides were air dried. Slides were stored in an airtight container for up to 1 month.

2.8.1.2 Lysis solution

Incomplete mix: NaCl 146.4 g, EDTA disodium salt 37.2 g, Tris 1.2 g, in 890 mL Purified water. pH was adjusted to 10 using 10 mol/L NaOH. The solution was stored at room temperature for up to 1 month.

Complete mix: Incomplete lysis solution 178 mL, Triton-X 2 mL, DMSO 20 mL

The complete mix was prepared fresh on the day of use and pre-cooled in a refrigerator prior to use.

2.8.1.3 Buffer F

40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin (BSA). pH was adjusted to 8.0 using 10 mol/L NaOH.

2.8.1.4 Electrophoressis solution

1 mmol/l sodium EDTA, 0.3 mol/l NaOH (pH 13)]

2.8.1.5 Neutralisation buffer

0.4 mol/l Tris–HCl (pH 7.5).

2.8.2 Compound exposure

See Section 2.2 Treatment of L5178Y mouse lymphoma cells with test chemicals. Duplicate cultures were used in the initial test and triplicate cultures were used in the two confirmatory assays. The known inducer of reactive oxygen species potassium bromate was used as the positive control.

2.8.3 Post-treatment slide preparation

As ultraviolet light can cause DNA damage, slide preparation was conducted under minimal light. After compound exposure, for each prepared gel, approximately 1.2 x 10^5 cells were centrifuged at 200 x *g* and washed with 1 mL ice cold PBS. Tubes were re-centrifuged at 200 x *g* and resuspended in 0.18 mL 0.5% w/v low melting point agar (LMA). 30 µL of cell suspension in LMA (containing approximately 20000 cells) was add to pre-agar coated microscope slides on a chilled surface and covered with a dry coverslip to spread the agar (up to 3 gels per slide). Once set, the coverslips were removed; the slides were immersed in freshly prepared pre-cooled complete lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris buffer (pH 10), 10% DMSO, 1% Triton X-100 adjusted to pH 10 with 10 mmol/L NaOH) and stored refrigerated overnight.

2.8.4 Addition of human 8-hydroxyguanine DNA-glycosylase (hOGG1) restriction enzymes

Following lysis, the slides were immersed in two changes of buffer F for 5 minutes each time at room temperature. For gels treated with hOGG1 restriction enzyme (New England Biolabs, Herts, UK), hOGG1 (1:1000 dilution), was added to the gel in 50 µl of buffer F, this was equivalent to 0.08 hU hOGG1 per gel (previously titrated by Smith *et al* (Smith *et al.,* 2006)). Gels were coversliped to spread enzyme and incubated in a humidified chamber for 10 minutes at 37°C.

2.8.5 DNA Unwinding & Electrophoresis

The following procedures were conducted in a refrigerator with minimal light. The slides were removed from lysis solution and randomly placed horizontally on a electrophoresis platform. The buffer reservoir of the unit was filled with freshly prepared electrophoresis buffer until the surfaces of the slides were covered. Cells were left to unwind for 20 minutes, followed by electrophoresis at 0.7 V/cm (calculated between the electrodes), 300 mA, for 20 minutes. Following electrophoresis slides were neutralised in three changes of neutralisation buffer.

2.8.6 Comet analysis

The slides were assessed by staining with 60 µL propidium iodide per slide and visualised using an Olympus BX51 fluorescence microscope fitted with a Texas red excitation filter and a barrier filter. This was linked via a CCD camera to a computer running Perceptive Instruments Comet IV image analysis software. Fifty morphologically normal nuclei were scored per slide using the following criteria:

a) only clearly defined non overlapping nuclei were scored

b) nuclei with no defined head, 'clouds', were not scored

c) cells with unusual staining artefacts were not scored

The amount of DNA in the comet tail was assessed by measurement of tail intensity i.e. the intensity of the fluorescence detected by image analysis of the head and tail, and is proportional to the amount of DNA that has moved from the head region into the comet tail.

2.8.7 Comet evaluation criteria

Statistical significant increases in tail intensity were analysed. Each plus and minus hOGG1 treatments were analysed separately. The data was log transformed and the average of repeat values used in the analysis.

As only increases in tail intensity above control were of interest, a one-sided test was used. Differences from control are presented as percentage changes (geometric mean changes). Effects are reported as statistically significant if the P-value was less than 0.05 (at the 5% level). Levels of significance were also assessed at the 1% and 0.1% level. One-way analysis of variance (ANOVA) was performed with the dose of test compound fitted as categorical variable. Contrasts were used to estimate the pair-wise differences between each compound dose and control. The contrasts were equivalent to t-tests, but used the pooled estimate of the variability across all groups (not the pooled variability from just the 2 groups being compared). This was considered to give a more robust estimate of the standard error. Methods were as recommended by Karen Oldman, AstraZeneca Alderley Park, Safety Assessment UK Statistics.

2.9 Cell free decatenation assay for detection of topoisomerase II inhibition

Method supplied and tests were kindly performed by Guo Chen AstraZeneca R&D, Boston USA. Data were sent to author for subsequent comparison analysis.

2.9.1 Preparation of treatment plates

1 µL of compound in DMSO solution was aliquoted into a V-bottom 96 well plate, filling columns 2 to 11 with 2-fold dilutions of compound concentration. Where possible the maximum test compound concentration was 0.2 mM in the reaction mixture. Column 1 and Column 12 contained positive and negative controls. The positive control was 1 µL of DMSO. The negative control was 1 µL of DMSO plus 5 µL of 0.5 M EDTA / 0.005% Brij-35. One plate was for the reaction (assay plate). The other was a control plate.

Assay plate: The assay reaction mixture was prepared by adding 25 µL of a premix into the wells of assay plate. After 5 min of mixing, 24 µL of 20 mM MgCl2 was added to initiate the reaction. The reaction was carried out at room temperature for 30 minutes. The premix was composed of 2 U/mL human topo II (USB (www.usbweb.com), Catalog number: 78303Y), 6.3 µg/mL circlet kinetoplastids DNA (kDNA) (TopoGEN (www.topogen.com/), Catalog number: 2013-3), 1 mM ATP, 50 mM Tris.HCl, pH 7.5, 125 mM NaCl, 5 mM DTT, 0.5 mM EDTA, 0.1 mg/mL BSA. At the end of reaction 5 µL of EDTA / 0.005% Brij-35 was added into the wells of assay reaction and the wells of the positive control.

Control plate: The control plate was prepared in the same way as the assay plate except that there was no enzyme included in the premix. At the end of incubation time, 5 µL of EDTA / 0.005% Brij-35 was added to each well.

2.9.2 Plate filtration

Set-up: The filtration system was set-up by placing a standard black flat-bottom 96-well plate as the receiver plate, a filter holder was placed on top of the receiver plate, and a filter plate (PN 5042) was placed on top of the plate holder. The filter system was connected to a vacuum line.

Filtration: 50 µL of the assay plate solution was transferred to each well of the filter plate prior to applying the vacuum. Once filtration was complete, the filter was rinsed with 150 µL of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl. The same procedure was performed for the control plate using a new receiver plate and a new filter plate. The procedure left undecatenated DNA retained on the filter and only decatenated DNA present in the receiver plates.

2.9.3 Decatenation detection

50 µL of SYBR® Green II solution (prepared by 1000 fold dilution of the stock in water) was placed into each well of the receiver plates. Fluorescence intensity was measured at 535 nm (excitation at 485 nm).

2.9.4 Data processing

A standard IC_{50} calculation tool (activity base) was used to calculate decatenation IC_{50}s.

2.10 Measurement of ClogP

Method supplied and analysis kindly performed by Guo Chen AstraZeneca R&D, Boston USA. Data were sent to author for subsequent comparison analysis.

ClogP was the calculated log of the ratio of the compounds solubility in octanol compared to water, analysed by Daylight Chemical Information Systems Inc., Laguna Niguel, CA.

2.11 Trapped in Agarose DNA ImmunoStaining (TARDIS) assay for detection of topoisomerase induced stabilised cleavage complexes

Basic method kindly supplied by Dr Caroline Austin, Newcastle University.

2.11.1 Buffers and other reagents

2.11.1.1 Preparation of agarose pre-coated slides

See as for *in vitro* comet assay Section 2.8.1.1.

2.11.1.2 Preparation of lysis buffers

See as for *in vitro* comet assay Section 2.8.1.1.

2.11.1.3 Protease inhibitors

Reagent:	Stock concentration in DMSO	Volume of stock	Final concentration in buffer
Benzamidine	100 mM	2ml	1%
PMSF	100 mM	2ml	1%
Leupeptin	2 mg/ml	200µl	0.1%
Pepstatin	2 mg/ml	200µl	0.1%
DTT	1 M	200µl	0.1%

Table 2.1 Preparation of protease inhibitors (to be added to 200 mL of solution)
PMSF = phenylmethylsulfonyl fluoride
DTT = dithiothreitol

2.11.1.4 Antibody buffers

1% BSA+PBS-T: 1%BSA+ PBS+ 0.1% tween 20 (e.g. 100ml PBS + 1g BSA+ 100µl tween20).

2.11.2 Compound exposure

See Treatment of L5178Y mouse lymphoma cells with test chemicals, Section 2.2

2.11.3 Post-Treatment slide preparation

After compound exposure, 3 x 10^5 cells in 1.5 mL were centrifuged at 200 x *g*, washed with 1 mL ice cold PBS and transferred to eppendorf tubes. Tubes were re-centrifuged at 200 x *g* and resuspened in 0.3 mL 0.5% w/v LMA. Two x 0.04 mL of cell mixture was placed onto pre-coated slides i.e. two gels containing 8000 cells from each concentration were prepared per slide. A dry coverslip was placed over the gels to spread agar. Slides were placed on cooling tray and coverslip removed once agar was set. Slides were placed in complete lysis buffer and refrigerated overnight.

2.11.4 Salt stage and antibody incubation

Slides were removed from lysis buffer and incubated in 1 M NaCl + protease inhibitors for 30 minutes at room temperature to remove any unbound protein.
Slides were washed 3 times in PBS + protease inhibitors for 5 minutes each.
Slides were placed inside a humidified tray and a 1:100 dilution in 1% bovine serum albumin (BSA) + PBS-with tween (PBS-T) of primary anti-rabbit topoisomerase II α and / or β antibody, was added (~50 µL per gel). In initial tests the antibodies used were kindly supplied by Newcastle University and were raised against human

topoisomerase II. Later tests used in house designed antibodies raised against mouse topoisomerase II (see Chapter 7). A flexible plastic coverslip was placed over gels to evenly distribute antibody. Slides were incubated at room temperature for 2 hours. Slides were washed 3 times in PBS-T + protease inhibitors, first dipped in and out quickly, next two washes of 5 minutes each. Slides were returned to humidified tray. From this point on slides were protected from light.

Secondary antibody (anti-rabbit FITC, using 1:100 dilution in 1% BSA + PBS-T) was added as for the primary antibody and incubate for 2 hours.

Sides were washed twice in PBS-T+ protease inhibitors, then washed one more time in PBS-T+ protease inhibitors and left for at least 20 minutes.

2.11.5 Propidium Iodide (PI) staining and scoring

Approximately 30 µL of 20 µg/mL PI was added to each gel, and slides coverslipped. Slides were visualised using an Olympus BX51 fluorescence microscope fitted with a Texas red excitation filter and a barrier filter and the amount of FITC fluorescents from each nucleus scored using Comet IV software (supplied by Perceptive Instruments).

2.11.6 Evaluation criteria

As the occasional cell from control and treated cultures can signal with very high intensity, thus biasing any mean score, for data analysis in the TARDIS assay median values for cellular relative antibody FITC signal intensity were used. As only increases in FITC signal above control were of interest, a one-sided test was used. Accordingly, a one-sided two sample paired mean t-Test on square root transformed data was performed as recommended by Karen Oldman, AstraZeneca Alderley Park, Safety Assessment UK Statistics. Effects were reported as statistically significant if the P-value was less than 0.05 (at the 5% level). Levels of significance were also assessed at the 1% and 0.1% level.

2.12 The Isolating *in vivo* Complexes of Enzyme to DNA (ICE) bioassay for detection of topoisomerase induced stabilised cleavage complexes

Basic methodology adapted from TopoGEN Inc Manual for *in vivo* link kit Catalogue Number 1022.

2.12.1 Compound exposure

See basic cell treatment methodology, with the exception that cells were washed in serum free RPMI media centrifuged and then lysed with 1.5 ml of 1% sarkosyl lysis buffer in Tris-EDTA (buffer pH 8.0).

2.12.2 Caesium chloride density centrifugation and DNA preparation

Initial tests used the following CsCl density preparations. Later tests used a single preparation of 1.5 g/mL CsCl (see Chapter 5 results & discussion):

Solution	mL of 1.86 g/mL CsCl stock solution	mL of Tris EDTA	Density (g/mL)
A	13.013	0.98	1.82
B	11.452	2.548	1.72
C	7.756	6.244	1.5
D	6.02	7.98	1.37

Table 2.2 CsCl density solution used during initial assay set-up

In preliminary tests 1 mL of each of the above solutions (Solution A up to Solution D) was carefully layered in an ultracentrifuge tube. For later tests 4 mL of Solution C was used (see Chapter 5 results & discussion). 1 mL of cell lysate was carefully layered on top of the gradient. Tubes were ultracentrifuged for 18 hours at 31000 rpm ($1x10^5$ x *g*). In preliminary experiments 200 µl fractions were then taken from the top of each tube using a 1 mL pipette with ~3 mm cut off pipette tips. This gave 25 fractions. In later tests where a single CsCl concentration was used, the caesium chloride layers were discarded. The DNA pellet remaining at the bottom of the tube was resuspended in 1 mL Tris-EDTA buffer pH 8.0. Spectrophotometric analyses (using a Thermo Biomate 5) at absorbance 260 nm was performed on fractions to determine DNA content, assuming that one absorption unit at 260 nm equals 50 µg/mL DNA. In preliminary tests, fractions containing DNA were all used to prepare slot blot membranes. In later tests the resuspended DNA was normalised such that ~10 µg DNA was loaded onto each slot. Prior to spectrophotometric analysis and slot blot membrane loading, samples were thoroughly vortexed, sonicated and for later tests

rotated on an haematological rotator overnight to ensure adequate DNA solutions were maintained (see Chapter 5 for further details of method development).

2.12.3 Slot blot and membrane preparation

Relevant fractions were loaded onto a nitrocellulose membrane using a slot blot (BioRad) as follows.

Nitrocellulose membranes were pre-wetted by incubation in 28 mM sodium phosphate buffer for at least 30 minutes and then 10 minutes in Tris Buffered Saline with Tween20 (TBST). Membranes were then loaded onto the slot blot apparatus along with appropriate wetted filters. The DNA containing fractions were added to each slot and diluted with 25 mM sodium phosphate. Vacuum was applied for approximately 10 minutes. A minimum of three, and where possible six, slots were prepared for each sample.

After membrane preparation was completed, membranes were removed, rinsed briefly with 25 mM sodium phosphate buffer and then incubated for at least 1 hour with casein based blocking buffer at room temperature. Membranes were then incubated overnight at 4°C with the appropriate primary antibody. The tests described in Chapter 5 used rabbit polyclonal primary antibodies against topoisomerase IIα and IIβ (TopoGEN inc) optimally diluted 1:1000 in casein based blocking buffer diluted in TBST. The topoisomerase IIβ antibody was also used for the work described in Chapter 8. For detail of work with in house prepared mouse antibodies to topoisomerase IIα see Chapter 7.

After overnight incubation, membranes were washed 3 times in TBST and incubated for 1 hour with a secondary anti-rabbit antibody optimally diluted 1:5000 in TBST, either conjugated to horseradish peroxidase (Promega) in preliminary tests or an infra red (IR) chromophore (Li-Cor R680 secondary antibody) in later tests. For horseradish peroxidase conjugated secondary antibodies, reagents from ECL™ (enzyme-linked chemiluminescence) advance western blotting detection kit (GE-Healthcare) were added as per manufacturer instructions and then imaged with Syngene charged-coupled device (CCD) and slots were quantified using Syngene software. For membranes treated with the IR secondary antibody (R680), the membrane was imaged with Li-Cor Odyssey IR scanner and quantified signal using Li-Cor Odyssey software.

2.12.4 Preliminary antibody titration and dilution

All antibody dilutions are in relation to the antibody's supplied concentration. TopoGEN's primary antibody was raised and extracted from rabbit serum. It was supplied in 2.5 units/ml (250 units in 100 µl) and their definition of a unit is as follows: "The antibody concentration is in the western blotting units; one unit corresponds to a 1:1000 dilution of antibody required to make 1 ml of diluted probe for a routine western blot. Thus 10 units of antibody will make 10 ml of diluted probe".

For the ECL based approach, a secondary goat-antirabbit antibody conjugated to horseradish peroxidase was used. 300 µl of antibody was supplied by Promega at 1 mg/ml.

For the IR based imaging approach, a secondary antibody conjugated to an IR chromophore was used. The antibody was supplied by Li-Cor under the product description goat-antirabbit R680. It was provided as a powder that was resuspended in 0.5 ml double distilled water and the final concentration was then 1 mg/mL. The fluorophore has a molecular weight of 950 g/mol. The excitation and emission wavelength is at 683 nm and 710 nm respectively. The ratio of the fluorophore to protein is 2.7 moles IR Dye 680: 1 mole IgG.

For initial antibody titration experiments varying combinations of primary antibody anti-topoisomerase IIα (TOPOGEN) concentration (1:250, 1:500 and 1:1000) were assessed against varying combinations of secondary antibody concentrations (1:2500, 1:5000 and 1:10000). Samples were loaded onto a slot blot held nitrocellulose membrane and combinations of antibody concentrations were tested against the following: a negative control, crude cell lysate containing free TOPOII (positive control) and relevant fractions from a 100 µM etoposide treated sample that had been through CsCl density ultracentrifugation. Dilutions of 1:100 primary antibody and 1:5000 secondary antibody were found to be appropriate. Data from the titration work is not presented.

2.12.5 Statistical Analysis for the ICE bioassay

Due to the limitation of ultracentrifugation it was only possible to run single cultures for each concentration tested in each test. Accordingly analysis was performed to measure inter-experimental variance and increase in ICE signal across at least triplicate experiments. A one-sided two sample paired mean t-Test on square root

transformed data was performed as recommended by Karen Oldman, AstraZeneca Alderley Park, Safety Assessment UK Statistics.

2.13 SDS-PAGE and Western Blot experiments

For confirmation of reactivity of in house prepared antibodies SDS-PAGE followed by Western Blots was performed. As mouse topoisomerase II enzyme was not commercially available initial Western Blots were performed looking at reactivity of each antibody with human topoisomerase II alpha (supplied by Inspiralis UK, HT205 500U). To confirm the response with mouse enzyme, cell extracts from L5178Y mouse lymphoma cells were prepared and also run through SDS-PAGE and Western Blot.

2.13.1 SDS-PAGE Western blots

2.13.1.1 Analysis of antibodies against commercially supplied human topoisomerase IIα

Human Topoisomerase II alpha (Topogen inc) was prepared at a concentration 10 U/µL. Information from the supplier indicated that 1 U was 20-40 ng protein. Samples were denatured at 95°C for 5 minutes and 0.2-0.4 ng was added to each SDS-PAGE gel lane (gel type was 4%-12% Criterion Bis-Tris 18 well gels). 10 ul Seeblue plus 2 marker and magic marker to determine size and location of protein were loaded as required.

2.13.1.2 Analysis of antibodies against L5178Y mouse lymphoma extracts

Two different cell extracts were prepared. Tubes containing 1 x 10^7 cells were extracted using 1% sarkosyl, this gave a general lysate of all cellular material hence total protein content. Additional specific cytosol and nuclear extracts were prepared using BioVision nuclear/cytosol fractionation kit (BioVision, USA). Briefly; cells were lysed with supplied lysis buffers, samples centrifuged at 16,000 x *g* for 5 minutes. After which the supernatant contained the cytosol fraction and the pellet contained the nuclear fraction. Lysates were prepared in the presence of protease inhibitors. Preparations were diluted with sample loading buffer and heated to 95°C for 5 minutes before being loaded on to SDS-PAGE gels as above.

All gels were run in 1 x BioRad 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer at 200 V for 55 minutes.

2.13.1.3 Western Blot

Proteins were transferred onto Amersham Hybond nitrocellulose membranes in 1 x Tris-Glycine (TG) buffer using Criterion blotting module. Transferred at 30 V overnight.

2.13.1.4 Antibody staining and ECL imaging

Following preparation, membranes were blocked with blocking buffer (4% Marvel powder in PBS+Tween) for 1 hour. Membranes were cut such that single lanes containing loaded topoisomerase II alpha enzyme and markers were separated. 12 cut membranes were prepared in this way and each cut membranes was incubated with one of the 12 prepared mouse antibodies (see Chapter 7) at 1 ug protein/mL in blocking buffer. Incubation was overnight at 4°C.

The secondary antibodies (Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat) was prepared as a 1:5000 in blocking buffer and membranes incubated for 1 hour at room temperature. Membranes were than incubated in Amersham ECL Prime substrate for 5 minutes before imaging on the Fuji scanner for 20 or 30 seconds, 1 minute and 5 minutes.

2.13.1.5 Antibody staining and Odyssey imaging

For later work to demonstrate SiRNA knockdown of topoisomerase α and β, 4 x 10^6 L5178Y mouse lymphoma cells were lysed using 1% sarkosyl or using BioVision nuclear/cytosol fractionation kit (from which only the nuclear extract was used) and were assessed for protein content and up to 10 µg total protein loaded per required number of GEL lanes and run as above with a BioRad 161-0374 rainbow coloured marker which shows up in the 700 channel for Odyssey or magic marker for ECL. This procedure was performed with control L5178Y mouse lymphoma cells and cells that had been had been pre-treated with SiRNA to topoisomerase II alpha and allowed to recover for two days after knockdown (See Chapter 8 for methodology employed for SiRNA knockdown). After Western blotting membranes were split and treated separately with antibodies to topoisomerase II α or β (diluted 1:1000 in blocking buffer) at 4°C overnight, prior washing in TBST and 2 hour incubation with (IR) chromophore (Li-Cor R680 secondary antibody) (diluted 1:5000 in blocking buffer) and imaged with Li-Cor Odyssey IR scanner and quantified signal using Li-Cor Odyssey software or Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat produced in goat secondary antibody than incubated in Amersham ECL Prime substrate for 5

minutes and imaged with the Biorad Chemi Doc MP. For further details of this method and method development See Chapter 8 Section 8.2.

2.14 General statistical analysis evaluation criteria

For all of the statistical analysis methods used for individual assay results were evaluated with confidence limits of 95% ($P<0.05$), 99% ($P<0.01$) and 99.9% ($P<0.001$) which are generally represented as *, ** and *** respectively.

2.15 General calculation of error within data sets

Where appropriate on graphical representations of data where 3 or more data points were available, the error within each mean data point is represented by the standard error of the mean which was calculated as:

$$\text{Standard error of mean} = \frac{\text{Standard deviation of mean}}{\text{Square root of number of replicates}}$$

This error is represented on graphs as bars equal to -/+ standard error of mean.

This method was recommended by Karen Oldman, AstraZeneca Alderley Park, Safety Assessment UK Statistics. It was considered to be the most appropriate estimate of variance for three or more replicates.

In preliminary experiments using only duplicate cultures standard error was calculated as half the difference between the two replicates.

CHAPTER 3

The incidence of positive results in the mouse lymphoma tk assay (MLA) in pharmaceutical screening and could aneugenicity be a plausible mechanism?

CONTENTS

3 The incidence of positive results in the mouse lymphoma tk assay (MLA) in pharmaceutical screeing and could aneugenicity be a plausible mechanism?

3.1 Introduction

The aim of the work described in this chapter was to put into perspective the number of positives responses seen in the MLA during pharmaceutical screening and to investigate whether aneugenicity could contribute to this number.

Along with other *in vitro* mammalian cell genotoxicity assays, the predictivity of the MLA for identifying carcinogens has often been questioned. As recently as 2005, David Kirkland *et al*'s retrospective review of genotoxicity data from the International Conference on Harmonisation (ICH) recommended tests indicated that out of 105 non-carcinogens, the MLA unequivocally correctly identified only 41 as being negative (Kirkland *et al.,* 2005). This would suggest the 'false positive' rate for the MLA to be as high as 61%. Whilst Kirkland *et al*'s review did not focus on pharmaceutical testing, there was a perception that the 'over sensitivity' of the MLA was a general phenomenon. An assay with such a high 'false positive' rate would seem to be of little value in a pharmaceutical screening paradigm. The paradox being that the MLA is still routinely used for screening in many pharmaceutical safety assessment laboratories. Accordingly, either the lack of specificity suggested by Kirkland *et al* is not seen during the genotoxicity testing of novel pharmaceuticals in a commercial environment (which is the perception gained by the author over the last 25 years) or even in these laboratories a surprisingly large number of novel pharmaceuticals are positive in *in vitro* mammalian cell genotoxicity tests. There is anecdotal evidence for the latter, and some groups have suggested this as a reason for using Option 2 in the recently adopted update of ICH S2 (ICHS2(R1), 2011). Option 2 includes the opportunity to test two *in vivo* genotoxicity tests (usually the rodent haematopoetic micronucleus assay and the rodent comet assay) instead of one *in vivo* test and an *in vitro* mammalian cell assay. Those in favour of this option maintain that it would reduce the number of 'false positives' seen in genetic toxicity screening. Furthermore, during the long discussion process surrounding the ICH S2 update, there was much debate on whether the highest concentration to be tested in *in vitro* mammalian cell genotoxicity tests should

be reduced from 10 mmol/L to 1 mmol/L. Part of the reasoning behind this initiative was also to help reduce the number of 'false positives'.

Assuming there really is an issue with an excessive number of false positives seen in the MLA, one option available to pharmaceutical discovery chemists is to use *in silico* structural alert relationships (SAR) to predict the MLA result. SAR models have also been validated by scientists within regulatory authorities to help predict mammalian cell genotoxicity with development pharmaceutical's and their metabolites and impurities (Matthews *et al.,* 2006, Contrera *et al.,* 2008). So can *in silico* analysis be of help in predicting the likelihood of a MLA positive result, and hence reduce the number of actual positive tests conducted?

Kirkland *et al*'s review on specificity in the MLA considered the inability of the MLA to correctly identify non-carcinogens as being non-genotoxic. Unfortunately, oncogenicity data would not be available for the vast majority of compounds tested in the MLA for pharmaceutical screening. However, it would be possible to consider a compound's primary pharmacological target and mechanism and relate this to any positive result, with the possibility of deducing beyond reasonable doubt the mechanism of a 'false positive' finding, hence changing a 'false positive' into a mechanistically interpretable result.

Furthermore, one mechanism that could be responsible for generation of uninterpretable positive responses in the MLA is aneugenicity. Aneugenicity is the loss or gain of a whole chromosome. In general, aneugens act via perturbation of mitotic spindle or associated proteins. Aneugens structures do not usually contain easily recognisable toxiphores, such as the aromatic amines, nitrosamines or epoxides that are associated with many classic DNA reactive mutagens. Accordingly, anuegens cannot be predicted by classic structural alert relationships, hence the first indication of a genotoxic effect may a perceived 'false positive' result obtained during routine pharmaceutical screening. However, although the ICH guidance states that the MLA is able to detect compounds that induce numerical chromosomal damage and that the detection of aneuploidy inducers is enhanced if a 24 hour treatment regimen is used with the microtitre method (ICHS2(R1), 2011), over the last decade there has been some debate about whether, or not, the MLA responds to aneugens as a class. Theoretically, if an aneugen induces loss of the copy of the mouse lymphoma cell chromosome 11b containing the functional tk^+ allele*,* this should be expressed as a trifluorothymidine (TFT)-resistant mutant. Several studies have reported positive MLA

results with aneugens including colchicine (Honma *et al.,* 2001), vinblastine (Honma 2001 *et al.,*) carbendazim (O'Donovan *et al.,*1999) and taxol (Wang *et al.,* 2009). However, with the exception of carbendazim, these positive results have not been easy to reproduce in different laboratories (O'Donovan *et al.,* 1999). Furthermore, although a strain of L5178Y cells with only one intact copy of chromosome 11 is known (Evans *et al.,* 1986), it has been suggested that L5178Y $TK^{+/-}$ clone 3.7.2c cells (the cell line used in the MLA) that are monosomic for chromosome 11 are not viable (Liechty *et al.,* 1998; Fellows *et al.,* 2005). This would be a prerequisite if TFT-resistant mutants were to be induced by simple chromosome loss. If aneugens do induce TFT-resistant mutant cells, it is possible that they are the result of more complex mechanisms, for example, chromosome loss and subsequent recombination and duplication (de Nooij-van Dalen *et al.,* 1998, Wijnhoven *et al.,* 2003) or simply the induction of chromosome damage since it is known that most aneugens can also induce structural changes to some extent.

To help answer some of these questions, a retrospective review was undertaken of the results of compounds tested in the MLA that the author study directed at AstraZeneca, Alderley Park, UK between 2001 and 2010. During this time 355 pharmaceutical compounds were evaluated in the MLA. The number of MLA positive conclusions from these tests was calculated, along with the *in silico* prediction using MultiCase (MCASE) MC4PC analysis, which has been reported to have 'good specificity, sensitivity and coverage for prediction of genotoxicity test results' (Contrera *et al.,* 2008). For compounds that were positive in the MLA at AstraZeneca during this period, consideration of the compounds primary pharmacological target and the response in other genetic toxicity assays was made to help elucidate the probable mechanism of the positive response. Furthermore, to help put into context the number of positive responses seen in the MLA, a similar review was made of the Ames assay results from the same data set. Finally, in order to investigate the response of the MLA to aneugens, and to see whether it was possible that aneugenicity could explain hitherto uninterruptable positive responses, seven compounds with differing modes of anuegenic action were tested in the MLA using conditions acceptable for pharmaceutical development (OECD, 1997) (Figure 3.1).

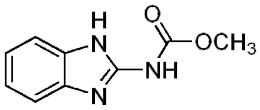
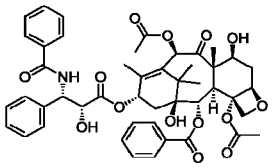
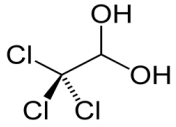
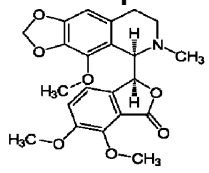
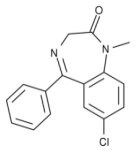
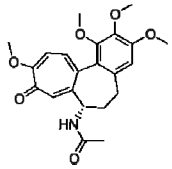
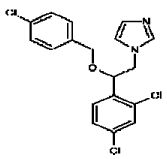
Carbendazim 	Uses: Broad-spectrum benzimidazole fungicide. MOA: Associated with the inhibition of tubulin polymerisation (Kirsch-Volders *et al.,* 2003).
Taxol (paclitaxal) 	Uses: Treatment of ovarian, breast and lung cancer. MOA: Binds to the β-subunit of tubulin, hyper stabilizing the spindle microtubules. The resulting microtubule/taxol complex does not have the ability to disassemble (Schiff *et al.,* 1979).
Chloral Hydrate 	Uses: As a hypnotic and sedative. MOA: Interferes with tubulin (Faust *et al.,* 2003) assembly and shortens microtubules (Lee *et al.,* 1987).
Noscapine 	Uses: As an antitussive. MOA: Disrupts the function of mitotic spindle (Schuler *et al.,* 1999), binds to tubulin and alters the conformation and assembly properties (Aneja *et al.,* 2007).
Diazepam 	Uses: As a sedative, muscle relaxant, anticonvulsant and anxiolytic. MOA: Inhibits centriole (Andersson *et al.,* 1981) and spindle pole separation. Prevents the separation of centrioles at prometaphase (Parry and Sors,1993).
Colchicine 	Uses: Treatment of inflammatory conditions, e.g. Gout. MOA: Interacts with α tubulin at two sites (Wallin *et al.,* 1988), depolerising microtubules and stabilizing microtubule dynamics at high and low concentrations respectively (Jordan and Wilson, 2004).
Econazole 	Uses: As an antifungal. MOA: Known to damage cell membranes (Gudi *et al.,* 1992). Inhibits ergosterol synthesis, and therefore microsomal and mitochondrial cell membranes (Parry and Sors, 1993).

Fig. 3.1. The aneugens used in these investigations MOA = Mode of action

3.2 AstraZeneca mouse lymphoma assay review results

3.2.1 MLA results

Out of 355 compounds tested in the MLA at AstraZeneca, Alderley Park, UK, between 2001 and 2010, 303 compounds were concluded to be negative (85% of the total number of compounds tested). Only 52 compounds were concluded to be positive (15% of the total compounds tested) (Figure 3.2). All of these 52 compounds were positive at concentrations of less than 1 mmol/L, the ICH limit concentration for *in vitro* genotoxicity testing of pharmaceuticals (ICHS2(R1), 2011). Of the 52 compounds that were positive, 36 were positive in the presence and absence of exogenous metabolism (S9) (69% of total positive compounds), 12 were uniquely positive in the presence of S9 (23% of total positive compounds) and 4 were uniquely positive in the absence of S9, short term and/or 24 hours exposure (8% of total positive compounds).

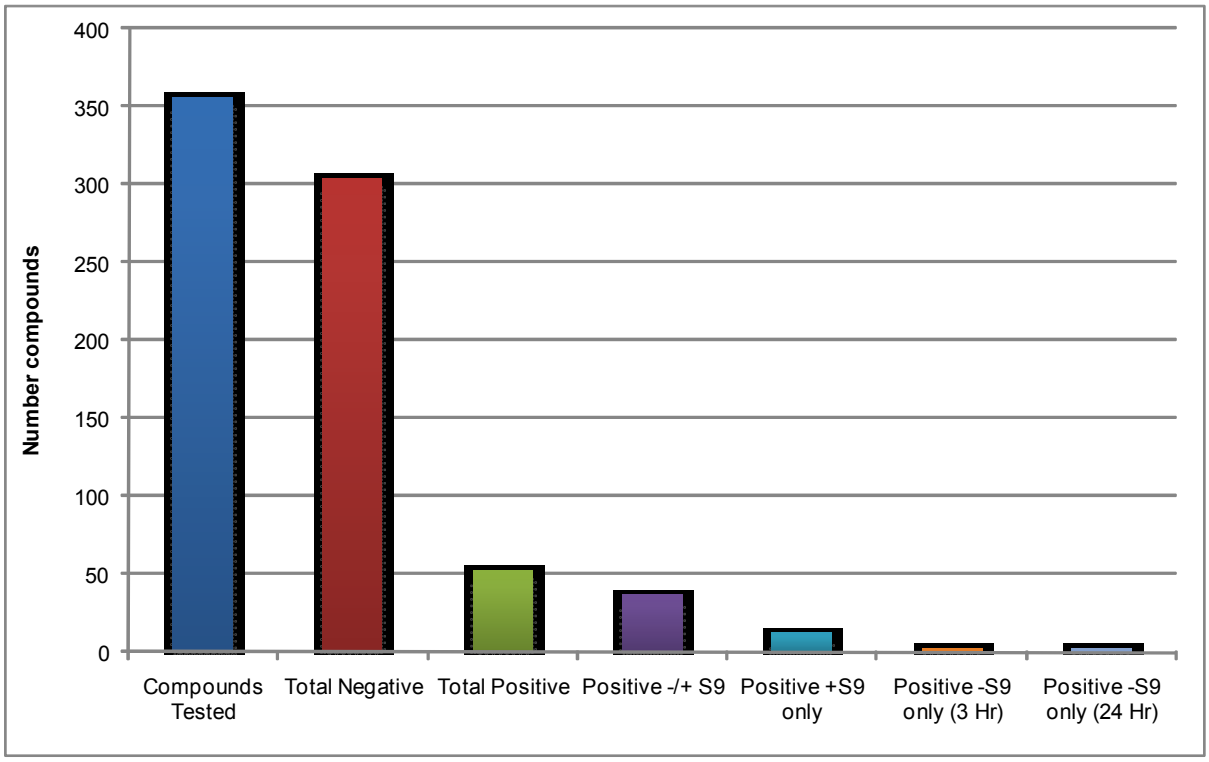


Figure 3.2: Conclusions from 355 MLA studies tested at AstraZeneca Alderley Park between 2001 and 2010

The mechanism of genotoxicity for the 12 compounds that were uniquely positive in the presence of S9 was considered to be unlikely to be due to the compounds primary pharmacological target (none of these compounds were pro-drugs). Of the remaining 40 positive compounds that were positive in the absence of metabolic activation, 19 were cancer therapy drugs for which the primary pharmacological target was either

inhibition of a kinase or inhibition of DNA polymerase, both of which are known to be mechanisms for *in vitro* genotoxicity (Marzin, 2007, Olaharski *et al.,*2009). A further 10 of the 40 positive compounds were anti-infectives for which the primary pharmacological target was either inhibition of topoisomerase II (9 compounds) or protein synthesis (1 compound), both of which are again known to be mechanisms for *in vitro* genotoxicity (Smart *et al.,* 2008, Tweats *et al.,* 2007). One further compound was a known genotoxic metabolite. Hence, including those compounds uniquely positive in the presence of S9, incredibly only 22 compounds out of 355 tested (6%) were positive by a mechanism that was unlikely to be explainable by the compounds primary pharmacological target (Figure 3.3).

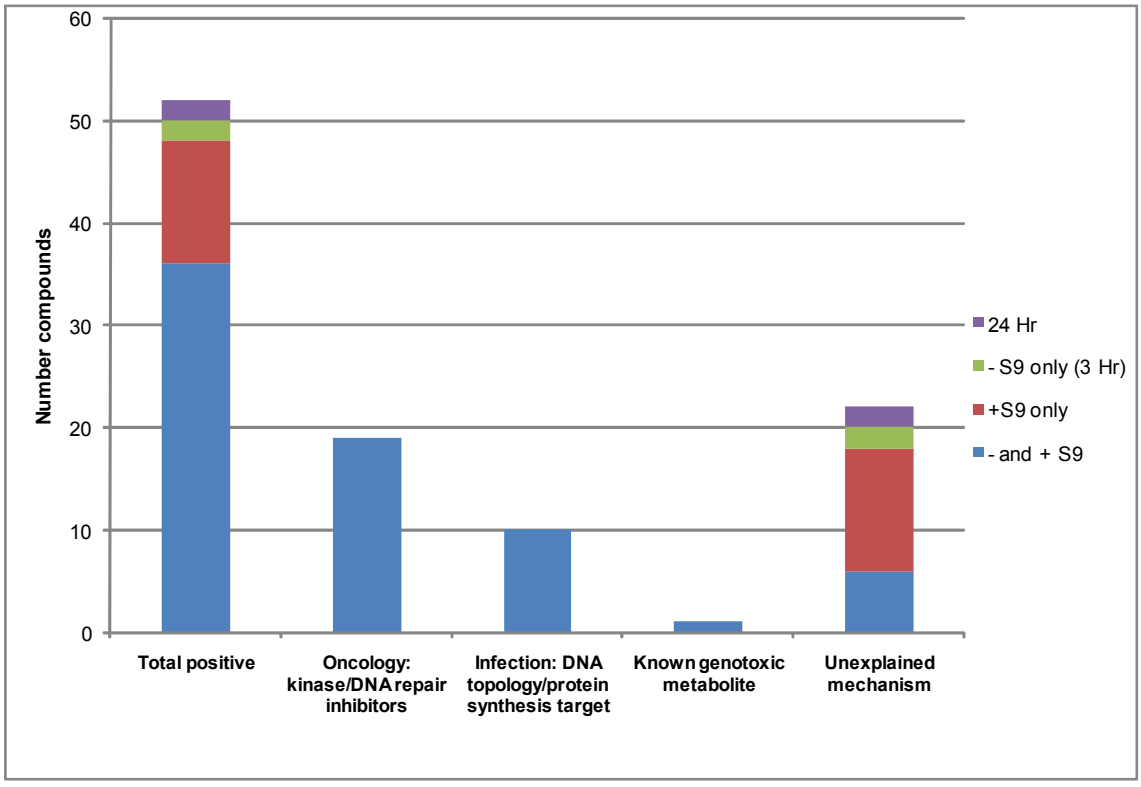


Figure 3.3: Pharmacological target of S9 independent MLA positive compounds out of the 355 compounds reviewed

Furthermore, 3 of the 22 mechanistically unexplainable positive compounds were also positive in an Ames or an *in vivo* bone marrow micronucleus test, adding weight to the likely biological relevance of the MLA positive finding (Figure 3.4).

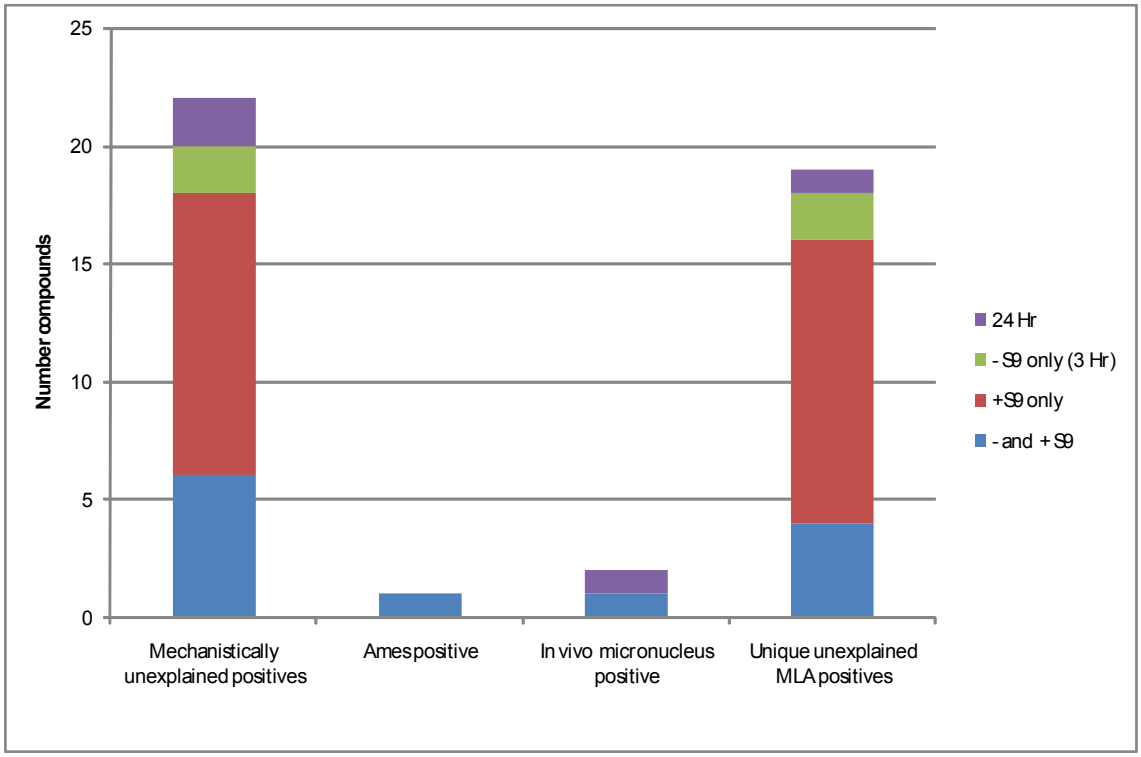


Figure 3.4: Response in Ames and *in vivo* micronucleus test of mechanistically unexplained MLA positives out of the 355 compounds reviewed

Accordingly, out of 355 compounds tested over a 10 year period at AstraZeneca, Alderley Park, UK, there were only 19 unique MLA positives for which the genotoxic mechanism was unlikely to be related to the compounds primary pharmacology (i.e. 5% of total compounds assessed).

3.2.2 Ames results

Of the 355 compounds tested in the MLA, only 10 were positive in the Ames test (3%). The vast majority of the Ames positives (7 out of 10) were from AstraZeneca's infection portfolio for which the primary pharmacological target was inhibition of bacterial topoisomerase II (gyrase or topoisomerase IV). As was expected for compounds targeting bacterial topoisomerase II, all of these anti-infective compounds were positive in the excision repair proficient *Salmonella* strain TA102 (Gocke, 1991). The other positive compounds were two small aromatic amines, for which the positive Ames response was not unexpected (Ashby and Tennant, 1988) and one other compound of unknown genotoxic mechanism (Figure 3.5). Seven of the 10 Ames positive compounds were also positive in the MLA. The three exceptions were the two small aromatic amines and one anti-infective, which may have been positive in the MLA if tested to higher concentrations, but in view of the positive Ames result this additional testing was not deemed necessary.

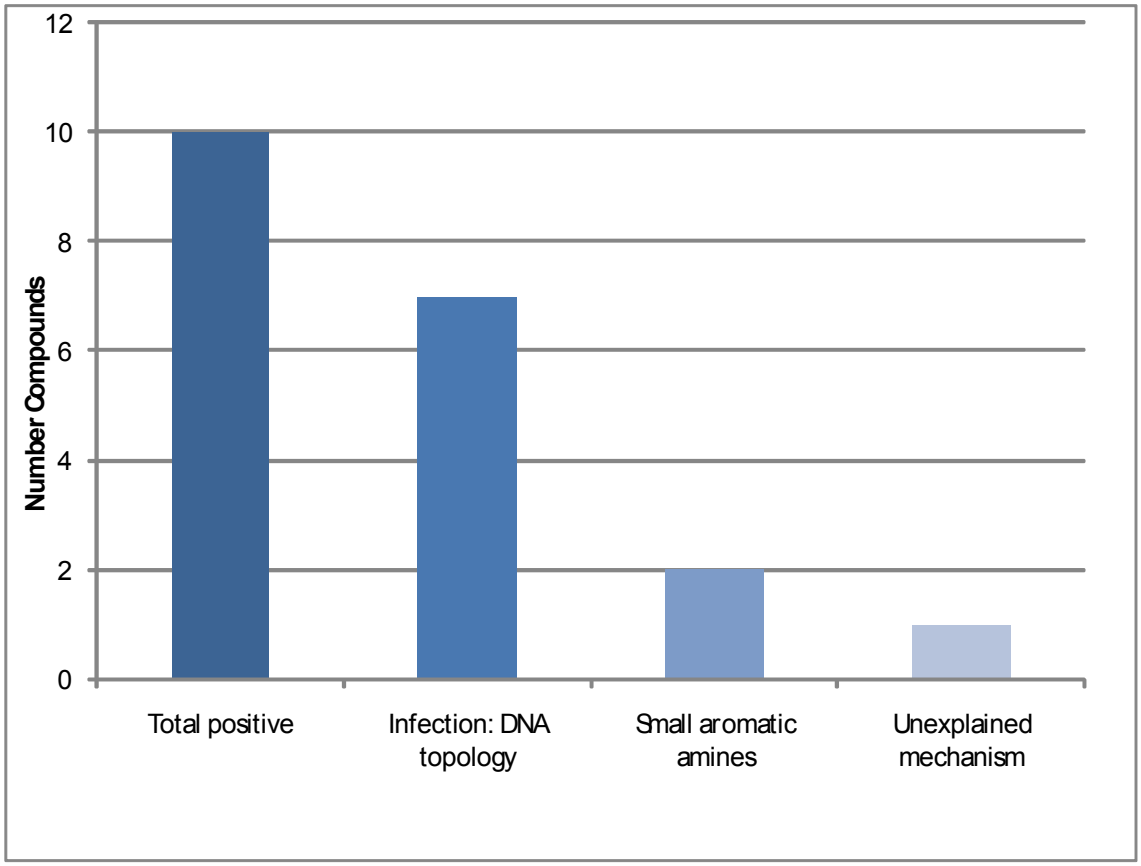


Figure 3.5: Number of positive Ames assays out of 355 compounds and their probable mechanism

3.2.3 MCASE MC4PC Analysis Results

338 of the compounds tested in the MLA at AstraZeneca, Alderley Park, UK, were analysed for structural alert relationships using Multicase module 4PC (MC4PC), a structural activity relationship programme designed to predict positive results in the mosuse lymphoma assay from previous results on similar structures. Results are shown in (Figure 3.6). Of these 338 compounds, MC4PC predicted 209 to be positive (62%). Surprisingly, there was no difference in the MC4PC positive prediction for those compounds negative in the MLA, for which MC4PC predicted 177 out of 286 to be positive (62%), or positive in the MLA, for which MC4PC predicted 32 out of 52 to be positive (62%). Hence the predictivity of MC4PC for the AstraZeneca Alderley Park, UK, MLA results were:

- Sensitivity (correct identification of an MLA positive response) 62% (n of 52)
- Specificity (correct identification of a MLA negative response) 38% (n of 286)
- Overall concordance 42% (n of 338)

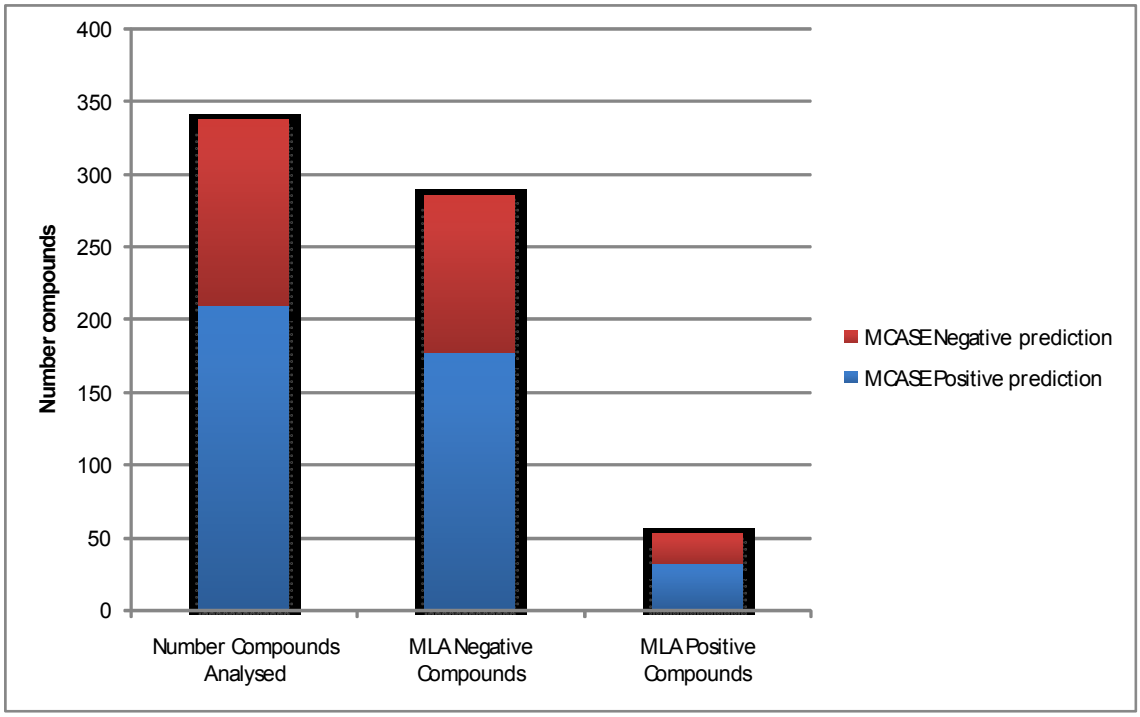


Figure 3.6: MCASE MC4PC MLA predictions for 338 compounds

3.3 The potential of an aneugenic mechanism for MLA positive responses

3.3.1 Results

The aneugens were all tested using 24 hours exposure, this exposure condition is considered to be the most suitable to identify division dependent mutagens and aneugens (Honma *et al.,* 1999). The following Table 3.1 shows the results for econozale, chloral hydrate, noscapine, diazepam and colchicine when tested in the MLA. In line with ICH recommendations for pharmaceutical development ICHS2(R1), 2011), single experiments were performed with the exception of colchicine which had previously been reported to be positive in the MLA (Honma *et al.,* 2001).

Econazole				**Chloral hydrate**			
Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones
0	100	66	43	0	100	88	40
14	45	75	53	1700	40	92	44
17	29	107	31	2100	27	127	59
21	14	154	52	2700	14	116	76
27	7	242*	63	3400	2	249*	84
4-NQO 0.33	39	734*	43	4-NQO 0.33	29	877*	40
Noscapine				**Diazepam**			
Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones
0	100	59	45	0	100	52	52
34	62	95	21	330	61	98	63
42	44	43	50	410	54	67	58
52	20	88	29	510	52	74	36
66	7	103	36	640	0	130	100
4-NQO 0.33	29	636*	44	4-NQO 0.33	40	540*	34
Colchicine Test 1				**Colchicine Test 2**			
Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones
0	100	107	50	0	100	55	33
0.0125	64	137	42	0.04	78	46	46
0.025	60	118	47	0.06	52	49	51
0.031	32	115	44	0.07	20	54	49
0.038	13	128	35	0.08	5	38	21

Table 3.1: MLA results for econozale, chloral hydrate, noscapine, diazepam and colchicine

RTG = Relative Total Growth
MF = Mutant Frequency x 10^{-6}, mutant frequency is pooled data from two replicates
* Significantly different from the control (Induced Mutant Frequency ≥ 126 x 10^{-6})

The following Table 3.2 shows the results for carbendazim when tested in the MLA. As carbendazim had previously been reported to be positive in the MLA (O'Donovan *et al.,* 1999) and as the results from the initial tests were inconclusive, triplicate tests were performed.

Carbendazim Test 1				**CarbendazimTest 2**				**CarbendazimTest 3**			
Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones
0	100	65	44	0	100	120	48	0	100	74	61
5	91	70	59	5	83	83	65	8	65	66	85
8	32	107	39	8	53	139	43	10	27	188	36
10	15	137	27	10	17	231	30	13	10	229*	74
13	1	430*	43	13	8	318*	37	16	3	474*	77
				16	2	568*	41	18	1	370*	88
4-NQO 0.33	11	1594*	38	4-NQO 0.33	42	909*	42	4-NQO 0.33	32	835*	64

Table 3.2: MLA results for carbendazim
RTG = Relative Total Growth
MF = Mutant Frequency x 10^{-6}, mutant frequency is pooled data from two replicates
* Significantly different from the control (Induced Mutant Frequency ≥ 126 x 10^{-6})

The following Table 3.3 shows the results for taxol when tested in the MLA. As taxol had previously been reported to be positive in the MLA (Wang *et al.,* 2009) and as the results from the initial tests were inconclusive, quadruplicate tests were performed.

Taxol Test 1				**Taxol Test 2**				**Taxol Test 3**			
Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones	Conc (µmol/L)	RTG	MF	% small clones
0	100	66	49	0	100	88	34	0	100	97	68
0.017	62	80	53	0.028	39	104	58	0.018	63	75	41
0.021	35	92	55	0.036	25	90	16	0.023	31	102	32
0.026	14	178	34	0.045	7	310*	109	0.028	12	174	37
0.033	2	521*	64	0.056	2	293*	126	0.036	2	652*	49
4-NQO 0.33	39	734*	43	4-NQO 0.33	29	877	40	4-NQO 0.33	50	762*	40

Taxol Test 4			
Conc (µmol/L)	RTG	MF	% small clones
0	100	74	61
8	65	66	85
10	27	188	36
13	10	229*	74
16	3	474*	77
18	1	370*	88
4-NQO 0.33	32	835*	64

Table 3.3: MLA results for taxol
RTG = Relative Total Growth
MF = Mutant Frequency x 10^{-6}, mutant frequency is pooled data from two replicates
* Significantly different from the control (Induced Mutant Frequency ≥ 126 x 10^{-6})

Significant increases in mutant frequency as indicated by exceeding the global evaluation factor (increase ≥ 126 x 10^{-6} above concurrent control) were seen with taxol, carbendazim, econazole and chloral hydrate (Tables 3.1 to 3.3) but only at highly toxic concentrations (≤17% RTG). Only taxol and carbendazim, at single concentrations in one of four and one of three tests, respectively, induced significant increases in mutant frequency at concentrations giving ≥ 10% survival as measured by reduction in relative total growth (RTG). In the single tests performed with econazole and chloral hydrate, increases in mutant frequency were only seen at concentrations giving 7% and 2% survival, respectively, i.e. concentrations below 10% survival which is the lowest acceptable level of toxicity for analysis in the MLA (Moore *et al.,* 2006). Also, where there were significant increases in mutant frequency, there was no clear evidence of a preferential increase in either the number of small or large mutant clones. Accordingly, the aneugens; taxol, carbendazim, econazole and chloral hydrate, did not give significant, concentration-related or reproducible increases in mutant frequency at concentrations of acceptable toxicity (> 10% survival). Hence they would not be considered to be unequivocally positive using the accepted evaluation criteria for the MLA (Moore *et al.,* 2006).

No significant increase in mutant frequency was seen following exposure to noscapine, diazepam or colchicine, when tested up to highly toxic concentrations (Table 3.1).

In all tests where it was used, the positive control 4-NQO induced a highly significant increase in mutant frequency.

3.4 Discussion

3.4.1 AstraZeneca mouse lymphoma assay review

Although cancer bioassay data are not available for these compounds, because 97% are not bacterial mutagens and all were tested as potential drug substances, it must be assumed that the majority are probably not DNA-reactive carcinogens. However, additional data are not available to assess any risk they might present. Even if they were all non-carcinogens, the data generated in this laboratory contradict the conclusion from the literature review that shows the MLA has a false positive rate of up to 61%. In fact, the incidence of positive results from this laboratory is lower than, but more consistent with, those for pharmaceuticals in general. Of marketed drugs listed in the 2008 US Physicians' Desk Reference, only 20% (32/163) are reported to be

positive in the MLA although these excluded most cytotoxic anticancer and antiviral drugs (all with known mechanistic genotoxicity), steroids with class-specific genotoxicity and biological or peptide drugs (Snyder, 2009). A slightly higher incidence of MLA positives, 27% (28/104), was seen in all drugs submitted to the German regulatory authority (BfArM) during the 1990's and no specific drug types were omitted from this analysis (Muller and Kasper, 2000). It should be noted that in both the Physicians' Desk Reference and BfArM datasets the incidences of positive results in *in vitro* cytogenetics assays is directly comparable with those in the MLA. At least for pharmaceuticals, it appears that the MLA does not generate as many positive results as commonly believed. This should be taken into consideration when comparing the performance of other *in vitro* genotoxicity tests, and perhaps more importantly, it is against this incidence that the performance and validation of novel *in vitro* genotoxicity tests should be judged.

The reasons for the discrepancy seen between the incidence of positive results in the MLA at AstraZeneca and that in the literature are not clear but there appear to be several possibilities. First, the quality of particularly some of the older studies included in retrospective reviews can be questioned. A recent alternative review of the studies performed at the National Toxicology Programme (NTP) (Schisler et al., 2010), which includes a significant number of the results cited by Kirkland *et al*, in his review of 2005 (Kirkland et al., 2005) has shown that 211 of 342 results (61%) were uninterpretable due to inappropriate concentration selection or control responses. Hence in this re-analysis, only 74 of the original 163 positive calls were considered likely to be appropriate. This is not a censure of Kirkland's review; he and his colleagues accurately and diligently assessed the available published data from a variety of sources and evaluated the results with respect to negative data from carcinogenicity tests. However, it does highlight the difficulty with making conclusions from retrospective analyses. Second, and possibly contributing to the poor quality of some of the published data, is the provenance of the mouse lymphoma L5178Y cells used in different laboratories. Recent work has shown that the sensitivity of L5178Y cells to test agents as indicated by *in vitro* micronucleus induction can depend on the source. For example, the non-carcinogen anthranilic acid was found to be genotoxic when tested using L5178Y cells from an established commercial repository, but negative when tested using cells supplied by AstraZeneca (Pfuler *et al.,* 2011). The cells from AstraZeneca have been shown to have the appropriate karyotype and can be shown to have been grown for no more than 4 weeks in continuous culture from a sample

provided by Dr Don Clive over 30 years ago (see Chapter 2), but the provenance of cells used in many laboratories may not be so well known. Finally, it is possible that the incidence of positives with compounds in the chemical space occupied by potential drug substances differs from that with the compounds in the literature that have been tested for other reasons.

Even though the rate of positive responses in the MLA is somewhat lower than some published reviews suggest, it would still be advantageous to be able to predict MLA activity using *in silico* screening. Furthermore, it appears that some regulatory authorities may have requested MLA testing on impurities in drug substances on the basis of alerts in unspecified SAR systems (noted by the author from experience with AstraZeneca drug submissions). Consequently, results for the compounds tested in this laboratory were analysed using MC4PC and it was disappointing that its predictivity was such that, in its current state of development it cannot be considered sufficiently accurate to be used to predict the MLA activity of pharmaceuticals, their metabolites or potential impurities. The sensitivity of MC4PC was similar to that previously reported by Matthews and Contrera, approximately 60% to 70%, but the specificity (38%) and overall concordance (42%) were much lower than Matthews and Contrera's reported approximately 70% for both specificity and concordance (Matthews *et al.,* 2006; Contrera *et al.,* 2008). In fact, MC4PC predicted 62% of all compounds, whether actually positive or negative, to be positive. It is perhaps not surprising that *in silico* screening is not very predictive of the actual MLA result. It is likely that the SAR database used by MCASE to construct the MC4PC module contains the same seemingly flawed NTP dataset as reviewed by Kirkland *et al*. It is probably coincidental, but at the same time intriguing, that Kirkland's review suggested a positive rate of 61% in the MLA, and MCASE analysis of AstraZeneca compounds predicted a positive rate of 62%. Regulatory requests for further mammalian cell genotoxicity evaluation based on SAR analysis could lead to delays in drug development. It is clearly important to have proven that these analyses should at best be used with caution, and, in the author's opinion, should not be used at all until their predictivity for *in vitro* mammalian cell genotoxicity can be greatly improved.

The results of the analysis of the positive responses of AstraZeneca pharmaceuticals in the Ames assay were also very interesting. Only 10 out of the 355 compounds assessed were positive in the Ames test. This is in no small way a tribute to the success of genetic toxicology research over the last 3 decades. Pharmaceutical discovery chemists are no longer developing chemicals that are potently DNA reactive.

Furthermore, AstraZeneca uses an in house *in silico* SAR system that very effectively predicts Ames positives; hence they can be eliminated in early drug discovery before the requirement for 'wet testing'. Unlike trying to predict mammalian cell genotoxicity based on flawed databases, Ames SAR systems can accurately predict compounds that are likely to bind to DNA, hence predict whether a compound will be positive in the Ames test. It was also noteworthy that 9 out of 10 of the Ames positives could have been predicted as likely to be positive due to either their chemistry (2 out of the 10 positives were small aromatic amines) or their primary pharmacology (7 out of 10 positives were anti-infectives targeting bacterial topoisomerase II).

3.4.2 Could aneugenicity be the mechanism responsible for some of the uninterpretable positive findings seen in the MLA?

Whilst the review of the number of positive responses seen in the MLA during routine screening at AstraZeneca demonstrated that the assay generates far fewer unexplainable positives than may have been expected, there were still a significant number (5% of 355 compounds tested) for which the genotoxic mechanism of the positive response could not be readily explained. There are of course several mechanisms that may be responsible for the non-pharmacologically related positives seen in the MLA that do not involve direct drug covalent DNA binding, for which aneugenicity is one plausible example. However, the data generated clearly demonstrates that seven aneugens with different modes of aneugenic action cannot be routinely identified as positive in the MLA, with noscapine, diazepam and colchicine giving negative responses even when tested up to concentrations reducing survival to < 10%. Although some evidence of mutagenicity was seen with taxol, carbendazim, econazole and chloral hydrate, the increases were generally at levels of survival < 10% and it is likely that, if tested as unknown compounds, the majority of these results would not be considered positive using currently accepted mouse lymphoma assay evaluation criteria (Moore *et al.,* 2006) .

It should be noted that the results obtained with taxol, colchicine and chloral hydrate do not agree with the positive responses previously reported for these compounds (Harrington-Brock *et al.,* 1998; Honma *et al.,* 2001, Wang *et al.,* 2009). In the current study, toxicity above and below 10% survival was seen at the same concentrations of taxol in different experiments and this may account for the apparent disagreement between laboratories i.e. results were called positive or negative depending on whether

the increases were seen just above or below 10% survival. Furthermore, in the laboratory where taxol has been reported to be positive, consistent results were not obtained between experiments using different lots of test material (Dr Martha Moore, Division of Genetic and Molecular Toxicology, Federal Drug Administration, personal communication). For colchicine, concentration-related increases in mutant frequency from 0.01 to 0.1 µmol/L have been reported (Honma *et al.,* 2001) but, again, these were most apparent at concentrations giving < 20% survival. It is possible that the karyotype of cells used may also have influenced the response. The cells used by Honma's group contained a population of 2.7% with three copies of chromosome 11 (Honma *et al.,* 2001), but no such cells were seen in 600 metaphases in AstraZeneca's laboratory (data not shown). There are data to indicate that cells monosomic for chromosome 11, which would obviously result from loss of a single chromosome from a disomic, are not viable (Liechty *et al.,* 1998; Fellows *et al.,* 2005). However, it is theoretically possible that loss of a single chromosome 11b from a trisomic cell from Honma's laboratory (i.e. 11a,11a,11b) could give rise to a viable disomic TFT-resistant clone (i.e. 11a,11a -11b).

3.4.3 Could topoisomerase II inhibition be responsible for some of the uninterpretable positive findings seen in the MLA?

The data from the selection of aneugens tested suggested that it was unlikely that anuegenicity was a mechanism that could readily explain some of the positive responses seen in routine MLA screening. Given that 29 out of 40 (73%) of the S9 independent MLA positives in the data set were from projects with compounds designed to target topoisomerase II, kinase or DNA repair enzyme inhibition, it would seem at least possible that some of the unexplainable unique MLA positives may be caused by off target effects on these enzymes. As has previously been reviewed in the introduction to this thesis, inhibition of topoisomerase II enzymes in particular induces potent positive effects in *in vitro* mammalian cell gene mutation assays, and this mechanism was also likely to be responsible for 70% of the Ames positives seen out of the 355 compounds analysed. This highlights the value of establishing and evaluating the performance of practical screening assays for off target mammalian cell topoisomerase II inhibition and associated genotoxic potential. If better assays were available either to predict the potential of a novel compound to inhibit topoisomerase II or to help explain hitherto unknown mechanisms of *in vitro* mammalian cell genotoxicity, it may be possible to further reduce the number of unexplainable positives generated by *in vitro* mammalian cell gene mutation assays.

3.5 Conclusion

In conclusion, of 355 compounds tested in the MLA in this laboratory, only 52 (15%) gave positive results; even if it is assumed that all of these are non-carcinogens, the incidence of "false positive" predictions of carcinogenicity must be much lower than the 61% apparent from analysis of the literature. Furthermore, only 19 compounds (5% of total) were positive by a mechanism that could not be related to the compounds primary pharmacological activity or positive response in other genotoxicity assays.

It was considered unlikely that direct DNA reactivity or off target aneugenicity was responsible for generation of the MLA positive responses. Although not certain, it was considered likely that the positive results obtained with compounds known to inhibit topoisomerases, kinases or DNA repair was due to their pharmacology. Currently there are few practical screens for these activities and, if better tests were to be developed it is possible that the occurrence of inexplicable positive results in the MLA could be reduced further. The conclusions from this work were published by the author in two peer reviewed papers in 2011 (Appendix I)

Having put into perspective the prevalence of positive responses in *in vitro* mammalian cell gene mutation assays, the aim of the rest of this research project will focus on investigating methods to determine drug induced topoisomerase II inhibition and relate this to the drug's potential for *in vitro* genotoxicity.

CHAPTER 4

Use of the cell free decatenation assay to measure drug induced topoisomerase II inhibition and its potential as a screening and problem solving tool for AstraZeneca discovery pharmaceuticals

CONTENTS

4 Use of the cell free decatenation assay to measure drug induced topoisomerase II inhibition and its potential as a screening and problem solving tool for AstraZeneca discovery pharmaceuticals

4.1 Introduction

The aim of the work described in this chapter was to see if a cell free assay could be used as a screen for topoisomerase II inhibitor induced genotoxicity.

The simplest screening tools for analysis of topoisomerase II activity and it's inhibition by chemical agents are cell free techniques. These techniques are relatively high throughput, hence are ideal for early compound selection in pharmaceutical discovery. In light of this, AstraZeneca developed a modified version of the topoisomerase II decatenation assay. Decatenation assays have been routinely used for many years for assessment of both the activity of topoisomerase II enzymes and evaluation of a drug's potency to inhibit activity (Pan and Fisher, 1999). The principal of the assay relies on the ability of purified topoisomerase II enzyme to bind with circular DNA and decatenate in the presence of ATP. The substrate is a network of linked DNA circles (catanes) which are released after decatenation. For measurement of a compounds ability to inhibit topoisomerase II, catenated DNA is treated with concentration ranges of topoisomerase II inhibitors and or poisons in the presence of topoisomerase II enzyme and ATP. Products of this reaction are then run on electrophoresis gels, broken circlets migrate further though the gel than the original linked DNA. The amount of migrated broken DNA can then be measured, giving an indication of decatenation efficiency. Calculation of a compounds IC_{50} (i.e. the concentration of drug that induces a 50% inhibition in decatenation) is used as the comparator between the efficiency of individual drugs to inhibit topoisomerase II (Fisher and Pan, 2008). In the modified version of the assay developed at AstraZeneca, rather than running decatenated DNA through gels, the product is loaded onto filter wells. After filtering, catenated DNA is retained on the filter, only decatenated DNA will pass through (see Chapter 2 for further details). The advantage of this microwell technology is an improvement in both the ease of sampling, measurement and assay throughput.

The principal use of the decatenation assay at AstraZeneca was to assess novel bactericidal drugs for their efficiency to inhibit bacterial topoisomerase IV and/or gyrase. However, the assay is amenable to using mammalian topoisomerase II, hence has the potential to screen drugs for their cross activity against the mammalian enzyme. The initial aim of the work for this research project was to demonstrate the effectiveness of the assay to measure the mammalian topoisomerase II inhibition of etoposide and gemifloxacin. The work progressed to screen a range of AstraZeneca anti-infective drugs designed to target bacterial topoisomerase and measure their activity against the mammalian enzyme. The inhibitory potency of each drug was then compared to their genotoxic potency as measured in the semi-automated *in vitro* micronucleus assay using L5178Y mouse lymphoma cells. The ultimate aim of this work was to see if topoisomerase II induced genotoxicity could be predicted by a simple high throughput cell free test.

The secondary aim of this work was to see if the decatenation assay could be used to elucidate the mechanism of unexpected positive results in *in vitro* mammalian cell genotoxicity tests. To this end, the technique was used to measure the potential for topoisomerase II inhibition of compounds from a series of novel kinase inhibitors developed for an anti-inflammatory therapy. In the early development of these kinase inhibitors, as part of the standard AstraZeneca genotoxicity screening paradigm, compounds from the lead chemical series were initially evaluated for genotoxicity using the in house AstraZeneca Genetox Warning SAR System to predict bacterial mutagenicity; there were no alerts (data not shown). From this chemical series a lead candidate was discovered, AZ101 (Figure 4.6). This compound had excellent drug-like properties and consequently was advanced to *in vitro* genotoxic screens in the course of normal screening progression at AstraZeneca. AZ101 was found to be negative in the bacterial reverse mutation (Ames) test but unexpectedly positive in the mouse lymphoma assay (MLA) at all concentrations analysed (55 to 111 µmol/L) in the absence and presence of exogenous metabolism (Aroclor induced rat liver S9). This potent response was considered to be a significant risk to the development of AZ101 and consequently its progress was stopped.

In order to assess whether genotoxicity was inherent to the chemical series and to attempt to understand the mechanism involved, a series of further investigations were initiated. A number of additional compounds were selected for testing with varied substituents around the core isoquinolinone (Compounds AZ102-AZ107 Figure 4.7).

These investigations were to determine if minor structural changes could modulate the genotoxic activity or whether the core isoquinolinone was inherently linked to the positive MLA result. The MN(vit) was used as a surrogate for the MLA for these investigations. As the genotoxicity of AZ101 was independent of exogenous metabolism, the MN(vit) was performed only in the absence of S9.

With regard to the potential mechanism of the genotoxicity seen with this chemical series, a number of possibilities were considered but, given the absence of in silico warning and the negative Ames test result, it was thought unlikely that AZ101 was directly DNA reactive. Furthermore, although some kinase inhibitors are inherently genotoxic (e.g. compounds that target cell checkpoint kinases (Marzin, 2007)), AstraZeneca had previously developed molecules to target the same kinase as AZ101. Compounds with similar target potency but with different chemistry were not mammalian cell mutagens. Accordingly, the genotoxicity of AZ101 was not considered likely to be linked to its target pharmacology. A possible alternative mechanism was based on the slight structural similarity of parts of the chemistry to fluoroquinolone antibiotics which are known to target mammalian cell topoisomerase II (Smart *et al.,* 2008). Accordingly, the decatenation assay was used to screen these compounds for potential topoisomerase II inhibition.

4.2 Results and discussion

4.2.1 Range-finding for the decatenation assay with reference compounds

The results for IC_{50} decatenation determination for etoposide and gemifloxacin are presented in Figures 4.1 and 4. 2.

Concentration (µmol/L)	Inhibition %	IC_{50} (µmol/L)
200	102.89	3.84
100	98.52	
50	92.31	**Slope**
25	86.50	0.878
12.5	73.22	
6.25	59.60	
3.13	38.49	
1.56	29.86	
0.781	25.21	
0.391	17.83	

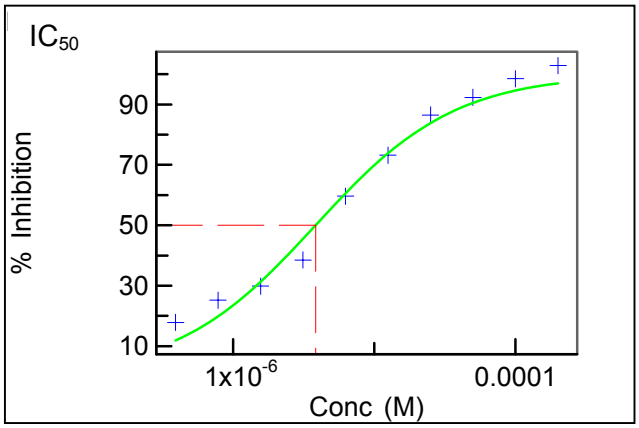


Figure 4.1. Etoposide decatenation results
Slope and graph derived from Activity Base calculation tool

Concentration (µmol/L)	Inhibition %	IC_{50} (µmol/L)
200	101.71	22
100	86.35	
50	76.47	**Slope**
25	52.42	1.302
1.25	26.93	
6.25	22.05	
3.13	7.19	
1.56	2.48	
0.781	5.24	
0.391	19.91	

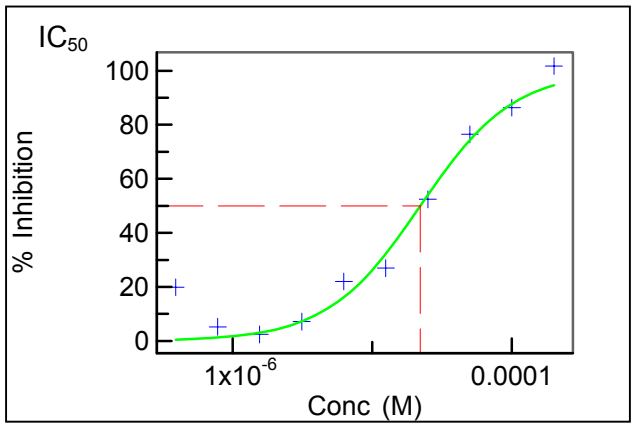


Figure 4.2. Gemifloxacin decatenation results
Slope and graph derived from Activity Base calculation tool

Prior to the commencement of this research project, etoposide had been tested in the decatenation assay during which an IC_{50} of 6 µmol/L was generated. Furthermore, the decatenation assay with gemifloxacin was repeated and an IC_{50} of 19 was obtained. Accordingly the IC_{50}s for etoposide and gemifloxacin were estimated to be approximately 4-6 and 19-22 µmol/L, respectively. As expected, the decatenation IC_{50} for etoposide was significantly higher than the LOGEL in a variety of *in vitro* mammalian cell genotoxicity assays (see Introduction Section 1.3.6.1 and Introduction Table 1.3). LOGEL of gemifloxacin was not available from the literature, hence a similar preliminary comparison could not be made. However, the basic drug induced genotoxic predictivity of the decatenation assay was correct in as much as the decatenation IC_{50} for the potent mutagen etoposide was four-fold lower than the decatenation IC_{50} for the less potent mutagen gemifloxacin. Accordingly, whilst the decatenation assay did not seem to possess the potential for quantitatively predicting *in vitro* mammalian cell genotoxic potency, there was a suggestion that it may be useful for qualitatively ranking compounds i.e. the lowest decatenation IC_{50} equating to the highest genotoxic potency.

4.2.2 The decatenation assay validation with AstraZeneca compounds design to target bacterial topoisomerase II

To further investigate the genotoxic predictivity of the decatenation assay it was used to predict the genotoxic potential in the MN(vit) for a series of compounds from AstraZeneca's infection portfolio. The primary target for all of these compounds was bacterial topoisomerase II. In all, 40 AstraZeneca early discovery compounds were tested. Each of the compounds had a similar basic core chemical structure but with minor changes to the chemistry of their side chains. Due to patent and intellectual property issues the structures of these compounds cannot be disclosed. The results of the semi-automated MN(vit) 'no observable genotoxic effect level' (NOGEL) and decatenation assay IC_{50} for the 40 AstraZeneca infection discovery compounds plus etoposide and gemifloxacin are presented in Table 4.1. In the MN(vit), these compounds were tested up to a maximum concentration of 400 µmol/L, or the compounds solubility limit in tissue culture media. In the decatenation assay they were tested up to a maximum concentration of 200 µmol/L. It was hoped that the relationship between decatenation IC_{50} and NOGEL was such that a low IC_{50} could be used to accurately predict a positive response in the MN(vit). It should be noted that

for this analysis, NOGEL was used rather than LOGEL. This was because some of the compounds tested were negative in the MN(vit), hence LOGEL could not be calculated.

Compound	MN(vit) Result	MN(vit) NOGEL (µmol/L)	Decatenation IC_{50} (µmol/L)
AZ1	Negative	≥400	>200
AZ2	Negative	≥400	>200
AZ3	Positive	100	>200
AZ4	Positive	200	>200
AZ5	Positive	100	>200
AZ6	Negative	100[1]	>200
AZ7	Negative	≥400	184
AZ8	Positive	12	170
AZ9	Negative	≥400	116
AZ10	Negative	≥400	112
AZ11	Negative	≥400	105
AZ12	Positive	<50	72.9
AZ13	Positive	<50	59.8
AZ14	Positive	50	56.8
AZ15	Negative	≥400	41.6
AZ16	Positive	<50	35.6
AZ17	Positive	50	32.2
AZ18	Positive	200	30.1
AZ19	Positive	< 100	26.9
AZ20	Negative	200[1]	25.1
AZ21	Negative	200[1]	23.2
AZ22	Negative	≥400	23.0
Gemifloxacin	Positive	10	19.0-22.0
AZ23	Positive	<50	17.6
AZ24	Positive	<50	15.6
AZ25	Positive	12.5	15.1
AZ26	Positive	50	14.3
AZ27	Positive	100	14.1
AZ28	Positive	100	12.4
AZ29	Positive	200	11.3
AZ30	Positive	50	8.7
AZ31	Positive	<50	8.0
AZ32	Negative	200[1]	7.8
AZ33	Positive	<50	6.4
AZ34	Negative	≥400	6.3
Etoposide	Positive	0.01	4.0-6.0
AZ35	Positive	<50	5.7
AZ36	Positive	6	3.5
AZ37	Positive	<50	3.3
AZ38	Positive	<100	3.0
AZ39	Positive	<50	2.0
AZ40	Positive	<50	1.4

Table 4.1. MN(vit) NOGEL and cell free decatenation IC_{50} results for AstraZeneca pharmaceuticals designed to target bacterial topoisomerase II.
Compounds are ranked in order of descending IC_{50}
[1] Highest concentration determined by solubility in tissue culture media
Maximum 400 and 200 µmol/L tested in Mn(vit) and decatenation assay, respectively

Even without the use of statistical analysis Table 4.1 shows that there was clearly a relationship between low decatenation IC_{50} and a positive response in the MN(vit). This relationships is represented in the scatter plot in Figure 4.3, which shows a reasonable clustering of positive MN(vit) responses associated with low decatenation IC_{50}.

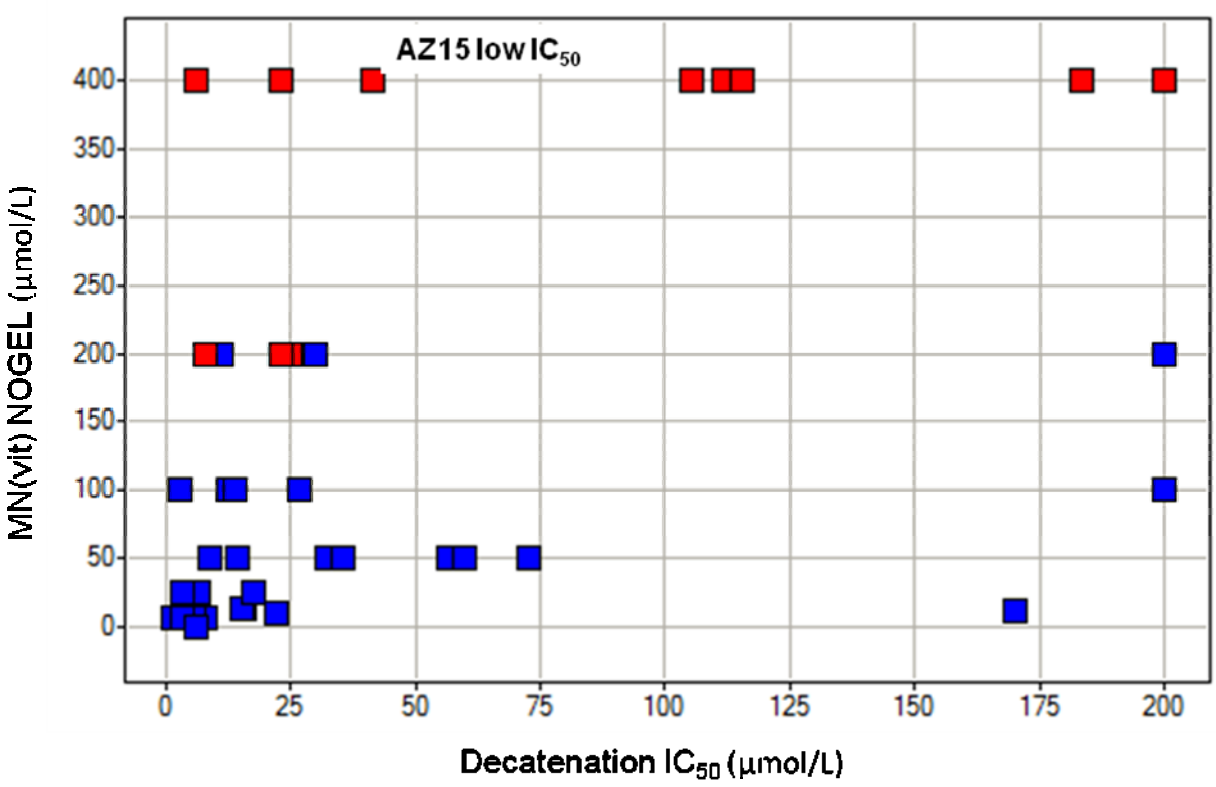


Figure 4.3. Spotfire scatter plot of MN(vit) NOGEL against cell free decatenation IC_{50} results for AstraZeneca pharmaceuticals designed to target bacterial topoisomerase II. Each square representing compounds AZ1-AZ40
Red square negative MN(vit), blue square positive MN(vit)

If the decatenation assay had potential for use as a predictive screen for genotoxicity, it would be important to establish an IC_{50} cut-off value below which with some assurance a compound could be considered positive and above which, with similar assurance, a compound could be considered to be negative. With the acceptance that it was a fairly arbitrary cut-off, based on the generated data, if an IC_{50} for decatenation of 20 µmol/L or below was considered to be indicative of a positive response in the MN(vit), 18 out of 20 compounds would have been correctly predicted as positive. However, it should be noted that with this cut-off the potent genotoxicant gemifloxacin with an IC_{50} of 19-22 would be a pretty borderline positive prediction. Unfortunately the negative prediction

would have been less impressive, with only 13 out of 22 compounds with a decatenation IC_{50} of above 20 µmol/L being correctly predicted as negative in the MN(vit). Furthermore, using this cut off the prediction of a negative MN(vit) included compounds AZ20, AZ21 and AZ22, which were all reasonably potent decatenation inhibitors (IC_{50} 20 to 25 µmol/L) i.e. pretty similar to the IC_{50} of gemifloxacin. Three compounds (AZ3, AZ4 and AZ5) were also positive in the MN(vit), albeit with a relatively high NOGEL (≥ 100 µmol/L), but did not demonstrate inhibition of decatenation at 200 µmol/L. It was of course possible that these compounds did inhibit decatenation at higher concentrations. Furthermore, it was perhaps surprising that such a low IC_{50} (IC_{50} < 20 µmol/L) was needed to be achieved before there was anything like an acceptable correlation for a positive response in the MN(vit). It is logical to assume that any reduction in decatenation IC_{50} is indicative of inhibition of topoisomerase II, but why was it that only the very potent decatenation inhibitors could be predicted with reasonable accuracy to be positive in the MN(vit)?

Clearly, the major difference between the MN(vit) and decatenation assay was that the MN(vit) used whole cells and the decatenation assay used naked DNA circles. It may have been that the lack of clear predictivity was due to differences in cellular uptake of the drugs tested. It would seem plausible that potent decatenation inhibitors that are not readily taken up by cells would be negative in the MN(vit), similarly weak decatenation inhibitors that are very readily absorbed may be more potent mutagens than could be predicted from a cell free assay. Accordingly, it was feasible that the predictivity of the decatenation assay could be improved if it was combined with an assessment of cellular uptake. Using a similar cell free scenario, the simplest method to estimate cell membrane partitioning was by measuring a compound's lipophilicity. Furthermore, there is precedence to suggest that lipophilicity may play a role in the potency of topoisomerase II inhibitors with respect to the genotoxicity of the bisdoxopiperazines ICRF187 and ICRF193. These are both topoisomerase II inhibitors with similar potencies against the enzyme. However, the more lipophilic ICRF193 is a far more potent mammalian cell mutagen (See Introduction Table 1.3; Andoh and Ishida, 1998). Lipophilicity may be calculated by assessment of the partitioning coefficient ClogP, which is the log of the ratio of the compounds solubility in octanol compared to water. The higher the ClogP the more lipophilic the compound and the more likely it will be absorbed by biological membranes. Using the example of the bisdoxopiperazines; the ClogP of ICRF187 is -2.65 (Drug Bank, 2011), whereas the

ClogP of the more potently genotoxic ICRF193 is -0.56 (Chem Spider, 2011). Whilst it was accepted that ClogP is an imperfect measure of cellular uptake (octanol : water partition coefficients cannot completely predict cell membrane : water partition coefficients) it's calculation is still routinely used as a preliminary screening tool for cell partitioning (Bergstrom et al., 2013). Accordingly, if cell permeability was a limiting factor for a compound's inherent genotoxicity there would be expected to be a reasonable correlation between the lipophilicity of the positive decatenation inhibitors and a positive response in the MN(vit). Hence, as a surrogate for uptake, the lipophilicity of the 40 AstraZeneca drugs was assessed by calculating ClogP. The correlation between ClogP and decatenation is shown in Figure 4.4 and the correlation between ClogP and genotoxicity is shown in Figure 4.5.

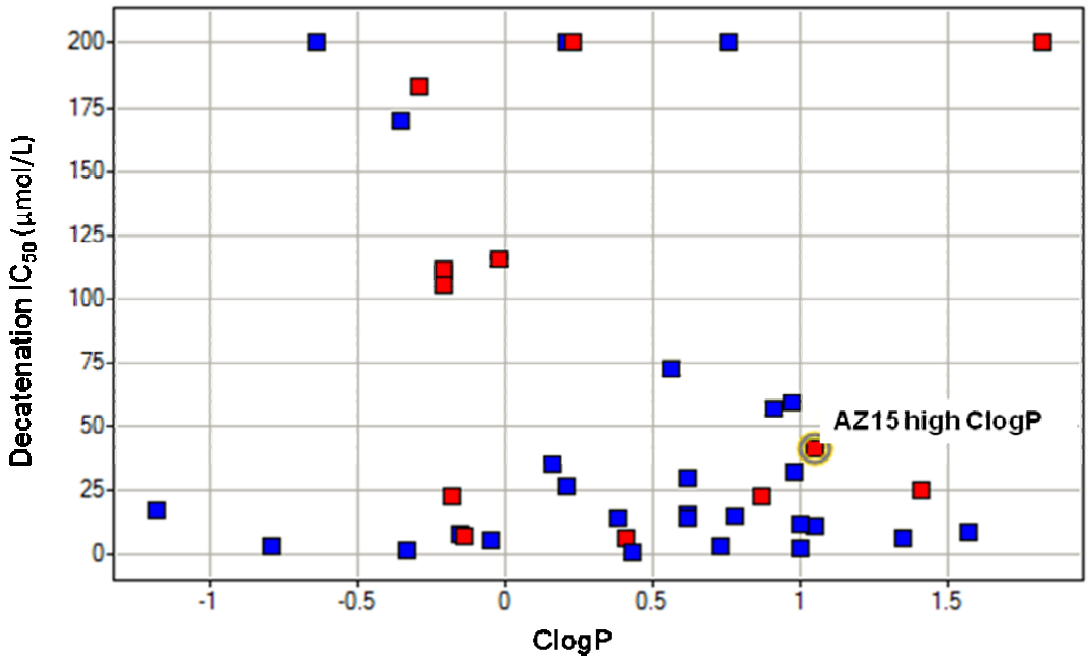


Figure 4.4. Spotfire scatter plot of cell free decatenation IC_{50} against ClogP (calculated ratio octanol to water solubility) results for AstraZeneca pharmaceuticals designed to target bacterial topoisomerase II. Each square representing compounds AZ1-AZ40 Red square negative MN(vit), blue square positive MN(vit)

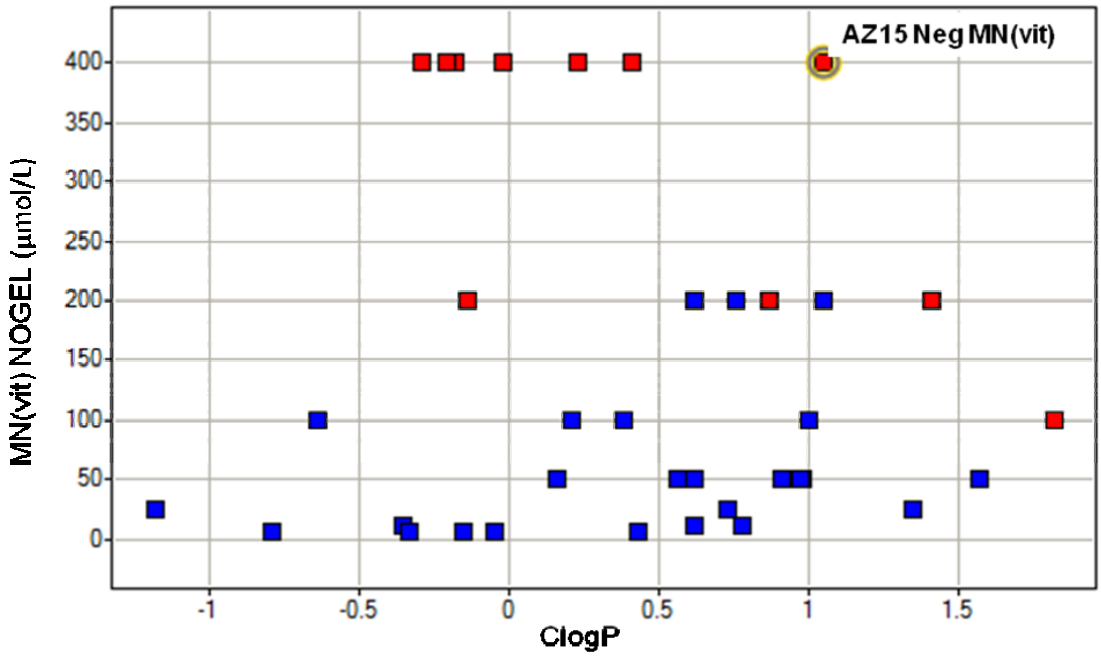


Figure 4.5. Spotfire scatter plot of MN(vit) NOGEL against ClogP (calculated ratio octanol to water solubility) results for AstraZeneca pharmaceuticals designed to target bacterial topoisomerase II. Each square representing compounds AZ1-AZ40. Red square negative MN(vit), blue square positive MN(vit)

Unsurprisingly, there was no relationship between ClogP and a compounds ability to decatenate naked DNA circlets (Figure 4.4). However, perhaps more surprisingly, there was no better correlation between ClogP and potency in the MN(vit) as determined by NOGEL (Figure 4.5). Accordingly, these plots clearly show that there was no relationship between ClogP and genotoxicity. For example AZ15 had high ClogP (> 1) hence would be considered likely to freely pass through cell membranes, AZ15 was also a reasonably potent decatenator with an IC_{50} of 42 µmol/L. However, AZ15 was negative in the MN(vit) even when tested in a freely soluble state in tissue culture medium up to 400 µmol/L, indicating that the compounds lipophilicity and hence presumed intra-cellular availability may not be a determining factor for its *in vitro* mammalian cell genotoxic response. It should not be considered that the ClogP work definitively confirms or refutes the contribution cellular uptake has to the genotoxicity of some of the compounds investigated. However, it has shown that the predictivity of the decatenation assay could not be improved by including this simple estimate of partitioning as an additional determining factor.

4.2.3 The decatenation assay used in an attempt to elucidate the mechanism of genotoxicity from a series of AstraZeneca kinase inhibitors

During the initial genotoxicity screening at AstraZeneca, the lead compound (AZ101) from this series was shown to be a mammalian cell specific metabolic activation independent mutagen, in that it was negative in the Ames test but positive in the MLA in the presence and absence of S9. These original data are shown in Tables 4.2 and 4.3.

	AZ101 Ames Test -S9 Revertants/plate					AZ101 Ames Test +S9 Revertants/plate				
Conc (µg/plate)	TA1535	TA1537	TA98	TA100	E.coli *uvrA*/pKM101	TA1535	TA1537	TA98	TA100	E.coli *uvrA*/pKM101
0	17	4	21	112	151	17	13	34	112	151
16	14	2	NT	125	149	14	11	NT	125	149
50	18	1	NT	148	142	18	12	NT	148	142
160	14to	4to	18	125to	130	14to	9to	20	125to	130
300	NT	NT	31	NT	NT	NT	NT	24	NT	NT
500	14to	2to	20	133to	131to	14to	10to	36	133to	131to
900	NT	NT	25	NT	NT	NT	NT	30	NT	NT
1600	12to	6to	25to	73to	104to	12to	4to	32to	73to	104to
5000	0to	0to	0to	0to	32to	0to	0to	0to	0to	32to
Pos Cnt[1]	385	12	284	496	901	385	232	932	496	901

Table 4.2. AZ101 original Ames test results obtained as part of AstraZeneca standard genotoxicity screening strategy

Pos Cnt[1]: Positive controls in the absence of S9; TA1535 Sodium azide 0.5 µg/plate, TA1537 9-Aminoacridine.HCl 50 µg/plate, TA98 2-Nitrofluorene 0.5 µg/plate, TA100 Sodium azide 0.5 µg/plate, E.coli *uvrA*/pKM101 Potassium dichromate 25 µg/plate

Positive controls in the presence of S9; TA1535, TA1537, TA98 and TA100 2-Aminoanthracene 2 µg/plate, E.coli *uvrA*/pKM101 2-Aminoanthracene 20 µg/plate.

NT = Not Tested. to = Toxicity seen as indicated by a reduction in background lawn

No significant increase seen (doubling over background)

AZ101 MLA -S9				AZ101 MLA +S9			
Conc (µmol/L)	RTG	MF	Small clone MF (%)	Conc (µmol/L)	RTG	MF	Small clone MF (%)
0	100	92	40 (45)	0	100	74	44 (60)
55	58	433+	320 (79)	55	11	1313+	772 (68)
67	26	898+	560 (72)	67	2	1658+	1355 (84)
86	9	1366+	927 (77)				
110	2	1720+	1432 (86)				
4-NQO 10	39	658+	357 (63)	CPA 1	37	835+	328 (50)

Table 4.3. AZ101 Test 1 original MLA results obtained as part of AstraZeneca standard genotoxicity screening strategy

RTG = Relative Total Growth

MF = Mutant Frequency x 10^{-6}

4-NQO = 4-nitroquinoline-N-oxide

CPA= cyclophosphamide

+ MF significantly different from the control (Induced MF ≥ 126 x 10^{-6})

In view of this highly potent response in the MLA (up to 20-fold increase in mutant frequency), AZ101 was dropped as a candidate drug. The search was then on to see if the chemistry of AZ101 could be modified to find a drug with similar drug target efficacy but without the genotoxic liability and to elucidate the mechanism of the observed genotoxic response. As the MLA has a high compound requirement and takes up to 4 weeks before results are available, the higher throughput semi-automated MN(vit) was proposed as the genotoxicity screening tool for this series. Initially AZ101 was

tested in a comparative MLA/MN(vit) test to ensure the genotoxic response was similar in the two assays. The data from this test are shown in Table 4.4.

AZ101 MLA/MN(Vit) -S9				
Conc (µmol/L)	**RTG**	**MF**	**Small clone MF (%)**	**MN per 1000 cells**
0	100	91	64 (72)	1.9
3.7	88	130	77 (62)	4.2
5.8	100	96	60 (64)	4.2
9.0	77	135	56 (44)	7.0
14	79	159	98 (64)	7.3
22	76	211	118 (60)	11.0*
27	58	378+	258 (74)	30.8***
34	26	1037+	636 (72)	57.8***
43	9	2038+	1271 (75)	99.8***
55	2	1507+	672 (49)	78.5***
64	0	1427+	689 (50)	55.6***
4-NQO 10	35	1153+	476 (57)	71.8***

Table 4.4. AZ1 Test 2 MLA and MN(vit) results
RTG = Relative Total Growth
MF = Mutant Frequency x 10^{-6}
4-NQO = 4-nitroquinoline-N-oxide
+ MF significantly different from the control (Induced MF ≥ 126 x 10^{-6})
*,**,*** Increase in micronuclei outside historical control range and statistically significant ($p<0.05$, 0.01, 0.001)

These data confirmed that AZ101 was positive in the MLA and semi-automated MN(vit) over similar concentration ranges. Accordingly, the semi-automated MN(vit) was used for the further screening of candidate drugs from this project. As the positive MLA result with AZ101 was surprising in that the compound had no genotoxic alerts and the drug target was not considered likely to be responsible, all regions of the molecule were initially considered for substructure variation and subsequent testing for genotoxicity. Regions of the molecule were delineated as regions 1-4 (Figure 4.6).

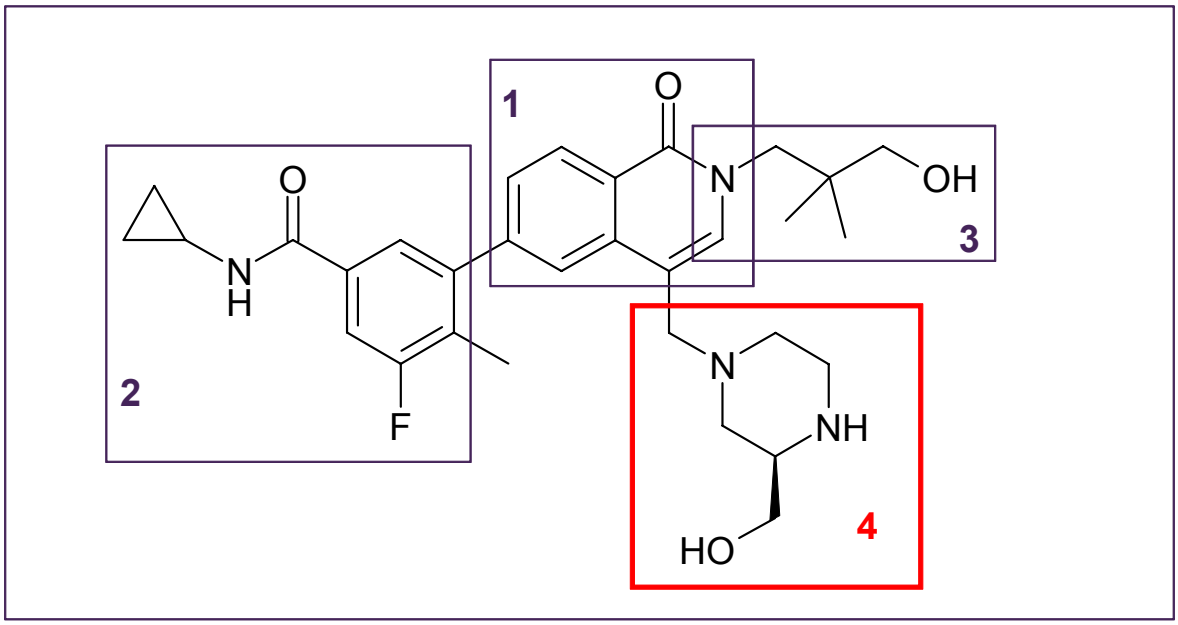


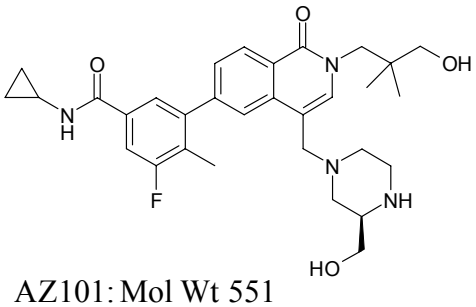
Figure. 4.6: AZ101 structural chemistry.

Region 1 isoquinolinone core. Regions 2 and 3 provided kinase potency. Region 4 di-amine side chain, focus of structural changes

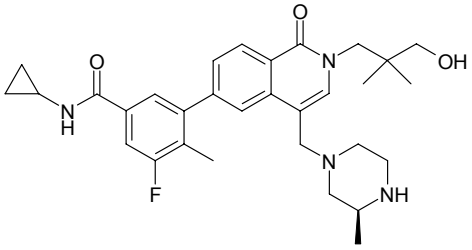
However, given the high value of the potent and novel core structure (region 1) and the difficulty of maintaining primary biological activity through such scaffold hopping (e.g. changes to region 1), the initial focus was to elucidate whether the genotoxicity could be modulated by small variation in the peripheral side chain structures (regions 2-4). Given additional constraints provided by the kinase potency structure activity relationships within regions 2 and 3 (data not shown), the di-amine side chain in region 4 was viewed as the most profitable area for preliminary investigation, and the results of these changes are detailed herein. Di-amine analogues of AZ101 were prepared with structural changes that retained the approximate distance between the two nitrogen atoms but modulated basicity, shape, size and the steric and chiral environment around either of the nitrogen atoms. The molecular core (i.e. the isoquinolinone) was relatively flat, and the di-amine substructure provided an ideal opportunity to instil additional 3D substructural atoms perpendicular to this plane to disrupt potential intercalation into narrow grooves and at other biological targets. The consideration being that genotoxicity may have been driven by DNA intercalation and related topoisomerase II inhibition. Such changes to the structure could ameliorate this potential. Although at this stage this approach was highly speculative it was considered useful because off-target mammalian cell topoisomerase II inhibition was

thought to be one of the potential effects that could have been responsible for such potent mammalian cell genotoxicity in the absence of a response in bacterial cells. Furthermore, preliminary molecular modelling performed by AstraZeneca R&D also indicated that AZ101 might bind to the minor groove of DNA. The fluoroquinolone gyrase targeting antibiotic norfloxacin is known to bind to DNA in this way (Ma *et al.,* 2005). Although the molecular modelling provided several orientations of the flat core entering the groove, a precise docking mode could not be found hence this work was used only as a potential insight into mechanism rather than providing definitive data on the genotoxic mode of action of the kinase inhibitor series (data are not shown).

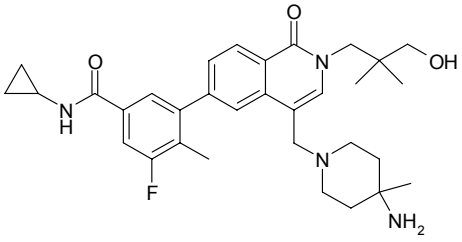
The chemistry changes around region 4 were suggested by and made in association with AstraZeneca Pharmaceutical Research and Development Chemists Tim Luker and Anne Cooper. A new series of potential candidates was generated, each of which were screened in the semi-automated MN(vit). Furthermore, confirmatory MLA tests were performed on the initial candidates and on candidates when a negative response was seen in the MN(vit). The structures of the new candidate drugs and their response in the semi-automated MN(vit) and the MLA are shown in Figure 4.7.



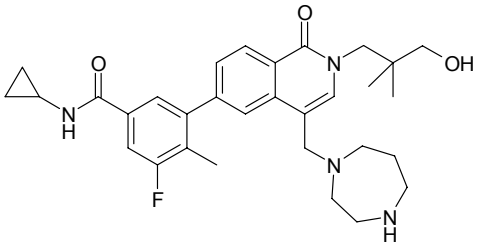
AZ101: Mol Wt 551
Negative Ames Test. Positive MLA and MN(vit)



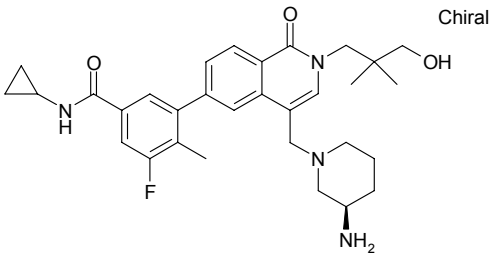
AZ102: Mol Wt 535
Positive MLA and MN(vit)



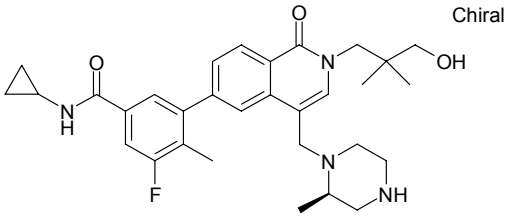
AZ103: Mol Wt 549
Positive MLA and MN(vit)



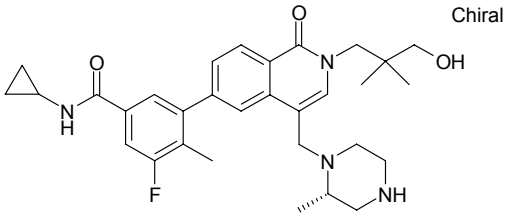
AZ104: Mol Wt 535
Positive MN(vit). Not tested MLA



AZ105: Mol Wt 535
Positive MN(vit). Not tested MLA



AZ106: Mol Wt 535
Negative MLA and MN(vit)



AZ107: Mol Wt 535
Negative MLA and MN(vit)

Figure 4.7: Structures, molecular weights and genotoxicity test results of the kinase inhibitors tested

These results demonstrated that with relatively minor changes to the chemistry around region 4, the genotoxicity of this chemical series could be modified. However, given that structural modelling work did not provide clear insight into potential DNA docking of these molecules the mechanism of genotoxicity was still not proven. Hence the decatenation assay was used to assess the potential of both the positive and negative compounds from this series to inhibit mammalian cell topoisomerase II. The results of the decatenation assay, along with the target kinase efficacy (kindly provided Tim Luker and Anne Cooper, AstraZeneca Pharmaceutical Research and Development) and the LOGEL for each of the kinase inhibitors investigated is shown in Table 4.5.

AstraZeneca Compound	**Kinase IC_{50} (nmol/L)**	**Decatenation IC_{50} µmmol/L**	**MN(vit) LOGEL µmol/L**
AZ101	0.16	125	22
AZ102	0.79	43	< 17
AZ103	0.13	33	< 17
AZ104	0.32	>200	< 26
AZ105	0.79	>200	21
AZ106	0.25	>200	Negative in MN(vit)
AZ107	0.79	>200	Negative in MN(vit)
Etoposide	NT	6	< 0.01

Table 4.5. IC_{50} for target kinase and DNA decatenation and LOGEL for a positive response in the MN(vit) of kinase inhibitors
LOGEL = Lowest observable genotoxic effect level in the MN(vit)
NT = Not Tested

As expected, the genotoxicity was not related to the compounds target kinase activity. For example, AZ102 which was potently positive in the MN(vit) had the same kinase IC_{50} as AZ107 which was negative in the MN(vit). The relationship between cell free decatenation and genotoxicity was more puzzling. As may have been expected if topoisomerase II inhibition was the mechanism of genotoxicity, the two compounds that were negative in the MN(vit) (AZ106 and AZ107) demonstrated no ability to inhibit mammalian topoisomerase II in the cell free assay up to the maximum concentration analysed (200 µmol/L). Furthermore, three of the MN(vit) positive compounds (AZ101, AZ102 and AZ103) did show some evidence of topoisomerase II inhibition with

decatenation seen at concentrations ranging between 33 and 125 µmol/L. However, the IC_{50} for decatenation was at least two-fold higher than the LOGEL in the MN(vit). Furthermore, the other two equally potent MN(vit) positive compounds (AZ104 and AZ105) did not show any ability to decatenate when tested up to 200 µmol/L. Accordingly, use of this simple cell free system could not clearly establish whether inhibition of topoisomerase II was responsible for the genotoxicity of these kinase inhibitors.

4.3 Conclusion

The validation work with compounds from AstraZeneca infection portfolio did show that highly potent inhibitors of cell free DNA decatenation (IC_{50} < 20 µmol/L) were associated with positive responses in the MN(vit). Accordingly, this simple cell free system did have some potential as an early screening tool for associated genotoxicity. However, the lack of correlation between reasonably potent decatenation inhibitors (IC_{50} 20 to 100 µmol/L) and genotoxicity demonstrated that the cell free system was a far from perfect tool.

Whilst there were MN(vit) positives from the screened infection compounds that did not decatenate (AZ3, AZ4 and AZ5), unlike the kinase inhibitors AZ104 and AZ105, these were all relatively weak mutagens (NOGEL ≥ 100 µmol/L). Accordingly whilst the decatenation work with the kinase inhibitors gave a tantalising indication that topoisomerase II inhibition may have been at least partly responsible for the genotoxicity of some of the compounds tested, it was far from proven. The conclusions from this work were published by the author in 2012 (Appendix 1).

The decatenation assay has some potential as an early screen for genotoxicity associated with mammalian cell topoisomerase II inhibition and hinted at the likely genotoxic mechanism of the kinase inhibitors investigated. However, because of the lack of correlation between relatively potent decatenation inhibitors from AstraZeneca infection portfolio compounds and positive responses in the MN(vit), because of the difference in dosimetry between decatenation inhibition and MN(vit) LOGEL/NOGEL and because two of the five positive kinase inhibitors did not inhibit decatenation, there is clearly a requirement for a more sensitive assay to be able to definitively screen for mammalian cell topoisomerase II inhibition and associated genotoxicity. The following part of this research project investigated whether cellular assays held a better prospect

for accurate detection of topoisomerase II inhibition and whether these assays could be used either as screening tools and/or to elucidate genotoxic mechanisms.

CHAPTER 5

Assessment of the genotoxicity of topoisomerase poisons II and cellular methods to determine topoisomerase II inhibition in L5178Y mouse lymphoma cells

CONTENTS

5 Assessment of the genotoxicity of topoisomerase poisons and cellular methods to determine topoisomerase II inhibition in L5178Y mouse lymphoma cells

5.1 Introduction

The preliminary aims of the work described in this chapter were two-fold.

1. To assess the lowest observed genotoxic level (LOGEL) for compounds whose primary mode of topoisomerase II poisoning was by stabilisation of the topoisomerase II/DNA cleavage complex. Gemifloxacin and etoposide were selected for this assessment. The drugs were tested in the *in vitro* micronucleus test (MN(vit)) and in the mouse lymphoma assay (MLA) using L5178Y mouse lymphoma cells, and the LOGEL was calculated for each assay. As a reference, the LOGEL for the DNA reactive mutagen 4-nitroquinolone-1-oxide (4-NQO) was also evaluated. 4-NQO is metabolised to the carcinogenic and mutagenic metabolite 4-acetoxyaminoquinoline 1-oxide, which forms guanine adducts leading to base substitution mainly by guanine to adenine transitions (Galiègue-Zouitina *et al.,* 1986, Fronza *et al.,* 1992). However, there is also data to suggest that when tested *in vitro* a proportion of the observed mammalian cell genotoxicity is by induction of reactive oxygen species (ROS), 4-NQO is known to induce oxidative adducts (Bailleul *et al.,* 1989). There are no published data to indicate that 4-NQO directly poisons topoisomerase II.

2. The second aim was to assess the performance of published methods for the detection of topoisomerase II poisoning in mammalian cells. At present, the most sensitive assays available for estimation of drug induced stabilised cleavage complex are considered to be the TARDIS and ICE (Cowell *et al.,* 2011). These both have advantages and disadvantages. The TARDIS assay is relatively easy to perform so multiple concentrations can be tested. However, sensitivity may be compromised by the unwound state of the analysed DNA and the background fluorescence on the agar slide (Lynch *et al.,* 2003). The ICE assay overcomes these issues by analysis of isolated DNA. However, the methodology is quite complicated, requiring overnight caesium chloride gradient

ultra-centrifugation, hence only a limited number of samples can be analysed from any given test (Subramanian *et al.,* 2001). It would clearly be useful to be able to improve either or both the sensitivity and throughput of these assays. The overall intention of this work being to confirm whether these methods are capable of identifying chemically induced topoisomerase II inhibition at concentrations approximately equal to or below the calculated LOGEL in this laboratory for etoposide and gemifloxacin. Hence demonstrating beyond reasonable doubt that topoisomerase II inhibition is the only mode of genotoxic action of these chemicals. Where appropriate, as a comparator, the response of 4-NQO was also assessed in the relevant assays.

L5178Y mouse lymphoma cells were selected for this work because this cell line is probably the most common mammalian cell line used in pharmaceutical *in vitro* genotoxicity safety assessment (OECD 1997; ICHS(R1), 2011) and was the cell line used for the *in vitro* mammalian cell genotoxicity work that was reviewed in Chapter 3 and the MN(vit) decatenation assay comparative work detailed in Chapter 4. Use of this cell line in the MN(vit) is common and studies using L5178Y cells in the MN(vit) have been widely published, including by the author (Fellows *et al.,* 2008, Fellows *et al.,* 2010). The author and co-workers at AstraZeneca UK developed a semi-automated version of the MN(vit) using MetaSystems' Metafer 4, comprising of a Zeiss Axioplan Imager Z1, (Doherty *et al.,* 2011). This system identifies micronuclei, which were then confirmed by eye to be acceptable to score i.e. were separate from the nucleus and within the cytoplasm, to have intact cytoplasmic membrane and to be less than one third of the diameter of the nucleus (Fenech, 2000). Furthermore, L5178Y mouse lymphoma clone 3.7.2C has been demonstrated to be mutant at both *Typ53* alleles hence either has no functional p53 or any p53 is mutant (Clark *et al.,* 2004). Whilst L5178Y cells are known to be capable of apoptosis (Velasco *et al.,* 2007) it is considered that because of the presence of the mutant *Typ53* gene, p53 mediated programmed cell death would not be a principal response to DNA damage in this cell line. Accordingly, the generation of mutation and specifically micronucleus induction should directly reflect the level of DNA damage hence making L5178Y mouse lymphoma cells particularly suitable for comparative investigations into LOGEL (Lynch *et al.,* 2003).

5.2 Results and discussion

In all tests triplicate cultures were scored.

5.2.1 Assessment of LOGEL using the MN(vit)

Initial experiments to assess LOGEL used the MN(vit) with Metafer semi-automated scoring. The results of micronucleus induction and associated cytotoxicity as measured by relative population doubling (RPD) for etoposide and gemifloxacin are presented in Figures 5.1 and 5.2, respectively.

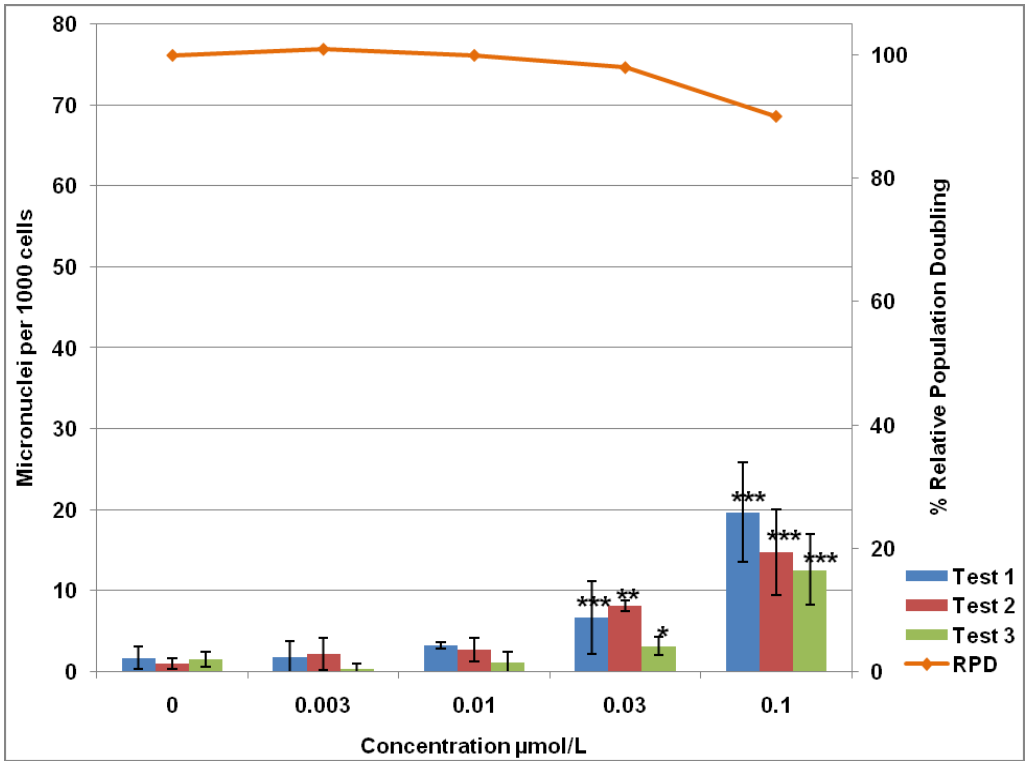


Figure 5.1: Results from three Metafer scored MN(vit) tests with etoposide to determine LOGEL

n per test = 3. Statistics: continuity adjusted Chi-squared test. Significance Level: *P<0.05 **P<0.01 ***P<0.001

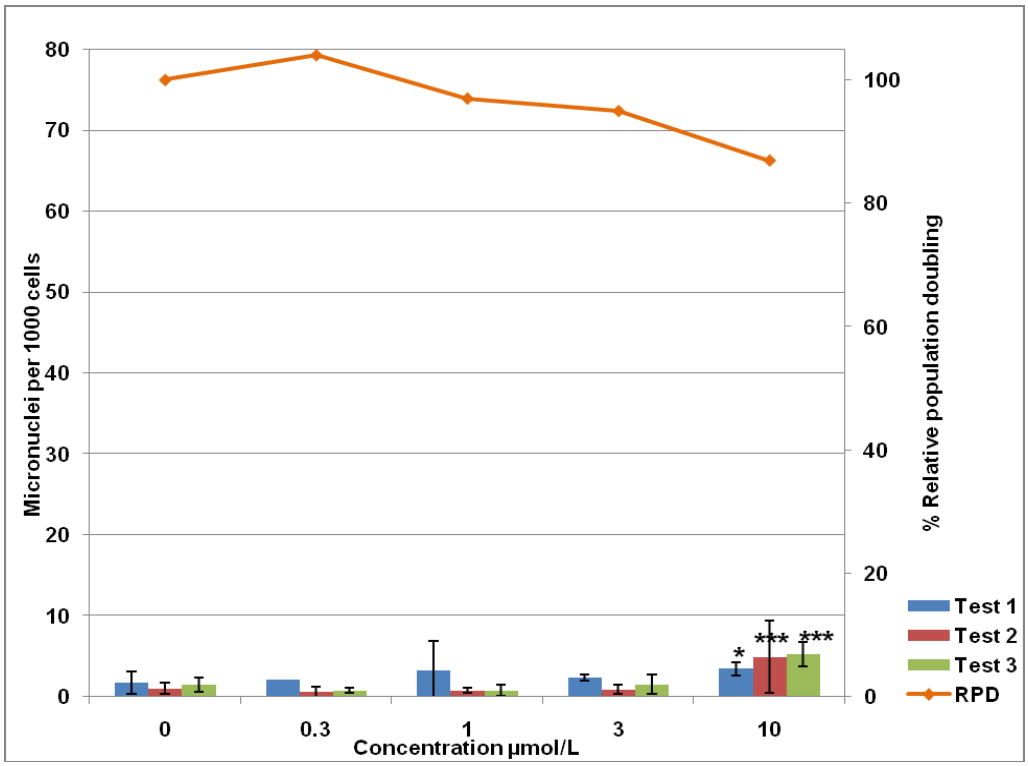


Figure 5.2: Results from three Metafer scored MN(vit) tests with gemifloxacin to determine LOGEL

n per test = 3. Statistics: continuity adjusted Chi-squared test. Significance Level: *P<0.05 **P<0.01 ***P<0.001

Over the concentration ranges analysed using the Metafer MN(vit) semi-automated scoring system, a statistically significant LOGEL for etoposide was established to be **0.03 µmol/L** and a statistically significant LOGEL for gemifloxacin was established to be **10 µmol/L**. However, the increases in mutant frequency seen at these concentrations were small and were close to the limits of the observed historical solvent control range for the Metafer MN(vit) in this laboratory, which was 0 to 4 micronuclei per 1000 mononucleated cells. Accordingly, to confirm these LOGELs and to ensure the Metafer analysis system was providing an appropriate level of sensitivity, further experiments were performed with manual microscope analysis of micronucleus induction. The results of duplicate confirmatory tests are presented in Figures 5.3 and 5.4.

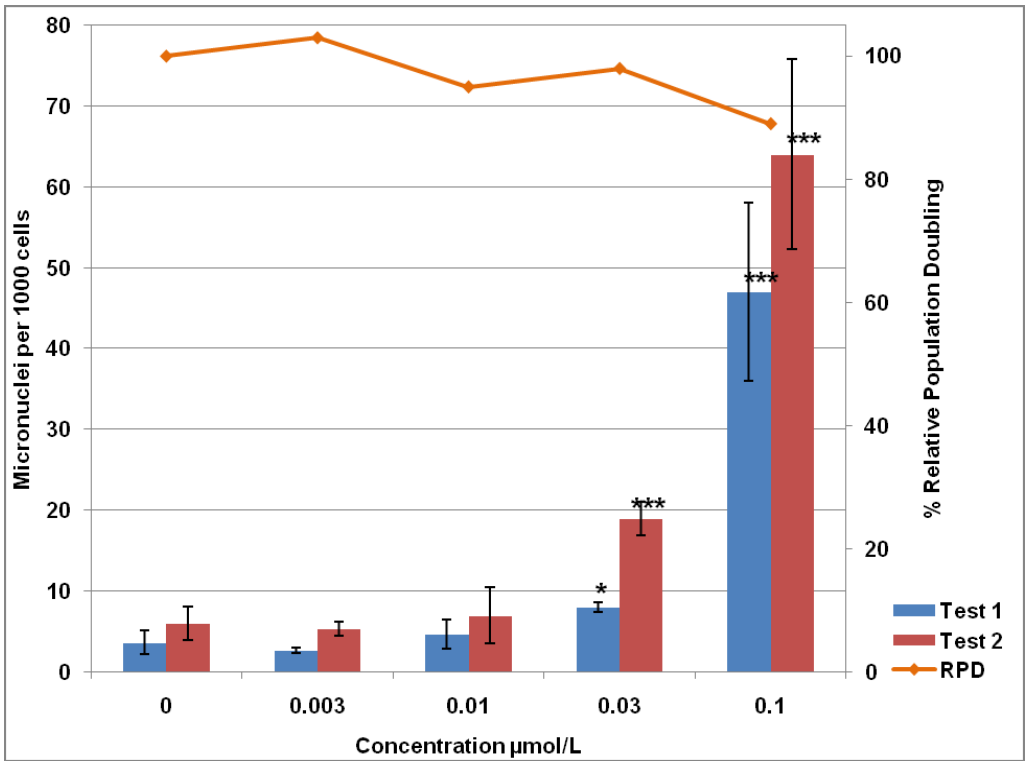


Figure 5.3: Results from two manually scored MN(vit) tests with etoposide to determine LOGEL

n per test = 3. Statistics: continuity adjusted Chi-squared test. Significance Level: *P<0.05 **P<0.01 ***P<0.001

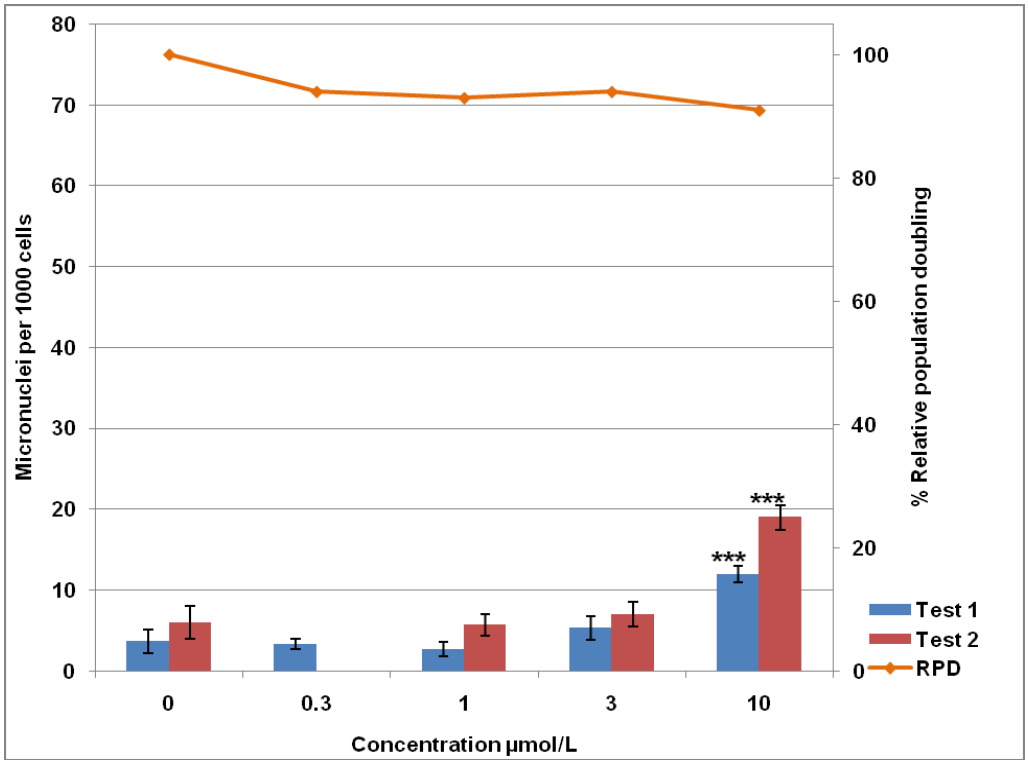


Figure 5.4: Results from two manually scored MN(vit) tests with gemifloxacin to determine LOGEL

n per test = 3. Statistics: continuity adjusted Chi-squared test. Significance Level: *P<0.05 **P<0.01 ***P<0.001

Scoring manually did identify more micronuclei per 1000 mononucleated cells than using the semi-automated scoring system. However, between the two systems, the increase was generally proportional across the tested concentration ranges and controls. Accordingly, the statistically significant LOGELs for etoposide and gemifloxacin were confirmed to be **0.03 µmol/L** and **10 µmol/L**, respectively. For both drugs, statistically significant increases in micronuclei were seen at essentially non-cytotoxic concentrations as determined by decrease in RPD. Interestingly, the confirmed LOGEL for etoposide was some 5-fold lower than values from the published literature (Ashby *et al.,* 1994, Lynch *et al.,* 2003), and demonstrated what a potent *in vitro* mutagen etoposide is.

Although no quantitative difference was seen between the LOGEL as determined by Metafer and manually, the fact that more micronuclei were identified by scoring by eye did suggest that the automated scoring system was less accurate at micronucleus detection. Accordingly all subsequent MN(vit) tests used manual scoring. The precise reason why Metafer underscored micronuclei was not clear. However, during manual scoring it was observed that micronuclei often appear in clumps of cells, possibly because these cells are damaged hence become more 'sticky'. The Metafer system only scores cells that are clearly single, hence may miss the increased number of micronucleated cells in more clustered regions of the slide.

The results of LOGEL assessment for the reference genotoxin 4-NQO are presented in Figure 5.5.

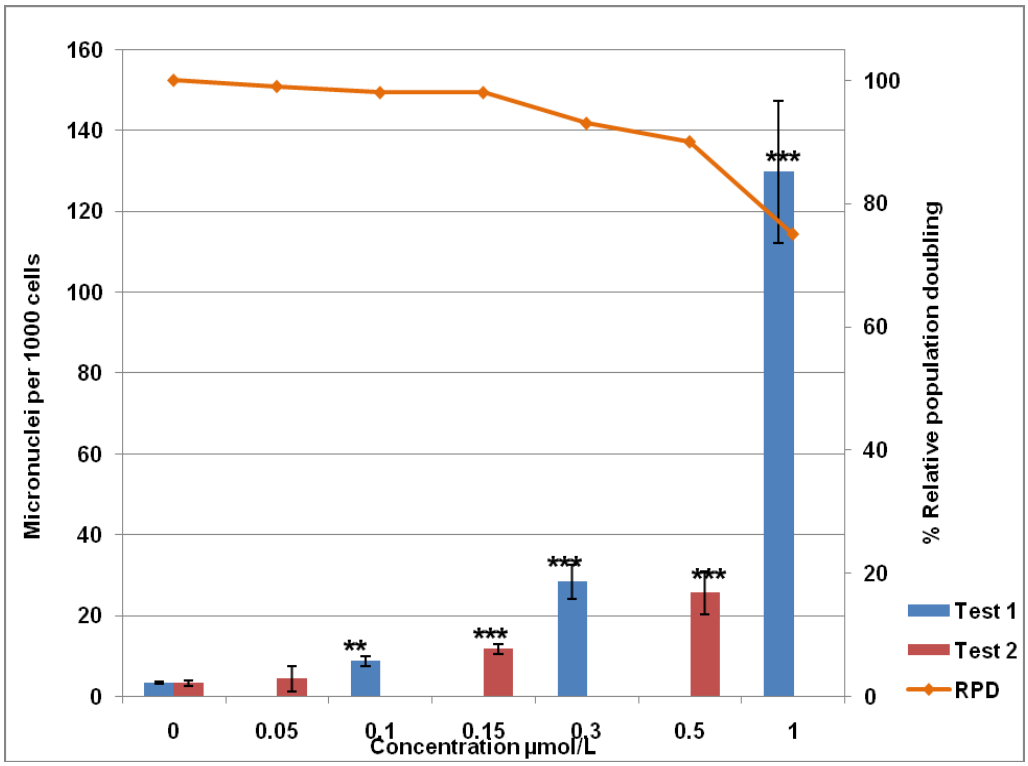


Figure 5.5: Results for two manually scored MN(vit) tests with 4-NQO to determine LOGEL
n per test = 3. Statistics: continuity adjusted Chi-squared test. Significance Level: *P<0.05 **P<0.01 ***P<0.001

The statistically significant LOGEL for 4-NQO was **0.1 µmol/L**.

5.2.2 Confirmation of the calculated LOGEL of etoposide and gemifloxacin using the MLA

Whilst clastogenicity is the most likely mechanism of topoisomerase II poison induced genotoxicity, it is possible that inefficient repair by non-homologous end-joining of topoisomerase inhibitor induced DNA lesions may generate point mutations and small intragenic deletions that do not form micronuclei. Hence the LOGEL of etoposide and gemifloxacin was also assessed in the MLA which detects a wider spectrum of DNA damage (Moore *et al.,* 1985). The results from a single confirmatory MLA experiments with etoposide and gemifloxacin are presented in Figures 5.6 and 5.7. It should be noted that for the MLA relative total growth (RTG) rather than RPD was used as the measure of cytotoxicity.

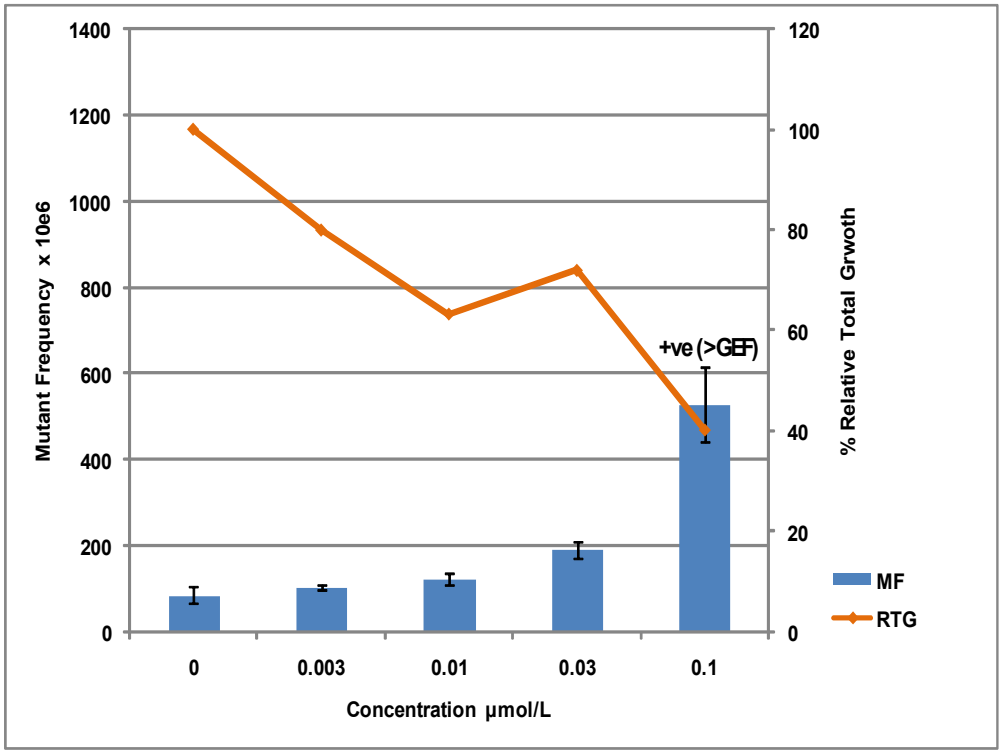


Figure 5.6: Results from a single MLA test with etoposide to determine LOGEL
n = 2. +ve Significant by global evaluation factor (GEF)

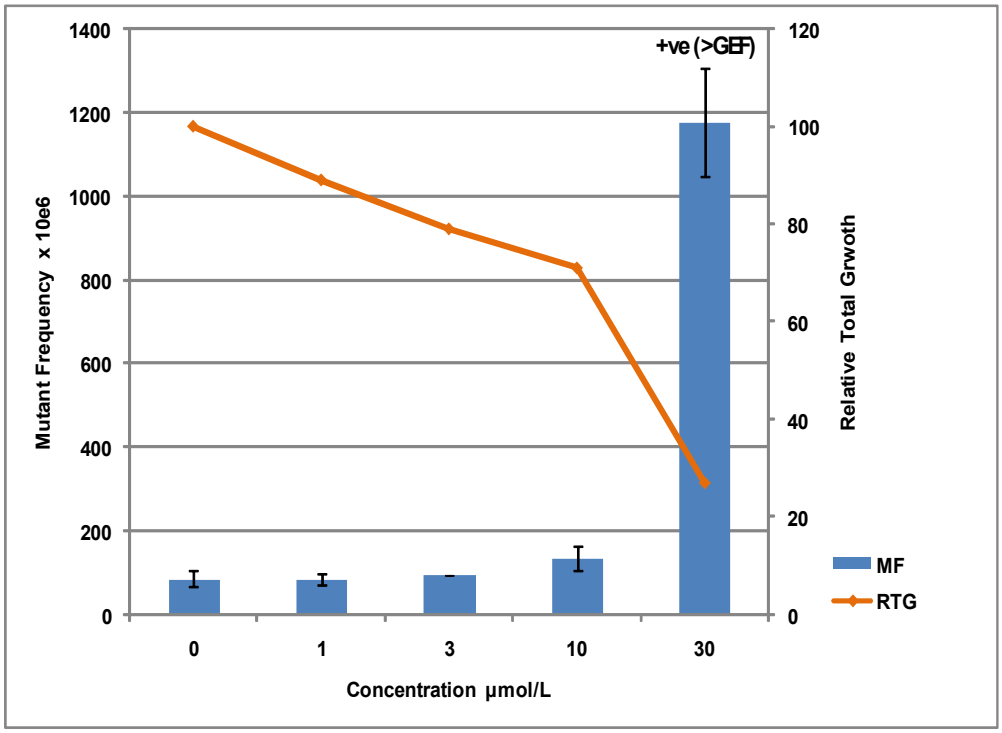


Figure 5.7: Results from a single MLA test with gemifloxacin to determine LOGEL
n = 2. +ve Significant by global evaluation factor (GEF)

Although the original intention of testing etoposide and gemifloxacin in the MLA was to see if the wider spectrum of mutation identified in this assay type gave a lower LOGEL than had been seen in the MN(vit), the LOGEL in the MLA was actually higher, with

etoposide and gemifloxacin giving MLA LOGELs of 0.1 µmol/L and 30 µmol/L, respectively. However, it should be noted that at 0.03 µmol/L etoposide and 10 µmol/L gemifloxacin, increases in mutant frequency were seen in the MLA, but they were not sufficient to be considered positive when evaluated using the global evaluation factor (GEF). The requirement of an increase in mutant frequency of at least 126 x 10^{-6} for a positive response by GEF is a high bar to achieve. Smaller increases in mutant frequency are likely to be statistically significant. It is also possible that the slight shift in LOGEL between the two assay types is because the MLA will only identify mutants in viable cells i.e. TFT resistant cells need to form viable clones before they can be scored in the MLA. Chromosome breakage as identified by the MN(vit) may be a lethal event and hence dead or dying cells may be scored. This hypothesis is supported by the observation that both etoposide and gemifloxacin have a delayed toxic effect, as demonstrated by the toxicity seen as measured by RPD (which only reflects growth for one day) compared to the toxicity seen as measured by RTG (which reflects two days growth and subsequent plating to determine cloning efficiency). The top concentrations tested of both etoposide and gemifloxacin would appear to be essentially non-cytotoxic as measured by RPD (Figures 5.1 to 5.4), but reduce RTG to 40% and 27% of concurrent control, respectively, indicating that these concentrations are highly cytotoxic (Figures 5.6 and 5.7).

Whilst intuitively it would seem logical to use the MLA LOGEL in viable cells as the comparator for assessment of the contribution of topoisomerase II inhibition to the genotoxicity of etoposide and gemifloxacin, it should be realised that both TARDIS and ICE measure stabilised cleavage complex formation immediately after treatment. Hence both of these assays will measure stabilised cleavage complex formation in viable, dying and dead cells. Accordingly, the LOGEL in the MN(vit) was considered to be the more appropriate comparator.

5.2.3 The TARDIS assay to measure induction of topoisomerase II/DNA stabilised cleavage complexes

Following the establishment of accurate LOGELs for etoposide and gemifloxacin, the next requirement was to estimate the contribution topoisomerase II/DNA stabilised cleavage complex formation makes to the observed genotoxicity of these drugs. To this end, etoposide and gemifloxacin were tested in the TARDIS assay using L5178Y mouse lymphoma cells to establish lowest observable cleavage complex formation effect levels (LOCCEL). Figure 5.8 shows the increase in FITC signal from antibody

labelled stabilised cleavable complexes following treatment with solvent control and 100 µmol/L etoposide.

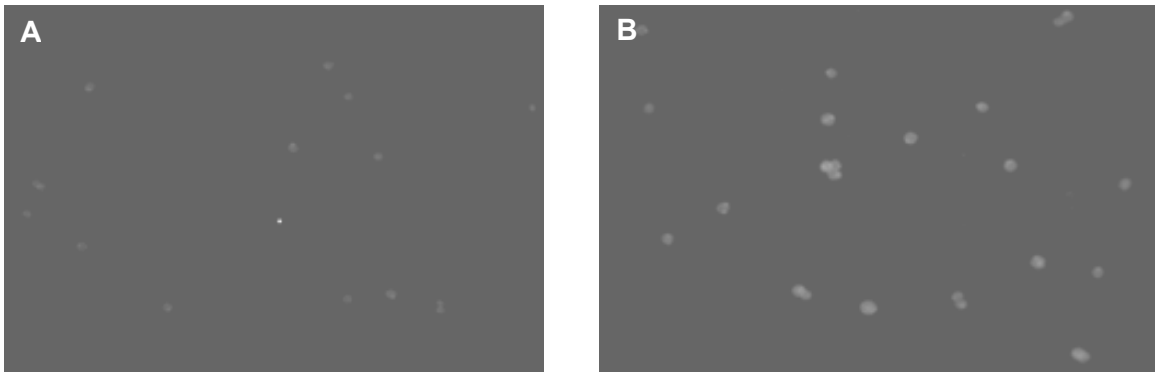


Figure 5.8: Images of analysed TARDIS slides. A: vehicle control. B: 100 µmol/L etoposide following 3 hours exposure

The results from TARDIS assays with etoposide and gemifloxacin are shown in Figures 5.9 and 5.10. Triplicate cultures were tested.

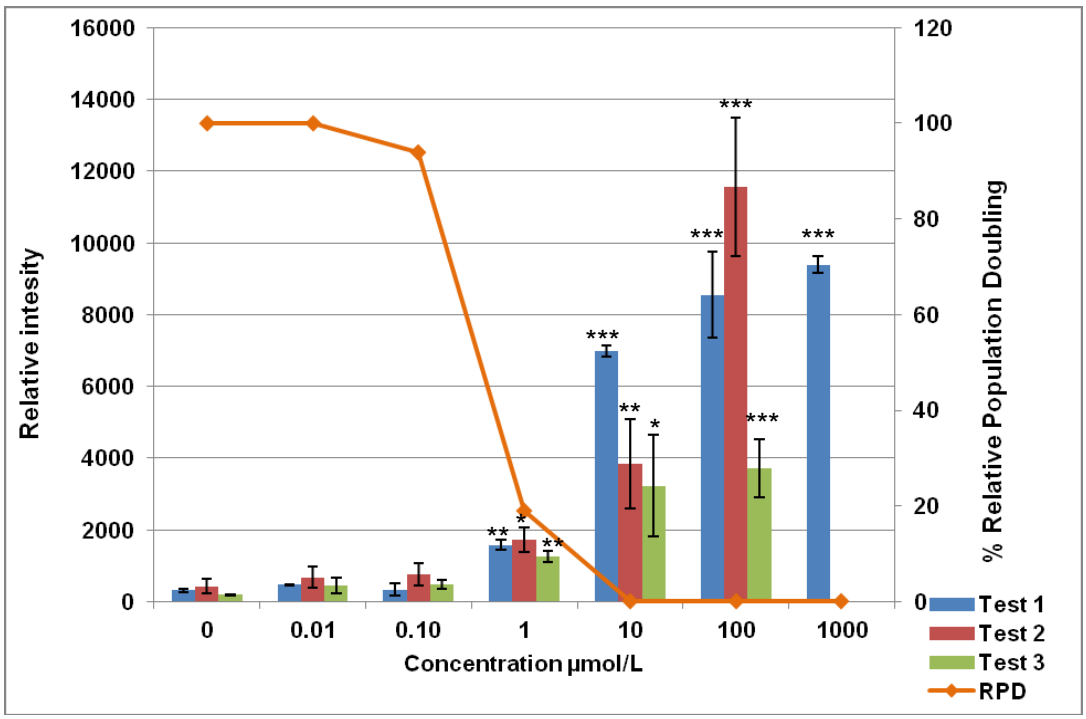


Figure 5.9: Median results from three TARDIS tests with etoposide to determine LOCCEL
Relative intensity = FITC signal from analysed nuclei
n per test = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

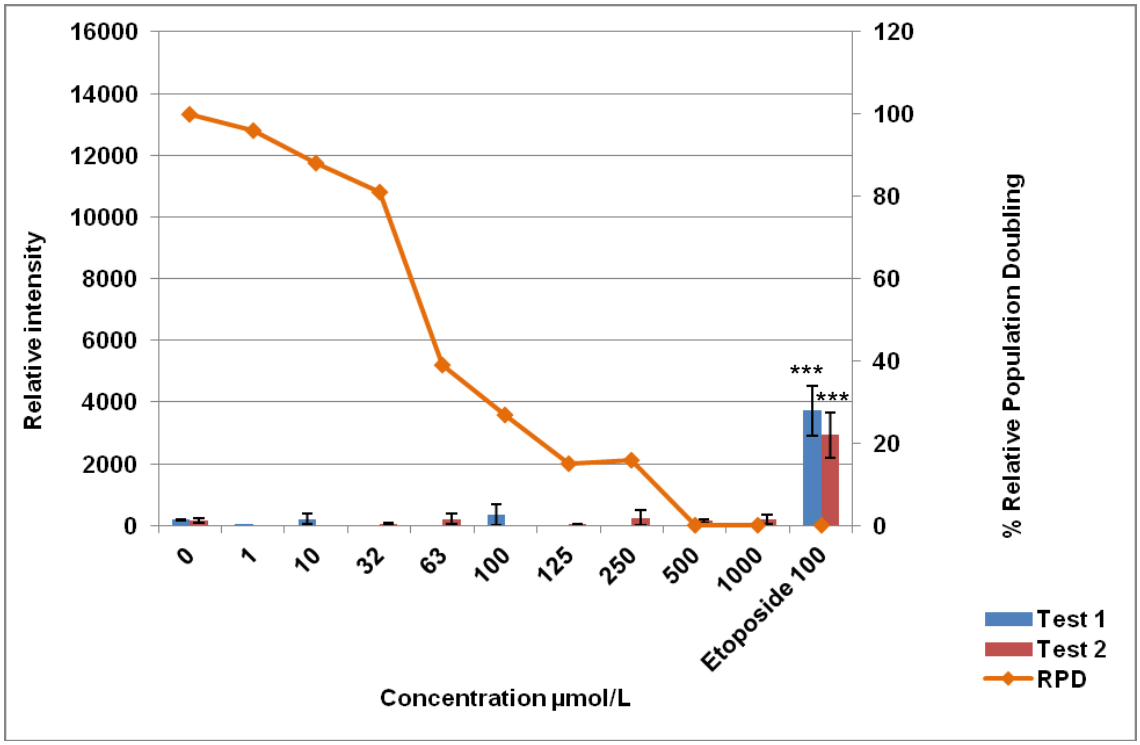


Figure 5.10: Median results from two TARDIS tests with gemifloxacin to determine LOCCEL
Relative intensity = FITC signal from analysed nuclei
n per test = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

A concentration-related increase in the amount of stabilised cleavage complex formation was seen with etoposide. However, very high concentrations of the drug were required before a statistically significant increase in FITC signal was seen and all of the concentrations where a statistically significant increase was seen were highly cytotoxic. An etoposide LOCCEL was established to be **1 µmol/L,** which was in agreement with published data from the TARDIS assay with other cell lines (Wilmore *et al.,* 1998). RPD at this concentration was less than 20%, which would indicate this population is not viable (RPD of less than 45% is considered to be excessively cytotoxic (OECD, 2010). The three higher concentrations where an increase in stabilised cleavage complex formation was seen gave no survival as measured by RPD. Accordingly, whilst a LOCCEL of 1 µmol/L was seen following etoposide treatment in the TARDIS assay, stabilised cleavable complex formation could not be seen in cells considered likely to be viable.

When gemifloxacin was tested in two TARDIS tests no significant increase in the formation of stabilised cleavage complexes was seen even though gemifloxacin was

tested up to concentrations giving zero survival. In both of these tests a high concentration of etoposide (100 µmol/L) did give statistically significant increases in stabilised cleavage complexes. The antibody used for the TARDIS work was a mixed topoisomerase IIα and β antibody, hence the lack of response of gemifloxacin could not be explained by any isoform specificity. The gemifloxacin response was also in general agreement with work at Newcastle University, where only a small increase in stabilised cleavage complexes was seen following exposure to very high (over 1 mmol/L) and probably lethal concentrations of gemifloxacin (Rance *et al.,* 2010). As there was clearly no response with gemifloxacin in duplicate tests, further testing using the same methodology was not considered to be warranted. Additional TARDIS assays with gemifloxacin are discussed in Chapter 7.

5.2.4 The ICE assay to measure induction of topoisomerase II/DNA stabilised cleavage complexes

The TARDIS assay evaluates cleavage complex formation in nuclei held in agar. It has previously been noted that the background fluorescence seen in this assay may compromise its sensitivity (Lynch *et al.,* 2003). It is also possible that the DNA organisation in nuclei held in agar is such that the available epitope for antibody binding to DNA bound topoisomerase II is limited; hence further compromising the sensitivity of the assay. The fact that TARDIS utilises a high salt concentration wash (1 M NaCl) to remove unbound topoisomerase II shortly before antibody application may contribute to this. Such high salt concentrations will mean DNA adopts a more compact and interwound state (Schlick *et al.,* 1994). The ICE assay does not have the same limitation as it uses isolated DNA to investigate topoisomerase II binding.

5.2.4.1 ICE assay method development

The initial ICE assay development work presented in this section (5.2.4.1) was validated and performed in collaboration with Martin Tran from the Karolinska Institute, Stockholm, Sweden.

The preliminary ICE assays were performed using four caesium chloride densities for DNA separation (1.37, 1.50, 1.72 and 1.82 g/mL). Cell lysate from a treated L5178Y culture was carefully placed on top of the gradients, which were then centrifuged for 18 hours at 31000 rpm ($1x10^5$ *g*). 200 µL aliquots were removed from the top of each tube. The first fraction was discarded and subsequent fractions were saved and analysed spectrophotometrically at 260 nm to assess content of DNA. DNA was

generally found in fractions between approximately 13-24. These fractions were used subsequently for measurement of topoisomerase II bound stabilised cleavage complexes.

Preliminary work was performed to investigate the qualitative response of topoisomerase IIα and β primary antibodies to detect etoposide induced stabilised cleavage complexes after 3 hours exposure. Figure 5.11 shows representative data.

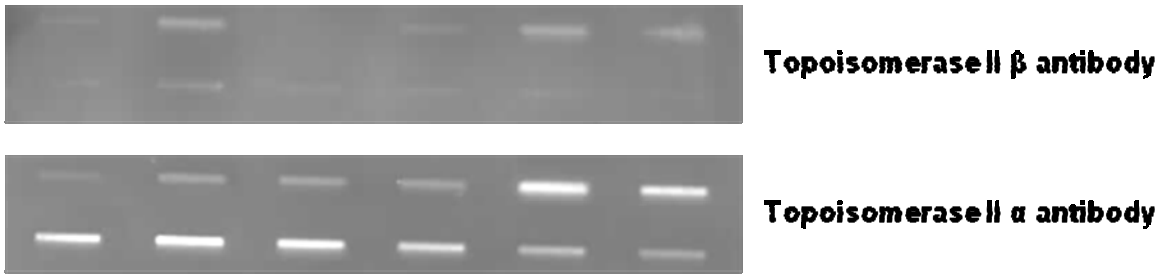


Figure 5.11: Formation of topoisomerase II α and β stabilised cleavage complexes from L5178Y cells exposed to 100 µmol/L etoposide for 3 hours then lysed. Each slot represents a fraction taken from caesium chloride gradient. Fractions 13 (top left) through to fraction 24 (bottom right)

This work clearly showed that topoisomerase IIα stabilised cleavage complexes were more prevalent than topoisomerase IIβ complexes following etoposide treatment. As this was in agreement with previously published data (Wilmore *et al.,* 1998), only topoisomerase IIα antibody was used for later ICE assays.

In initial tests, DNA was separated from etoposide treated cultures using the method described above. Samples from the appropriate fractions were slot-blot loaded onto a nitrocellulose membrane, antibody exposed and imaged using ECL™ (enzyme-linked chemiluminescence). Figure 5.12 shows representative data from an early tests.

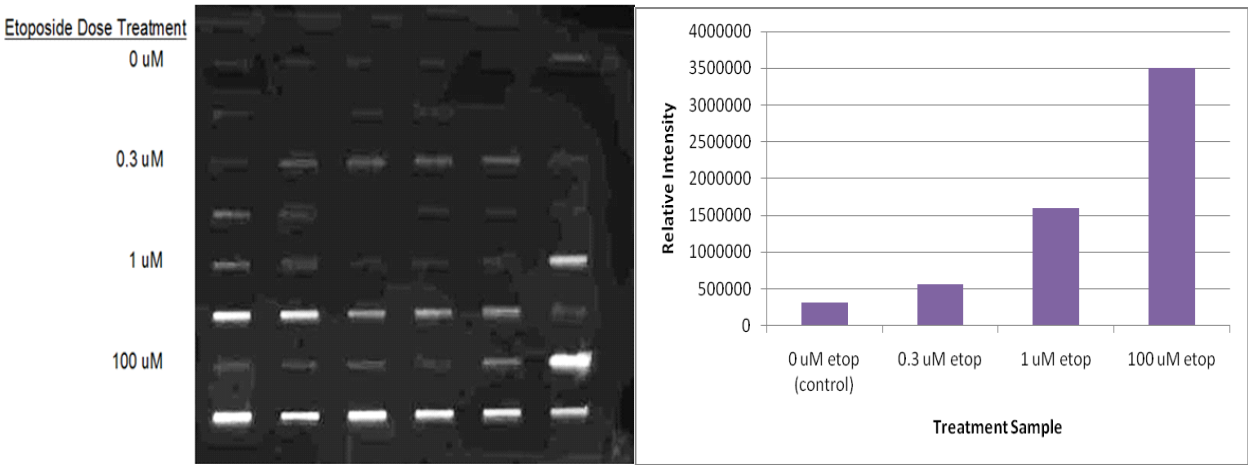


Figure 5.12: Image on left is topoisomerase II α ECL imaged caesium chloride fractions 13-24 of L5178Y cells exposure to 0, 0.3, 1 and 100 µmol/L etoposide for 3 hours then lysed. Bar chart on right is combined chemiluminescence signal for all 12 slots for each concentration

The data represented in Figure 5.12 proved difficult to repeat. Not least because of the technical difficulties with preparation of caesium chloride density gradients. Furthermore, preparation and analysis of 24 aliquots was time consuming and this technique did not allow for the improvement of inter-assay reproducibility by normalisation of DNA content between experiments. Accordingly, later tests used a modified DNA separation technique in which a single 1.5 g/mL density of caesium chloride was used, as described in Hawtin *et al.,* 2010. This technique meant that unbound protein was retained in or above the caesium chloride layer and DNA and DNA bound protein pelleted to the bottom of the tube. This could then be resuspended in Tris-EDTA buffer. Figure 5.15 shows an example of DNA and protein content of the subsequent DNA solution prepared using this modified method. For all experiments using this technique where absorbance of the resuspended DNA pellet was measured at 260 nm and 280 nm, the ratio between A_{260}/A_{280} was always between 1.7 and 2.0, indicating that the purity of the DNA extracted was of a high quality (Maciver, 2012). DNA was also normalised between tests such that ~10 µg DNA was loaded onto each slot-blot hence improving intra experimental comparability.

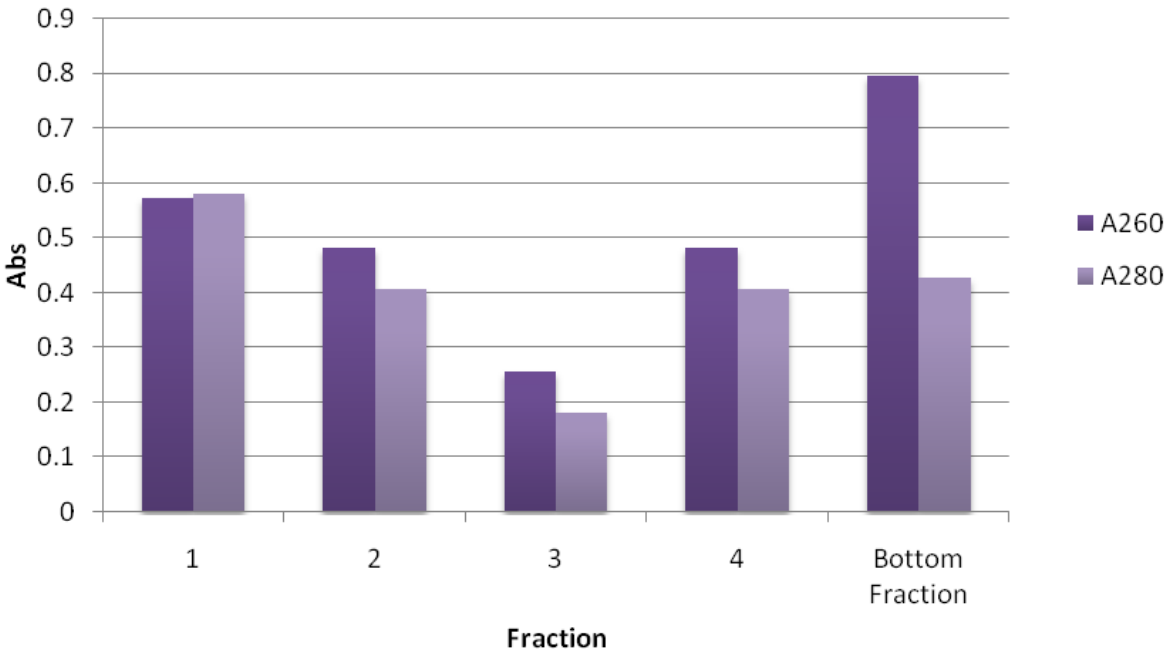


Figure 5.13: Spectrophotometric analysis at 260 nm (DNA content) and 280 nm (protein content) from 1 mL fractions following ultra-centrifugation of cell lysate using 1.5 g/mL caesium chloride
DNA shown to be in bottom fraction (ratio A_{260}/A_{280} >1.8)

In an additional attempt to improve the reproducibility and sensitivity of the ICE assay for detection of topoisomerase II bound stabilised cleavage complexes, later tests used a novel infra-red (IR) imaging technique (Odyssey^{TM}). The original ECL imaging technique relied on contrast and multiple exposure in a dark room to evaluate antibody content. Hence, depending on the signal on any individual membrane, the contrast and measured level of topoisomerase II was highly reliant on both the background luminescence and luminescence from other samples on the same membrane. Accordingly, both intra- and inter- experiment membrane comparisons may have been unreliable. The IR imaging technique did not rely on similar contrast imaging, hence weak and strong signals could be accurately measured on a single membrane. The initial IR imaging experiment was very encouraging and is shown in Figure 5.14. In this test topoisomerase II bound DNA was detected at etoposide concentrations as low as 0.01 µmol/L. Unfortunately, this preliminary experiment used only duplicate slots and in future experiments detection of etoposide induced stabilised cleavage complexes at this concentration proved to be impossible to repeat. The reason for this was unclear, but it may have been due to control signal variability seen when only duplicate slots were analysed.

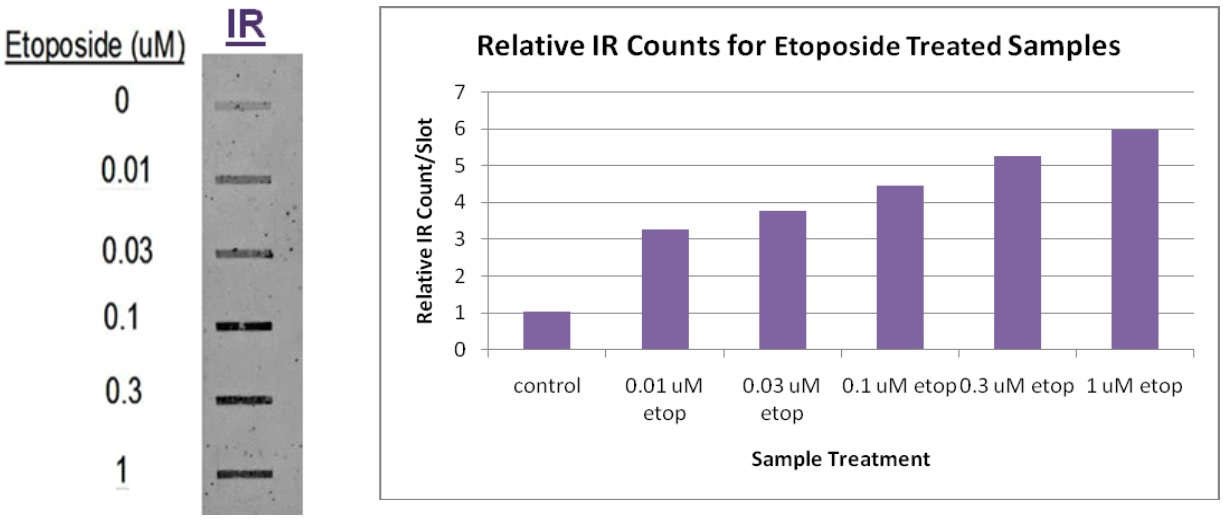


Figure 5.14: Preliminary experiment showing levels of topoisomerase II α bound to DNA in L5178Y cells following 3 hour exposure to 0.01, 0.03, 0.1, 0.3 and 1 µmol/L etoposide then lysed and ultra-centrifuged in 1.5 g/mL caesium chloride. Image on left is example of Odyssey™ infra red imaged slots. Bar chart on right is combined data from duplicate slots.
IR = Infra red

However, the single caesium chloride gradient/IR imaging method was easier to perform and gave the prospect for at least as good sensitivity with better inter experiment reproducibility than the earlier used ECL method, so this method was used for all later ICE assays. Results of which are as follows.

5.2.4.2 Results of etoposide, gemifloxacin and 4-NQO tested in the ICE bioassay

Using the modified ICE protocol with IR imaging, at least triplicate ICE tests were performed with etoposide, gemifloxacin and 4-NQO. The results of these tests are presented in Figures 5.15 to 5.21. In view of the limitation of the number of tubes that could be ultra-centrifuged at one time, only single cultures could be analysed for each experiment. Accordingly, as well as presenting individual experimental data (Figures 5.16, 5.18 and 5.20) mean data and the standard error of the mean is presented from all tests performed (Figures 5.17, 5.19 and 5.21).

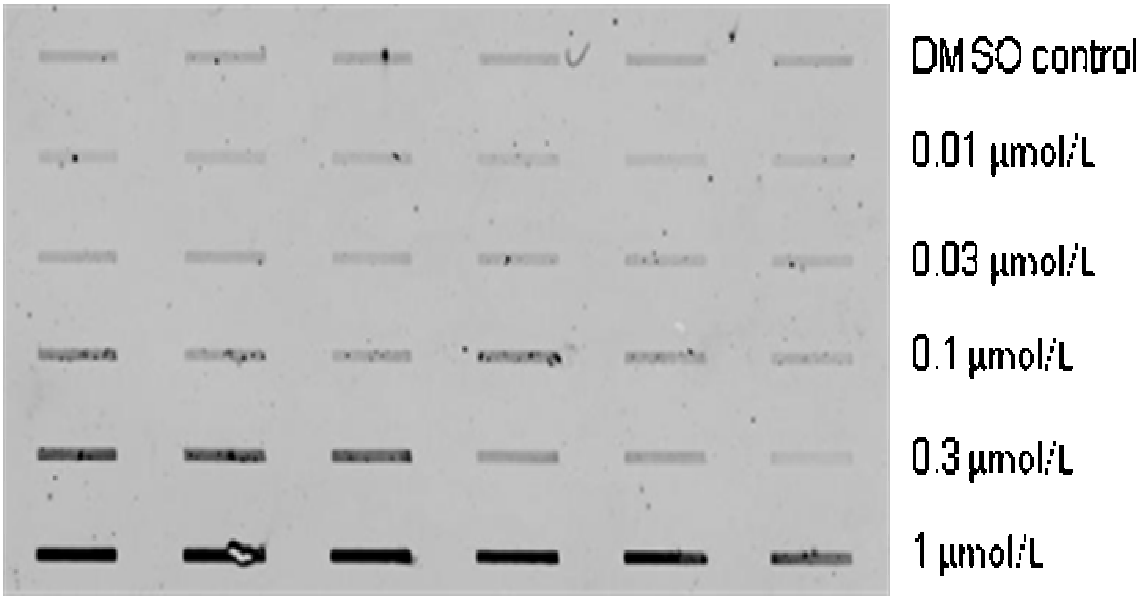


Figure 5.15: Example of ICE Odyssey™ IR imaged membrane.
Following 3 hours exposure of L5178Y mouse lymphoma cells to etoposide Above example is 6 slots prepared from single treatments

Figures 5.16 and 5.17 show data obtained from four individual ICE experiments with etoposide and the combined mean data from these tests, with statistical significance (Figure 5.17).

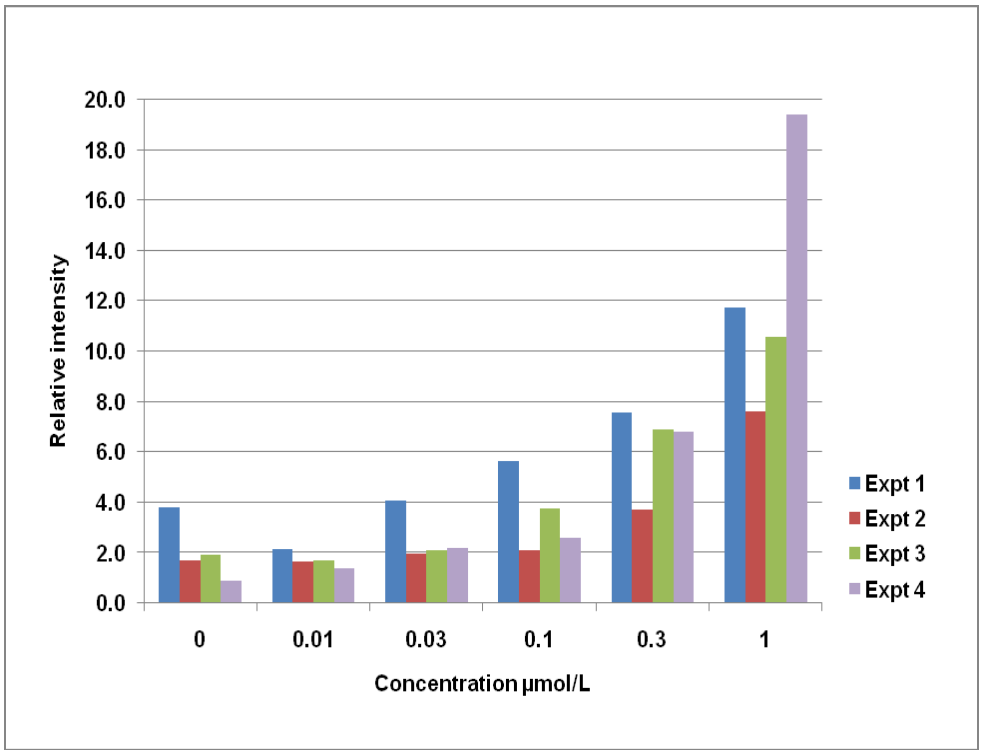


Figure 5.16: Results from four ICE tests with etoposide
Relative Intensity = Mean Odyssey IR signal

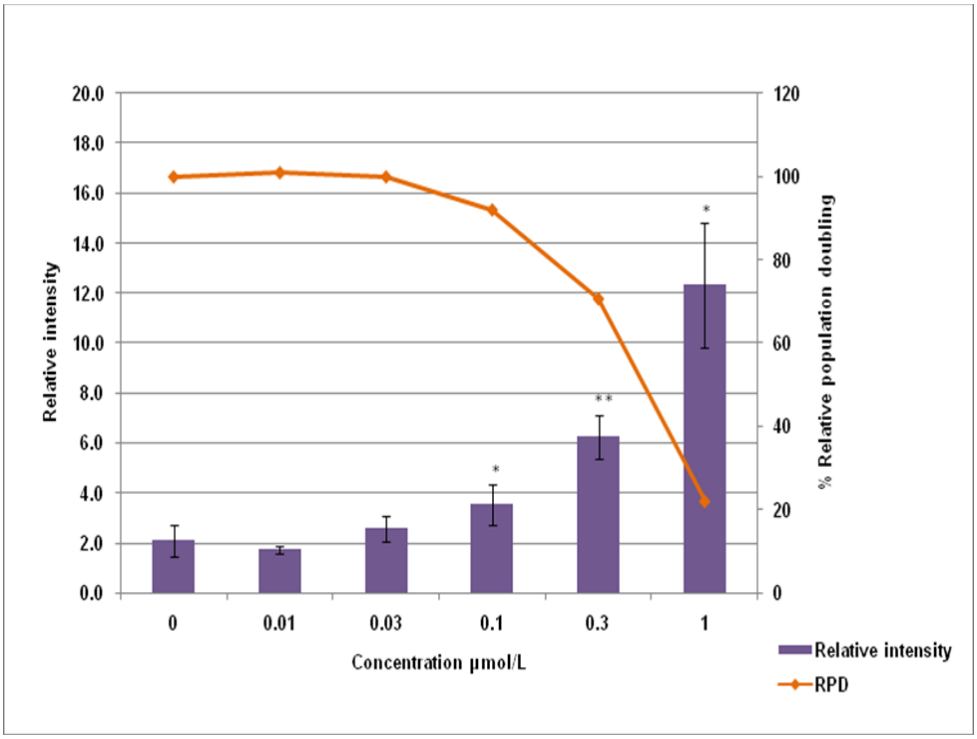


Figure 5.17: Mean cytotoxicity and ICE assay data from four ICE tests with etoposide to determine LOCCEL
Relative Intensity = Mean Odyssey IR signal
n = 4. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

Figures 5.18 and 5.19 show data obtained from four individual ICE experiments with gemifloxacin and the combined mean data from these tests, with statistical significance (Figure 5.19).

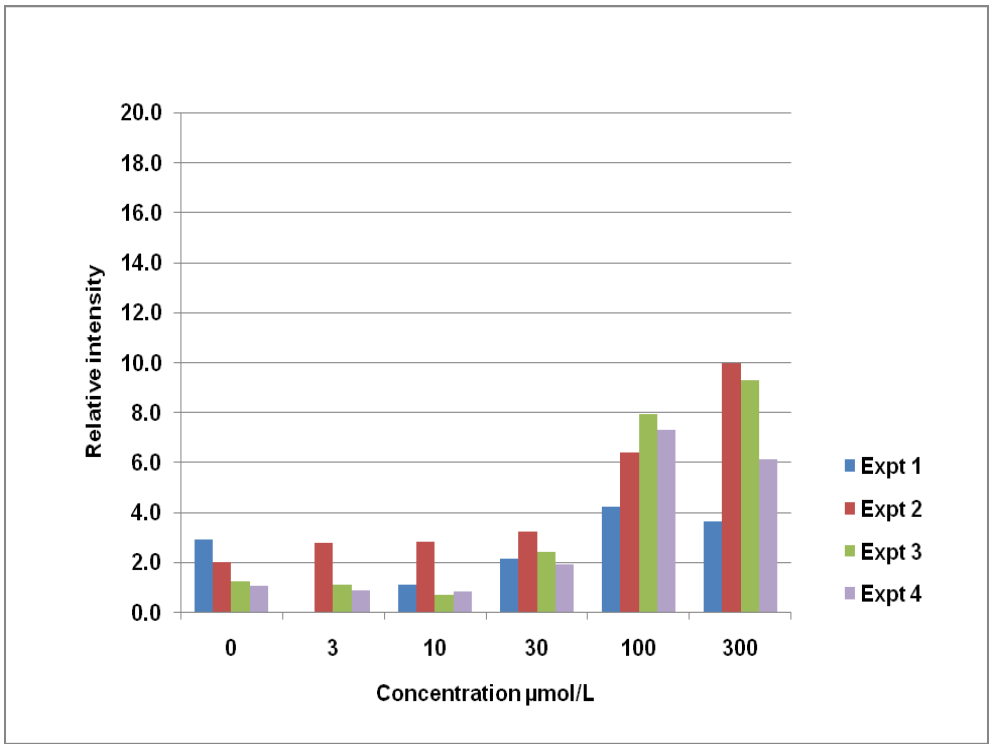


Figure 5.18: Results from four ICE test with gemifloxacin
Relative Intensity = Mean Odyssey IR signal

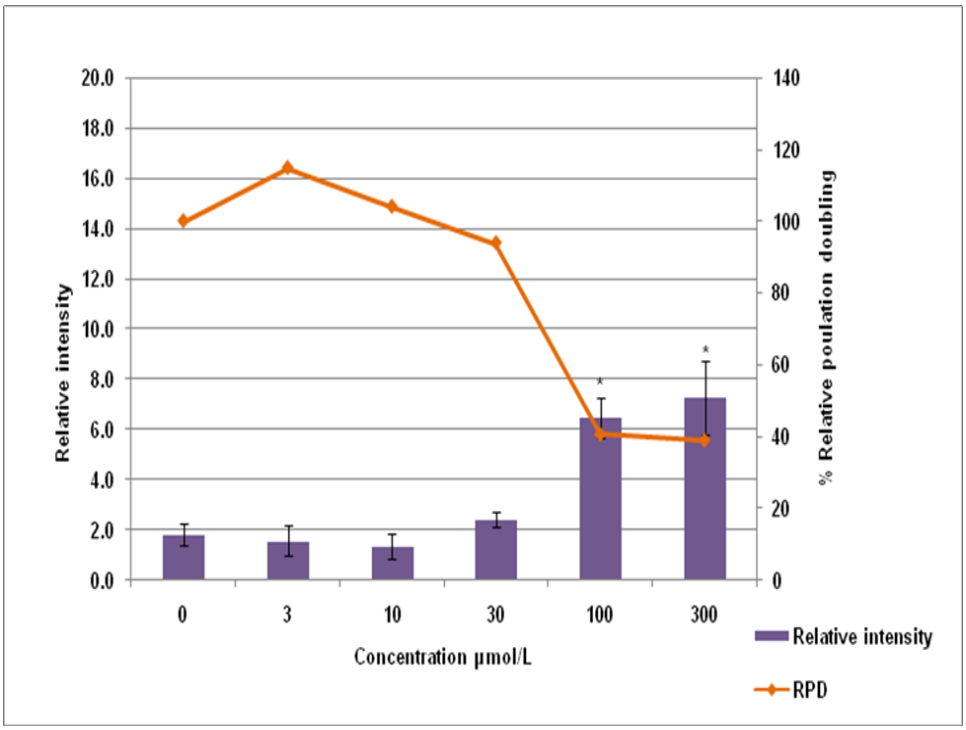


Figure 5.19: Mean cytotoxicity and ICE assay data from four ICE tests with gemifloxacin to determine LOCCEL
Relative Intensity = Mean Odyssey IR signal
n = 4. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

Figures 5.20 and 5.21 show data obtained from three individual ICE experiments with 4-NQO and the combined mean data from these tests with statistical significance (Figure 5.21).

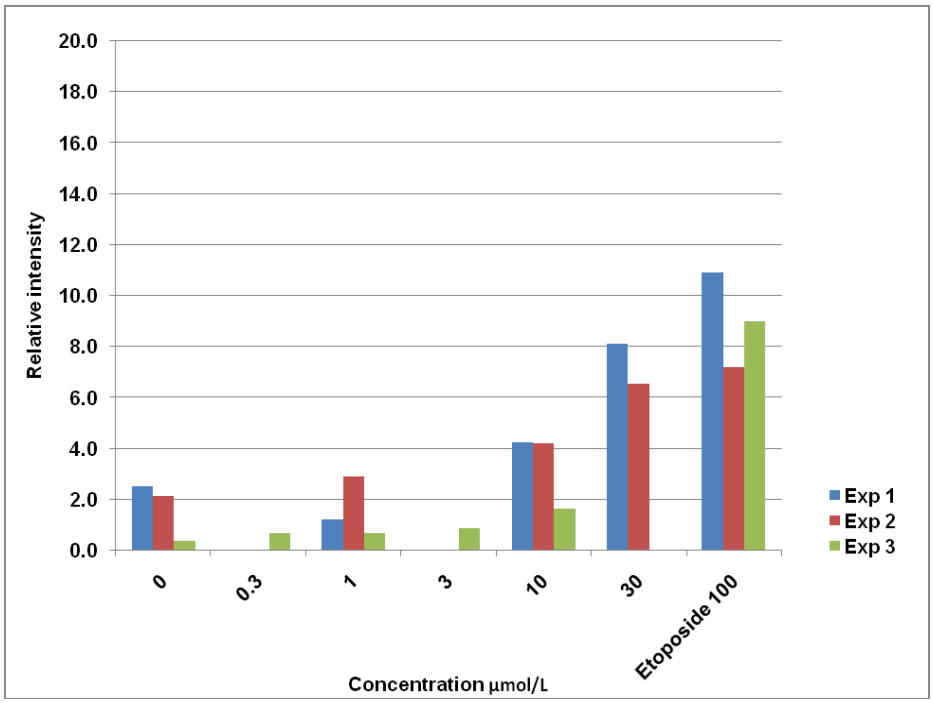


Figure 5.20: Results from three ICE test with 4-NQO
Relative Intensity = Mean Odyssey IR signal

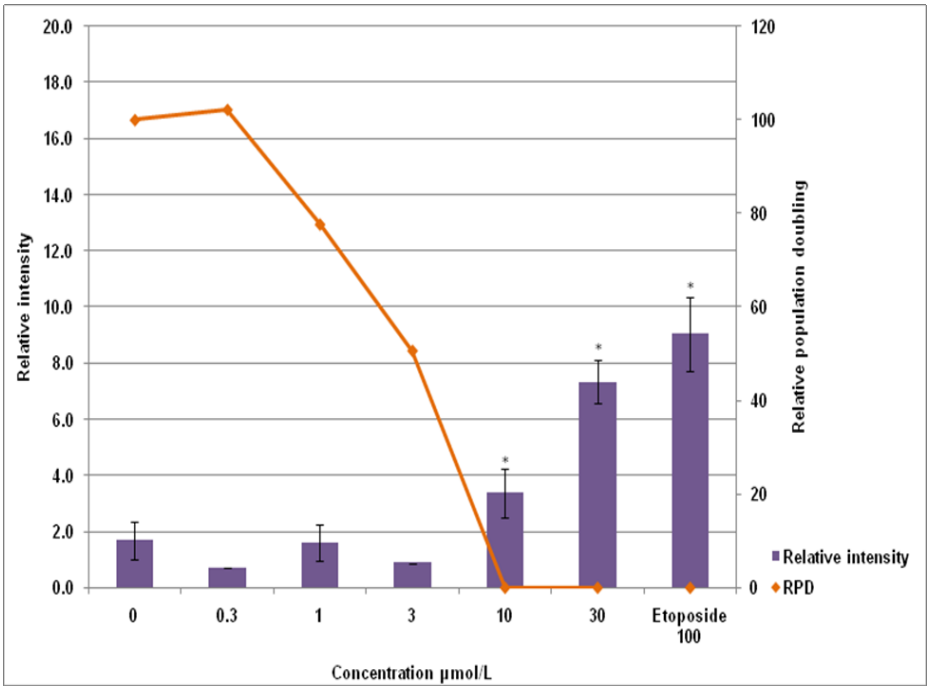


Figure 5.21: Mean experimental cytotoxicity and ICE assay data from tests with 4-NQO to determine LOCCEL
Relative Intensity = Mean Odyssey IR signal
n = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

The two known topoisomerase II poisons etoposide and gemifloxacin both showed statistically significant increases in the number of stabilised cleavage complexes at the

highest concentrations analysed in the ICE bioassay. Etoposide gave a LOCCEL of **0.1 µmol/L**. This concentration was relatively non-cytotoxic, yielding a RPD of 92%. The LOCCEL for gemifloxacin was **100 µmol/L**, and this concentration gave markedly more cytotoxicity, yielding a RPD of 41%. The response of these chemicals in the ICE assay was in contrast to that seen in the TARDIS assay. In TARDIS, etoposide gave statistically significant increases in stabilised cleavage complexes only at extremely cytotoxic concentrations at and above 1 µmol/L (which gave 20% RPD) and no increases were seen following gemifloxacin treatment even when tested up to lethal concentrations (0% RPD). These data indicate that at least when using L5178Y mouse lymphoma cells at AstraZeneca UK, the ICE assay was more sensitive for detection of topoisomerase II / DNA stabilised cleavage complexes than the TARDIS assay. Furthermore, with the modifications made and the improved reproducibility of the IR imaging, the sensitivity of the ICE assay was improved 10-fold for detection of etoposide over the previously published data (etoposide induced stabilised cleavage complex detection reduced from the previously published concentration of 1 µmol/L (Hawtin *et al.,* 2010) to 0.1 µmol/L at AstraZeneca). However, over the concentrations ranges explored in these tests, even with this improved level of detection in the ICE assay, the LOCCEL for etoposide was still 3-fold higher than the LOGEL in the MN(vit) (0.03 µmol/L) and the LOCCEL for gemifloxacin was 10-fold higher than the LOGEL in the MN(vit) (10 µmol/L). Of course it should be noted that the values of 3-fold and 10-fold differences were very much dependent on the concentration ranges tested. If closer spaced concentrations had been analysed, these fold increases may have been narrower. However, the principal that cleavage complexes could not be detected at similar concentrations to genotoxcicity is clear and it was clear that there was a greater difference between LOGEL and LOCCEL for gemifloxacin than etoposide. Accordingly, it could not be definitively concluded from these data that the formation of stabilised cleavage complexes was the primary mechanism of *in vitro* genotoxicity for etoposide and gemifloxacin.

4-NQO also showed statistically significant increases in detection of stabilised cleavage complexes at the highest concentrations tested, giving a LOCCEL of **10 µmol/L**. Initially it may perhaps seem to be surprising that a mutagen with no known direct interaction with topoisomerase II gave a positive response in the ICE assay. However, the concentrations where statistically significant increases in the formation of stabilised cleavage complexes were seen were all completely lethal (RPD 0%). 4-NQO is a potent inducer of reactive oxygen species (ROS) and 8-hydroxydeoxyguanosine

(8OhDG) adducts in mammalian cells (Arima *et al.,*2006). It has previously been shown that chemicals that induce ROS (e.g. hydrogen peroxide) recruit topoisomerase II and topoisomerase II is elevated at cytotoxic concentrations in cells undergoing apoptosis (Perillo *et al.,*2008, López-Lázaro *et al.,*2011). Furthermore, oxidative lesions (e.g. 8OhDG) are themselves known to be weak topoisomerase II poisons (Sabourin and Osheroff, 2000). Hence, it would seem to be plausible that at extremely cytotoxic concentrations of 4-NQO, recruitment of topoisomerase II is sufficient to be measurable in the ICE assay. However, this observation in L5178Y cells is in contrast to that reported for REC-helicase deficient fibroblasts where following 1 hour exposure to 30 µmol/L 4-NQO, an increase of topoisomerase I but not topoisomerase II stabilised cleavage complexes was seen in the ICE assay (Miao *et al.,*2006). It may be that this discrepancy was due to the shorter treatment duration, under which condition apoptosis would be less likely, and the specific response of the cell line used. Whatever the specific reason for this discrepancy, the detection of 4-NQO in the ICE assay with L5178Y mouse lymhoma cells does illustrate that methods to determine induction of stabilised cleavage complexes do have to be used with caution when attempting to elucidate genotoxic mechanisms. These data show the importance of using concurrent estimates of cytotoxicity alongside analysis of stabilised cleavage complexes and ensuring that concentrations giving excessive cytotoxicity are reviewed with caution when attempting to attribute mechanisms of genotoxic action. It should also be noted that the the LOCCEL of 4-NQO was some 100–fold higher than its LOGEL in the MN(vit), the difference between LOGEL to ICE LOCCEL for etoposide and gemifloxcin is only 3 to 10-fold. This indicates that the ratio of LOCCEL to LOGEL may be of use when attempting to determine if a compounds primary mode of genotoxic action is via direct interaction with topoisomerase II / DNA or if recruitment of topoisomerase II is via an alternative mechanism only seen at concentrations of excessive toxicity.

5.2.5 Results of etoposide, gemifloxacin and 4-NQO tested in the chloroquine and novobiocin topoisomerase II activity blocked MN(vit)

To further evaluate the contribution formation of stabilised cleavage complexes made to the genotoxicity of etoposide and 4-NQO, MN(vit) assays were performed in the absence and presence of sub-lethal concentrations of chloroquine. The chloroquine block micronucleus test has previously been used to investigate the genotoxic activity of topoisomerase II inhibitors using Chinese hamster V79 cells by Snyder *et al*.

Chloroquine blocks the activity of topoisomerase II poisons by DNA intercalation, which prevents subsequent topoisomerase II binding to DNA. Hence in the presence of chloroquine the genotoxic effect of topoisomerase II poisons is mitigated (Snyder, 2000). The current experiments were performed to estimate the proportion of DNA damage that could be attributed directly to formation of stabilised cleavage complexes i.e. if etoposide was not genotoxic following chloroquine block it could be assumed that the only mechanism responsible for the observed genotoxicity in *in vitro* mammalian cell tests was due to the chemicals activity as a topoisomerase II poison. The results of the chloroquine block MN(vit) using L5178Y cells with etoposide and 4-NQO are shown in Figures 5.22 and 5.23. Whilst it is not shown on the below graphs it should be noted that all concentrations of etoposide and 4-NQO induced statistically significant ($p<0.01$) increases in micronucleus frequency above the concurrent control when tested both in the presence and absence of chloroquine.

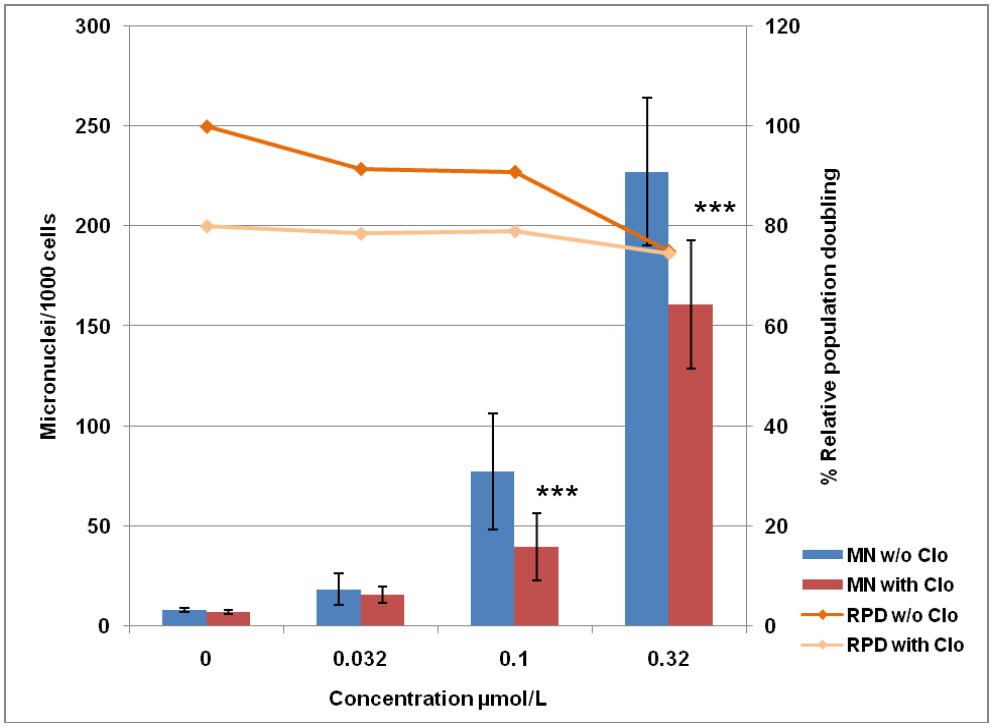


Figure 5.22: Etoposide cytotoxicity and MN(vit) with and without chloroquine block of toposisomerase II. Combined data from duplicate experiments. n = 2. Statistics: 2-sided Continuity-Adjusted Chi-Square Test to assess decrease in micronuclei in the presence of chloroquine. Significance decrease in micronuclei indicated on relevant bar: *p<0.05 **p<0.01 ***p<0.001

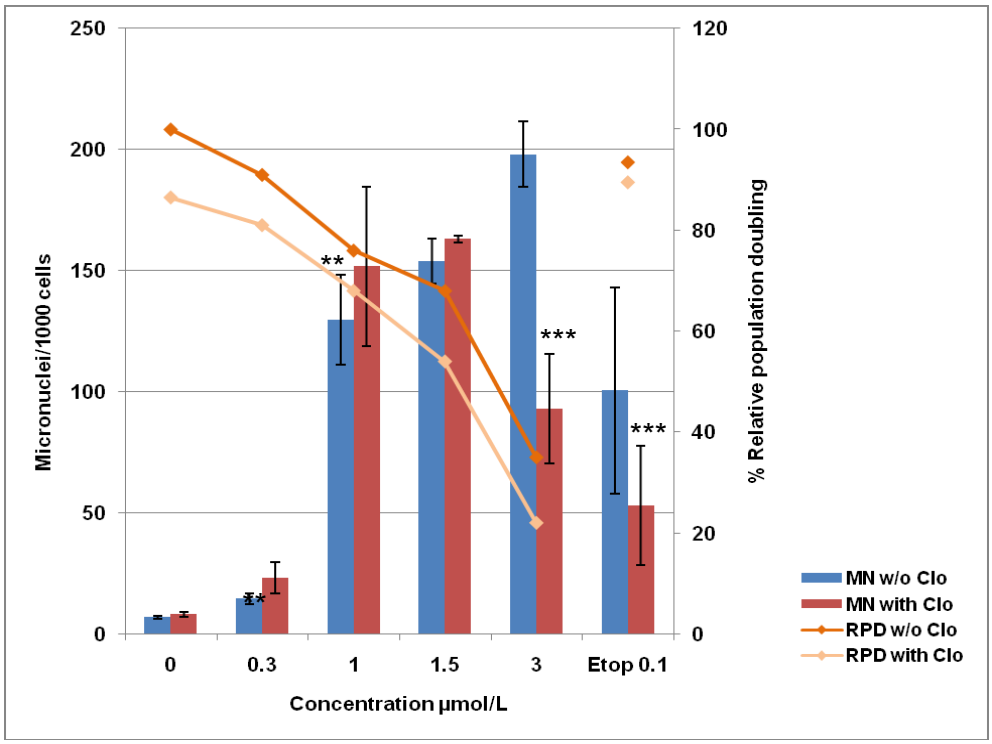


Figure 5.23: 4-NQO cytotoxicity and MN(vit) with and without chloroquine block of topoisomerase II. Combined data from duplicate experiments. n = 2. Statistics: 2-sided Continuity-Adjusted Chi-Square Test to assess decrease in micronuclei in the presence of chloroquine. Significance decrease in micronuclei indicated on relevant bar: *p<0.05 **p<0.01 ***p<0.001

Chloroquine did reduce etoposide induced micronucleus formation and this was statistically significant at the two highest concentrations analysed (p<0.001). However, the reduction was far from complete. A maximum decrease in micronucleus frequency was seen at the interim concentration of 0.1 µmol/L, but this was only a 48% reduction over non-chloroquine treated cultures. The reduction at the higher concentration of 0.32 µmol/L was only 29%. Chloroquine had a variable effect on the genotoxicity of 4-NQO. At the two lowest concentrations tested a statistically significant increase in micronucleus formation was seen in the presence of chloroquine (p<0.01). However, at the highest concentration tested (3 µmol/L) a statistically significant reduction in micronucleus formation was seen (p<0.001). However, 3 µmol/L was considered to be an excessively cytotoxic concentration (RPD < 40%). The response of 4-NQO in the chloroquine blocked MN(vit) was suggestive of its response in the ICE assay in as much as the reduction in micronucleus formation at a highly cytotoxic concentration could possibly be attributable to chloroquine blocking ROS induced topoisomerase II poisoning. However, there may be other explanations. As chloroquine inactivates topoisomerase II indirectly, i.e. by blocking enzyme DNA binding, it may be that the

lack of a more complete reduction of the effect of etoposide was because the concentration of chloroquine used did not reach saturation. However, the chloroquine concentration was titrated to ensure the compound itself did not induce excessive cytotoxicity (data not shown). Higher concentrations or longer exposure of chloroquine would have significantly slowed down cell division, which would have inevitably reduced micronucleus formation (micronuclei are only formed in dividing cells). This may be a more likely explanation for the reduction in NQO genotoxicity at the highest concentration tested where RPD in the presence of chloroquine was 22%, indicating very few cells had divided during the 24 hours following treatment. At the same concentration the RPD in the absence of chloroquine was 35%.

In view of the non-specific nature of the chloroquine block and the lack of a complete block of etoposide induced micronucleus formation, an additional set of experiments were performed using the topoisomerase II ATP competitive inhibitor novobiocin as the blocking agent. As novobiocin directly targets the topoisomerase II molecule it was hoped that it would be more effective than chloroquine. Similar to chloroquine, novobiocin concentrations were titrated to ensure minimal cytotoxicity in the absence of the test agents (data not shown). The results of the novobiocin block assays are presented in Figures 5.24, 5.25 and 5.26. Similar to the chloroquine experiments, it should be noted that all concentrations of etoposide and 4-NQO and the highest two concentrations of gemifloxacin induced statistically significant ($p<0.001$) increases in micronucleus frequency above the concurrent control when tested both in the presence and absence of novobiocin. The lowest concentration of gemifloxacin only induced a statistically significant increase in micronucleus frequency ($p<0.01$) in the absence of novobiocin.

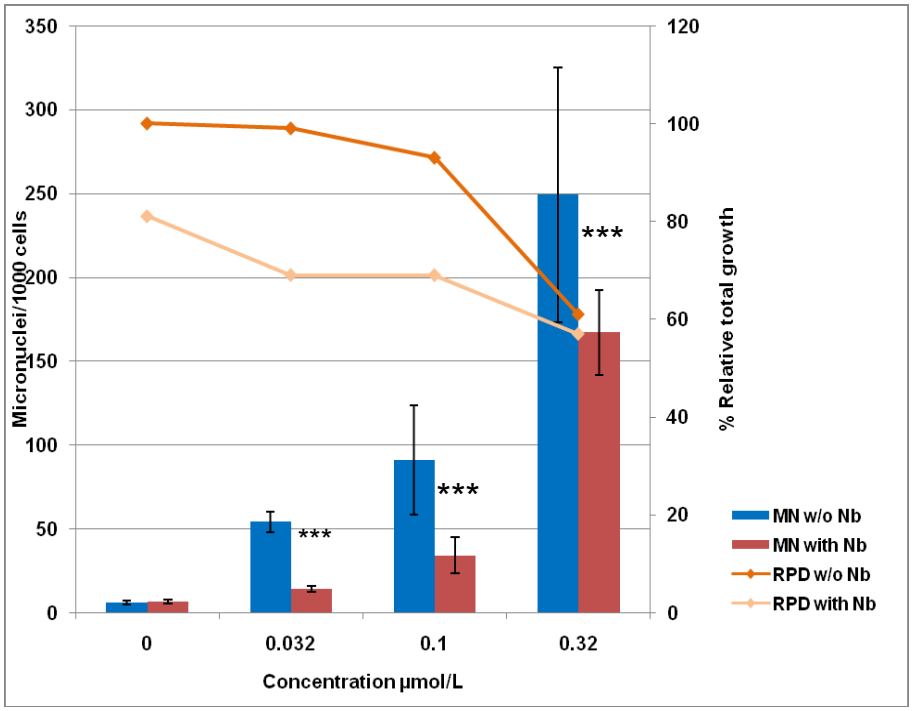


Figure 5.24: Etoposide cytotoxicity and MN(vit) with and without novobiocin block of topoisomerase II. Combined data from duplicate experiments. n = 2. Statistics: 2-sided Continuity-Adjusted Chi-Square Test to assess decrease in micronuclei in the presence of novobiocin. Significance decrease in micronuclei indicated on relevant bar: *p<0.05 **p<0.01 ***p<0.001

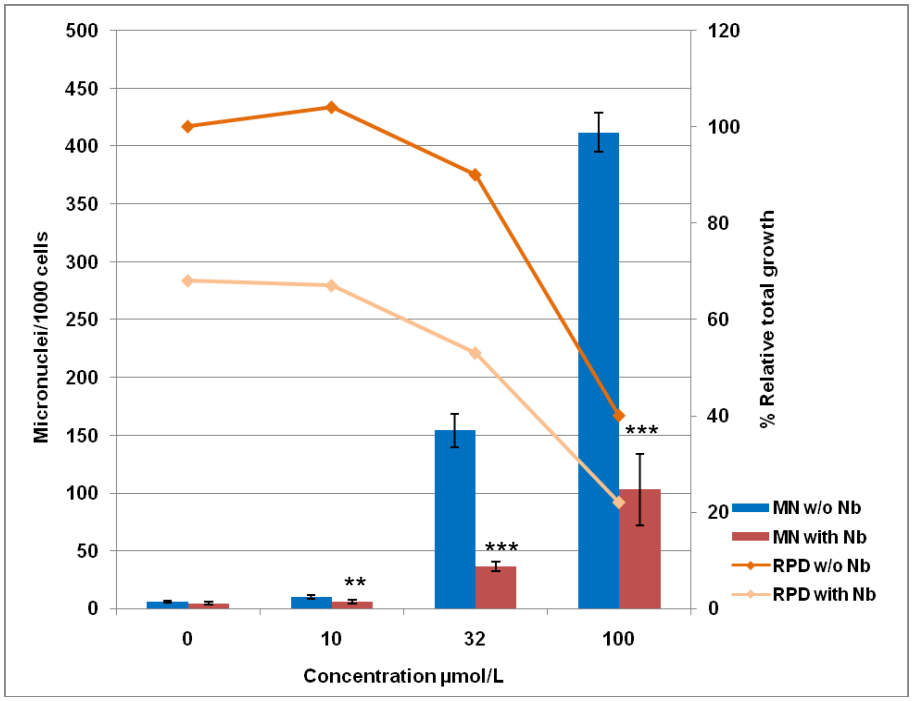


Figure 5.25: Gemifloxacin cytotoxicity and MN(vit) with and without novobiocin block of topoisomerase II. Combined data from duplicate experiments. n = 2. Statistics: 2-sided Continuity-Adjusted Chi-Square Test to assess decrease in micronuclei in the presence of novobiocin. Significance decrease in micronuclei indicated on relevant bar: *p<0.05 **p<0.01 ***p<0.001

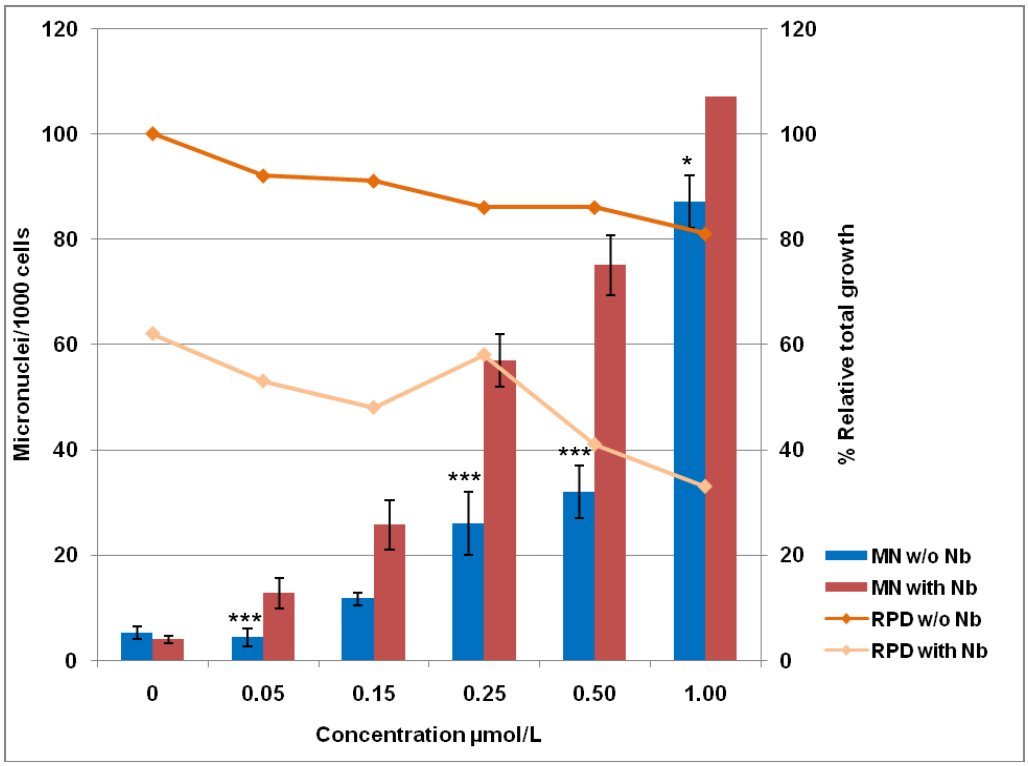


Figure 5.26: 4-NQO cytotoxicity and MN(vit) with and without novobiocin block of topoisomerase II. Combined data from duplicate experiment. n = 2. Statistics: 2-sided Continuity-Adjusted Chi-Square Test to assess decrease in micronuclei in the presence of novobiocin. Significance decrease in micronuclei indicated on relevant bar: *p<0.05 **p<0.01 ***p<0.001

At the lower concentrations tested, novobiocin blocked the genotoxic activity of etoposide more effectively than chloroquine. The greatest reduction in micronucleus frequency was seen at 0.032 µmol/L, which gave a 74% decrease in the presence of novobiocin, compared to the greatest reduction seen in the presence of chloroquine at 0.1 µmol/L, which gave a 48% decrease. Although the reduction in micronuclei in the presence of novobiocin was statistically significant at all etoposide concentrations analysed, at the higher concentrations the block was less complete i.e. at 0.32 µmol/L the reduction in micronucleus frequency in the presence of novobiocin was only 30%. Novobiocin also effectively blocked the genotoxic activity of gemifloxacin. The greatest reduction was seen at 32 µmol/L, which reduced micronucleus frequency by 76% in the presence of novobiocin and a similar reduction was seen (75%) at the highest concentration tested (100 µmol/L).

Similar to the response seen when testing low 4-NQO concentrations in the presence of chloroquine, 4-NQO in combination with novobiocin seemed to have a synergistic effect, where a statistically significant increase in the number of micronuclei was seen

at four of the five 4-NQO concentrations tested. The reason for this has not been elucidated. It should be noted that 4-NQO was not tested up to such high concentrations with novobiocin as had been tested with chloroquine. This was due to the additional cytotoxicity seen with 4-NQO/novobiocin combined treatments. Accordingly, the apparent reduction in micronucleus formation following treatment with 3 µmol/L 4-NQO in the presence of chloroquine could not be tested with novobiocin.

The data generated from both the chloroquine and novobiocin experiments clearly demonstrate that these topoisomerase II inhibitors partly mitigate the genotoxicity of the topoisomerase II poisons etoposide and gemifloxacin. Whereas, other than at a highly toxic concentration tested in combination with chloroquine, they do not mitigate the genotoxicity of 4-NQO. However, as statistically significant increases in micronucleus frequency were seen for all etoposide concentrations and two out of three gemifloxacin concentrations when tested in both the presence and absence of the topoisomerase II inhibitors, it was clear that neither chloroquine nor novobiocin was capable of completely removing the genotoxicity of these topoisomerase poisons, even at relatively low concentrations i.e. concentrations that were at or below the LOCCEL in the ICE assay. Novobiocin and chloroquine were amongst the most potent inhibitors of topoisomerase II activity used by Dr Ron Snyder in his original studies (Snyder, 2000). Snyder concluded from this work that:

'It is also shown that topo (topoisomerase II) poison dependent micronucleus production, presumably arising from cleavable complex formation, is strongly antagonized by a variety of catalytic topo II inhibitors of various structural and functional classes'.

Whilst the current studies have demonstrated that catalytic inhibitors are antagonistic to the genotoxicity of etoposide and gemifloxacin, if the reduction in genotoxicity is due to blocking cleavage complex formation hence blocking subsequent DNA strand breakage and micronuclei formation, this still means a significant amount of genotoxic damage is not blocked.

It is perhaps surprising that neither measurement of stabilised cleavage complex formation by TARDIS or ICE assays nor the MN(vit) using topoisomerase II blocking agents can definitively quantitatively link etoposide and gemifloxacin induced *in vitro* mammalian cell genotoxicity with their expected mechanism of action. This is also partly in contradiction to the conclusions from Smart *et al*'s 2008 publication, in which they considered etoposide induced formation of stabilised cleavage complexes were

formed 'at a similar time' to double strand breaks as measured by the *in vitro* Comet assay and γH2AX foci using V79 Chinese hamster lung fibroblast cells (Smart *et al.,* 2008). However, this conclusion was based on a virtually indiscernible increase in stabilised cleave complex formation at approximately 0.2 µmol/L etoposide as compared to a LOGEL of approximately 1 µmol/L, which was clearly a lot higher than the MN(vit) LOGEL of 0.03 µmol/L seen in the current investigations. Statistical analysis was not applied to the stabilised cleavage complex data and the authors conceded that 'clear detectable levels' were only seen at above approximately 2 µmol/L. Furthermore, the increase in comet tail DNA at 0.2 µmol/L was virtually the same as that seen at 1 µmol/L. Hence, at closer inspection, these data support rather than contradict the observations made in the current investigations.

5.3 Conclusion and the potential use of the topoisomerase II inhibition assays investigated

The intention of the work outlined in this chapter was to use cellular assays to attempt to establish whether the formation of stabilised cleavage complexes by topoisomerase II poisons could be directly related to their inherent genotoxicity. The ultimate aims being to provide better screening tools for topoisomerase II genotoxicity and to help elucidate genotoxic mechanisms from hitherto unexplained positives in *in vitro* mammalian cell genotoxicity assays. However, none of the methods used to investigate the genotoxicity of etoposide and gemifloxacin could definitively demonstrate that formation of stabilised cleavage complexes was their only mode of genotoxic action. Furthermore, the reference genotoxin 4-NQO was also shown to induce stabilised cleavage complexes.

Assays to directly measure formation of stabilised cleavage complexes and the TARDIS assay in particular were shown to be relatively insensitive when comparing LOCCEL with LOGEL. Following methodology and imaging improvements, the ICE assay did show better sensitivity, and the novobiocin and chloroquine block MN(vit) assays both showed that the genotoxicity of topoisomerase II poisons could be partly mitigated in the presence of topoisomerase II inhibitors. However, all of these assays could only link genotoxicity with formation of stabilised cleavage complexes. It was still not possible to demonstrate that formation of stabilised cleavage complexes occurred at similar or lower concentrations than genotoxicity, as would seem to be intuitive, and neither of the topoisomerase blocking agents used completely negated the genotoxic activity of etoposide or gemifloxacin at the majority of the concentrations tested.

Furthermore, although the improved ICE assay was relatively more sensitive when compared to the TARDIS assay at AstraZeneca, the ICE assay was a very time-consuming and technically quite difficult procedure. Given the requirement in the current protocol for ultracentrifugation and hence the limited number of cultures that could be routinely tested (maximum of six per day at AstraZeneca) the assay has no place in any early pharmaceutical screening paradigm to assess topoisomerase II inhibition.

The alternative potential use of the assays investigated would be to help mechanistically interpret unexpected positive responses in *in vitro* genotoxicity tests. However, the lack of sensitivity of both the TARDIS and ICE assays questions their value for the derivation of genotoxic mechanisms. At best these assays can demonstrate an association between a compounds ability to form stabilised cleavage complexes and genotoxicity. The question remains whether this is sufficient when trying to demonstrate that an unexplainable positive response in a genotoxicity screen is due to topoisomerase II poisoning. The work with NQO shows that a DNA reactive and ROS inducing mutagen will also form stabilised cleavage complexes in the ICE assay, albeit at highly cytotoxic concentrations. Accordingly, it should be accepted that when trying to attribute genotoxicity to formation of stabilised cleavage complexes it is important to take account of both the cytotoxicity seen and the ratio between LOGEL and LOCCEL. What ratio would be considered to be appropriate to unequivocally confirm that observed genotoxicity was due to topoisomerase II inhibition? In these investigations the MN(vit) LOGEL to ICE LOCCEL ratio was 1:3 for etoposide, 1:10 for gemifloxacin and 1:100 for 4-NQO. If a compound gave a ratio of 1:20 could it be definitively claimed to be genotoxic via topoisomerase II inhibition without further investigation? If further investigations included use of the topoisomerase II block MN(vit) in which genotoxicity was only partially moderated, this cannot be used as conclusive evidence that other genotoxic mechanisms are not at least partly responsible for the induced genotoxicity. This all demonstrates the need to gain further insight into why stabilised cleavage complexes cannot be seen before genotoxicity can be measured. Hence the question remains, is this due to the insensitivity of the assays currently available or are other mechanisms responsible for some of the genotoxicity of topoisomerase II poisons. The aim of the rest of this research project will be to elucidate why the link between formation of stabilised cleavage complexes and the *in vitro* mammalian cell genotoxicity of etoposide and gemifloxacin cannot currently be experimentally quantitatively confirmed.

CHAPTER 6

The potential of etoposide and gemifloxacin to induce reactive oxygen species (ROS) and associated genotoxicity

CONTENTS

6 The potential of etoposide and gemifloxacin to induce reactive oxygen species (ROS) and associated genotoxicity

6.1 Introduction

The aim of the work described in this chapter was to see if potential ROS induction by etoposide and gemifloxacin could contribute to the *in vitro* genotoxicity seen with these agents and hence explain the discrepancy between concentrations where micronuclei are induced and concentrations where topoisomerase II poison stabilised cleavable complexes can be detected.

Chapter 5 showed that when using methods designed to be sensitive for detection of stabilised cleavable complexes it was still not possible to measure etoposide or gemifloxacin induced complexes at directly comparable concentration to where genotoxicity was observed, as indicated by micronucleus formation in L5178Y mouse lymphoma cells. The two most likely explanations are that the methods used for detection of stabilised cleavable complexes were not sufficiently sensitive, or one or more alternative mechanism of genotoxicity (i.e. other than strand breakage caused directly by topoisomerase II poisoning) was partly responsible for the clastogenicity seen. As there are no reliable published data or obvious structural activity relationship to indicate that either gemifloxacin or etoposide could covalently bind to DNA, the most likely alternative mechanism that may contribute to their genotoxicity is induction of reactive oxygen species (ROS).

Endogenous and exogenous chemical induced ROS is a well defined mechanism of both genotoxicity and carcinogenicity (Kurokawa *et al.,* 1990; Dreher and Junod, 1996) including mutagenicity in L5178Y mouse lymphoma cells (Harrington-Brock *et al.,* 2003). For example the hydroxyl radical generated by both gamma radiation and certain chemical species interacts with guanine, preferably at the nucleophilic sites C8 and C4 giving characteristic oxidative lesions (Cadet *et al.,* 2003). Furthermore, the topoisomerase poison doxorubicin is known to induce secondary cardio toxicities via ROS induction (Sawyer *et al.,* 2010).

The mechanisms of exogenous chemical free-radical generation in biological systems are varied and may involve redox cycling with possible cytochrome p450 metabolism or mitochondrial interactions. For example, both mitochondrial and redox activity is

thought to be involved in the formation of ROS by doxorubicin. i.e. 1. Mitochondrial reductase mediated single electron reduction of doxorubicin is thought to generate semiquinone free radicals which are further reduced to the ROS superoxide anion. 2. Molecular oxygen may be reduced following doxorubicin and iron III interactions with subsequent redox generation of free radicals (Deavall *et al.,* 2012).

ROS induction has also been associated with the activity of fluoroquinolone antibiotics. The suggestion is that following formation of cleavable complexes in bacteria, the subsequent chromosome breakdown leads to a cascade of free radical generation which leads to further chromosome breakdown and cell death (Drlica *et al.,* 2009). Could this mechanism contribute to the mammalian cell genotoxicity of topoisomerase II poisons and hence explain why there was a discrepancy between the lowest concentrations of etoposide and gemifloxacin capable of inducing cleavable complexes and mammalian cell genotoxicity?

Oxidative DNA damage gives characteristic DNA lesions e.g. 8-oxoguanine, 8-oxoadenine and abasic sites. These may block replicative machinery or cause mutation following inefficient base excision repair. The characteristic lesions can be used as markers of oxidative DNA damage; for example by use of the modified restriction endonuclease *in vitro* Comet assay (Smith *et al.,* 2006) or by mass spectrometric technologies to measure the number of modified DNA bases (Farmer and Singh, 2008; Singh *et al.,* 2010). The restriction endonuclease modified Comet assay uses the principal that endonucleases, such as human 8-hydroxyguanine DNA-glycosylase (hOGG1), will cleave DNA strands at recognised lesions, hOGG1 being particularly sensitive for recognition of oxidative lesions e.g. induced 8-oxo-7,8-dihydroguanine (8-oxoGua) and methyl 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Under standard Comet electrophoresis conditions, these cleaved strands will form Comet tails (Smith *et al.,* 2006). Accordingly, if the primary mechanism of DNA damage was by chemically induced oxidative stress it would be expected that a significant increase would be seen in comet tail DNA in post-treatment cells when measured following incubation with hOGG1 endonuclease. Although hOGG1 is the commercially available human form of the endonuclease, the hOGG1 modified Comet assay has previously been used to measure gamma radiation and chemically induced oxidative damage in L5178Y mouse lymphoma cells (Smith *et al.,* 2006). Furthermore, preliminary work in this laboratory has demonstrated that when compared to concurrent solvent controls, clear increases in Comet tails were seen when L5178Y mouse lymphoma cells were treated with 1 µmol/L etoposide (Figure 6.1). As this methodology had previously been successfully used for identification of oxidative DNA

lesions in the cell type of interest, it was selected as the tool to be used for determination of whether oxidative stress contributes to the genotoxicity of the topoisomerase II poisons etoposide and gemifloxacin. The aim of the work described in the following chapter was to elucidate the potential of etoposide and gemifloxacin to cause genotoxicity by ROS induction.

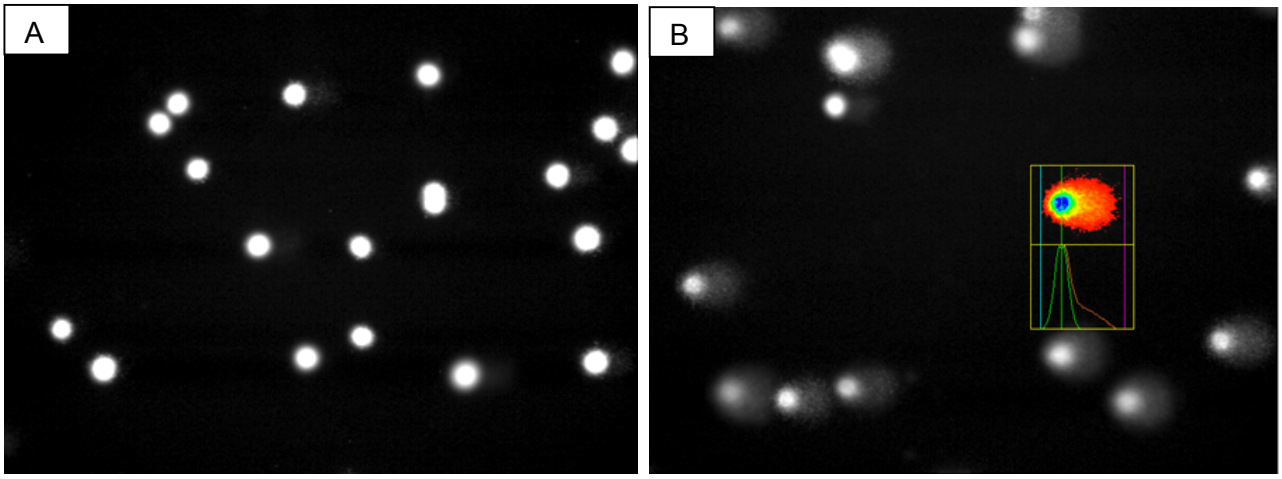


Figure 6.1. L5178Y mouse lymphoma cell Comets after 3 hours treatment with:
A. Solvent control (DMSO)
B. 1 µmol/L etoposide, image also shows image analysis of Comet tail. Red area indicates amount of DNA in tail = Tail Intensity

6.2 Results

The principal of the restriction endonuclease Comet assay relies on the ability of hOGG1 to cleave DNA at 8-oxoGua sites, hence yielding significantly larger amounts of DNA in the subsequently formed Comet tails as opposed to testing under standard Comet conditions. Accordingly, the concentration selection for etoposide and gemifloxacin in the preliminary test was made to ensure only a limited amount of damage would be detected in the absence of hOGG1, i.e. the ability of etoposide and gemifloxacin to cleave DNA following cleavage complex formation would not mask any earlier or associated formation of oxidative adducts that could be subsequently cleaved by the restriction endonuclease. The results of the preliminary test when duplicate cultures were tested with etoposide and gemifloxacin in the presence and absence of hOGG1 are shown in Figures 6.2 and 6.3, respectively.

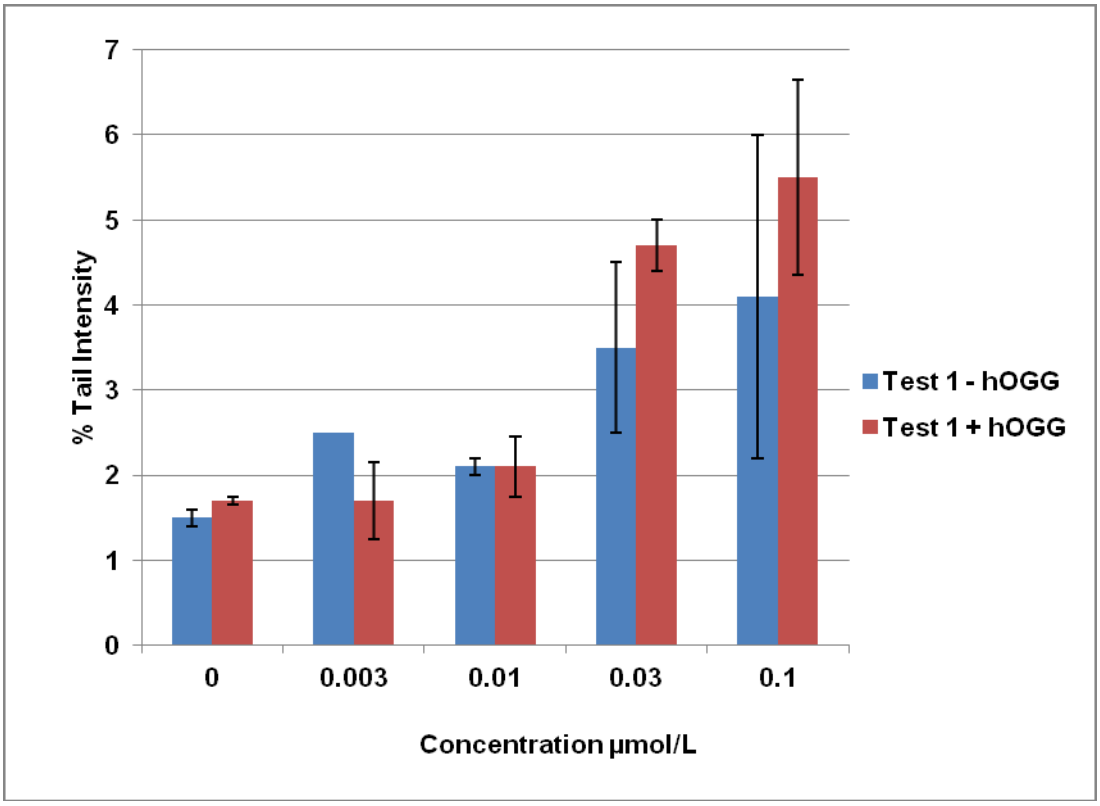


Figure 6.2: Preliminary restriction enzyme modified Comet assay with etoposide in the absence and presence of hOGG1. n = 2

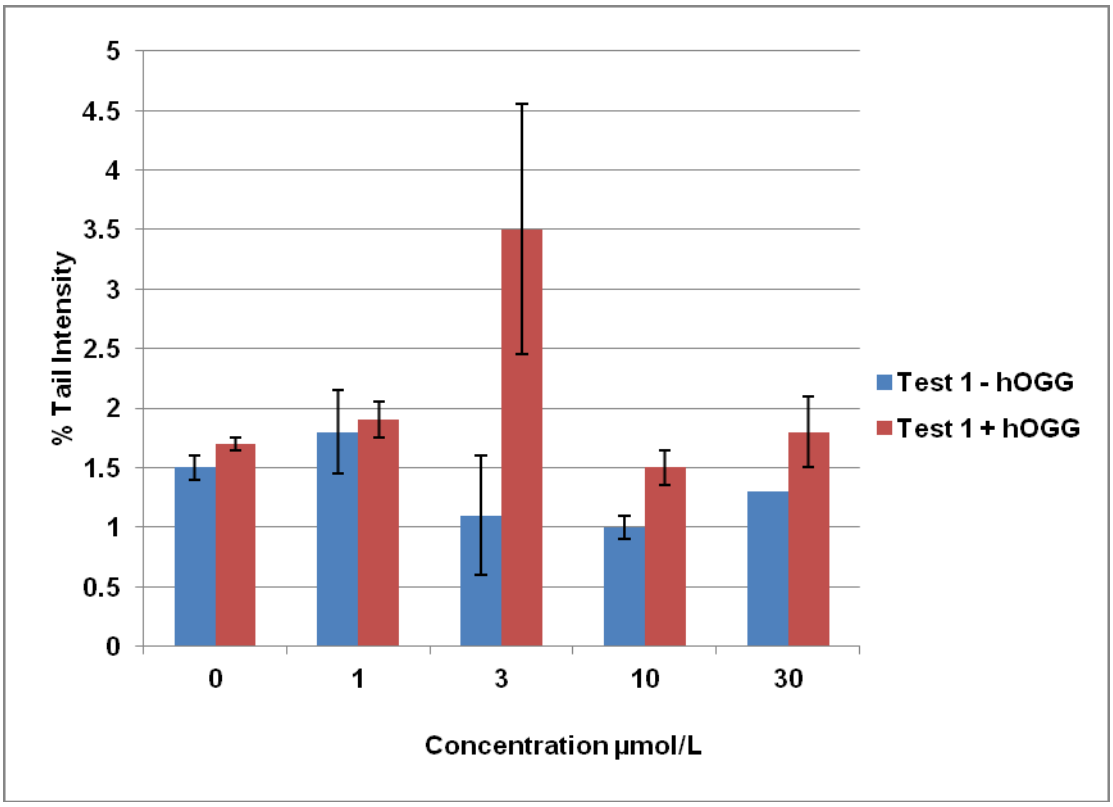


Figure 6.3 Preliminary restriction enzyme modified Comet test with gemifloxacin in the absence and presence of hOGG1. n = 2

In the preliminary test, at the majority of the concentrations tested of both etoposide and gemifloxacin, a small increase in Comet tail intensity in the presence of hOGG1 was seen when compared to treatment in the absence of hOGG1. Furthermore, a surprisingly large difference was seen at the intermediate concentration of 3 µmol/L gemifloxacin. However, there was no indication of a concentration-related increase in the difference in Comet tail intensity between treatments in the absence and presence of hOGG1 for either of the test agents. These tests used only duplicate cultures and there was a high degree of variability between most of the tested replicates. Accordingly, there was not considered to be a concentration-related or marked biologically significant increase in the difference in tail intensity following treatment with etoposide up to 0.1 µmol/L or genmifloxacin up to 30 µmol/L in the presence of hOGG1 when compared to treatments in the absence of hOGG1. All of the concentrations tested were essentially non-toxic (data not shown). As previously mentioned, one of the aims of the concentration selection for the preliminary test was to avoid testing up to concentrations where an increase in 8-oxoGua cleavable sites could be masked by the general increase in Comet tail following mutagen treatment. However, to ensure any potential effect was not being missed at low concentrations, higher concentrations of both etoposide and gemifloxacin were selected for the confirmatory tests. Furthermore, as no clear increase in cleaved oxidative lesions was seen with either etoposide or gemifloxacin, potassium bromate ($KBrO_3$), a known inducer of DNA oxidative damage and an *in vitro* mutagen (Priestley *et al.,* 2010) was included as a positive control for hOGG1 cleavage. The results from cytotoxicity analysis of etoposide, gemifloxacin and potassium bromate from two confirmatory tests are shown in Table 6.1.

Etoposide (µmol/L)	**0**	**0.03**	**0.1**	**0.3**	**1**
Test 2 (Survival %RPD)	100	97	88	66	6
Test 3 (Survival %RPD)	100	106	94	73	5
Gemifloxacin (µmol/L)	**0**	**8**	**24**	**80**	**240**
Test 2 (Survival %RPD)	100	98	86	46	23
Test 3 (Survival %RPD)	100	103	97	64	9
$KBrO_3$ (2000 µmol/L) RPD 91%					

Table 6.1 Cytotoxicity as mesured by Relative Population Doublings (RPD) from restriction enzyme modified Comet tests 2 and 3

The results for Comet tail intensity, including statistical analysis of increases, from two confirmatory tests where triplicate concentrations of etoposide, gemifloxacin and potassium bromate were tested in the presence and absence of hOGG1 are shown in Figures 6.4 and 6.5.

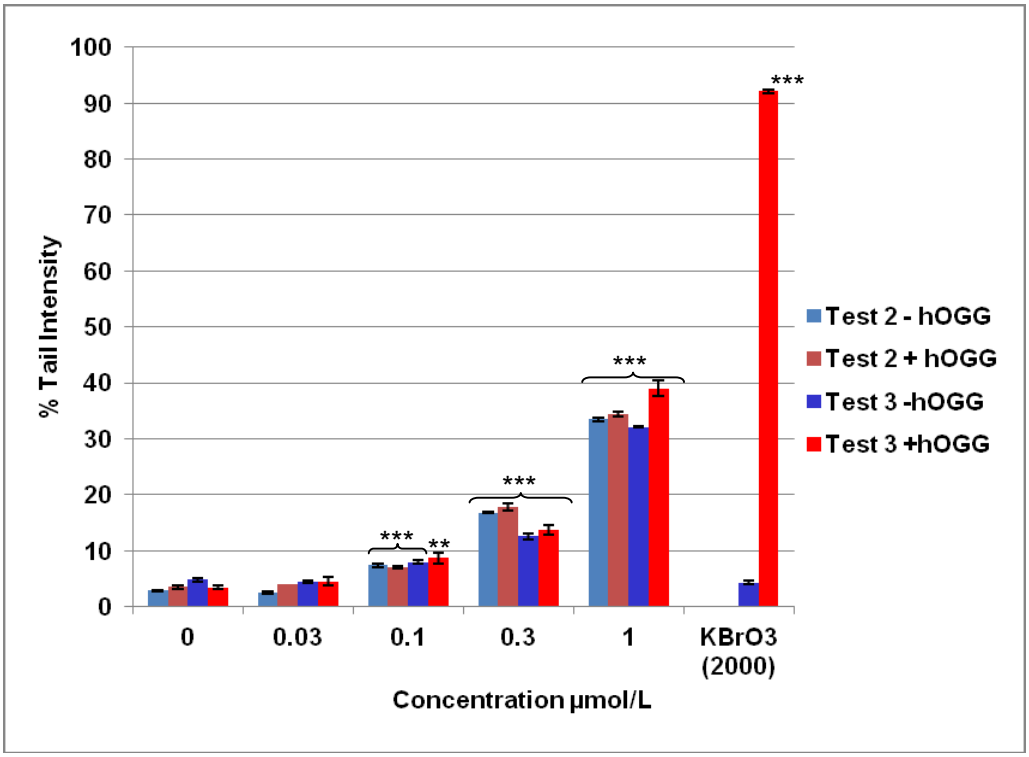


Figure 6.4 Restriction enzyme modified Comet tests 2 and 3 with etoposide in the absence and presence of hOGG1. $KBrO_3$ was used as a positive control
n = 3. Statistics: One-way analysis of variance (ANOVA). **, *** Significant increase in tail intensity over concurrent control ($p<0.01$, $p<0.001$)

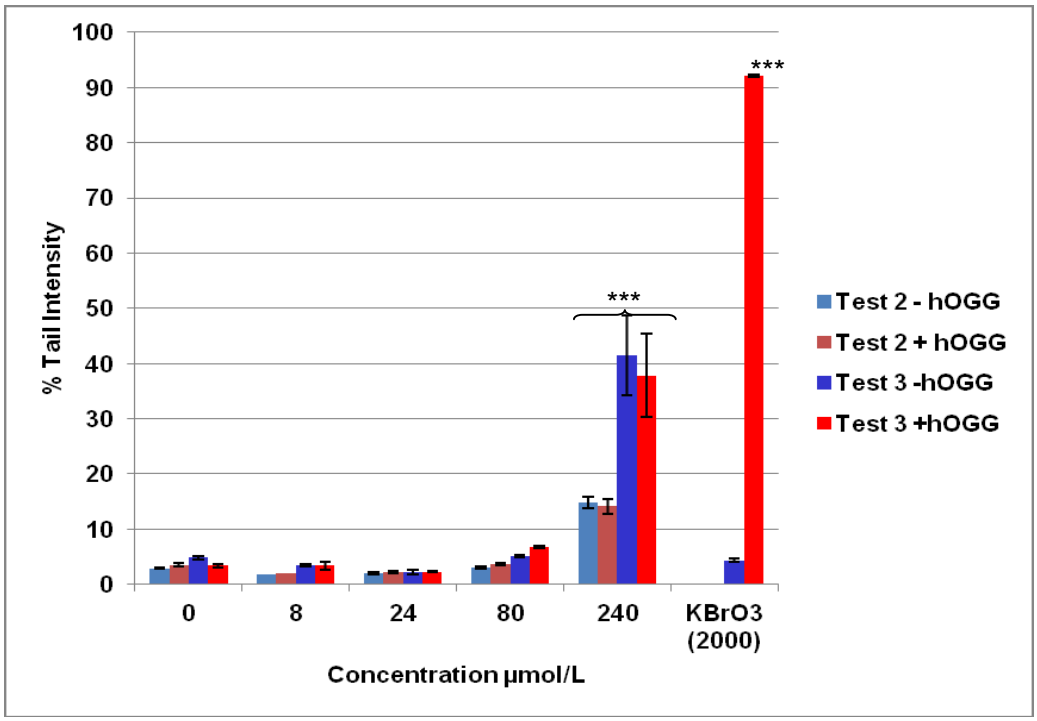


Figure 6.5 Restriction enzyme modified Comet tests 2 and 3 with gemifloxacin in the absence and presence of hOGG1. $KBrO_3$ was used as a positive control
n = 3. Statistics: One-way analysis of variance (ANOVA). *** Significant increase in tail intensity over concurrent control ($p < 0.001$)

The results from Tests 2 and 3 were in general agreement with those from Test 1.

For etoposide, at the highest concentration tested (0.3 and 1 µmol/L), there was a very small increase in tail intensity in the presence of hOGG1 when compared to the absence of hOGG1, but all increases above concurrent control gave a similar level of statistical significance ($p<0.001$). Furthermore, 1 µmol/L, where the largest difference was seen between tail intesity in the absence and presenece of hOGG1, was an extremely cytotoxic concentration (giving only 5%-6% relative survival as measured by RPD) and hence any difference could not be considered to be biologically significant. At the lower concentration of 0.1 µmol/L, in Test 3, the increase in tail intensity was actually more statistically significant in the absence of hOGG1 ($p<0.001$) than in its presence ($p<0.01$), although it should be noted that this was mainly due to the increased level of intra-replicate variability seen in the presence of hOGG1.

For gemifloxacin, at the only concentration where a statistically significant increase in tail intensity were seen (240 µmol/L, $p<0.001$) the increase was lower in the presence of hOGG1 than in its absence.

The very small differences in tail intensity seen beween treatments in the absence and presence of hOGG1 were put into perspective when compared with the difference seen

with the known inducer of oxidative DNA damage, potassium bromate. At an essentially non-toxic concentration (2000 µmol/L giving 91% relative survival) a 21-fold higher level of tail intensity was seen in the presence of hOGG1 than in its absence.

6.3 Discussion

The restriction endonuclease Comet work with etoposide and gemifloxacin unequivocally demonstrated that these topoisomerase II poisons did not induce hOGG1 cleavable 8-oxoGua sites on the DNA of L5178Y mouse lymphoma cells. The restriction endonuclease Comet is recognised as being specific for identifying oxidative DNA damage (Smith *et al.,* 2006) and has the advantages over alternative techniques such as high performance liquid chromatography (HPLC) with electrochemical detection and HPLC-tandem mass spectrometry in so much as it is less prone to false positives from detection of artefactual oxidative products. This was confirmed by the European Standards Committee on Oxidative DNA Damage inter-laboratory trial, which to date is probably the most thorough international validation of methods to identify oxidative DNA damage (Gedik *et al.,* 2005). Accordingly, it seems highly unlikely that oxidative damage significantly contributed to the observed *in vitro* genotoxicity of etoposide and gemifloxacin in L5178Y mouse lymphoma cells.

This result was surprising, especially for etoposide, where there is a significant amount of published literature indicating that its cellular toxicity is at least partly mediated by an oxidative stress response. Dumay and his co-workers demonstrated that when using HeLa cells, etoposide ROS induction was initially a precursor of apoptosis and ROS levels were further increased following apoptotic cell signalling and mitochondrial membrane dysfunction, with subsequent mitochondrial ROS production accelerating programmed cell death. It has also been shown that etoposide apoptotic activity could be increased by addition of an inhibitor of the free radical scavenger super oxide dismutase (Dumay *et al.,* 2006), indicating that etoposide induced apoptosis is at least partly driven by ROS generation. Furthermore, later work, again with HeLa cells, demonstrated that etoposide induced apoptosis was reduced by the addition of a variety of antioxidants (Rincheval *et al.,* 2012). As these researchers have shown that antioxidants can ameliorate etoposide activity and free-radical scavengers can increase etoposide activity it is probable in mammalian cell culture etoposide induced ROS is at least partly responsible for etoposide cytotoxicity. So why weren't 8-oxoGua cleavable sites found in L5178Y mouse lymphoma cells? The most likely explanation is the p53 status of HeLa and L5178Y mouse lymphoma cells. HeLa cells are known to contain wild type and functional p53 (Hwang *et al.,* 1996) whereas L5178Y mouse

lymphoma cells are known to have a dysfunctional mutant variant p53 (Storer *et al.,* 1997). The exact mechanism of etoposide induced ROS is complex, but it is known that inhibition of caspase significantly reduces etoposide induced intracellular ROS levels (Rincheval *et al.,* 2012). Etoposide damaged DNA leads to p53 activation and subsequent mitochondrial dysfunction (Karpinich *et al.,* 2002), p53 controls caspase activity and release in mitochondria (Schuler *et al.,* 2000). Accordingly, it is at least plausible that the mutant variant p53 in L5178Y mouse lymphoma cells means that caspase activity is low and hence there are lower levels of intracellular ROS. Further work would be required to confirm the exact mechanism.

6.4 Conclusion

Whilst it was surprising that etoposide did not generate ROS induced 8-oxoGua sites on the DNA of L5178Y mouse lymphoma cells, it was clear that the associated genotoxicity of the topoisomerase II inhibitors investigated could not have been influenced by this mechanism. Hence it seems probable that the potent genotoxicity of etoposide and gemifloxacin seen *in vitro* when using L5178Y mouse lymphoma cells, is due to the agent's direct inhibition of topoisomerase II and the subsequent formation of stabilised cleavable complexes. Accordingly the observed discrepancy between concentrations which induce micronuclei and concentrations at which stabilised cleavage complexes can be measured, cannot readily be explained by an additional genotoxic contribution from chemically induced ROS.

CHAPTER 7

Preparation of antibodies to mouse topoisomerase II and assays to determine if use of these antibodies can improve detection of cleavable complexes in L5178Y mouse lymphoma cells

CONTENTS

7 Preparation of antibodies to mouse topoisomerase II and assays to determine if use of these antibodies can improve detection of cleavable complexes in L5178Y mouse lymphoma cells

7.1 Introduction

The aim of the work described in this chapter was to design mouse specific antibodies and to see if use of these antibodies could improve the sensitivity of detection of topoisomerase II poison induced stabilised cleavable complexes in the TARDIS and ICE assays.

The majority of the work in Chapter 5 focussed on trying to determine the relationship between topoisomerase II poison induced formation of cleavable complexes and mammalian cell genotoxicity in L5178Y mouse lymphoma cells and the work in Chapter 6 demonstrated that it was unlikely that ROS induction contributed to the genotoxicity of etoposide and gemifloxacin. However, there was one potential flaw with the antibody methods that were used for cleavable complex detection. As there are currently no commercially available antibodies to mouse topoisomerase II, the cleavable complex work used the commercially available antibodies to human topoisomerase II as a surrogate. This may not be a significant an issue. The topoisomerase II enzymes are very well conserved across most eukaryotes, with even greater conservation across mammalian species. The homology between mouse and human is over 89%, with the vast majority of the sequence variability being towards the C-terminal domain, see Figure 7.1 (Uni Prot, 2012). Furthermore, the human antibody previously used for TARDIS (kindly provided by Newcastle University) were derived from the N-terminal domain, hence would be expected to have at least 90% homology with mouse. Unfortunately the coding of oligopeptides used for preparation of this antibody was not available; hence the exact sequence homology could not be determined. Furthermore, the commercially available human topoisomerase II alpha antibody used for the ICE assay (supplied by TOPOGEN) was derived from a 16 residue oligopeptide at the C-terminus. From figure 7.1 we can estimate that this human antibody would have approximately 80% homology with mouse. With such reasonably high homology, it would be expected that this antibody would probably react with mouse topoisomerase II. Indeed, from the results seen in the ICE assay (see Chapter 5) the antibody used signalled well and demonstrated more sensitivity for

detection of topoisomerase II poisons than the antibody used in TARDIS. However, with only 80% homology it was at least feasible that cleavable complex detection could be improved if a more specific antibody to mouse topoisomerase II was available. Accordingly the preparation of a series of specific antibodies for mouse topoisomerase II was undertaken. Following preparation of these antibodies and following confirmation of their specificity, they were used to investigate whether the sensitivity of one or both of TARDIS and ICE could be improved for detection of stabilised cleavable complexes following treatment with etoposide and gemifloxacin.

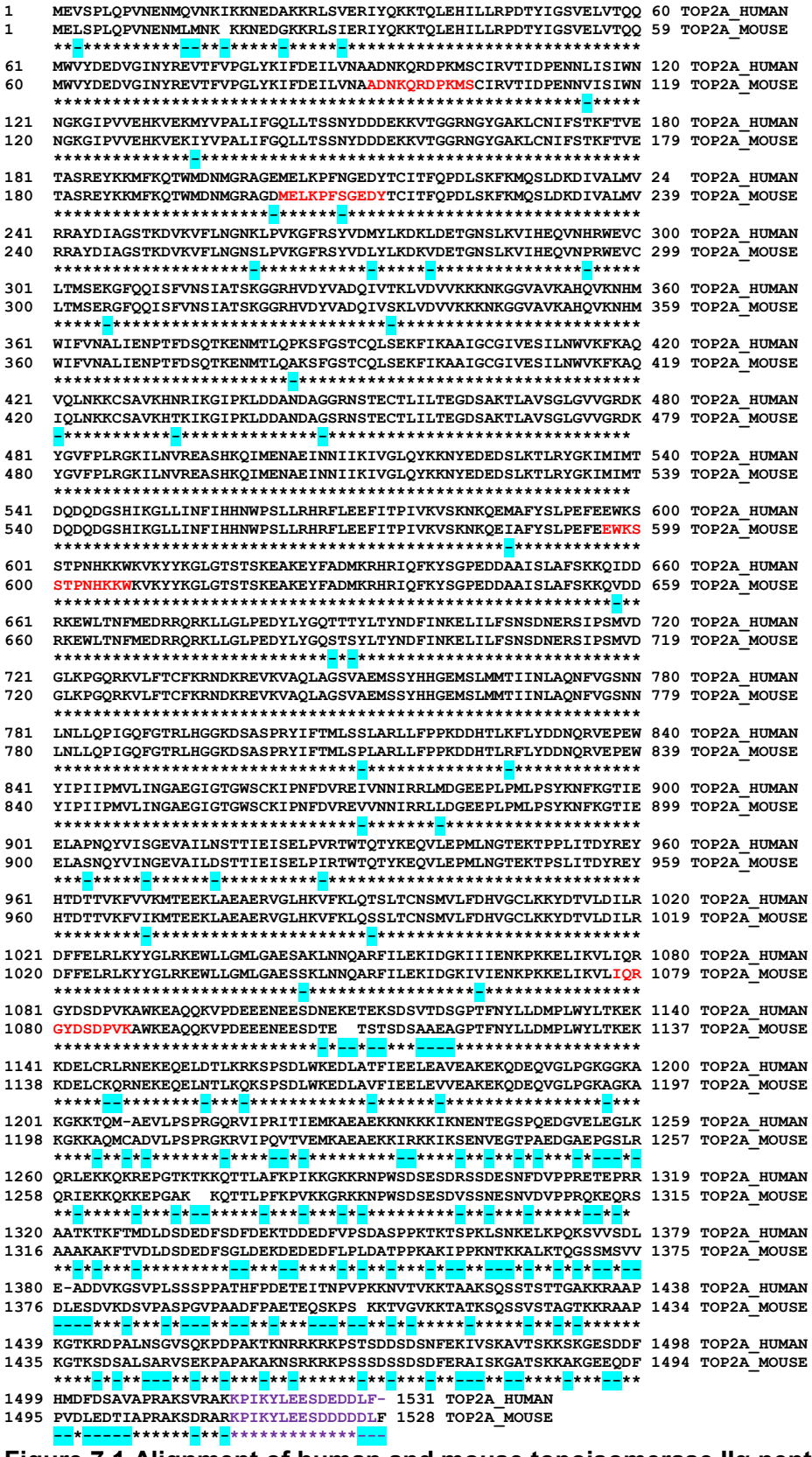


Figure 7.1 Alignment of human and mouse topoisomerase IIα peptides (Uni Prot, 2012)

* **Asterisk indicated similarity in human and mouse peptide sequence**

\- **Blue shading shows differences in human and mouse peptide sequence**

A **Red text are positions of peptides sequences used to develop mouse specific antibodies**

A **Purple text is probable position of peptide sequence of TOPOGEN human antibody**

7.2 Materials and methods

7.2.1 Design of antibodies

Figure 7.1 and Table 7.1 shows the sequence alignments of antigens designed for preparation of mouse specific topoisomerase II antibodies. These sequences were selected with the help of Sonia Houghton, AstraZeneca antibody group with the intention of having hydrophobic residues below 50%, good epitope accessibility and good target specificity. Table 7.1 and Figure 7.2 also show the specificity and homology between these sequences and topoisomerase II alpha and beta. As it was considered possible that epitope availability may be compromised when the enzyme is bound to DNA in a stabilised cleavage complex, the antibodies were also designed to span the whole protein. The intention being to see if antibody performance was relative to sequence position either at N-terminus, in the ATP or DNA binding regions or towards the C-terminus. It was realised that it would have been ideal to have antibodies specific to both topoisomerase II alpha and beta and a combined antibody with dual reactivity. However, antibody preparation is not a flawless procedure and given the time constraints of the project it was only possible to design and develop four antibodies. Accordingly, for confirmation of the work already conducted with antibodies to topoisomerase II alpha, it was considered to be of primary importance to ensure at least one highly sensitive mouse specific alpha antibody was obtained. Hence the sequences were not designed to obtain a specific beta antibody. It was hoped that the homology between alpha and beta sequences from antigen codes 3008415 and 3008424 would provide usable antibodies if there was a requirement to investigate topoisomerase II beta effects. Antibodies prepared from antigen codes 3008416 and 3008423 were designed to be topoisomerase II alpha specific (Table 7.1).

Antigen code	Peptide sequence	Specificity	Approximate position
3008415	ac-ADNKQRDPKMSC-nh2	Alpha 100% Beta 82%	N-terminus
3008416	ac-MELKPFSGEDYC-nh2	Alpha 100% Beta 55%*	N-terminus
3008423	ac-EWKSSTPNHKKWGC-nh2	Alpha 100% Beta 55%*	ATP binding domain
3008424	ac-IQRGYDSDPVKGC-nh2	Alpha 100% Beta 82%	DNA binding domain C-terminus

* Designed to be topoisomerase II alpha specific

Table 7.1 Design of mouse topoisomerase II antigens

The exact sequence specificity of these sequences for mouse and human topoisomerase alpha and beta is shown in Figure 7.2.

```
Peptide        ADNKQRDPKMS
Mouse 2a       ADNKQRDPKMS
Mouse 2b       ADNKQRDKNMT
Human 2a       ADNKQRDPKMS
Human 2b       ADNKQRDKNMT

Peptide        MELKPFSGEDY
Mouse 2a       MELKPFSGEDY
Mouse 2b       AKIKHFDGEDY
Human 2a       MELKPFNGEDY
Human 2b       AKIKHFNGEDY

Peptide        EWKSSTPNHKKW
Mouse 2a       EWKSSTPNHKKW
Mouse 2b       EWKKHIENQKAW
Human 2a       EWKSSTPNHKKW
Human 2b       EWKKHIENQKAW


Peptide        IQRGYDSDPVK
Mouse 2a       IQRGYDSDPVK
Mouse 2b       VQRGYESDPVK
Human 2a       IQRGYDSDPVK
Human 2b       VQRGYESDPVK
```

Figure 7.2 Sequence specificity of designed peptides
Yellow shading indicates peptide differences
Mouse 2a: Mouse topoisomerase II alpha
Mouse 2b: Mouse topoisomerase II beta
Human 2a: Human topoisomerase II alpha
Human 2b: Human topoisomerase II beta

7.2.2 Preparation of antibodies

Oligopeptide constructs of the sequences shown in figure 7.1 were prepared by Bachem, St Helens, UK (a contract research group who specialise in peptide sequence construction). These constructs contained an additional C-terminal cysteine to aid binding. The prepared peptide sequences were sent to the contract research laboratory Harlan, UK, who were responsible for the *in vivo* antibody preparation. For the initial immunisation, three New Zealand White rabbits received a sub-cutaneous injection of 100 µg of each antigen (i.e. 12 rabbits used) in the immunostimulant Freund's complete adjuvant. Four further boosts of 100 µg antigen in Freunds incomplete adjuvant (immunostimulant without mycobacteria) were administered sub-cutaneously at the time points indicated in Table 7.2. Over the course of dosing two test bleeds were performed and samples sent back to AstraZeneca, Antibody group for initial confirmation of antibody production (data not shown).

Day	Procedure
0	Pre Bleed & Immunisation
14	Boost 1
28	Boost 2
35	Test Bleed 1
42	Boost 3
56	Test Bleed 2
77	Boost 4
84	Terminal Bleed

Table 7.2 dosing schedule for antibody preparation

On Day 84 the production bleed was taken and the serum harvested and antibodies prepared and purified by AstraZeneca, Antibody group, and the protein content of each antibody determined. The given codes and protein concentration of each antibody is shown in Table 7.3. Preliminary work by AstraZeneca, Antibody group, demonstrated that for most standard usage (e.g. Western Blots) a 1:1000 dilution of each antibody was appropriate and this dilution was used for the Western Blot and ICE bioassay work performed for this project. As the sensitivity of TARDIS is known to be low, a dilution of 1:100 (as previously used with the human antibody) was considered appropriate. For TARDIS work where comparison of antibody performance was required antibodies were normailsed to a final concentration of 10 µg protein per mL. For other work on antibody comparisons, antibodies were normalised to 1 µg protein per mL.

Rabbit ID	Peptide sequence	Antibody code	Protein concentration (mg/mL)
3661-3	ADNKQRDPKMS	AGG-H3661	1.29
3661-3	ADNKQRDPKMS	AGG-H3662	0.9
3661-3	ADNKQRDPKMS	AGG-H3663	0.91
3664-6	MELKPFSGEDY	AGG-H3664	1.27
3664-6	MELKPFSGEDY	AGG-H3665	0.9
3664-6	MELKPFSGEDY	AGG-H3666	1.05
3667-9	EWKSSTPNHKKW	AGG-H3667	0.82
3667-9	EWKSSTPNHKKW	AGG-H3668	0.98
3667-9	EWKSSTPNHKKW	AGG-H3669	0.64
3670-21	IQRGYDSDPVK	AGG-H3670	1.17
3670-21	IQRGYDSDPVK	AGG-H3671	1.35
3670-21	IQRGYDSDPVK	AGG-H3672	1.03

Table 7.3 Antibody coding and protein content

7.2.3 SDS-PAGE and Western Blot experiments

SDS-PAGE Western blots were performed as detailed in the Material and Methods section 2.13.1.

7.3 Results and discussion

7.3.1 Affinity of mouse antibody to human topoisomerase II alpha

The results of a Fuji 1 minute scan of membranes prepared with each antibody against human topoisomerase II alpha (mouse topoisomerase II alpha was not commercially available) and the relevant Magic Marker ladder are shown in Figure 7.3. This assay was repeated and qualitatively similar results were obtained.

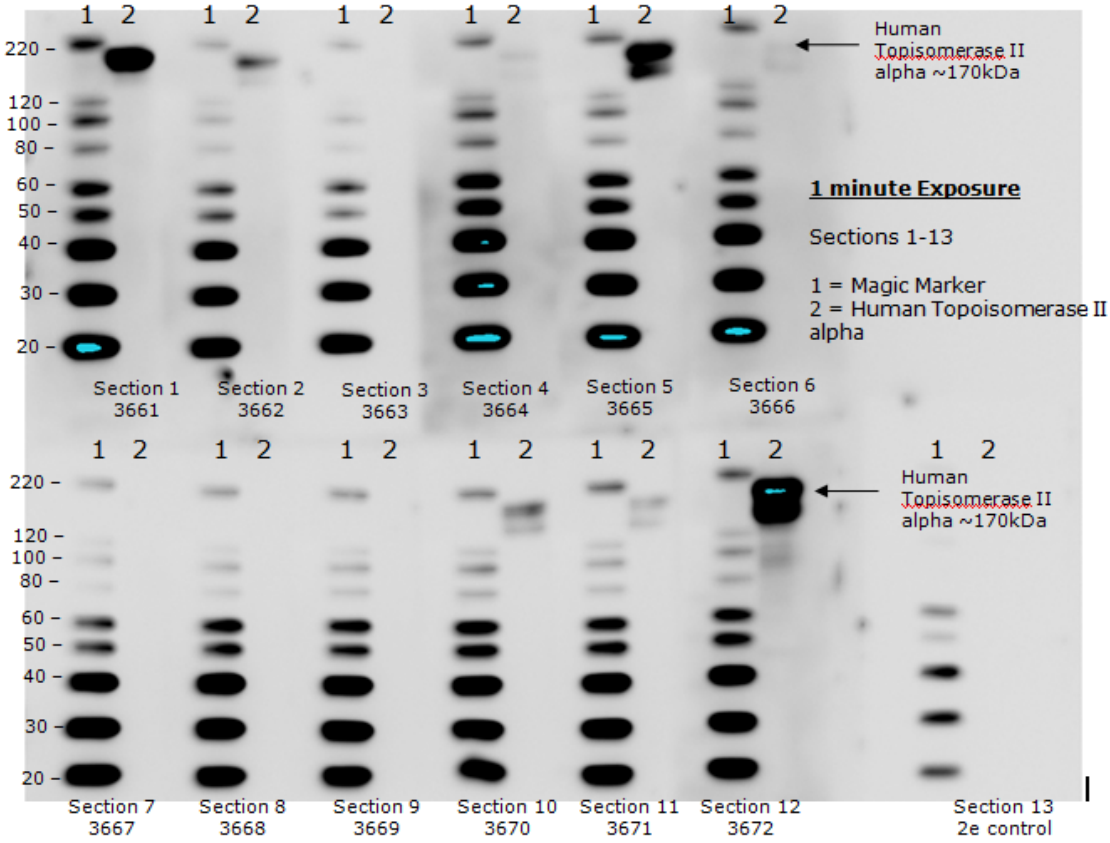


Figure 7.3 Image of ECL (chemeluminescence) stained membranes of each prepared mouse antibody against human topoisomerase II alpha (1 minute exposure)
Lane 1 Magic Marker ladder for peptide size
Lane 2 Human topoisomerase II alpha
Arrow indicates approximate position of 170 KDa human topoisomerase II alpha

The results of the antibody affinity check showed that the majority of the antibodies were effectively binding to topoisomerase II alpha. However, these data suggested there was both intra-peptide and inter-peptide sequence binding variability. No discernable response was seen with antibodies 3667, 3668 and 3669 in either of the tests performed. These antibodies were all prepared from rabbits dosed with peptide series EWKSSTPNHKKW. This sequence shared 100% homology between human and mouse; hence this cannot explain the lack of binding of these antibodies against human topoisomerase II alpha. From each of the other three peptide sequences used, there appeared to be one antibody that bound with significantly higher affinity, these being 3661 for sequence ADNKQRDPKMS, 3665 for sequence MELKPFSGEDY and 3672 for sequence IQRGYDSDPVK. Although peptide sequence ADNKQRDPKMS did not share 100% homology between human and mouse this did not appear to affect its affinity. Given the binding variability of these antibodies, all 12 were used to

investigate the response of 10 µmol/L etoposide to form cleavable complexes as detected by the TARDIS assay.

7.3.2 Mouse antibodies used in the TARDIS assay

As the TARDIS assay had previously showed low sensitivity, hence had 'more room for improvement', and as the assay was less time consuming and technically demanding than the ICE assay, it was used for the initial screening of the 12 mouse specific antibodies. Figure 7.4 shows the results of the preliminary screen using solvent control and 10 µmol/L etoposide in the TARDIS assay.

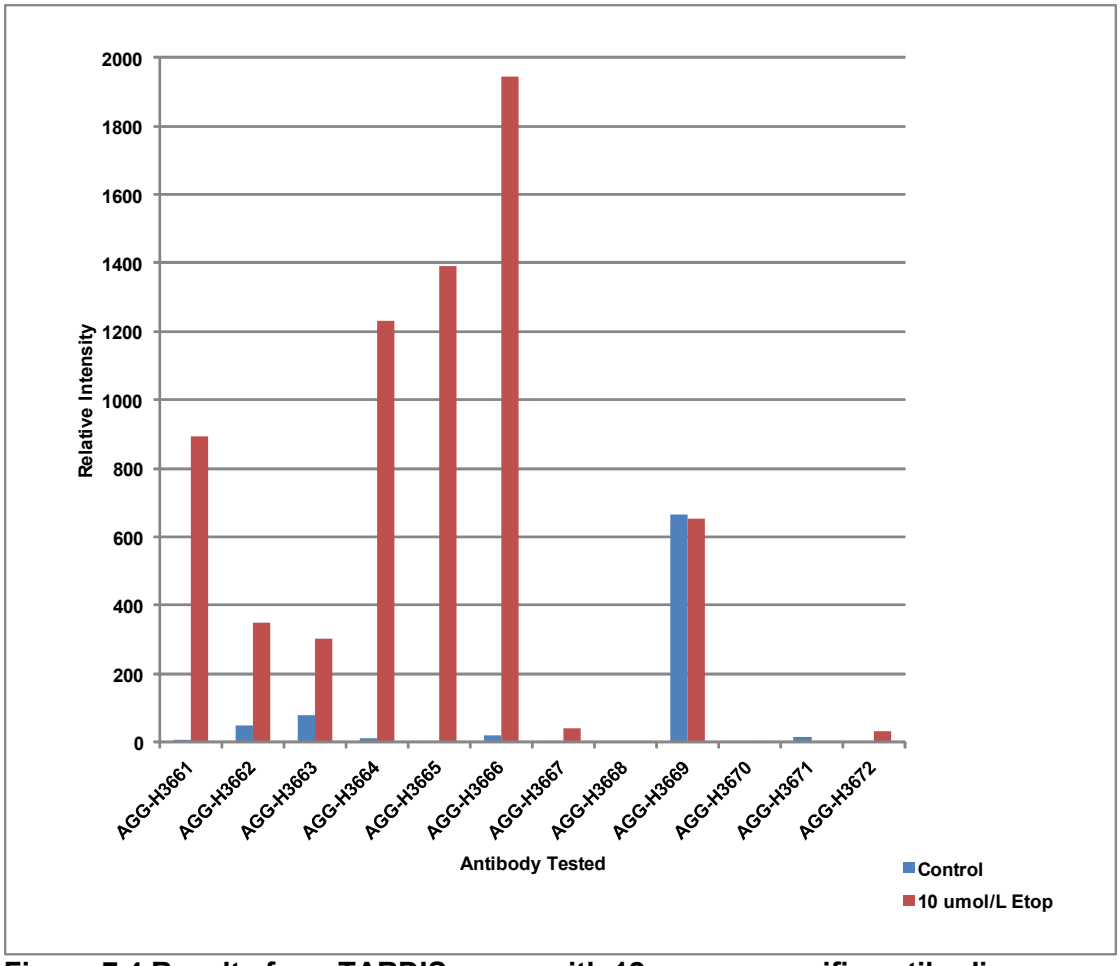


Figure 7.4 Results from TARDIS assay with 12 mouse specific antibodies. Single cultures tested so error bars not applicable
Relative intensity = FITC signal from analysed nuclei

The TARDIS response with antibodies 3661-3666 generally agreed with the PAGE-SDS Western work in as much as these antibodies all signalled well with the high concentration of etoposide used. There were some not unexpected variance in the qualitative response between the Westerns and TARDIS, but this was considered to be within the realms of variability expected between two assay types when targeting proteins from two different species. Similarly the response of antibodies 3667-3669

was in general agreement with the low/non-existent response in the Westerns, in as much as 3667 and 3668 did not really respond at all in TARDIS and 3669 appeared to have some non-specific binding with the signal being as high in the absence of etoposide as it was in the presence. Perhaps the most interesting response in this preliminary TARDIS work was the response of antibodies 3670-3672. These antibodies responded well in the Western work with human topoisomerase II, but did not signal in the TARDIS assay with mouse lymphoma L5178Y cells.

To confirm the response in the initial test with all 12 antibodies, the antibody that responded best in TARDIS from each peptide sequence was selected to determine an etoposide concentration response. For comparison, the human antibody used for the original TARDIS experiments as detailed in Chapter 5 was also included in this test. The results of the etoposide concentration response with mouse antibodies 3661, 3666, 3667, 3672 and the topoisomerase II human antibody supplied by Newcastle University are shown in Figure 7.5.

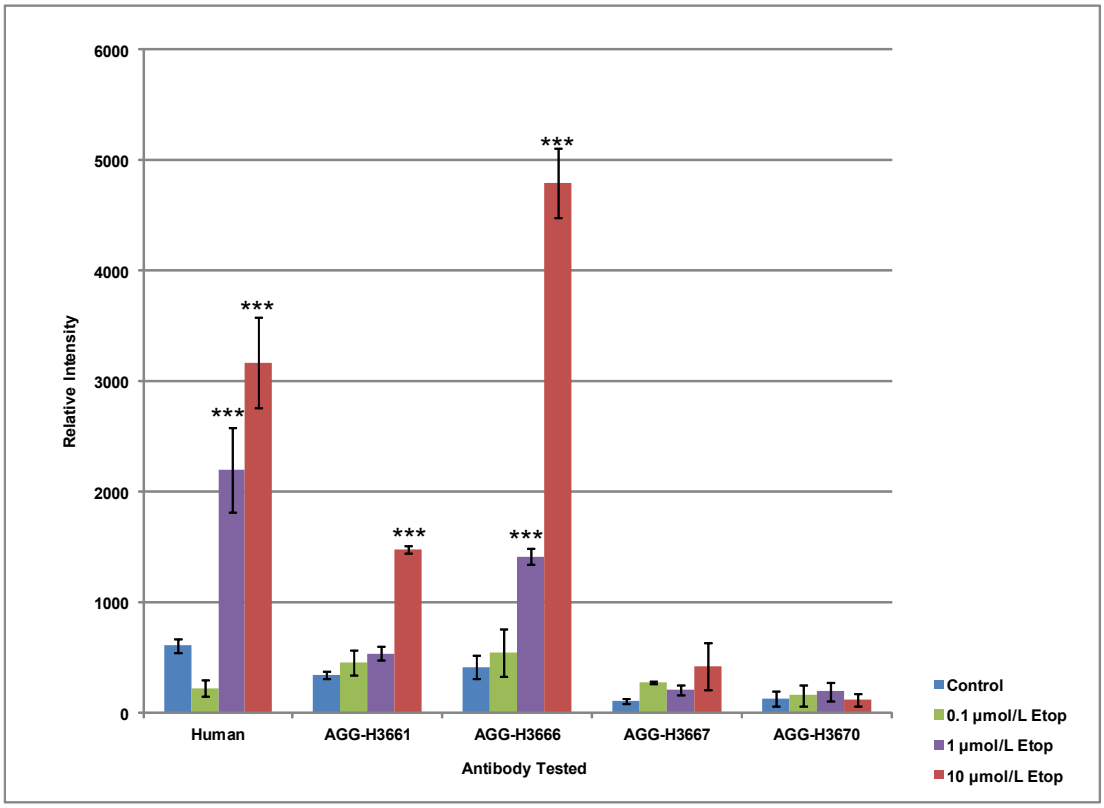


Figure 7.5 Results from TARDIS assay using etoposide with mouse antibodies derived from each peptide sequence and the human antibody supplied by Newcastle University.
Relative intensity = FITC signal from analysed nuclei
n per test = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

The results of this second experiment confirmed that in TARDIS, antibodies derived from sequences ADNKQRDPKMS and MELKPFSGEDY performed better than those derived from EWKSSTPNHKKW and IQRGYDSDPVK. The lack of response of the antibody derived from IQRGYDSDPVK was still surprising as this antibody had performed well against free human topoisomerase II alpha. Whilst two of the four mouse antibodies did signal etoposide induced cleavable complex formation in the TARDIS, they did not consistently outperform the human antibody previously used. However, antibody 3666 did show a somewhat increased signal at the highest concentration of etoposide (10 µg/mL). Accordingly, this antibody was used in a further test to see if use of a mouse specific antibody in TARDIS could indentify gemifloxacin cleavable complex formation. This had not been possible with the previous work with the human antibody (see Chapter 5). The results of the test for gemifloxacin induced cleavable complex formation using mouse antibody 3666 are shown in Figure 7.6. In this test 10 µmol/L etoposide was used as a positive control.

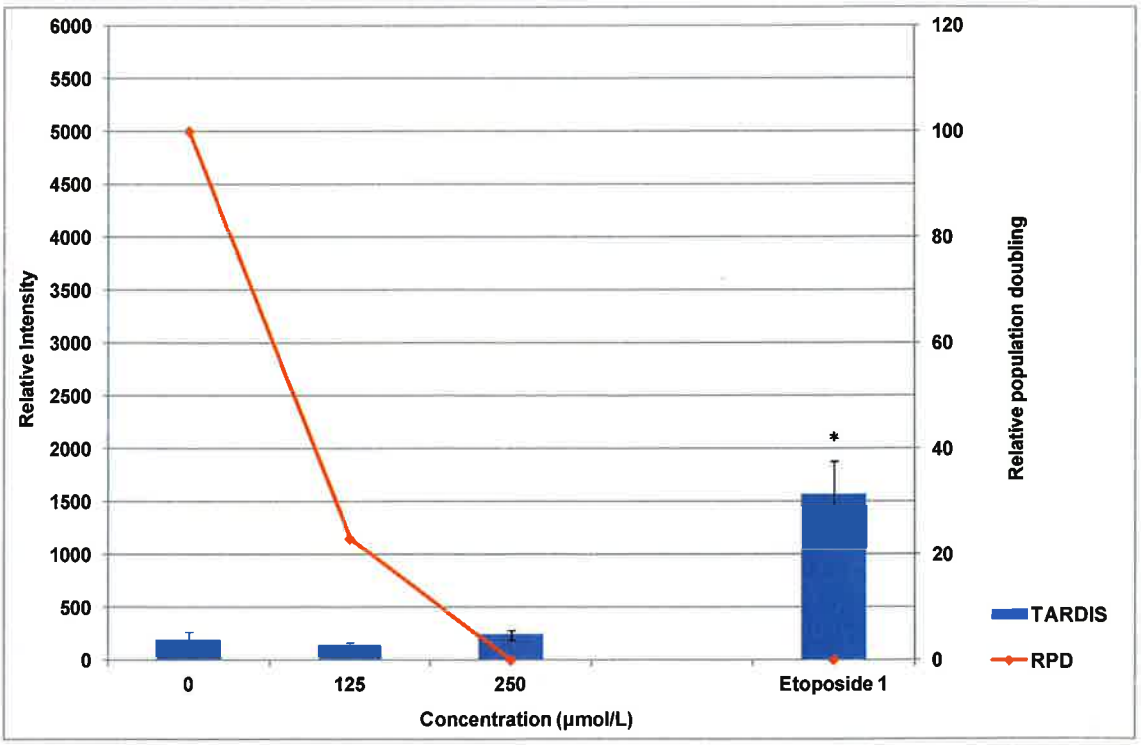


Figure 7.6 Results from TARDIS assay using gemifloxacin with mouse antibody 3666

Relative intensity = FITC signal from analysed nuclei
n per test = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

In this test cleavable complex formation could not be detected with gemifloxacin. It should be noted that two higher concentrations of gemifloxacin were tested that are not presented in Figure 7.6, these were 500 and 1000 µg/mL. Both of these

of etoposide in this test confirmed that the antibody used was performing well. Accordingly, when tested up to highly cytotoxic concentrations, with a mouse specific antibody, gemifloxacin induced formation of stabilised cleavable complexes could not be detected by the TARDIS assay. Although only a single confirmatory assay was performed, the generated data was in agreement with the earlier tests described in Chapter 5. Accordingly, further repeat testing was not considered to be necessary.

7.3.3 Mouse antibodies used in the ICE assay

Whilst the mouse antibodies did not improve the overall sensitivity of the TARDIS assay, because of the high response of antibody 3666 it was considered to be worthwhile pursuing whether use of this antibody could improve the sensitivity of the ICE assay. Furthermore, it was also considered worthwhile to investigate whether the lack of response of antibody 3670 for detection of cleavable complexes in nuclear preparations was mirrored by its activity in the ICE assay. Accordingly, a preliminary ICE assay was performed with etoposide using antibodies 3666 and 3670, the results of which are presented in Figures 7.7 ad 7.8, respectively.

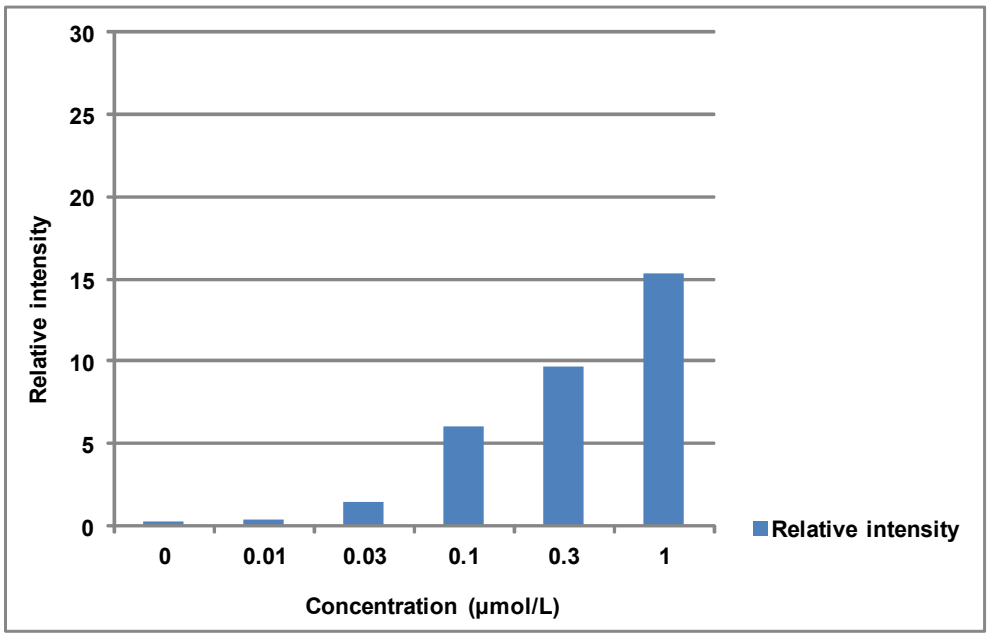


Figure 7.7 Results from preliminary ICE assay using etoposide with mouse antibody 3666

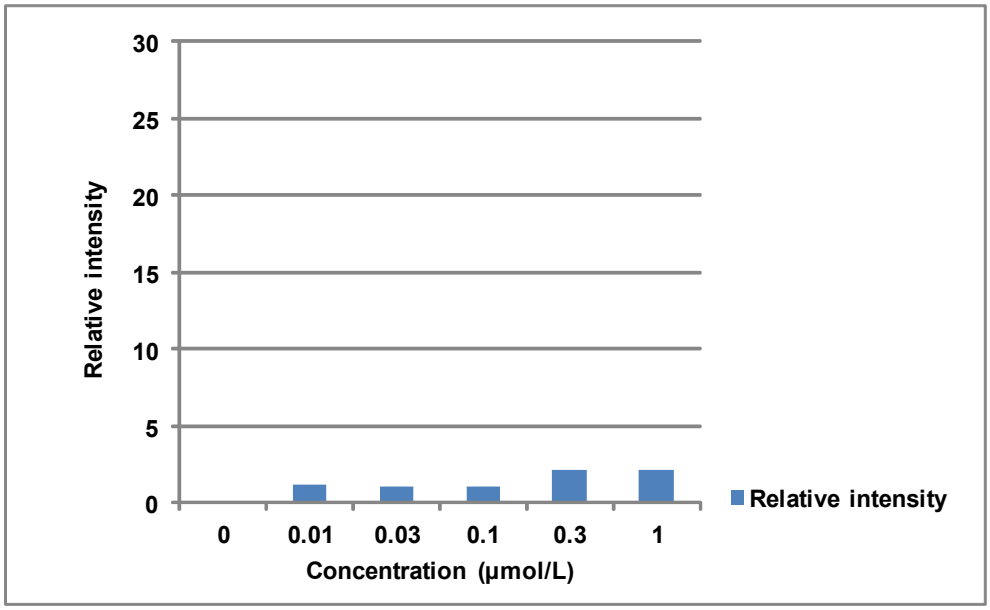


Figure 7.8 Results from preliminary ICE assay using etoposide with mouse antibody 3670
Relative Intensity = Mean Odyssey IR signal

The preliminary work in the ICE assay with antibodies 3666 and 3670 confirmed the TARDIS response with these enzymes, in so much as a clear etoposide concentration-response in relative intensity was seen with antibody 3666 but no consistent concentration-related increase in relative intensity was seen with 3670. The very low control response seen in this preliminary work also gave an indication that this antibody may improve the sensitivity of the ICE assay for detection of etoposide induced cleavable complexes. Accordingly, further ICE tests were performed with antibody 3666. The complete data from triplicate ICE assays performed with etoposide using antibody 3666 are shown Figures 7.9 and 7.10.

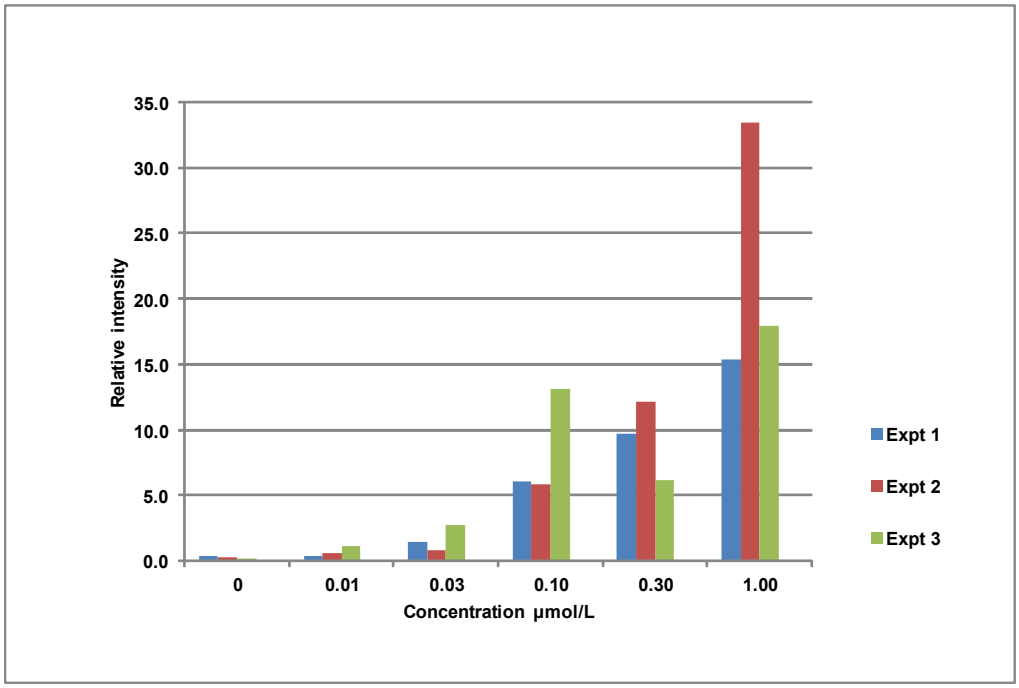


Figure 7.9: Results from three ICE tests with etoposide using antibody 3666
Relative Intensity = Mean Odyssey IR signal

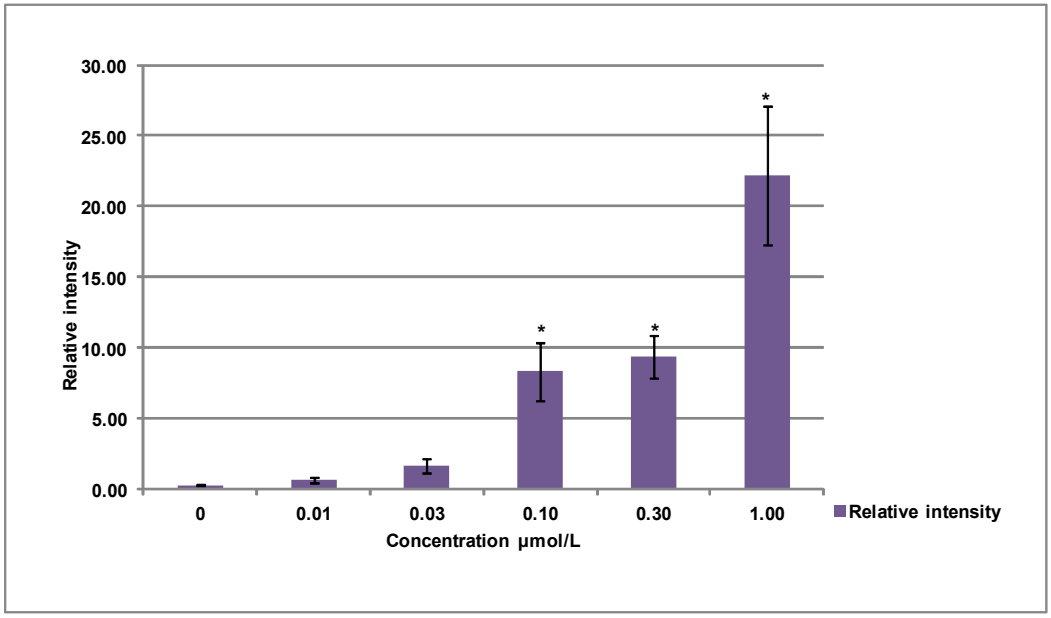


Figure 7.10: Mean ICE assay data from three ICE tests with etoposide using antibody 3666
Relative Intensity = Mean Odyssey IR signal
n = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

The lowest concentration of etoposide where a statistical significant increase in relative intensity was seen was 0.1 µmol/L, similar to the previous work with the human antibody (Chapter 5). However, although 0.03 µmol/L etoposide did not quite achieve statistical significance at the 5% level, the p value of the increase was 0.07. Accordingly, the marked increase in response in three independent ICE assays with

etoposide at 0.03 µmol/L was considered to be biologically significant. Hence using mouse antibody 3666 the LOCCEL for etoposide was considered to be at the same concentration as the LOGEL for etoposide. This being the first time that a clear concentration equivalence could be proven for genotoxicity and cleavable complex formation with this agent. Furthermore, the sensitivity of the ICE assay had been improved by use of the designed mouse specific antibody. Further work was then performed to see if a similar improvement in the detection of cleavable complexes in the ICE assay could be seen following gemifloxacin treatment. Figures 7.11 and 7.12 show data from three ICE assays with gemifloxacin using antibody 3666.

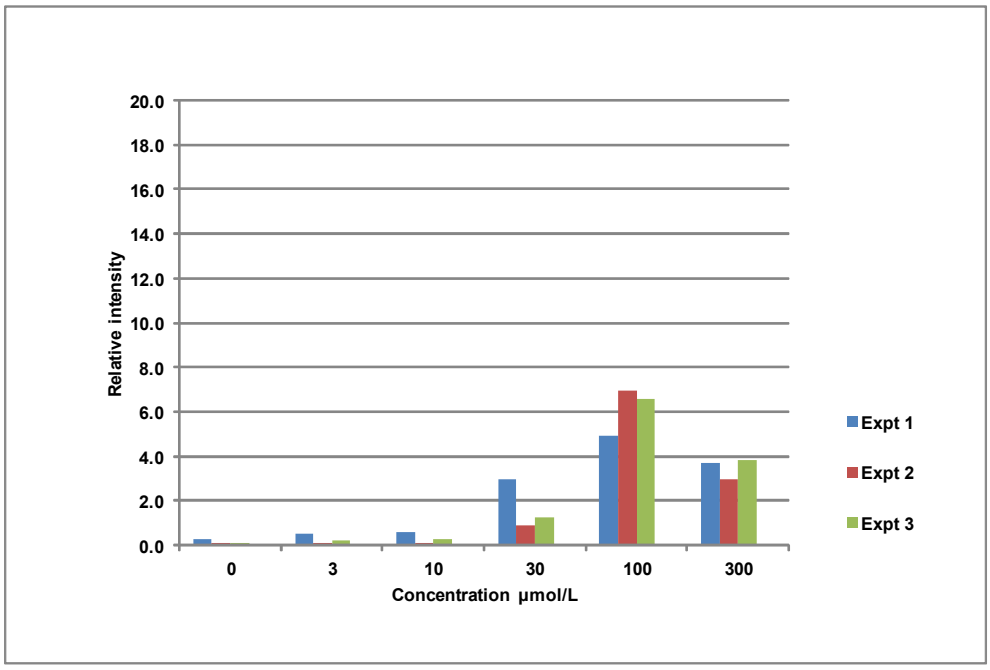


Figure 7.11: Results from three ICE tests with gemifloxacin using antibody 3666
Relative Intensity = Mean Odyssey IR signal

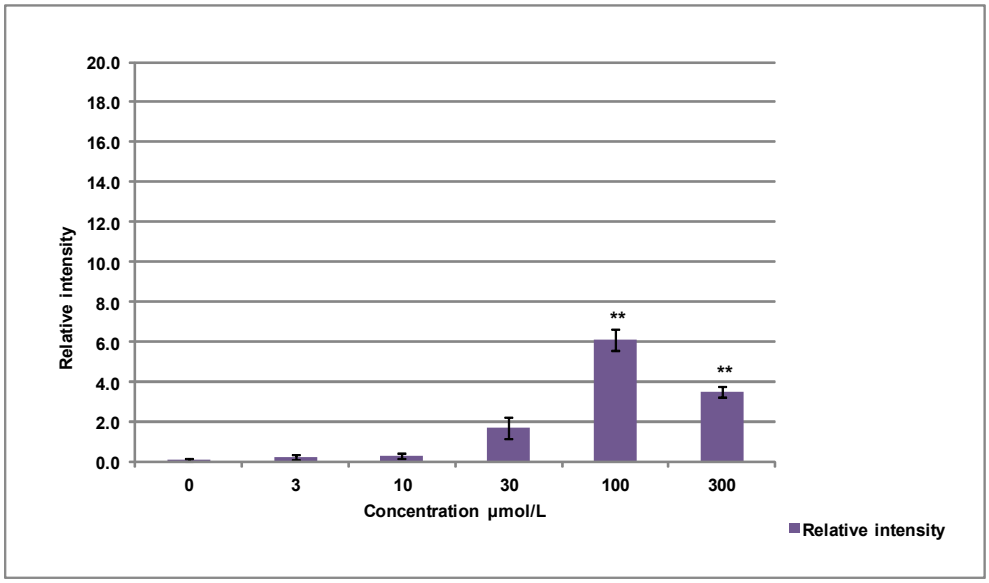


Figure 7.12: Mean ICE assay data from three ICE tests with gemifloxacin using antibody 3666
Relative Intensity = Mean Odyssey IR signal
n = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

The lowest concentration of gemifloxacin where a statistical significant increase in relative intensity was seen was 100 µmol/L, which once again was similar to the previous work with the human antibody (Chapter 5). However, similar to the response of etoposide at 0.03 µmol/L, although 30 µmol/L gemifloxacin did not quite achieve statistical significance at the 5% level, the p value of the increase was only just above i.e. 0.053. Accordingly, the marked increase in response in three independent ICE assays with gemifloxacin at 30 µmol/L was considered to be biologically significant. However, unlike etoposide this LOCCEL for gemifloxacin was still above the determined LOGEL (i.e. 10 µmol/L) and 10 µmol/L was clearly a non-responding concentration in the ICE assay even when the mouse antibody 3666 was used.

To ensure that the improvement in the sensitivity of detection of topoisomerase II poisons in the ICE assay using the mouse antibody was not just due to confounding factors such as non-specific binding which may not be related to topoisomerase II cleavable complex recognition, three further independent tests were run with 4-NQO. The results of these tests are shown in Figures 7.13 and 7.14.

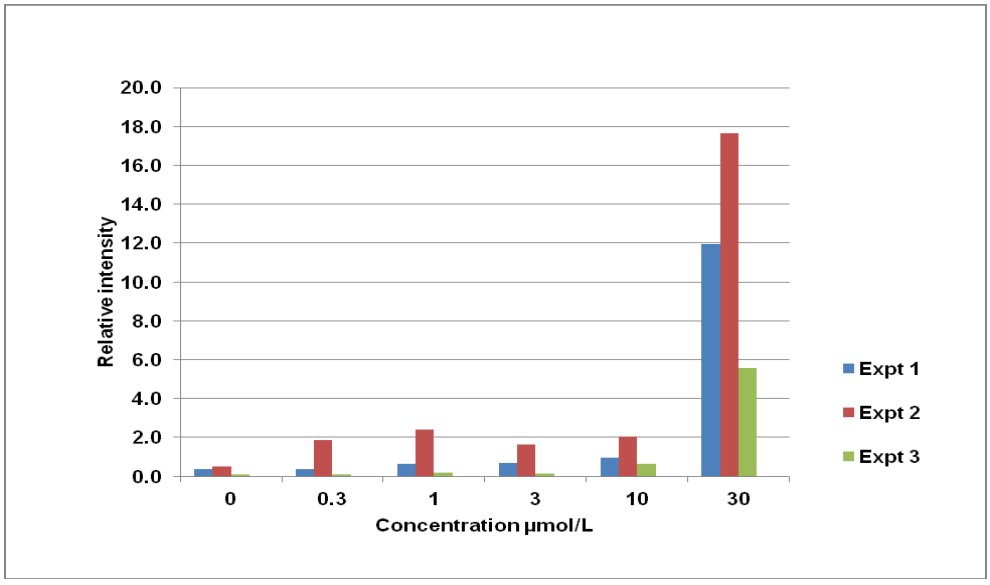


Figure 7.13: Results from three ICE tests with 4-NQO using antibody 3666
Relative Intensity = Mean Odyssey IR signal

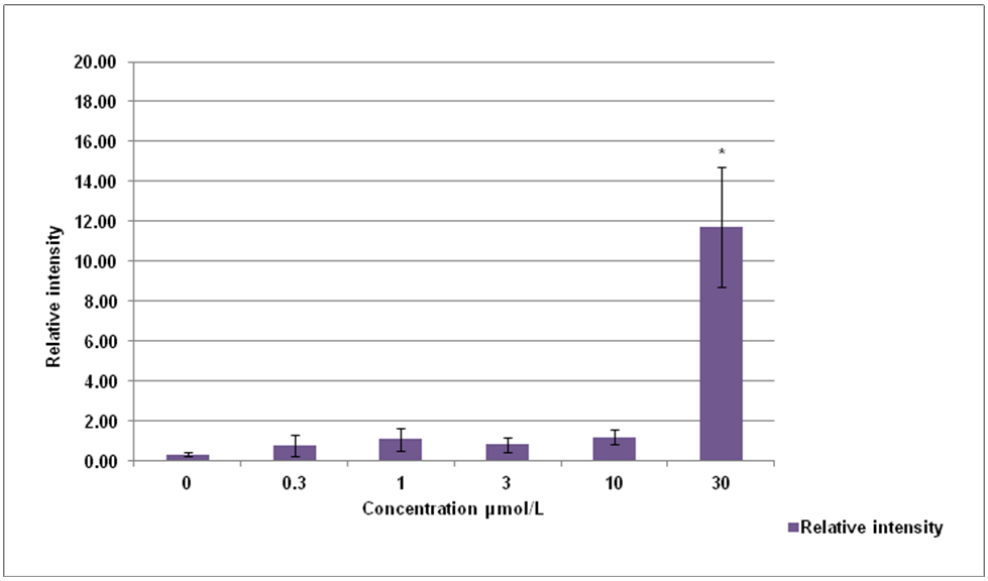


Figure 7.14: Mean ICE assay data from three ICE tests with 4-NQO using antibody 3666
n = 3. Statistics: T-test was used with square root transformation. Significance Level: *P<0.05 **P<0.01 ***P<0.001

The ICE assay with 4-NQO using the designed mouse antibody 3666 confirmed the results previously seen with the human antibody (Chapter 5). That is; a significant increase in topoisomerase II stabilised cleavable complexes was detected at the highest concentration tested (300 µmol/L), a concentration which had previously been shown to be highly cytotoxic. However, no significant increase was seen at any of the lower concentrations. Previously, with use of the human antibody, a significant increase in stabilised cleavable complexes had also been seen at 10 µmol/L (Chapter 5), no significant increase was seen at 10 µmol/L using mouse antibody 3666.

This may have been further evidence of the improved sensitivity for detection of specific topoisomerase II poison induced stabilised cleavable complexes.

It was demonstrated that the improved ICE sensitivity with antibody 3666 for detection of stabilised cleavable complexes induced by topoisomerase II poisons (etoposide and gemifloxacin) was not seen with the reference mutagen 4-NQO. Indicating that use of the specifically designed mouse antibodies gave a real improvement in the ICE assays ability to measure cleavable complex formation and to distinguish between induction of stabilised cleavable complexes by topoisomerase II poisons and other non-specific mechanisms such as those induced by the mutagen NQO.

7.3.4 Affinity of each prepared mouse antibody to mouse topoisomerase II alpha from L5178Y Mouse Lymphoma cell lysates

Whilst the TARDIS work with the mouse specific antibodies did not demonstrate improved sensitivity for detection of stabilised cleavable complexes, it had shown an intriguing discrepancy for the affinity of the antibodies to the DNA bound topoisomerase II in TARDIS as compared to the free human topoisomerase II used for the preliminary SDS-PAGE Western blot screening, particularly for the antibody derived from IQRGYDSDPVK. Furthermore, this discrepancy was confirmed in the ICE assay for antibodies 3666 and 3670. There was of course one simple explanation for this, in as much as one target was mouse and the other human, although it should be noted that the particular peptide sequence used to derive the antigen (IQRGYDSDPVK) did share 100% homology between human and mouse (see Figure 7.2). However, to confirm that it was not species specificity that was affecting the performance of the antibodies in TARDIS, cell extracts were prepared from L5178Y mouse lymphoma cells and SDS-PAGE Western blots were performed against all of the 12 antibodies. See Material and Methods section 2.13.1.2 for details of lysate preparation. The human topoisomerase IIα antibody that had previously been used for the Western work presented in Figure 7.3 was used for comparison. The results of a Fuji 30 second scan of membranes prepared with each antibody against L5178Y mouse lymphoma cell lysates and the relevant Magic Marker ladder are shown in Figure 7.15. This assay was repeated with similar results.

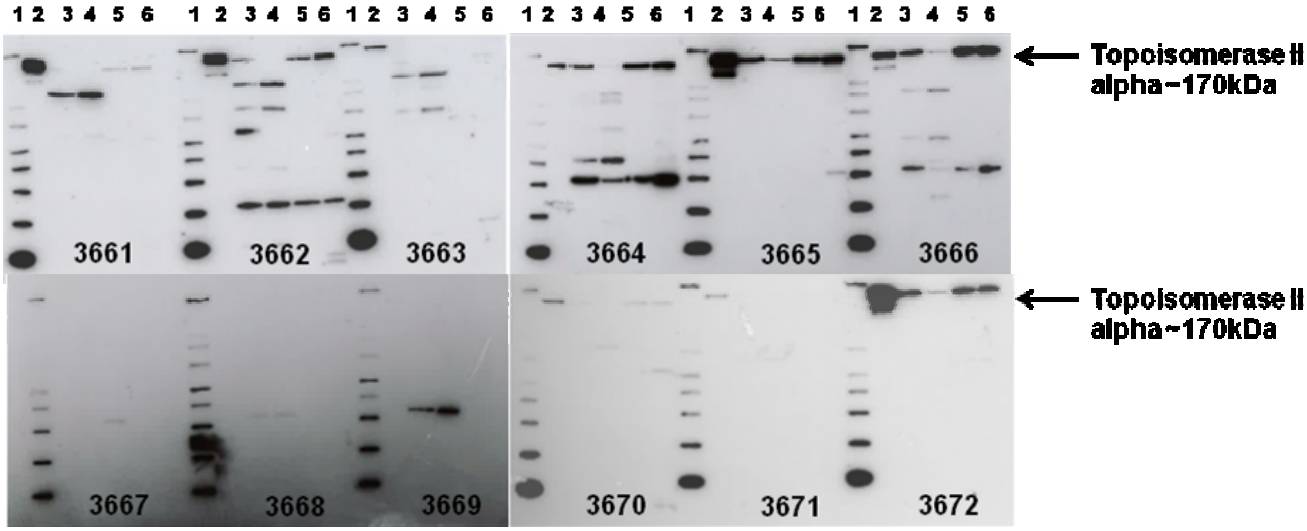


Figure 7.15 Image of ECL stained membranes of each prepared mouse antibody (3661-3670) against L5187Y mouse lymphoma cell lysates (30 second exposure)
Lane 1 Magic Marker ladder for peptide size
Lane 2 Human topoisomerase IIα
Lane 3 L5178Y cell sarkosyl lysate
Lane 4 L5178Y cell cytosol preparation
Lane 5 L5178Y cell nuclear preparation
Lane 6 L5178Y cell residual pellet after nuclear and cytosol preparations
Arrow indicates approximate position of 170 KDa human topoisomerase II alpha

The work with cell lysates confirmed that, as expected, the majority of topoisomerase II is found in the nuclear fraction (Mirski *et al.,* 2007). It also demonstrated that two of the antibodies (3665 and 3672) responded very well and were very specific for mouse topoisomerase IIα, although as 3672 did not work well in the cleavable complex assays, 3665 was considered to be the better candidate for future work. It should be noted that these data were not considered to have compromised any of the work that had already been performed in the cleavable complex assays with the seemingly slightly less specific antibody 3666, in so much as the sensitivity in the TARDIS assay was not improved by use of any of the mouse antibodies over the human enzyme indicating non-specific protein binding was not affecting the results. Furthermore, the DNA fraction used for analysis in the ICE assay was confirmed to be of very high purity ($Abs_{260}/Abs_{280} \approx 2$), hence results would not likely to have been affected by protein contaminated DNA. The very low background control level seen in the ICE work with the mouse antibodies used (3666 and 3670) also confirmed non-specific protein binding was not an issue.

In general the work with the mouse lymphoma cell lysates confirmed the work performed with the commercially available human topoisomerase II, albeit that antibodies 3661-3663 performed a little worse than expected against the cell lysates.

The antibodies that did not perform well in TARDIS (3667-3672) and ICE (only 3670 tested) generally demonstrated low affinity for mouse lysate topoisomerase II and at least for 3670-3672 performed better against human topoisomerase II enzyme. However, 3672 did perform well against both the human enzyme and mouse cell lysates, hence, its lack of response in TARDIS was unlikely to be due to species specificity in binding. Whilst the data do not support definitive conclusions, in general those antibodies designed to target sequences towards the n-terminal of the enzyme performed better. The lack of binding of 3672 seen in TARDIS and ICE could be due to the conformation of the protein during DNA binding. The targeted sequence of 3372 is just beyond the ATP binding domain and towards the C terminus where in its closed clamp form the enzyme would bind with the gate DNA strand, it is at least possible that this would directly affect availability of the epitope.

7.4 Conclusion

It was shown to be possible to prepare highly specific antibodies for mouse topoisomerase alpha and there was limited evidence to suggest that when preparing antibodies for detection of topoisomerase II induced stabilised cleavage complexes, sequence targets towards the n-terminus of the enzyme were preferable. Some improvement of the sensitivity of the ICE assay was seen when using antibodies of high affinity for mouse topoisomerase II alpha and for the first time a direct dose relationship between formation of stabilised cleavage complexes and micronucleus induction was demonstrated for etoposide. However, little improvement was seen with the sensitivity of the TARDIS assay and it was still not possible to detect gemifloxacin in this test. Accordingly, for workers interested in elucidating the potential of unknown agents to induce genotoxicity via inhibition of topoisomerase II, the use of highly specific antibodies (possibly designed to target n-terminal sequences) in the ICE assay is recommended.

CHAPTER 8

The relationship between the genotoxicity of the topoisomerase II poisons etoposide and gemifloxacin and their activity against the alpha isoform of the enzyme

CONTENTS

8 The relationship between the genotoxicity of the topoisomerase II poisons etoposide and gemifloxacin and their activity against the alpha isoform of the enzyme

8.1 Introduction

The aim of the work described in this chapter was to demonstrate that the *in vitro* mammalian cell genotoxicity of etoposide and gemifloxacin was driven by their activity against the alpha isoform of topoisomerase II.

Since 2012 several papers have been published indicating a link between the genotoxicity of etoposide and its activity against the beta isoform of topoisomerase II (Cowell and Austin., 2012a; Cowell and Austin., 2012b; Cowell *et al*., 2012, Smith *et al.,* 2014). This seriously questioned much of the research undertaken in the current project. The assumption had been made that due to the rapid division rate of L5178Y cells and the apparent significantly higher intracellular level of topoisomerase II alpha when compared to beta (Chapter 5 Figure 5.11), genotoxicity was driven by the alpha isoform of the enzyme. Accordingly the vast majority of the comparative work between the genotoxicity of etoposide and gemifloxacin and their ability to poison topoisomerase II and induce stabilised cleavage complexes, used antibodies to topoisomerase II alpha. This assumption appeared to be backed up by the Western, TARDIS and ICE work detailed in Chapter 7, where the most active antibodies (3664, 3665 and 3666) were highly topoisomerase II alpha specific. For these antibodies sequence homology to topoisomerase II alpha was 100% but only 55% to topoisomerase II beta (Chapter 7 Table 7.1). However, if the mammalian cell *in vitro* genotoxicity of topoisomerase II poisons was actually driven by their activity against the beta isoform of the enzyme, this would go some way to explaining why it has proven to be difficult to observe stabilised cleavage formation (using antibodies specific to topoisomerase II alpha) at concentrations similar to or lower than those that are genotoxic.

The recent publications linking topoisomerase II beta to genotoxicity have in the main involved investigating the activity of toposimerase II poisons in inducing the chromosome translocations that have been associated with secondary leukemias (Cowell and Austin., 2012a; Cowell and Austin., 2012b; Cowell *et al.,* 2012, Smith *et al.,* 2014). Generally, this work has focussed on the *in vitro* activity of the enzyme

isoforms and has used human pre-B leukemia Nalm-6 cells including; wild type, topoisomerase II alpha heterozygous and topoisomerase II beta null. For example Cowell *et al* demonstrated etoposide *in vitro* genotoxicity was significantly reduced in Nalm-6 cells without topoisomerase II beta activity (i.e. Nalm-$6^{topo2\beta-/-}$) when compared to Nalm-6 wild type or Nalm-6 cells heterozygous at the topoisomerase II alpha locus (i.e. Nalm-6 $^{topo2\alpha+/-}$). It was interesting to note that Cowell *et al* used cell lines with specific mutations in the coding genes for the topoisomerase II isoforms rather than using knockdown of the gene. Accordingly, as topoisomerase IIα null cells would not be viable, Cowell *et al* were not able to, or did not, demonstrate directly comparable activity between the two topoisomerase II isoforms. Nor did they actually report the relative intracellular enzyme levels of each isoform. For the purpose of the current research it was clearly important to establish whether the data reported for the activity of topoisomerase II isoforms and genotoxicity in Nalm-6 cells was also relevant for topoisomerase II poison associated genotoxicity in L5178Y mouse lymphoma cells i.e. does topoisomerase II alpha really not play a significant role in driving the genotoxicity of etoposide and gemifloxacin in L5178Y cells? To investigate the role of topoisomerase II alpha in the associated genotoxicity of topoisomerase II poisons, a topoisomerase II alpha specific small interfering RNA (siRNA) was custom made and the genotoxic activity of etoposide and gemifloxacin was investigated in cells following transfection of this siRNA or transfection of a negative control random scrambled siRNA.

8.2 Topisomerase IIα knockdown by siRNA transfection: materials, methods and the results of method development

8.2.1 Topoisomerase IIα specific siRNA knockdown

To accomplish knockdown of topoisomerase IIα, a Silencer®Select siRNA was custom made by Ambion® Life Technologies (Thermo Fischer Scientific, UK) to specifically target this isoform of the protein. SiRNA's are double strand sequences which, in an ATP dependent process, can be incorporated into an interfering RNA / protein silencing complex (RISC). This complex interacts with a complimentary target mRNA sequence (Nykanen *et al.,* 2001). Within this complex the siRNA duplex unwinds such that the antisense strand remains bound within the RISC and within the mRNA target. This is recognised by a combination of endonucleases and exonucleases, thus instigating degradation of the complementary mRNA strand (Martinez *et al.,* 2002). Such targeted

destruction of a specific mRNA will of course lead to reduction in production of the specific translation protein product of interest e.g. for the current research, topoisomerase IIα.

Ambion® claim that use of their enhanced off-target effect prediction algorithms they are able to make their Silencer®Select siRNA highly specific. For the purpose of the current work this was extremely important as the requirement was to knockdown topoisomerase IIα but not effect topoisomerase IIβ. Before the work commenced, a sequence check was made on the sense topoisomerase IIα target of the custom made sequence and any possible homology to topoisomerase IIβ mRNA. Figure 8.1 shows the sense sequence of the custom made siRNA and the best match to sequences on the topoisomerase IIβ transcript.

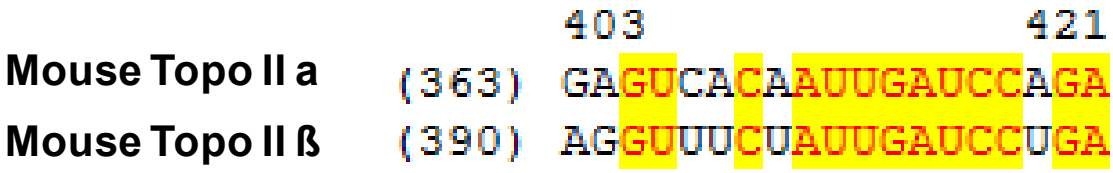


Figure 8.1 Sequence homology between siRNA topoisomerase IIα mRNA transcript target and similar sequence on topoisomerase IIβ transcript

With over 30% mismatched sequencing it was considered unlikely that the custom made silencing siRNA would target topoisomerase IIβ, hence it was considered to be suitable for use to establish L5178Y mouse lymphoma cells with effective and specific knockdown of topoisomerase IIα.

In line with Ambion® recommended protocols for use of their siRNAs a Silencer® Negative Control No. 1 (Ambion®) random scrambled siRNA was used. The specific sequence of this negative control has been identified by Ambion® algorithms to have minimal sequence similarity to any functional genes and to be proven by Ambion® to have minimal effects on cell viability. However, the sequence was not disclosed.

8.2.2 Transfection of L5178Y cells with Silencer®Select siRNA targeting topoisomerase IIα, method development

Transfection of L5178Y mouse lymphoma cells with topoisomerase IIα specific and negative control siRNA was accomplished by electroporation using a Lonza, UK, supplied Amaxa 96 well Shuttle™ Nucleofector. Electroporation involves passing small electrical charges across cell membranes. These have the effect of creating temporary conductive pores which allow for the cellular uptake of larger charged molecules (e.g.

plasmid or double stranded RNA) which would otherwise not be able to readily cross the cell lipid membrane (Ho and Mittal., 1996). However, it is essential to ensure that whilst sufficient current is applied for an appropriate time to allow uptake of the oligonucleotide or plasmid of interest, cell viability is not compromised. Furthermore, complete knockout of topoisomerase IIα would be likely to be a lethal event. For the purposes of the current investigations, the aim was to knockdown topoisomerase IIα by approximately 50%. This was considered not likely to be lethal but would be sufficient knockdown to investigate the activity of the enzyme in driving the genotoxicity of topoisomerase II poisons. Accordingly extensive method validation was undertaken to ensure an appropriate electroporation protocol was utilised and to ensure an appropriate concentration of topoisomerase II knockdown siRNA was titrated.

8.2.2.1 Electroporation method and results of method development

Following technical discussions with Lonza regarding their most appropriate proprietary Nucleofector™ Solution to use for suspension cells such as the L5178Y mouse lymphoma line, their SG Cell Line 96-well Nucleofector™ Solution kit was selected. To prepare the nucleofector solution; to each 20 µL of SG solution, 4.44 µl SG supplement was added. The ingredients of these solutions are proprietary to Lonza and were not disclosed. For the initial validation tests, supplemented SG solution was made up containing 0.4 µg of a positive control plasmid containing green fluorescent protein (pmaxGFP™ Control Vector (Lonza)). For later tests investigating topoisomerase IIα knockdown, supplemented SG solution was made up containing up to 2000 nmol/L negative control or topoisomerase IIα specific siRNA.

L5178Y mouse lymphoma cells were grown in exponential phase as described in Chapter 2 Section 2.1. Cells were centrifuged at 200 '*g*', washed in PBS and resuspended in supplemented SG solution containing pmaxGFP™ Control Vector or, for later tests, the relevant control or knockdown siRNA. 6 x 10^5 cells in 20 µL SG solutions were plated into an appropriate number of wells of a 96-well Nucleocuvette™ Plate (Lonza). Electroporation was carried out immediately after plating by placing the Nucleocuvette™ Plate onto the Amaxa 96 well Shuttle™ Nucleofector and exposing each well to a predetermined electrical pulse.

8.2.2.1.1 Results of method development for appropriate electroporation pulse

As a specific electroporation protocol for L5178Y mouse lymphoma cells was not available from Lonza, a series of preliminary tests were conducted using a wide range of electrical pulses. The exact voltage and duration of these pulses is coded by Lonza and was not disclosed.

The positive control plasmid (pmaxGFP™ Control Vector (Lonza)) was used as a marker of transfection efficiency. 24 hours after transfection, microscope slides were prepared with cells and, where possible, 1000 cells scored for presence of green fluorescent using x 200 magnification on a Zeiss Axioplan microscope. Furthermore, 24 hour post-treatment relative population doubling (RPD) was assessed as a marker for any toxicity associated with each specific pulse (Table 8.1).

Electroporation pulse (EP) code	% RPD	% cells positive for GFP
Control (no EP)	100	0
CA137	56	14
CM138	88	26
CM137	44	34
CM150	81	19
DN100	75	46
DS138	56	50
DS137	**34**	**47**
DS130	63	40
DS150	63	55
DS120	78	32
EH100	31	31
EO100	**78**	**15**
EN138	41	56
EN150	44	68
EW113	53	40

Table 8.1 Preliminary titration work of electroporation pulse for transfection of pmaxGFP™ Control Vector, measuring cells with GFP and survival by %RPD Blue highlighted row indicates best transfection efficiency with 75% survival (75% survival considered to be acceptable as viable cells would be required for subsequent micronucleus analysis)

The preliminary electroporation pulse work indicated that good transfection efficiency (46%) with reasonable survival could be achieved with pulse DN100. It should be noted that whilst this pulse only gave 75% RPD, cells were recovering well 24 hours after treatment and Day 1 to Day 2 cell growth was good (approximately 2 divisions in 24 hours). Accordingly, DN100 was considered to be close to an appropriate pulse

level. In line with Lonza reccommendations, additional pulses were investigated with energies slightly more or less than the DN100 pulse. Lonza protocols indicated that pulses CY100, DA100, DH100 and DI100 should increase viability whilst DP100, EH100, ER100 and FA100 should increase transfection efficiency. These pulses, along with DN100, were used in an additional electroporation titration experiment (Table 8.2).

Electroporation pulse (EP) code	% RPD	% cells positive for GFP
Control (no EP)	100	0
CY100	59	50
DA100	44	100
DH100	71	50
DI100	68	50
DN100	38	100
DP100	25	100
EH100	6	0[1]
ER100	0	0[1]
FA100	0	0[1]

Table 8.2 Additional titration work of electroporation pulse for transfection of pmaxGFP™ Control Vector, measuring cells with GFP and survival by %RPD. As GFP uptake was low, estimation of uptake either 0, 50% or 100% was made.
[1] Too toxic too score

The additional electroporation titration work indicated that DN100 gave good transfection efficiency, but quite high toxicity (only 38% RPD). However, pulses DH100 and DI100 both gave 50% transfection efficiency with good viability (approximately 70% RPD). As the aim of the siRNA work was to knockdown topoisomerase II alpha by approximately 50%, this level of transfection efficiency and viability was considered to be acceptable. Accordingly, DH100, DI100 and DN100 pulses were selected for titration of siRNA concentrations.

8.2.2.1.2 Results of method development for appropriate siRNA concentration

In an initial test, L5178Y mouse lymphoma cells were electroporated using pulses DH100 and DI100 with 0, 62.5, 125, 250 and 500 nmol/L topoisomerase II alpha specific siRNA. Triplicate cultures were used. One and two days after electroporation, from each treatment condition, 2 x 10^6 cells were lysed with sarkosyl and slot blot membranes prepared and incubated with topoisomerase II alpha specific antibody 3665. After incubation with Odyssey secondary antibody, membranes were analysed using the Odyssey IR and associated software (for methods see Chapter 2 section

2.12.3 and Chapter 7). Toxicity was estimated by RPD. In this preliminary test there was no clear concentration related decrease in the enzyme (data not shown). Accordingly a second test was performed using pulses DH100, DI100 and DN100 and concentrations of 0, 250, 500 and 1000 nmol/L topoisomerase II alpha siRNA (Figure 8.2 and Table 8.3).

Figure 8.2 Example of slot blot from Topisomerase II alpha knockdown one day after siRNA transfection. DH, DI and DN are electroporation pulse codes. Triplicate slts prepaed per treatment. Numbers on slot blot are relative infra-red intensity of each slot.

Electroporation pulse (EP) code	Topo IIα siRNA (nmol/L)	% RPD	Day 1 Relative topo IIα level	Day 2 Relative topo IIα level
Control no EP	0	100		
DH100	0	89	100	100
	250	99	86	80
	500	99	76	75
	1000	110	81	63
DI100	0	99	100	100
	250	111	85	59
	500	111	65	45
	1000	111	85	33
DN100	0	82	100	100
	250	93	65	62
	500	104	62	64
	1000	102	71	69

Table 8.3 Survival and topoisomerase IIα knockdown one and two days after topoisomerase IIα specific siRNA trasfection with electroporation pulses DH100, DI100 and DN100

From the work with electroporation pulses DH100, DI100 and DN100 and concentrations of siRNA up to 1000 nmol/L, topoisomerase IIα levels were generally lower when analysed on the second day after electroporation. There was limited evidence for topoisomerase IIα knockdown when using pulse DH100. Pulse DI100 appeared to be most effective at achieving approximately 50% topoisomerase IIα knockdown, whilst pulse DN100 also demonstrated evidence of knockdown for all concentrations of siRNA used. To confirm these results a third siRNA titration experiment was performed analysing topoisomerase IIα knockdown two days after electroporation; using the two best performing pulses (DI100 and DN100), using a higher concentration of siRNA and including use of negative control scrambled siRNA. Accordingly, in the third test 0, 500, 1000 and 2000 nmol/L of both topoisomerase IIα specific and negative control siRNA were used (Table 8.4).

Electroporation pulse (EP) code	siRNA (nmol/L)	% RPD	Day 2 Relative topo IIα level
Control no EP	0	100	
DI100	0	76	100
	500 Control	106	82
	1000 Control	77	92
	2000 Control	97	126
DI100	0	74	100
	500 TopoIIα	83	45
	1000 TopoIIα	86	37
	2000 TopoIIα	79	38
DN100	0	55	100
	500 Control	51	123
	1000 Control	57	123
	2000 Control	51	112
DN100	0	56	100
	500 TopoIIα	62	83
	1000 TopoIIα	53	54
	2000 TopoIIα	52	86

Table 8.4 Survival and topoisomerase IIα knockdown two days after siRNA trasfection with electroporation pulses DI100 and DN100

The data from the third titration experiment confirmed that effective siRNA induced topoisomerase IIα knockdown could be achieved when electroporation pulse DI100 was used. When DN100 pulse was used, knockdown was inconsistent across the siRNA concentration range and cytotoxicity was higher than seen with pulse DI100 (for DN100 treatments, survival as measured by RPD was generally less than 60% of the survival of non-electroporated control cells). Accordingly, pulse DI100 was considered

to be appropriate for future use. Furthermore, following DI100 pulse, toposomerase IIα knockdown was effectively achieved with all concentrations of siRNA. A slightly higher level of knockdown was seen with the two higher concentrations of siRNA, but as there was no clear advantage with using 2000 nmol/L, 1000 n/mol/L was selected to be used in follow on tests. Following electroporation with control scrambled siRNA, topoisomerase IIα knockdown was not seen. Furthermore, when DI100 pulse was used, the control siRNA was not cytotoxicity. This confirmed that the control was suitable for use.

In summary, the method validation work confirmed that the topoisomerase IIα specific siRNA was effective at achieving topoisomerase IIα knockdown in L5178Y mouse lymphoma cells. Approximately 50% knockdown, with associated high cell viability, could be achieved by using electroporation pulse DI100 with 1000 nmol/L siRNA and allowing recovery of cells for two days post electroporation. These conditions were used to prepare cells for the follow on work in which the genotoxicity of etoposide and gemifloxacin was investigated using L5178Y mouse lymphoma cells with and without knockdown of topoisomerase IIα.

8.2.3 Confirmation of the specificity of the siRNA for topoisomerase II alpha

Earlier work (see Chapter 6) had shown that antibody 3665 was highly specific for topoisomerase II alpha. Hence, use of slot blotting rather than full Western blotting was considered to be a suitable quick and easy way to estimate the relative levels of topoisomerase IIα following siRNA knockdown. However, this did not give any indication of the potential cross reactivity of the siRNA to topoisomerase II beta. Whilst cross reactivity was unlikely from the derived sequence specificity, given that the whole basis of this work was to demonstrate the causal link between topoisomerase II poisons genotoxicity and their specific activity against the alpha isoform of the enzyme, it was considered important to demonstrate, beyond reasonable doubt, that the siRNA used did not have any effect on the translation of topoisomerase II beta.

As specific mouse topoisomerase II beta antibodies had not been prepared for this research, nor were any commercially available, an antibody raised against human topoisomerase II beta was purchased from Topogen. A preliminary test using whole cell sarkosyl extraction followed by Western blotting and Odyssey analysis was performed to demonstrate whether this antibody was reactive with topoisomerase II beta extracted from L5178Y mouse lymphoma cells (Figure 8.3).

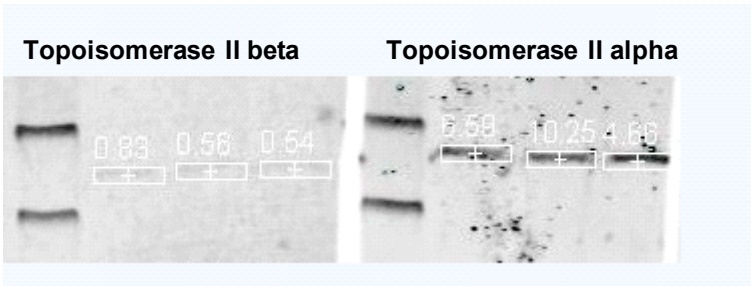


Figure 8.3 Topisomerase beta and alpha levels in L5178Y mouse lymphoma cells following sarkosyl whole cell lysis. 10 µg protein loaded (maximum available). Topoisomerase II beta membrane probed with Topogen human topoisomerase II beta antibody and topoisomerase II alpha membrane probed with mouse topoisomerase II alpha antibody 3665

The preliminary work using sarkosyl lysis demonstrated that the Topogen human antibody was reacting with mouse topoisomerase II beta. However, the detection levels were extremely low, only approximately 10% of that for topoisomerase II alpha. This level of detection was considered to be too low for any meaningful interpretation of topoisomerase II beta knockdown. Accordingly, other whole cell extraction and lysis methods were used to see if recovery of topoisomerase II beta could be improved. Methods investigated included use of RIPA buffer rather than sarkosyl and use of UV rather than infra red sceondary antibodies. However, none of these adaptations facilitated a significant improvement in the yield of topoisomerase II beta (data not shown). In view of the previously noted observation that topoisomerase II is more prevalent in the nuclear fraction of lysed cells (See Chapter 7), it was considered possible that the topoisomerase II beta yield may be improved if nuclear extracts were prepared instead of whole cell extracts. Accordingly, the BioVision nuclear/cytosol fractionation kit was used to prepare nuclear fractions of L5178Y mouse lymphoma cells pre-treated with scrambled or topoisomerase II alpha specific siRNA. Furthermore, at this point of the research, access to a higher resolution UV membrane scanner was made available, hence Anti-Rabbit IgG (whole molecule)–Peroxidase secondary antibody was used along with Amersham ECL Prime substrate. Results from a preliminary experiment are shown in Figure 8.4 and Table 8.5.

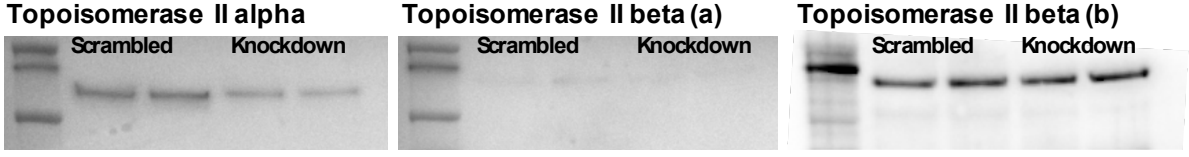


Figure 8.4 Images of Western blots from nuclear lysates from L5178Y cells pre-treated with scrambled or topoisomerase II alpha specific siRNA.
Images Topoisomerase II alpha and Topoisomerase II beta (a) taken at same ressolution. Image Topoisomerase II beta (b) taken at higher ressolution

Topoisomerase II alpha		**Topoisomerase II beta**	
L5178Y cells	**Band intensity**	**L5178Y cells**	**Band intensity**
Scrambled	**2633240**	**Scrambled**	**4538153**
Knockdown	**1064728**	**Knockdown**	**5553718**
% Topo α in knockdown compared to scrambled	**40%**	**% Topo α in knockdown compared to scrambled**	**122%**

Table 8.5 Band intensity of Western blots from nuclear lysates from L5178Y cells pre-treated with scrambled or topoisomerase II alpha specific siRNA measured by Biorad Chemi Doc MP software

Good signals were seen with both topoisomerase II alpha and beta antibodies. In fact the signal from the alpha antibody was so strong imaging resolution had to be reduced to a very low level to allow analysis. The preliminary work with Western blots from nuclear extracts of L5178Y mouse lymphoma cells electroporated with scrambled or topoisomerase II alpha specific siRNA indicated that the siRNA used did not cross react with topoisomerase II beta. In this test the level of topoisomerase II beta was actually slightly higher in cells pre-treated with siRNA. It should be noted that direct comparisons should not be made between band intensity of alpha compared to beta as these were measured using different resolution times.

To confirm the response of the preliminary test a repeat experiment was tested including an increased number of replicates and (for increased assurance of appropriate loading and knockdown of topoisomerase II alpha) an antibody to the nuclear housekeeping gene lamin B1 (a nuclear membrane matrix protein). Prior to antibody incubation, membranes were cut such that the top half was incubated with the relevant topoisomerase II antibody and the bottom half with lamin B1 antibody. Furthermore in the repeat test the amount of protein loaded for the topoisomerase II alpha knockdown extracts was reduced to 2.5 µg per lane (previously 10 µg per lane had been used). This reduction was made to take account of the previous observation that much higher levels of topoisomerase II alpha were present in nuclear lysates. 10 µg protein per lane was loaded for topoisomerase II beta analysis. The results of the repeat test are shown in Figure 8.5 and Table 8.6.

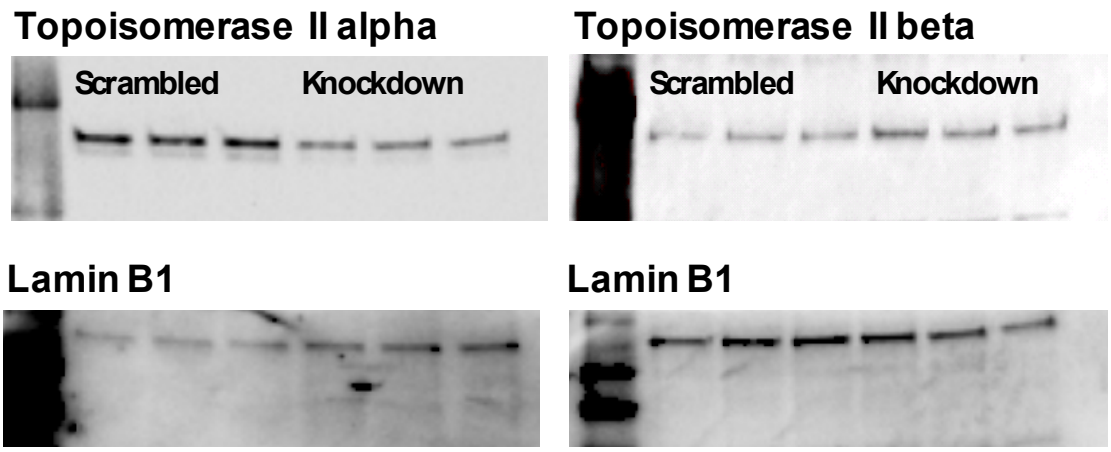


Figure 8.5 Images of Western blots from nuclear lysates from L5178Y cells pre-treated with scrambled or topoisomerase II alpha specific siRNA.

Topoisomerase II alpha			Topoisomerase II beta		
L5178Y cells	Band intensity		L5178Y cells	Band intensity	
	Mean	SD		Mean	SD
Scrambled	606843	34366	Scrambled	786520	129862
Knockdown	259841	14851	Knockdown	1144377	344443
% Topo α in knockdown compared to scrambled	43%		% Topo α in knockdown compared to scrambled	145%	
Lamin B1 (Topoisomerase II alpha membrane)			Lamin B1 (Topoisomerase II beta membrane)		
L5178Y cells	Band intensity		L5178Y cells	Band intensity	
	Mean	SD		Mean	SD
Scrambled	417678	104874	Scrambled	1626665	265088
Knockdown	830616	235750	Knockdown	1311962	394304
% Lamin B1 in knockdown compared to scrambled	199%		% Lamin B1 in knockdown compared to scrambled	81%	

Table 8.6 Band intensity of Western blots from nuclear lysates from L5178Y cells pre-treated with scrambled or topoisomerase II alpha specific siRNA measured by Biorad Chemi Doc MP software

The results of this confirmatory test clearly demonstrated that the siRNA used specifically targeted topoisomerase II alpha with no cross reactivity against topoisomerase II beta. In both tests, siRNA treated cells demonstrated an approximate 60% reduction in the level of topoisomerase alpha compared to cells treated with scrambled RNA. As had previously been observed, the measured levels of beta actually appeared to be higher in the cells treated with siRNA. The results of the analysis of levels of the house keeping gene clearly showed that loading was not responsible for the observed reductions or increases. It should be noted that it proved difficult to achieve good signalling for lamin B1 on the topoisomerase II alpha membrane. This was likely to be due to the low level of protein loaded (2.5 µg per lane). This may be why a seemingly higher level of lamin B1 was recorded in the cells treated with scrambled siRNA. This result was not considered to be biologically significant.

In both tests where topoisomerase II alpha levels were reduced, topoisomerase II beta levels were seen to increase. This may imply a compensatory mechanism but more work would be required to prove this.

In summary, it was considered that any effect on the genotoxic response seen when etoposide and gemifloxacin were tested on cells previously electroporated with the topoisomerase II alpha specific siRNA as compared to cells treated with scrambled RNA could not be attributed to the activity of topoisomerase II beta.

Given the time constraints of Western preparation and the need for simultaneous preparation of micronuclei, for the following experiments slot blotting was used to estimate topoisomerase II alpha levels at the time on treatment. The earlier work with slot blotting had indicated that similar levels of knockdown (approximately 60%) were measured using this technology when compared to the results from Western blotting (Tables 8.4, 8.5 and 8.6).

8.3 Determination of MN(vit) following exposure to topoisomerase II poisons using L5178Y mouse lymphoma cells with and without topoisomerase IIα knockdown

For determination of micronuclei induction, L5178Y mouse lymphoma cells were electroporated using pulse code DI100 in the presence of topoisomerase IIα knockdown or negative control siRNA and allowed to recover for 2 days. To assess comparative topoisomerase IIα knockdown, 2×10^6 cells from each condition were

lysed with sarkosyl and slot blot membranes prepared and exposed to topoisomerase IIα specific antibody 3665. After washing and further incubation with Odyssey secondary antibody, membranes were analysed using the Odyssey IR image capture system and associated software (for methods see Chapter 2 section 2.12.3 and Chapter 7). At the same time 2 x 10^6 cells (topoisomerase IIα knockdown or control) were treated with a range of concentrations of etoposide or gemifloxacin for 3 hours and determination of induced micronuclei and survival by RPD was made (for methods see Chapter 2, Section 2.6).

8.3.1 Determination of micronuclei following topoisomerase II alpha knockdown, results and discussion

The level of topoisomerase IIα knockdown as assessed by slot blot preparations using the topoisomerase II alpha specific antibody 3665 and Odyssey IR analysis is shown by representative data from Test 3 (Figure 8.6) and mean data from quadruplicate analysis in Table 8.7.

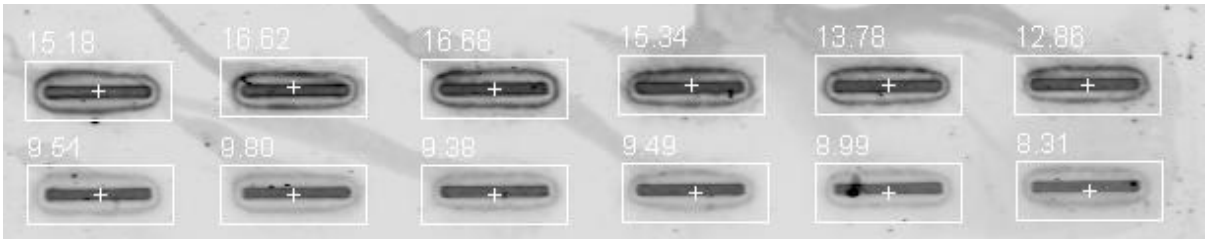


Figure 8.6 Slot blot analysis of L5178Y cells from Test 3 electroporated with negative control (top) or topoisomerase II alpha knockdown (bottom) siRNA. Analysis was conducted with the use of topoisomerase II alpha specific antibody 3665. Numbers on slot blot are relative infra-red intensity of each slot.

Test	Compounds Tested	n	Negative control Mean IR Intensity (SD)	Topo IIα Knockdown Mean IR Intensity (SD)	Topo IIα Knockdown (%)
1	Etoposide	6	7.46 (2.3)	4.34 (0.6)	58
2	Etoposide and gemifloxacin	5	9.42 (1.9)	5.29 (0.9)	56
3	Etoposide and gemifloxacin	6	15.11 (1.6)	9.08 (0.63)	60
4	Gemifloxacin	6	6.33 (0.13)	4.69 (1.0)	74

Table 8.7 Percent knockdown of topoisomerase II alpha in L5178Y mouse lymphoma cells electroporated with negative control (scrambled siRNA) or topoisomerase II alpha knockdown siRNA.

In all four tests, topoisomerase II alpha levels in L5178Y mouse lymphoma cells were reduced following electroporation in the presence of the topoisomerase II alpha specific siRNA. The overall mean knockdown across all 4 tests was approximately 38% (± 8%).

At the end of the 2 day recovery period following electroporation, a range of concentrations of etoposide and gemifloxacin were tested on cells that had not undergone electropration (untransfected), cells that had been electroporated with negative control siRNA and cells that had been electroporated with topoisomerase IIα knockdown siRNA (Topo IIα knockdown). The mean survival data (as measured by 24 hour post treatment relative population doubling (RPD)) for etoposide and gemifloxacin treatments in triplicate tests are detailed in Table 8.8.

Etoposide (µmol/L)	0	0.03	0.1	0.3
Untransfected cells (Survival %RPD)	100 (3.9)	97 (7.8)	100 (9.5)	33 (17)
Negative control (Survival %RPD)	100 (4.3)	98 (8.2)	86 (13)	52 (9.6)
Topo IIα knockdown (Survival %RPD)	100 (11)	101 (9.9)	100 (8.3)	83 (11)
Gemifloxacin (µmol/L)	**0**	**3**	**10**	**30**
Negative control (Survival %RPD)	100 (9.7)	91 (15)	100 (12)	82 (20)
Topo IIα knockdown (Survival %RPD)	100 (6.9)	97 (9.9)	96 (15)	92 (13)

Table 8.8 Cytotoxicity as mesured by Relative Population Doublings (RPD) from *in vitro* micronucleus tests with untransfected L5178Y cells and cells either electroporated with negative control (scrambled siRNA) or Topo IIα knockdown siRNA. Mean of triplicate replicates / experiments. Standard deviation in parenthesis

The results of analysis for etoposide induced micronuclei following 3 hour treatment of control L5178Y mouse lymphoma cells and cells used after electroporation with negative control or topoisomerase II alpha knockdown are shown in Figure 8.7.

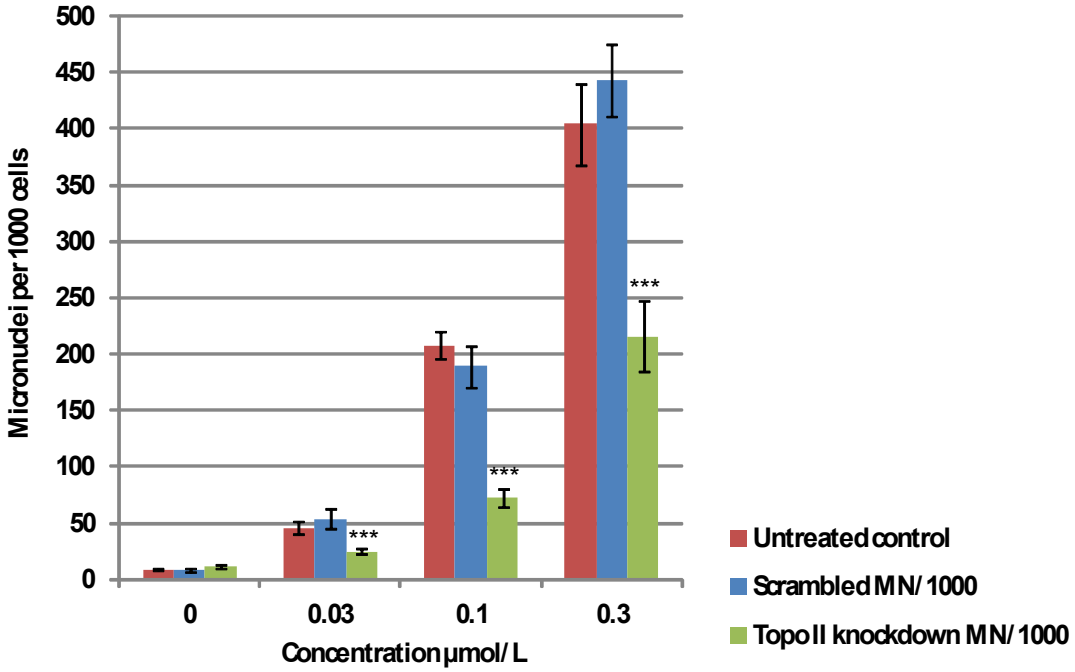


Figure 8.7 Mean data from triplicate experiments when etoposide was tested for induction of micronuclei (MN) using control L5178Y cells and cells electroporated with topoisomerase II alpha knockdown or negative control siRNA
Combined data from triplicate experiments
n = 3. Statistics: 2-sided Continuity-Adjusted Chi-Square Test. Significance decrease in micronuclei comparing scrambled with topoisomerase IIα knockdown: ***p<0.001
There was no significant difference between untreated control and scrambled

The results of analysis for gemifloxacin induced micronuclei following 3 hour treatment of L5178Y mouse lymphoma cells used after electroporation with negative control or topoisomerase II alpha knockdown siRNA are shown in Figure 8.8.

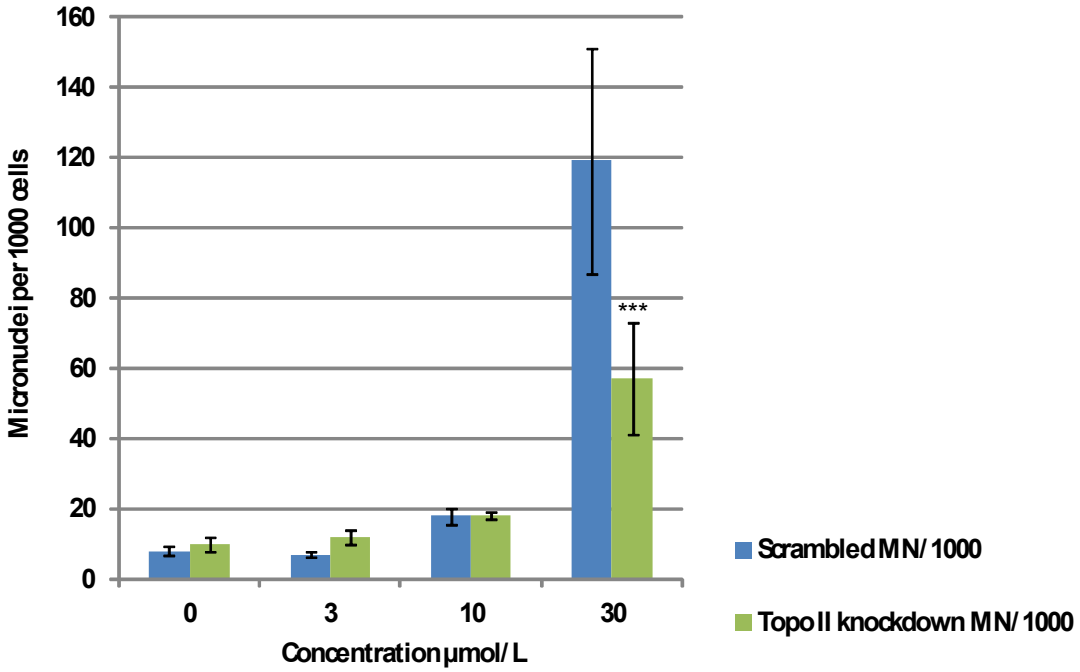


Figure 8.8 Mean data from triplicate experiments when gemifloxacin was tested for induction of micronuclei (MN) using L5178Y cells electroporated with topoisomerase II alpha knockdown or negative control siRNA
Combined data from triplicate experiments
n = 3. Statistics: 2-sided Continuity-Adjusted Chi-Square Test. Significance decrease in micronuclei comparing scrambled with topoisomerase IIα knockdown: ***p<0.001

The data presented in Figures 8.7 and 8.8 clearly show that the genotoxicity of 0.3 to 3 µmol/L etoposide and 30 µmol/L gemifloxacin was reduced when tested on cells that have reduced levels of topoisomerase IIα activity.

It should be noted that there was a degree of variability in the micronuclei induced at the highest concentration of gemifloxacin over the triplicate tests. However, this was due to a high response in Test 1 compared to the other two tests. There was little variability in the actual reduction of micronuclei when topoisomerase IIα knockdown cells were used (i.e. the relative number of micronuclei in 30 µmol/L gemifloxacin treated topoisomerase IIα knockdown siRNA transfected cells when compared to control siRNA transfected cells was 48%, 54% and 43% in Tests 1, 2 and 3, respectively). Accordingly, the variability seen did not prejudice the observation that the genotoxic activity of 30 µmol/L gemifloxacin was clearly reduced when tested with cells that had reduced levels of topoisomerase IIα.

Whilst it would be wrong to read too much into the actual numbers, it was interesting to note that following a mean knockdown of approximately 40% over 3 tests, etoposide genotoxicity was reduced by approximately 50% to 60% over the range of concentrations tested and gemifloxacin genotoxicity was reduced by approximately 50% at the highest concentration analysed. For example It was shown that for 0.3 µmol/L etoposide, topoisomerase IIα knockdown of 42% (± 2%) was associated with a reduction in micronuclei of 49% (± 9.7%). For 30 µmol/L gemifloxacin, topoisomerase IIα knockdown of 37% (± 9.5%) was associated with a reduction in micronuclei of 48% (± 0.2%). Accordingly, there was a clear indication of a direct relationship between reduced topoisomerase IIα levels and the level of genotoxicity induced by etoposide and gemifloxacin. This is believed to be the first time such a relationship has practically been established.

There was also limited data to suggest that as well as reducing genotoxicity, knockdown of topoisomerase II alpha also reduced cytotoxicity (e.g. at 0.3 µmol/L etoposide, untransfected cells gave 33% RPD whereas topoisomerase II alpha siRNA transfected cells gave 83% RPD).

8.4 Conclusion

The data presented clearly demonstrate that the genotoxicity of etoposide and gemifloxacin was directly associated with the nuclear levels of topoisomerase II alpha. This confirms two major points:

1. With some certainty we can relate the genotoxicity of these agents to their activity against the alpha isoform of topoisomerase II. This is the first time such a direct link has been definitively established.

2. These data contradict some of the earlier work from Cowell's laboratory (Cowell and Austin., 2012a; Cowell and Austin., 2012b; Cowell *et al.,* 2012) which suggested the genotoxicity of etoposide in Nalm-6 cells was driven by the beta isoform of the enzyme whereas its cytotoxicity was driven by the alpha isoform of the enzyme. In L5178Y cells, both the genotoxicity and cytotoxicity of etoposide was linked to the activity of the alpha isoform.

CHAPTER 9

General discussion and future possibilities

CONTENTS

9 General discussion and future possibilities

9.1 Introduction

The belief that mammalian cell gene mutation assays are prone to false positives is widespread and has been noted in several influential papers over the last decade (Kirkland *et al.,* 2005; Kirkland *et al.,* 2007; Kirkland and Speit, 2008; Matthews *et al.,* 2006a; Matthews *et al.,* 2006b). However, from personal experience of nearly 3 decades in *in vitro* genotoxicology, this perception seems to be unfounded, at least in the environment of pharmaceutical research. Preliminary work was performed to ascertain that the cells used for this research were of an appropriate provenance and karyotype (Chapter 2, Appendix 1). Once this was established, the initial aim of this thesis was to put into perspective the real incidence of positive findings in the mouse lymphoma assay (MLA) from a decade of screening at AstraZeneca UK (Chapter 3, Appendix 1). This showed that the rate of unexplained positives was only 5%, vindicating the use of the test in screening paradigms. However, there were still 5% of positive results that could not be readily explained by primary pharmacology or by likely DNA reactivity. The further aims of this thesis were to investigate the contribution that off target aneugenicity (chromosome loss or gain) or topoisomerase II poisoning might make to this. It was shown that the MLA is not an appropriate screen for aneugenicity, so this mechanism cannot contribute to the number of unexplained positive results in this test system (Chapter 3, Appendix 1). However, topoisomerase II poisoning is a known potent genotoxic mechanism and topoisomerase II poisons are readily identified in the MLA and other mammalian cell *in vitro* genotoxicity screens (Lynch *et al.,* 2003). There are several screens than can be used to identify topoisomerase II poisons (Fisher and Pan, 2008; Willmore *et al.,* 1998; Subramanian *et al.,* 2001), but little work has been done to directly link the ability of these tests to predict mammalian cell genotoxicity. The ability of topoisomerase II poisons to prevent cell free DNA decatenation in the presence of the enzyme and whether this could be used as a screen for genotoxicity, along with the ability of this test to identify the genotoxic mechanism of a pharmaceutical research compound which had an unexpected positive finding in the MLA, was investigated (Chapter 4, Appendix 1). This simple cell free test was not an ideal screen for genotoxicity. Accordingly the potential value of cell assays for topoisomerases II poisoning were considered. However, previous data from the TARDIS assay had shown that for reference compounds (e.g. etoposide) concentrations where topoisomerase II poisoning was seen were higher than concentrations inducing genotoxicity (Lynch *et al.,* 2003; Austin *et al.,* 1995). This seemed illogical, so work was undertaken to establish the

lowest observable genotoxicity level of the topoisomerase II poisons etoposide and gemifloxacin and equate this to the lowest observable concentration inducing a response in the TARDIS and ICE bioassays for measurement of topoisomerase II induced DNA stabilised cleavage complexes (Chapter 5). Unfortunately, despite initial assay improvements, cleavage complex formations could still not be detected at concentrations equal to or below which genotoxicity was seen. This suggested that either these assays were not sensitive or that etoposide and gemifloxacin were genotoxic by other mechanisms, the most likely of which was reactive oxygen species (ROS) induction. Investigation of the potential of these compounds for ROS induced genotoxicity using a modified version of the Comet assay showed that this mechanism could not contribute to the measured genotoxicity (Chapter 6). Hence the most likely explanation for the discrepancy between concentrations inducing measurable genotoxicity and measureable topoisomerase poisoning was the sensitivity of the cleavage complex assays. Attempts were made to further improve this sensitivity by preparation of mouse specific antibodies targeting sites spanning the topoisomerase II enzyme. Using these antibodies little improvement was seen in the response of the TARDIS assay, but for the first time the ability of etoposide to induce stabilised cleavage complexes and genotoxicity was seen at similar concentrations in the ICE assay. Furthermore, it was shown that antibodies targeting sequences towards the n-terminus of the topoisomerase II enzyme were generally the most efficient (Chapter 7). Finally, data emerging over the period of this research linked the genotoxicity of topoisomerase II poisons to their activity against the beta isoform of the enzyme (Cowell and Austin., 2012a; Cowell and Austin, 2012b; Cowell *et al*., 2012, Smith *et al.,* 2014). As this project had concentrated on the activity of the alpha isoform, these new data seriously questioned much of the research undertaken. To demonstrate that etoposide and gemifloxacin poisoning of topoisomerase II alpha in mouse lymphoma cells could induce genotoxicity, a topoisomerase II alpha siRNA was used to specifically knock down the alpha isoform of the enzyme by approximately 50%. This lead to a similar reduction in genotoxicity, for the first time indicating a direct relationship between the intra nuclear level of topoisomerase II alpha and the ability of topoisomerase II poisons to induce genotoxicity (Chapter 8).

9.2 The importance of authentication of cell lines used in research

At the beginning of the research within this thesis it was considered to be of primary importance to confirm the provenance of the cell line used. There are all too many reported cases of research projects advancing to a late stage only to discover that the cell lines used were either contaminated, or worst still, not actually the correct cell line the researchers

believed they were investigating (MacLeod *et al*., 1999; Capes-Davis *et al*., 2010; Derxler, 2010). Plus by default there must be many research projects that have used contaminated cells without being aware of the problem. For example, work at the German Collection of microorganisms and Cell Cultures showed that out of 598 leukaemia or lymphoma cell lines analysed, 31% were either contaminated with mycoplasma, with another cell line, with both or were not the cell line described by the supplier (MacLeod *et al*., 1999; Capes-Davis *et al*., 2010; Derxler, 2010). Over passaging or culturing can also lead to genetic drift and instability of karyotype (Hughes *et al*., 2007). The provenance of the mouse lymphoma L5178Y $tk^{+/-}$ cells, clone 3.7.2c, used for this research was very well established and could be traced back to the laboratory where the MLA was first developed, with minimal culturing since (Chapter 2, Table 2.1). These cells had also been demonstrated to be mycoplasma free by PCR analysis (data not shown). However, it was still considered to be prudent to confirm the cells karyotype. The spectral karyotyping confirmed that the cell stock had the same spectral karyotype as had been previously published (Sawyer *et al*., 2006), with the caveat that the origin of some of the stable translocations was refined. It was considered that the spectral karyotype defined in the current investigations should be used as the definitive karyotype for L5178Y $TK^{+/-}$ mouse lymphoma cells clone 3.7.2C. Furthermore, as part of an International Life Sciences Institute / Health and Environmental Sciences Institute (ILSI/HESI) Genetic Toxicology Testing Committee (GTTC) project to provide well characterised cells as close to the original isolates as possible, these cells have now been expanded and deposited at the European Collection of Animal Cell Cultures (ECACC) and Japanese Collection of Research Bioresources Cell Bank (JCRB) and are internationally available for any group wishing to establish or re-establish the MLA.

Continual culturing L5178Y cells for up to six months did not markedly affect the modal chromosome number of 40 nor did it induce marked increases in the percentage of cells with higher or lower chromosome numbers. Accordingly, the karyotype of L5178Y mouse lymphoma cells in continuous culture was considered to be surprisingly stable for a transformed cell line. This work was considered to be a gold standard for establishing that the cell lines used in a research project were of an appropriate provenance (Chapter 2, Appendix 1).

9.3 The predictivity of the Mouse Lymphoma Assay (MLA)

It was hoped that the work looking into the predictivity of the MLA in pharmaceutical research would significantly contribute to the debate on the over sensitivity of mammalian cell genotoxicity screens (Chapter 3, Appendix 1). This work was the first in its kind where a thorough review of the pharmacology of over 400 research medicine compounds was

reviewed and related to the mammalian cell genotoxicity of the agents and their activity in bacterial cell and *in vivo* assays. The 'real' MLA unexplainable positive rate of only 5% was perhaps surprising, but was also a reflection of how improved testing protocols for *in vitro* genotoxicity screens means uninterruptable positives from testing to excessively high concentration, high toxicity, high pH or high osmolality are now avoided (Moore., *et al* 2002; Moore., *et al* 2003; Moore., *et al* 2006; Moore., *et al* 2007; ICH, 2011). However, this work has not prevented other groups still questioning the relevance of *in vitro* genotoxicity screens. This is perhaps understandable in industries outside of pharmaceutical research where the pharmacology of research compounds is generally not so well understood, and it is clearly a high priority topic in the cosmetic industry where *in vivo* testing in Europe is no longer allowed. Recent publications by Fowler *et al* have continued to work on the predictivity of *in vitro* genotoxicity screens for cosmetics (Fowler *et al.,* 2012a; Fowler *et al.,* 2012b; Fowler *et al.,* 2014). They focussed on a subset of compounds that have previously been reported to be 'false' positives and concluded that use of P53 competent cell lines were preferable for the MN(vit). However, it is questionable whether focussing on a sub-set of difficult compounds is of real value when assessing the worth of screening assays. No screen is perfect, hence the requirement in most genotoxicity testing paradigms to test a battery. There is also some question regarding Fowler *et al*'s conclusions, a close inspection of his data suggests that there was as much difference in the predictivity of the MN(vit) between assays with or without cytokenisis block as there was between P53 competent or non-competent cell lines. It was considered that the 'real life' data reviewed in this thesis gives a broader analysis of the usefulness of the MLA as a screening tool. There are also several other on-going initiatives looking into the predictive power of *in vitro* genotoxicity tests, including those run by ILSI/HESI, from which there are emerging data showing the predictivity of *in vivo* genotoxicity tests for carcinogenic potency and even the predictivity of *in vitro* tests for the same (Gollapudi *et al* 2013; ILSI/HESI, 2014). This is a really valuable exercise for the future to genuinely put into perspective the worth of genotoxicity screening. It is hoped that the work on the predictivity of the MLA described in this thesis has helped with some of these initiatives.

Whatever the overall predictive power of the MLA, it was clear that the assay cannot routinely identify a range of aneugens with diverse mechanisms of action (Chapter 3, Appendix 1). This work was the first time a convincing data set had been published showing the insensitivity of the MLA for this mechanism and demonstrated that monosomy at chromosome 11 is likely to be a lethal event. This ended over two decades of debate as to whether the MLA could be used as a screen for aneugenicity. Unfortunately, the work was not available in time to influence the most recent ICH guidance (ICHS2(R1), 2011).

However, it is hoped that it will be of use for determining future regulatory screening paradigms.

It was clear that aneugenicity could not contribute to the 5% of mechanistically unexplainable positive responses seen in the retrospective review of MLA data, but topoisomerase II inhibition was a known target for 9 of the 40 MLA positives and 7 of the 10 Ames positives. Accordingly off-target topoisomerase II inhibition seemed to be a plausible mechanism to explain some of the hitherto unexplained responses. From reviews of the available literature it was clear that few laboratories had convincingly dosimetrically linked topoisomerase II inhibition to genotoxicity (Lynch *et al.,* 2003; Smart *et al.,* 2008; Smart, 2008; Cowell and Austin, 2012a). The work in this thesis aimed to prove this link and devise methodologies for screening compounds for topoisomerase II potential and perhaps more importantly devise tools which could be used to determine if genotoxic compounds were targeting topoisomerase II.

9.4 Methods used to detect topoisomerase II cleavable complexes and their relationship to *in vitro* genotoxicity

Perhaps unsurprisingly, the work detailed in Chapter 4 confirmed that cell free assays are not very useful tools for stoichiometric comparisons between effects at the DNA/enzyme level and mammalian cell genotoxicity. The complexities of biological systems compared to reactivity with naked DNA circlets were clearly very different. However, it was perhaps difficult to understand why such high concentrations were required to inhibit the enzyme in the decatenation assay as compared to genotoxicity (e.g. for etoposide; LOGEL was 0.03 µmol/L compared to decatenation IC_{50} of 4 to 6 µmol/L). That being said the assay did have some potential for the bold ranking of compounds e.g. as an early screen of new chemistry and its possible effect on mammalian topoisomerase II. The assay is quick and easy to run and could possibly have a role in identifying the real bad actors as far as drugs made to target bacterial topoisomerase II and their activity against the mammalian form of the enzyme i.e. compounds with a decatenation IC_{50} below 20 µmol/L did correlate well with mammalian cell genotoxicity (16 out of 18 AstraZeneca compounds correctly identified as potential mammalian cell mutagens). This could be of value in the early development of antibiotics designed to target bacterial gyrase in is as much as chemistry that clearly has potent crossover activity with the mammalian form of the enzyme could be detected and avoided. Unfortunately the assay performed poorly for less potent compounds and hence had little use as a definitive screen or as a tool for mechanistic investigations into compounds that may have off target effects on topoisomerase II e.g. the series of kinase inhibitors with an unexplained response in the MLA discussed in Chapter 4.

Accordingly, if there was a requirement to confirm beyond reasonable doubt that the mechanism of genotoxicity was not by direct DNA covalent binding (i.e. a mechanism for which there is considered to be no safe threshold dose that could be given to healthy volunteers in clinical trials) but was driven by inhibition of topoisomerase II (a mechanism which is considered to have a safety threshold (Tweats *et al*., 2007)), the cell free assay was not a very good screen. The logic that any screen for activity against the enzyme should be able to detect lower effect concentrations than those inducing measurable genotoxicty drove much of the research for the later part of this thesis.

Measurement of topoisomerase II cleavable complexes after treatment with topoisomerase II poisons in cells was likely to be of greater biological relevance than looking at interactions at the molecular level in a cell free system. Whilst the TARDIS and ICE assays had previously been reported as being the most sensitive assays for measurement of cleavage complex formation (Cowell *et al.,* 2011), the vast majority of the work in this thesis indicated that these assays generally identified cleavage complex formation at concentrations higher than those inducing micronuclei in L5178Y mouse lymphoma cells (Chapter 5). The TARDIS assay appeared to be particularly insensitive, at least when run at AstraZeneca using the available image analysis software, where TARDIS failed to identify cleavage complex formation with gemifloxacin. It is possible that using improved image analysis the sensitivity of the assay may be increased. However, the data generated for this thesis were generally supported by TARDIS data from other laboratories (including Newcastle University where the assay was developed), which showed the requirement for testing etoposide and other fluoroquinolone antibiotics to very high (and most probably lethal) concentrations before any relevant signal could be detected (Lynch *et* al., 2003; Rance *et al*., 2010). Following the preparation of mouse specific antibodies, a significant improvement was seen in the sensitivity of the ICE assay. Measurement of cleavage complex for etoposide from concentrations as low as 0.03 µmol/L was achieved (giving a greater than 5-fold increase above background). This lowest effect level was far lower than had previously been reported. A recent review by Nitiss *et al* indicated an increase in cleavage complex formation of only approximately 2.5-fold above background at 10 µmol/L etoposide (Nitiss *et al*., 2012), a concentration some 300-fold above the lowest effect level detected in the work for this thesis. This indicated just how much the sensitivity of the ICE assay could be improved by using specifically designed antibodies and imaging techniques. The observation that antibodies targeting areas of the enzyme towards the n-terminus were preferable, presumably because this area is more available once the closed clamp topoisomerase II / DNA cleavage complex is formed, will also provide guidance on future antibody design when investigating topoisomerase II DNA interactions.

Whilst the improvements in the sensitivity of the ICE assay were welcome, it should still be noted that the assay is very time consuming and low throughput. The requirement for overnight ultra-centrifugations means generally no more than 6 cultures can be run at any one time. This would not preclude its use as a screen for off target topoisomerase II effects, but would mean that it could only realistically be used for investigating a very limited number of compounds. The assay also uses a relatively large amount of material. To provide sufficient DNA for analysis, at least 10 mL of cell culture is required. Hence, when testing up to a highest concentration of 500 µg/mL (common in pharmaceutical research), approximately 20 mg of compound would be required for a single test spanning 4 concentrations. In early pharmaceutical development this amount of compound is often not available; for example, it would have been useful to have used the ICE assay to investigate whether the off-target topoisomerase II poisoning of the kinase inhibitors discussed in Chapter 4 was responsible for the observed mammalian cell genotoxicity, unfortunately sufficient material was not available.

Furthermore, even with the improvements in the sensitivity of the ICE assay, it was still not possible to detect cleavage complex formation at concentrations lower than those inducing micronuclei. However, other than simple consideration of the sensitivity of the assays it should also be noted that the sensitivity of the various statistical analysis methods used could also have influenced the calculated LOGEL for micronucleus formation or the LOCCEL for cleavage complex formation. The data generated for micronuclei are not continuous hence conform to a Poisson like distribution; accordingly a continuity adjusted Chi-squared test was used for analysis. However, the fluorescence intensity data from ICE are continuous, hence more closely fitting normal distribution; as such a T-test was used with square root transformation to even out variance. There was a concern that using such different analyses could influence the significance of the relevant increases. Emerging methodologies for analysis of genotoxic data suggested that rather than using no effect levels based on significance from particular statistical models, a better approach was to use a benchmark dose (Gollapudi *et al.,* 2013; Johnson *et al.,* 2014; Cao *et al*., 2014). In short, this benchmark dose (BMD) approach analyses the nature of dose-response curves and calculates a concentration which induces a modelled 10% increase above background (calculated by using the estimated lower limit of the one-sided 95% confidence interval of a concentration that induces a calculated 10% increase in effect above background ($BMDL_{10}$)). This can be calculated for concentrations below those analysed and is considered to be a good estimate of a point of departure (POD), below which there is considered to be no observable effect (Gollapudi *et al.,* 2013). Software to calculate BMD (PROAST) has been developed by the Netherlands National Institute for Public Health and the Environment and

is publically available (PROAST, 2014). With the help of AstraZeneca Discovery Sciences statistics department, using PROAST software, the $BMDL_{10}$ was calculated for etoposide and gemifloxacin ICE and MN(vit) data. The ICE $BMDL_{10}$ was 0.0011 µmol/L for etoposide and 5.65 µmol/L for gemifloxacin. The MN(vit) $BMDL_{10}$ was 0.0017 µmol/L for etoposide and 1.26 µmol/L for gemifloxacin. It should be noted that the estimated etoposide MN(vit) $BMDL_{10}$ was calculated on an exponential curve fitting model only, the Hill model also used by the software could not be calculated. This was believed likely to be due to a lack of fit at the top of the curve. The $BMDL_{10}$ did estimate a slightly lower POD for ICE than MN(vit) for etoposide, whilst for gemifloxacin the POD for the MN(vit) was lower. The calculated LOGEL and LOCCEL (from the work presented in Chapter 7) and $BMDL_{10}$ for etoposide and gemifloxacin are summarised in Figures 9.1 and 9.2 and Table 9.1.

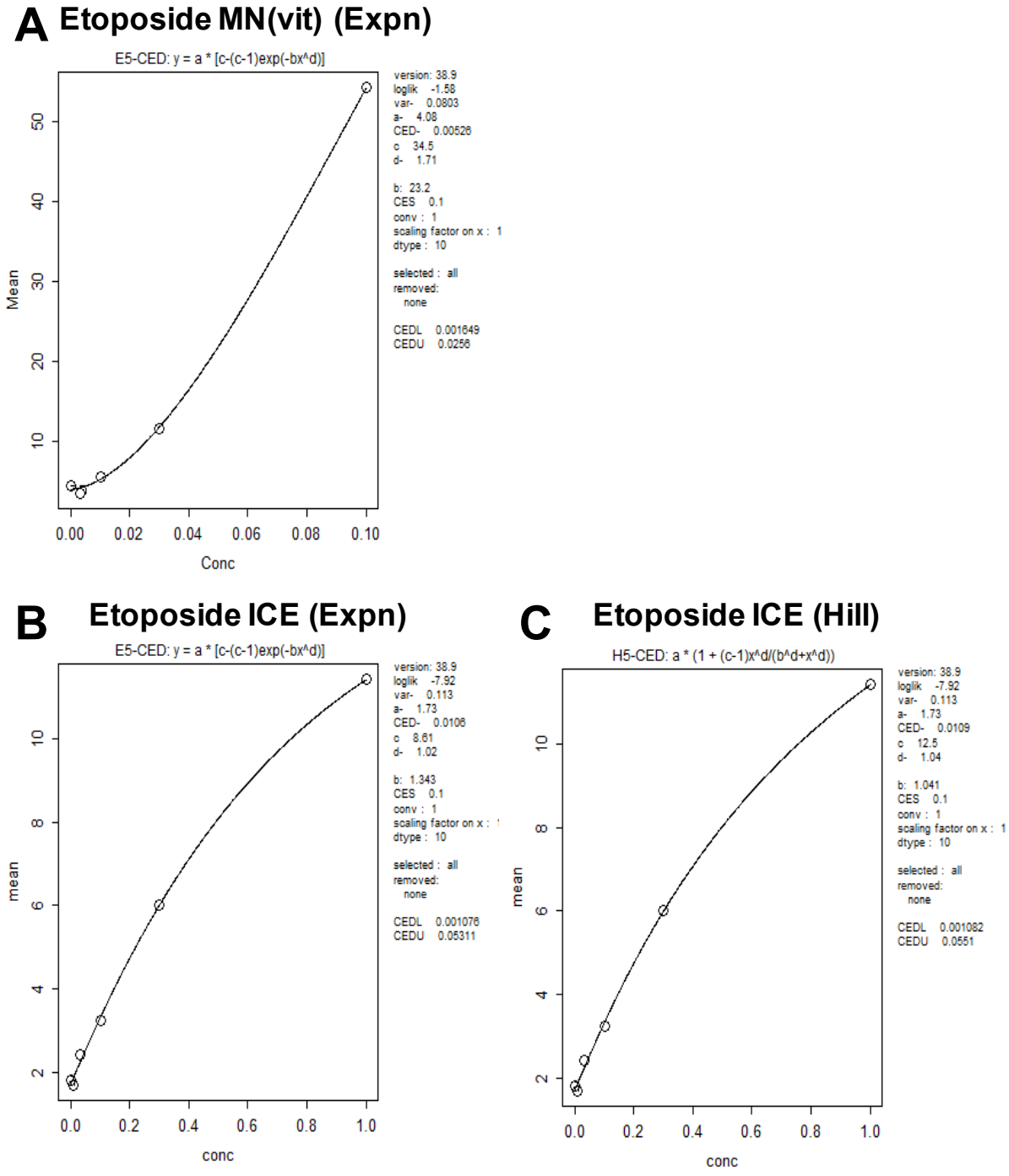


Figure 9.1: PROAST curves for bench mark dose analysis of etoposide MN(vit) (graph A) and ICE data (graphs B and C)
Expn = Exponential curve fitting model. Hill = Hill curve fitting model
CES = Critical Effect Size (or Benchmark Response) - set at a 10% increase above the background response
CED = Critical Effect Dose (or Benchmark Dose)- is the concentration at which a 10% increase over background is estimated to occur
The lowest Benchmark Dose from the 2 models was used as the calculated lowest Benchmark Dose ($BMDL_{10}$). For etoposide MN(vit), only the exponential model fitted

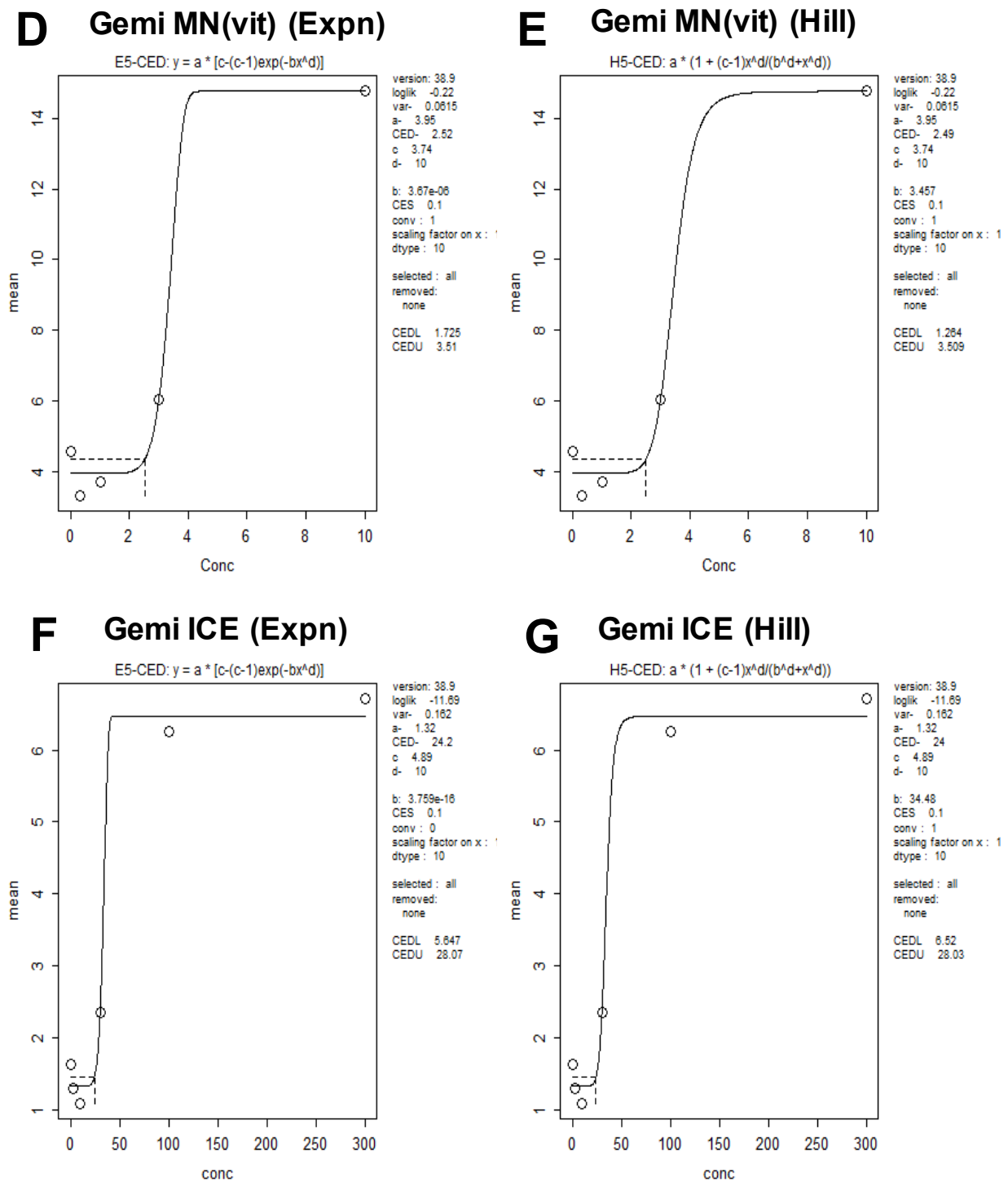


Figure 9.2: PROAST curves for bench mark dose analysis of gemifloxacin MN(vit) (graphs D and E) and ICE data (graphs F and G)
Expn = Exponential curve fitting model. Hill = Hill curve fitting model
CES = Critical Effect Size (or Benchmark Response) - set at a 10% increase above the background response
CED = Critical Effect Dose (or Benchmark Dose)- is the concentration at which a 10% increase over background is estimated to occur
The lowest Benchmark Dose from the 2 models was used as the calculated lowest Benchmark Dose ($BMDL_{10}$)

Compound	LOGEL (µmol/L)	LOCCEL (µmol/L)	MN(vit) $BMDL_{10}$ (µmol/L)	ICE $BMDL_{10}$ (µmol/L)
Etoposide	0.03	0.03	0.0016	0.0011
Gemifloxacin	10	30	1.26	5.56

Table 9.1: Etoposide and gemifloxacin lowest observable effect levels genotoxicity (LOGEL) or cleavage complex (LOCCEL) and benchmark doses ($BMDL_{10}$)

As expected from the use of BMD modelling, the actual $BMDL_{10}$ concentration was lower than the lowest observable effect levels. However, it was reassuring that both methods were in agreement in so much that detection of etoposide induced cleavable complexes was at similar concentrations to induction of micronuclei, but there was a greater difference between these concentrations for gemifloxacin. By whichever analysis method used, cleavable complex detection could not clearly be seen at concentrations markedly below those for which an increase in micronuclei was seen. The improvements in the ICE assay and the use of $BMDL_{10}$ have demonstrated for the first time that cleavage complex formation and genotoxicity can be seen at equivalent concentrations for etoposide. However, during this research it was not possible to determine why cleavable complex formation could not be detected at concentrations similar to micronuclei induction for gemifloxacin. Other mechanisms of genotoxicity did not seem to be relevant for gemifloxacin, there is nothing published to suggest that gemifloxacin can form DNA adducts and the observation that ROS (as measured by the modified COMET assay (Chapter 6)) did not contribute to the genotoxicity of either etoposide or gemifloxacin in L5178Y mouse lymphoma cells confirmed the specificity of the mechanism of action of both of these two topoisomerase II poisons. A recent publication by Williams *et al* also investigated the LOCCEL of two other fluoroquinolone antibiotics (clinafloxacin and lomefloxacin). Their data appeared to suggest that cleavage complex formation occurred at lower concentrations than genotoxicity (Williams *et al*., 2013). This was surprising, especially as they reported a LOCCEL for etoposide of 0.17 µmol/L, some 6-fold higher than the LOCCEL achieved for this thesis. However, on closer examination the calculated NOGELs they used were from literature reviews, i.e. from experiments that were not specifically designed to calculate lowest effect levels using the most sensitive analysis methods available. Accordingly, these data are not considered to contradict the data presented in this thesis.

9.5 The role of topoisomerase II alpha poisoning in causing mutation

Whilst a direct dosimetric link between genotoxicity and topoisomerase II poisoning was established for etoposide, this link was in effect circumstantial and the emerging data suggesting etoposide induced mutation and carcinogenicity was driven by the beta isoform of topoisomerase II questioned a great deal of the work for this thesis which had concentrated on the poisoning of topoisomerase II alpha. This may also have explained why there was a greater discrepancy between LOGEL and LOCCEL for gemifloxacin i.e. if gemifloxacin has a greater affinity for the beta isoform, it would not have been seen using cleavage complex assay with antibodies to topoisomerase II alpha. The recently published work on the poisoning of topoisomerase II beta and mutation included review of an earlier proposed mechanism for proteolytic degradation of topoisomerase II beta specific cleavage complexes (Xiao *et al.,* 2003). This mechanism, along with the elucidated role for topoisomerase II beta in transcription, lead to the hypothesis that double strand DNA breaks form following proteolytic degradation of topoisomerase II beta cleavage complexes during transcription. This being the suggested mechanism for the 11q23 translocation involving the *MLL* gene associated with etoposide therapy related secondary leukaemia (Cowell and Austin., 2012a; Cowell and Austin, 2012b). This possible role of topoisomerase II beta in mutagenesis has far greater consequences than invalidating some of the research for this thesis. It is driving the development of new cancer chemotherapeutic medicines to target topoisomerase II alpha with the hope that this will reduce the potential for secondary malignancies. This is a difficult task as the homology between topoisomerase II alpha and beta generally means compounds target both isoforms of the enzyme with similar potencies (Shapiro and Austin, 2014), hence a lot of research time and resource is required to pursue this. Accordingly, the work for this thesis in which it was clearly demonstrated that the mammalian cell genotoxicity of etoposide and gemifloxacin is driven by their activity against the alpha isoform of the enzyme was of particular significance (Chapter 8). This activity has been hinted at before (Lynch *et al.,* 2003; Williams *et al.,* 3013), but these data are the first to demonstrate a direct relationship between the knockdown of topoisomerase II alpha and a reduction in genotoxicity. It would clearly be premature to suggest that this invalidates the previous work linking the activity of poisoning topoisomerase II beta and secondary malignancies. All that has really been achieved is to demonstrate that in a particular cell line a particular genotoxic end-point (chromosome breakage as measured by formation of micronuclei) is linked to poisoning of topoisomerase II alpha. A great deal more research in leukemic cell lines of relevance to secondary malignancies and *in vivo* models would be required before it could be considered that the current hypothesis (i.e. proteolytic activity

against topoisomerase II beta cleavage complexes leading to etoposide induced cancers) is incorrect.

One other consequence of the work directly linking poisoning of topoisomerase II alpha and micronuclei induction is the potential for this to be used in a novel screening assay. All of the screens used for the research in this thesis have either been unable to unequivocally link genotoxicity to topoisomerase II poisoning (e.g. the cell free decatenation assay, the chloroquine or novobiocin block MN(vit) and the TARDIS assay) or are excessively time consuming and of potential only for low throughput screening (e.g. the ICE assay). The proposed new assay would involve the creation of a stabilised L5178Y mouse lymphoma cell line with approximately 50% knockdown of topoisomerase II alpha. Short hairpin RNA (shRNA) and siRNA technologies are now available to achieve this (Salazar *et al.,* 2014). Using these cells along with non-knockdown L5178Y mouse lymphoma cells in a side by side comparative MN(vit) assay could be used to determine whether genotoxicity associated with a compound was driven by its poisoning of topoisomerase II alpha i.e. if the compounds mode of action was by poisoning of topoisomerase II it would be expected that induction of micronuclei would be reduced by approximately 50% in the knockdown cell line. Combining the assay with a 96 well plate flow cytometric format MN(vit) would have potential for a high throughput screen using minimal test compound (1-2 mg) (Bryce *et al.,* 2013). Clearly a lot more validation work would be required before this proposed assay could be practically used in pharmaceutical research and development, but the potential is there.

9.6 Proposed screening paradigm to screen compounds for topoisomerase II inhibition and genotoxicity

The following Figure 9.1 illustrates a possible screening paradigm for early assessments of compounds with potential to interact with mammalian cell topoisomerase II (e.g. antibacterials designed to target gyrase).

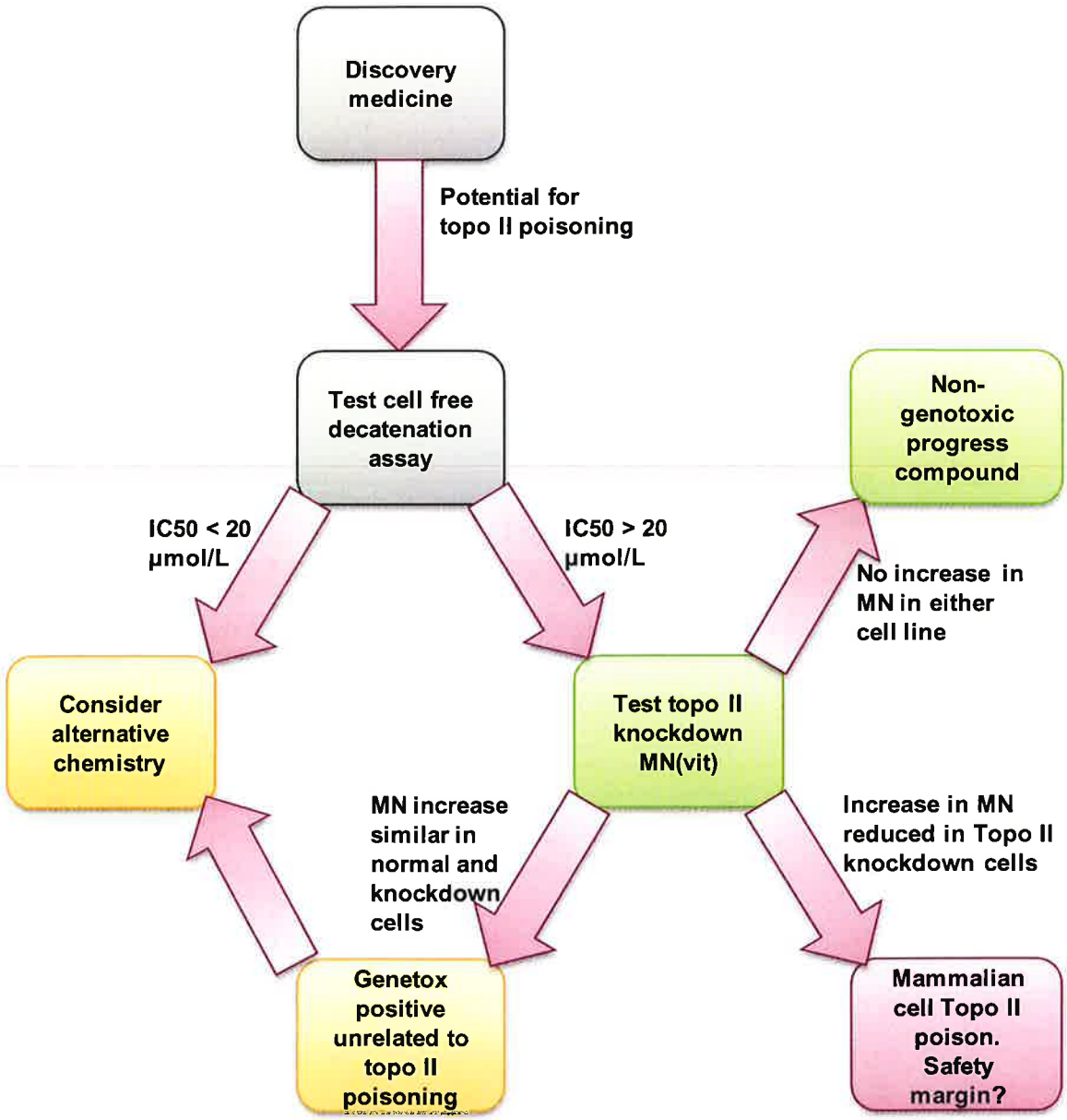


Figure 9.3: Screening paradigm for hazard identification of compounds with potential risk of topoisomerase II poison associated genotoxicity

The strategy outlined in Figure 9.3 utilises the high throughput decatenation screen as an early indicator of activity. Only very potent decatenators (IC_{50} less than 20 µmol/L) should be considered unsuitable for progression. If further investigations are required, comparing

the response of the compounds in a MN(vit) with L5178Y mouse lymphoma cells with a stabilised approximate 50% knockdown of topoisomerase II alpha and in cells without knockdown would provide information on:

a. Whether the compound is genotoxic by a mechanism not involving topoisomerase II poisoning (i.e. positive response similar in both cell lines). If the mechanism was via direct DNA adduct formation, the medicine could not be safely used in man.

b. Induces genotoxicity via interaction with mammalian call topoisomerase II (i.e. positive response reduced in knockdown cells). Such compounds could still make safe and efficacious medicines if it could be demonstrated that the effect was not seen *in vivo* or if there was a suitable safety margin between the genotoxic dose and the likely plasma exposure in man.

c. Is not genotoxic. Which for compounds designed to target bacterial gyrase, and assuming the medicine has potent bactericidal activity, would show that the compound has much higher affinity for the bacterial enzyme, indicating its value as an antibiotic.

9.7 Future investigations

Given the constraints of this research project it has not been possible to fully elucidate all of the mechanistic interactions between the topoisomerase poisons investigated and genotoxicity nor to complete all the work required to confirm the value of the screening paradigm detailed in Section 9.6. In particular, to bolster the data presented the following should be considered.

1. Confirm that etoposide and gemifloxacin do not form DNA adducts. There are no reliable literature reports that either of these compounds can form DNA adducts, and for gemifloxacin the response in the Ames assay (positive only with bacterial strains capable of DNA Repair (Gocke., 1991)), suggest direct DNA reactivity of these compounds is unlikely. However, if time had allowed this would have been confirmed. Unfortunately, radio-labelled drug was not available to perform direct DNA binding studies with these compounds, and facilities were not available to perform P32 post labelling for adduct detection. A small amount of preliminary work was performed in association with Leicester University using mass spectrometer technologies for adduct detection (Farmer *et al.,* 2008; Singh *et al.,* 2010). The initial data were promising and by using online column-switching liquid chromatography electrospray ionization tandem mass spectrometry, obvious etoposide or gemifloxacin adducts associated with naked DNA treatment were not observed (data

not shown). However, as it was not clear exactly what adducts should be looked for, this process potentially lacked sensitivity. In collaboration with Leicester, there is a project to improve the sensitivity of mass spectrometry for adduct detection by first removing endogenous nucleotides, hence analysing only modified bases. If this proves successful, etoposide and gemifloxacin will be further analysed to confirm that they do not directly form DNA adducts.

2. Confirm the predictivity of the topoisomerase II knockdown MN(vit) assay by running more compounds. Clearly the potential screening ability of the use of topoisomerase II knockdown cells has far from been established. Whilst the work with etoposide and gemifloxacin hints at its worth, several validation steps need to be completed. Including preparation of a stable knockdown cell line, confirmation that the assay correctly predicts other known mammalian cell topoisomerase II poisons including those with weak effects such as ciprofloxacin and confirmation that topoisomerase II knockdown does not also reduce the genotoxic effects of mutagens with different modes of action.

3. Extrapolation of the predictivity of topoisomerase II poisoning from *in vitro* to *in vivo*. The work for this thesis concentrated on the ability of topoisomerase II poisons to induce mutation *in vitro*. Clearly for risk assessment it is important to understand what the relationship is between *in vitro* and *in vivo* effects and ultimately the potential hazard to man. Both etoposide and gemifloxacin are potent *in vitro* and *in vivo* mutagens (Ashby *et al.,* 1994; Turner *et al.,* 2001; Rothfuss *et al.,* 2010), ciprofloxacin is a weak *in vitro* mutagen (Lynch *et al.,* 2003) and is negative *in vivo* (Herbold, 2001). Does this relationship between weak *in vitro* effects hold true for other topoisomerase II poisons and specifically for compounds that are positive for both topoisomerase II inhibition and genotoxicity in the screening assays detailed in Section 9.6? This idea has been pursued further with topoisomerase II (gyrase) targeting compounds from the AstraZeneca infection portfolio, where it has been demonstrated that weak *in vitro* mutagens are negative *in vivo*, whereas potent *in vitro* mutagens whose mode of action is targeting topoisomerase II are positive *in vivo* (data not shown). This work also hinted at a reduction in other *in vivo* toxicities (e.g. bone marrow toxicity) for compounds negative for *in vitro* genetic toxicity. Suggesting that mammalian cell genetic toxicity is a good biomarker for cross reactivity of compounds designed to target bacterial gyrase with the mammalian enzyme. This could potentially mean that the sort of screening cascade proposed in Section 9.6 may help to reduce the potential risk from other toxicity associated with topoisomerase II interactions as well as the potential for mutation and cancer.

Confirmation of this relationship would greatly aid the risk assessment of these compounds. Whilst the work on this has been completed and drafted for publication, unfortunately as propriety AstraZeneca compounds were used, permission to publish was not received in time for the data to be included in this thesis.

9.8 Final conclusions

The majority of the aims set out for this thesis were achieved. Data has been presented to provide reassurance that the mouse lymphoma assay is not prone to the generation of large numbers of false positive results when used in a pharmaceutical screening setting. However, it has been shown that the mouse lymphoma assay is not an appropriate screen for aneugenicity. Using the model compounds; etoposide and gemifloxacin, an improvement in the understanding of the relationship between topoisomerase II poisoning and genotoxicity has been made. The sensitivity of assays to measure topoisomerase II inhibition has been put into perspective and for the first time a thorough investigation into how the sensitivity of these assays relates to *in vitro* mammalian cell genotoxicity has been completed. Also for the first time the contribution of poisoning of topoisomerase II alpha to *in vitro* mammalian cell genotoxicity has been elucidated, potentially having a significant impact on the general understanding of the relationship between chemical interactions with the two isoforms of topoisomerase II and cancer. Finally, following on from the work investigating topoisomerase II alpha and associated genotoxicity, a novel screening paradigm has been proposed that could potentially de-risk chemical series designed to target bacterial topoisomerase II, thus significantly helping the development of important new medicines to tackle the unmet need of infectious disease.

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Appendix 1
Published papers relevant to this research

This text box is where the unabridged thesis included the following third party copyrighted material:

Fellows MD, Boyer S, O'Donovan MR. 2011 The incidence of positive results in the mouse lymphoma TK assay (MLA) in pharmaceutical screening and their prediction by MultiCase MC4PC. Mutagenesis. 26(4):529-32

This text box is where the unabridged thesis included the following third party copyrighted material:

Fellows M, Doherty AT, Priestley CC, Howarth V, O'Donovan MR. 2011 The ability of the mouse lymphoma TK assay to detect aneugens. Mutagenesis. 26(6):771-81.

This text box is where the unabridged thesis included the following third party copyrighted material:

Doherty AT, Hayes J, Fellows M, Kirk S, O'Donovan M. 2011. A rapid, semi-automated method for scoring micronuclei in mononucleated mouse lymphoma cells. Mutat Res. 726(1):36-41

This text box is where the unabridged thesis included the following third party copyrighted material:

Fellows M, Luker T, Cooper A, O'Donovan MR. 2012. Unusual structure–genotoxicity relationship in mouse lymphoma cells observed with a series of kinase inhibitors. Mutat Res. 746(1):21-8

This text box is where the unabridged thesis included the following third party copyrighted material:

Fellows MD, McDermott A, Clare KR, Doherty AT and Aardema MJ. 2014. The Spectral Karyotype of L5178Y TK+/- Mouse Lymphoma Cells Clone 3.7.2C and Factors Affecting Mutant Frequency at the Thymidine Kinase (tk) Locus in the Microtitre Mouse Lymphoma Assay. Environ Mol Mutagen. 55(1):35-42