RESOLUTION OF DNA ADDUCT FORMATION AT THE NUCLEOTIDE LEVEL AND CORRELATION WITH SITE SPECIFIC PROPENSITY TO MUTATION

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ABSTRACT

Tamoxifen a non steroidal antioestrogen (Z-*trans*-1-[4-(dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-butene) is widely used in the treatment of breast cancer, and is undergoing clinical evaluation as a chemopreventative in women thought to be at high risk of developing the disease. Tamoxifen is hepatocarcinogenic in rats, forming large numbers of tamoxifen DNA adducts when dosed over a period of time, but is inactive in standard genotoxicity tests. In this project I determined *in vitro* and *in vivo* DNA adduct formation at the nucleotide level from tamoxifen and selected metabolites. Sites of tamoxifen DNA adduct formation were mapped using the T4 DNA polymerase arrest and single stranded ligation assays. Following the reaction of plasmid DNA *in vitro* with α-acetoxytamoxifen or horseradish peroxidase/H_2O_2 activated 4-hydroxytamoxifen, DNA adduct formation occurred predominately on guanine. Preliminary studies *in vivo* also showed sites of tamoxifen DNA adduct formation on guanine with minor adduct formation on adenine. Plasmids reacted *in vitro* with activated 4-hydroxytamoxifen, were mutated 2 orders of magnitude more frequently than were plasmids reacted with α-acetoxytamoxifen in *E. coli*. This occurred despite α-acetoxytamoxifen forming a greater number of DNA adducts. A mutational hot spot in the bacterial *lacI* gene occurred in the region of the only adenine adduct for plasmids treated with activated 4-hydroxytamoxifen. These data indicated a lack of correlation between gross adduct number and mutagenic potential. This has implications for the interpretation of gross DNA adduct data.

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ERRATUM

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Typographical corrections

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Page 6:
Line 17 - betane should read betaine

Page 7:
Figure 6 legend: deoxyguanine should read deoxyguanosine; characteristicts should read characteristics and deoxythymine should read deoxythymidine

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Millard *et al.*, 1993 should read Millard & Beachy, 1993

Page 117:
Hender and Lutz, 1999 should read Ottender and Lutz, 1999

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Abbreviations

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
(γ^{32}P)- ATP	(γ^{32}P)-Adenosine 5'-triphosphate
NH_4OAc	Ammonium acetate
$(NH_4)_2SO_4$	Ammonium sulphate
[α-^{32}P]-UTP	[α-^{32}P]-Uridine 5'-triphosphate
8-oxo-dA	8-oxo-7,8-dihydro-2'-deoxyadenosine
8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
AAF	Acetylaminofluorene
AF	Aminofluorene
AFB_1	Aflatoxin B_1
AFB_1-epoxide	8,9-dihydro-8,9-epoxyaflatoxin B_1
AFB_1-FAPY	8,9-dihydro-8,9-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B_1
AFB_1-N^7-G	8,9-dihydro-2-(N^7-guanyl)-9-hydroxyaflatoxin B_1
AMV	Avian myoblastosis virus
AP	Apurinic/apyrimidinic
B(a)P	Benzo(a)pyrene
BER	Base excision repair
bp	base pair
C-D	Carbon-Deuterium
C-H	Carbon-Hydrogen
CPD	Cyclobutane pyrimidine dimer
CYP	Cytochrome P450
dCC	deoxycytosine-cytosine pyrimidine dimer
dCT	deoxycytosine-thymine pyrimidine dimer
dG-N^2-tam	N^2-deoxyguanosyl-tamoxifen
DMDO	Dimethyldioxirane
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphates
dsDNA	Double stranded deoxyribonucleic acid
dTT	deoxythymine-thymine pyrimidine dimer
DTT	Dithiothreitol
ε	Etheno
E. coli	*Escherichia coli*
EDTA	Ethylene diaminotetraacetic acid disodium
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
exo$^{+/-}$	exonuclease positive/negative
FAD	Flavinadeninedinucleotide
FAPY	Formamidopyrimidine
FCS	Foetal calf serum
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution

HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid]
HIV	Human immuno deficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
KCl	Potassium chloride
KOAc	Potassium acetate
lacI	Lactose initiator operon
LB	Luria broth
mdr1b	Rat multidrug resistance
$MgCl_2$	Magnesium chloride
$Mg(OAc)_2$	Magnesium acetate
$MgSO_4$	Magnesium sulphate
MOPS	3-[N-morpholino]-propanesulphonic acid
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
Nco I	Restriction enzyme isolated from *Nocardia corallina*
NER	Nucleotide excision repair
NP40	Nonylphenoxy polyethoxy ethanol
OH·	Hydroxyl radical
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pLIZ	Plasmid containing the *E. coli* lactose operon extracted from λLIZ shuttle vector of Big Blue™ rats
PMSF	Phenylmethylsulfonyl fluoride
Pwo	*Pyrococcus Wosei*
RAL	Relative adduct labelling
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SP6	Bacteriophage SP6
ssDNA	Single stranded deoxyribonucleic acid
T4	Bacteriophage T4
T7	Bacteriophage T7
Tam	Tamoxifen
Taq	*Thermus aquaticus*
TdT	Terminal deoxynucleotidyl transferase
TE	Tris-EDTA
TES	N-tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid
TFIIH	Transcription factor IIH
TLC	Thin layer chromatography
Tli	*Thermococcus litoralis*
TRC	Transcription repair coupling
TRCF	Transcription repair coupling factor
Tth	*Thermus thermophilus*
TWEEN 20	Polyoxyethylenesorbitan
U	Uracil
UV	Ultraviolet
Vsp I	Restriction enzyme isolated from *Vibro* species

XP	Xeroderma pigmentosum

GENERAL INTRODUCTION

1. Project Outline

In this project I have investigated DNA adduct formation at the nucleotide level after chemical exposure, particularly that formed by tamoxifen. At least two distinct metabolic pathways lead to adduct formation by tamoxifen, 1), via formation of α-sulphoxytamoxifen, and 2), via 4-hydroxytamoxifen. Both these metabolites form adducts with deoxyguanosine. I present new data on the DNA binding characteristics of these compounds at the nucleotide level, both *in vitro* and *in vivo*, and their sequence specific DNA binding properties. In particular I have correlated sites of DNA modification on plasmid DNA to the position of mutation using a bacterial mutation assay. In the discussion I review my data, in relation to published work, and draw conclusions concerning the mechanisms of DNA polymerase /adduct interactions, DNA adduct formation and mechanisms of mutation.

1.1 Adducts, Mutation and Cancer

Phenotypic change can result from accumulation of DNA mutations that modify gene expression. An example of such a phenotypic change is the gradual change from a normal cell to one that is neoplastic. The carcinogenic process involves multiple stages and is divided into initiation resulting from DNA damage, promotion and progression (Sukumar, 1990; Kinzler & Vogelstein, 1996; Barrett *et al.*, 1993). Experiments have shown that exposure to chemical carcinogens can be associated with DNA base changes to several critical genes. These results have lead to the concept that exogenous chemical adduction might cause mutations that activate or suppress proto-oncogenes and tumour suppressor genes respectively. It is now known that chemical carcinogens (genotoxins) react at multiple sites on DNA generating a variety of DNA adducts. DNA adduct formation is now recognised as the first critical molecular event in the initiation of chemical carcinogenesis (Barbacid, 1987; Balmain & Brown, 1988; Sukumar, 1990; Randerath *et al.*, 1989).

1.2 Role of DNA Damage in Mutation and Cancer

Molecular epidemiological studies have established a relationship of carcinogen exposure to DNA adduct formation in white blood cells of persons exposed to coke oven emissions and tobacco smoke with an elevated risk of developing lung and colon cancer (Motykiewicz *et al.*, 1995). Tobacco smoking is also correlated to DNA adduct formation in human tissues such as lung (Motykiewicz *et al.*, 1995; Perera *et al.*, 1992; Moller *et al.*, 1996; Binkova *et al.*, 1996; Nelson, 1996), cervix (Nelson, 1996), bladder (Binkova *et al.*, 1996; Nelson, 1996), breast (Grzybowska *et al.*, 1993), lymphocytes (Mooney *et al.*, 1997), larynx (Bartsch *et al.*, 1995, 1991) and placenta (Tang *et al.*, 1995; Bartsch, 1996). Molecular epidemiology has also indicated that DNA adduct formation from exposure of Chinese people to N-nitrosoamine elevates the risk of developing cancers of the oesophagus (Santella *et al.*, 1992) and in liver cancers of Japanese people (Savela & Hemminki, 1991). These associations all indicate a mutagenic risk is associated with DNA adduct formation.

1.3 DNA Damage

DNA damage may be defined as 'any modification of DNA that alters its coding properties or its normal function in replication or transcription', (Hanawalt, 1998). DNA is highly reactive and is subject to alteration by spontaneous change and interaction with physical and chemical agents. DNA adduct formation is recognised as a common property of most mutagens, with each class of mutagen producing a characteristic adduction pattern by reacting at specific sites in DNA (Dipple, 1995; Hemminki *et al.*, 1994).

Some mutagens are intrinsically reactive towards DNA, whereas others require metabolic activation (Hemminki *et al.*, 1994). Chemical mutagens that are not intrinsically electrophilic are activated to reactive electrophiles by phase I, and phase II metabolism and are capable of reacting with the numerous nucleophilic sites of DNA, RNA and protein. Phase I metabolism is performed by the multiple forms of hepatic cytochrome P450 (haem oxygenases) which have a capacity to activate xenobiotics to multiple DNA reactive species mainly by the oxidation of carbon or reduction of

nitrogen atoms in molecules (fig 1) (Nebert *et al.*, 1991; Guengerich, 1994; Nebert *et al.*, 1996). Additionally phase II metabolism by esterification or conjugation reactions of phase I metabolites may generate a more reactive electrophile (Dipple, 1995).

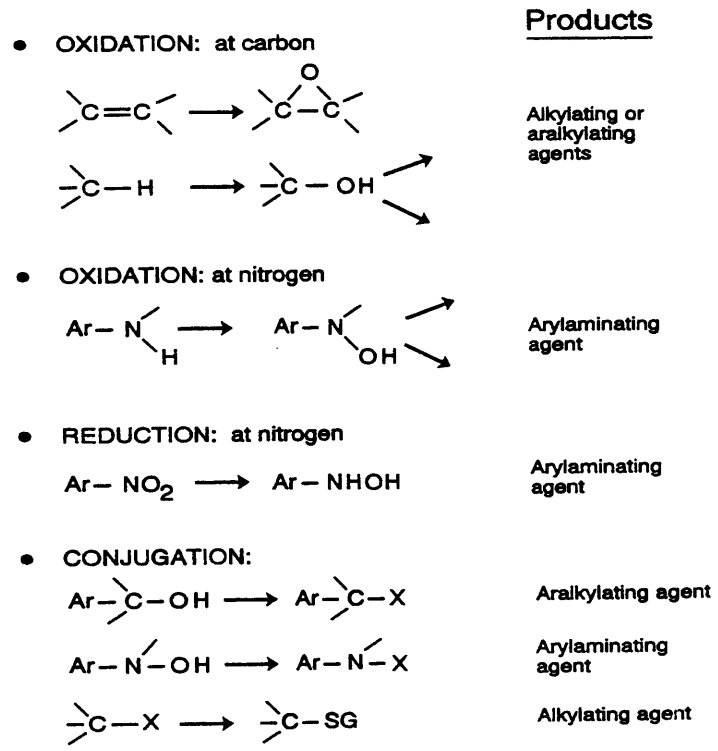


Fig 1 – metabolic reactions associated with the generation of DNA reactive electrophiles. Diagram taken from Dipple, 1995.

1.4 Chemistry of DNA Adduct Formation

The chemistry of DNA adduct formation is complex, and chemical mutagens are classed on the basis of their chemistry of DNA modification in aqueous solution.

1.4.1 Alkylation of DNA

Mutagens that transfer alkyl residues to the bases of DNA include the nitrosoamines, aliphatic epoxides, aflatoxins, lactones, nitrosoureas, mustards, haloalkanes, alkyl triazenes and sultones (Lawley, 1984). Non ionic alkylating agents acting through Sn2 mechanisms e.g. dimethylsulphate preferentially react with the ring nitrogen atoms of high nucleophilic strength eg N-7 of dG. Whereas those of greater ionic character react through Sn1 mechanisms e.g. N-methyl-N-nitrosourea. Sn1 reactions are less selective, and occur at the exocyclic oxygen atoms of DNA (fig 2) (Lawley, 1984; Moschel *et al.*, 1979; Hemminki, 1983). Depending on the alkyl subsituent of the electrophile,

adduction at N-7 of dG and N-3 of dA causes depurination of the adducted base generating an AP (apurinic) site. N-3 and N-7-alkyl-dA and N-7-alkyl-dG may also undergo imidazole ring opening (Lawley, 1966).

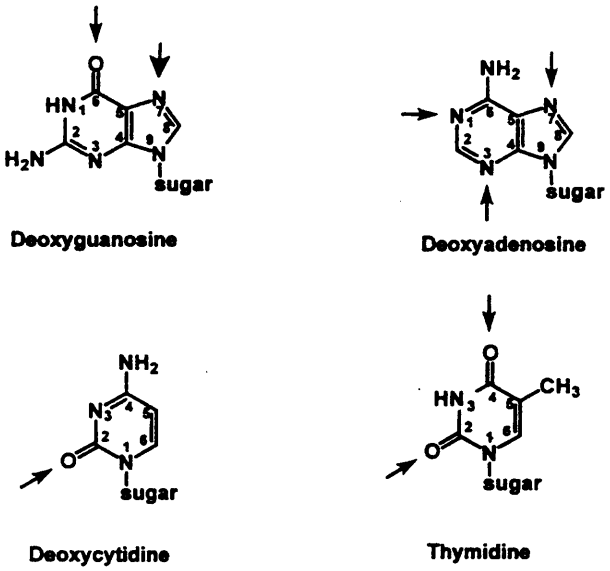


Fig 2 – sites of substitution of DNA by alkylating agents.

1.4.2 Arylamination of DNA

Electrophiles that transfer arylamine residues to DNA include aromatic amines, amides, aminoazo dyes, nitroaromatics and heterocyclic aromaticamines. The N-8 atom and amino groups of the purine nucleotides are the main targets (fig 3) (Humpherys *et al.*, 1992). These adducts are more stable than alkylated bases, but some N-8-dG adducts can undergo ring opening (Kadlubar, 1994).

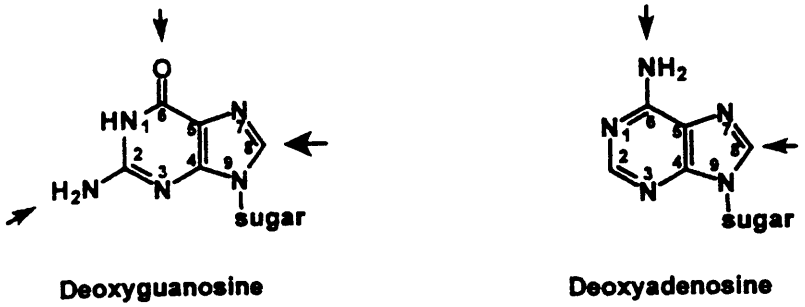


Fig 3 – sites of substitution of DNA bases by arylaminating agents

1.4.3 Aralkylation of DNA

The transfer of aralkyl groups to DNA occurs with the pyrrolizidine alkaloids, alkenyl benzenes and polyaromatic hydrocarbons (Weisman, 1985). Most adducts occur on the amino groups of the purines producing relatively stable adducts (fig 4) (Weisman *et al.*, 1985; Cheh *et al.*, 1993; Chadha *et al.*, 1989).

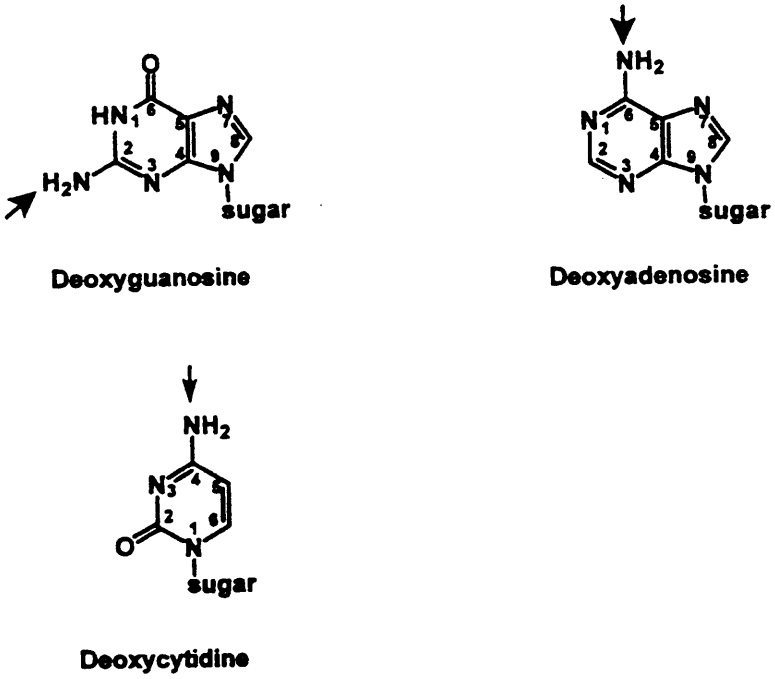


Fig 4 – sites of substitution of DNA bases by aralkyating agents

1.5 Mechanisms of Mutagenesis

Most chemical mutagens producing DNA adducts may cause mutation by inducing base pair substitution, additions and deletions (Loechler, 1996). These arise from the misreplication of adducted bases that either act as miscoding or noncoding lesions (Hemminki, 1993).

1.5.1 Spontaneous Mutation

Spontaneous mutagenesis results from a combination of factors, the most important being DNA polymerase errors (Loeb, 1991), and unrepaired endogenous DNA damage (Ames, 1989; Marnett & Burcham, 1993). Levels of endogenous DNA damage are species, sex, and age dependent. Endogenous adducts can form from reactive

electrophiles produced during normal cellular metabolism, oxidative stress and chronic inflammation (Ames & Gold, 1991). Endogenous DNA damage falls into five main categories; 1) deamination, 2) methylation, 3) depurination, 4) oxidative damage and 5) cyclic nucleotides (Rossman & Goncharova, 1998; Singer & Grunberger, 1993).

1.5.2 Deamination

Deamination of cytosine to uracil and 5-methyl-cytosine to thymine are the most common spontaneous mutations seen *in vivo* (Shapiro & Klein, 1966; Lindahl & Nyberg, 1974). Purine DNA bases are less susceptible to spontaneous hydrolytic deamination, with adenine forming hypoxanthine and guanine producing xanthine. Deamination is accelerated by the reaction of DNA with nitrous oxide (NO) which is formed endogenously (Nguyen *et al.*, 1992). Spontaneous and nitrosative deamination occurs via nucleophilic aromatic substitution, but the nitrosative pathway is favoured as better leaving groups are formed e.g. $-N_2OH_2^+$ or $-N_2^+$ rather than $-NH_3^+$ (Goul, 1959; Carey & Sunberg, 1990).

1.5.3 Alkylation

DNA can be alkylated from endogenous methylating agents including tertiary amines such as betane, choline and the trialkylsulphonium agent S-adenosylmethionine (SAM) (Rydberg & Lindahl, 1982; Barrows & Magee, 1982). The reactivity of nucleophilic oxygen and nitrogen atoms in DNA are variable towards alkylating agents (as described). The N-7 and N-3 of deoxyguanine and N-3 of deoxyadenine have the highest reactivity with Sn2 reacting alkylating agents, whereas, Sn1 reacting alkylating agents react more exclusively with the exocyclic base oxygens (Dipple *et al.*, 1982; Singer, 1982). SAM can methylate N-7, N-3 and O^6 of dG and N-3 of dA (fig 5) (Barrows & Magee, 1982; Nath *et al.*, 1992). These methylated bases vary markedly in their ability to induce mutations. A major product of methylation is Me-N-7-dG, but this position is not involved in base pairing (Saffhill *et al.*, 1985). However, this methylated base can undergo base catalysed ring opening of the imidazole ring to form 5-formamido-4,6-pyrimidine derivatives which can block DNA replication (Boiteux & Laval, 1983; O'Connor *et al.*, 1988; Tudek *et al.*, 1992). Methylation at N-3 of dA produces a cytotoxic lesion which blocks RNA polymerase progression (Lindahl *et al*,

In contrast, O^6Me-dG and O^4Me-dT are highly mutagenic lesions as methylation alters the hydrogen bonding pattern of the bases.

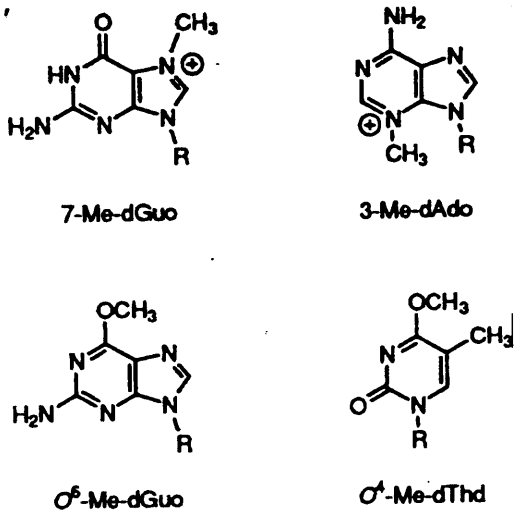
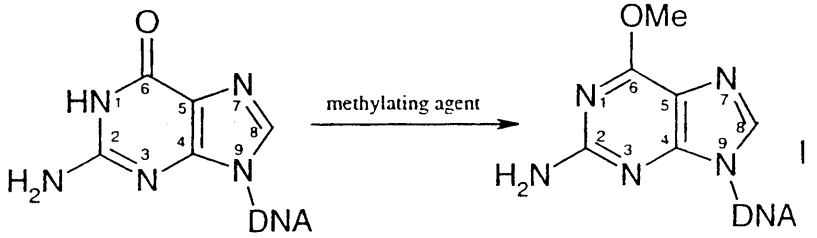


Fig 5 – structures of methylated bases

Methylation of O^6-dG fixes the enol tautomer of the base (fig 6) and this facilitates base pairing with thymine. O^6Me-dG produces G>A transitions in both bacterial and mammalian cells (Singer *et al.*, 1989; Singer, 1990; Loechler *et al.*, 1984; Rossi & Topal *et al.*, 1991; Pletsa *et al.*, 1992; Pauly *et al.*, 1991). O^4Me-dT is very mutagenic both *in vivo* and *in vitro* (Singer *et al.*, 1990) mainly inducing T>C transitions.



Keto form Enol form

Fig 6 - Methylation at the O^6 position of deoxyguanine fixes the normal keto base into an enol form. The enol form presents alternate hydrogen bonding characteristicts, allowing base pair fomation with deoxythymine

1.5.4 Depurination

Cleavage of the N-glycosyl bond between the base and deoxyribose generates an apurinic or apyrimidinic site (Basu & Essigmann, 1988). Spontaneous loss of purines occurs more rapidly than the loss of pyrimidines (Hurley *et al.*, 1994). Alkylation of N-7 of dG or N-3 of dA causes a delocalisation of the base and renders the N-glycosidic

bond sensitive to hydrolysis (Lawley, 1966). Abasic sites are non instructional DNA lesions and are therefore potentially mutagenic and cytotoxic. *In vitro* and *in vivo* studies of AP sites show that both DNA and RNA polymerases of prokaryotes normally pause at the site, but when bypassed, adenine is preferentially inserted opposite the lesion. AP sites give rise to α and β anomers of 2'deoxyribose which easily generate scissions in the phosphodiester backbone (Jones *et al.*, 1984; Kalnik *et al.*, 1988).

1.5.5 Oxidation

Endogenous sources of oxidants arise from the leakage of reactive oxygen species (ROS) from the mitochondria or endoplasmic reticulum (Cadenas, 1989). Although ROS are genotoxic, the precise nature of the DNA damaging species has not been elucidated (Rossman & Goncharova, 1998). ROS include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical ($\cdot O^{2-}$), nitric oxide (NO), organic oxyradicals ($RO\cdot$) and organic peroxyradicals ($ROO\cdot$) which can yield more than 30 different base adducts (fig 7), strand breaks, AP sites and cross links (Marnett & Barcham, 1993; Shigenaga *et al.*, 1989; Bonura & Smith, 1981). Hydrogen peroxide breaks down to form the hydroxyl radical ($\cdot OH$). This radical can attack purine and pyrimidine bases directly eg C^4, C^5 and N-8 of dG or dA resulting in ring opening, ring saturation or hydroxylation. Pyrimidine attack can yield a variety of alterations, mainly dT and dC glycols (Beckman *et al.*, 1990; Halliwell & Aruoma, 1991; Teebor *et al.*, 1988.

The mutagenic consequences of only a few of these adducts are known. The main oxidative product formed and characterised is 8-oxo-dG formed after hydroxyl radical attack (Floyd, 1990; Floyd *et al.*, 1986; Kasai *et al.*, 1986; Park *et al.*, 1989). 8-Oxo-dG is produced in large quantities after exposure to a variety of agents producing ROS eg. ionising radiation, 4-nitroquinoline, 2-nitropropane (Dizderglu, 1985; Kasai, 1993) as well as singlet oxygen, produced by hydrogen peroxide in the presence of Fe^{3+} or Cu^{2+} (Aruoma *et al.*, 1991, 1989). 8-oxo-dG lesion induces G>T transitions (Wood & Robins, 1989). Dexoyguanosine can also be oxidised forming 8-oxo dG-triosephosphate that can be incorporated opposite dA causing A>C transitions. The highly mutagenic lesion 5'hydroxy-2'-deoxycytidine produces T>C transversions (Feig

et al., 1994). Hydroxy radical attack on C5-C6 double bond of dT forms thymine glycol (5,6-dihydro-5,6-dihydroxythymidine) (Teebor, 1988) and blocks DNA polymerase progression *in vitro* (Ide *et al.*, 1985; Clark & Beardsley, 1989). This lesion may be weakly mutagenic *in vivo* (Hayes *et al.*, 1988), inducing T>C transitions (Basu *et al.*, 1989).

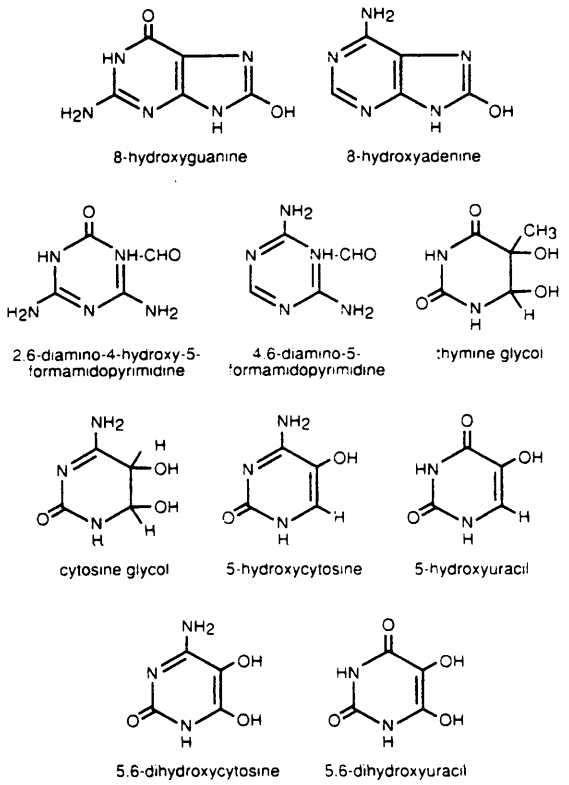


Fig 7 – chemical structures of the major oxidised adducts resulting from hydroxyl radical attack.

1.5.6 Cyclic Nucleotides

Polyunsaturated fatty acids contain one or more methylene groups positioned between *cis* double bonds that are highly reactive towards oxidising agents. They form carbon centered radicals which react with oxygen forming peroxyl radicals resulting in the formation of isoprostanes and malondialdehyde (MDA) (Marnett, 1999). MDA is a difunctional electrophile that can react with the exocyclic and ring nitrogens forming cyclic bases commonly called etheno (ε) adducts (fig 8) (Bartsch & Singer, 1985). This class of DNA lesion is formed by many genotoxic chemicals including vinyl

chloride and urethane (Bartsch, 1996). The four etheno bases produce mainly base pair substitution mutations. εdA induces A>G transitions, A>T and A>C transversions (Basu *et al.*, 1993; Pandya & Moriya, 1996); εdC causes C>A transversions and C>T transitions (Palejwala *et al.*, 1993; Moriya *et al.*, 1994); N^2,3-εdG produces G>A transitions (Cheng *et al.*, 1991); and 1,N^2-εdG induces G>T and G>C transversions, in addition to -1 and -2 frameshift mutations (Langouet *et al.*, 1997). The mutagenic efficiency of εdC and εdA is greater in mammalian cells than for *E. coli* (Pandya & Moriya, 1996; Shibutani *et al.*, 1996).

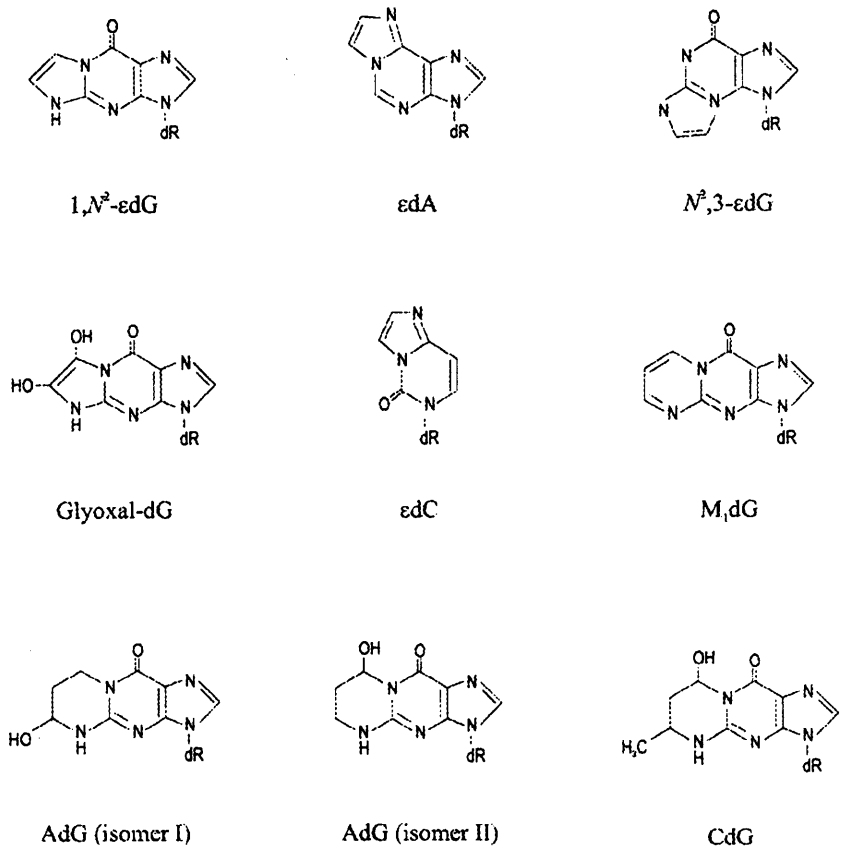


Fig 8 – Chemical structures of exocyclic base DNA adducts.

1.6 Physical DNA Damage and Mutagenesis

1.6.1 Ionising Radiation

Ionising radiation can transfer energy directly to DNA, exciting the macromolecule (Van Sonntag, 1987), yielding electron holes (radical cations) and electron adducts (radical anions). The charge generated migrates along the DNA chain localising at guanine bases and oxidising them (Becker & Sevella, 1993). Direct ionisation of DNA in the aqueous cellular environment is limited, therefore DNA damage arises mainly from the hydroxyl radical produced from the radiolysis of water (Melvin *et al.*, 1998, Pfeifer *et al.*, 1994).

In total ionising radiation produces more than 29 different DNA base and sugar adducts (Teoule, 1987). The hydroxyl radical is the major reactive species produced and is responsible for many DNA modifications particularly 8-oxo-dG (O'Connor *et al.*, 1993). Ionising radiation also produces intramolecular cyclisation of purine bases forming 8-5'-cyclodeoxyadenine and guanine (Dizderglu & Simic, 1984).

1.6.2 Ultraviolet Radiation

The most energetic part of natural solar UV radiation is the 280-320nm region (UVB) (Brash *et al.*, 1991). UVB is very efficient at producing direct DNA damage, mainly cyclobutane pyrimidine dimers (CPD's) and (6-4) photoproducts (fig 9) (Cadet *et al.*, 1992). Near UV radiation 320-380nm (UVA) DNA damage is dependent on the presence of molecular oxygen and thus DNA oxidation is prominent (Setlow & Woodhead, 1994).

Although 4 isomeric forms of photodimers are possible, only the *cis-syn* isomers are formed in reasonable yields in biological systems with dTT being the main lesion (Basu & Essigmann, 1988). In bacteria, mutation mainly occurs at the 3' side of dTT, dTC & dCC pyrimidine dimers inducing mainly G>A and A>G transitions. Dimer formation is sequence specific (Friedberg, 1985), generally dTT flanked by a 5'dA and 3' dG (Gordon & Haseltine, 1982). Pyrimidine-pyrimidine 6,4 photoproducts of mainly dC 3'

to a corresponding pyrimidine form from covalent bond formation between C6 and C4 of two pyrimidines (Mitchell *et al.*, 1989). The 6-4 photoproducts of dTC, dCC and dTT are formed at approximately one tenth of the frequency of cyclobutane dimers (Brash & Haseltine, 1982).

CPD's produce small local distortions in the DNA at the site of the lesion. They remain hydrogen bonded to their complimentary base pair but this bonding is weaker (Taylor & Oday, 1990). CPD's are effective blocks to DNA polymerase progression, but CPD's pair correctly when a bypass is forced *in vitro* with dA being inserted 95% of the time (Taylor, 1995). Most UV induced mutations occur at the dC containing bipyrimidine sites leading to C>T transitions (Fix & Bockrath, 1983; Lippke *et al.*, 1981; Tang *et al.*, 1986; Wood, 1985). The dTC CPD has been postulated to give rise to mutation during translesional synthesis by two potential mechanisms, the iminotautomer of dC pairing with dA or deamination of dC to dU (Jiang & Taylor, 1993; Pearson *et al.*, 1974; Taylor & O'day, 1990; Wood, 1985). dTC pyrimidine 6-4 photoproducts are potent pre-mutagenic lesions that can block translesional DNA synthesis *in vitro* (Chan *et al.*, 1985), but when bypassed induces a C>T transition (Horsfall & Lawence, 1994). The dTT 6-4 photoproduct is highly mutagenic in *E. coli* (LeClerc *et al.*, 1991) and mammalian cells (Jiang & Taylor, 1993), but not in yeast (Gibbs *et al.*, 1995).

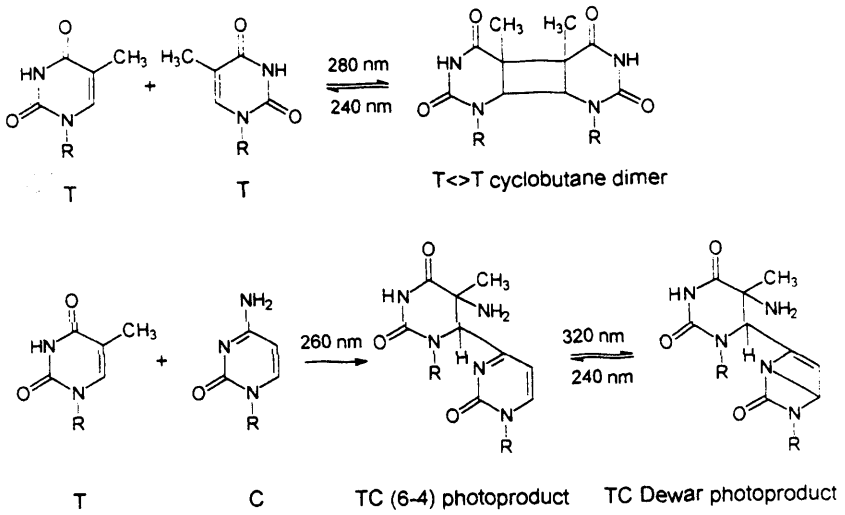


Fig 9 – The major UV-induced lesions in DNA.

1.7 Chemical Damage and Mutagenesis

DNA can be modified by several classes of DNA binding agents. In this section I will concentrate on tamoxifen and aflatoxin B_1.

1.7.1 Tamoxifen

Tamoxifen, a non steroidal anti oestrogen (Z-*trans*-1-[4-(dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-butene) (fig 10), is widely used in the treatment of breast cancer (Jaiyesimi *et al.*, 1995). It is currently undergoing clinical evaluation in the UK, USA, and Italy as a chemopreventative agent in women thought to be at high risk of developing oestrogen stimulated mammary tumours (Powles, 1992; Marques & Beland, 1997). Recent results in healthy women at high risk to breast cancer from the NSABP P1 study showed that tamoxifen treatment resulted in a 49% reduction in breast cancer incidence (Fisher *et al.*, 1999).

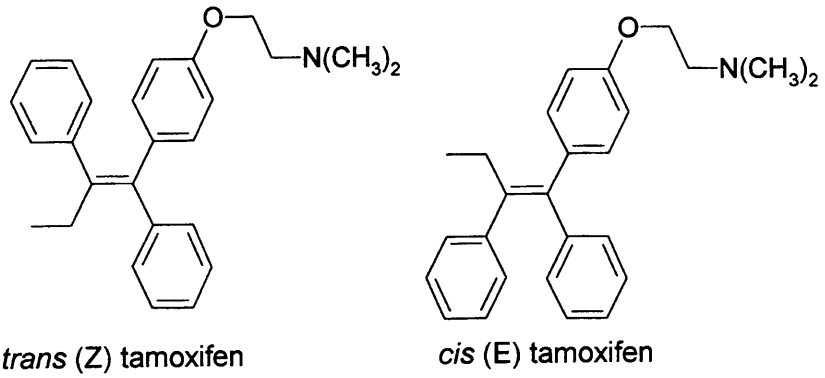


Fig 10 – Molecular structure of tamoxifen. *Trans* tamoxifen is an oestrogen antagonist which is used clinically whereas as the *cis* isomer is an oestrogen agonist.

Tamoxifen is inactive in standard short term genotoxicity tests (Tucker *et al.*, 1984). However, administration of tamoxifen to rats either for 1 year at 45.2mg/kg/day, or for 2 years, with doses ranging from 0.3 to 35mg tamoxifen/kg/day produces hepatocellular carcinoma in a dose dependent manner (Williams *et al.*, 1993; Greaves *et al.*, 1993; Hirsimaki *et al.*, 1993; Carthew *et al.*, 1995). Women have not been shown to develop hepatocellular carcinoma (HCC) from tamoxifen therapy, however, treatment is associated with an increased incidence of endometrial carcinoma (Fisher *et al.*, 1994; Mühlemann *et al.*, 1994; Curtis *et al.*, 1996; Rutqvist *et al.*, 1995; IARC, 1996). The risk of developing an endometrial tumour correlates with the duration of tamoxifen treatment (Curtis *et al.*, 1996; Fornander *et al.*, 1989; Leeuwen, 1994). In 1996, a working group of the IARC concluded that tamoxifen is carcinogenic in humans (IARC, 1996).

Mice are resistant to the carcinogenic effects of tamoxifen (Tucker *et al.*, 1984; Martin *et al.*, 1997), but tamoxifen DNA adducts are detected in the liver following oral administration (White *et al.*, 1992; Randerath *et al.*, 1994a; Martin *et al.*, 1997) and i.p. dosing (Randerath *et al.*, 1994a), but are present at lower levels than in the rat. Rats and mice treated similarly over 3 months with tamoxifen (40mg/kg/day) accumulate approximately 3000 adducts per 10^8 nucleotides in the livers of rats compared to 70 adducts per 10^8 nucleotides in mice (Carthew *et al.*, 1995; Martin *et al.*, 1997). Tamoxifen is also hepatocarcinogenic to hamsters (Montadon & Williams, 1994) and associated with hepatic DNA adduct formation (Phillips *et al.*, 1996a). Tamoxifen undergoes hepatic metabolism forming reactive electrophiles, which bind to macromolecules (Mani & Kupfer, 1991; Pathack & Bodell, 1994), with a 10 fold greater binding affinity towards protein (White *et al.*, 1997). Rats dosed with low concentrations of tamoxifen produce aneuploidy in the liver (Sargent *et al.*, 1994), with micronuclei formation in MCL5 cells[1] in a dose dependent manner and induction of unscheduled DNA synthesis in cultured rat hepatocytes (White *et al.*, 1992).

1.7.1a Metabolism

In humans, rats, and mice the major metabolites of tamoxifen are N-desmethyl tamoxifen, 4-hydroxytamoxifen and tamoxifen N-oxide with the latter two formed in smaller amounts (fig 11) (Robinson *et al.*, 1991; Mani *et al.*, 1993; Lim *et al.*, 1994; King, 1995). There is evidence from rat and human liver microsomal experiments that the CYP3A family is involved in the activation of tamoxifen (Foster *et al.*, 1985; Pathak *et al.*, 1995; Mani *et al.*, 1994). Tamoxifen administration induces CYP 2B1, CYP 2B2, CYP 3A1, epoxide hydrolase, aldehyde dehydrogenase in rat liver (Nuwaysir *et al.*, 1995; White *et al.*, 1993), and therefore, may be involved in its activation. Metabolism of tamoxifen by N-demethylation and 4-hydroxylation is associated with CYP1A, 3A, 2C, and 2D6 family in rats and humans (Jacolot *et al.*, 1991; Daniels *et al.*, 1992; Mani *et al.*, 1994). α-Hydroxy tamoxifen, 3,4 dihydroxy tamoxifen and

[1] These cells have been genetically engineered to express a battery of human CYP-P450 isoenzymes as well as epoxide hydrolase, but do not express the same balance of activating and detoxifying enzyme activities as normal cells (Crespi *et al.*, 1991).

metabolite E (fig 11) have also been detected in the media of hepatocyte cultures[2] incubated with tamoxifen (Phillips *et al.*, 1994b) and in the plasma of women treated with tamoxifen (Poon *et al.*, 1993; Murphy *et al.*, 1987; Stevenson *et al*, 1988). Microsomal metabolism in human, rat and mouse systems also produces N-didesmethyl, 4'-hydroxy, 4'-hydroxy,N-desmethyl, 4-hydroxy,N-oxide tamoxifen, tamoxifen 3,4 epoxide, 3,4 dihydrodiol tamoxifen, tamoxifen 3',4' epoxide, 3'4' dihydrodiol, α-hydroxy,N-oxide, α-hydroxy,N-desmethyl and 3,4 dihydroxy tamoxifen (Poon *et al.*, 1995; Lien *et al.*, 1991; Lim *et al.*, 1994; Foster *et al.*, 1980).

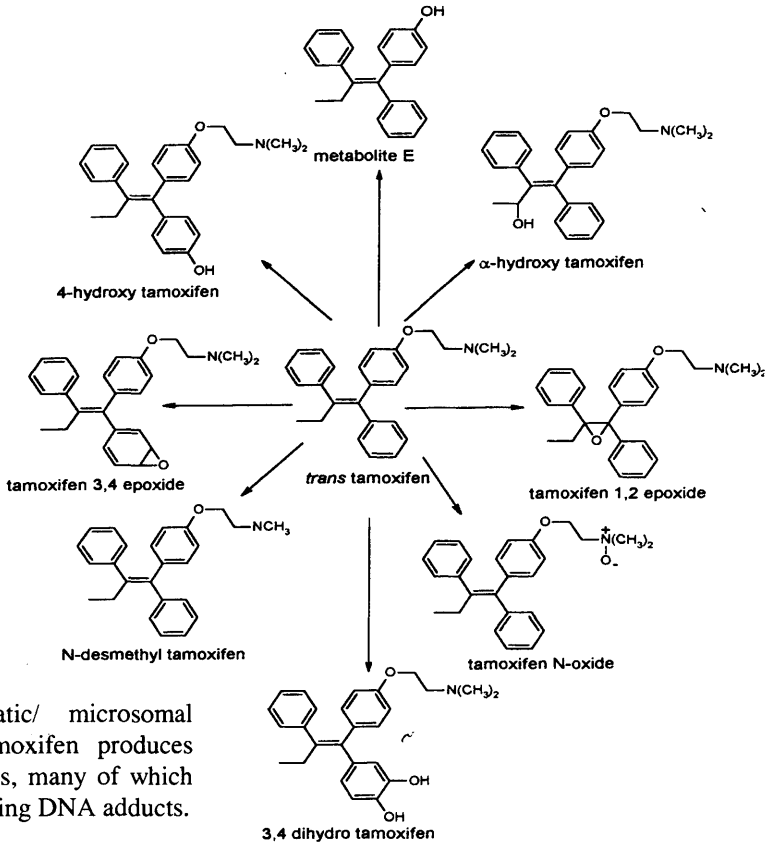


Fig 11 – Hepatic/ microsomal metabolism of tamoxifen produces multiple metabolites, many of which are capable of forming DNA adducts.

[2] Tamoxifen forms DNA adducts in primary cultures of rat hepatocytes with a pattern of adducts detected by ^{32}P-postlabelling being identical to those detected in rat liver *in vivo*, and these cells therefore may model whole animal liver metabolism

1.7.1b DNA Adduct Formation

1.7.1b(i) α-Hydroxylation

Tamoxifen and its analogue toremifene have been assessed for the ability to form DNA adducts. Screening of these analogues and metabolites of tamoxifen has indicated that the dimethylamine group is insignificant in producing reactive electrophiles capable of binding to DNA. However, the analogue toremifene strongly indicates that the ethyl side chain is crucial (White *et al.*, 1992; Chander *et al.*, 1991; Hasmann *et al.*, 1994). Toremifene (Z-2-[4-chloro-1,2,diphenyl-1-butenyl)-phenoxy]-N,N-dimethyl-ethanolamine) (fig 12) is different from tamoxifen by having a chlorine atom at the β position of the ethyl side chain. Toremifene increases the incidence of hepatocellular carcinoma only in diethylnitrosoamine initiated female Fisher rats at approximately one third of that seen for tamoxifen (Dragen *et al.*, 1995). Toremifene reduces the formation of hepatic DNA adducts, as measured by the ^{32}P-postlabelling assay resulting in no development of liver or endometrial tumours (White *et al.*, 1992; Li *et al.*, 1997). Rat and human microsomal activation of toremifene produces a reactive intermediate which forms DNA adducts at a reduced level (Hemminki *et al.*, 1995). Toremifene also produces dose dependent micronuclei formation in MCL5 cells *in vitro*, but at a lower level than for tamoxifen (Styles *et al.*, 1994). Peroxidase oxidation of tamoxifen and toremifene *in vitro* produces a free radical intermediate, but does not lead to a significant level of DNA adduct formation (Davies *et al.*, 1995b). Therefore, the proposed mechanism of the metabolic activation of tamoxifen to DNA binding species is via oxidation of either the ethyl moiety at the α or β carbon. This was confirmed by dosing rats with [ethyl-D_5]-tamoxifen which displays decreased ethyl side chain oxidation. This compound exhibits lower DNA binding properties, is less genotoxic, and induces fewer micronuclei formation in MCL5 cells than tamoxifen. This is because hydrogen extraction is the rate limiting step of this activation and C-D bonds have a slightly higher bond energy than C-H and so decrease ethyl side chain metabolism. This is called the primary deuterium kinetic isotope effect (Keefer *et al.*, 1973). Therefore this implicated that the ethyl side chain is hydroxylated as the main activating pathway for tamoxifen DNA adduct formation (Potter *et al.*, 1994).

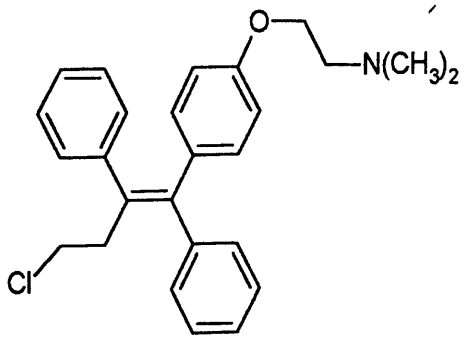


Fig 12 – Chemical structure of toremifene

Using α-D_2 ethyl and β-D_3 ethyl tamoxifen, an intermediate of α-hydroxy tamoxifen was identified (Phillips *et al.*, 1994c). Removal of the hydroxyl function from α-hydroxy tamoxifen produces a carbocation capable of alkylating DNA. α-Hydroxy tamoxifen is unlikely to be the ultimate reactive intermediate as it only has a low level of reactivity towards DNA *in vitro* (Shibutani *et al.*, 1997) producing approximately 5.6 adducts/10^8 normal nucleotides (Dasaradhi & Shibutani., 1997). In contrast allylic esters of α-hydroxy tamoxifen are expected to be highly reactive towards DNA and would react by a Sn1 addition reaction in which the ester group is substituted to the nucleophilic DNA via the formation of the allylic carbocation (Potter *et al.*, 1994). An ester of α-hydroxy tamoxifen, α-acetoxy tamoxifen was chemically synthesised since an acetoxy group is a better leaving group than the hydroxyl function. This reaction, of *trans* α-acetoxy tamoxifen with DNA, gave similar patterns of ^{32}P-postlabelled tamoxifen DNA adduct spots on a TLC plate to those produced by rats dosed with tamoxifen. When α-acetoxy tamoxifen postlabelled adducts are separated using HPLC, one major and 5 minor adduct peaks are generated that correspond to some adduct peaks identified from the liver of rats treated with tamoxifen (Martin *et al.*, 1998). Isolation by HPLC of two reaction products from hydrolysed DNA reacted *in vitro* with *trans* α-acetoxy tamoxifen produced a *trans* dG-N^2-tamoxifen adduct as the major product and *trans* and *cis* dA-N^2-tamoxifen as a minor product (Osborne *et al.*, 1997). The *trans* form of dG-N^2-tamoxifen adduct is the major species formed in the livers of rats treated with tamoxifen (Osborne *et al.*, 1996). Therefore, an ester formed by phase

II conjugation is likely to be the ultimate carcinogenic metabolite of tamoxifen. This is indicated by the use of sulphotransferase inhibitors pentachlorophenol and dehydroisoandrosterone-3-sulphate which inhibit tamoxifen DNA adduct formation in mice and rats respectively (Randerath *et al.*,1994a; Shibutani *et al.*, 1998a). Therefore α-hydroxy tamoxifen is expected to be O-sulphated prior to reaction with DNA (fig 13) (Osborne *et al.*, 1996). Chemically synthesised α-sulphoxy tamoxifen is highly reactive towards DNA *in vitro*, with *cis*-α-sulphoxy tamoxifen producing 8.7 adducts per 10^5 nucleotides and *trans*-α-sulphoxy tamoxifen producing 1 adduct per 10^5 nucleotides[3] (Brown *et al.*, 1998). Both α-*trans and cis* forms of sulphoxy tamoxifen when reacted with 2'-deoxyguanosine produce four diastereoisomers of α-(N^2-deoxyguanosyl) tamoxifen (dG-N^2-tamoxifen) adduct (the epimeric centre of each *trans* and *cis* form of dG-N^2-tamoxifen adduct is at the α-carbon). When the *trans* form of α-sulphoxy tamoxifen is reacted with 2'-dG the amount of the *trans* adduct formed is 60% greater than that of the *cis* form. In contrast when α-*cis*-sulphoxy tamoxifen is reacted with 2'-dG there is a nearly equal formation of *trans*/*cis* forms of the 2'-dG-N^2-tamoxifen adduct (Shibutani *et al.*, 1998b).

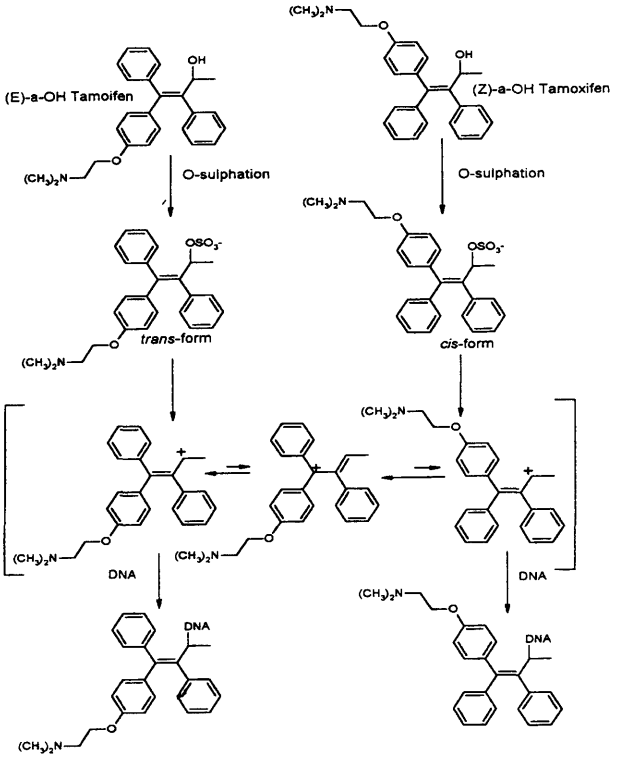


Fig 13 – Formation of α-sulphoxytamoxifen. This phase II metabolite can break down forming a putative reactive carbocation. This carbocation can undergo rotation around the central bond to producing *cis* and *trans* isomers capable of forming DNA adducts.

[3] α-*trans*-sulphoxy tamoxifen is highly reactive and produces fewer DNA adducts than the *cis* form due to the rapid hydrolysis in aqueous solution and exposure from the atmosphere when reacted with DNA *in vitro*.

Other phase I metabolites have been suggested as proximate carcinogens such as 3,4-dihydroxy tamoxifen (Dehal & Kupfer, 1996), 3,4 epoxy tamoxifen (Davies *et al.*, 1995b), (E,Z)-1,2-diphenyl-1-(4-hydroxyphenyl)-1-but-1-ene (*cis/trans* metabolite E), and 4-hydroxy tamoxifen. In addition, tamoxifen N-oxide, N-desmethyl tamoxifen and 4-hydroxy tamoxifen are capable of being further metabolised (α-hydroxylated) and form DNA adducts (Potter *et al.*, 1994; Phillips *et al.*, 1994b; Rajaniemi *et al.*, 1999; Phillips *et al.*, 1999; Brown *et al.*, 1999), therefore, giving the full spectrum of tamoxifen DNA adducts.

1.7.1b(ii) 4-Hydroxylation

4-Hydroxytamoxifen is a metabolite that has been detected in the serum of breast cancer patients (Robinson *et al.*, 1991), and has a binding affinity of 100 times that of tamoxifen for the oestrogen receptor (Jordon, 1976). HPLC analysis of ^{32}P-postlabelled adducts from DNA reacted *in vitro* with 4-hydroxy tamoxifen gave one major adduct peak that co-eluted with a very small adduct peak produced from rat liver (Martin *et al.*, 1998). 4-Hydroxytamoxifen can undergo subsequent oxidation to 4-hydroxytamoxifen quinone methide. This quinone methide is an intermediate that can yield DNA adducts via a Michael type addition reaction (fig 14) (Randerath *et al.*, 1994b; Pongracz *et al.*, 1995; Moorthy *et al.*, 1996b; Pathak *et al.*, 1996). Reactions of 4-hydroxy tamoxifen quinone methide with DNA gives two major adducts (E) and (Z) α-(deoxyguanosin-N^2-yl)-4-hydroxy tamoxifen. The structures of these adducts may be identical to those of the major adduct characterised from α-acetoxy and α-sulphoxy tamoxifen (Osborne *et al.*, 1996; Dasaradhi & Shibutani., 1997; Shibutani *et al.*, 1998a; Osborne *et al.*, 1997) differing only by the presence of a phenolic hydroxyl function.

Pentachlorophenol administration to rats along with tamoxifen increases the formation of more polar DNA adducts (Meerman *et al.*, 1983) and decreases the formation of adducts produced via α-hydroxylation (Randerath *et al.*, 1994a) due to the decreased sulphate conjugation indicating that α-hydroxytamoxifen may be the major proximate carcinogen in the rat. Furthermore, peroxy radicals may also be implicated in

peroxidase activation of 4-hydroxytamoxifen (Davies *et al.*, 1997) as well as formation of superoxide anions during rat hepatic metabolism of tamoxifen (Turner *et al.*, 1991). 3,4-Dihydroxytamoxifen is also formed by rat hepatic microsomal metabolism of 4-hydroxytamoxifen which can be subsequently oxidised to reactive semi and ortho-quinones (Dehal & Kupfer, 1996).

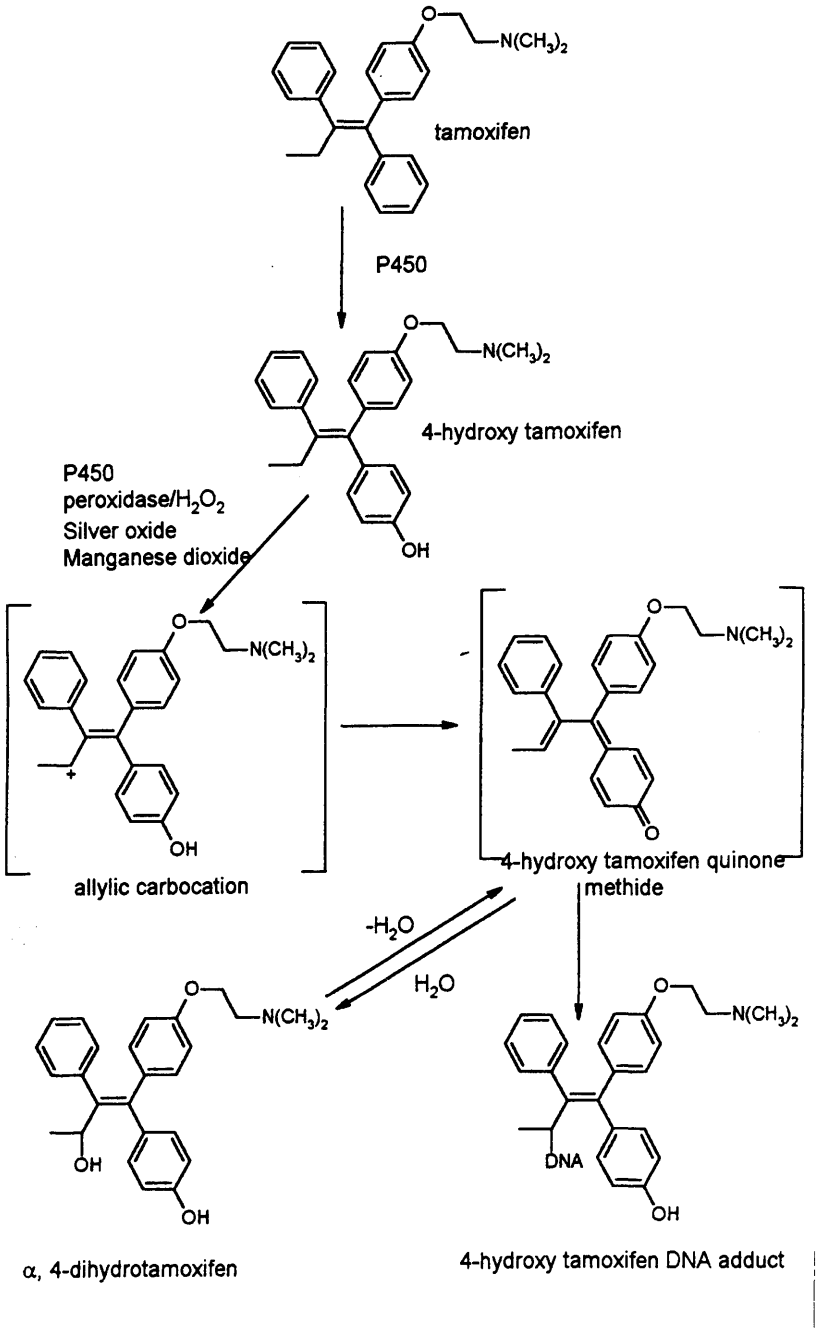


Fig 14 – Activation of tamoxifen to 4-hydroxytamoxifen quinone methide. An allylic carbocation is produced which can rotate around the central bond forming the *cis/trans* quinone methide which can react with DNA.

1.7.1c Tamoxifen Mutagenesis

Genetic analysis of hepatocellular carcinomas (HCC) from rats treated with tamoxifen has shown mutations clustering at codons 231 (CAC > CGC), and 294 (TGC > TGT) of the *p53* tumour suppressor gene with 37% of the analysed HCC's having miscoding mutations (Vancutsem *et al.*, 1994). Tamoxifen has also been found to be mutagenic in the livers of lambda/*lac I* transgenic rats (Davies *et al.*, 1998) with mutations mainly occurring at CpG sites (Phillips *et al.*, 1996a), and at the hypoxathine phosphoribosyl transferase (hprt) locus in Chinese hamster V79 cells (Rajah & Pento, 1995). Preliminary data from the mutational analysis of *p53* and the *k-ras* oncogene from endometrial carcinomas taken from women that had been treated with tamoxifen show an excess of G:C>A:T transitions at non CpG sites. Sporadic endometrial tumours are found to contain only 19% of this class of mutation (Dragan *et al.*, 1994).

1.7.1d Tamoxifen Carcinogenesis

Evidence suggests that the principal proximate carcinogen in rat liver is α-hydroxy tamoxifen, a metabolite also produced in mouse and human systems (Phillips *et al.*, 1994b; Poon *et al.*, 1995; Jarman *et al.*, 1995). *In vivo* data indicates that tamoxifen DNA adduct accumulation does not occur in the liver of women treated with tamoxifen (Martin *et al.*, 1995). However, α-hydroxy tamoxifen is detected in the culture media from human hepatocytes incubated in the presence of tamoxifen (table 1).

Species	Adducts/10^8 nucleotides Mean±SD	ng/ml α-hydroxy tamoxifen in culture media Mean±SD
Rat	89±19.9	26.8±10.1
Mouse	15±1.8	18.9±13.5
Human	Not detected[a]	0.41±0.55

Table 1 – hepatocytes treated with 10μM tamoxifen (adapted from Phillips *et al.*, 1996. [a] Limit of detection estimated at 0.04 adducts/10^8 nucleotides)

DNA adducts are not formed in human hepatocytes and this may be due to the fact that only low concentrations of α-hydroxy tamoxifen are produced, or it is being quickly metabolised to secondary inactive metabolites. However, human hepatocytes accumulate tamoxifen DNA adducts when incubated in the presence of α-hydroxy

tamoxifen itself, but there is very much less adduct formation than in the rat or mouse hepatocytes. Therefore, this may suggest that α-hydroxytamoxifen is efficiently detoxified or activated less by phase II metabolism in humans (Phillips *et al.*, 1996b).

Carmichael *et al.*, 1996 and other workers have found no evidence of endometrial or leucocytic tamoxifen DNA adducts from women receiving tamoxifen therapy. Hemminki *et al.*, 1996 reported human endometrial DNA and leucocyte adducts in patients treated with tamoxifen (Hemminki *et al.*, 1997) at very low levels, 2.7 and 5.5 adducts/10^9 nucleotides respectively using a HPLC detection system but this result has been questioned due to a lack of use of tamoxifen standards by Orton & Topham, (1997). However, Shibutani et al., (1999) has detected *trans* and *cis* α-N^2-dG-tamoxifen DNA adducts at concentrations ranging between 0.5 – 8.3 and 0.4 – 4.8 adducts per 10^8 nucleotides respectively. Moreover, the mechanism of tumourigenesis in the endometrium is not yet understood. This may be due to the complex pharmacology of tamoxifen, which may act as a complete carcinogen, initiating cells by genotoxic mechanisms through DNA adduct formation, and promoting cell growth via its oestrogenic agonist properties. Alternatively it may act by a hormonal mechanisms alone, where *trans* tamoxifen can act as an oestrogen agonist on the endometrium, initiating cell proliferation (Carmichael *et al.*, 1996; Dragan *et al.*, 1996; Dragan *et al.*, 1995). To support this purely hormonal mechanistic action, tamoxifen can promote cell division in oestrogen positive human endometrial carcinomas in the athymic mouse model (Gottardis & Jordan, 1988). In the rat, tamoxifen acts as both an oestrogen receptor agonist and antagonist in the uterus, and causes atrophy of the uterus upon prolonged exposure. Li *et al.*, (1997) found no formation of tamoxifen DNA adducts in rat uterus, but did find an enhancement of endogenous uterine DNA modifications. This suggests that there could be an indirect carcinogenic effect through the enhancement of endogenous DNA adduct formation. In mice, tamoxifen acts as a oestrogen receptor agonist in the endometrium at low doses causing cystic hyperplasia, but endometrial tumours have not been detected because of the high doses used that antagonise its oestrogenic properties.

1.7.2 Aflatoxin B_1

Aflatoxins are fungal mycotoxins produced by *Aspergillus flavus, parasiticus* and *nomius*. This class of compound belongs to a sub family of highly substituted coumarins which have a fused dihydrofuran configuration. Contamination of foodstuffs with aflatoxins is encouraged by conditions of high humidity and storage temperatures achieved in areas such as East Asia and sub Sahara Africa. These conditions facilitate mycotoxin production by *Aspergillus* which have a propensity to grow on cereal grains and nuts, (Busby and Wogan, 1984). There is concomitant production of four aflatoxins produced by these *Aspergillus* species. These are B_1, B_2, G_1 and G_2 (fig 15) of which B_1 and B_2 are cyclopentenone ring structures, and G_1 and G_2 have lactone ring structures. Aflatoxin B_1 and G_1 contain a double bond in the terminal furan ring which is absent in aflatoxin B_2 and G_2. These differences exert a large influence upon their carcinogenic potency which decreases in the order $B_1>G_1>B_2>G_2$ (Garner *et al.,* 1979). Aflatoxin B_1 (AFB_1) is the major aflatoxin produced by *Apergillus*, being the most carcinogenic and characterised (Swenson *et al.,* 1977). The IARC has now classified AFB_1 as a known human carcinogen (IARC, 1993).

Aflatoxin B_1 causes acute and chronic toxicity, the severity of which in animals is dependent on dose, length of exposure, species, breed, and diet (Eaton & Groopman, 1994). Acute effects are rare, constituting toxic hepatitis and Reyes syndrome (IARC, 1994, Reye *et al.*, 1963). The mechanism of toxicity is believed to be due to strong inhibition of DNA synthesis, nuclear RNA synthesis and RNA polymerase activity, and protein synthesis caused by the formation of aflatoxin B_1 nuclear DNA adducts (Busby & Wogan, 1985; McLean & Dutton, 1995; Clifford *et al.*, 1967; Gelboin, 1966; Saunders, 1972; Neal, 1973;Yu, 1977).

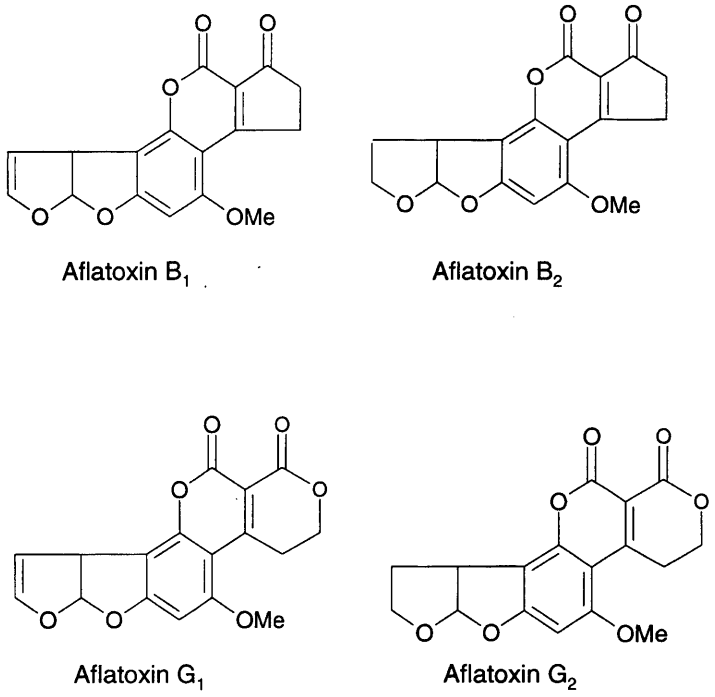


Fig 15 – Structure of the major aflatoxins produced by *Aspergillus flavus, paraciticus and nomius*

Chronic AFB_1 ingestion is associated with an increased incidence of hepatocellular carcinoma (HCC) in both humans and animals (IARC, 1994). Primary liver neoplasia in humans is multifactorial in origin, the geographical variation of HCC incidence being dependent on different aetiological agents. These include infection with hepatitis B, chemical carcinogens and alcoholic cirrhosis (Mace *et al.*, 1997). There is considerable epidemiological evidence that links the occurrence of HCC in these countries to high ingestion of AFB_1 (Busby & Wogan, 1984; Miller, 1994), that is synergistic with chronic infection of hepatitis B (Ross *et al.*, 1992). Analysis of primary liver tumours from countries where AFB_1 exposure is high indicated the presence of AFB_1 DNA adducts and an exclusive G:C to T:A transversion at the third position at codon 249 (AGG) of *p53* in a large percentage of these tumours (Hsu *et al.*, 1991; Ozturk *et al.*, 1991; Bressac *et al.*, 1991; Baily & Williams, 1993). The liver is the major target organ for AFB_1, but also significant numbers of tumours in the lung, kidney and colon result from AFB_1 exposure (Wang & Groopman, 1999). AFB_1 also induces chromosomal aberrations, micronuclei, sister chromatid exchange, unscheduled DNA synthesis, strand breaks and forms DNA adducts (IARC, 1993).

1.7.2a Metabolism

AFB_1 undergoes hepatic metabolism to its ultimate carcinogenic species which reacts with DNA. Phase I metabolism of AFB_1 is carried out by multiple forms of liver cytochrome P450, principally CYP 3A3, CYP 3A4 and CYP 1A2. There is also evidence of cyclo-oxygenase, prostaglandin H synthase contributing to this metabolism (Forrester *et al.*, 1990; Aoyama *et al.*, 1990; Crespi *et al.*, 1991). Oxidation of the 8,9 olefinic bond in the terminal furan ring yields both *exo* and *endo* isomers of AFB_1 8,9-epoxide, with the *exo* isomer appearing to be the only product of AFB_1 involved in reaction with DNA (Benasutti *et al.*, 1988; Raney, 1992). In rats approximately 1% of the administered AFB_1 dose is bound covalently to DNA, with binding peaking at 2 hours post dosing (Croy & Wogan, 1981). AFB_1 *exo*-8,9-epoxide principally reacts at N-7 of guanine in B-DNA (fig 16) with ≈20 fold more reactivity towards double stranded DNA than single via a Sn2 reaction from an intercalated state (Benasutti *et al.*, 1988; Johnson & Guengerich, 1997). Enhanced reaction of AFB_1 *exo*-8,9-epoxide with double stranded DNA by intercalation locks the B-DNA conformation by hydrogen bond formation, (Nordheim *et al.*, 1983). This hydrogen bond formation stabilises the AFB_1 transition state within the major grove prior to covalent attachment (Gopalakrishnan *et a.*, 1989, 1990; Iyer *et al.*, 1994), and orientates the epoxide allowing reaction with N-7-dG, (Jones & Stone, 1998). This reaction yields 95% *trans* 8,9 dihydrodiol-8-(deoxyguanosine-7-yl)-9-hydroxy AFB_1 (AFB_1-N-7-dG) adduct (Yu *et al.*, 1991; Essigman *et al.*, 1977). The position of the adduct at N-7 of dG results in a positively charged imidazole ring that is susceptible to depurination. A chemically and biologically stable adduct is produced when the AFB_1-N-7-dG adduct undergoes base catalysed opening of the imidazole ring. This leads to the formation of two formamidopyrimidine (AFB_1-FAPY) adducts, the major one being 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxy AFB_1 as well as 8,9 dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-yl)-9-hydroxy AFB_1 adduct (fig 16) (Hertzog *et al,*, 1982; Bailey *et al.*, 1993).

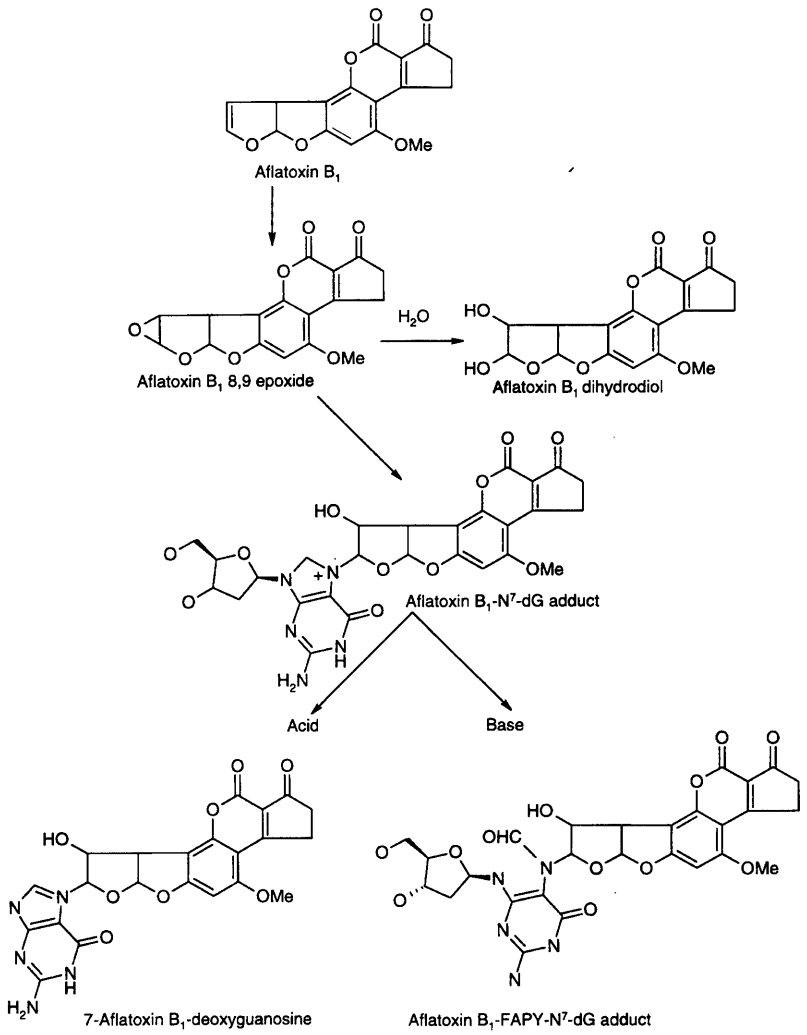


Fig 16 – Metabolic activation of AFB_1 to the reactive epoxide before reaction with N-7 of dG. The AFB_1-N-7-dG adduct can depurinate forming 7-AFB_1-G, leaving a AP site or can under go imidazole ring opening forming the main AFB_1-FAPY-N-7-dG adduct as depicted.

1.7.2b Mutagenesis

AFB_1 DNA adduct formation has been investigated on naked plasmid DNA containing *p53* sequences, (Puisieux *et al.*, 1991); and genomic DNA extracted from human HepG2 cells exposed to AFB_1 (Denissenko *et al.*, 1998). Using these systems, significant AFB_1 DNA adduct formation at the third base of codon 249 (AGG*[4]) of *p53* is observed (Puisieux *et al.*, 1991; Dennissenko *et al.*, 1998). Experimentally, AFB_1 is a very powerful rat hepatocarcinogen and a mutagen as shown by *in vitro* mutagenesis assays (Refolo *et al.*, 1998). It is known that the AFB_1-N-7-dG adduct causes G > T

[4] * denotes position of adduction

transversion (Bailey *et al.*, 1996). The mutation spectra of *p53* is tumour specific (Greenblatt *et al.*, 1994), and mutations at codon 249 of *p53* are rarely seen in any other tumour type. Aguilar *et al.*, (1994) compared AFB_1 exposure and mutation in *p53* in human hepatocelluar carcinoma in normal liver samples from the US, Thailand and Quidong in China where AFB_1 exposures are negligible and high respectively. The frequency of mutation at the third position of codon 249 paralleled the level of AFB_1 exposure, which may suggest that AFB_1 to be a causative agent or plays a role in this type of tumour. AFB_1 also induces GC>TA and CG>AT transversions in the adjacent codons of *p53*, but at a much lower frequency with AFB_1 preferentially inducing mutations at codon 249 (Hsu *et al.*, 1991; Ozturk *et al.*, 1991; Bressac *et al.*, 1991; Baily & Williams, 1993). In the *E. coli LacI* mutagenesis assay, AFB_1 produces >90%, GC>TA transversions (Foster *et al.*, 1983). Hot spots for AFB_1 mutagenesis were found to be in GC rich regions which are also hotspots for AFB_1-N-7-dG formation (Loechler, 1994). A similar result was obtained using the *sup*F mutation assay in human fibroblasts, >90% of mutations occurred at GC pairs which produced approximately 50% GC>TA transversions, with one third of the mutations occurring at GG sites (Levy *et al.*, 1992).

Rats dosed intraperitoneally with 0.125-1.0mg AFB_1 produce 12.5-110 adducts per $x10^6$ normal nucleotides two hours post injection. The major DNA adduct detected is AFB_1-N-7-dG (80%) with the second most common being AFB_1-FAPY adduct (7%). The biological half life of the AFB_1-N-7-dG adduct is approximately 7.5 - 12 hours, with 70%-80% of the AFB_1-N-7-dG adduct being removed over the next 24 hours, forming AP sites, and 20% converting to the FAPY adduct. *In vitro* loss of the AFB_1-N-7-dG adduct occurs to a lesser extent, owing to the lack of DNA repair (Groopman & Cain, 1988). The persistence of AFB_1-FAPY derivatives may be present during more than one round of DNA replication and therefore, may be important in tumour initiation (Harris *et al.*, 1993). Bailey *et al.*, (1996) has shown that the presence of an AFB_1 adduct, and not the AP site, induces the genetic requirements for mutagenesis in treated cells. However, differences in AFB_1 adduct levels rather than AFB_1 adduct types correlate to tumour formation (Wang & Groopman, 1999). AFB_1 *exo*-8,9-epoxide can also form minor DNA adducts with deoxyadenosine to produce the *trans* 8,9-dihydro-8-(N-7-adenyl)-9-hydroxy aflatoxin B_1 (AFB_1-N-7-dA) as well as an uncharacterised

deoxycytosine adduct, (Jones & Stone, 1998; Yu *et al.*, 1991). The AFB_1-N-7-dA adduct is more susceptible to depurination at ambient temperatures than the AFB_1-N-7-dG adduct (Iyer *et al.*, 1994; Stark & Liberman, 1991; Yu *et al.*, 1991). Whether these minor AFB_1 DNA adducts play a role in carcinogenesis is unknown at the present time. AFB_1 administration to rats also leads to the elevation of hepatic 8-oxo-dG adducts in a time and dose dependent manner which may enhance the carcinogenic effects of AFB_1 (Shen *et al.*, 1995; Yaborough *et al.*, 1996).

AFB_1 DNA adducts are capable of forming AP sites, base pair substitutions and frame shift mutations (Hsieh, 1986). DNA damage and hepatocarcinogenesis resulting from AFB_1 exposure can be modulated by a variety of factors including micronutrients, food restriction, viral infection and genetic polymorphism. How these additional factors work is still under investigation (Wang & Groopman, 1999).

1.8 Mechanisms for Tolerating DNA Damage

E. coli have evolved limited mechanisms that do not repair DNA damage but allows cell survival by increasing the mutation frequency.

1.8.1 Mutagenesis in Escherichia Coli

E. coli, growing under optimal conditions, have a low mutation frequency ($\approx 10^{-10}$ per bp per generation) (Bridges, 1998). The integrity of its genome is maintained by the fidelity mechanisms of base selection, proof-reading by DNA polymerase III and mismatch correction proteins. *E. coli* has evolved mechanisms to overcome chemical insult by raising its mutation rate in order to increase chances of survival. This important inducible phenotype is the *E. coli* SOS response (Fijalkowaska *et al.*, 1997). This pathway is induced when DNA damage is detected (Taddei *et al.*, 1997) as unrepaired bulky or non-instructive DNA lesions may block replication leading to lethality. The SOS response is mediated by the induced expression of ≈20 genes (Fijalkowaska *et al.*, 1997) of a regulon that is normally repressed by the Lex A protein controlled by Rec A. DNA damage leading to replication arrest generates single stranded DNA to which Rec A binds. Upon binding to ssDNA Rec A is activated in a ATP dependent manner to Rec A^* (Rec A co-protease) (Sassanfer and Roberts, 1990).

The proteins Rec F, Rec O and Rec R form a complex (Rec FOR) that facilitates SOS induction by improving the efficiency of Rec A binding to ssDNA (Calero *et al.*, 1991; Sassanfer & Roberts, 1990; Gimble & Sauer, 1989; Whitby & Lloyd, 1995). The proteins essential for SOS mutagenesis are Rec A, single strand binding protein (Ssb), UmuD and UmuC (Radman, 1975; Walker, 1984; Witkin, 1991). Rec A^* cleaves Lex A (Little, 1991) and proteolytically activates UmuD to UmuD' (Reuven *et al.*, 1998; Siegele & Hu, 1997; Simha *et al.*, 1991; Tang *et al.*, 1998; Sommer *et al.*, 1993; Sambamurti *et al.*, 1988). Upon dimerisation of UmuD', UmuC binds to form yet another complex, $UmuD'_2C$. It is postulated that $UmuD'_2C$, $RecA^*$ and Ssb act as a SOS mutasome (Echols & Goodman, 1991; Rajagopalan *et al.*, 1992) containing all or part of DNA polymerase III holoenzyme. This complex may catalyse translesional synthesis over the lesion by reducing the fidelity of replication by acting on either the nucleotide insertion step, or the extension step past a bulky or non instructional lesion (Rahman *et al.*, 1996). Some translesional synthesis can be mediated in the absence of SOS factors which usually leads to frameshift mutations. Therefore, these proteins may convert potentially lethal frameshifts into milder base pair mutations (Bridges, 1998).

The SOS response is presumed to mediate an inducible mutator phenotype, but certain DNA adducts such as ethenodeoxycytidine (εC) and ethenodeoxyadenine (εA) have revealed the existence of a novel ultraviolet light inducible mutagenic response in *E. coli*. This is termed UVM for UV modulation of mutagenesis (Palejwala *et al.*, 1994). The UVM response can be achieved with exposure of *E. coli* to either UV radiation, alkylating agents or hydrogen peroxide (Murphy *et al.*, 1996; Wang *et al.*, 1995; Wang & Humayun, 1996). Rahman *et al.*, (1996) determined that the UVM response does not significantly alter the mutation frequency of mis-pairing lesions such as O^6-methylguanine (O^6Me-dG). It is also known that the UVM response does not require a functional Rec A, UmuD or UmuC and can occur under conditions where the SOS response is not induced (Palejwala *et al.*, 1995). The mechanism of UVM mutagenesis is unknown at present, but may involve a reduction of post-translational mismatch repair (Humayun *et al.*, 1998; Murphy *et al.*, 1996).

1.8.2 Recombination in E. coli

Inhibition of DNA synthesis by the presence of a lesion leaves single stranded gaps which may break or be enzymatically cut producing a double strand break (DSB). DSB's are repaired by recombination (fig 17). Restoration of the original nucleotide sequence requires the interaction with an intact homologous DNA molecule which is facilitated by the multiple chromosomes present in growing *E. coli* cells or repeated sequences within a DNA molecule. The enzymes required for DSB and recombination repair are divided into three sets. The first set (presynapsis) consist of recBCD complex, recE, recJ and recQ that help convert double strand breaks into substrates for the second set (synapsis). The enzymes involved in these reactions are recA, recF, recO, recR, recT and ssb and act to pair homologous molecules before the final set (postsynapsis). The final set of enzymes consist of recN, sbcA, sbcB, subCD and lexA that convert the homologous molecules into recombined products (Kowalczykowski *et al.*, 1994; Smith, 1988; West, 1992; Resnick, 1976; Holliday, 1964).

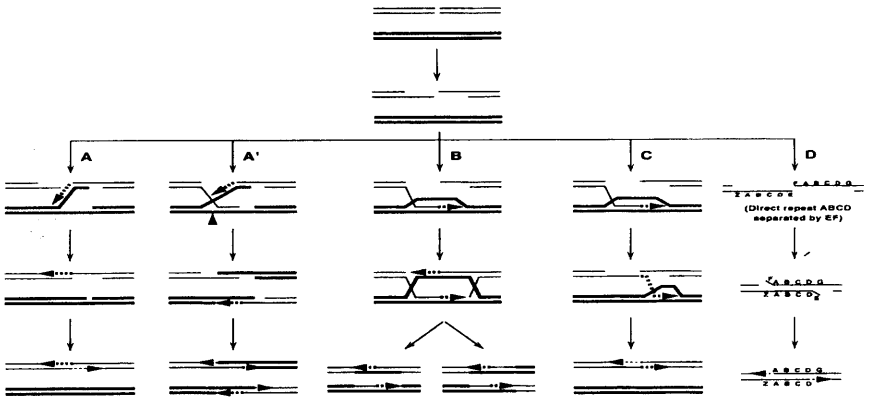


Fig 17 – General models for DSB repair and recombination. Each line represents a single strand of DNA, thick and thin lines being from homologs. Dotted lines represent newly synthesised DNA. At each double strand end a nuclease digests one strand to generate a 3' ssDNA tail. (A – A') after Resnick, 1976. (B) after Szostak *et al.*,1983. (C) after Formosa & Alberts, 1986. (D) after Lin *et al.*, 1984.

1.9 Repair of Genotoxic Lesions in Prokaryotes and Eukaryotes

DNA repair comprises of molecular reactions that remove DNA lesions increasing the chances of survival against DNA lesions that are cytotoxic and reduce the probability of mutation in critical genes prior to DNA replication. If a mutation (mismatch) occurs, then fixation is avoided by the removal of the inappropriate base prior to further DNA replication. The repair mechanisms are classed into four general categories: 1) direct repair, 2) base excision, 3) nucleotide excision and 4) mismatch repair (Sancar, 1995; Chaney & Sancar, 1996).

The four repair systems are found in both prokaryotes and eukaryotes, with enzymes performing direct repair and direct excisions being structurally homologous between the two systems. In general there is substantial overlap in the substrate specificities for direct repair, base excision and nucleotide excision repair, with nucleotide excision able to repair almost anything (Chaney & Sancar, 1996).

1.9.1 Direct Repair

Their are two examples of direct repair: 1) Photoreactivation and 2) Alkyl transfer.

1.9.1a Photoreactivation

Photoreactivation is the enzymatic reversal of pyrimidine cyclobutane dimers by photolyase of many species, except humans (Kato *et al.*, 1994; Ley, 1993; Li *et al.*, 1993). Dimers are reversed by electron transfer from $FADH_2$ at the active site of photolyase to the dimers breaking the dimer bond (Sancar, 1994).

1.9.1b Alkyl Transfer

In mammalian systems alkyl transfer is seen in the specific repair of the highly mutagenic O^6-methylguanine, O^6-methylguanine DNA methyltransferase (MGMT). MGMT can irreversibly transfer a methyl group from O^6-methyl-dG to a cysteine residue in its active site (Lindahl *et al.*, 1988; Mitra & Kaina, 1993; Samson, 1992). MGMT can also remove other alkyl groups from the O^6 atom of guanine, such as ethyl and butyl residues as well as from the O^4 atom of thymine but at lower efficiency (Zak

et al., 1994). MGMT is found in all species, with *E. coli* possessing two forms, Ada and Ogt (Samson, 1992).

1.9.2 Base Excision Repair (BER)

Substrates for BER include non bulky DNA adducts formed by methylating agents, and oxidised, reduced or fragmented bases produced by ionising radiation (Sancar, 1995; Freidberg, 1985). This type of repair involves removal of the damaged base by a DNA glycosylase producing a AP site (fig 18). The AP site is removed by an incision 3' of the AP site by AP lyase and 5' by AP hydrolase. The missing nucleotide is then replaced by DNA polymerase and ligated (Dodson, 1994). There are five well characterised mammalian DNA glycosylases and one major AP endonuclease in humans: Uracil, hydroxymethyluracil, thymine glycol, N-methylpurine and 8-hydroxyguanine DNA glycosylase (Sancar, 1996). The DNA glycosylases of *E. coli* and corresponding repair proteins release an array of damaged DNA bases (Wilson, 1998). Enzymes involved in *E. coli* BER are a glycosylase and AP lyase, respectively and remove oxidative damage (Jiang, 1997). Other proteins include, ung, a uracil glycosylase (Duncan & Weiss, 1982), xth and nfo are exonuclease III and endonuclease IV respectively that remove and repair AP sites (Kunz *et al.*, 1994).

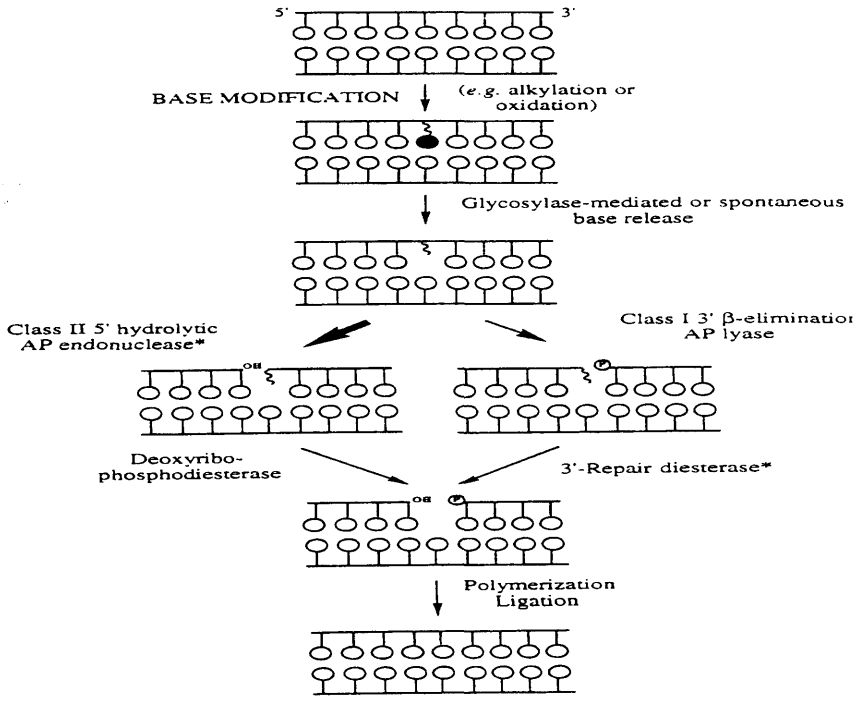


Fig 18 – Schematic steps in the BER pathway. The darkened circle represents a modified base that can be removed by a specific DNA glycosylase.

1.9.3 Nucleotide Excision Repair (NER)

1.9.3a Nucleotide Excision Repair in E. coli

An important adduct detection and removal system in *E.coli* involves the SOS induced UvrABC proteins for nucleotide excision (fig 19) (Sancar *et al.*, 1982). The promoter of *uvrA* contains a binding site for LexA with *uvrB* containing three promoters (P1, P2 and P3) of which LexA binds only to P2 (Sancar *et al.*, 1981; Vandenberg *et al.*, 1985). The gene product of *uvrC* is not under the control of LexA. This system is capable of recognising and repairing a large variety of DNA lesions (Ahn & Grossman, 1996), including AP sites, *cis*-diamminodichloroplatinum (*cis*-Pt) adducts (Alazard *et al.*, 1982), psoralen adducts, UV photo products (Sancar & Rupp, 1983), 2-acetyl aminoflourene (2-AAF) adducts (Tang *et al.*, 1982; Pierce *et al.*, 1989), and anthramycin adducts (Pierce *et al.*, 1989). The efficiency of DNA repair is influenced by the helix topology and DNA sequence in the proximity of the lesion and not the chemical structure of the adduct (Jones & Yeung 1988; Seeberg & Fuchs, 1990; Ramaswamy & Yeung, 1994). The mechanism of UvrABC adduct excision is achieved by a cascade of events (Lin & Sancar, 1992). The UvrA protein dimerises and forms a complex with UvrB on DNA ($UvrA_2B$). The UvrA protein guides the complex to the lesion site which it then dissociates (Orren & Sancar, 1989) allowing the UvrB protein to form a stable complex on DNA that locally denatures the helix around the lesion by 5-6 bp and bends the DNA by 130° (Sancar & Tang, 1993). The UvrB protein has a hydrophobic pocket which it fits the adduct moiety into. The size of the adduct and its hydrophobicity determine the overall efficiency of removal (Hsu *et al.*, 1995). UvrB then binds UvrC to form a protein-DNA complex, that allows UvrB cleavage of DNA on the 3' side of the lesion and UvrC cleavage 5' of the lesion. The resulting gap is filled in by DNA polymerase I (Sancar, 1996). The adduct structure, and the local sequence context, has a pronounced effect on the rate and extent of cleavage (Mekchovich *et al.*, 1998). Adducts are preferentially removed from the transcribed strand since a stalled RNA polymerase complex has a higher affinity for the $UvrA_2B$ complex via interactions with a transcription repair coupling factor (TRCF) (Ahn & Grossman, 1996; Seeley & Grossman, 1989).

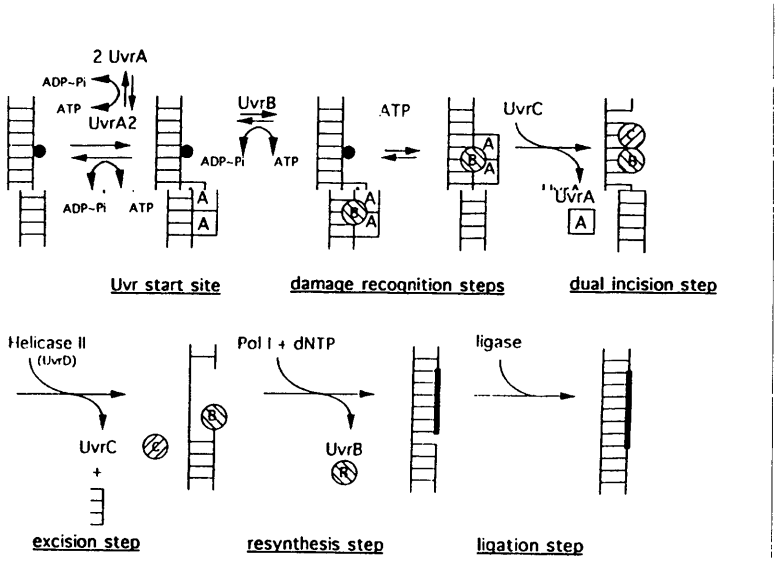


Fig 19 – Nucleotide excision repair pathway in *E. coli*. Diagram taken from Grossman *et al* in DNA Damage and Repair, vol I, 1998.

1.9.3b Nucleotide Excision Repair in Mammalian Cells

Nucleotide excision repair is the only repair pathway for bulky DNA adducts in eukaryotes and works by removing 27 to 29 nucleotide long oligomers containing the lesion (fig 20) (Sancar, 1995, 1996b). Humans defective in this system suffer from xeroderma pigmentosum (XP) characterised by skin tumours resulting from UV radiation damage (Engelberg *et al.*, 1994). Genetic analysis of XP patients has revealed seven genes named XPA to XPG as essential for nucleotide excision repair. A total of 14 polypeptides in 6 complexes are required for dual incision of DNA : 1) XPA (p31); 2) RPA (trimeric replication repair factor containing p70, p34 and p11); 3) TFIIH containing XPB, XPD, p62, p44 and p34; 4) XPC (p25 and p28); 5) XPF (p112), p33 and 6) XPG (p135) (Mu *et al.*,1995).

A simplified model has been proposed for the exonuclease function of the above complexes and is dependent upon ATP (Svoboda *et al.*, 1993). XPA binds to the damaged site and facilitates the entry of RPA. This complex recruits TFIIH, XPF/p33 and XPC complexes. The helicase like activity associated with TFIIH unwinds the DNA and XPF/p33 nicks the DNA at the 24th phosphodiester bond 5' to the lesion. XPG then makes an incision at the 5th phosphodiester bond 3' to the lesion (Freiberg, 1995; Habraken *et al.*, 1994; Matsunaga *et al.*, 1995; Harrington & Lieber,

1994;O'Donovan *et al.*, 1994; Sancar, 1994). Following excision the gap is filled by the replication proteins RPA, RFC (replication factor C), PCNA and DNA polymerase δ or ε and the repair patch is ligated.

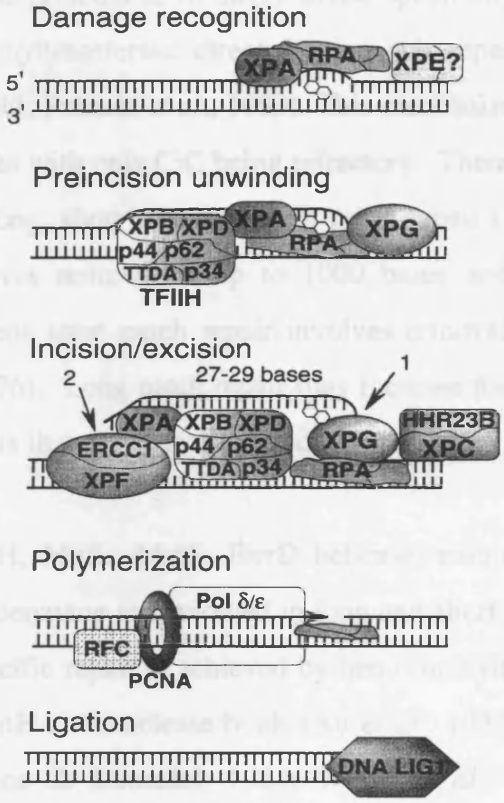


Fig 20 – Model of mammalian NER. Diagram taken from Thompson in DNA damage and repair, vol II. 1998.

1.9.4 Mismatch Repair

Mismatches in DNA resulting from replication errors, recombination and deamination are removed by special repair systems. Mismatches can also be removed by base excision or nucleotide excision as they distort the DNA structure (Freidberg, 1995; Modrich & Lahue, 1996).

1.9.4a Mismatch Repair in *E. coli*

If DNA adducts are not repaired by cellular repair mechanisms before DNA synthesis occurs, then mutations may arise. Mutations become fixed if the mismatch is not

removed and replaced with the correct nucleotide. Therefore, post replicative mismatch repair pathways have evolved in *E.coli* (Murphy *et al.*, 1996).

Mismatches are preferentially repaired on the transcribed strand. Strand specificity is achieved by the methylation of dA in 5'-dGA*TC by Dam methylase that provides signals to the repair proteins as to direct strand specificity (fig 21). This is known as DNA adenine methyltransferase directed mismatch repair (DDMR) (Kramer *et al.*, 1984; Lu *et al.*, 1983; Pukkila *et al.*, 1983). This mechanism can repair 11 out of the 12 possible mismatches with only C:C being refractory. There are three types of mismatch repair in *E. coli*, long, short and very short patch repair (Wagner *et al.*, 1976). Long patch repair involves removal of up to 1000 bases and recognises all single base mismatches, whereas short patch repair involves removal of approximately 20 bases (Wagner *et al.*, 1976). Long patch repair may increase the chances of the introduction of polymerase errors that may become fixed.

The proteins MutH, MutL, MutS, UvrD helicase, exonuclease VII & I, and DNA polymerase III holoenzyme are involved in long and short patch repair (Modrich *et al.*, 1991). Strand specific repair is achieved by hemi methylated adenine in the sequence dGATC where MutH endonuclease binds (Au *et al.*, 1992; Claverys & Mejean, 1988; Fishel, 1986; Lyons & Schendel, 1984; Welsh *et al.*, 1987). The MutS protein recognises and binds to sequences containing mismatches introduced during replication (Parker & Marinus, 1992; Su *et al.*,1988; Su & Modrich, 1986). MutL has no defined function, but is thought to bridge MutS and MutL, together to form an α shaped loop structure (Grilley *et al.*, 1989). MutH endonuclease is activated and nicks the nearest unmethylated dGATC sequence (newly synthesised strand) closest to the mismatch. The mismatch is then unwound by UmuD helicase and either exonuclease I, VII or Rec J to remove the surrounding bases. (Cooper *et al.*, 1993; Grilley *et al.*, 1989; Langlerouault *et al.*, 1987; Lu, 1987). DNA polymerase III fills in the gap and dA in dGATC is methylated again by Dam methylase. Once all the dGATC sites on the newly transcribed strand are fully methylated repair is halted.

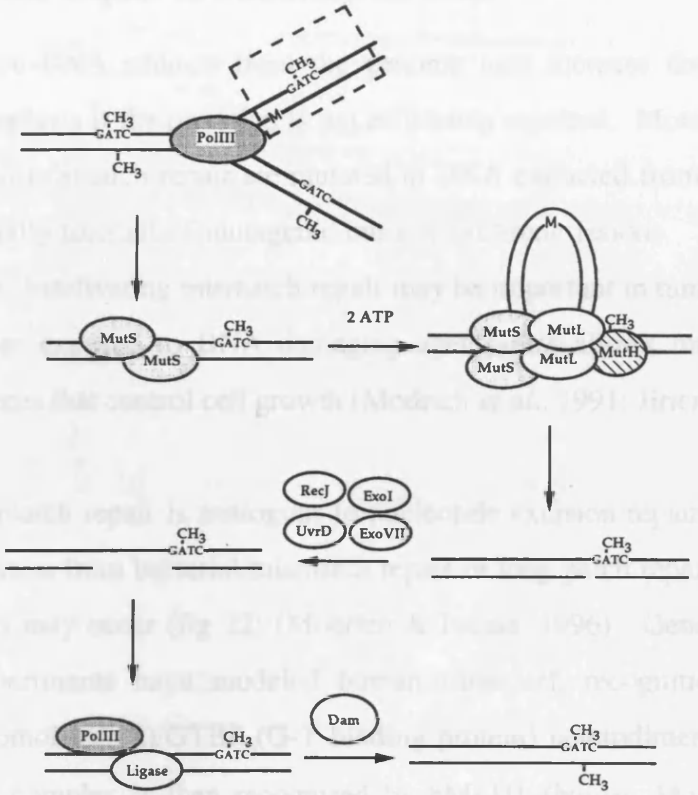


Fig 21 – Model for the initiation of DDMR. **M:** indicates base mismatch. Diagram taken from Rasmussen *et al* in DNA damage and repair, vol I, 1998.

Specifically, the Mut Y and Mut M gene products are glycosylases responsible for short patch removal of A:G, A:C, A:8oxoG mismatches (Au *et al.*, 1992; Baker *et al.*, 1995; Feinstein & Low, 1986; Fishel, 1986; Humbert *et al.*, 1995; Langlerouault, 1987), ring opened dG (Michaels *et al.*, 1991; Tchori *et al.*, 1991) and 8oxodG (Feinstein *et al.*, 1986; Feng *et al.*, 1996).

Methylation of dC^5 by Dcm methylase, of the internal cytosine residue in 5'-dCC*TGG can spontaneously deaminate forming thymine. This generates a T:G mismatch which is responsible for most of the background mutations seen in *E. coli* (Leib, 1991). This mismatched nucleotide is excised by Vsr endonuclease, with the accessory proteins Mut S and Mut L, and filled in by DNA polymerase I (Dzidic *et al.*, 1989; Jones *et al.*, 1997a,b; Lieb., 1987; Sohail *et al.*, 1990). This single nucleotide repair is also known as very short patch repair (Grilley *et al.*, 1989).

1.9.4b Mismatch Repair in Mammalian Cells

Failure to remove DNA adducts from the genome may increase the probability of mutation and neoplasia if the mutation is not efficiently repaired. Moreover, it is often seen that genes of mismatch repair are mutated in DNA extracted from tumours. This phenotype is usually tolerant of mutagenic but not cytotoxic lesions. Therefore, early mutagenic events, inactivating mismatch repair may be important in tumour initiation if the cell is further exposed to DNA damaging agents that allows mutation fixation within critical genes that control cell growth (Modrich *et al.*, 1991; Jiricny, 1998).

Mammalian mismatch repair is analogous to nucleotide excision repair and is referred to the MutLS system from bacterial mismatch repair or long patch repair as removal of up to 1000 bases may occur (fig 22) (Modrich & Lahue, 1996). Genetic studies and biochemical experiments have modeled human mismatch recognition by hMSH2 (human MutS homologue 2)/GTBP (G-T binding protein) heterodimer (referred to as hMutSα). This complex is then recognised by hMLH1 (human MutL homolougue 1)/hPMS (human homologue of yeast post-meiotic segregation gene 2) heterodimer (referred to as hMutLα), and DNA degradation is initiated from a nick (unligated Okazaki fragment) in the DNA strand with the mismatch (Chaney & Sancar, 1996).

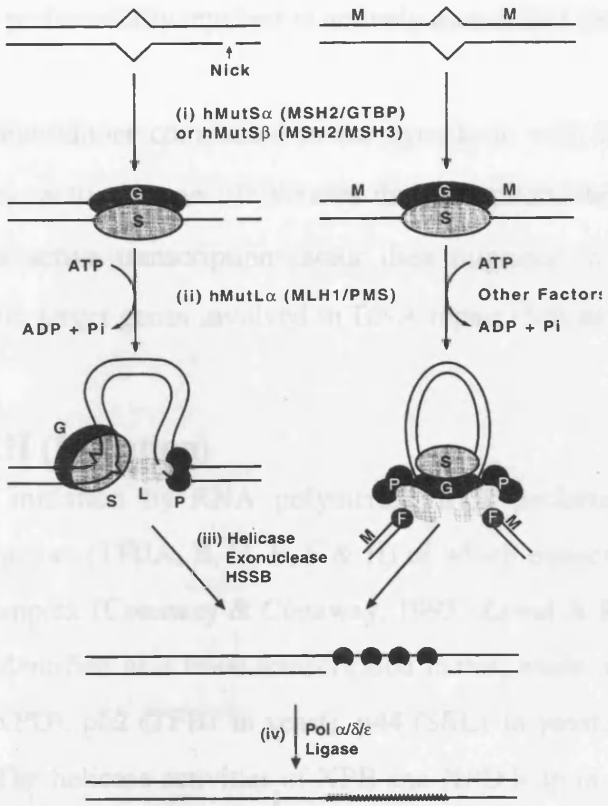


Fig 22 – Model for mammalian long patch mismatch repair. Firstly the mismatch site (bubble) is recognised by hMutSα or hMutSβ (S- hMSH2 or hMSH3 /G-GTBP) with high specificity. Secondly, hMutLα (L-hLMH1/P-PMS)binds to hMutSα or β to form a complex. If the heterduplex substrate contains a nick (left) a quartenary complex of MSH2/hGTBP/hMLH1,HPMS may bring the nick into the proximity of the mismatch site. If hemimethylated DNA (M) is the substrate (right), other unidentified factors (F) may be involved to search for and cleave a hemimethylated site. Thirdly, a helicase, exonuclease and ssb protein are required to generate intermediates with gaps that span the shorter patch between the strand break and the mismatch, irrespective of the polarity of the strand break. Finally, DNA polymerases ε or δ and DNA ligase are predicted to fill the gap and seal the nick respectively. (Diagram taken from Lu in DNA damage and repair, vol II, 1998.)

1.9.5 Transcription and Repair

Transcribed genes are believed to be more accessible to the repair proteins because of the loose chromatin structure (Hanawalt, 1994). However transcription and repair meet at all stages of transcription.

1.9.5a Activation

AP-1 is a heterodimer of jun and fos, binding to DNA upon reduction of cysteine residues in the DNA binding regions of both transcription factors. This reaction is catalysed by the Ref-1 activity of AP endonuclease (APE) (Xanthoudakis *et al.*, 1992). The precise physiological role of Ref-1 is not completely understood and may function independently to APE. Alternatively the interactions of APE with jun and fos, may allow recruitment to DNA damage of genes regulated by this transcription factor

(Sancar, 1995). The latter may be the case as Leadon & Cooper, (1993) have shown AP sites to be preferentially repaired in actively transcribed genes.

NF-κB is a heterodimer complexed in the cytoplasm with IκB as in inactive trimer. Oxidative stress activates specific kinases that phosphorylate IκB, liberating activated NF-κB. This active transcription factor then migrates to the nucleus which may activate specific target genes involved in DNA repair (Schmidt *et al.*, 1995; Anderson, 1994).

1.9.5b TFIIH (Initiation)

Transcription initiation by RNA polymerase II is performed with the aid of six transcription factors (TFIIA, B, D, E, F & H) of which transcription factor IIH (TFIIH) is the most complex (Conaway & Conaway, 1993; Zawel & Rosenberg, 1995). TFIIH was initially identified as a basal transcription factor, made up of seven subunits, p89 (XPB), p80 (XPD), p62 (TFB1 in yeast), p44 (SSL1 in yeast), p41 (cdk7), p38 (cyclin H) and p34. The helicase activities of XPB and XPD help in open complex formation, whereas cdk7 and cyclin H phosphorylate RNA polymerase II (Roy *et al.*, 1994; Shiekhattar *et al.*, 1995; Svejstrup *et al.*, 1995). The discovery of XPB and XPD indicated that TFIIH is a combined repair factor and a transcription factor (Drapkin *et al.*, 1994a+b; Schaeffer *et al.*, 1993; Schaeffer & Egly, 1994). Both functions requiring XPB, XPD, p62 and p44 subunits of TFIIH (Scheaffer *et al.*, 1994; Xiao *et al.*, 1994; Drapkin *et al.*, 1994). TFIIH, therefore, may be involved in transcription repair coupling (TRC) (Park *et al.*, 1995; Zawel & Rosenberg, 1995). Evidence for TRC come from observations that transcribed sequences are more rapidly repaired than nontranscribed sequences, with lesions in the transcribed strands repaired at an increased rate to those in the coding strand (Mellon *et al.*, 1987; Bohr & Anson, 1995; Mayne & Lehmann, 1982). This observation indicates that nontranscribed sequences may be repaired less efficiently than transcribed sequences (Sancar, 1995).

1.9.5c Elongation

TRC is well understood in *E. coli*. Stalling of an RNA polymerase at a lesion in *E. coli* is recognised by a TRC factor encoded by the *mdf* gene. This results in dissociation of the polymerase truncating the transcript. UvrA is then recruited to the lesion resulting

in a 10 fold enhancement in the repair rate of the template strand (Selby & Sancar, 1993).

For human TRC, two gene products, CSA and CSB complex to form a heterodimer that recognises a stalled RNA polymerase II (vanHouten *et al.*, 1993; Venema *et al.*, 1990). The CSA/CSB complex recruits XPA and TFIIH, initiating nucleotide excision repair (Selby & Sancar, 1994). TRC in humans is only observed in genes that are transcribed by RNA polymerase II and for bulky lesions repaired by nucleotide excision only (Reardon *et al.*, 1993; Venema *et al.*, 1990). Mutations in CSA and CSB are characterised by Cockayne's syndrome, where overall genome repair is normal, but there is a lack of preferential repair. This mechanism of human TCR does not involve the dissociation of RNA polymerase II. The stalled polymerase is recognised by TFIIS, enabling the polymerase to back up while CSA/CSB heterodimer and TFIIE recruits XPA and TFIIH to the site of the lesion. Restoration of the duplex allows the stalled polymerase to elongate the truncated transcript (Donahue *et al.*, 1994). TRC is known not essential for cell survival as global DNA repair is unaffected (Selby & Sancar, 1993; van Gool *et al.*, 1994; Venema *et al.*, 1990).

Summary

The human genome is constantly under assault from a wide variety of DNA damaging agents. DNA adduct formation may increase the probability of mutation which may become fixed if not corrected before the next round of replication. Therefore, an understanding of the biological mechanisms that lead to DNA adduct formation, mutation and events associated to the initiation of carcinogenesis will be major issues for cancer risk. DNA adduct nucleotide resolution studies may provide useful information about the binding characteristics of mutagens/carcinogens. This information when assessed with the position of mutation in critical genes may provide an improved risk assessment when used in conjunction with other DNA adduct studies. In this project attempts were made to determine DNA adduct formation by tamoxifen at the nucleotide level *in vitro* and *in vivo*, and assess the positions of mutation in an *in vitro* system in an attempt to correlate tamoxifen DNA adduct formation to mutation.

DETECTION OF SITE SPECIFICALLY PLACED AFB_1 DNA ADDUCTS AND SEQUENCE SELECTIVITY OF AFB_1 DNA ADDUCT FORMATION

2. Chapter Objectives

The objectives of this study were:

1. to optimise the polymerase arrest assay for the detection of site specifically placed AFB_1 adducts on an oligonucleotide template.
2. to determine the location of AFB_1 DNA adduction in plasmid DNA as to assess and confirm data from other studies of sequence specificity.

2.1 Introduction

Of the four aflatoxins produced by *Aspergillus* species, aflatoxin B_1 (AFB_1) is the most mutagenic and carcinogenic (Lee *et al.*, 1977). Oxidation of the terminal furan ring forms the ultimate carcinogenic species AFB_1-8,9-epoxide (Garner *et al.*, 1972; Swenson *et al.*, 1977; Lin *et al.*, 1977). Oxidative metabolism occurs in the liver by cytochrome P450, CYP 3A3 and 3A4 (Shimada & Guengerich, 1989). AFB_1 8,9-epoxide reacts at the N-7 position of dG forming the major adduct 2,3-dihydro-2-(N-7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N-7-dG) both *in vivo* (Croy *et al.*, 1978) and *in vitro* (Essigmann *et al.*, 1977). This adduct is chemically unstable and depurinates to leave a apurinic site (Groopman *et al.*, 1981; Croy and Wogan, 1981). The AFB_1-N-7-dG adduct can also undergo opening of the imidazole ring of guanine forming a chemically stable AFB_1-formamidopriymidine adduct (AFB_1-FAPY) (Lin *et al.*,1977).

It has been possible to map AFB_1-DNA adduct formation at the nucleotide level using a variety of PCR based reactions. *In vitro*, examination of the AFB_1 adduct spectra in

double stranded DNA, shows adduct formation to be non-random (Misra *et al.*, 1983; Muench *et al.*, 1983; Marien *et al.*, 1987). Analysis of the adduct pattern reveals that there is preferential reactivity towards certain deoxyguanines, that is influenced by the 5' and 3' flanking bases (Benasutti *et al.*, 1988). These hotspots are due to base neighbour effects forming the most stable transition state complex before covalent binding (Said & Shank, 1991). The highest degree of selectivity is found for paired guanines in double stranded DNA and non base paired guanines in single stranded DNA (Jocobsen *et al.*, 1987). The most reactive sequences are 5'-GG*G-3' > 5'-CG*G-3' > 5'-GG*T-3'[1]. Guanine residues that are flanked by dA and dT rich regions form poorer targets for adduct formation (Muench *et al.*, 1983). From these data, a set of empirical rules allow the prediction of relative reactivity of AFB_1 towards guanine to be 5'-G>C>A>T and 3'-G>T>C>A. AFB_1 8,9-epoxide binds very strongly in large runs of guanines in double stranded DNA. These runs of 10 or more dG's are seen in rat and human DNA within or near the 5' promoter regions of genes. This may imply that carcinogens that form adducts at runs of dG's not only have a possible role in promoting DNA mutation, but may also alter gene expression, (Said & Shank, 1991).

[1] * Indicates site of AFB_1 adduction.

2.2 Materials and Methods

2.2.1 Materials

Deoxyoligonucleotides were synthesised using standard phosphoramidite chemistry on an Applied Biosystems 394 DNA/RNA synthesiser (Protein & Nucleic acid Chemistry Laboratory, University of Leicester). The Plasmid pGEM® -T (Promega) is a standard cloning vector that carries the lacZ alpha-peptide, containing in addition to both SP6 and T7 RNA polymerase promoters flanking a multiple cloning site. Cloned into this plasmid was the target sequence from the *mdr1b* promoter (Dr Zhang, MRC Toxicology Unit, UK) (fig 22a). Aflatoxin B_1 and peroxymonosulphate (oxone) were obtained from Sigma (Poole, UK). *Taq* DNA polymerase was obtained from GibcoBRL, Life Technologies (Paisley, UK). *Klenow exo*$^+$ DNA polymerase was obtained from Promega. All other chemicals unless stated were obtained from Sigma (Poole, UK). Aflatoxin B_1 should only be used in a isolated fume hood, with all personnel using a double layer of gloves and a face mask. Any spilt aflatoxin B_1 solution should be mopped up with a 1% sodium hypochlorite solution.

2.3 Methods

2.3.1 Synthesis of dimethyldioxirane (DMDO), Murray & Jeyaraman, (1985); Adam *et al*, (1989)

Dimethyldioxirane (DMDO) synthesis was carried out in a 500ml reaction vessel containing 0.4075moles acetone and 0.2moles sodium bicarbonate in 50mls water, under a low pressure argon atmosphere. To this solution, was slowly added

0.0813moles of peroxymonosulphate (oxone) and 0.229moles acetone in 24mls water followed by vigorous stirring at room temperature for 1 hour. Dimethyldioxirane was drawn through glass wool into the air condenser which was cooled by dry ice and acetonitrile. The dimethyldioxirane/acetone solution was collected by condensation of the volatile liquids into two cold traps cooled on dry ice and acetone. Water was removed from the condensed solution using anhydrous magnesium sulphate and the mixture filtered. The dimethyldioxirane content of the solution was measured from the absorbance of the solution at 335nm and kept at -85°C until subsequent use.

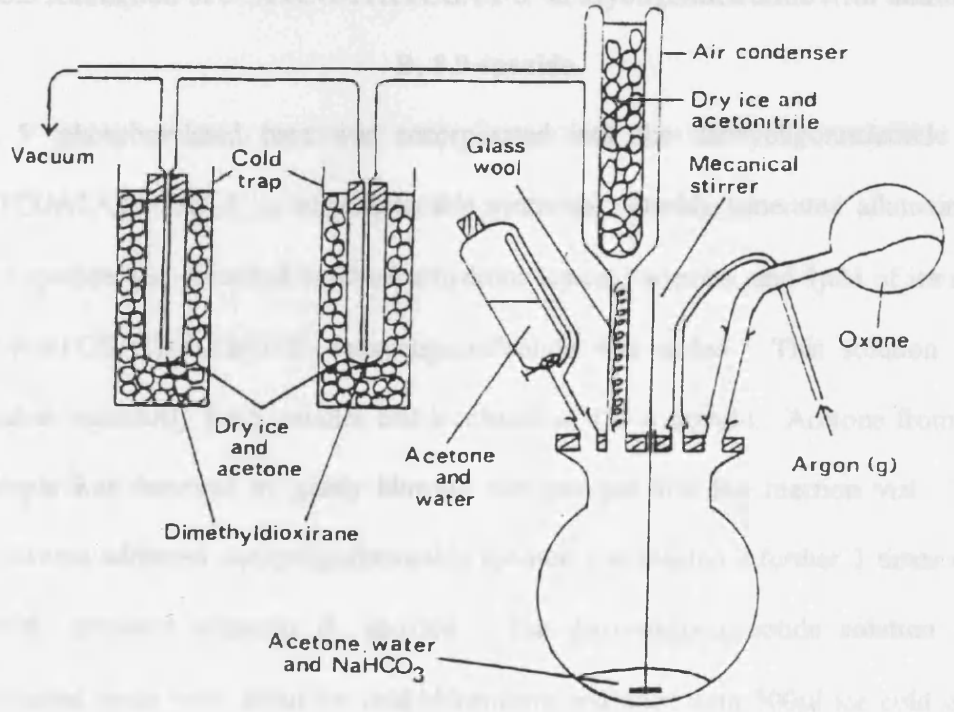


Fig 22a- Setup of equipment used for the synthesis of dimethyldioxirane.

2.3.2 Generation of aflatoxin B_1 8,9 epoxide by dimethyldioxirane, Baertschi et al (1988)

In a light proof glass vial, 1mg of aflatoxin B_1 was dissolved in 500µl anhydrous ice cold acetone. To this solution dimethyldioxirane in acetone cooled on dry ice was added to give a final concentration of 9.6mM and left at room temperature for 20 minutes with intermittent gentle shaking. Excess DMDO and acetone were removed by blowing nitrogen gas into the reaction to leave crystalline aflatoxin B_1 8,9 epoxide. Crystaline aflatoxin B_1 8,9 epoxide was kept on dry ice until use.

2.3.3 Adduction of 5'-ATCGATATCGAT-3' deoxyoligonucleotide with aflatoxin B_1 8,9 epoxide

A 5' phosphorylated base was encorporated into the deoxyoligonucleotide 5'-ATCGATATCGAT-3' at oligonucleotide synthesis. Freshly generated aflatoxin B_1 8,9 epoxide was dissolved in 400µl anhydrous ice cold acetone, and 1µM of ice cold 5'-P-ATCGATATCGAT-3' deoxyoligonucleotide was added. This solution was shaken vigorously for 5 minutes and incubated at 4°C overnight. Acetone from the sample was removed by gently blowing nitrogen gas into the reaction vial. The recovered adducted deoxyoligonucleotide solution was reacted a further 2 times with freshly prepared aflatoxin B_1 epoxide. The deoxyoligonucleotide solution was extracted twice with 500µl ice cold chloroform and once with 500µl ice cold ethyl acetate to remove any remaining unreacted aflatoxin B_1, and aflatoxin B_1 dihydrodiol. The crude deoxyoligonucleotide solution was partly purified using a NAP 5 (Pharmacia) gel filtration column equilibrated with ultrapure ice cold water. The deoxyoligonucleotide was eluted with a total of 3mls ultrapure ice cold water collected in 1ml fractions.

2.3.4 HPLC purification of aflatoxin B_1 adducted ATCGATATCGAT deoxyoligonucleotide

Aflatoxin B_1 modified deoxyoligonucleotide was separated from unmodified deoxyoligonucleotide in each 1ml fraction by reverse phase HPLC using a Techsphere 5 ODS column. An aliquot (100µl) of deoxyoligonucleotide solution was loaded onto the ODS column and separated using an organic solvent gradient of 5-50% CH_3CN in water over 30 minutes at 1ml/minute. The deoxyoligonucleotides were detected using an Applied Biosystems 1000s UV diode array detector set at 254nm for detection of DNA and 365nm for detection of aflatoxin B_1 adducts.

2.3.5 Formation of AFB_1-FAPY-N^7-dG adducted ATCGATATCGAT deoxyoligonucleotide, Chetsanga & Frenette, (1983)

The ring opened AFB_1-FAPY adduct was produced by incubating aflatoxin B_1 adducted P-ATCGATATCGAT deoxyoligonucleotides in 0.1M $NaHCO_3$ buffer pH9.6 overnight at 37°C . The deoxyoligonucleotides were precipitated from the aqueous solution by the addition of 0.1 volumes 3M sodium acetate pH5.2 and 2 volumes of ice cold ethanol.

2.3.6 Formation of a deoxyoligonucleotide template containing a site specific aflatoxin-B_1 DNA adduct

Deoxyoligonucleotide template ligation was performed in a total volume of 20µl containing 40 pmoles 5'phosphorylated d(CTATAGTGAGTCGTATTACTATAGTGTCACCTAAAT), 40 pmoles of biotinylated or alternatively 35 pmoles unbiotinylated and 5 pmoles ^{33}P labelled 5'-

d(CGCTTGATGAGTCAGCCGGAA), 40 pmoles scaffold d(ATTTAGGTGACACTATAGTAATACGACTCACTATAGATCGATATCGATTT CCGGCTGACTCATC) and 80 pmoles of purified 5'phosphorylated aflatoxin B_1 adducted/unadducted deoxyoligonucleotide. The oligonucleotides were annealed by heating to 95°C for 5 minutes in Hybaid Omnigene thermal cycler and cooled to 16°C at 0.01°C/second. Ligation was initiated by the addition of 66mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 1mM dithiothreitol, 1mM ATP (pH7.5), and 5 units of T4 DNA ligase (Boehringer Mannheim 5u/µl). The ligation reaction was allowed to proceed for 16 hours at 16°C. The double stranded template was purified by electrophoresis through a 20% polyacrylamide gel. An autoradiograph from the wet gel was overlaid back onto the gel to determine the position of the ligated template. The band was excised and the template recovered by incubation of the gel slice in 500µl 0.5M $(NH_4)OAc$, 10mM $Mg(Oac)_2$, 1mM EDTA (pH 8.0), 0.1% SDS buffer at 37°C for 4 hours. The template was recovered from solution by ethanol precipitation after all traces of polyacrylamide gel were removed. The radiolabel was removed by resuspending the template in water with the addition of 20 units calf intestinal alkaline phosphatase, and incubated for 2 hours at 37°C. Purified radiolabel free template was recovered from the reaction by ethanol precipitation.

A more efficient method for template purification was achieved by the addition of 10µg M-280 streptavadin coated paramagnetic beads (Dynal) to a biotinylated template in a total volume of 50µl containing 1M NaCl, 5mM Tris-HCl (pH 7.6), 5µM EDTA, and incubated at 37°C for 2 hours with intermittent gentle shaking. The

template were washed three times with Tris-EDTA (pH8.0) buffer and resuspended in 10µl ultrapure water.

Fig 24 - Ligated 69 base template. Arrows indicate points of ligation.

2.3.7 DNA polymerase arrest assay on site specific aflatoxin B_1 adducted DNA template

2.3.7a Taq DNA polymerase (Grimaldi *et al.*, 1994)

The arrest assay was performed using primer 5'-ATTTAGGTGACACTATAG-3' which corresponded to bases 1-19 of the ligated template. The primer was ^{32}P end labelled using γ–^{32}P-ATP, 3000Ci/mmol (Amersham) and 5 units T4 polynucleotide kinase (Promega), in a total volume of 10µl containing 200pmoles deoxyoligonucleotide primer, 70mM Tris-HCl (pH7.6), 10mM $MgCl_2$, 5mM dithiothreitol at 37°C for 30 minutes. The labelled primer was separated from unincorporated ^{32}P-ATP using a Chroma Spin™ -10 gel filtration spin column (Clontech, Heidelberg, Germany). Primer extension was carried out in a total volume of 20µl containing either 2µg paramagnetic beads bound with aflatoxin B_1 adducted/unadducted template or 1µg of unbiotinylated ligated aflatoxin B_1 adducted/unadducted template, 20mM Tris-HCl pH8.4, 50mM KCl, 2.5mM $MgCl_2$, 18pmoles labelled primer, 250µM of each dNTP, and 2 units Taq DNA polymerase. After gentle mixing, the samples were incubated in a Hybaid Omnigene thermal cycler. The deoxyoligonucleotide template was initially denatured at 95°C for 2 minutes

followed by linear amplification for 30 cycles each cycle consisting of 1 minute denaturation at 95°C, 1 minute annealing at 58°C and 1 minute chain elongation at 72°C. When amplification was completed, 5μL stop solution was added, (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole), and the extension products denatured by heating to 95°C followed by rapid cooling on ice. The DNA fragments were resolved on a 0.8mm by 15cm, 20% polyacrylamide gel containing 8M urea and a Tris-borate-EDTA buffer system which was run at 12 watts for approximately 1 hour. Gels were fixed in a 40% methanol, 10% glacial acetic acid, and 3% glycerol solution for 30 minutes and then dried onto Whatman 3MM paper at 50°C for 1 hour. Gels were visualised using a Molecular Dynamics Phosphorimager (Sunnyvale, CA). A 10bp DNA step ladder (Promega) was ^{32}P end labelled using 3μg ladder and $\gamma\,^{32}P$-ATP, as above and diluted to 50μl with ultrapure water and 10μl stop solution as above.

2.3.7b DNA polymerase I large (Klenow) fragment exonuclease$^+$

Primer extension using *Klenow* DNA polymerase *exo*$^+$ was performed in a total volume of 20 μl containing 2μg paramagnetic beads bound with aflatoxin B_1 adducted/unadducted template, 18pmoles labelled primer, 330μM of each dNTP, 50mM Tris-HCl (pH7.2), 10mM $MgSO_4$, 0.1mM dithiothreitol. The template was denatured by heating to 95°C for 2 minutes and the labelled primer annealed during subsequent cooling to 4°C. Extension was initiated by the addition of 2 units of Klenow exo$^+$ DNA polymerase I. Elongation was allowed to proceed for 30 minutes at 37°C before the addition of 5μl stop solution. Primer extension products were separated as above.

2.3.8 Adduction of plasmid DNA with aflatoxin B_1 8,9 epoxide

pGemT® *mdr1b* clone was incubated in a total volume of 100µl containing 6mM Tris-HCl, 6mM $MgCl_2$, 1mM NaCl, 1mM DTT and 20units *NcoI* at 37°C overnight. Restricted plasmid (100µg) was added to solutions of aflatoxin B_1 8,9 epoxide dissolved in ice cold acetone within a light proof glass vial to give final concentrations of 9.6, 28, 56, 96, 192, 960 & 1920nM. The plasmid DNA/AFB_1-epoxide solutions were shaken and incubated at 4°C over night. Samples were extracted twice with 500µl ice cold chloroform and once with 500µl ice cold ethyl acetate to remove aflatoxin B_1 and aflatoxin B_1 dihydrodiol. Plasmid DNA was precipitated from the aqueous phase by the addition of 0.1volumes of 3M sodium acetate pH5.2, and 2 volumes of ethanol.

2.3.9 Enzymatic hydrolysis of aflatoxin B_1 adducted plasmid DNA for detection of aflatoxin B_1 adducted nucleosides

Plasmid DNA (600µg) adducted with 1.92µM aflatoxin B_1 8,9 epoxide was precipitated and resuspended in 100µl of 5mM tris-HCl (pH 7.4), 2mM $MgCl_2$, 2mM $CaCl_2$ solution containing 63 units DNase I. The reaction was gently shaken and incubated at 37°C for 2.5 hours. To this solution 0.0016 units of calf spleen phosphodiesterase and 0.0036 units of snake venom phosphodiesterase were added and incubated at 37°C for 17 hours. The solution was made to pH8.0 using 50mM Tris-HCl. Calf intestinal alkaline phosphatase (0.14 units) was added and the mixture incubted for 7 hours at 37°C.

Aflatoxin B_1 modified nucleosides were separated from normal nucleosides by reverse phase HPLC using a Techsphere 5 ODS column. An 100µl aliquot of the enzymatically hydrolysed aflatoxin B_1 adducted plasmid DNA was loaded onto the ODS column. The DNA adducts were separated using an organic solvent gradient of 10-80% MeOH in HPLC grade water over 40 minutes at 1ml/minute. The nucleosides were detected using an Applied Biosystems 1000s UV diode array detector set at 254nm and 365nm. Deoxyguanosine and deoxyadenosine standards were also analysed.

2.3.10 DNA polymerase arrest assay on aflatoxin B_1 adducted plasmid DNA template

The polymerase arrest assay was carried out using primer 5'-TATTTAGGTGACACTATAG-3' which corresponds to bases 121-139 of the pGemT® plasmid SP6 promoter. The primer was ^{32}P end labelled using ^{32}P-ATP and purified, as above. Primer extension was performed in a total volume of 20µl containing 3µg aflatoxin B_1 adducted/unadducted plasmid DNA, 20mM Tris-HCl pH8.4, 50mM KCl, 2.5mM $MgCl_2$, 18pmoles labelled primer, 250µM of each dNTP, and 2 units Taq DNA polymerase. After gentle mixing the samples were incubated in a Hybaid Omnigene thermal cycler. The DNA was initially denatured at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 1 minute denaturation at 95°C, 1 minute annealing at 58°C and 1 minute chain elongation at 72°C. When amplification was completed, 5µl of stop solution was added, and the extension products denatured, and rapidly cooled on ice. The DNA fragments were resolved on a 0.4mm by 60cm, 6% polyacrylamide gel containing 8M urea and a Tris-

borate-EDTA buffer system. The gel was run at 60 watts for approximately 3-4 hours. Gels were fixed in a 10% methanol, glacial acetic acid solution for 20 minutes and dried onto Whatman 3MM paper at 80°C for 1 hour. Gels were visualised using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

DNA sequencing was performed using the Promega fmol® DNA sequencing system. Sequence reactions were performed in 6μL containing 100ng pGemT® *mdr1b* cloned plasmid DNA, 50mM Tris-HCl, pH 9.0, 2mM $MgCl_2$ buffer, 1.5pmoles ^{32}P labelled 5'-TATTTAGGTGACACTATAG-3' primer, 20μM dATP, dTTP, dCTP, 7-Deaza dGTP with either 30μM Deaza ddGTP, 350μM Deaza ddATP, 600μM Deaza ddTTP, 200μM Deaza ddCTP and 5 units sequencing grade *Taq* DNA polymerase. After gentle mixing the samples were incubated in a thermal cycler. After an initial denaturation at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 58°C and 1 minute chain elongation at 72°C. When amplification was completed, 3μl stop solution was added, and treated as above.

2.4 RESULTS

2.4.1 Reaction of Aflatoxin B_1-8,9-Epoxide with 5'-dATCGATATCGAT-3'

The 5'phosphorylated deoxyoligonucleotide dATCGATATCGAT was adducted with aflatoxin B_1-8,9-epoxide and separated from unadducted deoxyoligonucleotide by HPLC. After organic extraction to remove any aflatoxin B_1 and aflatoxin B_1 dihydrodiol a crude yellow solution was obtained. This solution was passed through a desalting gel column and the oligonucleotides eluted with water. Three 1 ml fractions were collected and each fraction was subjected to HPLC analysis. Fraction 1 (fig 23 – A) gave 3 distinct peaks, the first peak eluted at 4.58 minutes and absorbed only at 254nm. This peak was unadducted oligonucleotide as verified by HPLC analysis of unmodified oligonucleotide. Less polar second and third peaks eluted at 12.39 minutes and 16.71 minutes respectively absorbing at 254nm and 365nm which may indicate that these oligonucleotides are modified with aflatoxin B_1. Fraction 2 (fig 23 - B) gave similar results to fraction 1 with small a peak that eluted at 5 minutes. The two subsequent less polar peaks eluted at 11.73 and 17 minutes respectively and absorbed at both wavelengths. Fraction 3 (fig 23 - C) produced a large wide peak at 5.18 minutes that absorbed at 254nm and one less polar peak eluting at 16.95 minutes. The peaks eluting at approximately 12 minutes may be modified with one aflatoxin B_1 adduct whereas a less polar peak which eluted at approximately 17 minutes may be modified with two aflatoxin B_1 DNA adducts which would account for it being less polar.

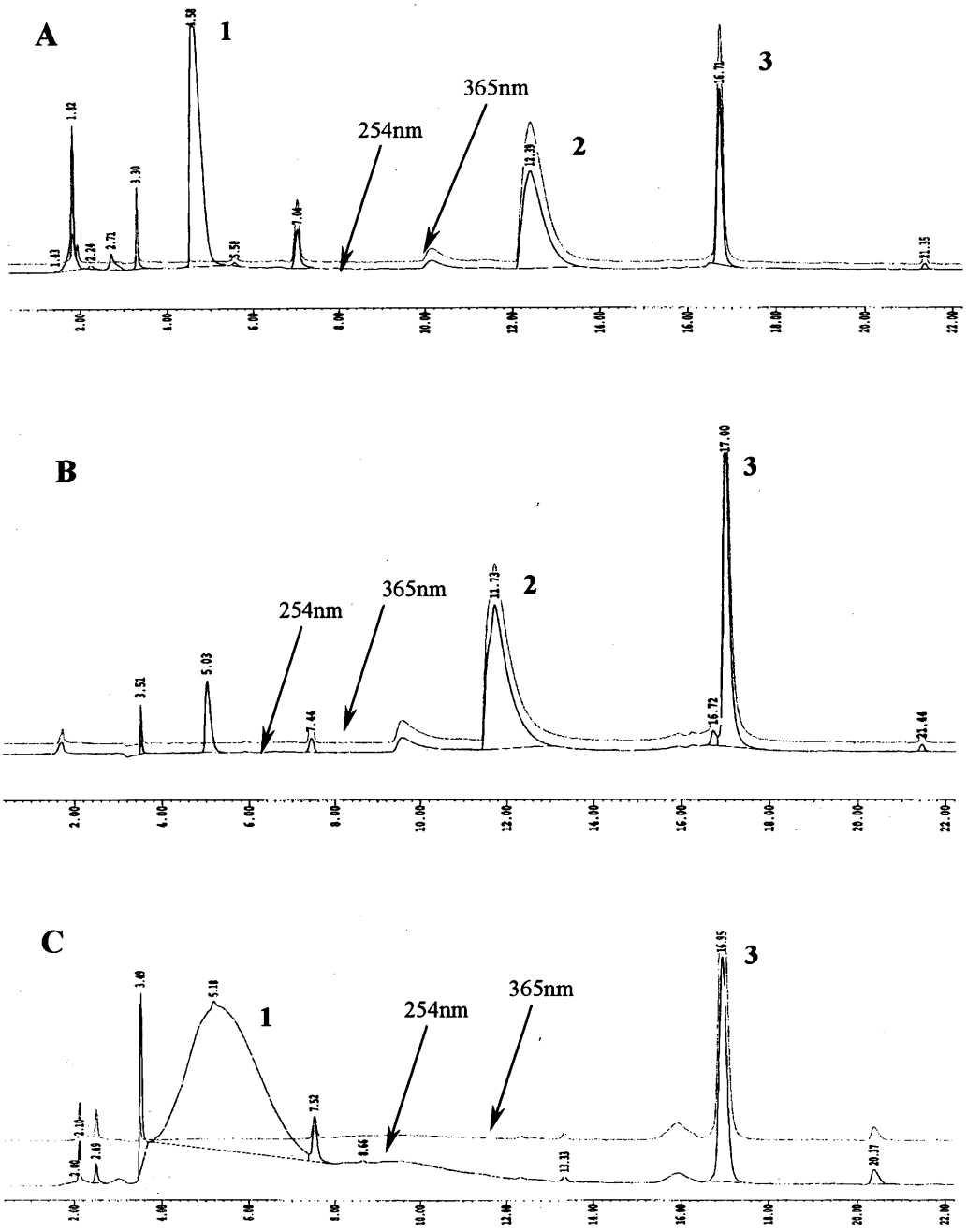


Fig 23 - HPLC separation of aflatoxin B_1 adducted dATCGATATCGAT. The crude reaction mix was subjected to gel column purification. Fraction 1 (A) peak 1 eluting at 4.58 minutes, peak 2, 12.39 minutes and peak 3, 16.71 minutes, fraction 2 (B) peak 2 eluted at 11.73 minutes, peak 3, 17.00 minutes and fraction 3 (C) peak1 eluting at 5.18 minutes and peak 3 at 16.95 minutes. One experiment typical of three is shown.

2.4.2 Ligation of adducted/unadducted dATCGATATCGAT to form the 69 base template

Peaks 1,2 and 3 were collected from the HPLC separations and used in ligation reactions to examine template formation. Ligation using the ^{33}P-labelled oligonucleotide was observed for all peaks collected as a weak band of radioactivity at 69 bases as shown in fig 24 lanes 1-7. The three peaks collected from fraction 1 were subjected to mild alkaline conditions, forming the AFB_1-formamidopyrimidine (AFB_1-FAPY) adduct. Similarly ligation occurred using these oligonucleotides Fig 24 lanes 8-10.

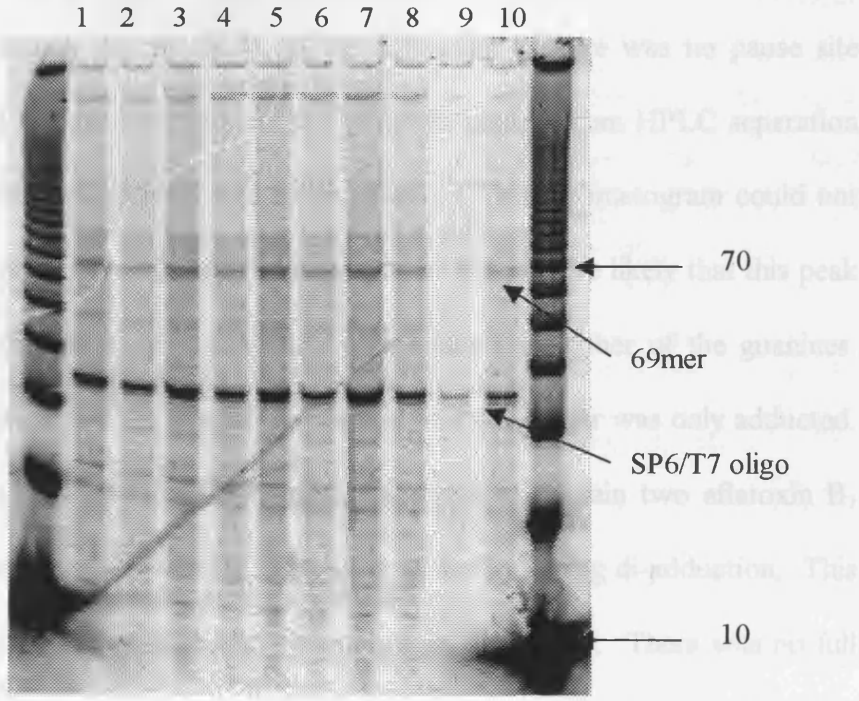


Fig 24- Ligation of major peaks from the HPLC separation of the adducted dATCGATATCGAT. Lane 1-3 fraction 1, peaks 1, 2, 3 respectively; Lanes 4-5 fraction 2, peaks 1 and 2 respectively; Lanes 6-7 fraction 3, peaks 1 and 3; lanes 8-10, fraction 1, peaks 1,2,3 respectively treated under mild alkaline conditions. One experiment typical of three is shown

The 69 base template was purified from the polyacylamide gel by determining its position using autoradiography. The band was eluted from the gel using a crush and soak method. After elution the template was 5' dephosphorylated to remove the radiolabel, and yields of approximately 600ng were obtained.

2.4.3 Polymerase arrest assay using the 69 base aflatoxin B_1 adducted template

Under optimal conditions using *Taq* DNA polymerase by single read through produced a pause site at base 40 from template produced from peak 2 of the HPLC separations. There was a reduction in full length extension, as seen in fig 25 lane 2 & 3. The exact locations of the adducts should be at 40bp and/or 46bp as the 12 mer was designed with only two guanines at positions 4 (G_1) and 10 (G_2). There was no pause site produced at 46 bp for this template. It was assumed peak 2 from HPLC separation contained one aflatoxin B_1 adduct due to its polarity. The chromatogram could not however, indicate as to which guanine was adducted. It would be likely that this peak would contain a mixture of oligonucleotide with adducts on either of the guanines. These results indicated that the first dG at position 4 of the 12mer was only adducted. However, using peak 3 to form the template, assumed to contain two aflatoxin B_1 adducts, pause sites were generated at both 40 & 46 bp indicating di-adduction. This data was therefore consistent with that obtained from the HPLC. There was no full length extension product produced most likely indicating that aflatoxin B_1 DNA adducts effectively block *Taq* DNA polymerisation.

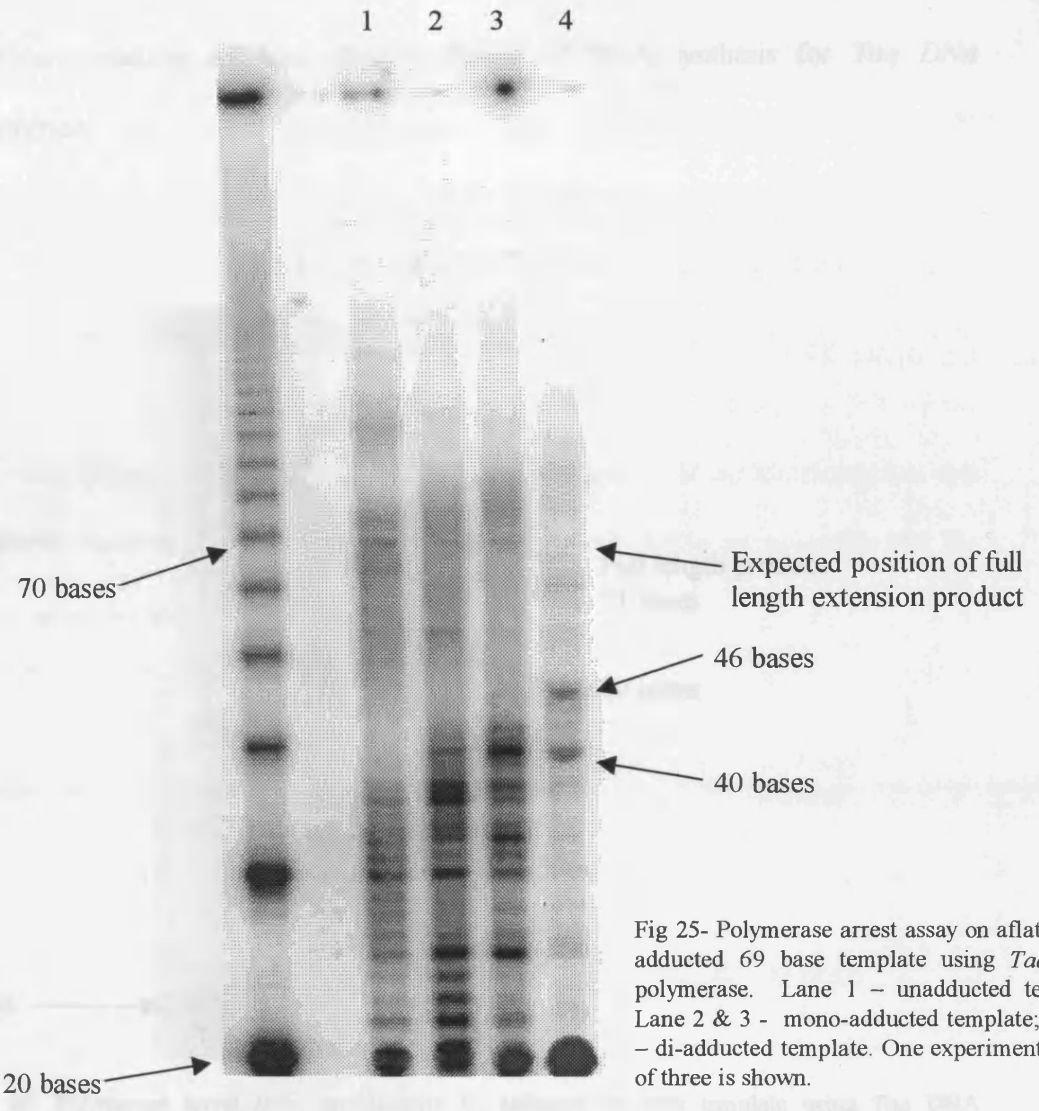


Fig 25- Polymerase arrest assay on aflatoxin B_1 adducted 69 base template using *Taq* DNA polymerase. Lane 1 – unadducted template; Lane 2 & 3 - mono-adducted template; Lane 4 – di-adducted template. One experiment typical of three is shown.

Reaction conditions of the arrest assay were changed to include 30 cycles of PCR. The method of template preparation used for these experiments was altered to include purification by capture of the 5' biotinylated template onto streptavadin coated paramagnetic beads. The mono-adducted 12mer was exposed to mild alkaline conditions to cause the imidazole ring of the aflatoxin B_1 adducted deoxyguanosine to open forming the AFB_1-FAPY-dG adduct. Pause sites were generated at position 40 for both ring closed fig 26 lane 2 and ring opened AFB_1-FAPY-dG adducts fig 26 lane 4 with a reduction in full length extension product formed. This indicated that AFB_1-

FAPY-dG adducts are also effective blocks of DNA synthesis for *Taq DNA* polymerase.

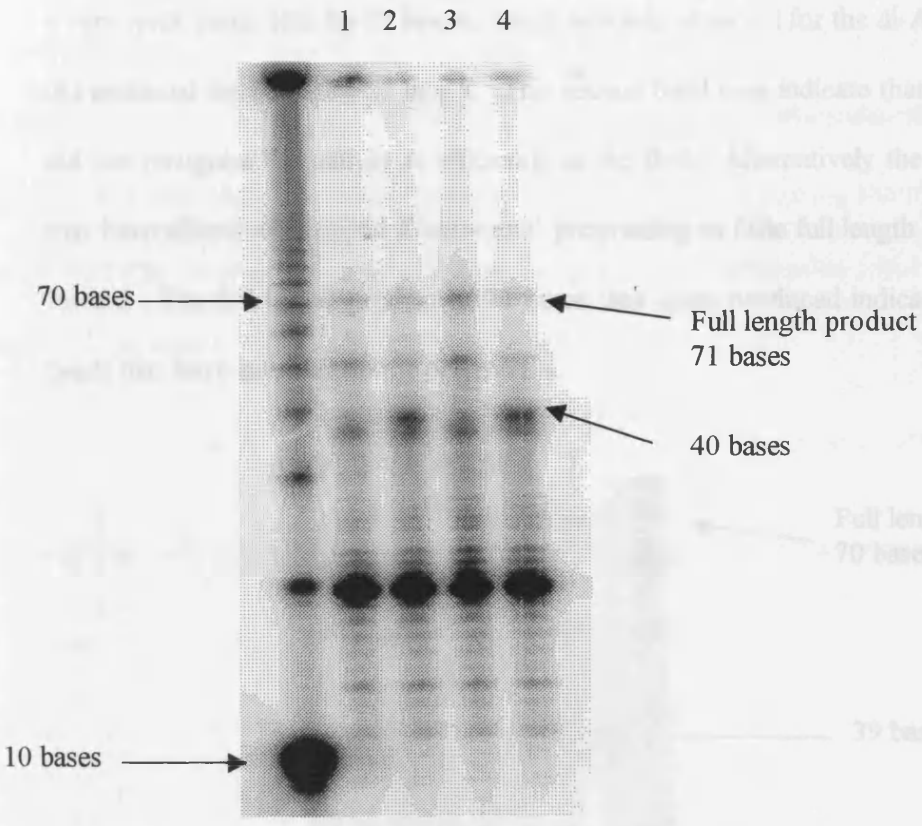


Fig 26- Polymerase arrest assay on aflatoxin B_1 adducted 69 base template using *Taq* DNA polymerase. Lane 1 unadducted template; lane 2 – AFB_1- mono-adducted template; lane 3 unnaducted template treated under mild alkaline conditions; lane 4 mono-AFB_1-FAPY-dG adducted template. One experiment typical of three is shown.

The size of the full length extension product was determined to be 71 bases. This may be artifactual due to primer extension being performed on a template bound to paramagnetic beads.

The arrest assay was also performed using the Klenow exo^+ fragment of *E.coli* DNA polymerase I. Using Klenow exo^+ DNA polymerase on the mono-adducted template a similar spectrum of adducts was identified fig 27 lane 1 and the AFB_1-FAPY dG

adduct fig 27 lane 4. The data also shows, Klenow exo$^+$ DNA polymerase halting one base before the adducted deoxyguanosine. The template containing two AFB_1-dG adducts showed pause sites at 40 bp. The second adduct at position 46 only produced a very weak pause site, fig 27 lane 6, which also was observed for the di-AFB_1-FAPY-dG adducted template, fig 27 lane 8. This second band may indicate that the enzyme did not recognise the adduct as efficiently as the first. Alternatively the first adduct may have effectively stopped *Klenow exo*$^+$ progressing as little full length product was formed. The full length product of 71 bases was again produced indicating that the beads may have an effect on the polymerase.

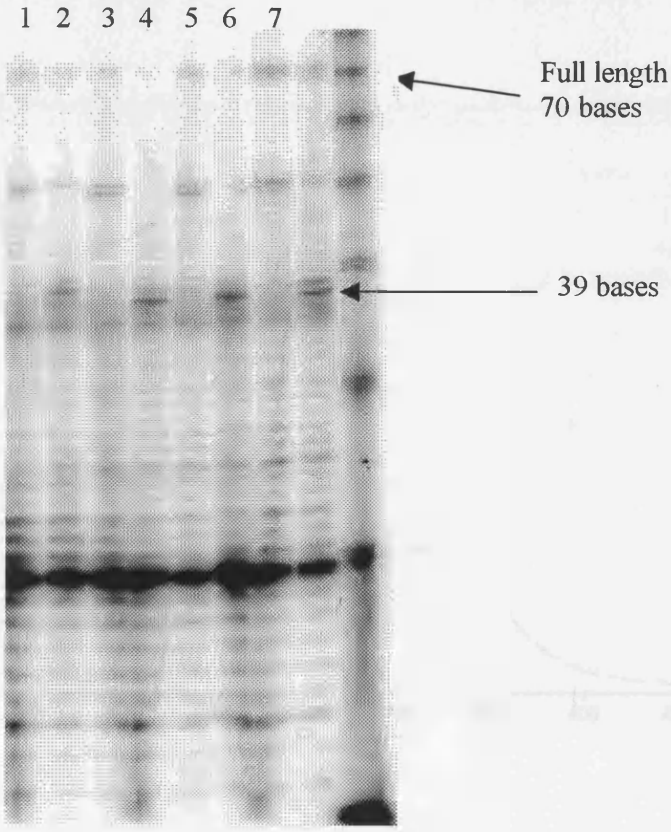


Fig 27- Polymerase arrest assay of aflatoxin B_1 adducted 5' biotinylated template using *Klenow exo*$^+$ fragment of DNA polymerase I. Lane 1,3,5&7 - unadducted template; Lane 2-AFB_1 mono-adducted template; Lane 4-AFB_1-FAPY mono adducted template; Lane 6 - AFB_1 di-adducted template; Lane 8 - AFB_1-FAPY di-adducted template. One experiment typical of three is shown.

2.4.4 Reaction of aflatoxin B_1-8, 9-epoxide with plasmid DNA

Determination of the level of adduction by aflatoxin B_1 using standard ^{32}P-post labelling is not possible due to the nature of the adduct. The absorption profile of the crude reaction mix devoid of free aflatoxin B_1 and aflatoxin B_1 dihydrodiol showed a typical absorption maxima of 365nm which identifies aflatoxin B_1 (fig 28). Conformation of adduction was sought by enzymatically hydrolysing the plasmid DNA to constituent nucleosides and resolving the adducted nucleosides from unadducted nucleosides using reverse phase HPLC (fig 29).

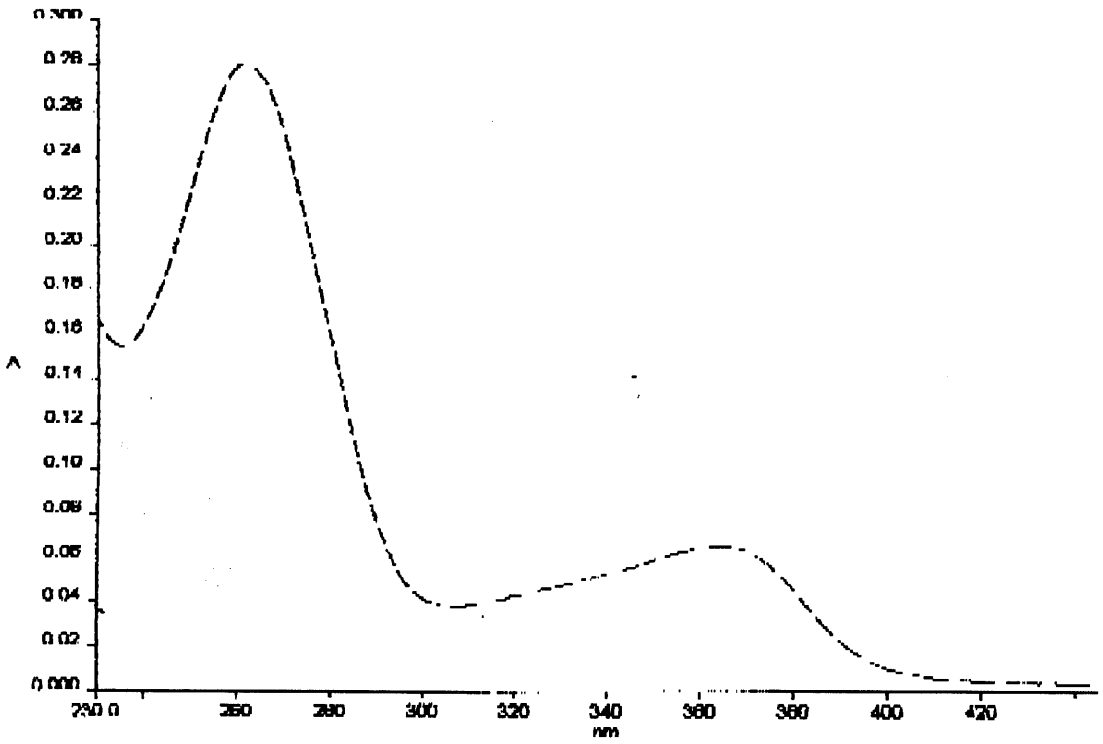


Fig 28- Absorption spectrum of aflatoxin B_1 adducted plasmid DNA showing λ max of 365nm for aflatoxin B_1 and 260nm for DNA.

Peaks eluting at 5.67 minutes and 12.87 minutes absorbing at 254nm were determined to be deoxyguanosine and deoxyadenosine respectively as analysed by comparison of retention times with standards. The peak absorbing at 254nm and 365nm with a retention time of 28.76 minutes was similar to that observed previously for the adducted base by Essigmann et al., (1977). It was therefore concluded that this was indicative of the AFB_1-N^7-dG species.

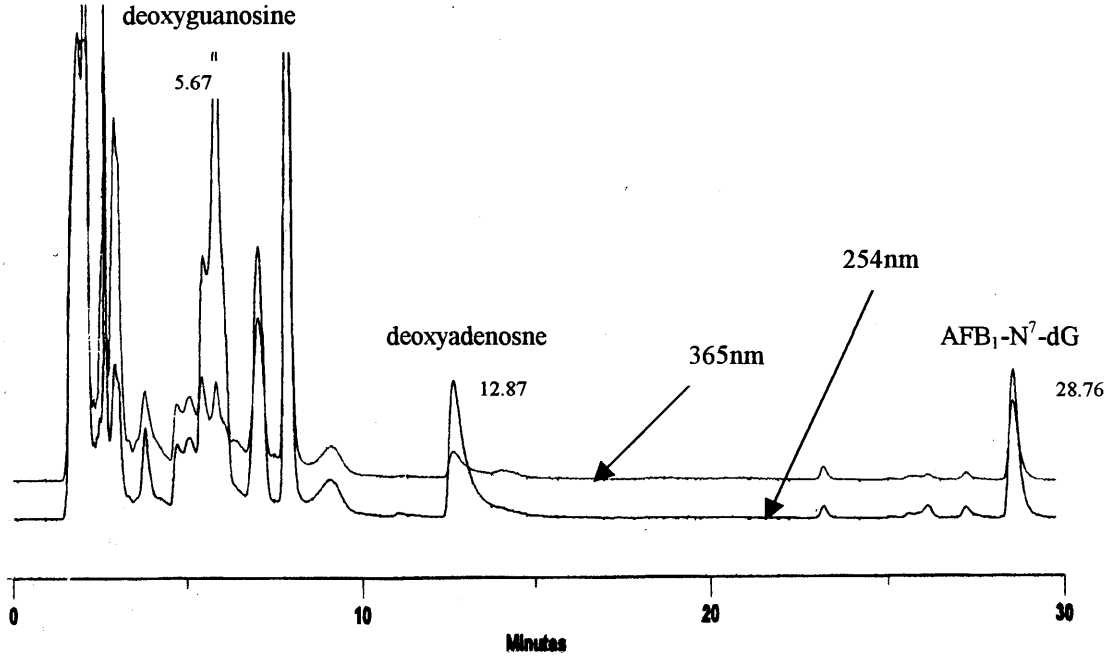


Fig 29- Chromatogram of enzymatically hydrolysed aflatoxin B_1 adducted plasmid DNA. Deoxyguanosine eluting at 5.67 minutes, deoxyadenosine eluting at 12.87 minutes and the AFB_1-N^7-dG adduct eluting at 28.67 minutes. One experiment typical of three is shown.

2.4.5 Polymerase arrest assay using aflatoxin B_1 adducted plasmid DNA

Having successfully shown that *Taq* DNA polymerase paused at aflatoxin B_1 adducts the experiments were extended to attempt detection of these adducts in plasmid DNA. Aflatoxin B_1 adducted plasmid DNA was treated under mild alkaline conditions to produce the stable AFB_1-FAPY-dG adduct. Initially plasmid DNA was adducted using

the same amount of aflatoxin B_1-8,9-epoxide as used for the oligonucleotide experiments producing heavily modified DNA. This was assumed as no extension products were detected in the adducted samples. To overcome this problem, adduction reactions were optimised for the polymerase arrest assay by adducting the plasmid with 9.6nM, 19nM, 28.5nM, 47.7nM, 95.5nM and 143nM of aflatoxin B_1-8,9-epoxide. Full length extension products were seen at all concentrations with possible pause sites detected in DNA adducted with 143nM aflatoxin B_1 8,9 epoxide (data not shown). The amount of epoxide in the adduction reaction was increased to 143nM, 286nM, 477nM, 954.5nM, 4.77μM and 9.5μM aflatoxin B_1 8,9 epoxide. Restriction digestion of the plasmid using *NcoI* was inhibited after adduction at these concentrations of aflatoxin B_1 8,9 epoxide and therefore the plasmid was linearised prior to adduction. Pause sites were detected from 286nM, with the strongest pause sites being observed for 4.77μM and 9.5μM (fig 30). Adduction was not seen at all guanines and the intensity of the pause sites were sequence specific (table 2) with the majority of guanine adducts having a 5' dC and 3' dA. The strongest pauses were with guanine next to either a 5' dG or dC and 3' dG or dC. Full length extension was achieved at the same intensity as control, up to 954.5nM aflatoxin B_1 8,9 epoxide, decreasing at 4.77μM and substantially reduced at 9.5μM aflatoxin B_1. These data indicated that at the highest AFB_1 8,9-epoxide concentrations, more guanines were becoming adducted therefore producing more pause sites and reducing the amount of full length product formed.

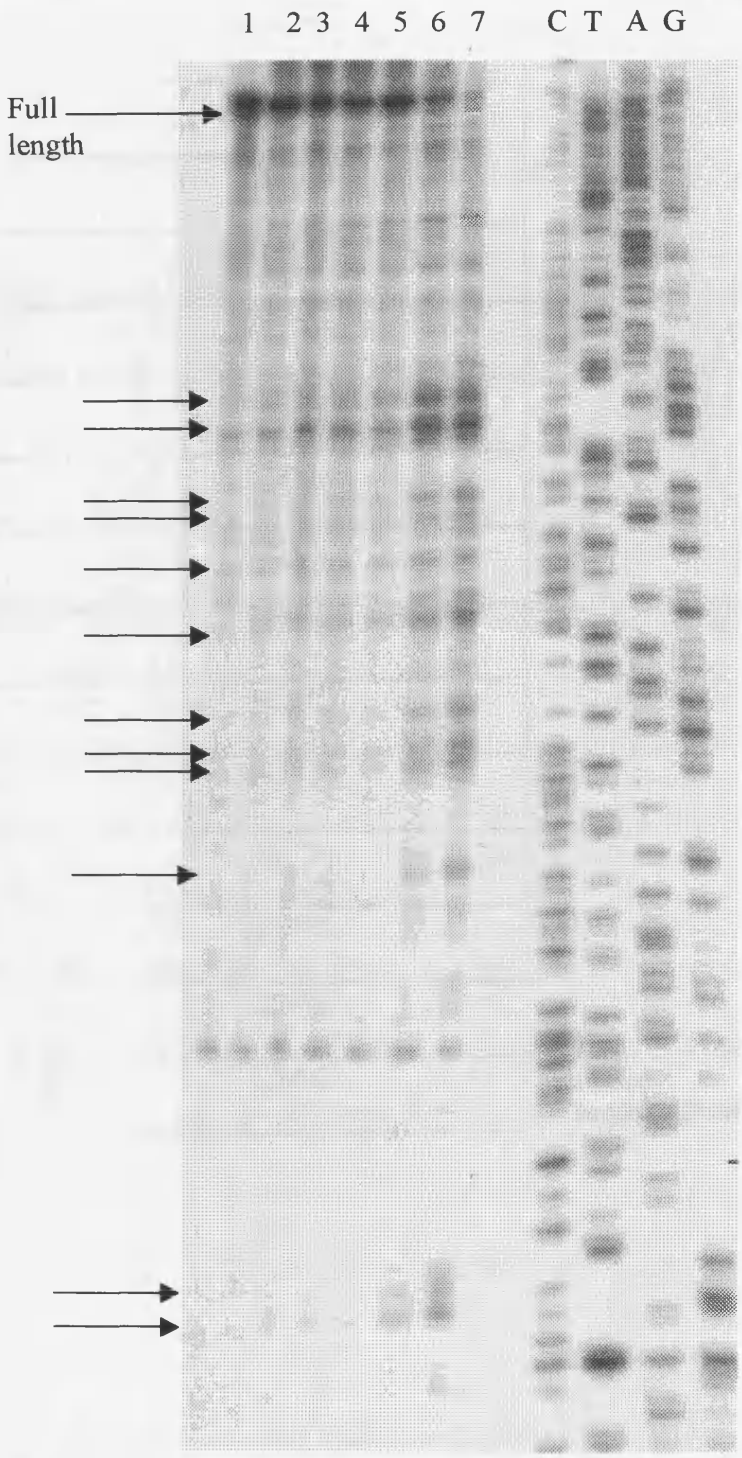


Fig 30 – Polymerase arrest assay on AFB_1 adducted plasmid DNA using Taq DNA polymerase. DNA was reacted with Lane 1 – Control; 2 - 143nM; 3 - 286nM; 4 - 477nM; 5 - 954.5nM; 6 - 4.77μM; 7 - 9.5μM AFB_1 8,9 epoxide. Arrows indicate sites of guanine AFB_1 DNA adduct formation as determined from the DNA sequencing ladder.

Pause site	Plasmid sequence
1	AGG*G
2	CG*A
4	CGG*T
5	TG*A
6	TG*C
7	CG*A
8	CG*A
9	CG*A
10	CG*A
11	AG*C
12	CG*T
13	AG*A
14	CG*C
15	CG*G
16	CG*G

Table 2 – Sequence context aflatoxin B_1 DNA adduct formation (* indicates adduct)

2.5 DISCUSSION

This study has sought the confirmation AFB_1 adduct formation on specifically placed deoxyguanines in an oligonucleotide template, and confirm the sequence specificity of AFB_1 DNA adduct formation in plasmid DNA using an optimised polymerase arrest assay.

2.5.1 Site Specific AFB_1 DNA adduct detection in a oligonucleotide template

Separation of the crude adducted oligonucleotide reaction mix by HPLC generated high intensity background and precise separation of the adducted and unadducted oligonucleotides could not be performed. Therefore, the reaction mix was partially purified by passage through a desalting gel column. Data from reverse phase HPLC, identified three major peaks, (fig 23). The peak 1, absorbing at 254nm only was determined to be unadducted. The two remaining peaks (2 & 3) were less polar and absorbed strongly at both 254nm & 365nm indicating adduction with AFB_1. The adducted deoxyoligonucleotide used in the study contained two guanines. Therefore peaks 2 and 3 may represent mono and di-adducted oligonucleotide respectively. Separation of AFB_1-FAPY adducted oligonucleotide was unsuccessful using the mobile phase as used to separate AFB_1-N^7-dG adducted oligonucleotides. An optimal mobile phase consisting of 40% methanol: 8% acetonitrile was ascertained to elute the AFB_1-FAPY adducted oligonucleotides after a range of mobile phases were tested.

Chromatographic separation cannot determine the positions of AFB_1 adduction. Therefore, identification of the adducted oligonucleotides was attempted using negative ion electrospray mass spectrometry. This failed to identify adduction due to

high levels of background ions as detected in all samples processed. Contamination of the samples by additional compounds eluting from the column could be the cause of this problem. Therefore, data derived from the chromatograms were inconclusive with respect to the number and position of AFB_1 adducts on the oligonucleotide, but conclusive of AFB_1 adduction.

AFB_1 adducted and unadducted 12mer oligonucleotide was utilised in the synthesis of the 69 base template to be used in the polymerase arrest assay. The results obtained using all major peaks collected from each fraction by HPLC confirmed template formation (fig 24). Data using single pass synthesis of *Taq* DNA polymerase in the arrest assay identified the site of adduct formation to be located on guanine, since the pause site correlated to base 40 of the mono-adducted template, and 40 and 46 bases of the di-adducted template (base no 4 (G_1) and 10 (G_2) respectively of the original 12mer oligonucleotide) fig 25. This data was consistent with previous studies, which have shown guanine to be the site of aflatoxin B_1 adduction. Full length extension of the template was achieved using *Taq* DNA polymerase in control reactions but was reduced on adducted templates. This indicated that aflatoxin B_1 is an effective block of polymerisation for this DNA polymerase. Purification of AFB_1-N^7-dG adducted template by polyacylamide gel electrophoresis may lead to the formation of AFB_1-FAPY-N^7-dG adducts as the gels were slightly alkaline. Adduct depurination may have also occurred during template synthesis and purification therefore, inhibition may have been due a AP site or the FAPY adduct.

An improved method of template purification was developed. The direct capturing of a biotinylated template onto streptavadin coated paramagnetic beads proved successful. Therefore, this latter method was exploited for the detection of the AFB_1-FAPY-N^7-dG adduct. Treatment of the AFB_1-N^7-dG adduct with alkali produces the ring opened FAPY adduct, which can form rotational isomers around the C^5 and N^5 bond of the pyrimidine moiety forming α and β configurations (Tomasz *et al*, 1987). Stacking structures may be enhanced with the FAPY lesion and may produce minimal helix distortion depending on the DNA sequence (Moa *et al*, 1998). Results generated by thermal cycling revealed that these adducts are capable of producing effective blocks to *Taq* DNA polymerase, with the formation of pause sites at 40 bases for both adduct forms on mono-adducted templates. Template bound to paramagnetic beads had no effect on adduct detection, but produced a full length extension product two bases larger than for unbound template. This discrepancy may be due to interaction of the beads with the polymerase and therefore, be an artifact. Thermal cycling may promote depurination of the AFB_1-N^7-dG adduct to form an AP site. Pause sites at G_1 were observed for both ring closed and opened forms of the AFB_1 adduct with full length extension product at about half the intensity of control reactions. This may be due to translesional synthesis across any AP sites produced in the early rounds of cycling (fig 26).

Aflatoxin B_1 DNA adduct detection was also assessed using the *Klenow exo*$^+$ fragment of DNA polymerase I, by single pass DNA synthesis. The polymerase paused at base 39 with the mono-adducted template, one base prior to the adducted guanine. This pause pattern correlated with data published by Refolo *et al*, 1995 & Jacobsen *et al*, 1987. The effective block of *Klenow exo*$^+$ DNA of the adduct at

39bp may be the cause of failure to detect the second adduct on the di-adducted template, as little full length extension product was produced. A potentially weak pause at 46 bases was observed for the FAPY di-adducted template with some full length extension product also being produced. This could indicate that the FAPY adduct may allow some translesional synthesis across the adduct (fig 27). A number of factors may contribute towards adduct detection and account for the differences in pause site production between *Taq* and *Klenow exo*$^+$ DNA polymerase. For example, lesions that affect Watson-Crick base pairing may alter the incorporation rates of the incoming nucleotide affecting the chemistry step of the polymerases. Aflatoxin B_1 adduction has no influence on base pairing but does produce a bulky DNA adduct that could sterically hinder the DNA polymerase. Changes in the local template structure, where adduction has occurred, and/or changes in the quaternary structure of the protein when the polymerase encounters the adduct, may alter the function of the active site. This may lead to alterations of amino acid residue interactions within the active site or associations with template at other sites of the protein. These changes may inhibit the polymerase enzyme catalytic function and restrict progression leading to termination of DNA synthesis. It is also possible that *Klenow exo*$^+$ incorporates a base complementary to the adducted base which is removed by 3'-5' exonuclease activity of the enzyme.

It was assumed that with oligonucleotides containing only one aflatoxin B_1 adduct, a mixed population on either of the two dG's, would be detected by the assay. The polymerase arrest assay showed pause sites at G_1 for the mono-adducted template with no other apparent sites of guanine adduct formation. The observed adduction spectrum may be influenced by the mechanism by which aflatoxin B_1 8,9-epoxide

forms DNA adducts with guanine. Yu *et al.*, (1990) investigated the binding affinity of aflatoxin B_1, using oligonucleotides that were self complimentary to those that remain single stranded. These data revealed a dramatic decrease in binding affinity to the single stranded oligonucleotides, and from these data concluded that aflatoxin B_1 requires intercalation into the template before covalent modification can occur. The oligonucleotide used in this study has the ability to self compliment (Td=27°C, Tm 32°C) or form a stable hairpin structure (ΔG= -3.6Kcal mol @ 4°C, loop Tm=57°C). Therefore, at 4°C a mixed population of ds template and hairpin could be produced in the adduction reaction. The efficiency of the reaction with aflatoxin B_1 8,9 epoxide and ds DNA is exceptionally high with preferential binding at particular reactive guanines being influenced by the 5' and 3' flanking bases i.e. nearest neighbour effects (Said *et al*, 1991).

Loechler *et al*, (1988) has performed molecular modelling of AFB_1 8,9 epoxide bound at N^7 of dG. This study suggested that the epoxide could either intercalate or bind externally to the major grove of B-DNA. This modelling revealed that AFB_1 adducts came into close contact with bases 5' and 3' to the reactive guanine and with the two complementary bases. This could, be the reason why AFB_1 shows preference for ds DNA over ss DNA for adduct formation. Explanation for externally bound AFB_1 in the major grove suggests the most preferential nearest neighbours, and their complimentary bases allow greater compatibility for interaction with the reactive guanine to form the most favourable interactions with the AFB_1 moiety. This allows the formation of a structurally favourable transition state complex before covalent binding which directs the adduct into the major grove (Muench *et al*, 1983; Weiner *et al*, 1994). For intercalation, the reaction was divided

into two steps. The initial step involves two adjacent base pairs separating to form a hole. This, in turn leads to AFB_1 8,9-epoxide entering the gap forming a energetically favourable transition state which is dependent on the 5' and 3' flanking bases before covalent reaction can occur. Studies have shown that the AFB_1 moiety of the adduct interacts with the major and minor grove of a self complimentary oligonucleotide (Gopalakrishnan *et al*, 1990a+b). A specific lock and key fit at particular sequences is possible to enhance the reaction of the epoxide with ds DNA, since non reactive structural analogues of AFB_1 diminish adduction and reduce sequence specificity (Misra *et al*, 1983; Kobertz & Essigmann, 1996; Kobertz *et al.*, 1997). Regioselectivity and ^1H NMR studies show consistency for non covalent interactions at the 5' face of the reactive guanine, placing the AFB_1 moiety at the 5' side of the reactive dG (Stone *et al*, 1988 & 1990; Gopalakrishnan *et al*, 1989 & 1990a+b). This suggests that intercalation of the epoxide above the 5' face of the reactive guanine produces a transition state placing the epoxide in a favourable orientation and in close proximity to the dG-N^7 leading to a back sided S_n2 reaction (Iyer *et al*, 1994). The 5' and 3' flanking sequences would determine the positioning of the transition state for in line nucleophilic attack of the dG-N^7 atom with the epoxide.

The above mechanism of adduction suggests aflatoxin B_1 intercalates into the self complimented oligonucleotide allowing reaction at G_1 and G_2 respectively producing the di-adducted oligonucleotide. Intercalation at the 5' side of dG orientates the AFB_1 moiety in such a way that the dG in the complementary strand is sterically inaccessible to attack by epoxide. Mono-adducted oligonucleotide is expected to be produced at G_1 in both stands. This would sterically inhibit further attack at G_2 in the

complementary stand and vice versa. However, this was undetected, therefore, di-adduction may be preferable for this complimentary oligonucleotide. Adduction at both G_1 and G_2 would also be expected to take place with the hairpin structure produced by the oligonucleotide. The small size of the hairpin may impose steric constraints that favour reaction at G_1. Therefore, adduction at the second guanine may be slow and di-hydrodiol formation may occur before intercalation takes place, indicating why only mono-adducted AFB_1 at G_1 occurs.

2.5.2 AFB_1 adduct detection in plasmid DNA

To determine sequence specificity of aflatoxin B_1 adduct formation, AFB_1 8,9 epoxide was incubated with plasmid DNA, initially in the same manner used in the oligonucleotide adduction reactions. Verification of adduction was sought by absorption spectra of the crude reaction mix devoid of free AFB_1 and AFB_1 8,9 dihydrodiol. Confirmation of AFB_1-N^7-dG adduct formation was gained by enzymatically hydrolysing adducted plasmid DNA to nucleosides and separating the adducts using reverse phase HPLC. This alternate technique to ^{32}P-post labelling was chosen to determine adduct formation because AFB_1 DNA adducted DNA does not digest efficiently enough to its nucleotide components to be used in the postlabelling procedure. There was slight variation from the results of Essigmann *et al.*, (1977) due to the differences in sample preparation and in the column used for HPLC. The peak eluting in the same area as Essigmann *et al.*, (1977) and was determined to be the AFB_1-N^7-dG adduct (fig 29). Other peaks absorbing at both wave lengths may be incomplete digestion products.

The polymerase arrest assay performed on plasmid DNA containing the AFB_1-FAPY DNA adduct does not depurinate during thermal cycling, therefore, this adduct would be advantageous as thermal cycling was used. Optimal adduction reactions were performed, that produced detectable pause sites in the assay. Data showed the minimum amount of epoxide that produced pause sites was 144nM and the maximum was 96μM at which no extension products were formed.

Data with the polymerase arrest assay shows that for AFB_1-FAPY-N^7-dG adducts, the major site of adduct formation was on guanine (fig 30). This correlated with our data using the oligonucleotides. Adduction on most guanines was detected, with no other sites of adduction observed. Aflatoxin B_1-dA adducts are produced in low abundance and are highly labile at ambient temperatures. These may have depurinated and therefore, not be detected by *Taq* DNA polymerase. The strongest pause sites were seen at 4.8μM and 9.6μM AFB_1 8,9 epoxide. Results show GG*C and CG*G to be the most intense bands and TG*A the weakest as determined by densitometry. The majority of adducted guanines detected were located next to a 5' cytosine and were of much the same intensity. Other workers have shown that AFB_1 8,9 epoxide reacts with guanine which give pause sites that vary in intensity (Essigmann *et al*, 1977 & 1983; Misra *et al*, 1983) so preferential binding to guanine is sequence context specific as the sequence determines favourable transition state complex formation. By analysis of a number of fragments of known sequences, (Misra *et al*, 1983, Muench *et al*, 1983 & Benasutti *et al* 1988) it has been possible to form a set of empirical rules to predict the relative reactivity of any given guanine within a known DNA sequence. The rules of reactivity are G>C>A>T for the 5' base and G>T>C>A for the 3' base next to the reactive guanine.

In summary it can be concluded that adduction of DNA by aflatoxin B_1 is dependent upon the intercalation of the *exo*-8,9 epoxide into a double stranded template before covalent bonding takes place. AFB_1 DNA adduct formation was found on guanine in a oligonucleotide specifically designed containing two dG's. Adduct were either formed on both guanines (G_1 and G_2) or just at G_1. *Taq* DNA polymerase paused at the site of the adducted guanine whereas *Klenow exo*$^+$ DNA polymerase paused one base before. AFB_1 adduct formation was also observed in plasmid DNA to be on guanine. Sequence specific binding in dsDNA was observed at CG or GG sequences. In general the sequence specificity is dependent on the 5' & 3' flanking bases facilitating the most stable transition states that orientate the epoxide for efficient nucleophic attack of the dG-N^7 atom.

DETECTION OF 4-HYDROXYTAMOXIFEN DNA ADDUCT FORMATION IN PLASMID DNA USING THE POLYMERASE ARREST ASSAY

3.1 Chapter Objectives

The objectives of this study were:

1. to optimise the polymerase arrest assay utilising DNA and RNA polymerases for the detection of adduct formation by 4-hydroxytamoxifen *in vitro*.
2. to determine sequence specific binding of 4-hydroxytamoxifen DNA adduct formation.

3.2 Introduction

Lesions that interfere with the progression of nucleotide polymerisation result in termination of DNA/RNA synthesis, either at the adducted base, or one or two nucleotides before (Bhanot & Ray, 1986; Bichara & Fuchs, 1985; Brash & Haseltine, 1982; Borowyborowski & Chambers, 1987; Drinkwater *et al.*, 1980). On occasions DNA synthesis terminates at a defined distance after replication of the adduct, but prior to complete primer extension (Latham, 1995).

The mechanism by which lesion bypass occurs has been elucidated from crystal structure analysis of DNA polymerases, including HIV I reverse transcriptase (Kohlstaedt *et al.*, 1992), rat DNA polymerase β (Davies *et al.*, 1994; Sawaya *et al.*, 1994), and Taq DNA polymerase (Kim, 1995). These polymerases share a tertiary structure analogous to a half open right hand with three distinct subdomains - palm, thumb and fingers (Joyce, 1994; Joyce & Steitz, 1995; Steitz, 1993a). The palm contains the polymerase catalytic active site, the thumb binds double stranded DNA template and the fingers bind the incoming nucleotide. Although all polymerases share this analogy, the organisation of

these subunits show structural variations which determine the overall interaction with a DNA adduct. Polymerases position their various substrates in an appropriate geometry that renders phosphoryl transfer energetically favourable (Steitz *et al.*, 1993a+b). Therefore, the efficiency of translesional synthesis past a DNA adduct will be dependent on how the polymerase-primer-template-nucleotide complex approximates the normal tertiary structure in the absence of a lesion (Hoffmann *et al.*, 1995).

Lesions that derange DNA secondary structure, alter base stacking properties or base pairing interactions with incoming nucleotides are generally greater obstacles to polymerase progression. Sequence context effects can influence how the polymerase anchors to the primer-template complex (Goodman *et al.*, 1993; Livneh *et al.*, 1993; Strauss, 1985). Therefore, adduct formation in specific DNA sequences may greatly alter DNA conformation compared with other sequences. So adducts in some sequences will reduce polymerase interactions with the template as well as changing the kinetic parameters for lesion extension more than in others (Dickerson, 1992). This effect may influence the detection of DNA adduction hot spots (Fowler & Skinner, 1986; Lai & Beattie,1988; Schaaper, 1996).

Polymerase extension assays detect adducts by measuring termination of chain elongation *in vitro*. However, *in vitro* assays will not determine the biological significance of DNA adduct toxicology *in vivo*. Polymerases unable to synthesise across damaged nucleotides, allow characterisation of the DNA adducting properties of specific mutagens/carcinogens at the nucleotide level (Moore & Strauss, 1979; Chan *et al.*, 1985). Extension of adducted templates by polymerases bearing one or more adducted nucleotides is reflected by the shorter pieces of DNA/RNA synthesised and a

proportional reduction in the amount of full length product (Gorden & Haseltine, 1982). The relative intensity of the polymerase pause sites indicates selectivity towards any sequence specific termination sites (Reardon, 1989).

3.3 Materials and Methods

3.3.1 Materials

The Plasmid pGemT® (Promega) is a standard cloning vectors that carries the *lacZ* alpha-peptide in addition to both SP6 and T7 RNA polymerase promoters flanking the multiple cloning site. Plasmid pGemT® was cloned with the target sequence from the *mdr1b* promoter (Dr Fhang Zhang, MRC Toxicology unit). Micrococcal nuclease, potato apyrase (grade VI), HEPES, hydrogen peroxide, horseradish peroxidase, and 4-hydroxytamoxifen were obtained from Sigma (Poole, Dorset, UK). T4 DNA polymerase, SP6 and T7 RNA polymerase, *AMV I* reverse transcriptase and *Tli* DNA polymerase, RNasin, and RQ1 Rnase free DNase were from Promega (Southampton, UK). *Tth, Pwo* DNA polymerase, HIV I reverse transcriptase, T4 polynucleotide kinase (3'-phosphatase-free), and calf spleen phosphodiesterase were from Boehringer Mannheim (Lewes, East Sussex, UK). T7 DNA polymerase was obtained from New England BioLabs (Hertfordshire, UK). *Taq* DNA polymerase was obtained from GibcoBRL, Life Technologies (Paisley, UK). [γ^{32}P]-ATP (>185 and 110 TBq/mmol, 370Mbq/ml), [α^{32}P]-UTP (110Tbq/mmol, 370Mbq/ml) was from Amersham International (Amersham, Buckinghamshire, UK). PEI-cellulose thin layer plates were from Macherey Nagel (supplied by Camlab, Cambridge, UK). The remaining chemicals of the highest available quality unless stated and were obtained from Sigma (Poole, UK).

3.4 Methods

3.4.1 Adduct Formation on pGEMT® Clone with Activated 4-Hydroxytamoxifen

100 µg of pGemT® *mdr1b* cloned plasmid DNA was dissolved in 340µL buffer containing 50mM HEPES-NaOH (pH6.2), 0.2mM EDTA, 0.03% TWEEN 20 and 500µM H_2O_2. 4-Hydroxytamoxifen was added to final concentrations 25, 50, 100, 160 and 250µM and the reaction pre-incubated at 37°C for 5 minutes. The reactive 4-hydroxytamoxifen quinone methide was generated by the addition of 10µl horseradish peroxidase (HRP) (5mg/ml) to the reaction, followed by incubation at 37°C for 30 minutes. After 30 minutes the reactions were stopped by the addition of 800µl ice cold chloroform. Unreacted 4-hydroxytamoxifen and 4-hydroxytamoxifen quinone methide was removed by extraction with water saturated ethyl acetate (3x400µl), and the DNA precipitated from the aqueous phase using 0.1 volumes 3M sodium acetate (pH5.2), 2 volumes ice cold ethanol. Control reactions were performed by incubating the plasmids in the absence of 4-hydroxytamoxifen in the presence and absence of H_2O_2±HRP.

3.4.2 ^{32}P-Postlabelling and TLC conditions

Adducted plasmid DNA samples were subjected to ^{32}P-postlabelling as described by Martin *et al.*, (1995). ^{32}P-Postlabelled samples were applied to 10x15 cm plastic backed PEI-cellulose TLC plates. TLC plates were developed firstly in D1 solvent (1.7 M sodium phosphate pH 7.0) onto filter paper wicks. Adducts were then separated by two dimensional chromatography using solvent systems D2 (2.6 M lithium formate, 6.4 M urea. pH 3.5) and D3 (0.6 M lithium chloride, 6.4 M urea, 0.4 M Tris-HCl, pH 8.0). Finally plates were developed again in D1 onto filter paper wicks. Adducts were visualised by autoradiography using OMAT-AR film with intensifying screens for

between 5 minutes and over night at room temperature. Adduct spots were excised from the plates and radioactivity quantified by scintillation counting. Adduct levels were determined by subtracting radioactivity in background regions from radioactivity in adduct spots. Adduct numbers are expressed as relative adduct labelling (RAL).

$$RAL = \frac{\text{d.p.m. in adduct peak}}{\text{sp.act. ATP x pmol dNp used for analysis}}$$

3.4.3 DNA Polymerase Arrest Assay on 4-Hydroxytamoxifen Adducted Plasmid DNA

The polymerase arrest assays were performed using primer 5'-TATTTAGGTGACACTATAG-3' which corresponds to bases 121-140 of the pGemT® plasmid SP6 promoter. The primer was ^{32}P end labelled using $\gamma^{32}P$-ATP, 3000Ci/mmol (Amersham) and 5 units T4 polynucleotide kinase (promega), in a total volume of 10µl containing 200pmoles deoxyoligonucleotide primer, 70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 5mM dithiothreitol at 37°C for 30 minutes. The labelled primer was separated from unincorporated ^{32}P-ATP using a Chroma Spin™-10 gel filtration spin column (Clontech, Heidelberg, Germany). Primer extension reactions were carried out in a total volume of 20 µl containing 3µg 4-hydroxytamoxifen adducted or unadducted pGemT® *mdr1b* cloned plasmid and 18pmoles labelled primer. The buffer and reaction conditions varied with the type of polymerase used.

For single pass DNA synthesis, all elongations were allowed to proceed for 30 minutes. The thermostable DNA polymerases have slight variations in elongation conditions and are listed below. When amplification was completd, 5µl stop solution was added (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole).

Extension products from each arrest assay were denatured by heating to 95°C followed by rapid cooling on ice. The DNA fragments were resolved on a 0.4mm by 60cm 6% polyacrylamide gel containing 8M urea and a tris-taurine-EDTA buffer system (Amersham). The gel was run at 60 watts which maintained a temperature of 50°C for approximately 2.5 hours. Gels were fixed in a 10% methanol, glacial acetic acid solution for 15 minutes and dried onto Whatman 3MM paper at 80°C for 1 hour. Gels were visualised using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

3.4.3a Taq DNA polymerase

The reaction buffer consisted of 250μM or 25μM of each dNTP, 20mM Tris-HCl pH8.4, 50mM KCl, 2.5mM $MgCl_2$, and 2 units Taq DNA polymerase. After gentle mixing the samples were then incubated in a thermal cycler. The DNA was initially denatured at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 1 minute denaturation at 95°C, 1 minute annealing at 58°C and 1 minute chain elongation at 72°C, 65°C, 60°C, or 55°C.

3.4.3b DNA polymerase I large (Klenow) fragment exonuclease$^-$ & exonuclease$^+$

The reaction buffer consisted of 330μM or 33μM of each dNTP, 50mM Tris-HCl (pH7.2), 10mM $MgSO_4$, 0.1mM dithiothreitol. The template DNA was denatured by heating to 95°C for 2 minutes and the primer annealed during subsequent cooling to 4°C. Extension was initiated by the addition of 2 units of either Klenow exo$^-$ or exo$^+$ DNA polymerase. Elongation was allowed to proceed for 30 minutes at either 25°C or 37°C.

3.4.3c T4 DNA polymerase

The reaction buffer consisted of 67mM Tris-HCl (pH8.8), 16mM $(NH_4)_2SO_4$, 6.7mM

$MgCl_2$, 6.7μM EDTA, 167μg/ml BSA, 10mM β-mercaptothanol buffer, 18pmoles labelled primer and 250μM of each dNTP. The DNA was denatured and the primer was annealed as above. Extension was initiated by the addition of 4 units of T4 DNA polymerase.

3.4.3d HIV I reverse transcriptase (adapted from Chary et al, 1997)

The reaction buffer consisted of 33mM Tris-acetate (pH7.8), 66mM KOAc, 10mM $Mg(Oac)_2$, 1mM dithiothreitol, 0.1mg/ml BSA, 18pmoles labelled primer, 300μM of each dNTP. The DNA was denatured and primer annealed as above. Extension was initiated by the addition of 2 units of *HIV I* reverse transcriptase and elongation was allowed to proceed for 30 minutes at 37°C before the addition of 5μL stop solution and treated as above.

3.4.3e AMV reverse transcriptase

The reaction buffer consisted of 50mM Tris-HCl (pH8.3), 50mM KCl, 10mM $MgCl_2$, 0.5mM spermidine, 10mM dithiothreitol, 18pmoles labelled primer, 250μM of each dNTP. The DNA was denatured and primer annealed as above and extension was initiated by the addition of 19 units of AMV reverse transcriptase.

3.4.3f T7 DNA polymerase

The reaction buffer consisted of 20mM Tris-HCl (pH7.5), 10mM $MgCl_2$, 1mM dithiothreitol, 5μg/ml BSA, 18pmoles labelled primer, 150μM of each dNTP. The DNA was denatured and primer annealed as above. Extension was initiated by the addition of 4 units of T7 DNA polymerase.

3.4.3g Pwo DNA polymerase

The reaction buffer consisted of 10mM Tris-HCl (pH8.85), 25mM KCl, 5mM

$(NH_4)_2,SO_4$, 2mM $MgSO_4$ buffer, 18pmoles labelled primer, 250μM of each dNTP, 2.5 units *Pwo* DNA polymerase.. The DNA was initially denatured at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 1 minute denaturation at 95°C, 1 minute annealing at 58°C and 45 seconds chain elongation at 72°C.

3.4.3h Tth DNA polymerase

The reaction buffer consisted of 10mM Tris-HCl (pH8.9), 0.1M KCl, 1.5mM $MgCl_2$, 50μg/ml BSA, 0.05% TWEEN® 20(v/v), 18pmoles labelled primer, 250μM of each dNTP, 2.5 units *Tth* DNA polymerase.. The DNA was initially denatured at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 1 minute denaturation at 95°C, 1 minute annealing at 58°C and 60 seconds chain elongation at 72°C.

3.4.3i Tli DNA polymerase

The reaction buffer consisted of 10mM Tris-HCl (pH9.0), 50mM KCl, 1% Triton® X100, 1.5mM $MgCl_2$, 18pmoles labelled primer, 200μM of each dNTP, 2.5 units *Tli* DNA polymerase. The DNA was initially denatured at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 1 minute denaturation at 95°C, 1 minute annealing at 58°C and 60 seconds chain elongation at 74°C.

3.4.3j T7 RNA polymerase

Transcription assays were performed using 600ng of either 4-hydroxytamoxifen adducted or adducted pGEMT® *mdr1b* cloned plasmid in a 100μl reaction mixture containing 40mM Tris-HCl (pH7.5), 6mM $MgCl_2$, 10mM dithiothretol, 2mM spermidine, 10mM NaCl, 20 units RNAsin ribonuclease inhibitor, 500μM each NTP, 12μM [α^{32}P]-UTP (3000Ci/mol, 10mCi/ml), and 50 units T7 RNA polymerase.

Transcription was allowed to proceed for 20 minutes at 37°C before the addition of 1 unit RQ1 DNase (RNase free) and incubated at 37°C for a further 15 minutes. RNA was precipitated from the aqueous phase by the addition of ice cold ethanol and the pellet washed to remove unincorporated [α^{32}P]-UTP. Transcription products were separated and visualised as above.

3.4.3k SP6 RNA polymerase

Transcription assays were performed using 600ng of either 4-hydroxytamoxifen adducted or unadducted pGEMT® *mdr1b* cloned plasmid in a 100µl reaction mixture containing 40mM Tris-HCl (pH7.9), 6mM $MgCl_2$, 10mM dithiothreitol, 2mM spermidine, 10mM NaCl, 20 units RNasin ribonuclease inhibitor, 0.5% Tween®-20, 500µM each NTP, 12µM [α^{32}P]-UTP (3000Ci/mol, 10mCi/ml), and 20 units SP6 RNA polymerase. Transcription was allowed to proceed for 20 minutes at 37°C and treated as above

3.5 DNA Sequencing Reactions

DNA sequencing was performed using the Promega fmol® DNA sequencing system. Sequence reactions were performed in 6µl containing 100ng pGEMT® *mdr1b* cloned plasmid DNA, 50mM Tris-HCl (pH 9.0), 2mM $MgCl_2$ buffer, 1.5pmoles ^{32}P labelled 5'-TATTTAGGTGACACTATAG-3' primer, 20µM dATP, dTTP, dCTP, 7-Deaza dGTP with either 30µM Deaza ddGTP, 350µM Deaza ddATP, 600µM Deaza ddTTP, 200µM Deaza ddCTP and 5 units sequencing grade *Taq* DNA polymerase. After gentle mixing the samples were incubated in a thermal cycler. Initial denaturation at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 30 seconds

denaturation at 95°C, 30 seconds annealing at 58°C and 1 minute chain elongation at 72°C. When amplification was completed, 3µl stop solution was added and treated as above.

3.6 RESULTS

3.6.1 Reaction of activated 4-hydroxytamoxifen with plasmid pGEMT *mdr1b*

Plasmid DNA was adducted by reaction of peroxidase activated 4-hydroxytamoxifen producing a yellow coloured solution due to 4-hydroxytamoxifen polymerisation products. DNA damage was demonstrated by TLC separation of ^{32}P-postlabelled adducted plasmid DNA prior to use in subsequent assays. TLC separation of postlabelled adducts from plasmid DNA reacted with activated 4-hydroxytamoxifen produced one major and two minor adduct spots (fig 31-C). The major adduct spot formed as a smear on the TLC plate eluting to the top right hand corner. Quantitation of the extent of DNA damage showed an adduct level of 21872±4039 adducts/10^8 normal nucleotides (mean±SD, n=4). Controls incubated with H_2O_2 and 4-hydroxy tamoxifen in the absence of HRP (Fig 31-A) and H_2O_2/HRP alone (fig 31-B) produced no DNA damage, indicating that 4-hydroxytamoxifen is devoid of DNA binding without being activated. Oxidative damage produced by H_2O_2/HRP activation produces DNA adducts that are not detectable in this system.

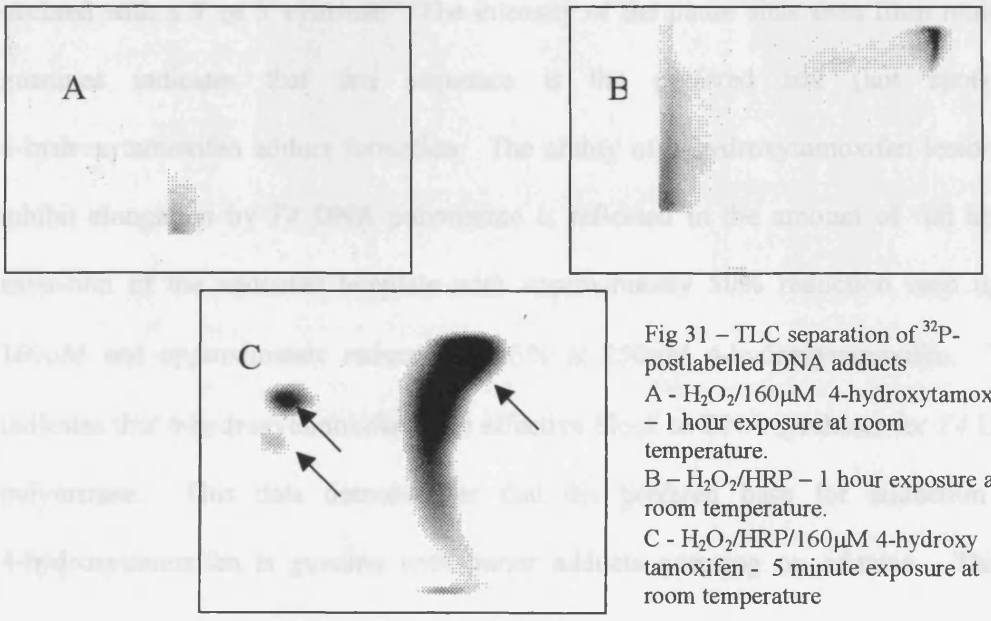


Fig 31 – TLC separation of ^{32}P-postlabelled DNA adducts
A - H_2O_2/160µM 4-hydroxytamoxifen – 1 hour exposure at room temperature.
B – H_2O_2/HRP – 1 hour exposure at room temperature.
C - H_2O_2/HRP/160µM 4-hydroxy tamoxifen - 5 minute exposure at room temperature

3.6.2 Polymerase arrest assays using 4-hydroxytamoxifen adducted plasmid pGEMT *mdr1b*

3.6.2a T4 DNA polymerase

T4 DNA polymerase (114kD) is a bacteriophage DNA dependent polymerase that requires a 5' protuding end and high concentrations of dNTP's for polymerisation to occur. *T4* DNA polymerase is highly processive and carries a strong 3' – 5' exonuclease function with no associated 5' – 3' endonuclease activity. This enzyme has a higher affinity for single stranded DNA than double stranded. The dG-4-hydroxytamoxifen lesion formed by the covalent attachment of 4-hydroxytamoxifen quinone methide to the N^2 position of guanine acted as a strong block to polymerisation by single readthrough of *T4* DNA polymerase (fig 32). Polymerase pause sites were mainly detected on guanine, with some adduct formation occuring on adenine. Dose related adduct formation was observed in runs of guanine. Sequence specific guanine adduct formation occured on guanines with a 3' or 5' adenine or cytosine. Guanine was also adducted in other sequences but to a lesser extent, with not all guanines being adducted. Adduct formation at adenine was not always seen in all experiments, but occured with a 3' or 5' cytosine. The intensity of the pause sites seen from multiple guanines indicates that this sequence is the prefered site (hot spot) of 4-hydroxytamoxifen adduct formation. The ability of 4-hydroxytamoxifen lesions to inhibit elongation by *T4* DNA polymerase is reflected in the amount of full length extension of the adducted template with approximately 50% reduction seen up to 160μM and approximately reduced by 75% at 250μM 4-hydroxytamoxifen. This indicates that 4-hydroxytamoxifen is an effective block to DNA synthesis for *T4* DNA polymerase. This data demonstrates that the prefered base for adduction for 4-hydroxytamoxifen is guanine with minor adducts occuring on adenine. This is

reflective of ^{32}P-poslabelling data showing guanine to be the major base for adduct formation (Osbourne *et al.*, 1996, 1997)

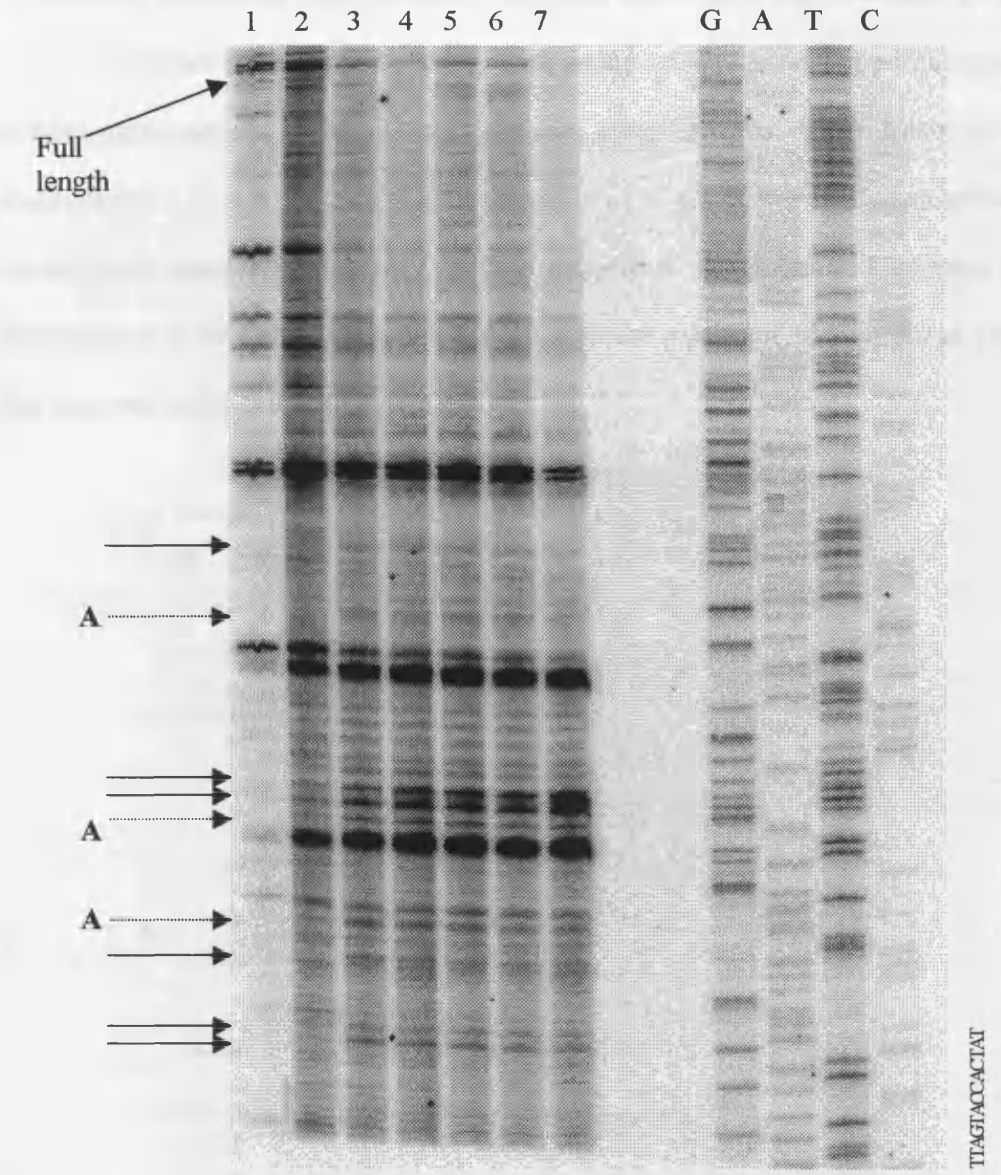


Fig 32 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *T4* DNA polymerase.. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 25μM 4-hydroxytamoxifen, 4 - 50μM, 5 - 100μM, 6 - 160μM, 7 - 250μM. Arrows indicate representative polymerase pause sites at guanine. The labelling of the sequence ladder reflects this with adenine adducts also marked. One experiment typical of three is shown.

Inhibition of *T4* DNA polymerase by 4-hydroxytamoxifen DNA lesions was found to be enzyme concentration dependent (fig 33). The minimum *T4* DNA polymerase concentration needed for maximal adduct detection was 4 units (fig 33 - lane 6). The lowest polymerase concentration used (1 unit) produced few extension products with no full length extension of the adducted template being achieved. The intensity of the pause sites seen for 2 and 3 units (fig 33, lanes 3 & 4) of polymerase was identical with the adduction pause sites remaining constant between 4 and 10 units. Increasing the concentration of *T4* DNA polymerase did not shift the pattern of adduct related pause sites or reveal additional adducted bases.

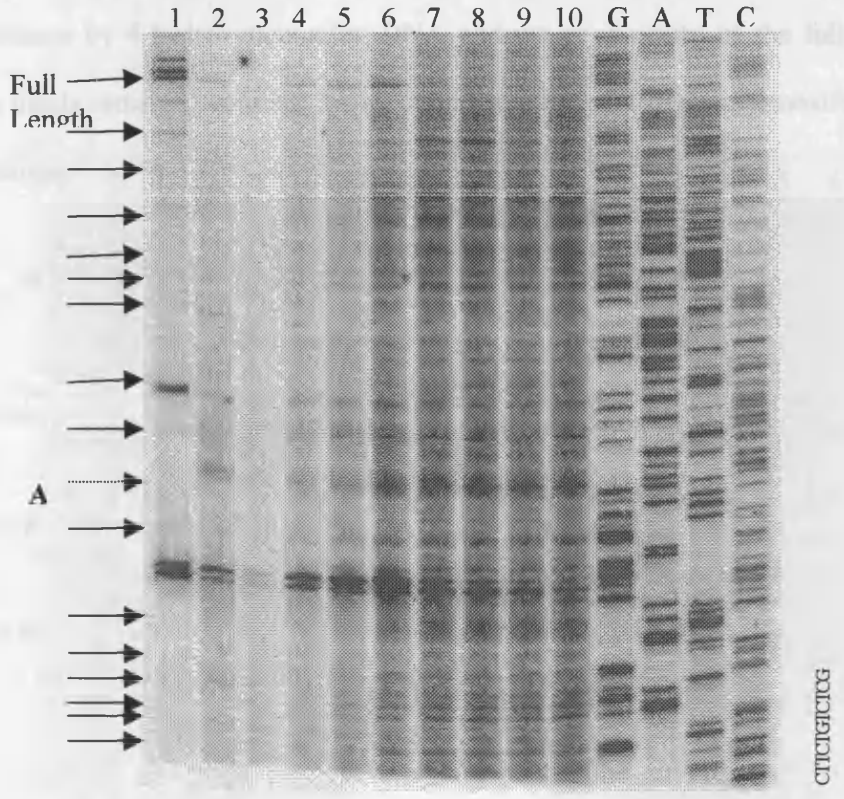


Fig 33 - Analysis of adduct sites on pGEMT-mdr1b clone after adduct formation *in vitro* with 4-hydroxytamoxifen by titrating the concentration of *T4* DNA polymerase. Titration of *T4* DNA polymerase with 160μM 4-hydroxytamoxifen adducted DNA. 1 – control/ H_2O_2 (2units), 2 - control H_2O_2/HRP (2units), 3 - 1 unit, 4 - 2units, 5 - 3units, 6 - 4units, 7 - 5units, 8 - 6units, 9 - 8units, 10 - 10units. The pause sites correspond to sites of inhibition of T4 DNA polymerase. The labelling of the sequence ladder reflects this. Efficient 4-hydroxytamoxifen adduct detection occurred using 4 units T4 DNA polymerase. Arrows indicate representative polymerase pause sites. One experiment typical of three is shown.

3.6.2b HIV I reverse transcriptase

HIV I (Human Immunodeficiency Virus I) reverse transcriptase is a RNA dependent DNA polymerase that can use DNA as a template under certain conditions. *HIV I* reverse transcriptase is a complex of a 66kD and 51kD subunits each containing a polymerase catalytic funtion. The 51kD subunit is a proteolytic product of the 66kD protein, devoid of RNase H activity but combines with the 66kD subunit to form the active polymerase (120 – 130kD). Results indicate that with a single read through of the adducted template, few pause sites were generated by the 4-hydroxytamoxifen lesions (fig 34). This indicates that the polymerase is of low sensitivity to inhibition of DNA synthesis by 4-hydroxytamoxifen DNA adducts as intensity of the full length extension bands remained constant for all concentrations of 4-hydroxytamoxifen used in the reactions.

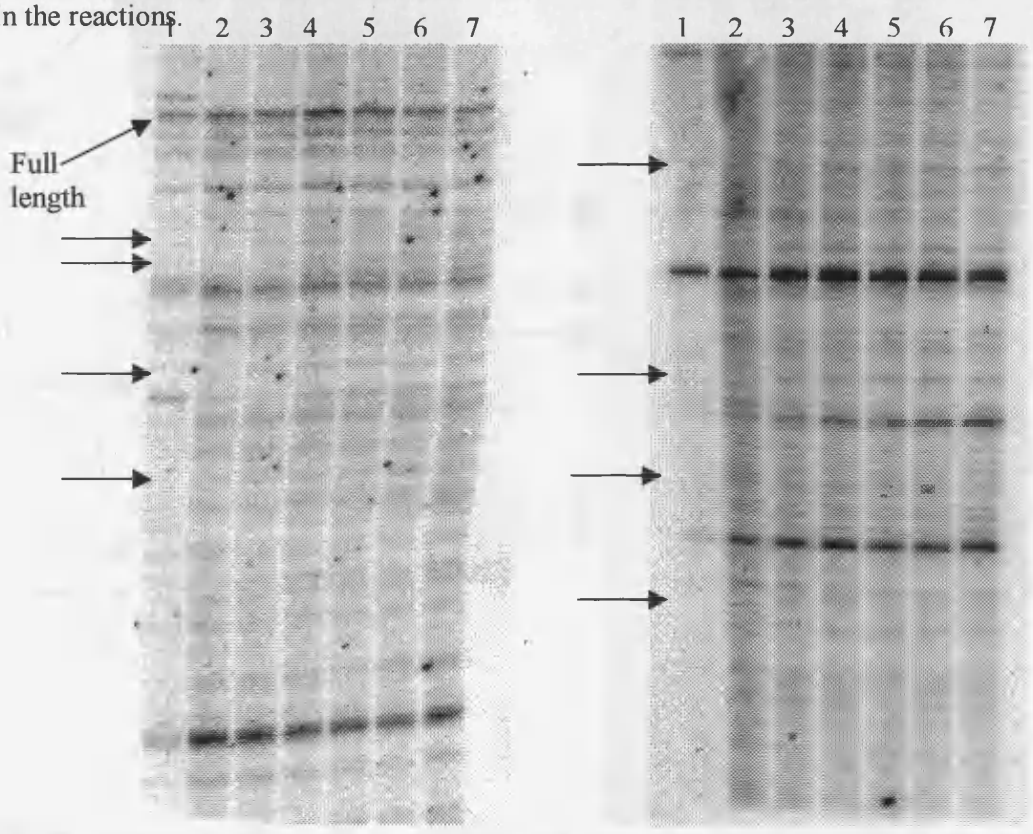


Fig 34 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *HIV I* reverse transcriptase. 1 – Control/ H_2O_2, 2 – Control H_2O_2/HRP, 3 – 25μM, 4 – 50μM, 5 – 100μM, 6 – 160μM, 7 – 250μM 4-hydroxytamoxifen. Arrows indicate pause sites in treated samples. One experiment typical of three is shown.

3.6.2c AMV reverse transcriptase

AMV (Avian Myeloblastosis Virus) reverse transcriptase is a DNA polymerase that uses DNA, RNA or DNA/RNA hybrids as templates for DNA synthesis. This polymerase consists of two polypeptide chains that have both a polymerase activity and powerful intrinsic ribonuclease H activity. *AMV* Reverse transcriptase in the polymerase arrest assay with a single read through of the 4-hydroxytamoxifen adducted DNA, showed that this enzyme was also of low sensitivity to inhibition of DNA synthesis by these DNA adducts (fig 35). The intensity of the bands indicating full length extension of the adducted template again remained constant indicating that this polymerase allows substantial bypass synthesis over the lesions.

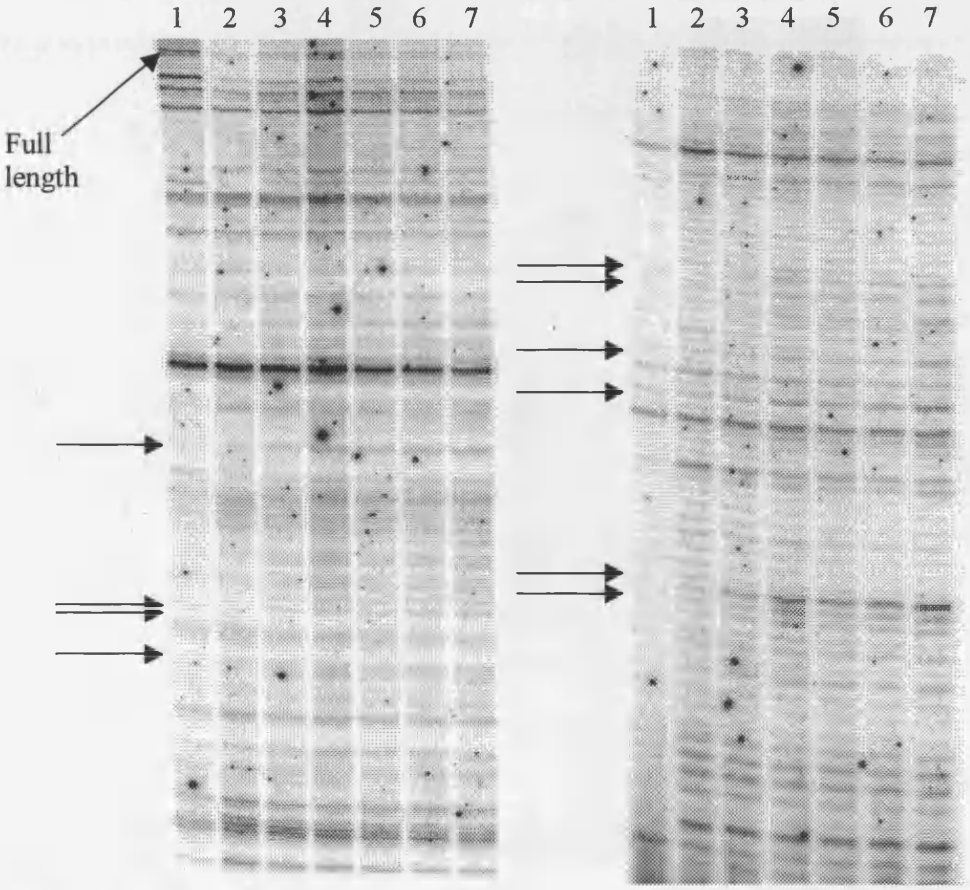


Fig 35 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *AMV* reverse transcriptase. 1 – Control/ H_2O_2, 2 – Control H_2O_2/HRP, 3 – 25μM, 4 – 50μM, 5 – 100μM, 6 – 160μM, 7 – 250μM 4-hydroxytamoxifen. Arrows indicate pause sites in treated samples. One experiment typical of three is shown.

3.6.2d Taq DNA polymerase

Taq DNA polymerase (95kD) consists of a single polypeptide chain that is a highly processive 5' – 3' DNA polymerase lacking any 3' – 5' exonuclease activity (proof reading function). Under optimal conditions *Taq* DNA polymerase produced no pause sites indicative of DNA adduct formation (fig 36). Bypass synthesis over the adducts occurred as full length extension of the adducted template was achieved at the same intensity as control samples. The adducts produced by 4-hydroxytamoxifen are thermally stable and therefore, thermal cycling of the linear PCR would not produce depurination of the adducts. *Taq* DNA polymerase is therefore insensitive under these conditions to inhibition of DNA synthesis by 4-hydroxytamoxifen DNA adducts.

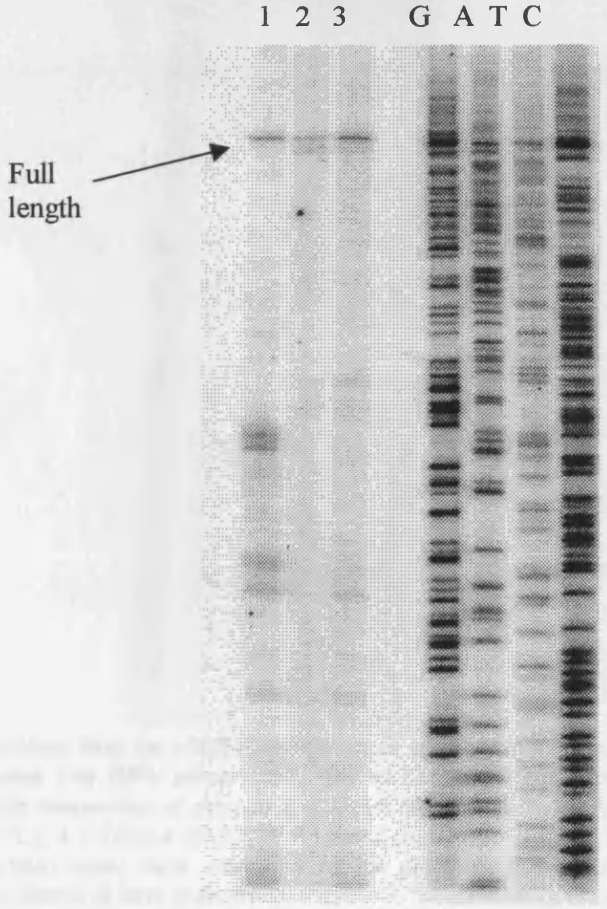


Fig 36 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Taq* DNA polymerase. 1- control/ H_2O_2, 2 - H_2O_2/HRP control, 3 - 160 μM 4-hydroxytamoxifen. *Taq* DNA polmerase was not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of three is shown.

Primer extension conditions were made less favorable by reducing the elongation temperature from 72°C to 65°C, 60°C, and 55°C. Under these suboptimal conditions, pause sites were not detected and bypass synthesis over the 4-hydroxytamoxifen DNA adducts occurred as full length extension product from adducted templates was observed at all temperatures of elongation (fig 37).

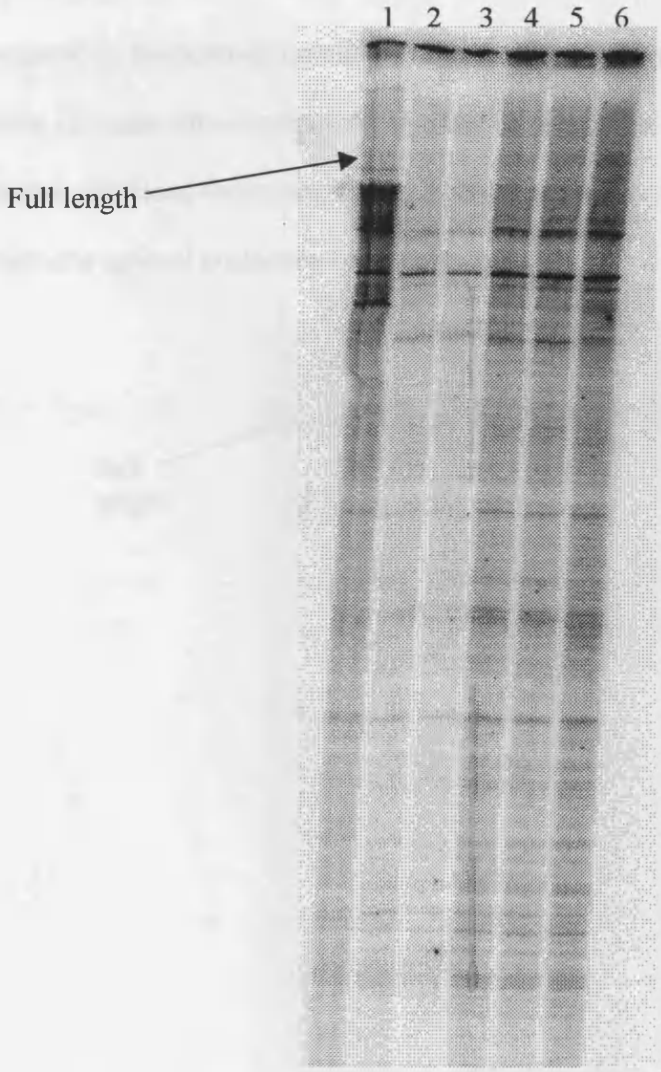


Fig 37 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Taq* DNA polymerase. Primer elongation conditions were made less favourable by reducing the temperature of elongation. Control/ H_2O_2, 2- H_2O_2/HRP control, 3 - 160μM 4-hydroxytamoxifen (72°C), 4 - 160μM (65°C), 5 - 160μM (60°C), 6 - 160μM (55°C). *Taq* DNA polmerase was not inhibited under these conditions by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of three is shown.

The nucleotide concentration in the primer extension reactions was reduced from 250μM to 25μM. Limiting the amount of nucleotide did not enhance 4-hydroxytamoxifen DNA adduct detection as pause sites indicative of adduct formation were not detected (fig 38). However, full length extension of the template was vastly reduced but equal in intensity at the lower nucleotide concentration of 25μM as compared to the optimal nucleotide concentration of 250μM, and may be due to the depletion of nucleotides limiting the number of extensions of linear PCR. *Taq* DNA polymerase produced fewer natural pause sites at this lower nucleotide concentration as seen with the optimal nucleotide concentration.

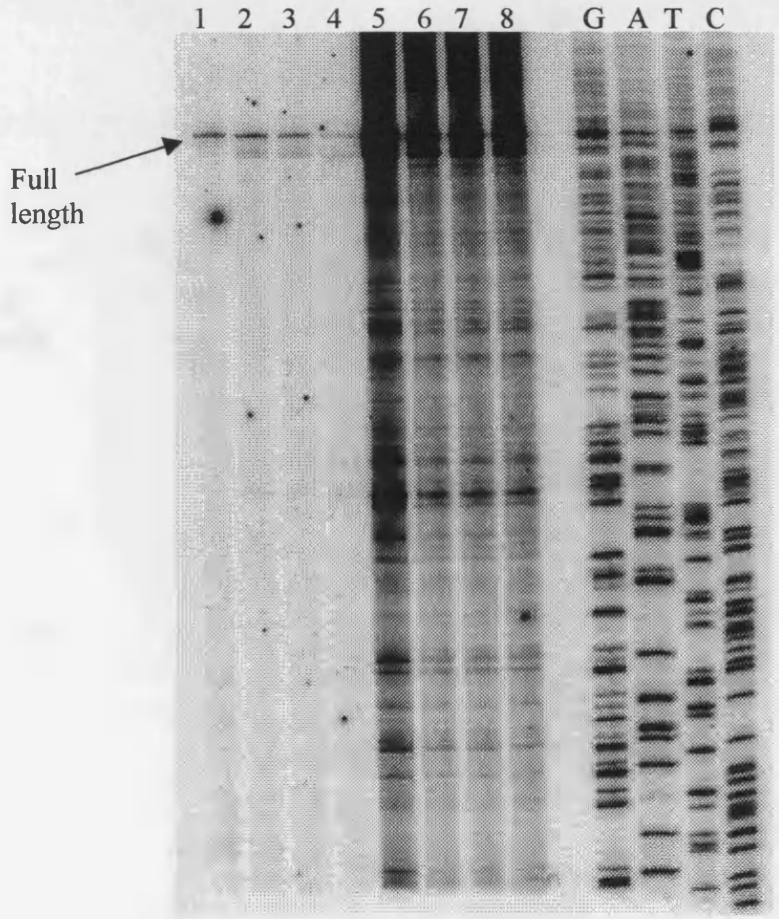


Fig 38 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Taq* DNA polymerase. The nucleotide concentration was reduced from 250μM to 25 μM in the primer extension reactions. Lanes 1 – 4 25μM Lanes 5 – 8 250μM . Lanes 1 and 4 – control/ H_2O_2, lane 2 and 6 – H_2O_2/HRP, 3 and 7 – 160μM 4-hydroxytamoxifen, 4 and 8 – 250μM 4-hydroxytamoxifen. No pause sites corresponded to sites of inhibition of *Taq* DNA polymerase using the reduced concentration of nucleotides in the arrest assay. One experiment of three is shown.

3.6.2e Klenow exo$^+$/exo$^-$ DNA polymerase

Klenow DNA polymerase is the large fragment of *E. coli* DNA polymerase I that lacks 5' – 3' endonuclease activity. *Klenow* DNA polymerase exists with either 3' - 5' exonuclease activity (exo$^+$) or contains a mutation which abolishes the 3' – 5' exonuclease activity (exo$^-$). Experiments using both forms of this polymerase under optimal conditions showed that *Klenow exo$^+$* and *exo$^-$* DNA polymerase were not sensitive to inhibition of polymerisation from lesions produced by 4-hydroxytamoxifen (fig 39). Bypass synthesis also occurred over the adducts as complete extension of the template was achieved

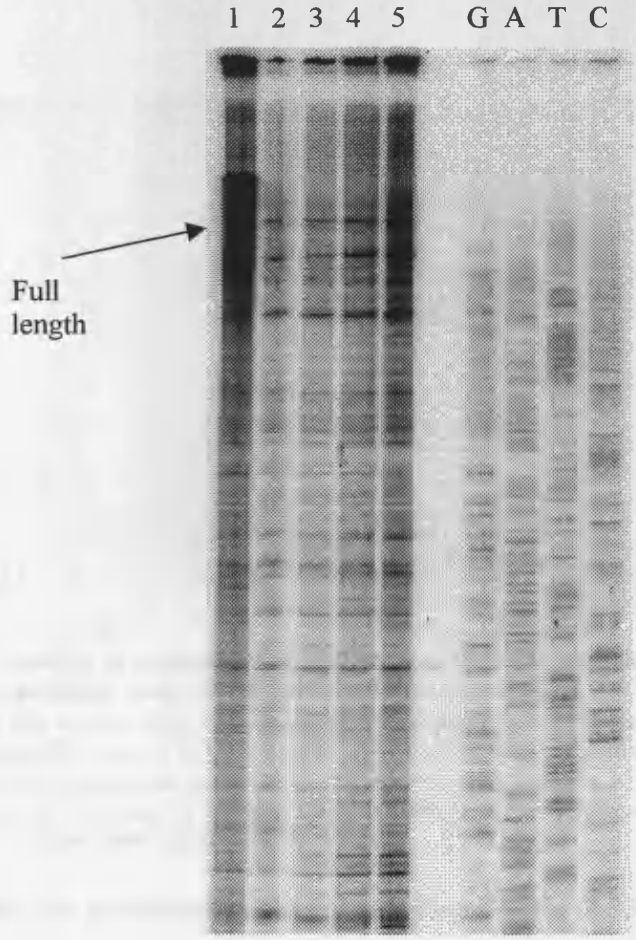


Fig 39 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Klenow* DNA polymerase *exo$^+$* and *exo$^-$*. 1 – Control/ H_2O_2 (*exo$^+$*), 2 - Control H_2O_2/HRP (*exo$^+$*), 3 - Control H_2O_2/HRP (*exo$^-$*), 4 – 160μM 4-hydroxytamoxifen (*exo$^+$*), 5 – 160μM 4-hydroxytamoxifen (*exo$^-$*). Both *Klenow exo$^+$* and *exo$^-$* DNA polymerase were not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of three is shown.

Primer elongation conditions were made less favourable by reducing the temperature of polymerase elongation from 37°C to 25°C (fig 40). Results indicated that no pause sites were generated under either of these conditions, with full length extension of the adducted template being achieved.

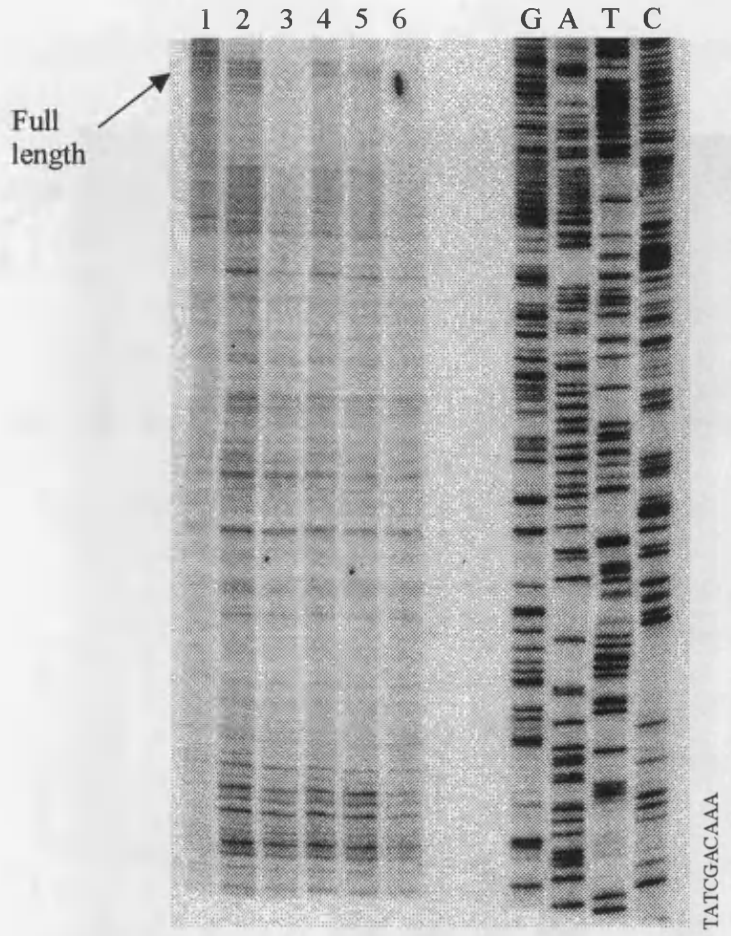


Fig 40 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Klenow* DNA polymerase *exo*$^+$ and *exo*$^-$. The temperature of primer elongation was reduced from 37 °C to 25 °C. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 160μM 4-hydroxy tamoxifen (*exo*+) 25°C, 4 - *(exo-)* 25°C, 5 – 250μM 4-hydroxytamoxifen (*exo*$^+$), 37°C, 6 – 250μM 4-hydroxytamoxifen (*exo*$^-$), 25 °C. *Klenow* DNA polymerase *exo*$^+$ and *exo*$^-$ DNA polmerase were not inhibited by reducing the temperature of elongation by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of three is shown.

The nucleotide concentration was reduced from 330μM to 33μM and primer extension repeated. Control reactions were performed at optimal nucleotide concentrations (330μM) for both forms of the DNA polymerase. The data indicates for both *Klenow*

exo$^+$ (fig 41 lane 3) and *exo*$^-$ (fig 41 lane 6) DNA polymerase that a reduction of nucleotide concentration had no effect of rendering the polymerase sensitive to inhibition of DNA synthesis by 4-hydroxytamoxifen DNA lesions. It can be concluded from these data that *Klenow* DNA polymerase is insensitive for the detection of 4-hydroxytamoxifen DNA adducts.

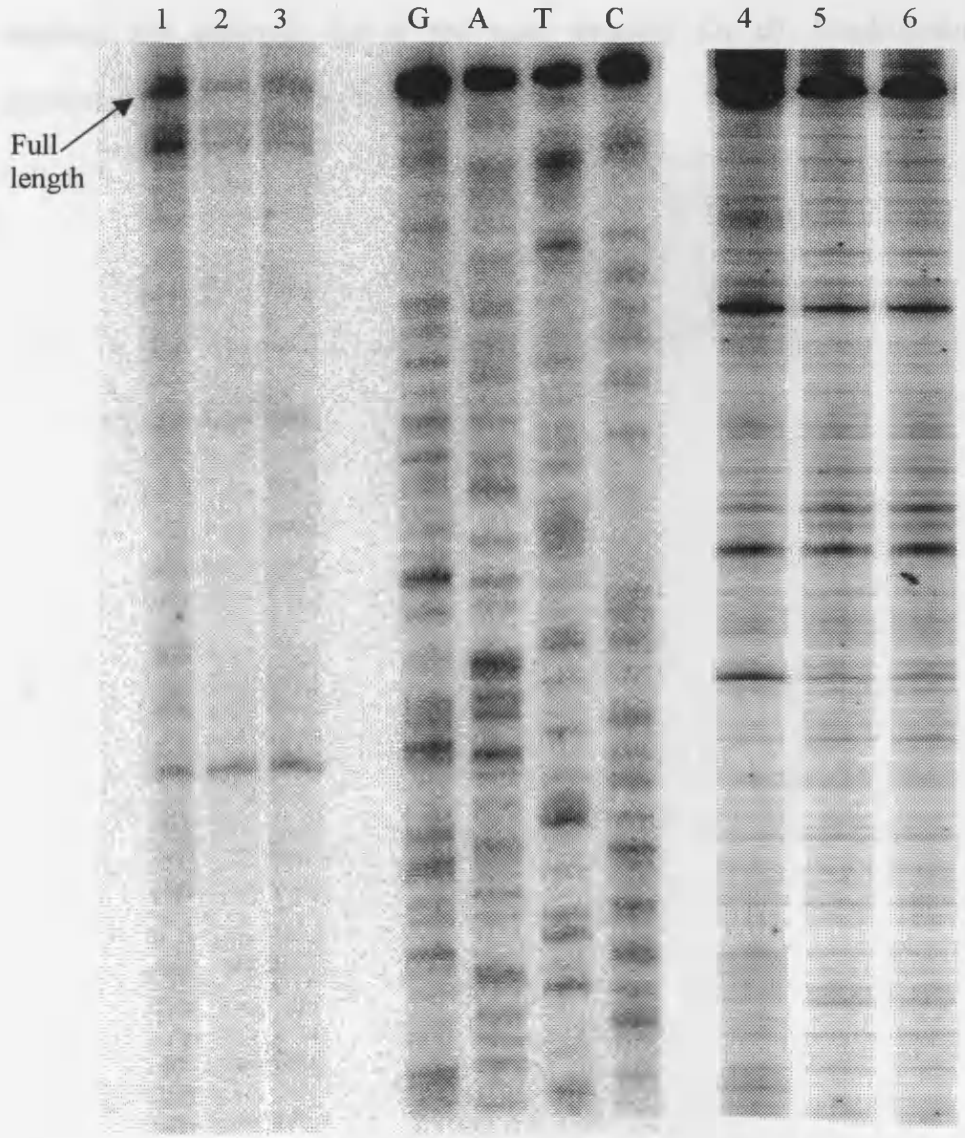


Fig 41 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Klenow* DNA polymerase. The nucleotide concentration was reduced from 330μM to 33μM in the primer extension reactions. Lames 1-3 *exo*$^+$, lanes 4-6 *exo*$^-$. Lanes 1 and 4- Control, 2 and 5- H_2O_2/HRP, lanes 3 using 330μM and lane using 33μM and 160μM 4-hydroxytamoxifen. Both *Klenow exo*$^+$ and *exo*$^-$ DNA polymerase were not inhibited by reducing the nucleotide concentration by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of three is shown.

3.6.2f Tli DNA polymerase

Tli DNA polymerase (Mr) is an extremely thermostable 5' – 3' DNA polymerase exhibiting a strong 3' – 5' exonuclease function. The polymerase arrest assay was performed using thermal cycling and data obtained indicated that this polymerase is insensitive to 4-hydroxytamoxifen lesions under optimal elongation conditions (fig 42). Bypass synthesis over the adducts occurred as full length extension of the adducted template was achieved, but at the same intensity for all 4-hydroxytamoxifen concentrations used in the assay.

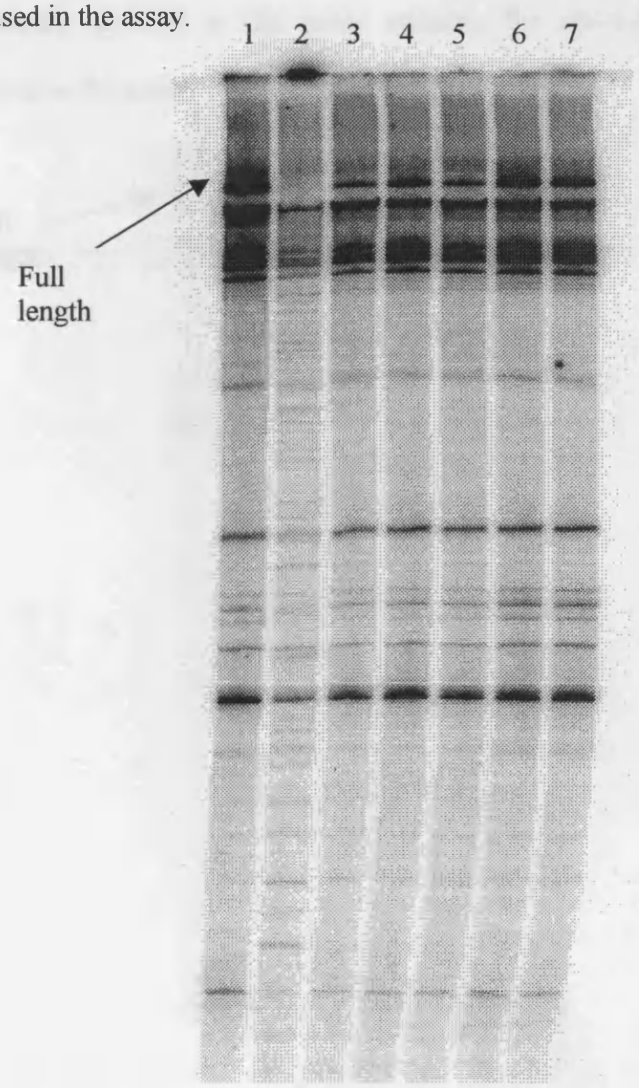


Fig 42 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Tli* DNA polymerase. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 25μM 4-hydroxytamoxifen, 4 - 50μM, 5 - 100μM, 6 - 160μM, 7 - 250μM. Tli DNA polymerase is not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of two is shown.

3.6.2g Tth DNA polymerase

Tth DNA polymerase (90kD) is a highly processive thermostable 5' – 3' DNA polymerase which lacks 3' – 5' exonuclease activity. *Tth* DNA polymerase has a very efficient intrinsic reverse transcriptase activity only in the presence of manganese ions. Results using thermal cycling for the polymerase arrest assay showed that under optimal enzyme conditions *Tth* DNA polymerase was insensitive to inhibition by 4-hydroxytamoxifen adducts on template DNA (fig 43). This polymerase synthesised full length extension product at the same intensity for all 4-hydroxytamoxifen concentrations used in the assay.

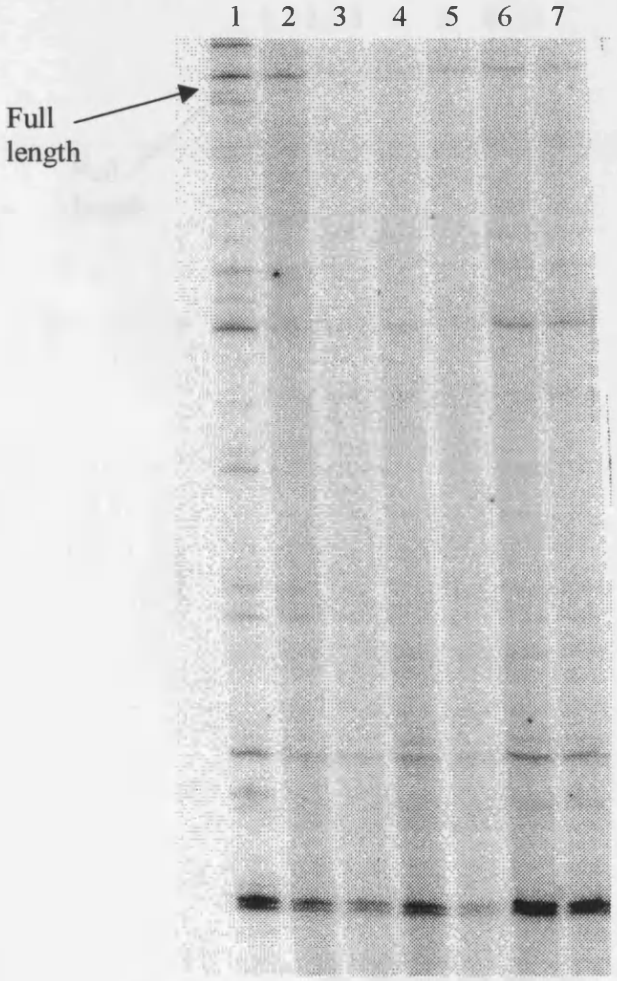


Fig 43 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Tth* DNA polymerase. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 25μM 4-hydroxytamoxifen, 4 - 50μM, 5 - 100μM, 6 - 160μM, 7 - 250μM. *Tth* DNA polymerase is not inhibited by the presence of 4-hydroxytamoixfen DNA adducts. One experimeny typical of two is shown.

3.2.6h SP6 and T7 RNA Polymerase

SP6 and T7 RNA polymerase are DNA dependent RNA polymerases, that are strictly specific for their respective phage promoters. These promoters are found in many standard cloning vectors and direct RNA polymerisation downstream of the specific promoter. Results using SP6 RNA polymerase indicated that under optimal conditions, RNA synthesis was not inhibited by the presence of 4-hydroxytamoxifen DNA adducts as no pause sites were produced (fig 44). Translational bypass over the adducts occurred as full length extension of the 4-hydroxytamoxifen adducted template was achieved.

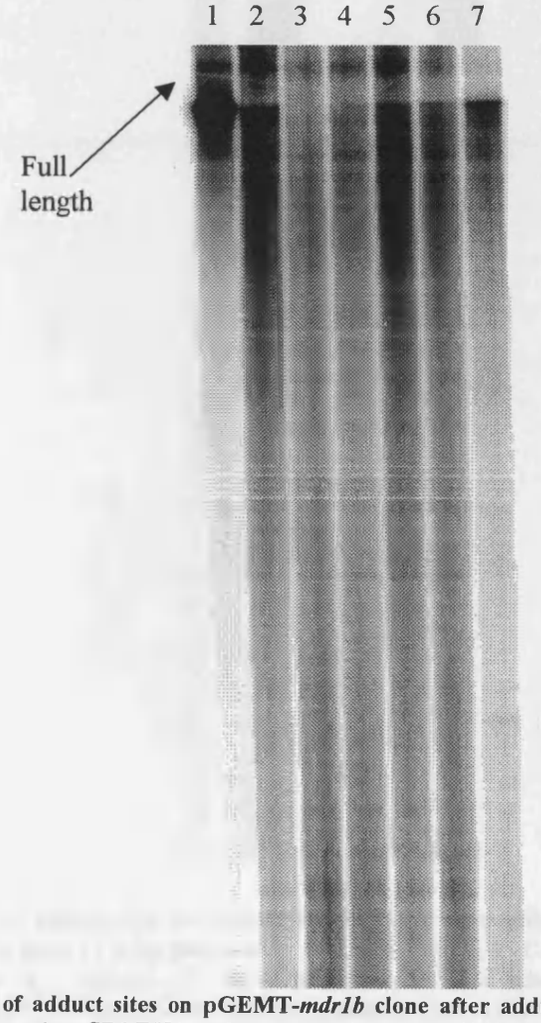


Fig 44 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *SP6* RNA polymerase. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 25μM 4-hydroxytamoxifen, 4 - 50μM, 5 - 100μM, 6 - 160μM, 7 - 250μM. *SP6* RNA polymerase was not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment of three.

T7 RNA polymerase gave similar results, with no inhibition of transcription from the 4-hydroxytamoxifen adducted DNA template (fig 45). Again translational bypass synthesis occurred over the adducts because full length extension of the adducted template was achieved.

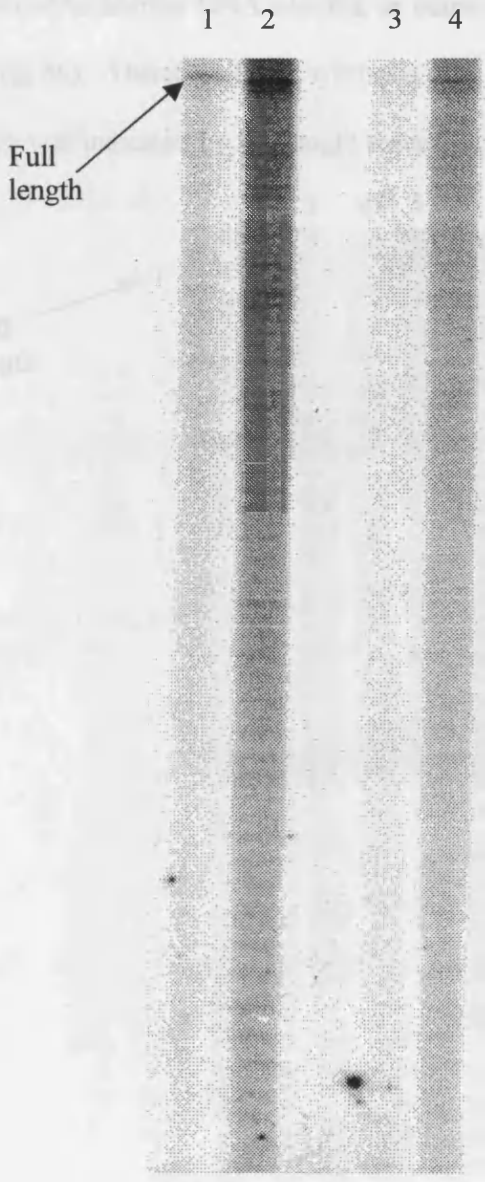


Fig 45 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *T7* RNA polymerase. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 160μM 4-hydroxytamoxifen, 4 - 250μM. T7 RNA polymerase is not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment typical of three is shown.

3.2.6i Pwo DNA polymerase

Pwo DNA polymerase (90kD) is an extremely thermostable enzyme. This enzyme is a highly processive 5' – 3' DNA polymerase and with 3' – 5' exonuclease activity. Under optimal conditions, Pwo DNA polymerase was insensitive to inhibition of DNA synthesis by 4-hydroxytamoxifen DNA adducts, as pause sites caused by these lesions are not detected (fig 46). Therefore DNA synthesis over 4-hydroxytamoxifen adducts had occurred which was indicated by full length extension of the template.

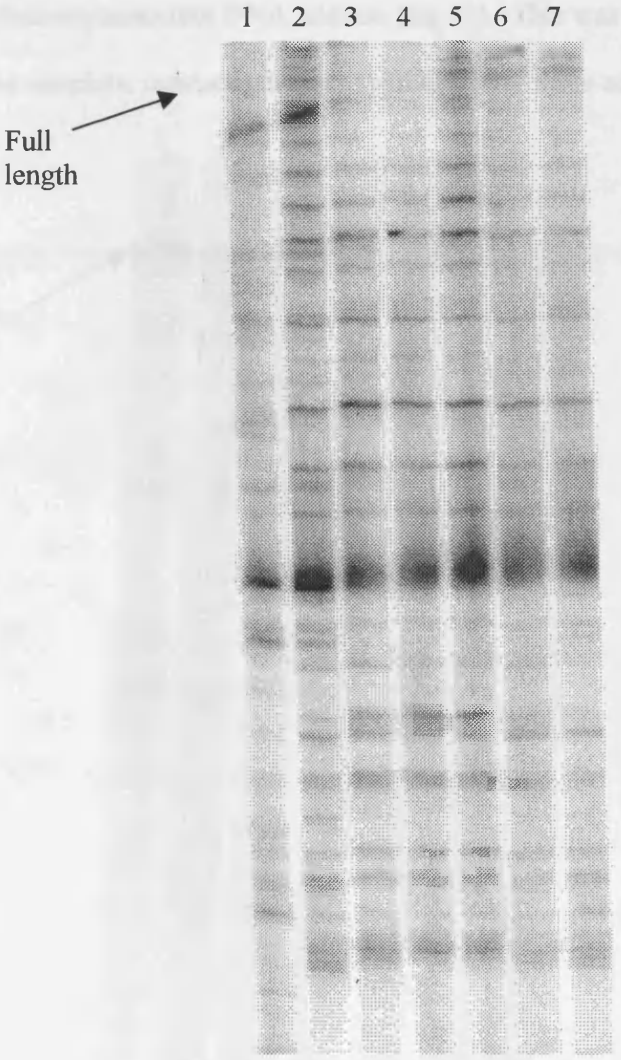


Fig 46 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *Pwo* DNA polymerase. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 25μM 4-hydroxytamoxifen, 4 - 50μM, 5 - 100μM, 6 - 160μM, 7 - 250μM. Pwo DNA polymerase was not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment of two is shown.

3.2.6j T7 DNA polymerase

T7 DNA polymerase is a bacteriophage DNA polymerase that catalyses the replication of T7 phage during infection. T7 DNA polymerase consists of two subunits, T7 gene 5 protein (84kD) and *E.coli* thioredoxin (12kD). This polymerase is the most processive of all DNA polymerases and contains a potent 3' - 5' exonuclease function. Single read through of the polymerase in the arrest assay indicated that this polymerase under optimal elongation conditions was insensitive to inhibition of DNA synthesis by the presence of 4-hydroxytamoxifen DNA adducts (fig 47). This was shown by full length extension of the template, indicating bypass synthesis over these adducts.

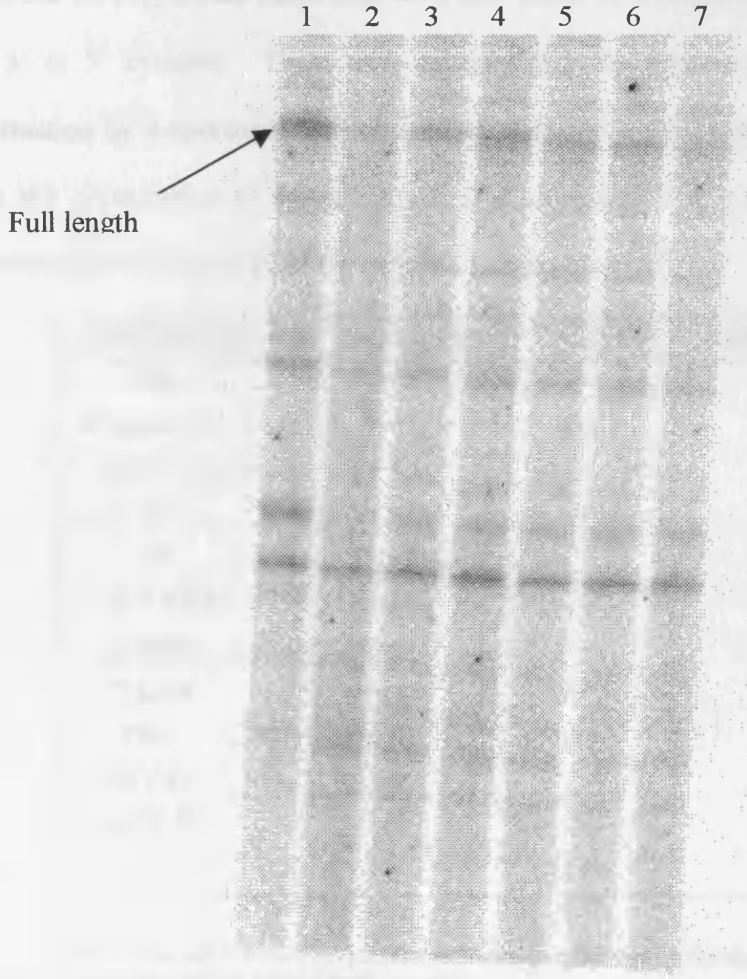


Fig 47 - Analysis of adduct sites on pGEMT-*mdr1b* clone after adduct formation *in vitro* with 4-hydroxytamoxifen using *T7* DNA polymerase. 1 – Control/ H_2O_2, 2 - Control/H_2O_2/HRP, 3 - 25μM 4-hydroxytamoxifen, 4 - 50μM, 5 - 100μM, 6 - 160μM, 7 - 250μM. *T7* DNA polymerase was not inhibited by the presence of 4-hydroxytamoxifen DNA adducts. One experiment of two is shown.

3.3 Summary

In summary relatively few polymerases were sensitive to inhibition by the presence of 4-hydroxytamoxifen DNA adducts on plasmid DNA. The exception was *T4* DNA polymerase which was strongly inhibited by the presence of these adducts, in a manner which was enzyme concentration dependent. *AMV* and *HIV I* reverse transcriptase were weakly inhibited by the presence of 4-hydroxytamoxifen DNA adducts. *T4* DNA polymerase pause sites occurred at the majority of guanines with slightly stronger pauses when the reactive guanine was located next to a 3' or 5' adenine or cytosine. Several *T4* polymerase pause sites were also observed at adenine when located next to a 3' or 5' cytosine. These data indicates that the identification of DNA adduct formation by 4-hydroxytamoxifen quinone methide is polymerase specific dependent on the organisation of the active site and associated functions of the polymerase, determines the sensitivity of the enzymes to inhibition (table 3).

Polymerase	*Processivity*	*Fidelity*	*Adduct detection*
Taq	++++	-	-
Klenow (exo$^+$)	++	+++	-
Klenow (exo$^-$)	++	-	-
Tli	++	+++	-
Tth	++++	-	-
SP6 RNA	++++	-	-
T7 RNA	++++	-	-
T7 DNA	+++++	+++++	-
Pwo	++++	++++	-
HIV I RT	++	-	+
AMV RT	++	-	++
T4	++	++++	+++++

Table 3- Characteristics of the polymerases used in this study for the detection of 4-hydroxytamoxifen DNA adduct formation

3.4 Discussion

Polymerases from different families share fundamental similarities in their tertiary structure and catalytic mechanisms (Arnold *et al.*, 1995; Joyce & Steitz, 1995; Sousa, 1996; Brautigam & Steitz, 1998). The majority of the amino acids that form the surface of the polymerase cleft are highly conserved with their respective families (Minnick *et al.*, 1999). Polymerases behave differently with respect to their kinetics and thermodynamic stability during replication of DNA adducts (Plum & Breslauer, 1994; Pilch *et al.*, 1995; Cai *et al.*, 1993; Lowe and Guengerich., 1996; Reha-Krantz *et al.*, 1996; Frey *et al.*, 1995). Kinetic effects if combined with the other properties of the polymerase, such as the presence and activity of a proof-reading function (3'-5' exonuclease) or processivity (Strauss, 1985; Clark & Beardsley, 1989; Shibutani *et al.*, 1991) are important contributing factors for the ability of a polymerase to bypass a lesion. In general, a polymerase with high processivity enhances adduct bypass, whereas an active 3'-5' exonuclease impedes lesion bypass (Shibutani *et al.*, 1997; Paz-Elizar *et al.*, 1996 & 1997; Mozzherin *et al.*, 1997; Rajagopalan *et al.*, 1992). Most bulky DNA adducts can potentially inhibit polynucleotide biosynthesis *in vitro* (Dzantiev & Romano, 1999). Therefore in this study DNA and RNA polymerases were chosen with different properties, which vary in their proof-reading activity, processivity, and thermostability. The aim of this study was to identify plasmid DNA bases adducted with 4-hydroxytamoxifen by the polymerase arrest assay.

3.4.1 Polymerase arrest assays

Plasmid DNA, when treated treated with activated 4-hydroxytamoxifen, produced DNA adducts that were separated by TLC (fig 31). DNA treated with 160μM 4-hydroxytamoxifen, produced a significantly large number of adducts as assessed by ^{32}P-postlabelling. 4-Hydroxytamoxifen adducts are thermostable and therefore, do not depurinate at high temperatures. This property of the adduct is advantageous as thermostable polymerases can be exploited in the arrest assay, using thermocycling conditions. *Taq* DNA polymerase is a processive enzyme, but lacks a proof-reading function. Results from this study using *Taq* DNA polymerase, under optimal conditions, identified no sites of polymerase pausing (fig 36). *Taq* DNA polymerase is blocked by many bulky DNA adducts such as aflatoxin B_1 and benzo[a]pyrene (Benasutti *et al.*, 1988; Comess *et al.*, 1992; Thrall *et al.*, 1992). However, this polymerase is known to bypass some forms of DNA damage such as *cis*-syn thymine dimers, 6-4 photoproducts (Wellinger & Thoma, 1996) and 7,8-dihydro-8-oxoadenine (Guschlbauer *et al.*, 1991). Altering the rate of reaction by decreasing the elongation temperature, or reducing the nucleotide concentration, had no effect on increasing the sensitivity of *Taq* DNA polymerase to 4-hydroxytamoxifen adducts. A reduction of the nucleotide concentration is known to increase the sensitivity to some bulky DNA adducts by *Vent exo*$^-$ DNA polymerase (Smith *et al.*, 1998).

The thermostable DNA polymerases, *Pwo*, *Tli*, and *Tth* were also tested in the assay under optimal reaction conditions. *Pwo* and *Tli* DNA polymerase possess proof-reading functions (*exo*$^+$), with *Pwo* being a processive polymerase. However, *Tth* is a processive polymerase but lacks a proof-reading function (*exo*$^-$). The results showed no pause sites were produced by the presence of 4-hydroxytamoxifen adducts with either

enzyme. This shows that the exonuclease functions and processivities associated with these thermostable polymerases did not influence elongation past 4-hydroxytamoxifen lesions.

The *Klenow* fragment of DNA polymerase I was also assessed in the arrest assay. *Klenow* DNA polymerase has two forms: exonuclease positive (exo^+) and exonuclease deficient (exo^-). Therefore, the effects of proof-reading on the ability of the polymerase to bypass 4-hydroxytamoxifen adducts could be determined. Under optimal conditions for both forms of the DNA polymerase no pause sites indicative to adduct formation were generated (fig 39). Lowering the elongation temperature or the nucleotide concentration did not increase the sensitivity of this polymerase to inhibition by 4-hydroxytamoxifen adducts. Studies have shown that *Klenow* exo^+ can bypass a variety of bulky lesions including those formed from 2-aminofluorene (Michaels *et al.*, 1987) and 2-acetylaminofluorene (Shibutani *et al.*, 1993a), oestrogens (Shibutani *et al.*, 1993b), styrene oxide (Latham *et al.*, 1995), and some isomers of benzo[a]pyrene diolepoxide. This enzyme shows the effects of proof-reading on bypass of thymine dimers. It has been shown that exo^- extended one base further than exo^+ which detected 50% fewer adducts than the exo^- (Smith *et al.*, 1998). *Klenow* exo^- can also synthesise beyond lesions, caused by ionising radiation and AP sites, whereas exo^+ is inhibited (Sagher *et al.*, 1994). From these results, it can be established that adducts formed from 4-hydroxytamoxifen are readily bypassed by both forms of *Klenow* DNA polymerase. Bypass over 4-hydroxytamoxifen lesions is therefore, not affected the 3'-5' exonuclease activity of the *Klenow* fragment of DNA polymerase I.

T7 DNA polymerase was insensitive to inhibition of elongation on 4-hydroxy tamoxifen adducted plasmid DNA. This polymerase has been reported to bypass a wide variety of bulky DNA adducts including intra-strand cross links formed from *cis*-diaminodichloro platinum II (Michaels *et al.*, 1987; Lindsley & Fuchs, 1994), actinomycin D, 2-aminofluorene, 7-bromoethylbenz[a]anthracene (Hruszkewycz *et al.*, 1992), and styrene oxide. However a 3'-5' exonuclease deficient form of *T7* DNA polymerase does not bypass 2-acetylaminofluorene, benz[a]pyrene diolepoxide adducts (Strauss & Wang, 1990, Lindsley & Fuchs, 1994), and thymine dimers. *T7* DNA polymerase is otherwise a highly processive polymerase due to its interactions with *E.coli* thioredoxin. Thioredoxin acts as an accessory protein and clamps the polymerase to the DNA template and is unable to bypass a variety of lesions in the absence of its processivity factor. Moreover, in the presence of proliferating cell nuclear antigen (PCNA), DNA polymerase δ is known to become less sensitive to abasic sites (Mozzherin *et al.*, 1997). *T7* DNA polymerase also has the strongest 3'-5' exonuclease function of all polymerases. The very high processivity or a combination of these features could explain the mechanism of bypass synthesis over many bulky DNA adducts.

In this study, *SP6* and *T7* RNA polymerase were the only two RNA polymerases used to assess 4-hydroxytamoxifen adduct formation. Both of these polymerases bypassed the 4-hydroxytamoxifen lesions. Adduct detection studies published to date, have not included *SP6* RNA polymerase, but it is known that *T7* RNA polymerase is relatively insensitive to inhibition of RNA synthesis by most bulky DNA adducts. Lesions formed by 2-amino and 2-acetylaminofluorene (Chen & Bogenhagen, 1993), several isomers of benzo[a]pyrene diolepoxide (Choi *et al.*, 1994), and pyrrolo[2,1-

c][1,4]benzodiazepines (Puvvada *et al.*, 1997) are known to block transcription. Many of these assays have reported that some DNA adducts stimulate rather than inhibit transcription (Puvvada *et al.*, 1997).

AMV and *HIV I* reverse transcriptase are mammalian viral polymerases that transcribe RNA into dsDNA. These polymerases may also utilise DNA as a template under certain conditions and both are 3'-5' exonuclease deficient (Goodman *et al.*, 1993). However, these polymerases had low sensitivity to inhibition of elongation on 4-hydroxytamoxifen adducted DNA. The relative intensity of the pauses were low with no reduction in intensity of the full length elongation product being observed. This indicates that there is a substantial amount of bypass over the 4-hydroxytamoxifen lesions. Few adduct studies have been performed using *AMV* reverse transcriptase, but it is found to bypass *cis*-thymine glycol adducts (Clark & Beardsley, 1989), and abasic sites (Takeshita *et al.*, 1987). *HIV I* RT is blocked by all but one of the six stereoisomers of benzo[a]pyrene diolepoxide adducts, actinomycin D (Dzantiev & Romano., 1999), and abasic sites.

Results from the polymerase arrest assay show that for 4-hydroxytamoxifen, *T4* DNA polymerase was strongly inhibited by the presence of these adducts under normal single pass conditions (fig 32). Therefore, this polymerase was chosen to determine the sequence specific formation of 4-hydroxytamoxifen adducts on plasmid DNA. The major site of 4-hydroxytamoxifen adduct formation was found to be on guanine with a high percentage of *T4* DNA polymerase showing arrest at guanine (fig 32). This correlates with studies that show guanine to be the main site of tamoxifen adduct formation (Osbourne *et al.*, 1996). Relatively few pause sites correlated to adenine,

which has already been identified as a minor adduct formed by the reaction of DNA with α-acetoxy tamoxifen (Osbourne *et al.*, 1997). Even though a high percentage of dG was adducted, the majority of reactive guanines were located 3' or 5' to dA or dC, with the intensity of arrest sites in other sequences being as above. The two guanines adducted in the sequence AG*TG*TGT gave an intense pause sites. This indicates that this sequence may be a preferred sequence 'hot spot' for 4-hydroxytamoxifen. The third dG in this sequence was as not heavily adducted as the previous guanines, which may indicate sequence specific modulations in the duplex DNA caused by the presence of the tamoxifen moiety. This may have inhibited adduct formation or influenced the ability of this polymerase to bypass the adduct in this sequence by altering the conformation of the lesion within the duplex. At the concentration of 250μM 4-hydroxytamoxifen, used in the adduction reaction, a substantial decline in the intensity of the full length extension of the primer was observed. This polymerase may not be efficient at detecting all DNA adducts in all sequences.

Detection of 4-hydroxytamoxifen DNA adducts by *T4* DNA polymerase was found to be enzyme concentration dependent. The minimum amount of polymerase required to efficiently detect these adducts was 4 units (58nM). At certain concentrations of polymerase and in the absence of DNA template it is known that the *T4* DNA polymerase molecules can interact with each other to form dimeric complexes in solution (Alberts *et al.*, 1982). This enzyme is found to also undergo dimerisation at the template/primer junction over a range of polymerase concentrations (27nM to 740nM), where the first enzyme molecule binds directly to the single strand-double strand junction, and the second binds to the single stranded portion of the template or is bound to the first polymerase (Munn and Alberts., 1991; Alberts *et al.*, 1975). At

58nM polymerase, a dimer complex will form and this seems to be an important factor in 4-hydroxytamoxifen DNA adduct detection. Other polymerases known to couple together forming dimers is the DNA polymerase III holoenzyme from *E. coli* (Kim & McHenry, 1996; Marians *et al.*, 1998). At the replication fork and in solution, *T7* DNA polymerase also dimerises (Lee *et al.*, 1998; Park *et al.*, 1998; Debyser *et al.*, 1994).

3.4.2 Mechanisms of 4-Hydroxytamoxifen DNA Adduct Induced Polymerase Inhibition

A basic mechanism of polymerisation of nucleotides into polynucleotides is defined as the binding of a polymerase to its respective nucleic acid template substrate. This is followed by the binding of a nucleotide and a conformational change of the polymerase to form a catalytically active complex for phosphodiester bond formation. Relaxation of this complex follows bond formation, allowing release of pyrophosphate and the translocation of the polymerase to the new primer terminus (Steitz *et al.*, 1993b; Pelletier *et al.*, 1994). Recently, it has been established that the presence of a DNA adduct, modified base or duplex secondary structure represents 'unusual' replication circumstances that may have a pronounced effect on the polymerase catalytic activity and fidelity. The ability of a polymerase to synthesise beyond an adduct is dependent on the combination of stereochemical, steric hindrance effects of the adduct, kinetic factors that are governed by the individual polymerase, processivity, proof-reading, and sequence context effects (Hatahet *et al.*, 1999). Most DNA reactive chemotherapeutic anti-cancer drugs are cytotoxic because they effectively inhibit DNA and RNA synthesis by sterically hindering the polymerases in actively dividing cells (Rill & Hecker, 1996).

Lowe and Guengerich, (1999), have suggested that when the DNA adduct is located within the active site of the polymerase after nucleotide binding has occurred, the formation of an additional tertiary complex of polymerase-DNA-nucleotide may arise. This complex may be catalytically incompetent and forms a non productive side pool of inactive polymerase. Depending on the individual kinetics of the polymerase, the inactive complex may be capable of overcoming this incompetency as to allow phosphodiester bond formation or move back to the ground state of polymerase-DNA-nucleotide. In addition incompetent tertiary complexes may form during the nucleotide binding step.

Alterations in tertiary structure are thought to be responsible for the production of catalytically incompetent complexes. The structure of the adduct moiety within the active site may interfere with critical regions in the polymerase. This may be due to new hydrogen bond formation or interference of existing hydrogen bonding as well as other interactions with the polymerase (Makay *et al.*, 1994; Hruszkewycz *et al.*, 1992; Rodriguez *et al.*, 1993; Shibutani *et al.*, 1993a; Jelinsky *et al.*, 1995; Moriya *et al.*, 1996; Hanrahan & Loechler, 1997). 4-Hydroxytamoxifen is a large moiety that has several sites for hydrogen bond formation and other non polar interactions that may have interfered with critical sites within the active sites of *T4* DNA polymerase, *AMV* and *HIV I* reverse transcriptase. After nucleotide binding and polymerase cleft closure, the bound nucleotide is delivered to the active site and aligned for nucleophilic attack by the 3'-hydroxyl group of the primer (Dzantiev & Romano, 1999). All polymerases are thought to reach a fully active catalytic configuration if the incoming nucleotide can adopt ideal Watson-Crick geometry within the active site. Therefore, the presence of an 4-hydroxytamoxifen DNA adduct within the active site of *T4* DNA polymerase, *HIV*

I and *AMV* reverse transcriptase upon conformational change may disrupt interactions of the polymerases with the DNA template, decreasing the complex stability producing a catalytically inactive state. These effects do not occur for the majority of polymerases tested in this study. This may be due to the movement of the 4-hydroxytamoxifen moiety in the active sites of the insensitive polymerases, which upon conformational change, may accommodate for correct hydrogen bond formation with the incoming nucleotide due to differences in the structures of the active sites, therefore, overcoming a replication block. Alternately, alterations in hydrogen bond formation with the DNA template may result in stabilisation of a complex that allows phosphodiester bond formation. Moreover, the entry of the incoming nucleotide may be impeded by the presence of the 4-hydroxytamoxifen DNA adduct moiety, resulting in loss of free energy of binding (Beard *et al.*, 1996; Kraynov *et al.*, 1997), obstructing access to the Watson-Crick edge of the templating base and possibly shifting the adducted guanine base from its normal position. This may result in steric hindrance effects of the adduct or disrupt hydrogen bond formation of this base in the active sites, reducing its polymerase stability with the template.

The perturbing effects on DNA secondary structure caused by the presence of a bulky adduct may be sensed by the polymerases up to 5 or more nucleotides away from the lesion (Miller & Grollman, 1997). There is an associated decrease in the rate of polymerisation (k_{cat}), which may be intentional in order to allow for correct pairing of the incoming nucleotide, or allow for correction of mismatches by the polymerase proof-reading function (Miller & Grollman, 1997). Adducts that present alternative hydrogen bond formation or obstruct the Watson-Crick edge of the templating base may cause deviations from ideal Watson-Crick geometry within the active site. This

results in energy differences of elongation past the lesion determined by simple kinetic parameters of K_m and V_{max} for right (Watson-Crick) versus wrong (Watson-Crick) geometry and k_{cat} for nucleophilic attack at the 3' hydroxyl group of the primer that allows inactive complex formation.

Insertion and extension of a correct/incorrect nucleotide is not only governed by the polymerase, but it is heavily influenced by the local sequence context and can significantly decrease the elongation rate (Eger *et al.*, 1991; Polesky *et al.*, 1992; Moran *et al.*, 1997; Liu, 1997). Lesions that are usually blocking such as thymine glycol, abasic sites, urea and β-ureidoisobutyric acid are completely bypassed within certain sequence contexes (Hatahet *et al.*, 1999; Maccabee *et al.*, 1994; Evans *et al.*, 1993; Hayes and LeClerc, 1986; Belguise-Valladier and Fuchs, 1995). Studies have shown that the stacking interactions between a lesion are nearest neighbour dependent. Therefore, the presentation of the 4-hydroxytamoxifen lesion within certain sequences may allow correct nucleotide insertion, but not in others causing devation of the best fitting Watson-Crick geometry for either correct or incorrect nucleotide insertion, which may result in a poor extendable primer terminus (Shibutani *et al.*, 1991). Results from the arrest assay with *T4* DNA polymerase indicated that most of the reactive guanines had a 3' or 5' dA or dC and the most intense site was seen in the sequence AG*TG*TGT. This sequence may have influenced the overall Watson-Crick geometry at the sites of 4-hydroxytamoxifen adduct formation that resulted in a poorly extendable 3' terminus. There were only several sites of adenine adduct formation detected from the T4 DNA polymerase arrest assay. The stalling of the T4 DNA polymerase complex at these sites may have been dependent on the sequence context where the adducted adenine was located.

The above processes may be crucial for *AMV* and *HIV I* reverse transcriptase as they are deficient in an active 3'-5' exonuclease function. In this study *T4* DNA polymerase is devoid of its accessory protein gp45, therefore, this polymerase is not processive. Many studies have shown that *T4* DNA polymerase is dependent on its processivity factor for translesional replication past a DNA adduct (Reha-Krantz *et al.*, 1996). The 3'-5' exonuclease function of this polymerase is very active and is located on the same polypeptide chain as the polymerase activity, whereas other polymerase proofreading functions are located on separate subunits. The exonuclease function may compete for polymerisation depending on the state of the primer terminus. The decision to proof read is determined by the kinetics of elongation past a lesion causing the primer terminus to be transferred to the exonuclease active site (Baker & Reha-Krantz, 1998; Johnson, 1993; Goodman *et al.*, 1993). The proof-reading function of a *T4* DNA polymerase may have interfered by competing with the activity of polymerisition and cause inhibition of elongation and polymerase disociation.

In conclusion, the majority of polymerases used in this study were insensitive to inhibition by 4-hydroxytamoxifen DNA adducts. *AMV* and *HIV I* reverse transcriptase were of only low sensitivity compaired to *T4* DNA polymerase. This polymerase shares a high degree of homology with mammalian viral polymerases which may have contributed to their weak inhibitory effects of elongation past 4-hydroxytamoxifen lesions. The combined effects of *T4* DNA polymerase with its active proof-reading function, lack of processivity factor, and the chemical structure of the adduct may have caused sufficient kinetic changes that impede elongation past 4-hydroxytamoxifen DNA adducts.

SITE SPECIFIC TAMOXIFEN-DNA ADDUCT FORMATION ON THE pLIZ LAC I GENE WITH COMPARISON TO SITES OF MUTATION FORMED IN SCS8 *E.COLI*

4.1 Chapter Objectives

1. To determine the sites of *in vitro* adduct formation on the pLIZ *lacI* gene following its reaction with α-acetoxytamoxifen and HRP/H_2O_2 activated 4-hydroxytamoxifen using the T4 DNA polymerase arrest assay.
2. To evaluate the mutation frequency resulting from gross DNA adduct formation of both metabolites of tamoxifen in *SCS8 E.coli*.
3. To determine if a correlation exists between the sites of DNA adduct formation to the sites of mutation in the *lacI* gene.

4.2 INTRODUCTION

Metabolism of exogenous xenobiotics to multiple reactive electrophiles capable of forming DNA adducts may be associated with an elevated risk for development of mutation (Groopman & Kensler, 1993; Hemminki *et al.*, 1994; Essigmann, 1991). The mutational consequences of DNA adduct formation may be dependent up on a number of factors, including persistence, adduct structure, relative quantity of each adduct formed, and replication potential of the target cells (Essigmann & Wood, 1993; Loechler, 1996). The interpretation of adduct data should also be viewed with reference to the adduct structure and its mutagenic efficiency in relation to its repair. This is because strongly mutagenic structures that are rapidly repaired *in vivo* are of less concern than weakly mutagenic adduct structures stable to the repair processes (La & Swenberg, 1996).

The gross adduct burden formed from acute dose studies will be dependent upon the length of time between administration of a single dose and analysis of DNA (Swenberg *et al.*, 1985). Therefore, to correlate DNA adduct formation to mutation, a period of

dosing producing a steady state concentration of DNA adducts should be sought. Steady state is determined by the rate of formation, repair, cell death and dilution of the adducts by DNA replication. However, this excludes repair resistant adducts in quiescent cells (Vanhoffen *et al.*, 1995).

The observed mutations in DNA should correlate with the binding characteristics of that particular mutagen. Singer and Essigmann, (1991) proposed that the 'ultimate goal' of an adduct study should be to investigate which adduct structure is responsible for a particular mutation. In biological systems the mutational spectra formed by a single mutagen is produced from a wide variety of adduct species that often display regions of high and low mutation frequency (Friedberg, 1985). This may imply that the mutagenic potency of a lesion is partially determined by the sequence context and partially by the chemistry of the lesion itself. The removal of lesions by base damage recognition proteins may be dependent upon lesions producing changes in the local DNA structure and chemistry surrounding the lesion (Nilsen *et al.*, 1995; Svoboda *et al.*, 1993; Basu & Essigmann, 1988).

The probability of mutation (Equation 1) describes the mutation frequency for any nucleotide in any given sequence to be the product of the lesion frequency, the repair rate, misreading of the adduct by the polymerase and mismatch repair error frequency (Nelson *et al.*, 1996).

[1]*Equation 1:*

$$P_{(mutation)} = P_{(hit\ by\ mutagen)} \times \overbrace{P_{(not\ repaired)} \times P_{(damage\ misread\ by\ polymerase)}}^{L_S} \times P_{(mismatch\ repair\ error)}$$

The term $P_{(not\ repaired)}$ is dependent upon the time of lesion formation in relation to DNA replication and will determine the mutation frequency as seen in acute dose studies. In chronic dosing, DNA damage reaches steady state, and therefore, overall lesion formation (L_S) will be quantified by equation 2:

[1]*Equation 2:* $L_S = L_{(frequency)} \times t_{½} / \ln 2$

Lesion frequency ($L_{(frequency)}$) is measured using nucleotide resolution studies either *in vitro* or *in vivo* from an acute dose and $t_{½}$ is the half life of the adduct due to the repair processes. Lesion frequency is found to be dose independent for many chemical mutagens or carcinogens, but the pattern of $t_{½}$ of individual nucleotide positions often varies with dose and biological system used (Tornaletti & Pfeifer, 1994; Vanhoffen *et al.*, 1995) due to saturation and /or induction of repair processes.

Therefore, nucleotide resolution studies *in vivo* and *in vitro* of lesion formation (Brash & Haseltine, 1982; Denissenko *et al.*, 1996) will help correlate the pattern of lesion frequency to the sites of mutation. To date, few studies have attempted to correlate gross DNA adduct formation to mutation fixation directly resulting from the presence of an adduct (Dessinenko *et al.*, 1997, 1998; Hatahet *et al.*, 1998). These studies may identify the main mutagenic lesions produced from muliple reactive electrophiles from the metabolism of a xenobiotic with respect to the effects of sequence context on mutation fixation (Holmquist, 1998).

[1] Adapted from Tornaletti & Pfeifer, 1994 and Vanhoffen *et al.*, 1995.

4.3 Materials And Methods

4.3.1 Materials

SCS8 *E. coli,* pLIZ plasmid extracted from Big Blue™ rat liver genomic DNA was obtained from Stratagene (La Jolla, CA). 4-Hydroxytamoxifen was obtained from Sigma (Poole,UK) and α-acetoxytamoxifen was a gift from Dr K. Brown (MRC Toxicology Unit, Leicester, UK), synthesized from α-hydroxytamoxifen according to the method of Osborne *et al., (1996).* All other chemicals were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated.

4.4 Methods

4.4.1 λ pLIZ Extraction from Big Blue™ Rat Genomic DNA

The ExAssist/SOLAR system (Stratagene, La Jolla, CA) was used to efficiently excise the pLIZ plasmid (fig 48). A λLIZ phage stock was made by coring a colourless (non-mutant) plaque from a plate used in the Big Blue™ *lacI* assay of rat liver (Dr R. Davies, MRC Toxicology unit, Leicester, UK) and transferred to a sterile microfuge tube containing 20μl chloroform and 500μl SM buffer containing 0.1M NaCl, 8mM $MgSO_4$, 0.05M Tris-HCl (pH7.5), 0.01% gelatin. The tube was vortexed and the plaque incubated at room temperature for 2 hours. In a conical tube, 200μl XL1-Blue *E. coli* (*endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, lac, [F', proAB, lacI^{q}ZΔM15, Tn10(tet^r)]*) in 10mM $MgSO_4$, 100μl λLIZ phage stock (>1x10^5 phage particles) and 1μl of ExAssist helper phage (>1x10^6pfu/ml) was incubated at 37°C for 15 minutes. XL1-Blue *E. coli* were prepared by growing an inoculate of XL1-Blue *E. coli* in 200mls LB broth (per liter: 10g NaCl, 10g Bacto-Tryptone, 5g Yeast extract) supplemented with 0.2% maltose and 10mM $MgSO_4$ at 30°C until an OD_{600} of 1.0 was reached. The

supplemented with 0.2% maltose and 10mM $MgSO_4$ at 30°C until an OD_{600} of 1.0 was reached. The cells were centrifuged at 2000g for 10 minutes and resuspended in 0.5 volumes $MgSO_4$. After incubation 3mls of 2x YT media (per liter: 10g NaCl, 10g yeast extract, 16g Bacto-Tyrptone) was added and the cell suspension was incubated for 2.5 hours at 37°C with shaking (225rpm). After incubation the solution was heated to 70°C for 20 minutes before centrifugation at 4000x g for 5 minutes. The supernatant containing the pLIZ phagemid packaged as filamentous phage particles was decanted off the cell debris and stored at 4°C until needed. The double stranded pLIZ plasmid was obtained by adding 1µl and 50µl phage stock solution from the supernatant above to two separate tubes containing 200µl SOLAR™ *E.coli* (*e14⁻(mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn5(kan^r), uvrC, lac, gyrA96, relA1, thi-1, endA1, λ^r, [F' proAB, lacI^qZΔM15,] Su⁻ (non-suppressing)*). SOLAR™ *E.coli* were prepared in same manner as for XL1-Blue *E. coli*. From each tube, 100µl of *E.coli* was plated on LB/ampicillin (50µg/ml) plates and incubated overnight at 37°C. The colonies appearing on the plate contain the pLIZ double stranded plasmid (phagemid) containing the *lac I* gene.

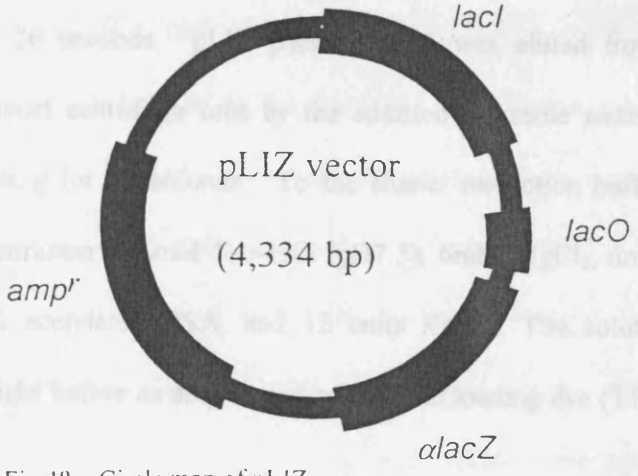


Fig 48 – Circle map of pLIZ

4.4.2 Verification of Plasmid pLIZ Formation

A single SOLAR™ *E. coli* colony harbouring the pLIZ plasmid was grown in LB broth containing ampicillin (100μg/ml) at 37°C for 16 hours. The bacterial culture was pelleted by centrifugation at 1900x g for 10 minutes at 4°C. The supernatant was removed and the bacterial pellet resuspended in 200μl of buffer, 50mM Tris-HCl (pH 7.6), 10mM EDTA, 100μg/ml RNAse A, and transferred to a 1.5ml eppendorf centrifuge tube. To the resuspended *E. coli*, 200μl of lysis solution was added, 0.2M NaOH, 1% SDS and the tube gently inverted 10 times. To this solution, 200μl of neutralisation buffer was added, 1.25M KOAc, 7% acetic acid and the solutions mixed by inversion. The precipitate of bacterial proteins and chromosomal DNA was removed by centrifugation at 14000x g for 5 minutes at room temperature. The supernatant was removed and added to 1ml of, 7M guanidine hydrochloride, 0.4M KOAc, 2.3% acetic acid adjusted to pH 5.5, 1.5g silica gel per 100ml total volume. Using vacuum suction this solution was loaded on to a Wizard® minicolumn (Promega) and washed with 2ml of wash buffer conatining, 200mM NaCl, 20mM Tris-HCl (pH7.6), 5mM EDTA, 50% ethanol. Excess wash buffer was removed from the column by centrifugation at 14000x g for 20 seconds. pLIZ plasmid DNA was eluted from the column into a sterile eppendorf centrifuge tube by the addition of sterile water at 70°C and centrifuged at 14000x g for 30 seconds. To the eluate, restriction buffer was added to give a final concentration of 6mM Tris-HCl (pH7.5), 6mM $MgCl_2$, 6mM NaCl, 1mM dithiothreitol 0.01% acetylated BSA, and 12 units *KpnI*. The solution was incubated at 37°C overnight before an aliquot was mixed with loading dye (TE buffer, 0.05% bromophenol

blue and 0.05% xylene cyanole) and electrophoresed on a 1% agarose gel (Promega). pLIZ formation was indicated by a band of 4300bp.

4.4.3 Caesium Chloride Density Gradient Purification of pLIZ Plasmid DNA

An inoculation culture of SOLAR™ *E.coli* harboring pLIZ plamid was grown in LB containing 100μg/ml ampicillin in a shaking incubator for 8 hours. This culture was transfered to 800ml LB containing 100μg/ml ampicillin and allowed to grow at 37°C in a shaking incubator for 16 hours. This culture was pelleted by centrifugation at 4000x g for 10 minutes at 4°C. The bacterial pellet was resuspended in 20mls buffer containing 50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA (pH8.0), and 5mg/ml lysozyme and incubated on ice for 15 minutes. To this solution, 60mls of lysis solution was added, 0.2M NaOH, 1% SDS on ice and incubated for 5 minutes with gentle stirring. Following incubation, 45mls of ice cold 5M potassium acetate was added and incubated on ice for 20 minutes with occasional gentle mixing. The flocculant of bacterial chromosomal DNA and protein was removed by centrifugation at 12000x g for 20 minutes at 4°C. To the supernatant, 0.6 volumes of room temperature isopropanol was added and the left to incubate at room temperature for 15 minutes. The precipitated RNA and plasmid DNA was recovered by centrifugation at 12000x g for 30 minutes at 25°C, rinsed with 70% ethanol and resuspended in 5ml Tris/EDTA (TE) buffer, 10mM Tris-HCl (pH8.0, 1mM EDTA (pH8.0). This solution was extracted twice against equal volumes of 1:1 phenol:chloroform and once with an equal volume of chloroform. To this solution, 4mg ethidium bromide, and caesium chloride was added until a specific gravity of 1.55 – 1.60 g/cm^3 was reached. Plasmid DNA was obtained from this solution by banding of the DNA by centrifugation at 24,0000x g for 18 hours. The plasmid DNA band was recovered and ethidium bromide removed by 5x extraction

against an equal volume of butan-1-ol. Plasmid DNA was precipitated from the caesium chloride solution by the addition of 2 volumes Tris/EDTA (pH8.0) and 3 volumes of isopropanol and incubated at –20°C for 2 hours before centrifugation at 12000x g for 30 minutes at 4°C. The pellet was washed with 70% ethanol and resuspended in 500µl sterile water.

4.4.4 Adduct Formation on pLIZ with Activated 4-Hydroxytamoxifen

pLIZ plasmid DNA (100µg) was dissolved in 340µl of reaction buffer containing 50mM HEPES-NaOH (pH6.2), 0.2mM EDTA, 0.03% TWEEN 20 and 500µM H_2O_2. Fresh 4-hydroxytamoxifen was added in ethanol to final concentrations of 10, 25, 50, 100, 160 and 250µM and the reaction pre-incubated at 37°C for 5 minutes. The reactive 4-hydroxytamoxifen quinone methide was generated by the addition of 10 µl of horseradish-peroxidase (HRP) (5mg/ml) to the reaction, followed by incubation at 37°C for 30 min. After 30 min the reactions were quenched by the addition of 800µl ice cold chloroform. Unreacted 4-hydroxytamoxifen/ 4-hydroxytamoxifen quinone was removed by extraction with water saturated ethyl acetate (3x200µl), and the DNA precipitated from the aqueous phase using sodium acetate/ethanol. Control reactions were performed by incubating the pLIZ plasmid without 4-hydroxytamoxifen, in the presence or absence of the HRP/H_2O_2 activating system.

4.4.5 Adduct Formation on pLIZ with α-Acetoxytamoxifen

pLIZ plasmid DNA (100µg) was dissolved in 100 µl of reaction buffer containing 10µM EDTA, 0.15M NaCl and 15mM SSC pH8.8. Fresh α-Acetoxytamoxifen was added in

ethanol to final concentrations of 10, 25, 50, 100, 160 & 250μM, and the reactions incubated at 37°C overnight. Controls contained ethanol alone. The samples were extracted three times with 100μl ethyl acetate to remove unreacted α-acetoxytamoxifen and the DNA precipitated as above.

4.4.6 ^{32}P-Postllabeling Analysis of Plasmid DNA

Plasmid DNA was subjected to ^{32}P-postlabelling as described previously (Gupta *et al.*, 1984). Briefly, DNA was digested to nucleotides using micrococcal nuclease and calf spleen phosphodiesterase (Boehringer-Mannheim) overnight at 37°C. The digestion mix was further treated with nuclease P1 to convert unadducted nucleotides to nucleosides. Adducted nucleotides were subsequently ^{32}P-radiolabeled by 5' phosphorylation using [γ-^{32}P]ATP (>5000 Ci/mmol, 10 mCi/ml; Amersham International) and 3'-phosphatase-free T4 polynucleotide kinase (Boehringer-Mannheim). ^{32}P-Labelled adducts were separated by HPLC with on-line radiochemical detection (Martin *et al.*, 1998). For each dose (10, 25 and 50μM) there were 3 plasmid DNA samples which were each analyzed twice (For each treatment dose, n = 6). Mean levels of adducts ± SD are expressed as relative adduct labeling, RAL x 10^8.

The thermal stability of the adducts produced by 4-hydroxytamoxifen and α-acetoxy tamoxifen was assessed by heating pLIZ adducted DNA to 95°C for 5 minutes and compared with untreated control by ^{32}P-postlabelling.

4.4.7 T4 DNA Polymerase Arrest Assay

The arrest assays were carried out using primer 5'-GTACCCGACACCATCGAATG-3' which corresponded to bases -82 to -62 where the adenine of the *lac I* translation initiation codon is +1. Using this primer the transcribed (non-coding) strand of the *lacI* gene acts as the template. The primer was ^{32}P end labeled using $\gamma^{32}P$-ATP, 3000Ci/mmol (Amersham) and 5 units T4 polynucleotide kinase (promega), in a total volume of 10µl containing 200pmoles deoxyoligonucleotide primer, 70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 5mM dithiothreitol at 37°C for 30 minutes. The labeled primer was separated from unincorporated ^{32}P-ATP using a Chroma Spin™-10 gel filtration spin column (Clontech, Heidelberg, Germany). Primer extension was carried out in a total volume of 20µl containing 3µg adducted or unadducted *pLIZ* plasmid DNA, 67mM Tris-HCl (pH8.8), 16mM $(NH_4)_2SO_4$, 6.7mM $MgCl_2$, 6.7µM EDTA, 167µg/ml BSA, 10mM β-mercaptoethanol buffer, 18pmoles labeled primer, and 250µM of each dNTP. The DNA was denatured by heating to 95°C for 2 minutes and the primer annealed during subsequent cooling to 4°C. Extension was initiated by the addition of 4 units of T4 DNA polymerase. Elongation was allowed to proceed for 30 minutes at 37°C before the addition of 5µl stop solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanole). DNA was denatured by heating 95°C followed by cooling on ice. The DNA fragments were resolved on a 0.4mm by 60 cm 6% polyacrylamide gel containing 8M urea using a tris-taurine-EDTA buffer system (Amersham). The gel was run at 60 watts that maintained a temperature of 50°C. Running time was approximately 2.5 hrs. Gels were fixed in a 10% methanol, glacial

acetic acid solution for 15 minutes and dried on Whatman 3MM paper. Gels were visualized using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

4.4.8 Production of the DNA Sequencing Ladder

DNA sequencing was performed using the Promega fmol® DNA sequencing system. Sequence reactions were performed in 6µl containing 100ng pLIZ plasmid DNA, 50mM Tris-HCl (pH 9.0), 2mM $MgCl_2$ buffer, 1.5pmoles ^{32}P labeled 5'-GTACCCGACACCATCGAATG-3' primer, 20µM dATP, dTTP, dCTP, 7-Deaza dGTP with either 30µM Deaza ddGTP, 350µM Deaza ddATP, 600µM Deaza ddTTP, 200µM Deaza ddCTP and 5 units sequencing grade *Taq* DNA polymerase. After gentle mixing the samples were incubated in a thermal cycler. Initial denaturation at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 58°C and 1 minute chain elongation at 72°C. When amplification was completed, 3µl stop solution was added and samples treated as above.

4.4.9 Formation of competent SCS8 *E. coli*

A single colony of SCS8 *E.coli* (*recA1, endA1, mcrA D(mcrBC-hsdRMS-mrr), D(argF-lac)U169?80dlacZDM15 Tn10 (Tet')*) was inoculated in 2mls L. Broth containing 12.5µg/ml tetracycline and grown at 37°C for two hours with shaking (225rpm). From this culture 0.1 volumes was used to inoculate 200ml of L. Broth (with 12.5µg/ml tetracycline) and the culture was allowed to grow to an optical density of 0.7 at 600nm. The culture was then poured into 50ml centrifuge tubes and cooled on ice for 15

minutes and the bacteria pelleted by centrifugation at 1900x g for 10 minutes at 4°C. The supernatant was discarded and the SCS8 *E. coli* were re-suspended in 40mls ice cold buffer 1, (100mM RbCl, 50mM $MnCl_2$, 30mM potassium acetate, 10mM $CaCl_2$, 15% glycerol; pH 5.8 with dilute acetic acid) and incubated on ice for 1 hour. After incubation the bacterial cells were pelleted by centrifugation at 1900x g for 10 minutes at 4°C and resuspended in 5ml ice cold buffer 2, (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% glycerol; pH 6.8 with dilute NaOH). The competent SCS8 *E. coli* were then immediately dispensed into 200µl aliquots and frozen on solid CO_2 and stored at -80°C until use.

4.4.10 Transformation of SCS8 E.coli with 4-hydroxy and α-acetoxtamoxifen adducted pLIZ

Competent SCS8 *E.coli* (100µl) were incubated with 50ng of unadducted or adducted pLIZ plasmid for 20 minutes on ice in pre-cooled plastic tubes. After incubation the tubes were placed into a water bath at 42 °C for 90 seconds and then incubated on ice for a further two minutes. To the transformed cells, 900µl SOC medium (2g Bacto-Tryptone, 0.5g yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$, 10mM $MgCl_2$, 20mM glucose per 100ml) was added and the bacteria incubated at 37°C for 60 minutes with shaking (300rpm). The transformation mixture was plated on to agar containing 100µg/ml ampicillin, 80µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Blue mutant colonies were counted and expressed as a percentage of non mutant white colonies. Mutant colonies were picked, grown, and the recovered pLIZ plasmid sequenced for *LacI* gene mutations. For each transformation 30-60 x 10^3 colonies were screened.

4.5 Automated DNA sequencing

Control and mutant *LacI* genes were cycle sequenced (PNACL, MRC Toxicology unit, Leicester) using the ABI prism™ big dye terminator cycle sequencing system with AmpliTaq® DNA polymerase using the same primer as used for the polymerase stop assay. Reaction products were analyzed on a Perkin Elmer Applied Biosystems 377 DNA sequencer.

```
   1 AAAGAACGTG GACTCCAACG TCAAAGGGCG AAAAACCGTC TATCAGGGCG ATGGCCCACT
  61 ACGTGAACCA TCACCCTAAT CAAGTTTTTT GGGGTCGAGG TGCCGTAAAG CACTAAATCG
 121 GAACCCTAAA GGGAGCCCCC GATTTAGAGC TTGACGGGGA AAGCCGGCGA ACGTGGCGAG
 181 AAAGGAAGGG AAGAAAGCGA AAGGAGCGGG CGCTAGGGCG CTGGCAAGTG TAGCGGTCAC
 241 GCTGCGCGTA ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCAGGTGG
 301 CACTTTTCGG GGAAATGTGC GCGGAACCCC TATTTGTTTA TTTTTCTAAA TACATTCAAA
 361 TATGTATCCG CTCATGAGAC AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA
 421 GAGTCCTGAG GATGAGTATT CAACATTTCC GTGTCGCCCT TATTCCCTTT TTTGCGGCAT
 481 TTTGCCTTCC TGTTTTTGCT CACCCAGAAA CGCTGGTGAA AGTAAAAGAT GCTGAAGATC
 541 AGTTGGGTGC ACGAGTGGGT TACATCGAAC TGGATCTCAA CAGCGGTAAG ATCCTTGAGA
 601 GTTTTCGCCC CGAAGAACGT TTTCCAATGA TGAGCACTTT TAAAGTTCTG CTATGTGGCG
 661 CGGTATTATC CCGTATTGAC GCCGGGCAAG AGCAACTCGG TCGCCGCATA CACTATTCTC
 721 AGAATGACTT GGTTGAGTAC TCACCAGTCA CAGAAAAGCA TCTTACGGAT GGCATGACAG
 781 TAAGAGAATT ATGCAGTGCT GCCATAACCA TGAGTGATAA CACTGCGGCC AACTTACTTC
 841 TGACAACGAT CGGAGGACCG AAGGAGCTAA CCGCTTTTTT GCACAACATG GGGGATCATG
 901 TAACTCGCCT TGATCGTTGG GAACCGGAGC TGAATGAAGC CATACCAAAC GACGAGCGTG
 961 ACACCACGAT GCCTGTAGCA ATGGCAACAA CGTTGCGCAA ACTATTAACT GGCGAACTAC
1021 TTACTCTAGC TTCCCGGCAA CAATTAATAG ACTGGATGGA GGCGGATAAA GTTGCAGGAC
1081 CACTTCTGCG CTCGGCCCTT CCGGCTGGCT GGTTTATTGC TGATAAATCT GGAGCCGGTG
1141 AGCGTGGGTC TCGCGGTATC ATTGCAGCAC TGGGGCCAGA TGGTAAGCCC TCCCGTATCG
1201 TAGTTATCTA CACGACGGGG AGTCAGGCAA CTATGGATGA ACGAAATAGA CAGATCGCTG
1261 AGATAGGTGC CTCACTGATT AAGCATTGGT AACTGTCAGC CTCAGGTTAC TCATATATAC
```

1321 TTTAGATTGA TTTAAAACTT CATTTTTAAT TTAAAAGGAT CTAGGTGAAG ATCCTTTTTG

1381 ATAATCTCAT GACCAAAATC CCTTAACGTG AGTTTTCGTT CCACTGAGCG TCAGACCCCG

1441 TAGAAAAGAT CAAAGGATCT TCTTGAGATC CTTTTTTTCT GCGCGTAATC TGCTGCTTGC

1501 AAACAAAAAA ACCACCGCTA CCAGCGGTGG TTTGTTTGCC GGATCAAGAG CTACCAACTC

1561 TTTTTCCGAA GGTAACTGGC TTCAGCAGAG CGCAGATACC AAATACTGTC CTTCTAGTGT

1621 AGCCGTAGTT AGGCCACCAC TTCAAGAACT CTGTAGCACC GCCTACATAC CTCGCTCTGC

1681 TAATCCTGTT ACCAGTGGCT GCTGCCAGTG GCGATAAGTC GTGTCTTACC GGGTTGGACT

1741 CAAGACGATA GTTACCGGAT AAGGCGCAGC GGTCGGGCTG AACGGGGGGT TCGTGCACAC

1801 AGCCCAGCTT GGAGCGAACG ACCTACACCG AACTGAGATA CCTACAGCGT GAGCTATGAG

1861 AAAGCGCCAC GCTTCCCGAA GGGAGAAAGG CGGACAGGTA TCCGGTAAGC GGCAGGGTCG

1921 GAACAGGAGA GCGCACGAGG GAGCTTCCAG GGGGAAACGC CTGGTATCTT TATAGTCCTG

1981 TCGGGTTTCG CCACCTCTGA CTTGAGCGTC GATTTTTGTG ATGCTCGTCA GGGGGGCGGA

2041 GCCTATGGAA AAACGCCAGC AACGCGGCCT TTTTACGGTT CCTGGCCTTT TGCTGGCCTT

2101 TTGCTCACAT GTTCTTTCCT GCGTTATCCC CTGATTCTGT GGATAACCGT ATTACCGCCA

2161 TGCATACTAG TCTCGAGTAC GTAC**GTACCC GACACCATCG AATG**GTGCAA AACCTTTCGC

2221 GGTATGGCAT GATAGCGCCC GGAAGAGAGT CAATTCAGGG TGGTGA**ATG**T GAAACCAGTA

2281 ACGTTATACG ATGTCGCAGA GTATGCCGGT GTCTCTTATC AGACCGTTTC CCGCGTGGTG

2341 AACCAGGCCA GCCACGTTTC TGCGAAAACG CGGGAAAAAG TGGAAGCGGC GATGGCGGAG

2401 CTGAATTACA TTCCCAACCG CGTGGCACAA CAACTGGCGG GCAAACAGTC GTTGCTGATT

2461 GGCGTTGCCA CCTCCAGTCT GGCCCTGCAC GCGCCGTCGC AAATTGTCGC GGCGATTAAA

2521 TCTCGCGCCG ATCAACTGGG TGCCAGCGTG GTGGTGTCGA TGGTAGAACG AAGCGGCGTC

2581 GAAGCCTGTA AAGCGGCGGT GCACAATCTT CTCGCGCAAC GCGTCAGTGG GCTGATCATT

2641 AACTATCCGC TGGATGACCA GGATGCCATT GCTGTGGAAG CTGCCTGCAC TAATGTTCCG

2701 GCGTTATTTC TTGATGTCTC TGACCAGACA CCCATCAACA GTATTATTTT CTCCCATGAA

2761 GACGGTACGC GACTGGGCGT GGAGCATCTG GTCGCATTGG GTCACCAGCA AATCGCGCTG

2821 TTAGCGGGCC CATTAAGTTC TGTCTCGGCG CGTCTGCGTC TGGCTGGCTG GCATAAATAT

2881 CTCACTCGCA ATCAAATTCA GCCGATAGCG GAACGGGAAG GCGACTGGAG TGCCATGTCC

2941 GGTTTTCAAC AAACCATGCA AATGCTGAAT GAGGGCATCG TTCCCACTGC GATGCTGGTT

3001 GCCAACGATC AGATGGCGCT GGGCGCAATG CGCGCCATTA CCGAGTCCGG GCTGCGCGTT

3061 GGTGCGGATA TCTCGGTAGT GGGATACGAC GATACCGAAG ACAGCTCATG TTATATCCCG

3121 CCGTTAACCA CCATCAAACA GGATTTTCGC CTGCTGGGGC AAACCAGCGT GGACCGCTTG

3181 CTGCAACTCT CTCAGGGCCA GGCGGTGAAG GGCAATCAGC TGTTGCCCGT CTCACTGGTG

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3241 AAAAGAAAAA CCACCCTGGC GCCCAATACG CAAACCGCCT CTCCCCGCGC GTTGGCCGAT
3301 TCATTAATGC AGCTGGCACG ACAGGTTTCC CGACTGGAAA GCGGGCAGTG AGCGCAACGC
3361 AATTAATGTG AGTTAGCTCA CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC
3421 TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACCA
3481 TGATTACGGA TTCACTGGCC GTCGTTTTAC AACGTCGTGA CTGGGAAAAC CCTGGCGTTA
3541 CCCAACTTAA TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT AGCGAAGAGG
3601 CCCGCACCGA TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGG CGCTTTGCCT
3661 GGTTTCCGGC ACCAGAAGCG GTGCCGGAAA GCTGGCTGGA GTGCGATCTT CCTGAGGCCG
3721 ATACTGTCGT CGTCCCCTCA AACTGGCAGA TGCACGGTTA CGATGCGCCC ATCTACACCA
3781 ACGTAACCTA TCCCATTACG GTCAATCCGC CGTTTGTTCC CACGGAGAAT CCGACGGGTT
3841 GTTACTCGCT CACATTTAAT GTTGATGAAA GCTGGCTACA GGAAGGCCAG ACGCGAATTA
3901 TTTTTGATGG CGTTAACTCG GCGTTTCATC TGTGGTGCAA CGGGCGCTGG GTCGGTTACG
3961 GCCAGGACAG TCGTTTGCCG TCTGAATTTG ACCTGAGCGC ATTTTTACGC GCCGGAGAAA
4021 ACCGCCTCGC GGTGATGGTG CTGCGTTGGA GTGACGGCAG TTATCTGGAA GATCAGGATA
4081 TGTGGCGGAT GAGCGGCATT TTCCGTGCTC TAGACGATCG CCCTTCCCAA CAGTTGCGCA
4141 GCCTGAATGG CGAATGGAGA TCTATACGCG TAAATTGTAA GCGTTAATAT TTTGTTAAAA
4201 TTCGCGTTAA ATTTTTGTTA AATCAGCTCA TTTTTTAACC AATAGGCCGA AATCGGCAAA
4261 ATCCCTTATA AATCAAAAGA ATAGACCGAG ATAGGGTTGA GTGTTGTTCC AGTTTGGAAC
4321 AAGAGTCCAC TATT
```

Fig 48a. Linear map of pLIZ plasmid, *LacI* transcription start bp 2269 (underlined), stop bp 3349; αLacZ transcription start bp 3477, stop bp 4111; Ampicillin resistance, transcription start bp 432, stop bp 1296. Boxed sequence indicates primer binding site.

4.6 RESULTS

4.6.1 ^{32}P-Postlabelling

DNA reacted with α-acetoxytamoxifen or HRP/H_2O_2 activated 4-hydroxytamoxifen was analysed by ^{32}P-postlabelling of tamoxifen adducted bisphosphates prior to use in the *T4* DNA polymerase arrest assay and subsequent mutagenesis assay in SCS8 *E.coli*. The HPLC profiles obtained following the reaction with either α-acetoxytamoxifen or activated 4-hydroxytamoxifen were clearly different. In this present study postlabelled pLIZ plasmid DNA reacted with α-acetoxytamoxifen produced one major peak (fig 49A, peak 6), proposed by Martin *et al.*, 1998 to be the geometric or sterioisomers of the deoxyguanosine adducts which elute together using this system. Peak 5 is a N-desmethyltamoxifen guanine adducts (Martin *et al.*, 1999). HRP/H_2O_2 activated 4-hydroxytamoxifen gave one major peak (b), more polar than those of the major peaks produced from α-acetoxytamoxifen (Fig. 49B). The tamoxifen DNA adduct producing peak b is presumed to be the same geometric isomer as for peak 6 of α-acetoxytamoxifen but more polar due to the presence of a hydroxyl group. Using this HPLC system a dose dependent increase in tamoxifen DNA adduct levels was observed for α-acetoxytamoxifen with levels that increased from 6571 ± 2432 adducts/10^8 nucleotides at 10μM, to 26838 ± 4796 adducts/10^8 nucleotides at 50μM. Unlike α-acetoxytamoxifen, activated 4-hydroxytamoxifen adduct levels did not increase over this dose range and were approximately 3601 ± 108 adducts/10^8 nucleotides at all three dose levels (Fig 49C).

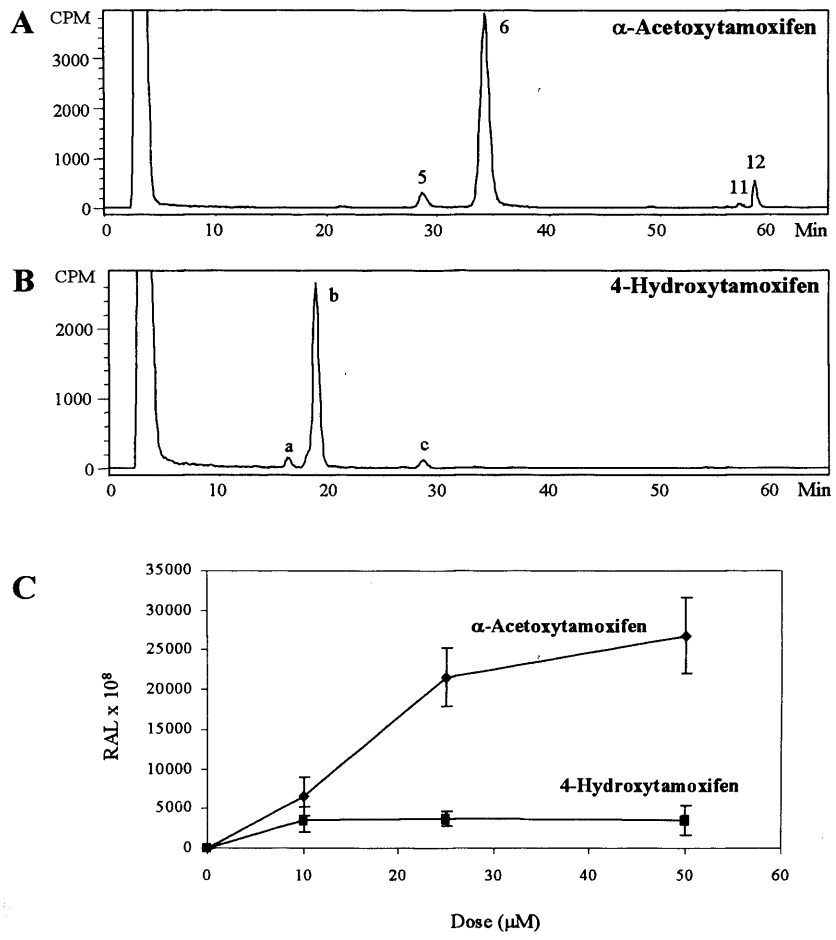


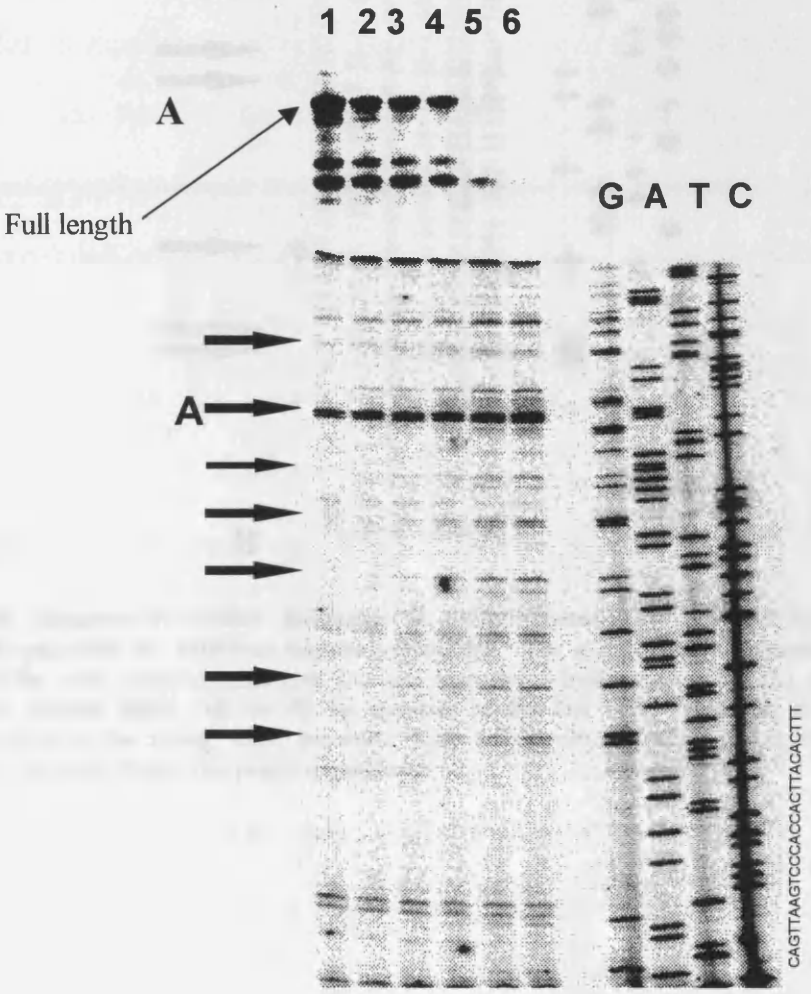
Fig 49. Comparison of HPLC separation of ^{32}P-postlabeled DNA adducts formed by α-acetoxytamoxifen and activated 4-hydroxytamoxifen. A: pLIZ DNA reacted with 10μM α-acetoxytamoxifen (0.5μg DNA). B: pLIZ DNA reacted with 10μM activated 4-hydroxytamoxifen (0.5μg DNA). C: Relationship between adduct levels and concentration of activated tamoxifen metabolite 0-50μM, correlating to the concentrations used in the mutation study, mean (n = 6) and SD shown. Numbering system from Martin *et al.*, 1998.

4.6.2 Analysis of DNA adduct formation by polymerase arrest assay

Optimal conditions for the analysis of 4-hydroxytamoxifen DNA adducts were obtained using *T4* DNA polymerase with a single read through of the DNA using an end labeled primer (chapter 3). This data is in common with previous results showing that *T4* DNA polymerase is more sensitive to inhibition of DNA synthesis progression by adducts (Latham *et al.*, 1995, Chary *et al.*, 1995). The chemical stability of the DNA adducts from both 4-hydroxytamoxifen and α-acetoxytamoxifen was assessed by heating adducted pLIZ plasmid DNA to 95°C for 5 minutes and adduct levels assessed by the ^{32}P-postlabelling system described above. No differences were found in gross DNA adduct levels or patterns of adduct formation in plasmid DNA that was heated to 95°C to non heated samples. This indicated that the DNA denaturing step in the arrest assay protocol was unlikely to have any effect of changing the DNA adduct structures.

Using *T4* DNA polymerase in the arrest assay a similar spectrum of DNA adducts was obtained from the *lacI* gene adducted with either activated 4-hydroxytamoxifen or α-acetoxytamoxifen (Fig. 50). It is not known for the adducts detected in the arrest assay, if *T4* DNA polymerase is stopping on the adducted base, or one base prior, or post to, the adduct. Data from the assays assumes *T4* DNA polymerase halts on the adducted base and identifies pause sites to be on most guanines and two adenines (Fig. 50A+B). Published data indicates that tamoxifen forms most adducts on guanine and adenine as a minor adduct (Osbourne *et al.*, 1996, 1997). If this data is modeled to *T4* DNA polymerase halting before or after the adduct base then the spectrum of adduct formation will not correlate to the adduct spectrum expected from the theoretical chemistry and published data.

Adduction by both α-acetoxytamoxifen and activated 4-hydroxytamoxifen was detected only on two adenines by *T4* DNA polymerase (Fig 50A+B). This multiple adduction site was located between two runs of three guanines. This region was also a natural pause site for the polymerase, which may be artifactual due to the presence of adducts on the neighboring guanines. It is not precisely clear if both adenines are adducted or if the polymerase halted before or at the second adenine. However, for activated 4-hydroxytamoxifen this area of the *lacI* gene was a very prominent mutational hot spot (Table 3).



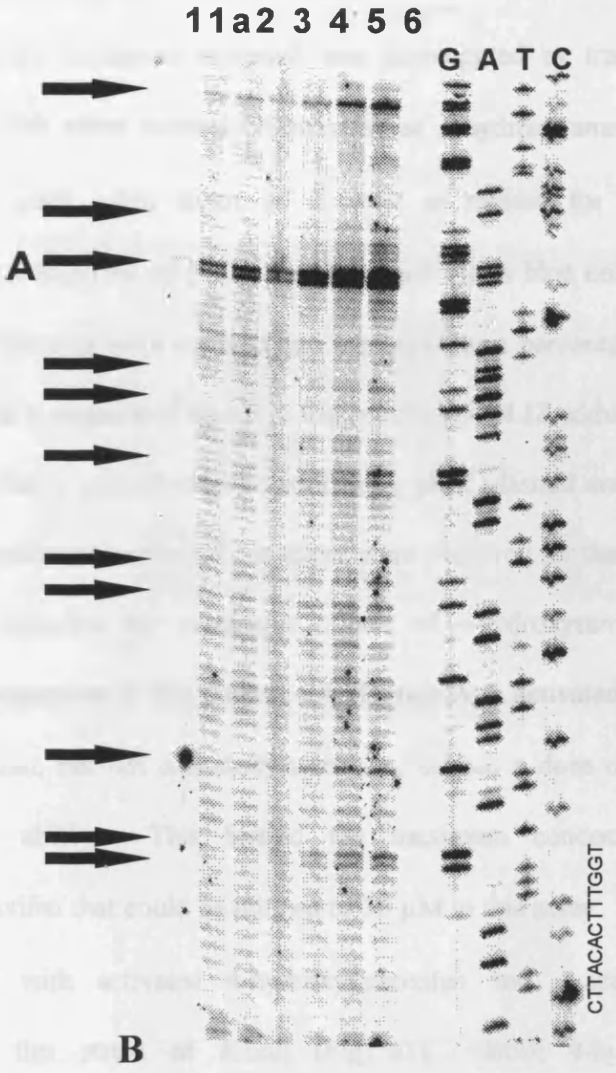


Fig 50. Analysis of adduct formation on pLIZ plasmid by *in vitro* reaction with α-acetoxytamoxifen or activated 4-hydroxytamoxifen. The adduct pattern generated with *T4* DNA polymerase with α-acetoxytamoxifen (A) and activated 4-hydroxytamoxifen (B). The primer was situated between bases -81 to -61 bp upstream of the *lacI* gene translation initiation site, and corresponded to the coding (sense) sequence. Lane 1, Control/H_2O_2, Lane 1a/H_2O_2/HRP, lanes 2 to 6, 25, 50, 100, 160, 250μM compound respectively.

4.6.3 Pattern of mutagenesis in SCS8 E.coli

Tamoxifen adduct mutagenic potential was investigated by transforming competent SCS-8 *E.coli* with either α-acetoxytamoxifen or 4-hydroxytamoxifen adducted pLIZ plasmid (0-30 μM). This strain of *E. coli* is mutant for *lacI* and the alpha complementation fragment of β-galactosidase, and forms blue colonies with a mutated plasmid *lacI*. Mutants were counted and expressed as a percentage of the white (wild type) colonies as a measure of mutation frequency. For pLIZ adducted with activated 4-hydroxytamoxifen, a control reaction containing pLIZ plasmid and HRP/H_2O_2 only was included. A significant number of mutations were observed for the HRP/H_2O_2 treatment of pLIZ and therefore the mutagenic effects of 4-hydroxytamoxifen adducts were assessed by comparison to this control. Adduction with activated 4-hydroxytamoxifen, but not α-acetoxytamoxifen, caused a dose dependent decrease in transformation ability. This limited the maximum concentration of activated 4-hydroxytamoxifen that could be utilized to 30 μM in this assay. DNA adducts formed from reaction with activated 4-hydroxytamoxifen and α-acetoxytamoxifen were mutagenic in this strain of *E.coli* (Fig 51). Both 4-hydroxytamoxifen and α-acetoxytamoxifen were significantly mutagenic at 1μM. The mutagenicity of 4-hydroxytamoxifen increased to 20 μM before decreasing in contrast to the α-acetoxytamoxifen adducted plasmid whose mutagenicity decreased from a peak at 1μM and remained constant even at 250μM. The mutation frequency observed with α-acetoxytamoxifen was about 2 orders of magnitude less than that for activated 4-hydroxytamoxifen (Fig 51).

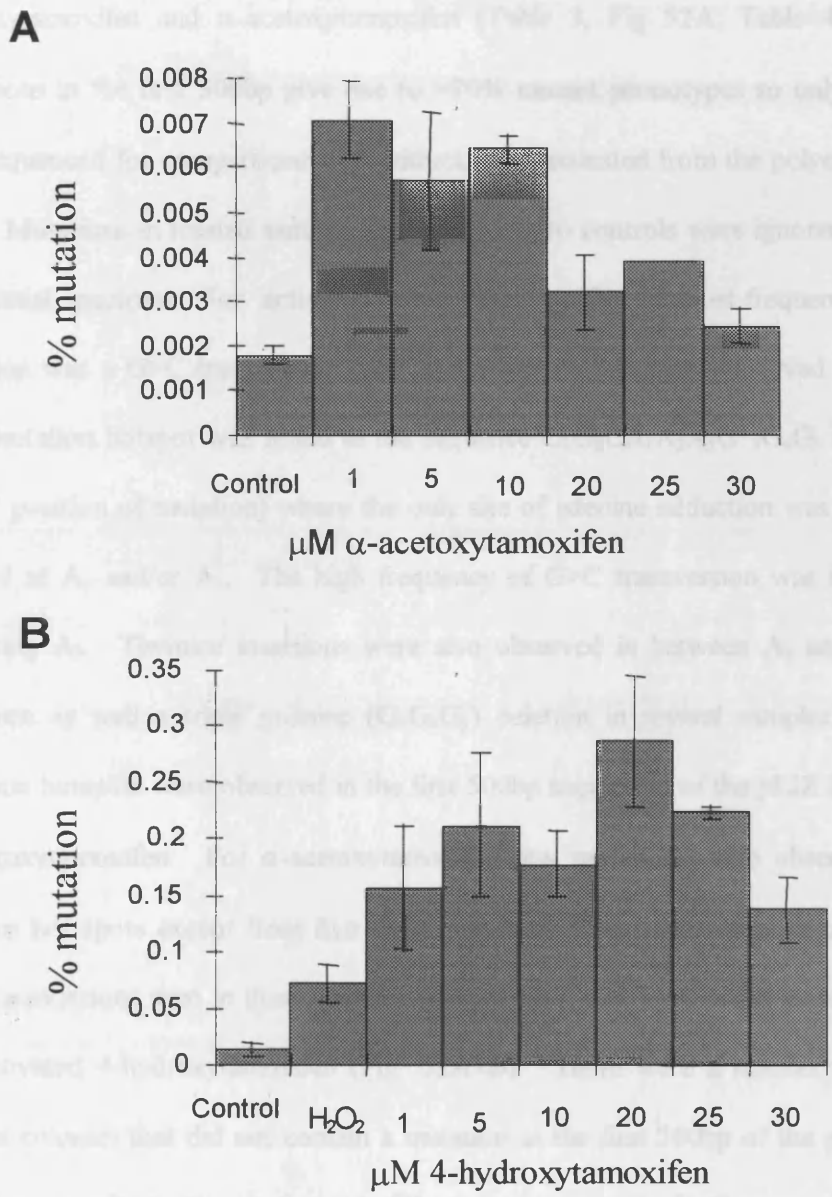


Fig 51. Frequency of mutation in SCS-8 *E.coli* of the *in vitro* adducted pLIZ plasmid containing the *lacI* gene. The pLIZ plasmid adducted *in vitro* with α-acetoxytamoxifen (A) or activated 4-hydroxytamoxifen (B) at the concentrations shown. This plasmids were transformed into competent SCS-8 *E.coli*. Mutant colonies were detected by complementation of β-galactosidase to hydrolyse the chromogenic x-gal as to form blue colonies. The number of mutant colonies are expressed as a percentage of the white (non mutant) colonies. The mean and SD of three experiments is shown.

4.6.4 Mutagenic spectrum of tamoxifen adducted *lacI* gene

The mutagenic spectrum of the *lacI* gene was determined by sequencing from mutant plasmids extracted from 'blue' SCS-8 *E.coli* colonies for both activated 4-hydroxytamoxifen and α-acetoxytamoxifen (Table 3, Fig 52A; Table 4, Fig 52B). Mutations in the first 300bp give rise to >70% mutant phenotypes so only this region was sequenced for comparisons with adduct data generated from the polymerase arrest assay. Mutations in treated samples corresponding to controls were ignored in the final mutational spectrum. For activated 4-hydroxytamoxifen the most frequently observed mutation was a G>C transversion (due to the mutation hotspot observed at base 64). This mutation hotspot was found in the sequence $G_1G_2CA_1A_2A_3G^*_3G_4G_5$ (+58 to +66 bp - * position of mutation) where the only site of adenine adduction was located was located at A_1 and/or A_2. The high frequency of G>C transversion was found on G_3 following A_3. Thymine insertions were also observed in between A_2 and A_3 of this sequence as well a triple guanine ($G_3G_4G_5$) deletion in several samples. No other mutation hotspots were observed in the first 500bp sequenced of the pLIZ *lacI* gene for 4-hydroxytamoxifen. For α-acetoxytamoxifen few mutations were observed and no obvious hot spots except from five observations of T>A transversion at position 163. The transversions seen in these samples were of G>T and T>A rather than the G>C as for activated 4-hydroxytamoxifen (Fig. 52A+B). There were a number of observed mutant colonies that did not contain a mutation in the first 500bp of the gene. These mutations may have arisen in the rest of the sequence or in the *lacO* operon itself.

[1]Position	Mutation	Occurrence
23	G>C	1
33	C>A	1
64	G>C	1
67	C>A	1
91-108	DELETION	1
93	ΔA	1
104	C>G	1
105	G>C	1
110	T>A	1
114	C>T	1
159	C>G	1
160	G>A	1
163	T>A	5
240-256	DELETION	1
244	G>A	1
315	C>G	2
388	+G	1
441	A>T	1
	No detected mutation	6

Controls

Pos	Mutation	Occurrence
	No detected mutation	4
94	G>A	1
116	C>G	1
304	C>A	1
327	C>A	2
475	C>G	1

Table 4 Mutations in daughter pLIZ (first 500 bp) plasmids after treatment with α-acetoxytamoxifen and extraction from 'blue' SCS-8 *E.coli*

[1] The numbering system assumes the A of the translation initiation site of the *lacI* gene to be +1

4-Hydroxytamoxifen PLIZ mutations

Position	Mutation	Occurrence
-20	C>G	3
22	G>A	1
23	C>A	1
59	+C	2
62	+T	1
64-66	ΔGGG	2
64	G>C	18
92	A>G	1
121	G>C	1
155	G>C	1
160	+G	1
203	ΔG	1
244	G>A	1
248	+A	1
289	+C	1
298	+C	1
319	+T	1
331	C>A	1
347	G>A	1
375	T>A	1
384	G>A	1
400	+C	1
414	C>T	1
464	T>A	1
490	+A	1

Controls: Spontaneous Mutations

Position	Mutation	Occurrence
	No detected mutations	9
30	C>T	1
31	G>T	1
460	T>G	1

HRP/H_2O_2 Mutations

Position	Mutation	Occurrence
29	G>T	1
58	G>C	1
59	ΔG	2
202	ΔG	1
243	C>T	2
332	C>T	1

345	G>A	1
362	+T	1
398	+C	1
418	ΔG	1
460	T>G	1
491	+A	1

Table 5 Mutations in daughter pLIZ (first 500 bp) plasmids after treatment with 4-hydroxytamoxifen and extraction from 'blue' SCS-8 *E.coli*

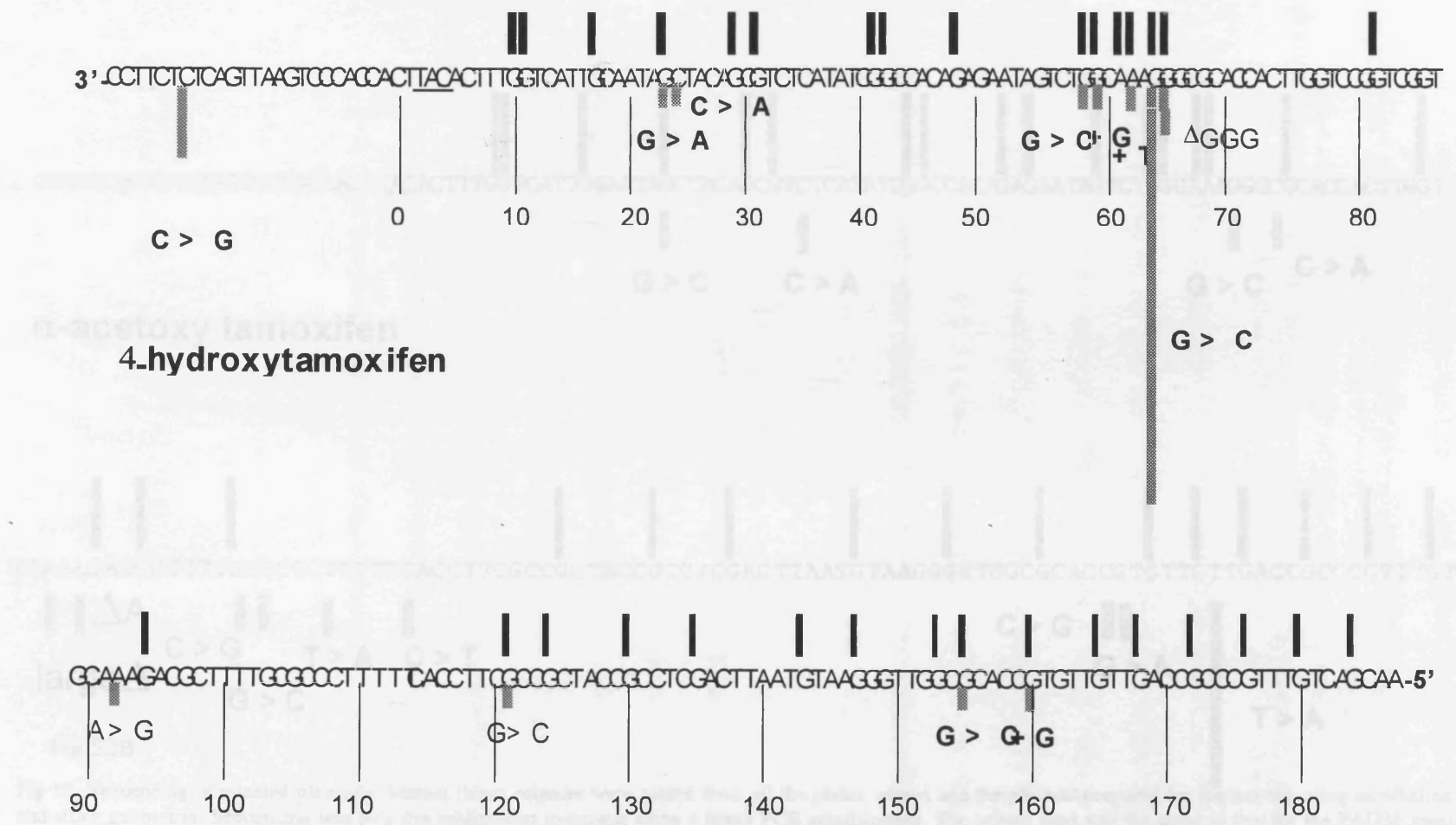

3'
C > A
G > A
G > C
G
ΔGGG
0
10
20
30
40
50
60
70
80
C > G
G > C
4-hydroxytamoxifen
A > G
G> C
G > G
90
100
110
120
130
140
150
160
170
180
-5'

Fig 52A

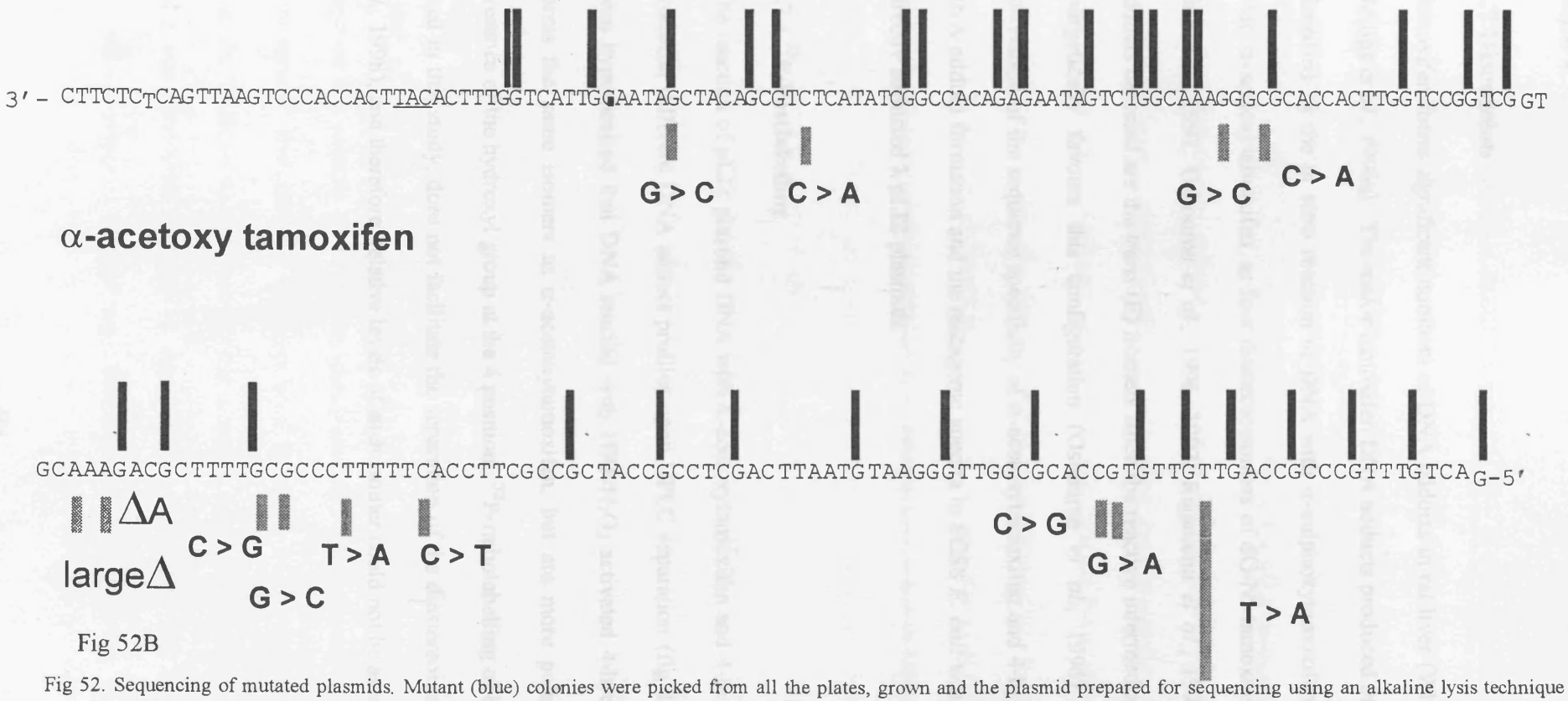


Fig 52B

Fig 52. Sequencing of mutated plasmids. Mutant (blue) colonies were picked from all the plates, grown and the plasmid prepared for sequencing using an alkaline lysis technique and silica gel matrix. Sequencing was by a dye sublimation technique using a linear PCR amplification. The primer used was the same as that for the PAUSE assay. The sequence shown is the antisense (transcribed) strand in the 3' to 5' direction of the *lac I* gene starting at base 34 downstream of the adenosine of the translation initiation codon (underlined). This is the sequence corresponding to that 'read' for the arrest assay. Only mutations detected within the region that corresponded to sequence analyzed for the arrest assay are shown. The black bars above the sequence mark the pause sites shown in figure 50A+B. The grey bars show sites of mutation with the type of mutation marked. The length of the bar indicated the number of times a mutation was observed with the shortest bars corresponding to one observation

4.7 Discussion

Tamoxifen forms significant numbers of DNA adducts in rat liver (White *et al.*, 1992; Phillips *et al.*, 1996a). The major tamoxifen DNA adducts produced *in vivo* have been identified by the *in vitro* reaction of DNA with α-sulphoxytamoxifen, and its model ester, α-acetoxytamoxifen, as four diastereoisomers of dG-N^2-tamoxifen (Dasaradhi & Shibutani, 1997, Osbourne *et al.*, 1996, 1997; Rajaniemi *et al.*, 1999). The major adducts detected are the *trans* (E) isomers since the reactive intermediate (carbocation) energetically favours this configuration (Osbourne *et al.*, 1996). In this study, correlation of the sequence specificity of α-acetoxytamoxifen and 4-hydroxytamoxifen DNA adduct formation and the mutagenic spectra in SCS8 *E. coli* was examined from directly adducted λ pLIZ plasmids.

4.7.1 ^{32}P-Postlabelling

The reaction of pLIZ plasmid DNA with α-acetoxytamoxifen and 4-hydroxytamoxifen produced different DNA adduct profiles upon HPLC separation (fig 49 A+B). It has been hypothesised that DNA reacted with HRP/H_2O_2 activated 4-hydroxytamoxifen, forms the same isomers as α-acetoxytamoxifen, but are more polar owing to the presence of the hydroxyl group at the 4 position. ^{32}P-radiolabelling of bi-phosphates as used in this study, does not facilitate the separation of the diastereoisomers (Martin *et al.*, 1998), and therefore, relative levels of each isomer could not be assessed.

4.7.2 T4 DNA Polymerase Arrest Assay

The T4 DNA polymerase arrest assay used with α-acetoxytamoxifen and 4-hydroxytamoxifen adducted DNA indicated that the major site of DNA adduct formation occurred on guanine, with most guanine bases being adducted. Thesse data correlated with my previous studies, (Chapter 3) and that of Osbourne *et al.*, (1996). Adduct formation also occurred on the first or second adenine in a triplet located between multiple guanines (fig 50A+B). No other sites of adenine adduct formation were observed in the *lacI* gene section examined. Tamoxifen adenine DNA adducts have been identified as minor adducts, following reaction of DNA with α-acetoxytamoxifen (Osbourne *et al.*, 1997). In the assay, the adenine adducts identified on pLIZ generated stronger stop sites for both α-acetoxytamoxifen, but more so for 4-hydroxytamoxifen, compared with the guanine adducts. Previous data from the T4 polymerase arrest assay, using the *mdr1b* promoter sequence cloned into pGEMT vector also indicated the presence of adenine adducts, which were in this case of comparable intensity to the guanine adducts (Chapter 3). The increased ability of T4 DNA polymerase to stall at the adenine tamoxifen adduct in pLIZ may be due to the positioning of the adduct in this sequence (GGCAAAGGG), that may facilitate a conformational change in the secondary structure of the DNA. This may impose a greater steric hindrance to T4 DNA polymerase. The intensity of the adenine adduct pause of 4-hydroxytamoxifen may be explained by the presence of the hydroxyl function that presents an alternative adduct structure within the active site of T4 DNA polymerase. Disruption of hydrogen bond formation by the hydroxyl function may alter the Watson-Crick geometry of the incoming nucleotide, increasing the likelihood of a non productive complex or insertion of a nucleotide that does not facilitate polymerase extension of a mismatch (Baker & Reha-Kranz, 1998). Sequence context

effects are observed for pauses generated by T4 DNA polymerase due to aminofluorene and acetylaminofluorene (Strauss & Wang, 1990).

A reduction in the bacterial transformation ability was observed when using concentrations of activated 4-hydroxytamoxifen higher than 30μM. This dose-dependent decrease in transformation efficiency has been described for 2-acetylaminoflourene, where a 30-fold reduction in the transformation ability is seen when compared to 2-aminoflourene, with *in vitro* adducted plasmids (Fuchs & Seeberg., 1984). This is related to the ability of AAF to block RNA polymerase progression, which therefore, acts as a cytotoxic lesion. 4-Hydroxytamoxifen may also be acting as a cytotoxic lesion at these higher concentrations, by blocking the *E. coli* RNA polymerase transcribing the ampicillin resistance gene.

4.7.3 Correlation of Gross Adduct Formation to the Mutation Frequency

Gross DNA adduct formation was assessed by ^{32}P-postlabelling and was related to the mutation frequencies obtained from both α-acetoxytamoxifen and 4-hydroxytamoxifen adducted pLIZ plasmid DNA. An increase in the mutation frequency was observed for both compounds, each peaking at different concentrations (fig 51A+B). It is clearly shown from the results that α-acetoxytamoxifen formed approximately four fold more adducts, at 50μM than at 10μM, whereas 4-hydroxytamoxifen gives no increase (fig 49C). This data showed that adducts produced by 4-hydroxytamoxifen plateau at 10μM. However, the α-acetoxytamoxifen mutation frequency was approximately two orders of magnitude lower than for activated 4-hydroxytamoxifen. Therefore, no correlation between gross adduct formation and mutation frequency exists, indicating that total adduct number may not be used to examine the mutagenic potential of

tamoxifen.

pLIZ plasmid DNA treated with HRP/H_2O_2 alone, produced a significant mutation frequency via the formation of oxidative damage and was used as the control to compare the mutation frequency from activated 4-hydroxytamoxifen treatment. Oxidative damage produced by the metabolism of hydrogen peroxide by horse radish peroxidase, results in the formation of the hydroxyl radical ($OH^{\cdot}$)(O'Conner *et al.*, 1985). The hydroxyl radical causes the formation of DNA strand breaks, DNA protein cross links, the generation of AP sites, and predominant lesion of 7-hydro-8-oxo guanine (8-oxo dG) as well as 5,6-dihydro-5,6-dihydroxythymine (thymine glycol) (Essigmann & Wood, 1993), and ring opened base products (Randerath *et al.*, 1996). 8-oxo dG, which is mutagenic *in vivo* (Fuciarelli, 1989; Gajewski, 1990; Glickman *et al.*, 1980) and *in vitro* (Grosovsky *et al.*, 1988; Guschlbauer *et al.*, 1991), exists as the 6,8-diketo tautomer at physiological pH in a *syn* conformation. Thymine glycol is also mutagenic and inhibits many DNA polymerases *in vitro* (Patel *et al.*, 1984, 1986a,b, 1985). Data from the T4 DNA polymerase arrest assay did not detect pause sites on thymine for HRP/H_2O_2 treated or HRP/H_2O_2 activated 4-hydroxytamoxifen adducted pLIZ plasmids and mutations from this control plasmid may have arisen from 8-oxo dG, AP sites and other minor oxidative lesions.

Adduct formation determined by the T4 DNA polymerase arrest assay was similar for both 4-hydroxytamoxifen and α-acetoxytamoxifen, but the mutation frequencies from both compounds indicated that adducts of 4-hydroxytamoxifen are more mutagenic. This may be because of the adduct structure, with the presence of the hydroxyl function ressiding in the active site of DNA polymerase III holoenzyme, producing an

alternative Watson-Crick geometry, that allows a higher mis-incorporation rate during translesional synthesis across the adduct. Alternatively, alteration in DNA secondary structure may alter the interactions of the adduct within the active site or perturb polymerase-template interactions.

4.7.4 Correlation Between Adduct Formation and Mutation

Mutations arising on plasmid pLIZ will occur rapidly because plasmids introduced by transformation, rapidly establish their copy number within the bacteria before chromosomal replication and cell division (Highlander & Novick, 1987; Thomas *et al.*, 1998). Therefore, 4-hydroxytamoxifen and α-acetoxytamoxifen adducted pLIZ plasmid will become diluted very quickly. This may effect the efficiency of mismatch repair, that the proofreading function of DNA polymerase III holoenzyme has missed, and allow plasmid replication before errors can be corrected (Singh *et al.*, 1998).

Sequencing data from mutant pLIZ plasmid DNA showed that the types of mutation between α-acetoxytamoxifen and 4-hydroxytamoxifen were different (table 3 and 4). This observation may be attributed to the presence of the hydroxyl group at position 4 of tamoxifen. This may present an alternative hydrogen bond pattern to incoming nucleotides within the active site of DNA polymerase III holoenzyme with the extension of the mismatch being kinetically favourable. The mutational hot spot at dG^{64} of *lacI* on 4-hydroxytamoxifen adducted pLIZ may be attributed, to the presence of the 4-hydroxytamoxifen adenine adducts at positions 61 or 62, which may distort the secondary structure of this sequence. This distortion may cause perbutation of the 6-8 base interactions between template and DNA polymerase III holoenzyme (Hatehet *et al.*, 1998) causing mutation by the mechanisms already described (chapter 3). It is also

possible that the dG-N^2-4-hydroxytamoxifen adduct at this position distorts the template in this sequence that only facilitates kinetic extension and/or insertion of dG opposite the adducted base, or may be acted upon in an SOS dependent manner.

The SCS8 *E. coli* used in this study are *rec A* negative, and therefore, do not pocess an inducible SOS response mechanism. Induction of the SOS response has been reported for *rec A* negative bacteria by the development of competence (Martin *et al.*, 1995). This is because Lex A cleavage occurs via intramolecular, as well as intermolecular reactions, in the absence of Rec A protein (Kim & McHenry, 1996; Little, 1984; Little., 1993; Little, 1991), which facilitates the efficiency of cleavage under physiological conditions. Development of competence may alter the pH of the bacterial periplasm facilitating Lex A cleavage at an alkaline pH. UmuD can also be post-translationally processed in the absence of Rec A, to generate catalytically active UmuD' (Peat *et al.*, 1996). Lex A repression could therefore be lifted in competent bacteria, mediating an SOS response. This response may be significant enough to allow mutasome complex formation, that will reduce the fidelity of DNA polymerase III holoenzyme to insert G opposite dG-N^2-4-hydroxy tamoxifen adducts. Low levels of mutation were observed for the adducts reported on the adenines. Activation of the SOS response may lead to the formation of the uvrABC repair proteins, which may efficiently repair α-acetoxytamoxifen DNA adducts. However, this is unlikely if these adducts are less distorting than the adducts produced by 4-hydroxytamoxifen, as discussed above. Also, inhibition of the uvrABC response is likely to arise from the transformation procedure which induces the heat shock protein (HSP) σ^{32}, a product of the *rpo H* (*htp R*) gene (Gross & Watson, 1996). HSP σ^{32} increases the expression of the *omp T* gene product, which inactivates uvrB by proteolytic cleavage into uvrB* (Caron &

Grossman, 1988).

The major *in vivo* adduct of tamoxifen produced in rat liver is derived from α-sulphoxytamoxifen, of which α-acetoxytamoxifen is a model ester producing identical adducts. The adducts formed by 4-hydroxytamoxifen quinone methide are formed in very low numbers in rat liver (Martin *et al.*, 1998). Data from the Big Blue™ rat *lacI* assay with α-hydroxytamoxifen showed that the precursor for α-sulphoxytamoxifen produced a much lower mutation frequency than tamoxifen alone (Davis *et al.*, 1999 unpublished data). This strongly correlates with data generated in this study, which indicates that the adducts of α-acetoxytamoxifen are the main adducts produced in the rat by tamoxifen and may not be strongly mutagenic. However, minor adducts, such as those formed by 4-hydroxytamoxifen are particularly mutagenic and cytotoxic and may contribute significantly to the mutagenic spectrum of rats dosed with tamoxifen.

4.7.5 Conclusion

This study has shown guanine to be the main site of adduct formation, *in vitro*, using the T4 polymerase arrest assay of both 4-hydroxytamoxifen and α-acetoxytamoxifen adducted pLIZ plasmid DNA. Moreover, the identification of an adduct hotspot for both compounds, of two adducted adenines flanked by guanines was detected in the *lacI* gene. This region of the gene was also a mutation hotspot for 4-hydroxytamoxifen. The mutation frequencies observed strongly suggest that 4-hydroxytamoxifen adducts are more mutagenic than those formed by α-acetoxytamoxifen. This indicates that the presence of the hydroxyl function at

position 4 increases the probability of inaccurate translesional DNA synthesis by the mechanisms described. Alternatively, 4-hydroxytamoxifen may be more disruptive to the secondary structure of the DNA helix, which may stall the replisome complex sufficiently to induce an SOS response, or mutation may occur from transcription coupled error prone repair. This study also highlights the lack of correlation of gross adduct formation and mutagenic potential and portrays the importance of minor DNA adduct formation.

SITE SPECIFIC TAMOXIFEN DNA ADDUCT FORMATION IN RAT LIVER AND PRIMARY HEPATOCYTE CULTURES

5.1 Chapter Objectives

The objectives of this study were:

1. to optimise the single strand ligation assay using T4 DNA polymerase for the determination of a) tamoxifen DNA adduct formation and sequence selectivity in the *lacI* gene of Big Blue™ and *mdr1b* promoter from rat liver, b) α-hydroxytamoxifen DNA adduct formation and sequence selectivity in the *mdr1b* promoter of Wistar rat liver.
2. to determine α-hydroxytamoxifen DNA adduct formation and sequence selectivity in primary Fischer F344 rat hepatocyte cultures.

5.1.1 INTRODUCTION

Carcinogen exposure has been correlated to DNA adduct formation, mutation, and tumour formation in animal models. An understanding of the mutagenic processes *in vivo* will be achieved from an awareness of the DNA adduction frequency and distribution in critical genes assessed against the types of mutation and location of mutational hotspots (Pfeifer & Riggs, 1996). These observations help illustrate the importance of studying DNA damage at the nucleotide level , in cellular DNA that exists in a highly ordered structure complexed with many proteins. The study of *in vivo* DNA adduct formation also takes into account all intracellular components that may interfere with carcinogen binding and detoxification pathways (Grimaldi *et al.*, 1994; Pfeifer & Riggs, 1996).

Several methods are used for the quantitative nucleotide resolution of DNA adduction in mammalian genomic DNA (Zaret, 1997; Pfeifer, 1996; Komura & Riggs, 1998). One such method is ligation mediated PCR (LMPCR) (Pfeifer *et al.*, 1989; Mueller & Wold, 1989; Pfeifer *et al.*, 1993). This method is sensitive but has limitations requiring direct measurement of nicks or breaks in DNA induced from chemical (piperidine) or enzymatic (UvrABC endonuclease) reactions (Pfeifer *et al.*, 1991; Tornaletti & Pfeifer, 1994), followed by exponential PCR of the fragmented double stranded DNA. However, even under optimal conditions UvrABC endonuclease incises DNA at low efficiency, converting a fraction of the adducts into strand breaks (Thomas *et al.*, 1988). Many lesions are not detected using this method due to the lack of formation of cleavable adducts such as the lesions produced by ionising radiation (Friedberg *et al.*, 1985; Ward, 1988).

The polymerase arrest assay is a versatile method, useful in mapping many kinds of DNA lesions that inhibit polymerase progression (Ponti *et al.*, 1991). Lindsley & Fuchs, (1994) proposed that the polymerase arrest assay may preferentially detect the most biologically relevant DNA adducts as highly mutagenic adducts inhibit polymerases progression more than less mutagenic adducts. However, for a DNA adduct to be mutagenic and not cytotoxic, efficient translesional DNA synthesis over the lesion is necessary. Therefore, the structural and kinetic characteristics of the polymerase will determine the overall effiency of DNA adduct detection.

The detection of DNA adducts in genomic DNA at the nucleotide level using the polymerase arrest assay is compromised by that of sensitivity. The measurement of sites of adduct formation requires the use of linear DNA amplification. Products of this

elongation reaction can only be detected for highly purified plasmid DNA bearing a significant proportion of DNA modification. The probability of adduct formation in a single copy gene is low (Ponti *et al.*, 1991). This problem is overcome by exponential amplification of terminated single stranded DNA extension products produced by the initial linear amplification (Grimaldi *et al.*, 1994; Komura & Riggs, 1998; Pfeifer & Riggs, 1996).

5.2 Materials and Methods

5.2.1 Materials

Female Wistar rat liver and Big Blue™ rat liver were gifts of Dr E.A Martin and Dr R. Davies respectfully. Female Fischer F344 rats (200g) were obtained from Harlan Olac (Oxford, UK). Deoxyoligonucleotide primers were synthesised using standard phosphoramidite chemistry from PNACL (Leicester, UK). Calcium chloride, EGTA, glutamine, dexamethasone, HEPES, TES, Tris, β-mercaptoethanol, PMSF, sucrose, magnesium chloride, sodium chloride, SDS, phenol and 5-(3-aminoallyl)-2'-deoxyuridine-5'triphosphate (AA-dUTP) were obtained from Sigma (Poole, Dorset, UK). HBSS, Williams media E, Weymouths 705/1 media, Dulbeccos modified Eagles media, sodium bicarbonate, gentamycin, insulin, Taq DNA polymerase and dNTP's were obtained from GibcoBRL, Life Technologies (Paisley, UK). Collagenase H, rat tail collagen, T4 DNA ligase, T4 RNA ligase, RNase A1, RNase T1 and proteinase K were obtained from Boehringer Mannheim (Lewes, East Sussex, UK). T4 DNA polymerase, terminal deoxynucleotidyl transferase (Tdt), *HinfI*, *VspI* were obtained from Promega (Southampton, UK). The remaining chemicals unless stated were of the highest grade available.

5.2.2 Methods

5.2.3 Animal Treatments

Female Big Blue™ rats (from Harlan Olac, Oxford, UK), 6-8 weeks old were dosed orally with 20mg/kg/day tamoxifen (dissolved in tricaprylin) for 6 weeks, and control group received tricaprylin (1ml/kg/day) for 6 weeks. Both animal groups were culled 6 weeks after the last dose and the livers stored at -80°C until DNA isolation. Female Wistar (LAC-P) rats 6 weeks old (bred on site) dosed orally with 40mg/kg/day α-hydroxytamoxifen (dissolved in tricaprylin) for 5 days and control group received tricaprylin (1ml/kg/day) for 5 days. Both animal groups were culled on day 6 and the livers stored at -80°C until DNA isolation.

5.2.4 Liver Perfusion for the Isolation of Primary Hepatocytes From Fischer F344 Rats

HBSS, 500mls devoid of phenol red, calcium and magnesium ions, containing 0.13% sodium bicarbonate was circulated through the sterilised perfusion apparatus (fig 53) and oxygenated with 95% oxygen, 5% carbon dioxide for 10 minutes at 37°C. After oxygenation, 100mls of this buffer was dispensed into a sterile bottle and 30mg of collagenase H, and 58mg $CaCl_2$ was added. This solution was kept at 37°C to be used for recirculation. To the remaining 400mls, 76mg of EGTA was added and kept at 37°C.

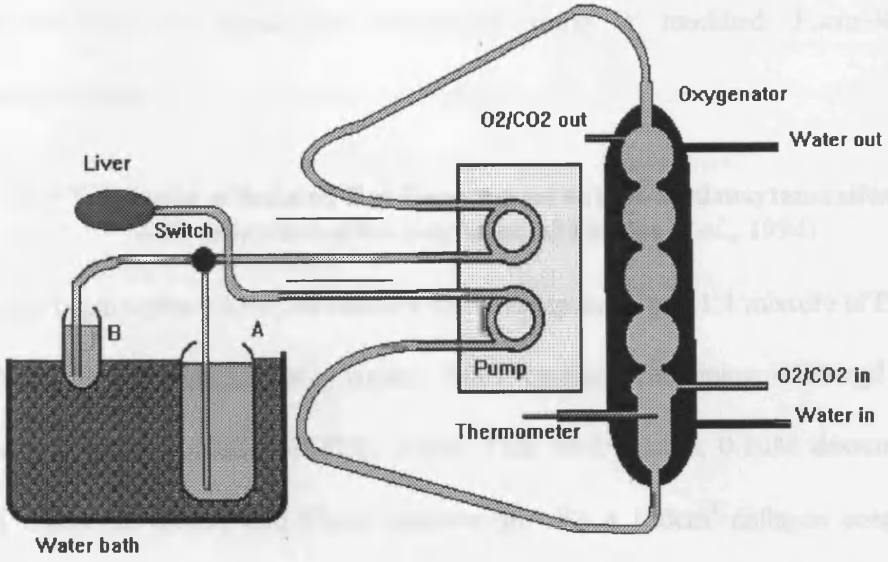


Fig 53 - Schematic diagram of the perfusion apparatus.

Female Fischer F-344 rats 8-10 weeks old (200g) were anaesthetised with 0.7ml Sagatal (May & Baker) ip. The hepatic portal vein was exposed and cannulated using a 16G Argyle Medicut intravenous cannula filled with heparin (1000units/ml) (CP Pharmaceuticals) and tied to the vein using strong thread. The descending vena cava was cut and the liver perfused with oxygenated HBSS/EGTA buffer at 50ml/min until all blood from the liver was cleared. The liver was then perfused with the oxygenated HBSS/collagenase H/$CaCl_2$ solution for 20 minutes or until the liver showed signs of breaking up. After this time the liver was excised and transferred to a sterile petri dish and scored with a scalpel. Ice cold Williams media E containing 10% foetal calf serum was added to the scored liver and gently agitated to separate the hepatocytes. The suspended hepatocytes were filtered through a nylon cloth (Nyblot N° 10.5, 125µm; John Stanier & Co Ltd, Manchester, UK) into a sterile centrifuge tube. Hepatocytes were sedimented by centrifugation at 50g for 3 minutes. This washing step was repeated to a total of three times. Finally the hepatocytes were resuspended into 30mls cold media. Viability and cell number was determined by the addition of 90µl trypan

blue to 10µl of hepatocyte suspension using a modified Fuchs-Rosenthall haemocytometer.

5.2.5 Treatment of Isolated Rat Hepatocytes with α-Hydroxytamoxifen and 4-Hydroxytamoxifen (variation of Phillips *et al.*, 1994)

Primary hepatocytes ≥80% cell viability were resuspended in a 1:1 mixture of Dulbeccos modified Eagles medium: Weymouths 705/1 medium containing 4500mg/l glucose, 2mM L-glutamine, 10mM HEPES, 10mM TES, 5mU insulin, 0.1µM dexamethasone, 10% foetal calf serum, and 25µg/l gentamycin. To a 120cm^3 collagen coated tissue culture dish, 13x10^6 cells were seeded and allowed to attach for 4 hours in a humidified incubator at 37°C with a 5% CO_2 atmosphere. After cell attachment, unattached cells were removed and the medium replaced as above but with out foetal calf serum. α-Hydroxytamoxifen was added in DMSO (<1% DMSO final concentration in media) to give final concentrations of 5, 10, 20 or 50µM and incubated for 18 hours before their nuclei were isolated.

5.2.6 Isolation of Nuclei from Primary Fischer F344 Rat Hepatocyte Cultures

To a 120cm^3 tissue culture dish containing 13x10^6 primary rat hepatocytes, 2mls of lysis buffer, 0.5M NaCl, 10mM Tris-HCl, pH8.0, 2mM EDTA, pH8.0, 0.1% NP40, 1% β-mercaptoethanol, and 0.1mM PMSF was added. The hepatocytes were scraped off the dish and the nuclei sedimented by centrifugation at 1000x g for 5 minutes at 4°C. The pellet was collected and resuspended in a buffer containing 0.25M sucrose, 5mM $MgCl_2$, 10mM Tris-HCl, pH8.0, 10mM dithiothreitol, and 0.1mM PMSF, and layered over the same buffer containing 1.1M sucrose. This was centrifuged at 12000x g for 15 minutes at 4°C. The sedimented nuclei were collected for high molecular weight DNA extraction.

5.2.7 Isolation of Genomic DNA

To homogenised rat liver samples or nuclei extracted from primary rat hepatocytes, 5.4mls DNA extraction buffer, 0.15M NaCl, 10mM EDTA, 10mM Tris-HCl pH 8.2, 1% SDS was added in a 15ml polypropylene centrifuge tube. All samples were inverted gently to mix. The samples were then heated to 65°C for 2 minutes followed by the addition of 1.2mg of RNase A1 and 1mg of heat inactivated RNase T1 and incubated at 37°C for 2 hours. After incubation, 1.2mg proteinase K was added and incubated at 37°C overnight before being transferred to 50ml capped polypropylene tubes. The DNA samples were then extracted against 11mls of Tris-HCl (pH8.0) buffered phenol inverted gently for 10 minutes before being centrifuged at 630x g for 10 minutes. Using a wide bore pipette tip, high molecular weight DNA was removed to a fresh tube and extracted with 6mls of 1:1 phenol:chloroform, then 6mls chloroform. If during the extraction steps, the DNA was too viscous, 1ml DNA extraction buffer containing 1% SDS was added. High molecular weight genomic DNA was precipitated by the addition of 600µl 7.5M ammonium acetate (pH7.5) and 12mls ice cold 95% ethanol. DNA was resuspended in DNA storage buffer containing 1.5mM NaCl and 0.15M sodium citrate (pH7.5).

5.2.8 Single Strand Ligation PCR (modified version, Grimaldi *et al*, 1994)

The following protocol assayed the transcribed strand of rat *mdr1b* promoter or the Big Blue™ *lacI* transgene. The *mdr1b* promoter was assayed from genomic DNA extracted from primary rat hepatocyte cultured in the presence of α-hydroxytamoxifen and α-hydroxytamoxifen treated female Wistar rats. Genomic DNA was incubated with 10 units *VspI* in a buffer containing 6mM Tris-HCl pH7.9, 6mM $MgCl_2$, 150mM NaCl, 1mM dithiothreitol at 37°C overnight. DNA extracted from Big Blue™ rats for analysis

of the *lacI* transgene was digested with 10 units *HinfI* in a buffer containing 6mM Tris-HCl pH7.5, 6mM $MgCl_2$, 50mM NaCl, 1mM dithiothretol at 37°C overnight. DNA was precipitated from solution by the addition of 0.1 volumes sodium acetate pH5.4 and 2 volumes ice cold ethanol.

Oligonucleotide primers

Rat *mdr1b* promoter

NT-1-B (5' biotin labelled): 5'-d(AGCGGCCTCCAGCCTCTTAC)

NT-1: 5'-d(CAGCCTCTTACAGCTTCAAG)

^{32}P-labelled primer: 5'-d(TTACAGCTTCAAGAGCCGCT)

LacI

NT-1-B (5'biotin labelled): 5'-d(ATCAGCCCACTGACGCGT)

NT-1: 5'-d(GTTGCGCGAGAAGATTGTG)

^{32}P-labelled primer: 5'-d(CGAGAAGATTGTGCACCG)

Common:

Ligation primer: 5'-P-d(TATGACTATGCATGATCTACGAT)

5.2.8a First Round PCR (linear PCR)

First round, linear PCR (fig 54) was carried out in a total of 20µl buffer containing 3µg adducted/unadducted genomic DNA, 20pmoles primer NT-1-B, 67mM Tris-HCl (pH8.8), 16mM $(NH_4)_2SO_4$, 6.7mM $MgCl_2$, 6.7µM EDTA, 167µg/ml BSA, 10mM β-mercaptothanol buffer, and 250µM of each dNTP. DNA was denatured by heating to 95°C for 2 minutes and the primer annealed by subsequent cooling to 4°C. Extension was initiated by the addition of 4 units of T4 DNA polymerase. Elongation was allowed to proceed for 30 minutes at 37°C then cooled to 4°C.

5.2.8b Capture and Ligation

Extension products from the linear PCR reaction were purified by the addition of 5µl 5x washing and binding buffer (WBB) (25mM Tris-HCl pH7.6, 5mM EDTA, 5M NaCl) to the PCR mixture containing, 20µl (200µg) WBB washed streptavidin M-280 Dynabeads (Dynal, UK). The suspension was incubated at 37°C for 2 hours with occasional shaking. The beads were sedimented in a magnetic rack and washed three times with 200µl TE (10mM Tris-HCl pH 7.6, 1mM EDTA) and resuspended in 10µl ligation mixture, 30mM Tris-HCl pH7.8, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25pmoles ligation oligonucleotide (5'P-ATCGTAGATCATGCATAGTCATA), 10units T4 DNA ligase, and 20 units T4 RNA ligase. The mixture was ligated overnight at 16°C, washed three times with 200µl TE pH7.6, and resuspended in 10µl ultrapure sterile water, and transferred to a 0.5ml thin walled PCR tube for second round PCR.

The ligation oligonucleotide was 3' aminoacyl blocked to stop self ligation in a total volume of 100µl containing 100mM cadodylate pH6.8, 1mM $CoCl_2$, 10mM $MgCl_2$, 0.1mM dithiothreitol, 10mM $MnCl_2$, 5µg ligation oligonucleotide, 0.4mM 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (AA-dUTP), and 50 units terminal deoxynucleotidyl transferase (Tdt) and incubated at 37°C for 2 hours. The reaction was stopped by the addition of 25mM EDTA. The reaction mixture was extracted twice against equal volumes of 1:1 phenol:chloroform once against an equal volume of chloroform. The aminoacyl blocked ligation oligonucleotide was further purified using a Chroma Spin™-10 gel filtration spin column (Clontech, Heidelberg, Germany).

5.2.8c Second Round PCR

Second round PCR (fig 54) was performed in a volume of 20µl containing, 20mM Tris-HCl pH8.4, 50mM KCl, 10µl captured and ligated extension products, 20pmoles NT-1 primer, 20pmoles ligation primer, 250µM dNTPs, 2.5mM $MgCl_2$, and 2.5 units Taq DNA polymerase. The cycling conditions consisted of an initial denaturation at 96°C for 5 minutes, then 28 cycles of 96°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute + 2 seconds extension per cycle with a final 5 minute extension at 72°C.

5.2.8d Third Round PCR

Third round PCR (fig 54) was carried out by the addition 10µl PCR reaction buffer containing 20mM Tris-HCl pH8.4, 50mM KCl, 250µM dNTPs, 2.5mM $MgCl_2$, 20pmoles ^{32}P labelled primer and 1 unit Taq DNA polymerase to the PCR mix from the second round PCR reaction. The primer was ^{32}P end labelled using $\gamma^{32}P$-ATP, 3000Ci/mmol (Amersham) and 5 units T4 polynucleotide kinase (Promega), in a total volume of 10µl containing 200pmoles deoxyoligonucleotide primer, 70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 5mM dithiothreitol at 37°C for 30 minutes. The labelled primer was separated from unincorporated ^{32}P-ATP using a Chroma Spin™-10 gel filtration spin column (Clontech, Heidelberg, Germany). This mixture was subjected to an initial denaturation step of 96°C for 5 minutes, then 10 cycles of 96°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute + 2 seconds per cycle with a final 5 minute step of 72°C. When amplification was completed, 5µL stop solution was added (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole). The extension products were denatured by heating to 95°C for 3 minutes followed by rapid cooling on

ice. The DNA fragments were resolved on a 0.4mm by 60cm 6% polyacrylamide gel containing 8M urea and a tris-taurine-EDTA buffer system (Amersham). The gel was run at 60 watts which maintained a temperature of 50°C for approximately 2.5 hours. Gels were fixed in a 10% methanol, glacial acetic acid solution for 15 minutes and dried onto Whatman 3MM paper at 80°C for 1 hour. Gels were visualised using a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

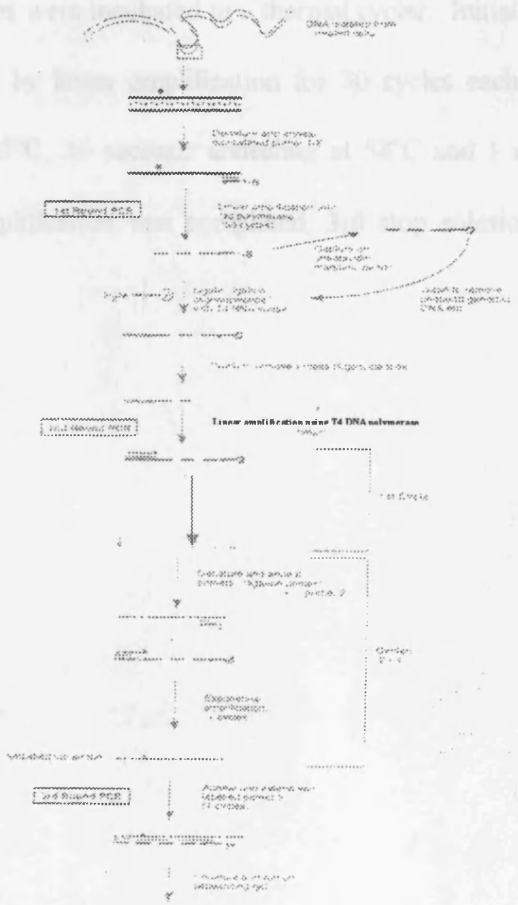


Fig 54 – Schematic diagram of the sslig PCR assay. Diagram taken from Grimaldi et al in Technologies for detection of DNA Damage and mutations, Plenium Press, NY, 1996

5.2.9 DNA Sequencing Reactions

DNA sequencing was performed using the Promega fmol® DNA sequencing system. Sequence reactions were performed in 6µl containing 100ng pLIZ plasmid DNA, 50mM Tris-HCl (pH 9.0), 2mM $MgCl_2$ buffer, 1.5pmoles ^{32}P labelled 5'-GTACCCGACACCATCGAATG-3' primer, 20µM dATP, dTTP, dCTP, 7-Deaza dGTP with either 30µM Deaza ddGTP, 350µM Deaza ddATP, 600µM Deaza ddTTP, 200µM Deaza ddCTP and 5 units sequencing grade *Taq* DNA polymerase. After gentle mixing the samples were incubated in a thermal cycler. Initial denaturation at 95°C for 2 minutes followed by linear amplification for 30 cycles each consisting of 30 seconds denaturation at 95°C, 30 seconds annealing at 58°C and 1 minute chain elongation at 72°C. When amplification was completed, 3µl stop solution was added and samples treated as above.

5.3 Results

5.3.1 Analysis of tamoxifen DNA adduct formation in the *lacI* gene of hepatic DNA extracted from Big Blue™ rats

Hepatic tamoxifen DNA adduct formation was assessed at the nucleotide level of the *lacI* gene from transgenic Big Blue™ rats. Rats dosed with 20mg/kg/day tamoxifen produced T4 DNA polymerase pause sites (fig 55). No pause sites were detected in the *lacI* gene from Big Blue™ rats dosed with 5 or 10mg/kg/day tamoxifen. Tamoxifen DNA adduct concentration measured by ^{32}P-postlabelling gave measurements of 601 ± 88 adducts/10^8 normal nucleotides for rats dosed with 20mg/kg/day tamoxifen and 37 ± 8.3, 132 ± 14.6 and 199.3 ± 31.6 adducts/10^8 normal nucleotides for control, 5 and 10mg/kg/day tamoxifen respectively.

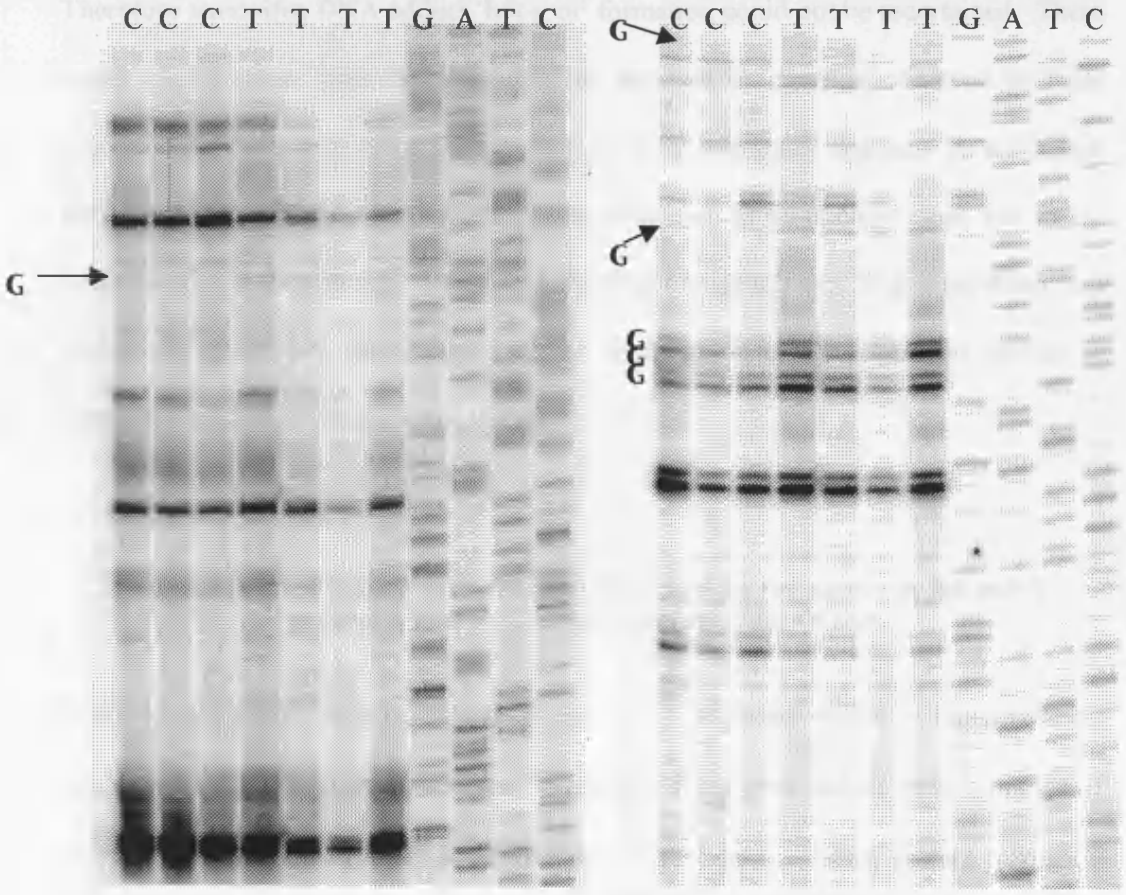


Fig 55 – **Single Strand Ligation Assay (sslig) performed on the *lacI* gene extracted from Big Blue™ rat liver.** C=control Big Blue™ rats, T= Big Blue™ rats dosed with 20mg/kg tamoxifen for 6 weeks and culled 6 weeks after the last dose. The DNA sequencing ladder does not correspond to sites of adduct formation, but is used to determine the position of the pause sites after the nested PCR steps. N=3

Exponential amplification of the linear extension products produced by T4 DNA polymerase from rats dosed with 20mg/kg/day tamoxifen indicated that this polymerase paused predominantly on guanine. Sequence specific guanine adduct formation mainly occurred when the reactive guanine was located 3' or 5' to a cytosine. Guanine pause sites were also observed in other sequences but to a lesser extent. Tamoxifen DNA adduct formation was not always observed at the same position from all experiments performed which may indicate limitations of the assay or loss of adducts from the DNA between experiments. The relative intensities of the pause sites between experiments were not consistent, probably due to variations in ligation and PCR efficiencies. Therefore tamoxifen DNA adduct 'hot spot' formation could not be ascertained. There were several pause sites corresponding to thymine or cytosine observed in most experiments. The amount of pausing at these sites was small and may be artifactual indicating that guanine was the only site of adduction as determined from this assay. Guanine adduct formation in the liver of Big Blue™ rats reflects ^{32}P-postlabelling data that shows guanine to be the major site of modification by DNA reactive species of tamoxifen (Osbourne et al., 1996, 1997).

5.3.2 Analysis of α-hydroxytamoxifen DNA adduct formation in the *mdr1b* promoter of female Wistar and Big Blue™ rats

DNA adduct formation was assessed in the *mdr1b* promoter region of female Wistar rats dosed with α-hydroxytamoxifen. ^{32}P-postlabelling gave adduct concentrations of 1444 ± 384 adducts/10^8 normal nucleotides respectively. DNA adduct formation primarily occurred on adenine and polymerase pause sites also corresponding to guanine were observed (fig 56). Sequence specificity of putative adenine adduct

formation occurred in runs of dA's. Adenine adducts of tamoxifen are only minor adducts (Osbourne et al., 1996, 1997). This may indicate that adenine is a preferred site of adduction for α-hydroxytamoxifen *in vivo* or these adducts are more resistant to the repair processes. In contrast the same region of the *mdr1b* promoter was also assayed from Big Blue™ rats dosed with 20mg/kg/day tamoxifen. Putative DNA adduct formation in this region were mostly found on guanine, but results were not consistent between experiments.

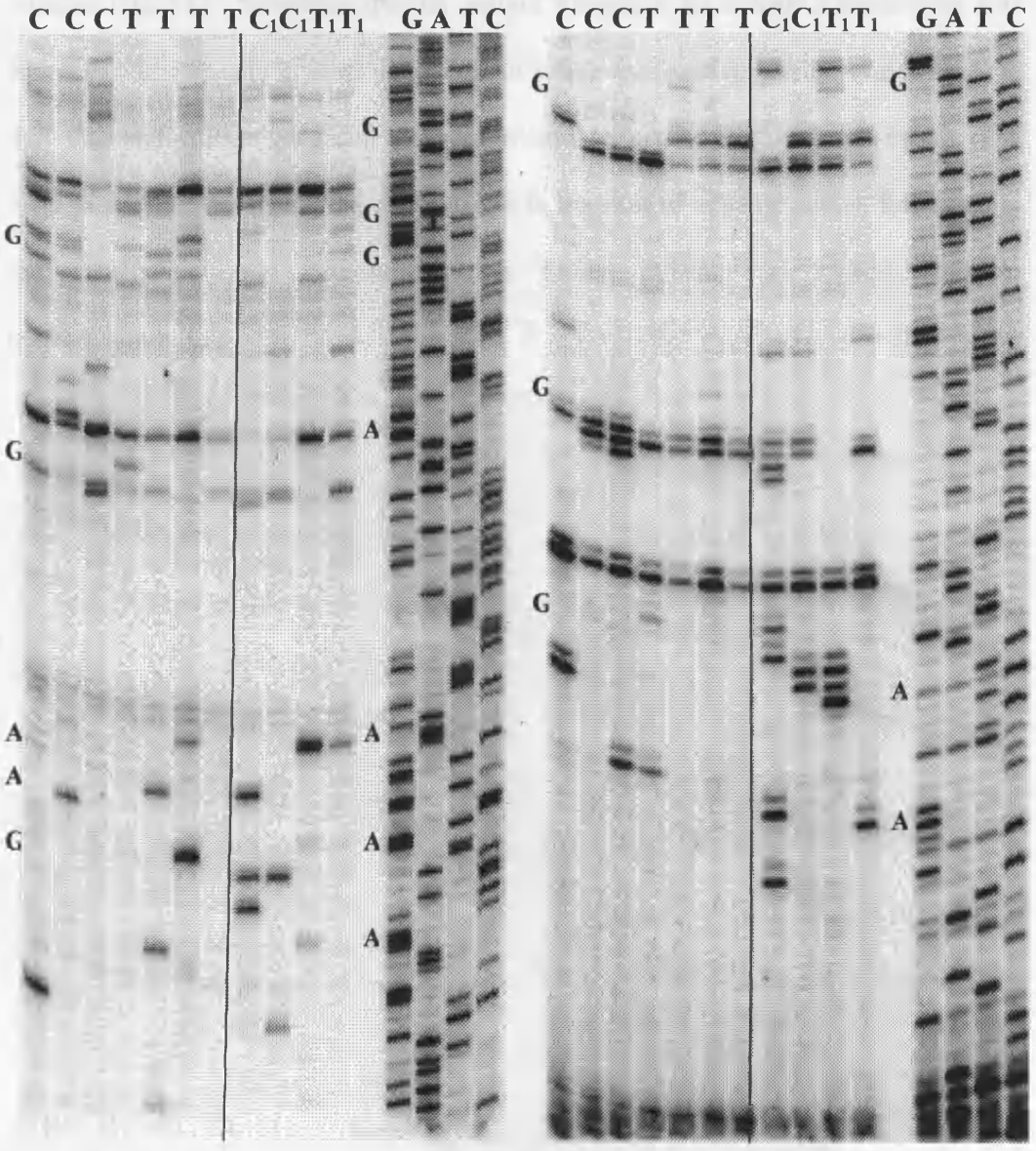


Fig 56 – **Sslig assay in the *mdr1b* promoter. DNA was extracted from both Big Blue™ rats treated with tamoxifen and Wistar rats treated with α-hydroxytamoxifen.** C= control (Big Blue™ rat), T= Big Blue™ rats dosed with 20mg/kg tamoxifen for 6 weeks and culled 6 weeks later; C_1= control (Wistar rat), T_1= Wistar rats dosed with 40mg/kg α-hydroxytamoxifen for 5 days and culled on the 6th day. Only pause sites corresponding to either dG or dA have been indicated. The sequencing ladder does not correspond to the sites of DNA adduct formation, but is used to determine the position of the pause sites after nested PCR. N=2

5.3.3 Analysis of DNA adduct formation in the *mdr1b* promoter from DNA extracted from Fischer F344 primary hepatocyte cultures exposed to α-hydroxytamoxifen

DNA adduct formation was assessed in the *mdr1b* promoter region from DNA extracted from hepatocytes cultured in the presence of α-hydroxytamoxifen. Putative DNA adduct formation was on guanine, with DNA adduct formation also occurring on adenine (fig 57). Sequence specific adduct formation by α-hydroxytamoxifen was found to occur equally in dGG sequences or where the reactive guanine was 3' or 5' to dC. Guanine adducts were also observed at other sequences but to a lesser extent. The level of adenine adduction did not correlate to the sites of adenine adduct formation in the *mdr1b* promoter determined from female Wistar rats treated with α-hydroxytamoxifen.

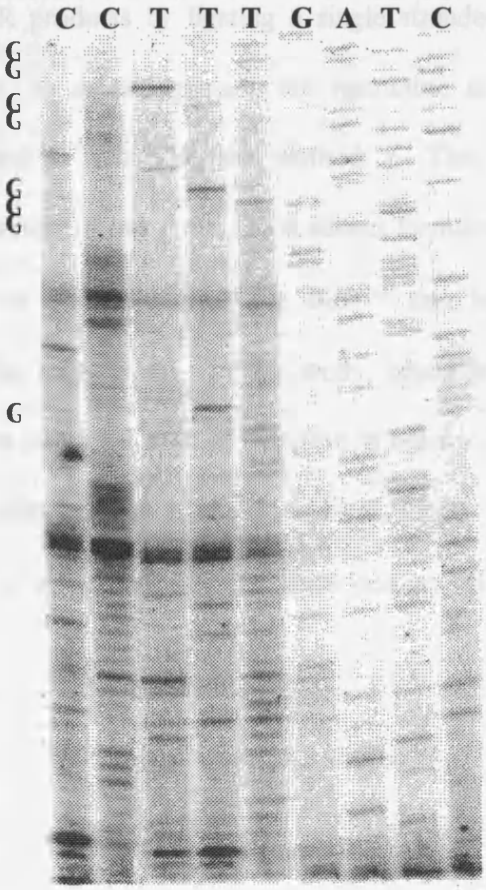


Fig 57 – **Sslig assay on the *mdr1b* promoter from DNA extracted from primary Female Fischer F344 rat hepatocytes**. C= control, T= hepatocytes incubated in the presence of 20μM α-hydroxytamoxifen for 18 hours. The sequencing ladder does not correspond to the sites of DNA adduct formation, but is used to determine the position of the pause sites after nested PCR. N=3

5.4 Discussion

The antioestrogen tamoxifen, has been shown to induce hepatic tumours in rats. It is commonly thought that tamoxifen exerts its tumourogenic action via genotoxic effects of tamoxifen DNA adduct formation (Greaves *et al.*, 1993; Han & Liehr, 1992; White *et al.*, 1992; Phillips *et al.*, 1994c Sargent *et al.*, 1994). LMPCR is one of the main methods employed to resolve DNA lesion formation of genomic DNA for *in vitro* studies of mammalian cells (Zaret, 1997; Pfeifer, 1996). This method is sensitive but limited since it is only able to detect DNA adducts that cause nicks or breaks in DNA. Grimaldi et al., (1994), determined binding sites for cisplatin using genomic DNA isolated from cells by combining the polymerase arrest assay with exponential amplification of the linear PCR products by ligating a single stranded linker to the extension products. To date, no studies present the resolution of DNA adduct formation at the nucleotide level *in vivo* using this method. This study seeks to determine the site specific sequences of tamoxifen DNA adduct formation *in vivo* from DNA extracted from the livers of Wistar and Big Blue™ rats treated with α-hydroxytamoxifen and tamoxifen respectively. In this study, tamoxifen DNA adduct formation was correlated to the published sites of mutation in hepatic DNA from Big Blue™ rats treated with tamoxifen (Davies et al., 1996, 1997, 1999). Data from this study facilitated a comparison of α-hydroxytamoxifen from primary rat hepatocytes *in vitro* to those obtained *in vivo*.

5.4.1 Analysis of Tamoxifen DNA Adduct Formation in the *LacI* Gene of Hepatic DNA Extracted From Big Blue™ Rats

Results from the sslig assay employing hepatic DNA extracted from Big Blue™ rats demonstrated tamoxifen DNA adduct formation at guanine in the *lacI* gene (fig 55). Pause sites, although present, were weak and detected in animals dosed with 20mg/kg tamoxifen only. Sequence specific tamoxifen DNA adduct formation was observed mainly at reactive guanine sites located 3' or 5' to a neighbouring cytosine. It was noted that Big Blue™ rats treated with this dose regimen of tamoxifen exhibited a 3 fold increase in total number of tamoxifen DNA adducts compared to Big Blue™ rats dosed at 10mg/kg/tamoxifen. This assay did not detect pause sites corresponding to adenine adduct formation in the region of the *lacI* gene examined. This may be explained by the fact that the numbers of tamoxifen adenine DNA adducts were below the lower limits of detection for this assay. Alternatively, lack of observable tamoxifen adenine DNA adduct formation may be due to the existence of unfavourable chromatin structure which may have a role in the determination of site selectivity for DNA binding of reactive electrophiles of tamoxifen. The sites of tamoxifen DNA adduct formation determined by the sslig assay within the *lacI* gene did not in fact correlate to positions of mutation observed in hepatic DNA taken from Big Blue™ rats dosed with tamoxifen. This may be explained by the low concentration of tamoxifen DNA adducts present in the samples being below the lower limits of detection of T4 DNA polymerase. Therefore, it is feasible to suggest that similar studies should be performed employing animals which have steady state levels of tamoxifen DNA adducts.

It is well established that the *lacI* gene has a high proportion of CpG sites. CpG sequences generally represent preferential binding sites for DNA reactive electrophiles

with consequent adduct formation. The high levels of CpG sites within the *lacI* gene may be a contributory factor to the lack of observable tamoxifen adducted adenines detected by the sslig assay. This may be explained by the inherent propensity of reactive tamoxifen metabolites to bind to these preferential sites which are present in high numbers. This will be further influenced by methylation status of these sequences which is known to affect DNA binding properties (Denissenko *et al.*, 1997), however, dC methylation status of the *lacI* gene remains unknown at present in the rat. Examples of the effect of methylation on adduct formation are adduction of dG at CpG sites by N-methyl-N-nitrosourea is inhibited when dC is methylated (Mathison *et al.*, 1993); the formation of UV photoproduct is reduced (Glickman *et al.*, 1996; Pfeifer *et al.*, 1991); conversely adduction by mitomycin C (Millard *et al.*, 1993; Johnson *et al.*, 1995) and benzo[a]pyrene diolepoxide (Denissenko *et al.*, 1997) is enhanced when dC is methylated.

Methylation of the C5 atom of dC causes electronic effects that may be transmitted to the N^2 amino group of the base paired guanine increasing its nucleophilicity (Johnson *et al.*, 1995). This effect may increase the reactivity of guanines in methylated CpG regions that enhanced the formation of N^2-dG-tamoxifen DNA adduct formation. Binding may in fact be enhanced when activated tamoxifen species intercalate at these methylated sequences which possibly serve to stabilise a conceptual transition state prior to covalent binding. This hypothesis is supported by the knowledge that α–acetoxytamoxifen only binds to single stranded DNA at very high concentrations (Shibutani *et al.*, 1997) and does not occur for HRP/H_2O_2 activated 4-hydroxytamoxifen (data not shown).

5.4.2 Analysis of α-Hydroxytamoxifen DNA Adduct Formation in the *mdr1b* Promoter of Wistar Rats

Wistar rats dosed with α-hydroxytamoxifen formed approximately double the total number of adducts in comparison to tamoxifen dosed Big Blue™ rats. Results from the *mdr1b* promoter sslig assay indicate that T4 DNA polymerase is strongly inhibited by DNA adducts derived from α-hydroxytamoxifen in this DNA region. Pausing was particularly common at adenine sites and much greater in number when compared to those associated with guanine (fig 56). However, further experiments employing larger numbers of animals are required if the sequence specificity of α-hydroxytamoxifen adenine adducted regions representing hot spots for adduct formation are to be confirmed. Tamoxifen DNA adduct formation was further assessed in the same region of the *mdr1b* promoter taken from Big Blue™ rats dosed with 20mg/kg tamoxifen (fig 55). Interestingly, the observable pattern of adduction was quite different from that obtained in Wistar rats which demonstrated pause sites predominantly at guanine in the same DNA region. The stronger pause sites attributable to tamoxifen adducted adenines suggests adenine adduct formation by α-hydroxytamoxifen may initially form at higher concentrations in studies using acute dosing protocols compared to steady state tamoxifen adduct formation *in vivo*. This difference may be explained by the total tamoxifen DNA adduct concentration and effects of DNA repair and dilution by DNA replication. Even though adenine adduct formation by the reactions of α-suphoxytamoxifen and α-acetoxytamoxifen with DNA are minor (Phillips *et al.*, 1996a) this may indicate that adenine adducts detected in the *mdr1b* promoter of Wistar rats dosed acutely with α-hydroxytamoxifen may initially form at adenine sites in high enough concentrations that can be readily detected by T4 DNA polymerase. Moreover, these adducts may be rendered more susceptible to DNA repair in comparison to

guanine adducts that form in abundance at steady state. The DNA adduction pattern may also be accounted for by the fact that tamoxifen is metabolised to other DNA reactive species of which α-hydroxytamoxifen may not form or may be more readily detoxified and excreted.

5.4.3 Analysis of DNA Adduct Formation in the *mdr1b* Promoter from DNA Extracted from Primary Fischer F344 Hepatocyte Cultures Exposed to α-Hydroxytamoxifen

Phillips *et al.*, (1994) showed that primary hepatocyte cultures incubated in the presence of α-hydroxytamoxifen, produce total adduct concentrations of approximately 5000 adducts per 10^8 normal nucleotides. Based on this information, this system was used to assess α-hydroxytamoxifen adduct formation in the *mdr1b* promoter from DNA extracted from hepatocytes cultured in the presence of α-hydroxytamoxifen. Results demonstrate that α-hydroxytamoxifen produces pause sites predominantly at guanine (fig 57) which are distinct from those formed in Wistar rats treated with α-hydroxytamoxifen. It was further noted that sequence specific guanine adduct formation by α-hydroxytamoxifen occurred in GG sequences. Results indicated that there are relatively few sites of adenine adduct formation creating pause sites compared to those obtained from experiments using hepatocyte cultures from Wistar rats. The differences in the pattern of adduct formation may be attributed to the whole animal effects of metabolism and detoxification, or species differences as the primary hepatocytes were produced from Fischer F344 rats.

5.4.4 Conclusion

This study has shown guanine to be the site of tamoxifen DNA adduct formation in the liver of Big Blue™ rats dosed with tamoxifen. However, the sites of tamoxifen DNA adduct formation in the livers of Big Blue™ rats did not correlate to the sites of mutation. Moreover, tamoxifen guanine and adenine adduct formation was observed in the liver of Wistar and primary rat hepatocyte cultures treated with α-hydroxytamoxifen. The sites of tamoxifen DNA adduct formation did not correlate between these studies. Overall, no tamoxifen DNA adduct hotspots could be assertained from these studies.

6 General Discussion

The principal aims of this study have been to determine the DNA binding sites of tamoxifen and selected metabolites at the nucleotide level *in vitro* and *in vivo*. The work was initiated following findings with certain carcinogens that DNA adduct formation is correlated to DNA mutations and subsequent cancer formation (reviewed in Greenblatt *et al.*, 1994). This project attempted to examine the links between DNA adduct formation and propensity to mutation.

DNA adduct formation is recognised as a common property of many mutagens and carcinogens. DNA adduct formation in relation to mutation frequency is governed by a number of factors such as their type and quantity, persistence, and the rate of target cell replication. *In vitro* and *in vivo* models have lead to the conclusion that some DNA adducts have no effect on DNA replication fidelity whereas, others lead to base mis-incorporations and polymerase blockage. Disruptive DNA adduct formation induces the initiation of DNA repair. DNA repair is an important component in the protection of the cell against DNA damage as it removes DNA adducts before DNA replication.

6.1 Detection of DNA adduct formation at the nucleotide level

Plasmid DNA was adducted with horseradish peroxidase/ H_2O_2 activated 4-hydroxytamoxifen. These adducts caused the inhibition of DNA synthesis by *T4* DNA polymerase, *HIV I* and *AMV* reverse transcriptase (chapter 3, figs 32, 34, 35). It has been shown that DNA adduct formation from many compounds can interfere with

polymerase extension of adducted bases by both prokaryotic and eukaryotic polymerases (Larson & Strauss, 1987; Brown & Romano, 1991; Thrall *et al.*, 1992).

Polymerases share many common catalytic and structural features. However, organisation of their distinctive sub-domains, and exonuclease activities show variations that render them sensitive to inhibition by different DNA adduct structures. The ability of a polymerase to bypass a DNA adduct is also dependent on a combination of stereochemical and steric-hindrance effects. Polymerase inhibition by *Taq* and *Klenow exo*$^+$ DNA polymerase on templates containing site specific aflatoxin B_1 DNA adducts were different (chapter 2, fig 27). However, translesional DNA synthesis occurred for 4-hydroxytamoxifen DNA adducts using these DNA polymerases (chapter 3 figs 36, 38). Previous work has identified guanine to be a major site of aflatoxin B_1 DNA adduct formation (Jacobsen *et al.*, 1987). The present study has shown that *Taq* DNA polymerase paused at the site of aflatoxin B_1 guanine adduct formation whereas *Klenow* DNA polymerase paused one base prior to the adducted guanine (chapter 2, figs 26, 27). This effect was also observed for the AFB$_1$-FAPY-N^7-dG adduct (fig 27). This may have been due to the presence of the 3'-5' exonuclease activity (proof-reading) of *Klenow exo*$^+$ DNA polymerase. The purpose of proof-reading is to remove mis-incorporated nucleotides to regenerate correctly base paired primer termini. The efficiency of proof-reading is reflected by the balance of the polymerase between polymerisation and proof-reading which is a competitive process (Johnson, 1993). From my work (chapter 2, fig 27), it may be concluded that aflatoxin B_1 DNA adducts may be miscoding lesions for *Klenow* as it was able to bypass these lesions. The exonuclease activity of *Klenow* may out compete polymerisation and remove the mis-incorporated base. This lead to chain termination *in vitro* at base 39 as opposed to base

40 for *Taq* DNA polymerase. Termination at the site of adduct formation is also determined by the kinetic effects of adduct geometry of correct Watson-Crick base pair formation. A correct or incorrect nucleotide may be incorporated opposite a lesion, but dependent on the conformation of this base pair, polymerase kinetic block may occur leading to chain termination.

Taq, Pwo, Tth, Tli, Klenow, T7 DNA polymerase and *SP6, T7* RNA polymerase were insensitive to inhibition of DNA and RNA synthesis by the presence of 4-hydroxytamoxifen DNA adducts (chapter 3). *T4* DNA polymerase was the most sensitive to inhibition, with *AMV* and *HIV I* reverse transcriptase showing inhibition to a lesser degree. *AMV* and *HIV I* reverse transcriptases share a high degree of structural homology with *T4* DNA polymerase but lack exonuclease activity. This may indicate that a 4-hydroxytamoxifen DNA adduct may interact at similar critical residues within these polymerase active sites. This may lead to the formation of a kinetically unfavourable 3'-primer-template junction or formation of an incorrect Watson-Crick base pair, that leads to an inactive polymerase complex.

The highly active exonuclease activity of *T4* DNA polymerase is found on the same polypeptide chain as the polymerase function. In the absence of the processivity factor gp45, exonuclease activity may have out competed polymerase activity of the enzyme leading the formation of an inactive complex, and template dissociation. *T4* DNA polymerase paused at sites of guanine and adenine on the *mdr1b* promoter indicating that these bases were adducted by 4-hydroxytamoxifen (chapter 3, fig 32). The same polymerase detected guanine adducts in the bacterial *lacI* gene and only one very strong pause site was observed at an adenine in the section of the gene assayed (chapter 4, fig

50). Pausing at guanine by *T4* DNA polymerase was slightly stronger in some sequences than others indicating sequence selectivity of binding. This effect may be due to enhanced binding at these sites via intercalation at specific sequences, that stabilised a non-covalently bound electrophile before covalent reaction took place. Alternatively, effects of duplex conformation at these sequences may determine how the adduct is presented within the active site of *T4* DNA polymerase that are more inhibitory (Wyatt *et al.*, 1997; Misra *et al.*, 1983; Kobertz *et al.*, 1996, 1997; Iyer *et al.*, 1994; Hargreaves *et al.*, 1997; Neidle *et al.*, 1999). Due to the intensity of the pause, the location of the adenine adduct within this sequence may have destabilised the duplex DNA. This may have enhanced exonuclease partitioning of the polymerase that acted as a kinetic barrier to adduct extension. This theory could be tested by utilising T7 DNA polymerase lacking *E. coli* thioredoxin as its processivity factor, as this polymerase has the most active exonuclease activity of all polymerases, albeit the exonuclease activity being located on a separate subunit.

In conclusion, inhibition of polynucleotide synthesis results from variations in polymerase structure, and kinetic effects that determine the efficiency of lesion extension. Geometrically incorrect base incorporation within the active site and alterations in the 3' termini base pair configuration can make elongation unfavourable, dependent on the nature and ability of the polymerase to deal with any resulting altered DNA conformation. This is shown in my work by aflatoxin B_1 deoxyguanine DNA adducts which inhibited primer elongation by *Taq* and *Klenow* DNA polymerase whereas these polymerases were insensitive to inhibition by 4-hydroxytamoxifen DNA adduct formation. Polymerases employed in the detection of 4-hydroxytamoxifen DNA adduct formation were mostly insensitive to inhibition by these DNA adducts. Of the

polymerases that were sensitive, inhibition was greater for *T4* DNA polymerase than either *AMV* or *HIV I* reverse transcriptase. *AMV* and *HIV I* reverse transcriptases are structurally similar with *T4* DNA polymerase, and the effects of the exonuclease function may have accounted for the increased sensitivity of T4 DNA polymerase to inhibition by 4-hydroxytamoxifen DNA adduct formation.

6.2 Tamoxifen DNA adduct formation and mutagenesis

pLIZ Plasmid DNA containing the bacterial *lacI* gene when reacted with either horseradish peroxidase/ H_2O_2 activated 4-hydroxytamoxifen or α-acetoxytamoxifen formed DNA adducts on most guanines and one site of adenine (chapter 4, fig 50). Tamoxifen DNA adduct formation in hepatic DNA occurred on guanine from Big Blue™ rats treated with tamoxifen, guanine and adenine with Wistar rats treated with α-hydroxytamoxifen, and Fisher F344 primary hepatocytes cultured in the presence of α-hydroxytamoxifen (chapter 5, figs 55, 56, 57).

^{32}P-Postlabelled DNA adducts from the liver of rats dosed with tamoxifen, resolved by TLC and HPLC produce 12 different adduct types (White *et al.*, 1992; Martin *et al.*, 1998). α-Hydroxytamoxifen, a minor *in vivo* metabolite of tamoxifen is suggested to be the major proximate rat carcinogen as it produces the same adduct profile in the liver as rats treated with tamoxifen. This metabolite is further activated to a reactive sulphate ester that binds to DNA (chapter 1, fig 13). α-Acetoxytamoxifen is a model of the sulphate ester and reaction with DNA *in vitro* forms 6 of the 12 adducts produced by tamoxifen in rat liver *in vivo* (Martin *et al.*, 1998; Jacolot *et al.*, 1991; Phillips *et al.*,

1995). However, α-acetoxytamoxifen produces a similar pattern of major ^{32}P-postlabelled adducts *in vitro* as tamoxifen in rat hepatocytes treated *in vitro* (Phillips *et al.*, 1994) and *in vivo* (Martin *et al.*, 1998), suggesting the main DNA adducts derived from α-acetoxytamoxifen are the same as the major adducts formed in rat liver. DNA adduct formation by either α-sulphoxytamoxifen and α-acetoxytamoxifen have been identified as four stereoisomers of tamoxifen and deoxyguanine in which the α position of tamoxifen is covalently linked to the exocyclic amino group of deoxyguanine forming N^2-dG-tamoxifen adducts (Osbourne *et al.*, 1996, 1997). A minor adduct of deoxyadenosine has also been identified. The major phase I metabolites of tamoxifen are N-desmethyltamoxifen and 4-hydroxytamoxifen. N-desmethyltamoxifen can be further metabolised and form N-desmethyltamoxifen DNA adducts at a slightly lower concentrations than the main α-sulphoxytamoxifen DNA adduct. DNA adduct formation by 4-hydroxytamoxifen is minor in rat liver (Martin *et al.*, 1998; Brown *et al.*, 1999; Rajaniemi *et al.*, 1999). It is postulated that DNA adducts formed from 4-hydroxytamoxifen are structurally similar to those formed by either α-sulphoxytamoxifen or α-acetoxytamoxifen differing only by an extra hydroxyl group (Marques & Beland, 1997). My data shows similar adduct profiles for the main adducts formed by α-acetoxytamoxifen and the minor adducts formed from 4-hydroxytamoxifen *in vitro* as those that form *in vivo* from rats treated with tamoxifen.

α-Acetoxytamoxifen reacts directly with DNA *in vitro*, and gave rise to substantially greater total amounts of DNA adducts than enzymatically activated 4-hydroxytamoxifen with a dose dependent increase in DNA adduct levels from 6571 ± 2432 adducts /10^8 nucleotides at 10μM to 4796 ± 2682 adducts/ 10^8 nucleotides at

50μM (fig 49). Unlike α-acetoxytamoxifen, activated 4-hydroxytamoxifen adduct levels did not increase over 3601 ± 108 adducts /10^8 nucleotides (chapter 4, fig 49). This difference in DNA adduct concentration may be explained by preferential reaction of 4-hydroxytamoxifen quinone methide with protein. 4-Hydroxytamoxifen quinone methide can also form polymers which would prevent its reaction with DNA. Therefore, the actual 4-hydroxytamoxifen concentration in the reaction will be lower than for the direct reacting α-acetoxytamoxifen. However, the pattern of α-acetoxytamoxifen and 4-hydroxytamoxifen adduct formation were the same (chapter 4, fig 50). Rats dosed with α-hydroxytamoxifen have 30 fold greater DNA adduct levels than rats treated with an equivalent dose of tamoxifen (White, personal communication). I have shown that plasmids adducted with 4-hydroxytamoxifen produced a two-fold greater increase in the mutation frequency in *E. coli* than plasmids similarly treated with α-acetoxytamoxifen, even though the total level of DNA adducts was approximately 4 fold greater with α-acetoxytamoxifen treatment. DNA synthesis over tamoxifen DNA lesions may be either non-mutagenic or mutagenic depending the kinetic favorability of correct or incorrect base incorporation. A DNA polymerase that permits translesional DNA synthesis will increase the chance of a mutagenic event occurring. *Klenow* is the large fragment of *E. coli* DNA polymerase I and was not inhibited by the presence of 4-hydroxytamoxifen DNA adducts (chapter 3, fig 40). Therefore, *in vitro* primer extension studies of α-acetoxytamoxifen adducted DNA utilising Klenow DNA polymerase may determine the effects of adduct chemistry for translesional DNA synthesis. Such a result may have allowed prediction of the observed mutagenic outcome from the *E. coli* mutagenesis system utilised in the project. Kinetic studies of polymerase mis-incorporation opposite a defined lesion can be quantitated

(Goodman *et al.*, 1993). Shibutani and Dasaradhi, (1997) reacted α-sulphoxytamoxifen with oligonucleotides producing epimers of *trans* and *cis* N^2-dG tamoxifen adducts. *In vitro* primer extension by DNA polymerase α, β, δ and Klenow *exo*$^-$ produced mis-incorporations and deletions with a frequency that varied depending on the polymerase used. This study should be performed using 4-hydroxytamoxifen to see if the rate of mis-incorporation increases. The level of total tamoxifen DNA adduct formation did not correlate to the observed mutation frequency indicating that in this system, α-acetoxytamoxifen DNA adducts are not as mutagenic as DNA adducts produced from 4-hydroxytamoxifen.

The majority of guanines were adducted with α-acetoxytamoxifen or 4-hydroxytamoxifen and correlated to the sites of guanine mutation in the bacterial mutation assay. The pause sites indicative of adenine adduct formation were located 1 and 2 bases from the mutational hotspot in 4-hydroxytamoxifen treated pLIZ plasmid DNA. However the mutated guanine was also adducted and may be responsible for the observed mutation, but the combination of the adenine and guanine adducts may have been such to distort the DNA structure so it enhanced mutagenesis at this position. Therefore, my observations suggest that the frequency of mis-incorporation is dependent upon the chemistry of the adduct and not on total DNA adduct concentration.

Sequence dependent formation and lack of repair of polycyclic aromatic hydrocarbon DNA adducts correlate with the positions of *p53* mutational hotspots in smoking related lung cancers (Denissenko *et al.*, 1996, 1998). A unique mutational hot spot at the third base of codon 249 (dG) of the *p53* gene is seen in primary liver tumours from people

exposed to aflatoxin B_1. Significant aflatoxin B_1 DNA adduct formation has been observed at this site, but this is not the major site of adduction and is repaired relatively quickly. Denissenko, concluded that additional mechanisms may be involved in mutation at this site as *p53* mutations are usually late events in tumour initiation. In the liver of Big Blue™ rats, tamoxifen produces a 3-fold increase in the *lacI* gene mutation frequency (Davies *et al.*, 1997). The mutations occur at CpG sites with a significant number also observed at TpA sites, and no one site appearing to be a hotspot for mutation (Davies *et al.*, 1996, 1997). From this study sites of tamoxifen DNA adduct formation did not correlate to the sites of DNA mutation and location in the *lacI* gene from Big Blue™ animals treated with tamoxifen (chapter 5). The types of mutation generated in the bacterial system will not directly relate to the mutation types formed *in vivo* due to differences in the kinetics of polymerase extension past DNA adducts and the effects of DNA repair. More experiments would need to be performed on animals where a steady state tamoxifen DNA adduct concentration had been reached to arrive at conclusions regarding dG adduct formation and mutagenesis. The mutation frequency in the *lacI* gene of Blue Blue™ rats dosed with α-hydroxytamoxifen produced a lower mutation frequency than for animals dosed with tamoxifen (Davies personal communication), although the total DNA adduct concentration was greater than for animals dosed with tamoxifen (Martin, Personal communication). This data adds weight to the hypothesis that the main adducts derived from α-hydroxytamoxifen form in greater number, but are significantly less mutagenic than the minor DNA adducts derived from the metabolism of tamoxifen itself.

6.3 Tamoxifen carcinogenesis

Results from rodent model systems suggest that tamoxifen treatment producing DNA adducts is causally related to liver tumour development (White, 1999). The bioactivation of α-hydroxytamoxifen to the sulphate ester results in the formation of significant numbers of tamoxifen DNA adducts (Phillips *et al.*, 1995). Isomers of dG-N^2-tamoxifen adducts formed by α-sulphoxytamoxifen have been shown to be mutagenic in simian kidney cells (Terashima *et al.*, 1999). When rats were dosed with α-hydroxytamoxifen (40mg/kg/day for 5 days) adduct levels of approximately $3000x10^8$ nucleotides formed, similar to those seen after 6 months exposure to tamoxifen. However, no hepatocellular carcinoma resulted at 13 months in contrast to a 50% incidence from the tamoxifen treated animals (White, Personal communication). This may suggest that adducts formed from the metabolism of α-hydroxytamoxifen did not produce the significant minor DNA adducts required to cause the critical mutations needed for the initiation of hepatocarcinogenesis. This includes 4-hydroxytamoxifen adduct formation as α-hydroxytamoxifen can be 4-hydroxylated to α, 4-dihydroxytamoxifen which is more reactive towards DNA than 4-hydroxytamoxifen quinone methide and α-hydroxytamoxifen but is rapidly detoxified. This may confirm my data that DNA adducts derived from α-acetoxytamoxifen are not as mutagenic as other tamoxifen metabolites producing minor DNA adducts.

Tamoxifen induced endometrial tumour formation has been attributed to the partial oestrogenic effect of this drug in the human uterus (Stearns & Gelmann, 1998; Kedar *et al.*, 1994). Tamoxifen may also have a genotoxic action in the uterus as human

metabolism of tamoxifen produces DNA reactive metabolites and metabolites that may be further activated in the uterus by the peroxidase activity in this tissue (Stearns & Gelmann, 1998; Pathak & Bodell, 1994, 1995; Hemminki *et al.*, 1995; Han *et al.*, 1992; White *et al.*, 1992; Osbourn *et al.*, 1996). However, Li *et al.*, (1997), has found an enhancement of endogenous uterine DNA adduct formation in rat uterus which may occur in human uterine tissue. If a genotoxic mechanism is responsible for the development of endometrial tumour formation, then tamoxifen DNA adducts should be formed in the uterus. Both 4-hydroxytamoxifen and α-hydroxytamoxifen metabolites are produced by women receiving tamoxifen therapy. However, the detection of tamoxifen DNA adduct formation in uterine tissues of women receiving tamoxifen therapy has produced conflicting results. Standard ^{32}P-postlabelling techniques utilising TLC separations have not detected human uterine tamoxifen DNA adduct formation (Carmichael *et al.*, 1996). Hemminki *et al.*, (1996) using a HPLC technique has reported 2.7 adducts per 10^9 normal nucleotides, but the study lacked standards which lead to the result being questioned (Orton *et al.*, 1997). A more reliable result, utilising a butanol extraction step has been obtained from the group of Shibutani *et al.*, (1999), who identified *cis* and *trans* epimers of α-(N^2-deoxyguanosinyl)-tamoxifen adducts. The levels of *trans* and *cis* adducts ranged from 0.5 to 8.3 and 0.4 to 4.8 adducts per 10^8 normal nucleotides respectively. Therefore, this group have suggested that DNA reactive metabolites of tamoxifen are potentially genotoxic in the human uterus.

No increase in uterine DNA adducts above control values could be detected in rats dosed with α-hydroxytamoxifen (Brown *et al.*, 1998). This suggests that α-hydroxytamoxifen is not further activated in this organ. Tamoxifen acts as an

oestrogen antagonist in rat uterus and thus may contribute to the lack of rat uterine tumours. α-Hydroxytamoxifen is activated to a reactive electrophile by sulphation. Human recombinant hydroxysteroid sulphotransferase (SULT2A1) can sulphate α-hydroxytamoxifen, producing DNA adducts, but its activity is 3 fold less than for the rat sulphotransferase (Shibutani *et al.*, 1998). However, in six uterine samples Shibutani *et al.*, (1999) found *trans* and *cis* forms of tamoxifen-N^2-dG adducts are the major adducts. This can lead to a conclusion that some women may form further metabolites of α-hydroxytamoxifen in the uterus. The principal metabolites of tamoxifen include tamoxifen N-oxide, N-desmethyltamoxifen and 4-hydroxytamoxifen (Moorthy *et al.*, 1996; Marques *et al.*, 1997; Phillips *et al.*, 1994, 1996). N-desmethyltamoxifen has a longer half life than tamoxifen and can also be hydroxylated to α-hydroxy-N-desmethyltamoxifen. This metabolite has been shown to produce the second major DNA adduct from rat liver (Rajaniemi *et al.*, 1999). Low level uterine DNA adducts are reported following short term dosing of either tamoxifen or 4-hydroxytamoxifen to rats (Pathak & Bodell, 1994) although this has not been confirmed by subsequent studies (Osborne *et al.*, 1999). 4-Hydroxytamoxifen can undergo subsequent oxidation to 4-hydroxytamoxifen quinone methide, an electrophile which can react with DNA (Randerath *et al.*, 1994[a+b]; Pongracz *et al.*, 1995; Moorthy *et al.*, 1996; Pathak *et al.*, 1996). A number of 4-hydroxytamoxifen derivatives have been suggested as proximate carcinogens. Cytochrome P450 mediated 3-hydroxylation of 4-hydroxytamoxifen producing 3,4-dihydroxytamoxifen can form the 3,4-hydroxy catechol that can be oxidised to reactive semi and ortho-quinones (Dehal & Kupfer, 1996).

Although the mechanism of action of tamoxifen in the formation of uterine tumours has been proposed to be hormonal, 4-hydroxytamoxifen can accumulate in human endometrium. Therefore, utilising my data obtained from the mutagenesis assays (chapter 4) may indicate a possible role of 4-hydroxytamoxifen DNA adduct formation in the human uterus for the induction of human mutation in the uterus. This organ has high specific peroxidase activities which may be increased by the oestrogenic effects of tamoxifen by inducing hyperplasia which may lead to 4-hydroxytamoxifen quinone methide formation (Pathak *et al.*, 1996; Robinson *et al.*, 1991). This suggests DNA adduct formation in the human endometrium by 4-hydroxytamoxifen is possible. The cytochrome CYP2D6 is involved in the 4-hydroxylation pathway of tamoxifen metabolism in human liver (Dehal & Kupfer, 1997) along with variable contributions of other CYP forms (Crewe *et al.*, 1997). This cytochrome is polymorphic in the human population, therefore the level of 4-hydroxytamoxifen DNA adduction in uterine tissue may be variable if present at all.

To conclude, my data suggests that DNA adducts formated from 4-hydroxytamoxifen are more mutagenic, and occur in much smaller quantities than the main adducts formed from α-hydroxylation. Therefore 4-hydroxytamoxifen may be genotoxic to the human endometrium in sub-populations where CYP2D6 activity is high or for metabolites that can be activated by peroxidase action. The oestrogenic agonist activities of tamoxifen and 4-hydroxytamoxifen may potentiate mutation fixation by promoting cell division in the uterus and therefore tamoxifen may act as a complete carcinogen in the human endometrium.

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